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Primary and spontaneously immortalized mouse embryonic fibroblasts (MEF) were derived from embryonal stem cells in which both alleles of the *BRCA2* gene have been mutated by gene targeting. The mutation deletes an extreme carboxy-terminal domain of the BRCA2 protein encoded by exon 27, one of two regions known to mediate binding with RAD51 protein. *BRCA2*-mutant MEF were markedly hypersensitive to the DNA crosslinking drug mitomycin-C, and showed evidence of severe chromosomal instability. While immortal wild-type MEF approximated tetraploidy, immortal *BRCA2*-mutant MEF showed subtetraploid chromosome numbers and one to several abnormally small chromosomes or "minichromosomes" in every metaphase. The majority of minichromosomes either lacked telomeres or lacked a centromere detectable by fluorescence in-situ hybridization. Primary MEF were then examined for chromosomal changes over the first few passages after dissociation from embryos. *BRCA2*-mutant primary MEF showed sharply elevated numbers of chromatid and chromosome aberrations, including breaks, deletions and exchanges. By the third passage, approximately half the metaphase cells in *BRCA2*-mutant populations had one or more chromosome abnormalities. This marked chromosomal instability in *BRCA2*-mutant cells, and their hypersensitivity to DNA interstrand crosslinks, may reflect a deficiency in DNA repair by homologous recombination.

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Introduction

The BRCA2 protein has been shown to physically interact with the DNA repair and homologous recombination protein RAD51, both in vitro and in vivo (Chen *et al.*, 1998b; Katagiri *et al.*, 1998; Marmorstein *et al.*, 1998; Mizuta *et al.*, 1997; Sharan *et al.*, 1997; Wong *et al.*, 1997) suggesting that BRCA2 participates in a recombination complex during cell division and DNA repair processes. Shortly after exposure of normal cells to ionizing radiation, RAD51 protein relocalizes to form discrete nuclear foci, which have been proposed to represent the assembly of multiprotein recombinational repair complexes at sites of DNA damage (Chen *et al.*, 1998a; Chen *et al.*, 1999; Haaf *et al.*, 1995). In Capan-1 human cells, which lack an intact *BRCA2* gene, formation of RAD51 foci is severely impaired (Yuan *et al.*, 1999). The latter cells are also hypersensitive to ionizing radiation and to drugs that cause DNA double-strand breaks, and show reduced repair of DNA double-strand breaks after radiation exposure (Abbott *et al.*, 1998). If loss of the interactions between BRCA2 and RAD51 results in reduced capacity for homologous repair of DNA double-strand breaks or homology-dependent repair of other lesions during DNA replication, this may in turn contribute to genomic instability, and result in the eventual mutation of genes required for control of cell growth and division.

Domains that mediate interaction with RAD51 have been identified in two parts of the BRCA2 protein. An extreme carboxy-terminal domain within exon 27, comprising amino acids 3196-3232, was first shown to mediate RAD51 binding (Mizuta *et al.*, 1997; Sharan *et al.*, 1997). Soon after, it was discovered that the eight conserved "BRC repeats" encoded by the large central exon 11 also can function individually to bind RAD51. Homozygous mutations in mouse that delete all of the identified RAD51-interaction domains of BRCA2 causes early embryonic death, apparently due to failure of cell proliferation (Ludwig *et al.*, 1997; Sharan *et al.*, 1997; Suzuki *et al.*, 1997). Cells from very early *BRCA2*$^{-/-}$ embryos show an exaggerated sensitivity to ionizing radiation (Sharan *et al.*, 1997). An essentially identical phenotype of early embryonic lethality and radiosensitivity has also been reported for homozygous null *RAD51* mutation in mouse (Lim & Hasty, 1996; Tsuzuki *et al.*, 1996). However, targeted mutations of the mouse *BRCA2* gene that preserve all or some of the BRC repeats in exon 11 can support embryonic development to term and the birth of viable pups (Connor *et al.*, 1997; Friedman *et al.*, 1998, and this report).

Morimatus has reported the generation of embryonal stem (ES) cells in which both alleles of the *BRCA2* gene have been truncated by gene targeting, using two different targeting vectors (Morimatus et al., 1998). Both truncations delete exon 27, which encodes the extreme carboxy-terminal RAD51-interaction domain of the BRCA2 protein. The two targeted alleles have been designated *BRCA2*^{lex1} and *BRCA2*^{lex2}. *BRCA2*$^{lex1/lex2}$ ES cells are hypersensitive to ionizing radiation but not to ultraviolet radiation. The *BRCA2*$^{lex1/lex2}$ ES cells were used to generate primary mouse embryonal fibroblasts (MEF). Primary *BRCA2*$^{lex1/lex2}$ MEF cells show an impaired growth rate and reduced cloning efficiency as compared to primary *BRCA2*+/+ MEF, and undergo premature replicative senescence as determined by colony size distribution and serial passage (3T3-equivalent) analysis.

Here we report characterization of these cells for sensitivity to mitomycin C, which produces DNA interstrand crosslinks; a type of damage thought to require repair through homologous recombination. Because mutations that impair homologous recombination have been associated with chromosomal instability, in yeast and in mammalian cells, we examined primary and immortalized *BRCA2*$^{lex1/lex2}$ MEF for effects of the mutation on chromosome stability.

Body

1) The BRCA2^{lex2} *allele is embryonic lethal when homozygous.* Embryonic stem (ES) cells bearing the *BRCA2*^{lex1} and *BRCA2*^{lex2} alleles were originally generated by two successive rounds of gene targeting (Morimatsu *et al.*, 1998). When first reported, neither targeted allele existed in the homozygous state. *BRCA2*$^{lex1/+}$ and *BRCA2*$^{lex2/+}$ mice have since been generated and bred. Crosses of *BRCA2*$^{lex1/+}$ mice yield viable *BRCA2*$^{lex1/lex1}$, *BRCA2*$^{lex1/+}$ and *BRCA2*$^{+/+}$ pups in approximately the expected 1:2:1 Mendelian ratio. In contrast, *BRCA2*$^{lex2/+}$ crosses have produced no *BRCA2*$^{lex2/lex2}$ pups at all. Of 56

mice genotyped from *BRCA2*$^{lex2/+}$ crosses, there were 32 *BRCA2*$^{lex2/+}$ and 24 *BRCA2*$^{+/+}$. We also genotyped embryonic day 10.5 embryos from a *BRCA2*$^{lex1/+}$ female crossed with a *BRCA2*$^{lex1/lex2}$ male. In addition to *BRCA2*$^{lex2/+}$ and *BRCA2*$^{lex1/lex2}$ embryos that were normal in appearance, one *BRCA2*$^{lex2/lex2}$ embryo was recovered, which was partially resorbed (Figure 1). This embryo appeared arrested at approximately embryonic day 6, similar to embryos homozygous for truncations of BRCA2 that delete all of the identified RAD51-interaction domains (Ludwig *et al.*, 1997; Sharan *et al.*, 1997; Suzuki *et al.*, 1997). These results indicate that the *BRCA2*^{lex2} allele is lethal, while the *BRCA2*^{lex1} allele is able to support embryonic development to term.

Figure 1. Day 10.5 embryos harvested from a *BRCA2*$^{lex1/+}$ female mated with a *BRCA2*$^{lex1/lex2}$ male. The larger embryo at center right was genotyped by PCR as a *lex1/lex2* compound heterozygote and appears morphologically normal. The much smaller (partially resorbed) embryo indicated by the red arrow at left was genotyped as a *lex2/lex2* homozygote.

2. *Immortalized BRCA2*$^{lex1/lex2}$ *MEF show reduced cloning efficiency.* *BRCA2*$^{lex1/lex2}$ ES cells were used to derive populations of primary *BRCA2*$^{lex1/lex2}$ MEF cells and, from these, a single clone of spontaneously immortalized *BRCA2*$^{lex1/lex2}$ MEF, as described in (Morimatsu *et al.*, 1998). Although the immortalized *BRCA2*$^{lex1/lex2}$ MEF could be propagated readily, their growth was slower than that of an isogenic wild-type (*BRCA2*$^{+/+}$) immortalized clone. To determine whether cellular viability is impaired in the immortalized *BRCA2*$^{lex1/lex2}$ MEF cells, their cloning efficiency was measured by colony formation assays. In eleven paired determinations, mean cloning efficiency for wild-type MEF was 24.9% (S.D. = 10.9%), but for the immortalized BRCA2$^{lex1/lex2}$ MEF, only 16.0% (S.D. = 4.8%), i.e. reduced by about one third. By a paired t-test, the difference is significant ($P < 0.002$).

3. *Immortalized BRCA2*$^{lex1/lex2}$ *MEF are moderately hypersensitive to ionizing radiation.* We have shown previously that *BRCA2*$^{lex1/lex2}$ ES cells are more sensitive than wild-type (*BRCA2*$^{+/+}$) ES cells to gamma radiation (Morimatsu *et al.*, 1998). To determine whether immortalized *BRCA2*$^{lex1/lex2}$ MEF retain the phenotype of hypersensitivity to ionizing radiation, their sensitivity was assessed in comparison to immortalized isogenic wild-type MEF. Sensitivity was measured by survival (colony formation) after acute exposure to gamma radiation at doses ranging from 2 to 10 Gy (Figure 2). At each dose tested, *BRCA2*$^{lex1/lex2}$ MEF were significantly more sensitive than wild-type MEF (P values ranging from 0.01 to 0.05). At 2 Gy, survival of *BRCA2*$^{lex1/lex2}$ MEF was reduced by only 1.5 - fold relative to wild-type. However, the difference became greater with increasing dose, so that at 10 Gy, survival of *BRCA2*$^{lex1/lex2}$ MEF was reduced by 10-fold relative to wild-type. Compared in terms of the dose required to reduce viability to 10% of non-irradiated controls, *BRCA2*$^{lex1/lex2}$ MEF were about 1.4-fold more sensitive to gamma radiation than wild-type MEF. Both the *BRCA2*$^{lex1/lex2}$ MEF and the wild-type MEF appear to be somewhat more resistant to gamma radiation than the corresponding ES cell lines (compare Figure 2, this report, to Figure 2 in Morimatsu et al (Morimatsu *et al.*, 1998)). Nonetheless, the relative difference in sensitivity between *BRCA2*$^{lex1/lex2}$ cells and wild-type cells, in terms of dose required to reduce survival by 90%, is comparable: about 1.4-fold for the immortalized MEF cells, and about 1.7-fold for the ES cells.

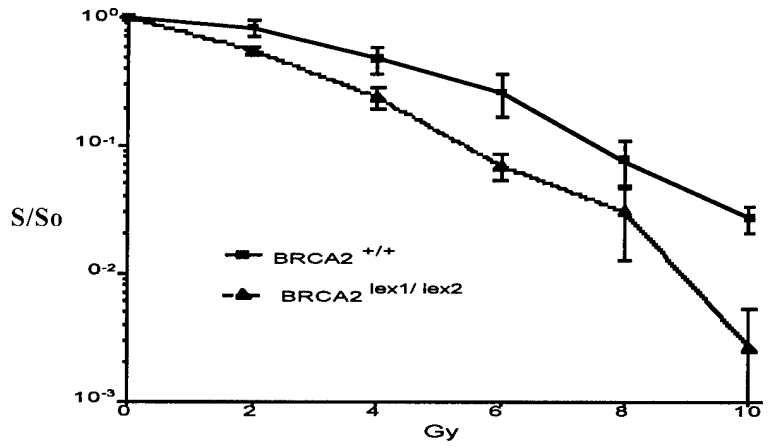

Figure 2. Sensitivity of *BRCA2*$^{lex1/lex2}$ and wild-type (*BRCA2*$^{+/+}$) immortalized MEF cells to gamma radiation was measured by a colony formation assay. Each data point represents the mean of three separate experiments. Each experiment was plated in triplicate. Error bars represent standard error of the mean.

4. Immortalized BRCA2$^{lex1/lex2}$ MEF are severely hypersensitive to mitomycin C. Extreme hypersensitivity to drugs that cause DNA interstrand crosslinks has been observed in two other mammalian cell lines now known to have defects in homologous recombinational repair; the XRCC2-mutant line irs1 and the XRCC3-mutant line irs1SF (Caldecott & Jeggo, 1991; Cui *et al.*, 1999; Tebbs *et al.*, 1995), but had not been examined previously in *BRCA2*-mutant cells. We compared immortalized *BRCA2*$^{lex1/lex2}$ MEF to immortalized wild-type MEF for sensitivity to the DNA crosslinking drug mitomycin C. Sensitivity was measured by survival (colony formation) after plating in the presence of mitomycin C at initial concentrations ranging from 3 x 10^{-9} M to 3 x 10^{-7} M (Figure 3). Mean survival after plating in mitomycin C at 3 x 10^{-9} M (or less) was not significantly lower for *BRCA2*$^{lex1/lex2}$ MEF than for wild-type MEF. But at progressively higher concentrations of mitomycin C (1 x 10^{-8} M, 3 x 10^{-8} M and 1 x 10^{-7} M), survival of *BRCA2*$^{lex1/lex2}$ MEF fell more sharply and was significantly lower than for wild-type MEF ($P < 0.02$, < 0.005 and < 0.01, respectively). At 3 x 10^{-8} M mitomycin C, survival of *BRCA2*$^{lex1/lex2}$ MEF was 5.6-fold lower, and at 1 x 10^{-7} M, 48-fold lower than for wild-type MEF. Compared in terms of the concentration of mitomycin C required to reduce survival to 50% of nontreated controls, *BRCA2*$^{lex1/lex2}$ MEF were approximately eight-fold more sensitive than wild-type MEF.

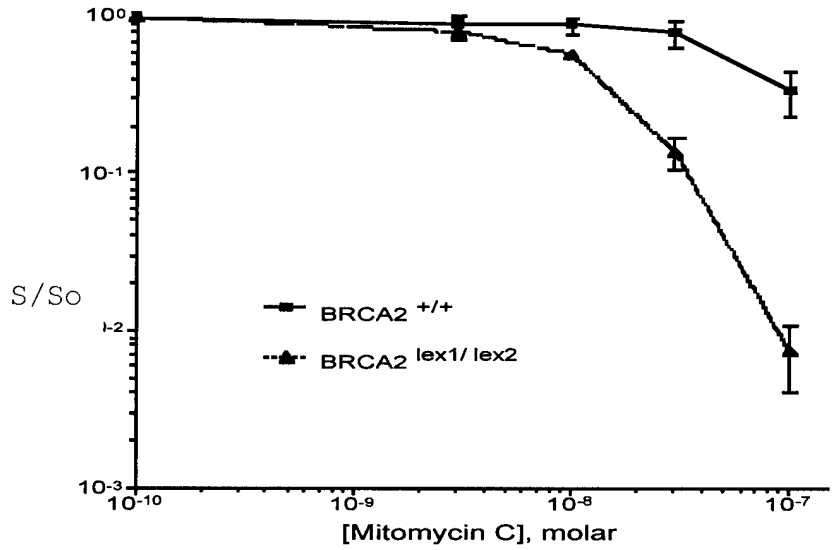

Figure 3. Sensitivity of *BRCA2*$^{lex1/lex2}$ and wild-type (*BRCA2*$^{+/+}$) immortalized MEF cells to mitomycin C was measured by a colony formation assay. Each data point represents the mean of three separate experiments. Each experiment was plated in triplicate. Error bars represent standard error of the mean.

***5. BRCA2*$^{lex1/lex2}$ *MEF exhibit chromosomal instability*.** Immortalized *BRCA2*$^{lex1/lex2}$ MEF and *BRCA2*$^{+/+}$ MEF were karyotyped. Single clones of wild-type (*BRCA2*$^{+/+}$) and *BRCA2*$^{lex1/lex2}$ MEF were harvested for metaphase spreads at approximately equal periods of time in culture after their emergence as immortalized cell colonies (about two months). In comparison to wild-type MEF, immortalized *BRCA2*$^{lex1/lex2}$ MEF showed an increased frequency of chromatid- and chromosome-type aberrations (breaks and deletions), and sharply increased occurrence of abnormally small chromosomes, or "minichromosomes" (Table 1). Every *BRCA2*$^{lex1/lex2}$ metaphase examined had two to six minichromosomes. Both the wild-type and *BRCA2*$^{lex1/lex2}$ MEF lines displayed abnormal chromosome numbers. The wild-type line showed a bimodal distribution of chromosome numbers, with most of the population approximating a tetraploid complement of 80 chromosomes, and a smaller part of the population near the diploid number of 40 chromosomes (Figure 4A). Tetraploidization is commonly associated with spontaneous immortalization of mouse cells (Todaro & Green, 1963). The *BRCA2*$^{lex1/lex2}$ line, in contrast, was sub-tetraploid, with a modal chromosome number of 60, and a smaller subpopulation of near-diploid cells (Figure 4B). The metaphase spreads of immortalized MEF were then examined by fluorescence in-situ hybridization (FISH) using probes for telomeric repeats and for the mouse major satellite sequence, which lies in close proximity to the centromeres of mouse chromosomes. A representative *BRCA2*$^{lex1/lex2}$ metaphase is shown in Figure 5. Most of the minichromosomes seen in immortalized *BRCA2*$^{lex1/lex2}$ MEF lacked detectable telomeres. Of those minichromosomes that retained telomeres, many lacked a detectable centromeric region. In addition to acentrics, dicentric chromosomes or minichromosomes were also present in most *BRCA2*$^{lex1/lex2}$ MEF metaphases (Figure 5), but were seen only rarely in wild-type immortalized MEF.

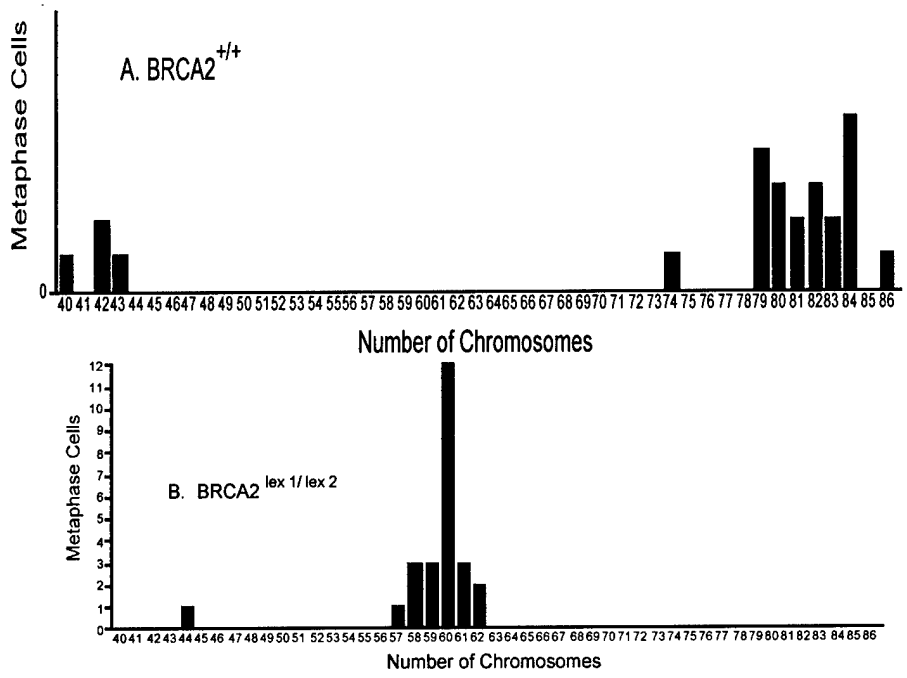

Figure 4. Chromosome number distributions in wild-type (A) and *BRCA2*$^{lex1/lex2}$ (B) immortalized MEF cells.

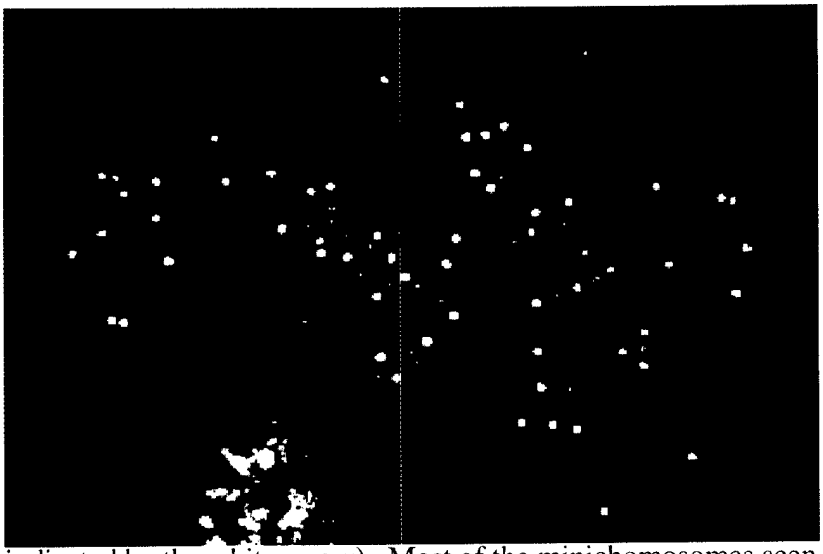

Figure 5. Fluorescence in-situ hybridization with probes for centromeres and telomeres. Chromosome spreads prepared from immortalized MEF cells were hybridized with fluorescent probes for centromeric and telomeric sequences. The centromeric probe appears white in this composite image; the telomeric probe is green. Chromosomes were counterstained with DAPI (blue). Because normal mouse chromosomes are acrocentric, only the telomeres of the long arms can be seen here; the telomeres of the short arms are obscured by the brighter centromeric probe (An example of a normal chromosome is indicated by the white arrow). Most of the minichomosomes seen in BRCA2-mutant metaphase cells lacked detectable telomeres. At least five of this type occur in this metaphase (an example is indicated by the red arrow). Acentric and dicentric chromosomes or minichromosomes were also seen frequently in BRCA2-mutant metaphase cells. Four acentrics (e.g. yellow arrow) and two dicentrics (e.g. green arrow) can be seen in this metaphase).

Conclusion and Discussion

We wished to determine whether the chromosomal instability seen in immortalized *BRCA2$^{lex1/lex2}$* MEF arose during the process of immortalization, or already existed in primary cells prior to senescent crisis. We therefore examined early-passage primary MEF for chromosomal abnormality. The derivation of primary MEF from chimeric embryos was as previously described (Morimatsu *et al.*, 1998). Two populations of *BRCA2$^{lex1/lex2}$* MEF and three populations of wild-type (*BRCA2$^{+/+}$*) MEF that had been frozen at the first passage after dissociation from embryos ("passage 1") were thawed and expanded for several passages, with metaphase spreads prepared at each passage. Passage 1 wild-type MEF grew vigorously after thaw and showed no appreciable slowing of growth over at least ten passages afterward (data not shown). However, the *BRCA2$^{lex1/lex2}$* MEF were seen to senesce prematurely in culture, as reported previously (Morimatsu *et al.*, 1998), and could not be expanded beyond the fourth passage. For one of the two primary *BRCA2$^{lex1/lex2}$* MEF populations examined (281.1), sufficient numbers of metaphase cells for scoring were recovered only at the second and third passages. Scoring of early passage primary MEF for chromosomal aberrations is summarized in Table 2. Compared to wild-type primary MEF, the *BRCA2$^{lex1/lex2}$* primary MEF had sharply elevated numbers of chromatid and chromosome aberrations, including gaps, breaks, deletions and exchanges. The *BRCA2$^{lex1/lex2}$* MEF had higher numbers of chromosomal aberrations even at passage 2, and accumulated further aberrations more rapidly than wild-type MEF. By the third passage, approximately half the metaphase cells in *BRCA2$^{lex1/lex2}$* populations had one or more visible chromosome abnormalities.

Deletion of the RAD51-interaction domain encoded by the carboxy-terminal exon 27 of the mouse *BRCA2* gene is associated with a cellular phenotype of reduced cloning efficiency, moderate hypersensitivity to ionizing radiation, a more severe hypersensitivity to the DNA crosslinking drug mitomycin C, and gross chromosomal instability. The phenotype seen in *BRCA2$^{lex1/lex2}$* MEF cells is probably attributable to the *BRCA2^{lex1}* allele. As described previously (Morimatsu *et al.*, 1998), the *BRCA2^{lex1}* allele retains all of the BRCA2 coding sequence except the carboxy-terminal exon 27, and splices from exon 26 to a new stop codon and polyadenylation signal within the *HPRT* minigene that was used as a positive selection marker in gene targeting. The resulting RNA transcript can be detected by RT-PCR, and is likely translated as a protein that omits the exon 27 RAD51-interaction domain. The *BRCA2^{lex2}* allele is deleted for most of

exon 26 as well as exon 27 and the native polyadenylation signal. Whether it produces a stable transcript or protein product is unknown. While the *BRCA2^{lex1}* allele can sustain embryonic development to term in the homozygous state, the *BRCA2^{lex2}* allele is an embryonic lethal when homozygous. Since other, more severely truncated alleles of BRCA2 can support development to term when homozygous (Connor *et al.*, 1997; Friedman *et al.*, 1998), the most plausible explanation is that the *BRCA2^{lex2}* allele fails to express a protein product, while the *BRCA2^{lex1}* allele codes for an altered protein with partial activity.

The immortalized *BRCA2$^{lex1/lex2}$* MEF cells are more sensitive than immortalized wild-type MEF to the DNA crosslinking drug mitomycin C. Moreover, the hypersensitivity to mitomycin C is more pronounced than the hypersensitivity to gamma radiation (compare Figure 2 and Figure 3). Mitomycin C can form monoadducts with DNA, and intrastrand as well as interstrand crosslinks. Monoadducts and intrastrand crosslinks involve bases on only one strand of the DNA duplex and leave the complementary strand intact. They can be repaired by nucleotide excision repair in much the same way as other bulky adducts or UV-induced pyrimidine dimers. Loss of the BRCA2 exon 27 RAD51-binding domain evidently has little effect nucleotide excision repair, since we previously found that *BRCA2$^{lex1/lex2}$* ES cells have no hypersensitivity to UVC irradiation (Morimatsu *et al.*, 1998). The hypersensitivity of *BRCA2$^{lex1/lex2}$* MEF to mitomycin C therefore indicates that the BRCA2 protein has some role in cellular response to DNA interstrand crosslinks. Because interstrand crosslinks covalently link bases on each strand of the DNA duplex, their repair necessarily involves cleavage and processing of both strands and hence the potential for loss of genetic information. In Escherichia coli, repair of interstrand crosslinks is apparently accomplished without loss of genetic information by a pathway that includes elements of both nucleotide excision repair and homologous recombination ((Friedberg *et al.*, 1995) and references therein). The process is dependent upon the bacterial RecA protein, the prototypical recombinational strand transferase to which the RAD51 family of eukaryotic recombination and repair proteins is related. There is substantial evidence that repair of interstrand crosslinks in mammalian cells also involves both nucleotide excision and homologous recombinational repair functions (reviewed in (Thompson, 1996)). The results obtained here suggest that BRCA2 participates in this process.

Immortalized *BRCA2$^{lex1/lex2}$* MEF cells differed from wild-type in the numbers of chromosomes present and in the occurrence of chromosomal abnormalities. Immortalized wild-type MEF approximated the near-tetraploid chromosome numbers that are commonly seen in spontaneously immortalized mouse cells within a few passages after their emergence from a primary cell population undergoing senescent crisis (Todaro & Green, 1963). The immortalized *BRCA2$^{lex1/lex2}$* MEF cells, in contrast, had a median chromosome number of 60. The meaning of this difference is uncertain. One possibility is that the subtetraploid numbers seen in *BRCA2$^{lex1/lex2}$* MEF cells resulted from tetraploidization followed by a process of chromosome loss. This interpretation is supported by the evidence of chromosomal instability seen in *BRCA2$^{lex1/lex2}$* MEF cells. Immortalized *BRCA2$^{lex1/lex2}$* MEF showed several-fold more chromatid- and chromosome-type breaks and deletions than wild-type, and very frequent occurrence of abnormally shortened "minichromosomes". By FISH analysis, the majority of minichromosomes appear either to lack telomeres or to lack centromeres, which implies that they are chromosome fragments. Chromosome fragments that lack centromeres are subject to loss by segregation errors. Chromosome fragments that lack telomeres can be expected to undergo progressive shortening, and further cycles of fusion and breakage events. The dicentric chromosomes and minichromosomes seen in immortalized *BRCA2$^{lex1/lex2}$* MEF may reflect the latter process.

Chromosomal instability was also demonstrable in primary *BRCA2$^{lex1/lex2}$* MEF, even at the earliest passage tested (passage 2) after dissociation from embryos. It was therefore not acquired by *BRCA2$^{lex1/lex2}$* MEF during immortalization, at least not wholly. However, chromosomal abnormalities were also seen (at much lower frequency) in wild-type primary MEF cells, suggesting that the phenomenon is partly due to the artificial environment of cell culture. In addition to chromosome instability, the primary *BRCA2$^{lex1/lex2}$* MEF underwent premature replicative senescence in culture. Perhaps because of this growth defect, it was more difficult to derive spontaneously immortalized *BRCA2$^{lex1/lex2}$* MEF. And although replicative senescence has been overcome in the single immortalized *BRCA2$^{lex1/lex2}$* MEF clone

obtained, it appears that the original growth defect has not been entirely suppressed, as cloning efficiency is still substantially reduced relative to wild-type immortalized MEF.

The phenotype of *BRCA2*$^{lex1/lex2}$ MEF cells can be compared with those of mouse cells bearing two other sublethal *BRCA2* mutations. Connor et al reported a targeted mutation that truncates the *BRCA2* open reading frame near the 3' end of exon 11, preserving the first six of the eight BRC repeats and most of the seventh (Connor *et al.*, 1997). Primary MEF cells homozygous for this mutation show a growth defect in culture, and overexpress both p21 and p53. These cells are also deficient in repair of ionizing radiation-induced double-strand breaks, as assessed by comet assay. Friedman et al (Friedman *et al.*, 1998) created a targeted mutation within exon 11 that retains only the first three BRC motifs. Primary MEF homozygous for this mutation also suffer a proliferative defect, as well as increased sensitivity to ultraviolet radiation and to methylmethanesulfonate, and a high frequency of spontaneous chromosomal breaks and aberrant chromatid exchanges (Lee *et al.*, 1999; Patel KJ, 1998). Together with the results reported here, these findings indicate that mutations in either of the regions of the BRCA2 protein shown to mediate interaction with RAD51 (The BRC repeats and exon 27) can produce cellular phenotypes of growth impairment, sensitivity to DNA damage, and gross chromosomal instability. That the *BRCA2* mutations reported by Connor et al and Friedman et al have deleterious phenotypes is perhaps unsurprising, given that these truncations eliminate more than a third, or more than half (respectively) of the BRCA2 protein. The BRCA2^{lex1} allele deletes only the last 189 residues of the 3328 amino acid mouse BRCA2 protein (less than 6% of its length), and leaves all eight of the BRC repeats intact, yet produces a very similar cellular phenotype as the more severe truncations. The results obtained here confirm that the extreme carboxy-terminal region encoded by exon 27, though not essential to viability, is important in the function of BRCA2. It is formally possible that the phenotypic effects associated with deletion of exon 27 (or, for that matter, with deletions of BRC repeats) result from something other than altered interactions with RAD51. However, interaction with RAD51 is the only function so far demonstrated for the region encoded by exon 27. The truncated BRCA2 protein encoded by the *BRCA2*^{lex1} allele presumably retains the capacity to bind RAD51 via the BRC repeats, since peptides that contain only a single BRC repeat have been shown to do so (Chen *et al.*, 1998b). This raises the question of why the BRCA2 protein should require so many RAD51 interaction domains, all eight of the BRC repeats as well as the region encoded by exon 27, to be fully functional in vivo.

The phenotypes of *BRCA2* mutations in mouse cells can be compared more generally to those of cell lines with mutations in other known or suspected homologous recombination genes, including the other major breast cancer susceptibility gene, *BRCA1*. As for BRCA2, homozygous null mutation of *BRCA1* causes early embryonic lethality in mouse (Gowen *et al.*, 1996; Hakem *et al.*, 1996; Liu *et al.*, 1996; Ludwig *et al.*, 1997). *BRCA1*-deficient mouse and human cells are more sensitive than wild type to ionizing radiation (Abbott *et al.*, 1999; Shen *et al.*, 1998). Antisense suppression of *BRCA1* causes increased sensitivity to cisplatin which, like mitomycin C, forms DNA interstrand crosslinks (Husain *et al.*, 1998). Recently, *BRCA1*-mutant mouse ES cells have been shown to be impaired for gene targeting and for homologous repair of chromosomal double-strand breaks (Moynahan *et al.*, 1999). Homozygous null mutation of the *RAD51* or *MRE11* genes also results in embryonic and/or cell lethality, just as for *BRCA2* and *BRCA1* (Lim & Hasty, 1996; Tsuzuki *et al.*, 1996; Xiao & Weaver, 1997). In DT40 chicken lymphoblastoid cells, conditional knockouts of *RAD51* or *MRE11* produce growth arrest and spontaneous chromosome breakage followed by cell death when *RAD51* or *MRE11* expression is shut down (Sonoda *et al.*, 1998; Yamaguchi-Iwai *et al.*, 1999). Knockout of *RAD54* in DT40 cells produces a milder phenotype of growth impairment and chromosome instability (Bezzubova *et al.*, 1997; Takata *et al.*, 1998). The hamster cell lines irs1 and irs1SF are mutated for *XRCC2* and *XRCC3* respectively (two members of the *RAD51* gene family). Both cell lines have been shown to be deficient in homologous recombinational repair of chromosomal double-strand breaks (Brenneman *et al.*, 1999; Johnson *et al.*, 1999; Pierce *et al.*, 1999). These two mutants in particular offer illuminating parallels to sublethal *BRCA2* mutation in mouse. Similarly to *BRCA2*$^{lex1/lex2}$ MEF cells, the *XRCC2* and *XRCC3* mutant hamster lines show reduced cloning efficiency relative to their wild-type counterparts. Like *BRCA2*$^{lex1/lex2}$ MEF, both of the hamster mutant lines show only moderate hypersensitivity to ionizing radiation (2 to 3-fold greater that wild-type), yet are extremely sensitive to mitomycin C (about 60-fold and 100-fold greater than wild-type). Both also suffer severe chromosomal instability, showing high frequencies of chromosome breakage and rearrangement even in the absence of exogenous DNA damage (Cui *et al.*, 1999; Fuller & Painter, 1988; Tebbs *et al.*, 1995). The phenotype of

BRCA2$^{lex1/lex2}$ MEF is less severe in each of these respects than those of the hamster XRCC2 and XRCC3 mutations, but it should be born in mind that *BRCA2*^{lex1} is not a null mutation. The similar phenotypes of sublethal *BRCA2* and *BRCA1* mutations and mutations in RAD51 homologs lend support to the idea that the physical interactions of BRCA2 protein with RAD51 reflect a critical involvement in homologous recombinational repair.

Key Researech Accomplishments

- The BRCA2^{lex2} allele is embryonic lethal when homozygous
- Immortalized BRCA2$^{lex1/lex2}$ MEF are moderately hypersensitive to ionizing radiation
- Immortalized BRCA2$^{lex1/lex2}$ MEF are severely hypersensitive to mitomycin
- BRCA2$^{lex1/lex2}$ MEF exhibit chromosomal instability

Reportable Outcomes

- We have generated mouse immortalized BRCA2$^{lex1/lex2}$ MEF
- A postdoctoral fellow, Dr. Mark Breenman was hired for this study
- A DOE postdoc fellowship was award for Dr. Breenman was based on this research
- A manuscript entitled "The Exon 27 RAD51-Binding Domain of BRCA2 is Required for Chromosomal Stability and Resistance to DNA Interstrand Crosslinks" is being prepared.

Conclusion

The primary *BRCA2*$^{lex1/lex2}$ MEF cells previously reported have been used to derive spontaneously immortalized MEF. Here we report characterization of these cells for sensitivity to mitomycin C, which produces DNA interstrand crosslinks; a type of damage thought to require repair through homologous recombination. We find that deletion of the BRCA2 exon 27 RAD51-binding domain results in a marked hypersensitivity to mitomycin C in immortalized MEF cells, indicating a defect in ability to repair DNA interstrand crosslinks. Because mutations that impair homologous recombination have been associated with chromosomal instability, in yeast and in mammalian cells, we examined primary and immortalized *BRCA2*$^{lex1/lex2}$ MEF for effects of the mutation on chromosome stability. Both immortalized and early-passage primary MEF cells deleted for the exon 27 RAD51-binding domain also exhibit severe chromosomal instability.

References

Abbott, D.W., Freeman, M.L. & Holt, J.T. (1998). *J Natl Cancer Inst*, 90, 978-85.

Abbott, D.W., Thompson, M.E., Robinson-Benion, C., Tomlinson, G., Jensen, R.A. & Holt, J.T. (1999). *J Biol Chem*, 274, 18808-12.

Bezzubova, O., Silbergleit, A., Yamaguchi-Iwai, Y., Takeda, S. & Buerstedde, J.M. (1997). *Cell*, 89, 185-93.

Brenneman, M.A., Weiss, A.E., Nickoloff, J.A. & Chen, D.J. (1999). *Mutation Research - DNA Repair (in press)*.

Caldecott, K. & Jeggo, P. (1991). *Mutat Res*, 255, 111-21.

Chen, J., Silver, D.P., Walpita, D., Cantor, S.B., Gazdar, A.F., Tomlinson, G., Couch, F.J., Weber, B.L., Ashley, T., Livingston, D.M. & Scully, R. (1998a). *Mol Cell*, 2, 317-28.

Chen, J.J., Silver, D., Cantor, S., Livingston, D.M. & Scully, R. (1999). *Cancer Res*, 59, 1752s-1756s.

Chen, P.L., Chen, C.F., Chen, Y., Xiao, J., Sharp, Z.D. & Lee, W.H. (1998b). *Proc Natl Acad Sci U S A*, 95, 5287-92.

Connor, F., Bertwistle, D., Mee, P.J., Ross, G.M., Swift, S., Grigorieva, E., Tybulewicz, V.L. & Ashworth, A. (1997). *Nat Genet*, 17, 423-30.

Cui, X., Brenneman, M., Meyne, J., Oshimura, M., Goodwin, E.H. & Chen, D.J. (1999). *Mutat Res*, 434, 75-88.

Friedberg, E.C., Walker, G.C. & Siede, W. *DNA Repair and Mutagenesis*. American Society for Microbiology, Washington, D.C.

Friedman, L.S., Thistlethwaite, F.C., Patel, K.J., Yu, V.P., Lee, H., Venkitaraman, A.R., Abel, K.J., Carlton, M.B., Hunter, S.M., Colledge, W.H., Evans, M.J. & Ponder, B.A. (1998). *Cancer Res*, 58, 1338-43.
Fuller, L.F. & Painter, R.B. (1988). *Mutat Res*, 193, 109-21.
Gowen, L.C., Johnson, B.L., Latour, A.M., Sulik, K.K. & Koller, B.H. (1996). *Nat Genet*, 12, 191-4.
Haaf, T., Golub, E.I., Reddy, G., Radding, C.M. & Ward, D.C. (1995). *Proc Natl Acad Sci U S A*, 92, 2298-302.
Hakem, R., de la Pompa, J.L., Sirard, C., Mo, R., Woo, M., Hakem, A., Wakeham, A., Potter, J., Reitmair, A., Billia, F., Firpo, E., Hui, C.C., Roberts, J., Rossant, J. & Mak, T.W. (1996). *Cell*, 85, 1009-23.
Husain, A., He, G., Venkatraman, E.S. & Spriggs, D.R. (1998). *Cancer Res*, 58, 1120-3.
Johnson, R.D., Liu, N. & Jasin, M. (1999). *Nature*, 401, 397-9.
Katagiri, T., Saito, H., Shinohara, A., Ogawa, H., Kamada, N., Nakamura, Y. & Miki, Y. (1998). *Genes Chromosomes Cancer*, 21, 217-22.
Lee, H., Trainer, A.H., Friedman, L.S., Thistlethwaite, F.C., Evans, M.J., Ponder, B.A. & Venkitaraman, A.R. (1999). *Mol Cell*, 4, 1-10.
Lim, D.S. & Hasty, P. (1996). *Mol Cell Biol*, 16, 7133-43.
Liu, C.Y., Flesken-Nikitin, A., Li, S., Zeng, Y. & Lee, W.H. (1996). *Genes Dev*, 10, 1835-43.
Ludwig, T., Chapman, D.L., Papaioannou, V.E. & Efstratiadis, A. (1997). *Genes Dev*, 11, 1226-41.
Marmorstein, L.Y., Ouchi, T. & Aaronson, S.A. (1998). *Proc Natl Acad Sci U S A*, 95, 13869-74.
Mizuta, R., LaSalle, J.M., Cheng, H.L., Shinohara, A., Ogawa, H., Copeland, N., Jenkins, N.A., Lalande, M. & Alt, F.W. (1997). *Proc Natl Acad Sci U S A*, 94, 6927-32.
Morimatsu, M., Donoho, G. & Hasty, P. (1998). *Cancer Research*, 58, 000-000.
Moynahan, M.E., Chiu, J.W., Koller, B.H. & Jasin, M. (1999). *Mol Cell*, 4, 511-8.
Patel KJ, Y.V., Lee HS, Corcoran A, Thistletwaite FC, Evans MJ, Colledge WH, Friedman LS, Ponder BAJ, Venkitaraman AR. (1998). *Molecular Cell*, 1(3), 347-357.
Pierce, A.J., Johnson, R.D., Thompson, L.H. & Jasin, M. (1999). *Genes Dev*, 13, 2633-8.
Sharan, S.K., Morimatsu, M., Albrecht, U., Lim, D.S., Regel, E., Dinh, C., Sands, A., Eichele, G., Hasty, P. & Bradley, A. (1997). *Nature*, 386, 804-10.
Shen, S.X., Weaver, Z., Xu, X., Li, C., Weinstein, M., Chen, L., Guan, X.Y., Ried, T. & Deng, C.X. (1998). *Oncogene*, 17, 3115-24.
Sonoda, E., Sasaki, M.S., Buerstedde, J.M., Bezzubova, O., Shinohara, A., Ogawa, H., Takata, M., Yamaguchi-Iwai, Y. & Takeda, S. (1998). *Embo J*, 17, 598-608.
Suzuki, A., de la Pompa, J.L., Hakem, R., Elia, A., Yoshida, R., Mo, R., Nishina, H., Chuang, T., Wakeham, A., Itie, A., Koo, W., Billia, P., Ho, A., Fukumoto, M., Hui, C.C. & Mak, T.W. (1997). *Genes Dev*, 11, 1242-52.
Takata, M., Sasaki, M.S., Sonoda, E., Morrison, C., Hashimoto, M., Utsumi, H., Yamaguchi-Iwai, Y., Shinohara, A. & Takeda, S. (1998). *Embo J*, 17, 5497-508.
Tebbs, R.S., Zhao, Y., Tucker, J.D., Scheerer, J.B., Siciliano, M.J., Hwang, M., Liu, N., Legerski, R.J. & Thompson, L.H. (1995). *Proc Natl Acad Sci U S A*, 92, 6354-8.
Thompson, L.H. (1996). *Mutat Res*, 363, 77-88.
Todaro, G. & Green, H. (1963). *Journal of Cell Biology*, 299-313.
Tsuzuki, T., Fujii, Y., Sakumi, K., Tominaga, Y., Nakao, K., Sekiguchi, M., Matsushiro, A., Yoshimura, Y. & MoritaT. (1996). *Proc Natl Acad Sci U S A*, 93, 6236-40.
Wong, A.K.C., Pero, R., Ormonde, P.A., Tavtigian, S.V. & Bartel, P.L. (1997). *J Biol Chem*, 272, 31941-4.
Xiao, Y. & Weaver, D.T. (1997). *Nucleic Acids Res*, 25, 2985-91.
Yamaguchi-Iwai, Y., Sonoda, E., Sasaki, M.S., Morrison, C., Haraguchi, T., Hiraoka, Y., Yamashita, Y.M., Yagi, T., Takata, M., Price, C., Kakazu, N. & Takeda, S. (1999). *Embo J*, 18, 6619-6629.
Yuan, S.S., Lee, S.Y., Chen, G., Song, M., Tomlinson, G.E. & Lee, E.Y. (1999). *Cancer Res*, 59, 3547-51.

Appendices: N/A