Disruption of *Brca2* increases the spontaneous mutation rate *in vivo*: synergism with ionizing radiation

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The breast cancer predisposition gene *BRCA2* encodes a protein involved in the repair of DNA double-strand breaks, which arise spontaneously and following exposure to ionizing radiation (IR). To develop a mouse model that examines the effect of *BRCA2* mutation and IR exposure on *in vivo* somatic mutation acquisition, we crossed mice with targeted disruption of *Brca2* with a *LacZ* transgenic mutation reporter strain. Loss of both wild-type *Brca2* alleles caused a 2.3-fold increase, equivalent to an extra 100 mutations per cell, in the *in vivo* acquisition of spontaneous somatic mutation by 2 weeks gestation. IR (4 Gy) had a disproportionate effect on animals homozygous for *Brca2* disruption, inducing 3.4-fold more mutations compared with wild-type animals. These data provide the first evidence that loss of *Brca2* increases *in vivo* somatic mutation acquisition and synergizes with IR exposure, with potential attendant implications for mammographic screening and therapeutic IR in mutation carriers.

INTRODUCTION

Human cancers require the acquisition of a number of somatic mutations in key oncogenes and tumour suppressors to achieve a malignant phenotype (Lengauer *et al.*, 1998); abnormal DNA repair and consequent genomic instability can accelerate this process (Loeb, 1991). Germline mutation in *BRCA2* considerably increases the risk of breast, ovarian and other cancers (Rahman and Stratton, 1998), and the *BRCA2* protein has been implicated in the repair of DNA double-strand breaks (DSBs) by homologous recombination (Kerr and Ashworth, 2001). Cell-based assays have shown that *BRCA2* is required for the error-free repair of DNA

DSBs by gene conversion (Moynahan *et al.*, 2001; Tutt *et al.*, 2001). Loss of *BRCA2* appears to result in the use of an alternative error-prone single-strand annealing (SSA) pathway (Karran, 2000; Tutt *et al.*, 2001). However, it is not yet known whether these *in vitro* results, suggesting an increase in mutation frequency caused by loss of *BRCA2*, are applicable *in vivo*. DNA DSBs arise spontaneously during DNA replication and are also induced by ionizing radiation (IR). Carriers of *BRCA2* mutations are subjected to IR during both screening mammography and adjuvant radiotherapy for breast cancer, raising concern about excessively increased mutation induction by IR (Bennett, 1999; Friedenson, 2001). Here we use *in vivo* mutation reporter mice (Boerrigter *et al.*, 1995) carrying a targeted disruption of *Brca2* (Connor *et al.*, 1997) to model this question. We found that *Brca2* heterozygotes undergo no elevated spontaneous or IR-induced mutation frequency compared with wild-type animals. However, mice homozygous for *Brca2* disruption had a substantial increase in spontaneous mutation frequency, which is considerably exacerbated by IR.

RESULTS

Somatic mutation reporter mouse model

We have described previously a mouse model to examine the *in vivo* phenotype of a truncating mutation (*Brca2*^{Tr2014}, hereafter referred to as *Brca2*^{Tr}; Connor *et al.*, 1997), which mimics cancer-predisposing mutations in humans. This mutation truncates the protein just before the final BRC repeat in exon 11

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Fig. 1. Schematic diagram of a *LacZ* plasmid transgenic mouse model for analysis of the effect of *Brca2* loss of function on *in vivo* somatic mutation frequency. Mice are shown of the three potential *Brca2* genotypes either treated or untreated with IR. These mice are also homozygous for two pUR288 *LacZ* plasmid concatamer chromosome integration sites on chromosomes 3 and 4 (marked as black chromosomes). Each concatamer contains ~10 copies of pUR288. The ampicillin^r gene is shown as a blue box, the bacterial origin of replication as a yellow box and the *LacZ* reporter gene in red. *Hind*III sites between the plasmids are marked (H). Flanking regions of genomic DNA are represented by the green line. Individual linear plasmids are shown after release from genomic DNA by *Hind*III digestion. The central plasmid has a point mutation within *LacZ* represented by a green dot. The plasmid on the right-hand side has suffered a deletion or genomic rearrangement removing the 3' *LacZ* sequence with a break point extending into flanking genomic DNA. This removes the original *Hind*III site and thus *Hind*III digestion recovers a *LacZ* mutant plasmid of different size to the wild type. Single reporter plasmids are recovered from genomic DNA, transformed into *E. coli* and mutation frequency determined by comparing colony numbers on mutant selection (P-gal) and non-selective rescue efficiency (X-gal) plates.

at a similar site to the common Ashkenazi 6174delT *BRCA2* truncation. Mice homozygous for the *Brca2^{Tr}* mutation are prone to tumorigenesis. We used *in vitro* cell culture systems to show an increase in error-prone DNA DSB repair after disruption of *Brca2* (Tutt *et al.*, 2001). To establish whether this defect seen *in vitro* is associated with increased somatic mutation *in vivo*, we have crossed mice heterozygous for the *Brca2^{Tr}* mutation with a *LacZ*-plasmid transgenic mutation reporter mouse (Boerriqter *et al.*, 1995; Figure 1). This system can detect spontaneous and

IR-induced somatic mutations including the large genomic deletions, insertions or translocations (Gossen *et al.*, 1995) predicted to occur from DSB misrepair using non-homologous end-joining (NHEJ) or SSA (Jasin, 2000). We generated *Brca2^{Tr/Wt}* mice that were additionally homozygous for the pUR288 transgene mutation reporter concatamer at each of two chromosomal loci, then intercrossed these mice to examine the effect of the truncating mutation in *Brca2* on spontaneous somatic mutation frequency using the *LacZ* reporter genes (Figure 1).

Effect of loss of *Brca2* on spontaneous *in vivo* somatic mutation acquisition

Most *Brca2*^{Tr/Tr} mice die at birth (Connor *et al.*, 1997), so to generate sufficient samples for analysis we examined the mutation frequency in littermate *Brca2*^{Tr/Wt}, *Brca2*^{Tr/Tr} and wild-type embryos at day 14.5 gestation (Figure 2A). We found that loss of both wild-type *Brca2* alleles causes a rapid and significant increase in spontaneous somatic mutation frequency at this embryonic stage: *Brca2*^{Tr/Tr}, $8.9 \pm 0.4 \times 10^{-5}$; *Brca2*^{Wt/Wt}, $3.9 \pm 0.6 \times 10^{-5}$ ($P = 0.0018$). This is equivalent to the somatic mutational load acquired by a 2-year-old wild-type mouse (Dolle *et al.*, 1997). Based on a *LacZ* reporter gene target size of 3 kb and a genome size of 6×10^9 bp, this equates to an additional 100 mutations in every cell after only 2 weeks gestation. As unrepaired DNA DSBs within the reporter gene will not be recovered from genomic DNA by this assay, the observed increase in mutation frequency therefore represents completed DNA repair by an error-prone mechanism. Heterozygosity for this *Brca2* mutation has no effect on somatic mutation frequency: *Brca2*^{Tr/Wt}, $3.34 \pm 0.3 \times 10^{-5}$; *Brca2*^{Wt/Wt}, $3.9 \pm 0.6 \times 10^{-5}$ ($P = 0.42$). This suggests that at this embryonic stage, at least, there is no mutator phenotype associated with loss of one wild-type copy of *Brca2* and that loss of the second wild-type allele drives the rapid acquisition of somatic mutation throughout the genome.

Characterization of *in vivo* mutations induced by *Brca2* mutation

A high proportion of the infrequent spontaneous somatic mutations in wild-type *LacZ*-plasmid transgenic reporter mice arise from deletions and genomic rearrangements rather than point mutations (Dolle *et al.*, 2000). This type of mutation can arise from DNA DSB misrepair events by error-prone NHEJ and SSA repair pathways (Dolle *et al.*, 2000). IR also increases this class of mutation (Gossen *et al.*, 1995). We have shown that loss of wild-type *Brca2* results in increased use of error-prone SSA to repair a chromosomal DSB in a cell culture system (Tutt *et al.*, 2001). Therefore, we asked whether somatic mutations induced by loss of *Brca2* *in vivo* are of the deletion/rearrangement type by analysing mutations present in the *LacZ* reporter plasmids recovered in the experiments described above (Figure 2A and B). Deletions, insertions and translocations, such as those induced by IR, cause restriction fragment size changes (Gossen *et al.*, 1995), whereas point mutations, such as those induced by ethyl nitrosourea, do not (Dolle *et al.*, 1996). We found no significant

Fig. 2. Analysis of the effect of loss of *Brca2* on *in vivo* somatic mutation frequency and mutation type. (A) Scatter plot showing the somatic *LacZ* mutation frequency in the pUR288 reporter in *Brca2*^{Wt/Wt} ($n = 4$), *Brca2*^{Wt/Tr} ($n = 5$) and *Brca2*^{Tr/Tr} ($n = 3$) day E14.5 embryos. Data points represent individual animals each based on $>10^5$ recovered reporter plasmids. Horizontal bars represent the mean. (B) *AvaI*–*PstI* restriction fragments from recovered *LacZ* mutant pUR288 plasmids run as an example of Size-change and No-change mutants compared with the wild-type pUR288 restriction digest pattern. (C) The bar chart shows the frequency of no-change, size-change and mouse genome rearrangement *LacZ* mutants in *Brca2*^{Wt/Wt} ($n = 4$), *Brca2*^{Wt/Tr} ($n = 5$) and *Brca2*^{Tr/Tr} ($n = 3$) day E14.5 embryos. Error bars represent the standard error of the mean (SEM).

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difference ($P = 0.75$) between the proportion of reporter plasmid mutants with detectable size changes recovered from *Brca2*^{Tr/Tr} embryos (76%; 51/67) compared with *Brca2*^{Wt/Wt} embryos (71%; 22/31) and *Brca2*^{Wt/Tr} embryos (78%; 43/55). We then used a PCR assay to distinguish size change mutations due to gross deletions/rearrangements involving flanking mouse genomic DNA from deletions internal to *LacZ* (Dolle *et al.*, 2000). Interestingly, deletions/rearrangements involving the mouse genome are more common in *Brca2*^{Tr/Tr} recovered mutants (96%; 49/51) than in *Brca2*^{Wt/Wt} and *Brca2*^{Wt/Tr} control mutants (78%; 51/65) ($P = 0.0065$). The character of these mutations is compatible with use of error-prone repair of DNA DSBs and shows that tissues lacking *Brca2* acquire somatic mutation via this mechanism at a greater rate than in tissues with wild-type *Brca2* (Figure 2C).

In vivo somatic mutation induction by exposure to IR

IR induces, amongst other lesions, DNA DSBs and is recognized as being carcinogenic to breast tissue (Goss and Sierra, 1998). An increase in error-prone repair of IR-induced DNA DSBs due to abnormal function of *BRCA2* would be predicted to increase radiation-induced mutagenesis. This is of clinical interest, as carriers of *BRCA2* are exposed to IR during mammography and adjuvant radiation therapy associated with breast conserving surgery for ductal carcinoma *in situ* and invasive breast cancer. To examine the effect of *Brca2* mutation on IR-induced somatic mutations, we compared littermate *Brca2*^{Wt/Wt}, *Brca2*^{Wt/Tr} and *Brca2*^{Tr/Tr} embryo sets irradiated with 4 Gy X-rays 24 h prior to embryo harvest on day 14.5 gestation. We found that 4 Gy induces a significant increase over the spontaneous mutation frequency in wild-type ($10.6 \pm 1 \times 10^{-5}$ versus $3.9 \pm 0.6 \times 10^{-5}$; $P = 0.0013$), *Brca2*^{Wt/Tr} ($10.6 \pm 0.6 \times 10^{-5}$ versus $3.34 \pm 0.3 \times 10^{-5}$; $P < 0.0001$) and *Brca2*^{Tr/Tr} embryos ($31.6 \pm 2.9 \times 10^{-5}$ versus $8.93 \pm 0.4 \times 10^{-5}$; $P = 0.0004$; Figure 3A). The induction of mutations (in absolute numbers) above the spontaneous background is therefore ~3-fold greater in *Brca2*^{Tr/Tr} embryos than in the wild type ($22.7 \pm 2.5 \times 10^{-5}$ versus $6.7 \pm 1 \times 10^{-5}$; $P < 0.0001$; Figure 3B). We estimate that loss of *Brca2* *in vivo* results in an extra 320 somatic mutations in every cell as a result of misrepair of 4 Gy IR-induced damage. We also asked whether any subtle mutator phenotype associated with haploinsufficiency due to mutation in one *Brca2* allele, not revealed in untreated *Brca2*^{Wt/Tr} embryos, might become apparent under the stress of DNA damage induced by 4 Gy of IR. In fact, we found no increase in radiation-induced mutagenesis in *Brca2*^{Wt/Tr} embryos compared with the wild type ($7.2 \pm 0.8 \times 10^{-5}$ versus $6.7 \pm 1 \times 10^{-5}$; $P = 0.68$). This further supports the contention that mutation or loss of both copies of *Brca2* is required before a mutator phenotype is acquired, after which *Brca2*-deficient tissues then suffer a greater number of somatic mutations per unit dose of IR.

Characterization of IR-induced in vivo mutations

Characterization of the size of recovered mutant plasmids reveals that increased mutation induction by IR in *Brca2*^{Tr/Tr} embryos is predominantly via a deletion/rearrangement mechanism (86%; 32/37). We found that although the mutation spectrum is similar to that in wild-type embryos (76%; 16/21) and *Brca2*^{Wt/Tr} embryos (74%; 32/43; $P = 0.39$), the frequency at which these

Fig. 3. Analysis of the effect of IR on *in vivo* somatic mutation frequency and mutation type. (A) The bar chart shows the somatic mutation frequency in the *LacZ* pUR288 reporter in 4 Gy irradiated *Brca2*^{Wt/Wt} ($n = 8$), *Brca2*^{Wt/Tr} ($n = 9$) and *Brca2*^{Tr/Tr} ($n = 6$) day E14.5 embryos compared with the unirradiated embryos of the same genotype. Error bars represent SEM. (B) Scatter plot showing the frequency of somatic *LacZ* mutation in the pUR288 reporter induced over the spontaneous background in 4 Gy-irradiated *Brca2*^{Wt/Wt} ($n = 8$), *Brca2*^{Wt/Tr} ($n = 9$) and *Brca2*^{Tr/Tr} ($n = 6$) day E14.5 embryos. (C) The bar chart shows the frequency of size-change and no-change *LacZ* mutants in 4 Gy-irradiated *Brca2*^{Wt/Wt} ($n = 8$), *Brca2*^{Wt/Tr} ($n = 9$) and *Brca2*^{Tr/Tr} ($n = 6$) day E14.5 embryos. Error bars represent the SEM.

somatic mutations occur is lower than in *Brca2*^{Tr/Tr} embryos (Figure 3C).

DISCUSSION

Homozygous mutation in *Brca2* has been shown in cell-based *in vitro* assays to decrease error-free DNA DSB repair by homology-directed gene conversion (Moynahan *et al.*, 2001) and increase error-prone homology-directed repair (Tutt *et al.*, 2001). Here we have used an *in vivo* mouse mutation reporter model, carrying a *Brca2*-truncating mutation, similar to those found in humans, to show that loss of both wild-type *Brca2* alleles causes rapid spontaneous acquisition of genome-wide somatic mutations. These are predominantly deletion/rearrangement mutations consistent with misrepair of DNA DSBs arising during DNA replication. We have also shown that the loss of both wild-type *Brca2* alleles from tissues *in vivo* causes an increase in the number of somatic mutations induced by a given dose of IR. Loss of the remaining wild-type *BRCA2* allele is presumed to be a very early event in familial breast cancer development and is found to occur in pre-invasive cancer, and even in histologically normal luminal epithelial tissue from carriers of the *BRCA2* mutation (S. Lakhani, personal communication). Our data suggest that, if these results are applicable to human breast epithelial cells, IR may induce genome-wide somatic mutations at a disproportionately high rate in these *BRCA2*-deficient tissues in carriers of the *BRCA2* mutation, thus providing the genomic diversity for selection of a more malignant phenotype. We also show that heterozygous tissues retaining a functional *Brca2* allele do not acquire radiation-induced somatic mutation more frequently than the wild type, suggesting that the 'normal' tissues of a human *BRCA2* mutation carrier may acquire IR-induced somatic mutations no faster than a non-carrier. However, these tissues may remain at risk as a result of random inactivation of the single remaining wild-type *BRCA2* allele by IR and consequent induction of a mutator phenotype.

There are two sources of iatrogenic IR to which carriers of *BRCA2* mutation are commonly exposed: screening mammography and radiation therapy used as an adjuvant to breast 'wide local excision'. Annual mammography is currently advised from the age of 25 or 10 years before the index case (Burke *et al.*, 1997), and concern has been raised about the effect this may have on somatic mutation induction (Bennett, 1999; Friedenson, 2001). In the absence of alternatives to mammographic screening, the benefits of early detection of an occult breast cancer probably outweigh the increased risk of radiation-induced mutagenesis. Alternative MRI screening strategies, which avoid use of IR, are currently being evaluated (Tilanus-Linthorst *et al.*, 2000). If these prove to be of equivalent screening efficacy to mammography, our data showing increased IR mutagenesis in *Brca2*-deficient tissue in a mouse model provide mechanistic evidence to support their use in preference to mammography for *BRCA2* mutation carriers.

Adjuvant radiation therapy is delivered to a much higher dose than that used for mammography. The increased acquisition of radiation-induced somatic mutation by a cell, which is also inactivated by this irradiation, is clearly of no concern. This phenomenon leads to a dose threshold, above which further increases in radiation dose are associated with reducing risk cancer induction (Hall, 2000). The nature of this dose-response

curve for tissues lacking *BRCA2* remains to be established. It is likely that *BRCA2*-deficient tissues within the treated breast that receive the full dose (50 Gy) will be protected by this effect. The untreated breast is exposed to a lower dose of scattered irradiation (2–6 Gy; Tercilla *et al.*, 1989) and, consequently, *BRCA2*-deficient cells that have acquired multiple IR-induced mutations may survive to form tumours. Studying the effect of scattered irradiation on long-term contralateral breast cancer risk in *BRCA2* mutation carriers requires a long follow-up and is currently unknown. If found to be significant, radiotherapy techniques that minimize the radiation dose to the untreated breast or the selective use of more extensive resection or mastectomy without radiation therapy may be preferred by some women. Our results, obtained in an embryonic mouse model of *BRCA2* mutation showing a 3-fold increase in *in vivo* radiation mutagenesis, suggest that these issues should be further investigated in both tissue-specific *Brca2* 'knockout' mouse models and in the clinic.

METHODS

Generation of *Brca2*^{Wt/Tr2014} LacZ pUR288 mutation reporter strain. C57BL/6-TgN(LacZpl)60vii mice were obtained from the Jackson Laboratory and were crossed with C57BL/10 *Brca2*^{Wt/Tr2014} mice (Connor *et al.*, 1997). These mice were then back-crossed to the C57BL/6-TgN(LacZpl)60vii mutation reporter strain and homozygosity for both pUR288 concatamer integration sites was confirmed by test breeding to wild type and genotyping with primer sets specific for the two pUR288 cloned integration sites. For generation of embryos, *Brca2*^{Wt/Tr2014} C57BL/6-TgN(LacZpl)60vii mice were intercrossed and the females killed on day E14.5 gestation measured from the 'plug' date.

Mouse irradiation and embryo harvest. For irradiation experiments, female mice received 4 Gy whole-body irradiation on day E13.5 gestation from a Pantak 250 kV X-ray set at 90 cm focus skin distance at a dose rate of 0.75 Gy/min. Embryos were harvested 24 h later. For all experiments, embryos were harvested on day E14.5, washed in phosphate-buffered saline at 4°C and were snap frozen on dry ice and stored at –80°C. Genomic DNA was prepared by phenol–chloroform extraction.

Plasmid rescue and mutant frequency determination. Complete protocols for this system have been described elsewhere (Vijg, 1996). Briefly, between 10 and 20 µg of genomic DNA were digested with *HindIII* for 1 h in the presence of magnetic beads (Dyna) pre-coated with lacI–lacZ fusion protein. The beads were washed three times to remove the unbound mouse genomic DNA. Plasmids were subsequently eluted from the beads with isopropyl-D-thiogalactoside. After circularization with T4 DNA ligase, plasmids were used to electrotransform *Escherichia coli* C (Δ LacZ, *galE*[–]) cells by electroporation. Transformations were plated on titration plates containing X-gal and LacZ mutant selection plates containing P-gal. Mutant frequencies were determined as the ratio of the number of colonies on the selective plates versus titration plates. Each determination point is based on at least 100 000 recovered plasmids. The *in vitro* background mutant frequency of this system is $\sim 1 \times 10^{-5}$ (Dolle *et al.*, 1999). Identification of size-change mutations was performed by digestion of rescued LacZ mutant pUR288 plasmids with *AvaI* and *PstI*. To screen for size-change mutants with a breakpoint in the mouse genome, a PCR amplification was performed by using a forward primer specific for the last 18 bp

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of *lacZ* before the *HindIII* site (pUR3285-F) in combination with a reverse primer after the *HindIII* site (pUR3421-R; Dolle et al., 2000). This procedure results in a 137 bp product only when both breakpoints are in the *lacZ* gene. As a positive control, a primer set (pUR4071-F and pUR4181-R) specific for the *Amp^r* gene was included in the same reaction.

Statistical analyses. Mutation frequencies were compared using a two-tailed unpaired Student's *t*-test. Proportions of recovered reporter plasmids containing restriction fragment size changes or with mouse genome rearrangements were compared using two-sided analysis of contingency tables by Fisher's exact test or the Chi squared test. All statistical comparisons were performed using Graphpad Prism software.

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