



Gene replacement with the human *BRCA1* locus: tissue specific expression and rescue of embryonic lethality in mice

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We have generated transgenic mice that harbor a 140 kb genomic fragment of the human *BRCA1* locus (TgN·*BRCA1*^{GEN}). We find that the transgene directs appropriate expression of human *BRCA1* transcripts in multiple mouse tissues, and that human *BRCA1* protein is expressed and stabilized following exposure to DNA damage. Such mice are completely normal, with no overt signs of *BRCA1* toxicity commonly observed when *BRCA1* is expressed from heterologous promoters. Most importantly, however, the transgene rescues the otherwise lethal phenotype associated with the targeted hypomorphic allele (*Brcal*^{Δex15A}). *Brcal*^{-/-}; TgN·*BRCA1*^{GEN} bigenic animals develop normally and can be maintained as a distinct line. These results show that a 140 kb fragment of chromosome 17 contains all elements necessary for the correct expression, localization, and function of the *BRCA1* protein. Further, the model provides evidence that function and regulation of the human *BRCA1* gene can be studied and manipulated in a genetically tractable mammalian system. *Oncogene* (2000) 19, 4085–4090.

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Introduction

Inherited mutations within *BRCA1* account for 1–4% of breast and 4–5% of ovarian cancers (Biesecker, 1993; Whittemore *et al.*, 1997). *BRCA1* kindreds have increased rates of other cancers as well, and carriers show lifetime cancer risks of 60–80%. Carrier frequencies have been calculated at one in 800 in the US population, and the number is considerably higher in women of Ashkenazi Jewish descent (Struwing *et al.*, 1997). Most mutations lead to premature termination of the nascent protein, but model systems to study these mutations have been slow to develop.

Brcal-null mice succumb to embryonic lethality early in life (E6.5–9) (Gowen *et al.*, 1996; Hakem *et al.*, 1997; Ludwig *et al.*, 1997), an effect which is at least partially dependent on *Trp53* (p53) gene status (Cressman *et al.*, 1999b; Hakem *et al.*, 1997; Ludwig *et al.*, 1997). In keeping with the high rate of *TP53* mutation in *BRCA1*-derived human cancers (Crook *et al.*, 1998;

Phillips *et al.*, 1999), mice with mutations in *Brcal* develop tumors which show allelic loss of *Trp53* (Xu *et al.*, 1999), and bigenic mice with mutations in both *Brcal*^{+/-} and *Trp53*^{-/-} are specifically prone to the development of breast cancers (Cressman *et al.*, 1999b; Xu *et al.*, 1999). Paradoxically, human cells that lack *BRCA1* are very difficult to obtain and doubly mutant mouse cells grow poorly due to high rates of chromosomal loss associated with defective G2 checkpoint control (Xu *et al.*, 1999). Mouse cells lacking functional *Brcal* are also more sensitive to ionizing radiation and oxidative stress (Shen *et al.*, 1998). One surprising result was that virtually all tumor-derived cell lines appeared to produce some *BRCA1* (Lane *et al.*, 1995; Scully *et al.*, 1997a; Wilson *et al.*, 1997). Thus, *BRCA1* displays some features which are atypical for tumor suppressor genes and there remains a significant need for informative and genetically tractable models of *BRCA1* function.

To date, there have been no effective strategies to replace *BRCA1* functionality *in vivo*. The process is complicated by cellular senescence or apoptosis in many cells engineered to overexpress the *BRCA1* cDNA from heterologous promoters (Aprelikova *et al.*, 1999; Shao *et al.*, 1996; Wilson *et al.*, 1997; Zhang *et al.*, 1998). Since mouse embryos deficient in *Brcal* die early in development, a straightforward assay of function is to analyse human *BRCA1* gene replacement vectors for their ability to rescue *Brcal*-deficient mouse embryos. To test this notion, we generated mice that carry a 140 kb fragment of the *BRCA1* locus (TgN·*BRCA1*^{GEN}) and find the transgene rescues the otherwise lethal phenotype observed in the homozygous *Brcal*^{-/-} mice. We isolated human *BRCA1* protein from these lines and found a correlation between expression level and rescue. Furthermore, the protein was correctly translated and stabilized following exposure to DNA damage. From these results, it is apparent that significant conservation of function exists between human and mouse genes. This is notable since the mature proteins are only 60% identical at the amino acid level (Lane *et al.*, 1995). These results provide strong evidence that hypotheses regarding human *BRCA1* function can be evaluated in mice. Candidate *BRCA1* replacement vectors can be tested for their ability to support embryonic or adult physiological processes, and more accurate models of human disease alleles can be studied. TgN·*BRCA1* mice can be manipulated experimentally (hormones, drugs, etc.) and genetically without the confounding effects of genetic background or the ethical issues associated with manipulating genes in humans.

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Results

Placement of a *Brca*^{AexIIISA} allele into an FVB/N genetic background creates a line of syngeneic mice suitable for analysis of *BRCA1* transgenes

A hypomorphic allele of murine *Brca1* (*Brca1*^{AexIIISA/129}) (Figure 1a) was generated through homologous recombination in embryonic stem (ES) cells derived from a 129^{SvEv} genetic background (C Deng and P Leder, unpublished). To remove issues of multiple genetic backgrounds from the analysis of *BRCA1* transgenic mice, the *Brca1*^{AexIIISA} mutation was backcrossed into FVB/N mice for 12 generations to produce a new line, *Brca1*^{AexIIISA^{FVB}}. As described for various *Brca1*-null alleles made by gene targeting in 129 backgrounds (Gowen *et al.*, 1996; Hakem *et al.*, 1997; Liu *et al.*, 1996; Ludwig *et al.*, 1997; Shen *et al.*, 1998), the majority of *Brca1*^{AexIIISA^{FVB}} homozygous (*Brca1*^{-/-}) embryos die before embryonic day 9 and never produce viable offspring. *Brca1*^{AexIIISA^{FVB}} heterozygotes (*Brca1*^{+/-}) are normal and have no apparent predisposition to breast cancer. Attempts to identify normal transcripts in *Brca1*^{-/-} embryos have failed, indicating that the mutation produces a null (or severely hypomorphic) allele of the *Brca1* gene.

The wild-type human *BRCA1* locus can be introduced into mice as a heritable transgene

In order to test the ability of human *BRCA1* to rescue embryonic lethality in *Brca1*^{-/-} embryos, we made repeated attempts to generate transgenic mice expressing full-length *BRCA1* cDNAs under the control of heterologous promoters. The promoter-cDNA constructs included well-characterized promoters derived

from the chick β -actin gene ($n=80$ embryos injected), cytomegalovirus ($n=40$), and the mouse mammary tumor virus ($n=160$). Pronuclear microinjection of these constructs resulted in very low yields of surviving founders, and none of these were shown to express human *BRCA1* mRNA. These results provide evidence suggestive that mis-expression of *BRCA1* has a phenotypic consequence (cellular arrest or lethality) that is not well tolerated during embryonic development.

To test whether genomic sequences from the human locus would mediate more appropriate expression in mice, we created transgenic mice carrying the entire human *BRCA1* locus derived from PAC103014 (Brown *et al.*, 1995). The fragment contained all 25 exons of the *BRCA1* gene (see Figure 1b), as well as elements of two flanking genes, *NBR2* and *ETA38*, as confirmed by exon specific probes (not shown). Following a single round of injection ($n=30$ embryos), five founders (henceforth referred to as TgN·*BRCA1* lines A, B, C, D or E) passed the transgene to offspring. The resulting lines could be mated to homozygosity.

The human *BRCA1* locus is able to rescue developmental arrest in *Brca1*^{-/-} mice

TgN·*BRCA1* founders were mated with *Brca1*^{+/-} mice (*Brca1*^{AexIIISA^{FVB}}) to produce pairs of *Brca1*^{+/-}; TgN·*BRCA1* bigenic animals. The bigenic mice were subsequently mated and their offspring were analysed for *Brca1* status. Viable *Brca1*^{-/-} mice were recovered quantitatively from crosses with line B and, to a lesser extent, with line A (Figure 2 and Table 1). In Figure 2, lanes 2, 6 and 12 represent 3-week-old *Brca1*^{-/-} mice rescued by the presence of the *BRCA1* transgene from line B. Subsequently, we established independent lines of *Brca1*^{-/-}; TgN·*BRCA1*-B mice (maintained through the fourth generation). Such mice appear completely normal with respect to development, litter size, longevity and disease-free survival.

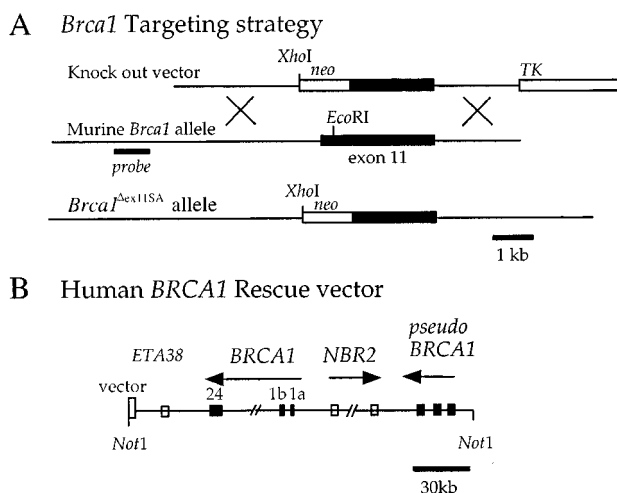


Figure 1 (a) Schematic diagram of the strategy for generating a targeted allele of the endogenous mouse *Brca1* gene. The targeting vector was introduced into ES cells and correctly targeted clones were identified by Southern blot analysis using an external flanking sequence as probe (probe) (Shen *et al.*, 1998). The targeting vector eliminates an *EcoRI* site in exon 11. (b) Schematic diagram of the genomic fragment used for generation of TgN·*BRCA1*^{GEN} transgenic mice. Solid boxes represent exons from *BRCA1* and the *BRCA1* pseudo gene, open boxes represent exons from *NBR2* and *ETA38* which are included in this fragment of DNA. The genomic fragment was isolated from a P-element clone (PAC103014) containing a 140 kb fragment of the *BRCA1* locus (Brown *et al.*, 1995)

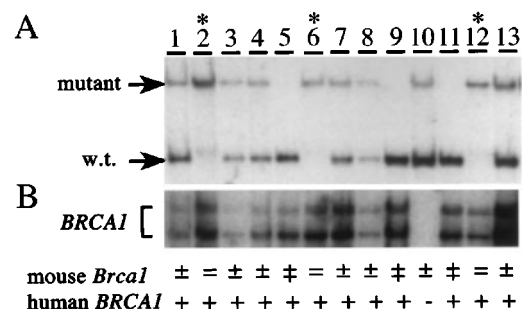


Figure 2 Embryonic lethality in *Brca1*^{-/-} mice rescued by the human *BRCA1* genomic fragment. (a and b) represent Southern blot analysis of *EcoRI* digested tail DNA from 13 littermates (3-week-old) produced from a cross of *Brca1*^{+/-};TgN·*BRCA1*-B^{+/-} bigenic parents. (a) Probed with a fragment of the endogenous murine *Brca1* gene and signals represent unique fragments generated from *Brca1* wild-type (wt) and *Brca1*^{AexIIISA} (mut) alleles. (b) Probed with the entire human *BRCA1* cDNA. The probe did not cross hybridize with mouse DNA (*c.f.* lane 10) and identified several unique genomic bands in all of the TgN·*BRCA1* transgenic lines. Note that lanes 2, 6 and 12 (*) represent viable animals containing no wild-type murine *Brca1* alleles and thus represent viable *Brca1*^{-/-} animals. All such surviving individuals also carry a TgN·*BRCA1*^{GEN} transgene ($n>400$ animals)

Table 1

Cross of double heterozygotes ^a Parental genotype	Brca1 genotype of offspring, observed (expected)			n
	+/+	+/-	-/-	
<i>Brca1</i> ^{+/-} ; BRCA1 ^{GEN} -A	19 (18)	52 (36)	2 (18)	71
<i>Brca1</i> ^{+/-} ; BRCA1 ^{GEN} -B	43 (63)	150 (126)	58 (63)	251
<i>Brca1</i> ^{+/-} ; BRCA1 ^{GEN} -C	8 (18)	62 (35)	0 (18)	70
<i>Brca1</i> ^{+/-} ; BRCA1 ^{GEN} -D	20 (14)	21 (18)	0 (14)	55
<i>Brca1</i> ^{+/-} ; BRCA1 ^{GEN} -E	22 (9)	15 (19)	0 (9)	37
<i>Brca1</i> ^{+/-} ; FVB ^b	65 (68)	205 (135)	0 (68)	270
<i>Brca1</i> ^{-/-} ; BRCA1 ^{GEN} -B ^c	0 (0)	0 (0)	44 (44)	44

^aBigenic parents doubly heterozygous for one of the TgN-BRCA^{GEN} integrations (A, B, C, D or E) and the targeted *Brca1*^{Aex115A/FVB} allele, were mated. One to two week old pups from the resulting litters were tested for the wild-type *Brca1* allele, and for the TgN-BRCA1^{GEN} transgene (not shown), by Southern analysis. ^bMatings between *Brca1*^{+/-} heterozygotes that did not carry a copy of the TgN-BRCA1^{GEN} transgene never produced viable *Brca1*^{-/-} offspring. ^cMatings between four pairs of bigenic *Brca1*^{-/-} homozygotes that carried the TgN-BRCA1^{GEN} line-B insertion resulted in litters of normal size

Brca1^{-/-} mice were never recovered in the absence of the human *BRCA1* transgene ($n > 270$) (Table 1) or from crosses with lines C, D and E. Analyses of offspring from additional *Brca1*^{+/-} matings provided evidence that a functional *BRCA1* allele is absolutely required for embryonic development of animals with an otherwise wild-type genotype ($n > 400$).

Studies of offspring from all *Brca1*^{+/-}; TgN-BRCA1 lines demonstrated that TgN-BRCA1-B and TgN-BRCA1-A lines were both capable of directing rescue of *Brca1*^{-/-} mice (Table 1). Rescue by transgenic line A was significantly less effective than for line B, indicating that line A mediated rescue with reduced penetrance. However, the fact that two independent transgene integrations mediated genetic rescue provides evidence that rescue was achieved through the actions of the inserted DNA and was not an artifact of the integration site. Because the penetrance of line-A mediated rescue was low, and lines C, D and E did not rescue, we hypothesized that these lines expressed either defective transcripts, or inappropriate levels, of *BRCA1* as compared to line-B. To look at this more closely, we isolated protein from tissues of several TgN-BRCA1 lines and examined the expression of human *BRCA1* protein.

Human *BRCA1* protein, is expressed in TgN-BRCA1^{GEN} transgenic mice and responds to ionizing radiation

Proteins extracted from the testis were analysed by immuno-precipitation followed by immuno-blotting with species-specific anti-human *BRCA1* antibodies (Figure 3a). Testis is a site of significant *BRCA1* expression (Lane et al., 1995; Scully et al., 1997b) due to a presumed role for the protein in homologous strand exchange (Snouwaert et al., 1999; Zabludoff et al., 1996). Antibodies were the generous gift of Drs C Wilson and D Slamon (UCLA School of Medicine, Los Angeles, CA, USA) and have been described elsewhere (Wilson et al., 1997, 1999). None of the antibodies used in this experiment cross-reacted with extracts derived from mice. Detection of protein required immuno-precipitation from 500 μ g of tissue due to low levels of protein expression (preliminary data not shown). The protein migrated as a doublet at 230–240 kD with a smaller band (approximately 98 kD) visible in transgenic lines A and B. Although the intensity varied considerably between individual lines, the 230–240 kD doublet was detected in all four

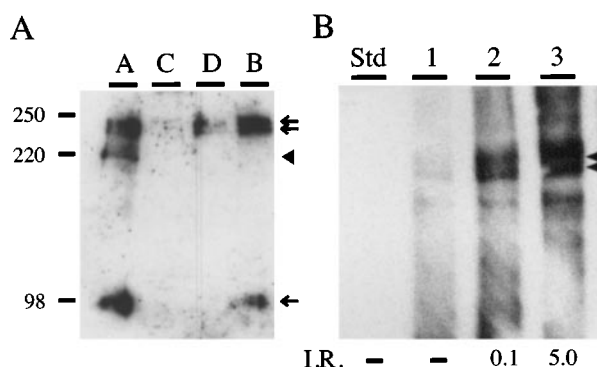


Figure 3 Human *BRCA1* protein is expressed in TgN-BRCA1 transgenic mice. (a) Extracts of testis were prepared from the indicated TgN-BRCA1 transgenic lines (A, C, D and B). NP40 extracts (500 μ g total protein) were prepared as described (Scully et al., 1997a) and subjected to immuno-precipitation with a rabbit anti-human *BRCA1* polyclonal antibody (115), followed by SDS-PAGE and immuno-blotting of precipitated proteins with an anti-human *BRCA1* monoclonal antibody (SD118). Small arrows indicate immuno-reactive species in the TgN-BRCA1-B extract which correspond to bands identified on extracts of human cells (not shown). The arrowhead identifies a unique band observed only in transgenic line A. The size markers indicate the migration (relative kD) of size standards included on the gel. (b) Testicular tissue from transgenic line B was placed in tissue culture media and exposed to the indicated dosages of ionizing-irradiation (IR) calibrated in Gray (0.1 or 5 Gy). Immuno-precipitates were prepared as above. Labeled proteins were visualized with HRP-labeled rabbit anti-mouse IgG followed by a chemiluminescence reaction (Pierce Chemical, Rockford, IL, USA)

TgN-BRCA1 transgenic lines tested (Figure 3a). Lines C and D expressed the 230–240 kD *BRCA1* doublet at relatively low levels compared to lines A and B. This may represent a rationale for the inability of lines C and D to mediate rescue (Table 1). Line A displayed an additional band migrating somewhat below the 230–240 kD doublet. The significance of the additional band in line A extracts is not clear, but might contribute, in part, to the difference in rescue activity between lines A and B. Other tissues were tested as well with similar results, but protein expression levels were generally much lower and detection less reproducible. The expression of full-length protein from four of five transgenic lines provides compelling evidence that pronuclear injection is a reliable mechanism for introducing large fragments of chromosome 17 for functional analysis in mice.

BRCA1 protein has been demonstrated to respond to DNA damaging agents by increased phosphorylation and movement out of nuclear dot structures (Chen *et al.*, 1999; Gowen *et al.*, 1998; Scully *et al.*, 1997a). To test whether proteins expressed from the TgN-*BRCA1*-B transgene respond to DNA damaging agents, we isolated fresh tissues and exposed them to ionizing radiation for various time periods. Tissues were then cultured for 1 h at 37°C. Proteins were extracted as described above. As demonstrated for testicular tissue, the 230–240 kD *BRCA1* doublet became more pronounced following exposure to gamma-irradiation (Figure 3b). These results are consistent with a rapid increase in stability of the protein following radiation exposure as has been reported for *BRCA1* in human tissue culture cell lines (Scully *et al.*, 1997a). The results also indicate that the human *BRCA1* protein can interact functionally with the DNA damage response machinery in murine tissues.

Human BRCA1 transcripts are differentially expressed in TgN-BRCA1^{GEN} transgenic mice and mirror the pattern of endogenous Brca1 transcription

We developed a multiplex RNase protection assay (mRPA) that allowed simultaneous measurement of transcripts originating from mouse *Brca1*, the human *BRCA1* transgene, and *L32*, an ubiquitously expressed ribosomal subunit protein (Figure 4). The three riboprobes were mixed (30:30:1 ratio) and hybridized to various RNA extracts. As shown in Figure 4b mRPA detected only human transcripts in extracts

from a human breast cell line (MCF7: lane 3) and only mouse transcripts in a mouse breast cell line (NuMG; lane 4). When the same RNA samples were mixed, both human and mouse transcripts were distinguishable (Figure 4b, lane 5).

When the mRPA assay was used to measure transcripts in tissues from TgN-*BRCA1*-B transgenic animals, human *BRCA1* mRNA was observed to parallel that of the mouse *Brca1* transcript (Figure 4c). RNA isolated from thymus, breast, spleen, kidney and testis all showed significant expression of both human and mouse *BRCA1* genes (Figure 4), while expression was low in muscle (Figure 4c, lane 3). Interestingly, human *BRCA1* transcripts were expressed in a 1:1 ratio with mouse *Brca1* in male tissues (Figure 4c, lanes 6–7), while the human transcript was always more elevated in female tissues; approximately 1.8:1 in mammary gland (Figure 4c, lane 2). While it was beyond the scope of this study to pursue the potential role of sex steroids in the regulation of *BRCA1*, it is worth noting that the human *BRCA1* gene contains a putative estrogen response element (Xu *et al.*, 1997) which appears to be absent from the mouse promoter. TgN-*BRCA1*-B transgenic animals could therefore play a role in the study of similarities and differences in the regulation of mouse and human *BRCA1* genes.

Discussion

BRCA1 has been implicated in a variety of biological settings, including double-strand break repair and homologous recombination in mitotic cells (Cressman *et al.*, 1999a; Snouwaert *et al.*, 1999), and DNA strand exchange in meiotic cell division (Chen *et al.*, 1998; Cressman *et al.*, 1999a; Scully *et al.*, 1997b; Zabludoff *et al.*, 1996). Depending on the severity of the deletion, murine embryos lacking *Brca1* die between 7.5 and 11 days of development due to accumulated DNA defects (Xu *et al.*, 1999). We have shown that a fragment of human chromosome 17, containing a wild-type human *BRCA1* allele, is capable of supporting development and a full array of adult functions in *Brca1*^{-/-} deficient mice. The observation that rescued mice live a full lifespan and rear normal litters, provides evidence that the human gene product is capable of complementing the absence of the mouse gene in both meiotic and mitotic cells.

Prior to generation of the TgN-*BRCA1*^{GEN} transgenic lines, we conducted experiments in which a series of heterologous promoters were used to direct *BRCA1* in transgenic animals. The failure of these promoter-*BRCA1* constructs to support development indicates that overexpressed, or mis-expressed, *BRCA1* blocks development. This result was anticipated, in part, because of studies which demonstrate very low levels of *BRCA1* expression in normal cells, and other studies that show cellular growth-arrest or apoptosis in many cell lines which overexpress *BRCA1* (Shao *et al.*, 1996; Wilson *et al.*, 1997; Zhang *et al.*, 1998). When we transfected mammary cell lines with *BRCA1* cDNAs linked to *BRCA1* promoter sequences, we did not observe apoptosis or cellular arrest (T Lane and E Solomon, preliminary observations). These results support the need to better understand *BRCA1*

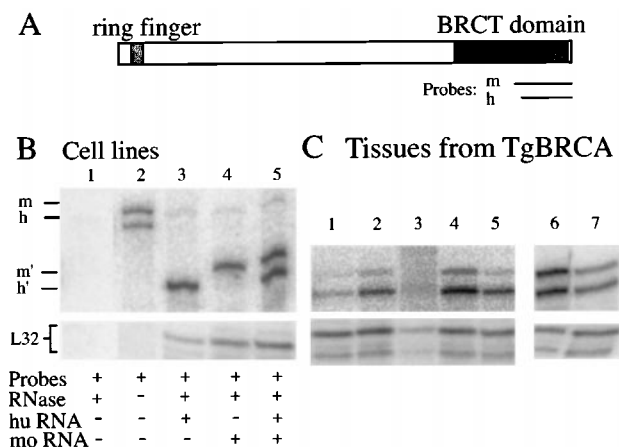


Figure 4 Human *BRCA1* mRNA is expressed at appropriate levels in TgN-*BRCA1* transgenic mice. (a) Schematic diagram of the *BRCA1* cDNA and location of sequences used as riboprobes. m=full-length mouse *Brca1* riboprobe, h=full-length human *BRCA1* riboprobe. (b) Detection of human and mouse *BRCA1* mRNA in extracts of mammary epithelial cell lines. Distinct bands representing the protected human (h') *BRCA1* and mouse (m') *Brca1* riboprobes are observed in RNA isolated from human (MCF7, lane 3 and 5) and mouse (NuMG, lane 4 and 5) breast cell lines. (c) RNA extracted from various tissues of TgN-*BRCA1*-B mice contain appropriate amounts of mouse and human *BRCA1* mRNA. Tissues examined: adult kidney (1), mammary gland (2), muscle (3), spleen (4 and 6), thymus (5) and testis (7). Lanes 1–5 represent tissues from female, and lanes 6–7 from male, FVB/N;TgN-*BRCA1*-B mice. For both b and c, an *L32* riboprobe is added to each reaction to show equivalence of RNA

regulatory domains and suggest due caution in interpreting experiments which assess phenotypes of *BRCA1* overproducing cells.

Over 140 distinct *BRCA1* mutations have been identified in breast and ovarian cancer-prone families worldwide (BIC, 1999). In addition to inherited mutations, other means of regulating BRCA1 function may be important in sporadic tumors. The gene is down-regulated at the transcriptional level in sporadic cancer (Catteau *et al.*, 1999; Mancini *et al.*, 1998; Wilson *et al.*, 1999) and the BRCA1 protein becomes mislocalized following infection with certain transforming viruses (Maul *et al.*, 1998). There are currently no models which allow testing for the consequences of individual mutations in the human *BRCA1* gene in an intact animal system. By creating lines of mice that express human *BRCA1*, assessment of interactions with other transforming pathways and analysis of specific mutations can be greatly accelerated.

Materials and methods

Mouse strains and nomenclature

FVB/N (Taconic, Germantown, NY, USA) is an inbred strain with characteristics that include large pronuclei amenable for microinjection, good parental care, and large litter size. Some of the mice described in this report were originally created on strain 129^{SvEv} but were then backcrossed into FVB/N to create FVB/N congenic strains as described below. TgN·BRCA1^{GEN} (abbreviated TgN·BRCA1) is a transgenic line generated in FVB/N carrying a 140 kb genomic fragment of the human *BRCA1* locus. A targeted hypomorphic allele of *Brcal* (the mouse BRCA1 locus) was generated as described below (*Brcal*^{AexIIISA}). *Brcal*^{+/-} heterozygotes carry one copy, while *Brcal*^{-/-} animals carry two copies, of the *Brcal*^{AexIIISA} allele.

Constructs used to create specific transgenic mice

Two transgenic lines are described in this paper: (i) For generation of TgN·BRCA1^{GEN}, PAC103014 (Brown *et al.*, 1995) was digested with *NotI* and gel purified by pulse-field electrophoresis. Prior to microinjection into pronuclei of one-cell FVB/N embryos, the 140 kb insert was electroeluted, concentrated by precipitation, and dialyzed against PIPES injection buffer (10 mM PIPES pH 7.0, 0.15 M KCl, 5 mM NaCl) supplemented with 70 μ M spermidine and 30 μ M spermine; (ii) A hypomorphic allele of murine *Brcal* (*Brcal*^{AexIIISA}) was generated by introducing the neomycin resistance gene in lieu of the endogenous splice acceptor for exon 11 of the murine *Brcal* gene through homologous recombination in 129^{SvEv} derived TC-1 embryonic stem cells. The targeting vector created in pPNT (Tybulewicz *et al.*, 1991) is identical to that described by Shen *et al.* (1998), while the ES cells are derived from an independent transfection (C Deng and P Leder, unpublished). Chimeric founder animals were mated to FVB/N females and were backcrossed for 12 generations into FVB/N to create *Brcal*^{AexIIISA/FVB}. All targeted mice used in the present study represent *Brcal*^{AexIIISA/FVB} animals. *Brcal*^{AexIIISA} carriers are denoted as *Brcal*^{+/-} or *Brcal*^{-/-} depending on the number of wild-type alleles present.

DNA isolation and Southern analysis

DNA was isolated from tails (1 cm), digested with *EcoRI*, resolved on 0.8% agarose/TAE gels, and transferred to nylon membranes, as described (Lane and Leder, 1997). DNA

probes included: (i) For genotyping at the *Brcal* genomic locus, a 420 bp fragment of *Brcal* genomic DNA lying just upstream of the sequences used to create the *Brcal*^{AexIIISA} mutation; and (ii) For TgN·BRCA1 genotyping, a 5 kb fragment of the human *BRCA1* cDNA. Probes were labeled by random priming (³²P]CTP, Prime-It labeling kit, Stratagene, La Jolla, CA, USA) and specific hybridization was detected after washing the membranes at 62°C in 0.1% SSC, 0.1% SDS. Positive signals were visualized by autoradiography in the presence of intensifying screens. Images were recorded on pre-flashed X-ray film (Hyperfilm-MP, Amersham, Arlington Heights, IL, USA).

Antibodies and immuno-precipitation analysis

The anti-BRCA1 antisera used have been characterized extensively as described (Wilson *et al.*, 1997, 1999). 115 is a rabbit polyclonal antibody useful for immuno-precipitation and SD118 is a mouse monoclonal antibody useful for immuno-blot analysis. Immuno-precipitations from tissue extracts were performed as follows. Tissues were extracted in RIPA buffer: 150 mM NaCl, 1.0% NP40, 0.5% DOC, 0.1% SDS, 50 mM Tris-HCl (pH 8.0). The following inhibitors were added just before use: 0.25 mM EDTA, 0.1 mg/ml pepstatin A, 0.4 mM PMSF, 1 mM NaF, 1 mM NaVanadate, 1 mM NaPPI. Protein concentration was determined by Bradford Assay (Pierce, Rockford, IL, USA) and 500 μ g aliquots were used for immuno-precipitation with 1 μ l of antibody 115. Immuno-precipitates were dissolved in SDS-loading buffer containing 4 mM DTT and run on either 8% or 3–8% gradient minigels (NuPAGE, Novex, San Diego, CA, USA) following the manufacturer's guidelines. 8% gels were run for 6 h and 3–8% gradient gels were run for 90 min at 150 mA/gel. Proteins were then transferred to Immobilon P membranes, blocked in MT buffer (PBS containing 1% nonfat dried milk, 0.05% Tween-20), and incubated with primary antibody (SD118). Blots were developed with HRP-labeled anti-mouse IgG (Pierce) and a chemi-luminescence detection substrate (SuperSignal, Pierce). Labeled proteins were detected with X-Omat-AR X-ray film, and regular X-Omatic intensifying screens (Kodak, Rochester, NY, USA).

RNase protection assays

RNA was isolated from tissues and cell lines by the acid-phenol method (Trisol, Gibco/BRL, Grand Island, NY, USA) and 10 μ g of total RNA were analysed by multiplex RNase protection assays (mRPA). Riboprobe vectors protected a 367 bp fragment of human *BRCA1* cDNA, a 384 bp fragment of mouse *Brcal* cDNA (nts) and an 112 bp fragment of mouse *L32*. cDNA sequences were introduced into pBSII-KS (Stratagene, La Jolla, CA, USA) and riboprobes were synthesized from linearized templates using T7 RNA polymerase in the presence of [³²P]UTP. Equal molar amounts of the two BRCA1 riboprobes were mixed with 10-fold less of the *L32* probe before hybridization with RNA at 56°C following the manufacturer's specifications (RPA kit, Pharmingen, San Diego, CA, USA). Mixtures were then treated with RNase and precipitated. Protected fragments were separated on 6% UREA-PAGE sequencing gels. Labeled fragments were identified by exposure to phosphor-imager screens (Storm 860, Molecular Dynamics, Sunnyvale, CA, USA). Band intensities were normalized to the *L32* signal.

Cells, tissue culture and γ -radiation

All cells and tissues were maintained in complete medium containing DME medium (GIBCO Laboratories, Grand Island, NY, USA), 10% by volume FCS, 250 μ g/ml amphotericin, 100 U/ml penicillin G and 100 U/ml strepto-

mycin sulfate. For DNA damage studies, mouse tissues were harvested in serum-free medium and dissected into 3 mm fragments. Fragments were then washed by brief centrifugation and resuspended in serum-free medium prior to brief exposure to gamma-irradiation using a calibrated [^{137}Cs] source (maintained by the UCLA School of Medicine). Dosages were 0, 0.1 or 5 Gray (Gy) as reported in previous studies of human breast cell lines (Abbott *et al.*, 1999; Scully *et al.*, 1997a). Tissues were then rinsed, transferred to complete medium, and incubated with agitation in a tissue culture incubator, 37°C, 5% CO₂. After 1 h, tissues were rinsed with serum-free media and extracted for protein analysis.

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