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A delayed chemically induced tumorigenesis in *Brca2* mutant mice

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***BRCA2* is a breast cancer susceptibility gene. Germline mutations of *BRCA2* account for about 10–30% of familial breast cancer cases. Consistent with its tumor-suppressor activity, BRCA2 plays an important role in DNA repair. To assess the susceptibility of carriers of mutant *BRCA2* to tumorigenesis induced by DNA-damaging carcinogens, we generated a *Brca2* knockout mouse strain and studied its susceptibility to chemically induced tumorigenesis. Similar to previously reported *Brca2* knockout mice, our $Brca2^{-/-}$ embryos die at E8.5–9.5, while the $Brca2^{+/-}$ mice are tumor-free and fertile. Unexpectedly, $Brca2^{+/-}$ mice developed tumors slower than did their wild-type littermates when treated with a potent carcinogen 7,12-dimethylbenz[a]anthracene (DMBA). *In vitro* experiments showed that $Brca2^{+/-}$ mouse cells and Capan-1 cells, a human pancreatic cancer cell line deficient of BRCA2, were more sensitive to DMBA-induced apoptosis, than were $Brca2^{+/+}$ mouse cells and a derivative of Capan-1 cells that expressed exogenous wild-type BRCA2, respectively. Our results suggest that enhanced sensitivity of *Brca2* mutant cells to DMBA-induced apoptosis at the dose of DMBA we used contributes to the delayed tumorigenesis of $Brca2^{+/-}$ animals. This suggestion may also provide a rational explanation for a previous unexpected finding that cigarette smoking appears to reduce the breast cancer risk of *BRCA2* mutation carriers.**
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Introduction

Approximately 10–30% of familial breast cancer patients harbor heterozygous germline mutations on the *BRCA2* gene. The finding that the wild-type *BRCA2* allele is frequently lost in breast cancer from mutant *BRCA2* carriers suggests that *BRCA2* is a tumor-suppressor gene for breast cancer in these patients (Welcsh and King, 2001; Schwab *et al*., 2002; Scully and Puget, 2002). BRCA2 has been found to be involved in several fundamental cellular processes such as DNA repair and recombination, and cell cycle checkpoint control (Orelli and Bishop, 2001; Venkitaraman, 2001a, b, 2002). Unexpectedly, heavy smoking appears to reduce the breast cancer risk of mutant *BRCA2* carriers (Brunet *et al*., 1998). This finding has raised an important issue concerning the relationship between *BRCA2* mutants and environmental carcinogens with respect to breast tumorigenesis (Brunet *et al*., 1998).

To study the functions of BRCA2, several *Brca2* knockout mouse lines have been generated (Connor *et al*., 1997; Ludwig *et al*., 1997; Sharan and Bradley, 1997; Suzuki *et al*., 1997; Friedman *et al*., 1998; McAllister *et al*., 2002). *Brca2* appears to have a critical role in embryo development. $Brca2^{-/-}$ animals of three *Brca2* knockout lines die early in embryogenesis (Ludwig *et al*., 1997; Sharan and Bradley, 1997; Suzuki *et al*., 1997), while only some $Brca2^{-/-}$ animals of another three *Brca2* knockout lines survived to birth (Connor *et al*., 1997; Friedman *et al*., 1998; McAllister *et al*., 2002). A correlation between the location of *Brca2* truncations and the viability of $Brca2^{-/-}$ embryos has been suggested based on the different viability of $Brca2^{-/-}$ embryos of these *Brca2* knockout mouse lines (Friedman *et al*., 1998; Morimatsu *et al*., 1998). Consistent with *BRCA2* being a tumor-suppressor gene, surviving $Brca2^{-/-}$ mice frequently develop thymic lymphoma and other types of tumor (Connor *et al*., 1997; Friedman *et al*., 1998; McAllister *et al*., 2002). Moreover, mice carrying mammary gland-specific *Brca2* knockout develop mammary gland tumors frequently (Ludwig *et al*., 2001). Different from humans carrying heterozygous *BRCA2* mutations, $Brca2^{+/-}$ mice do not have increased risk for spontaneous tumor. However, there has been no published study on the susceptibility of $Brca2^{-/-}$ animals to carcinogen-induced tumorigenesis.

Our goal has been to understand the role of *BRCA2* mutations in carcinogen-induced tumorigenesis. Here

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we report the intriguing findings from our investigation on treating heterozygous *Brca2* knockout mice and their wild-type littermates with an environmental carcinogen, DMBA.

Results and discussion

Generation of Brca2 knockout mice

We generated a *Brca2* knockout mouse strain using a homologous recombination replacement technique to study the susceptibility of *BRCA2* mutant carriers to environmental carcinogens (Figure 1a). Figure 1b shows the typical result of genotyping $Brca2^{+/+}$ and $Brca2^{+/-}$ mice when probe A was used in a Southern blot analysis, which is consistent with the expected results. Similar to

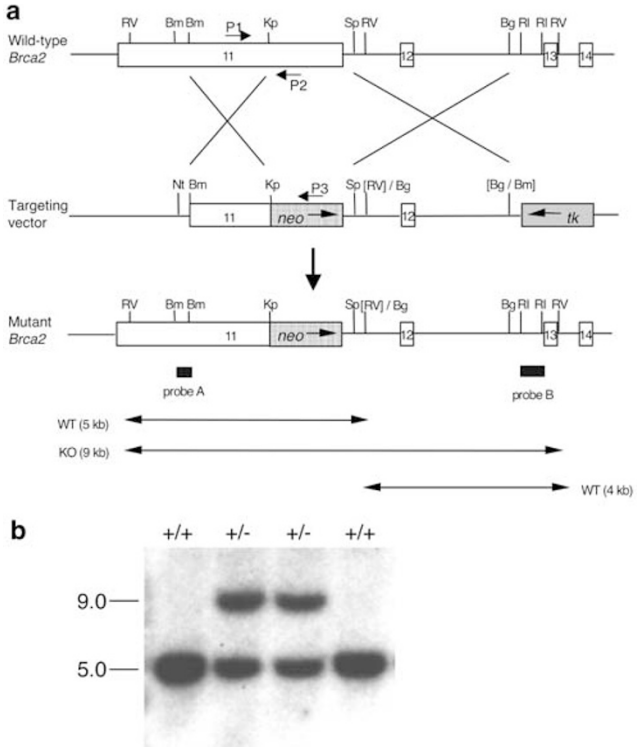

Figure 1 Targeting mouse *Brca2* by homologous recombination. (**a**) Schematic representations of the wild-type *Brca2* locus, the targeting vector, and the *Brca2* mutant locus after targeting. Exons 11, 12, 13, and 14 are shown as open boxes and introns are shown as solid lines between them. A double-selection replacement vector was constructed in pLG1, substituting a 2-kb 3′ region of exon 11 with a neomycin-resistance (neo) gene flanked by 1.3 (5′) and 1.9 kb (3′) of homologous sequences and the thymidine kinase (tk) gene. The expected sizes of *Eco*RV fragments from the wild-type and mutant (KO) alleles are 5 and 9 kb, respectively, for probe A, and 4 and 9 kb, respectively, for probe B. The restriction enzyme sites are abbreviated as follows: Nt (*Not*I), Bm (*Bam*HI), RV (*Eco*RV), Kp (*Kpn*I), Sp (*Spe*I), RI (*Eco*RI), and Bg (*Bgl*II). Brackets indicated deleted sites during construction. The PCR primers (P1, P2, and P3) used for genotyping are indicated as arrows. The above schemas are not drawn to scale. (**b**) Genotyping of the *Brca2* locus by Southern blot analysis. DNA samples prepared from tails of wild-type (+/+) and $Brca2^{+/-}$ (+/−) mice were digested with *Eco*RV and hybridized with probe A. The DNA bands specific to the wild type (5 kb) and the KO (9 kb) are shown

previously reported $Brca2^{+/-}$ mice (Connor *et al*., 1997; Ludwig *et al*., 1997; Sharan and Bradley, 1997; Suzuki *et al*., 1997; Friedman *et al*., 1998; McAllister *et al*., 2002), our $Brca2^{+/-}$ mice did not develop spontaneous tumors and have remained tumor-free for more than one year. Crosses between $Brca2^{+/-}$ mice yielded about two-thirds $Brca2^{+/-}$ mice and one-third of $Brca2^{+/+}$ mice. Examination of embryos showed that $Brca2^{-/-}$ embryos died at E8.5–E9.5. Based on the observation of previously reported *Brca2* mutant mouse lines (Ludwig *et al*., 1997; Sharan and Bradley, 1997; Suzuki *et al*., 1997), it was suggested that embryos carrying *Brca2* mutants that did not encode any BRC repeat could not survive, whereas embryos carrying *Brca2* mutants that encoded at least three BRC repeats were partially viable (Connor *et al*., 1997; Friedman *et al*., 1998; McAllister *et al*., 2002). However, the mutant *Brca2* allele of our knockout mice encodes a truncated protein containing the N-terminal 1593 amino acids of Brca2, which includes four BRC repeats. The viability of $Brca2^{-/-}$ embryos could also be influenced by their genetic background and whether the mutant *Brca2* allele is expressed or not (Connor *et al*., 1997; Friedman *et al*., 1998; Bennett *et al*., 2000; McAllister *et al*., 2002).

Delayed DMBA-induced tumorigenesis in $Brca2^{+/-}$ mice

To assess the cancer susceptibility of *Brca2* mutant carriers exposed to environmental DNA-damaging agents, we treated female virgin $Brca2^{+/-}$ mice and their wild-type littermates with DMBA in the presence of a hormone, medroxyprogesterone acetate (MPA) (Aldaz *et al*., 1996a, b). Based on the two-hit tumor-suppressor model (Knudson, 1971), the inactivation of one *Brca2* allele in $Brca2^{+/-}$ mice may lead to faster tumorigenesis than in the wild-type mice. In a pilot experiment, we unexpectedly found that $Brca2^{+/-}$ mice ($n=15$) developed tumors significantly slower than did $Brca2^{+/+}$ mice ($n=6$) ($P=0.0058$, log-rank test) (Figure 2a). Consistent with the reported observations (Aldaz *et al*., 1996a, b), we also found that MPA/DMBA treatment caused predominantly mammary tumors. $Brca2^{+/-}$ mice developed mammary carcinoma (13/15), lymphoma (7/15), and ovarian carcinoma (2/15). The wild-type mice developed mammary carcinoma (5/6), lymphoma (2/6), and ovarian carcinoma (1/6). To confirm the unexpected finding described above, we performed a second experiment with a larger sample size and found that $Brca2^{+/-}$ mice ($n=39$) indeed developed tumors significantly slower than did wild-type mice ($n=22$) ($P=0.0227$) (Figure 2a). The combined data from these two experiments were plotted using the Kaplan and Meier method, and showed a significant delay of tumorigenesis in $Brca2^{+/-}$ mice, as compared with that of wild-type mice ($P=0.0007$) (Figure 2a, b).

Enhanced apoptosis induced by DMBA in $Brca2^{+/-}$ cells

DMBA is a DNA-damaging agent, and is known to induce both tumorigenesis and apoptosis (Pena *et al*., 1998). Limited DNA damage may induce tumorigenesis;

a

	1st		2nd		Combined	
	n	*P*	*n*	*p*	*n*	*p*
Brca2 $^{+/+}$	6		22		28	
Brca2 $^{+/-}$	15	0.0058	39	0.0227	54	0.0007

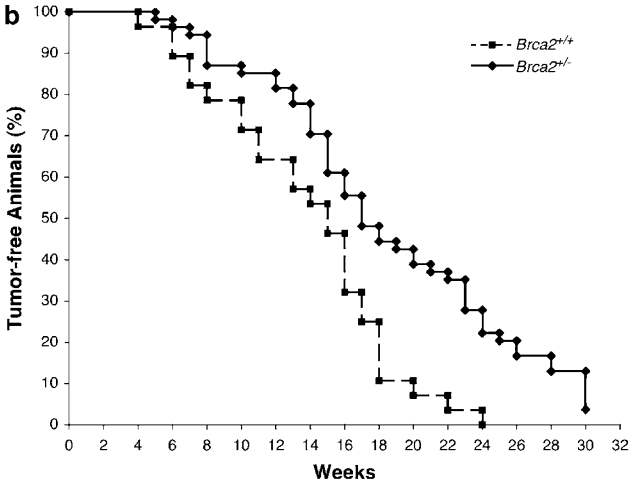

Figure 2 Delayed DMBA-induced tumorigenicity in *Brca2*$^{+/-}$ mice. (**a**) Summary of data obtained from two independent experiments. The tumor incidence was calculated based on the time of tumor occurring from the last DMBA treatment in relation to the total number of animals in each group, and was analysed by the Kaplan and Meier method (Kaplan and Meier, 1958). The reduced tumorigenesis in *Brca2*$^{+/-}$ mice is significant in each experiment, and in the combination of both experiments, as indicated by the *P*-values (log-rank test). (**b**) The data from two experiments are combined and shown as a Kaplan and Meier plot

however, extensive DNA damage could cause cell death. Thus, the unexpected results shown in Figure 2 prompted us to hypothesize that there was a difference in the DMBA-induced apoptosis between *Brca2*$^{+/-}$ and wild-type cells that contributed to the slower rate of tumorigenesis in *Brca2*$^{+/-}$ mice. To test this hypothesis, we established cell strains from a mammary tumor, each from two *Brca2*$^{+/-}$ mice and a wild-type mouse. The *Brca2* genotypes of these cell lines were confirmed by PCR (Figure 3a). The growth rates of these cell lines were measured by direct cell count. As shown in Figure 3b, although there is no significant difference in the growth rates among these cell lines on day 2, *Brca2*$^{+/-}$ cell lines grew significantly faster than *Brca2*$^{+/+}$ cell line after day 3 (Figure 3b and data not shown). This

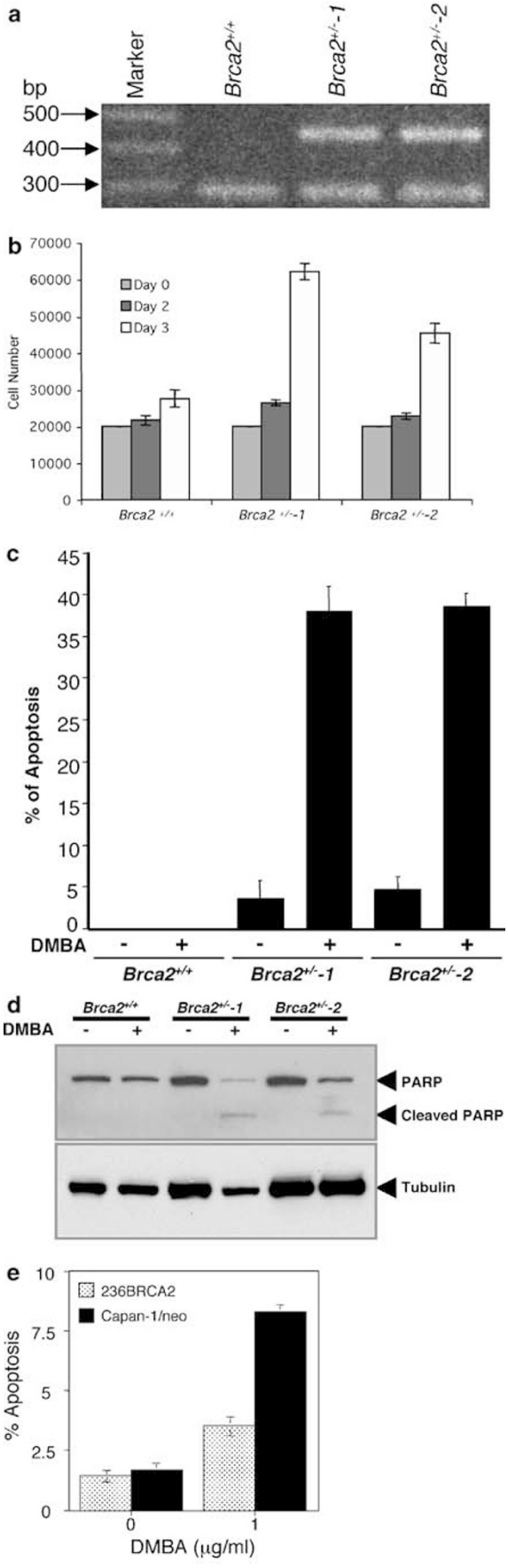

Figure 3 Enhanced apoptosis in *Brca2* mutant cells treated with DMBA. (**a**) The *Brca2* genotyping of mammary tumor cells derived from *Brca2*$^{+/-}$ (*Brca2*$^{+/-}$*–1* and *Brca2*$^{+/-}$*–2*) and wild-type (*Brca2*$^{+/+}$) mice was performed by PCR. (**b**) Growth rates of *Brca2*$^{+/+}$ and *Brca2*$^{+/-}$ cells. Cells (2×10^4) were plated and cell numbers were scored by using a Z1 Coulter Particle Counter (Beckman) at the indicated time points. The experiment was performed in triplets. (**c**, **d**) Enhanced apoptosis of *Brca2*$^{+/-}$ cells induced by DMBA. Cells were treated with DMBA (1 μg/ml) for 36 h and then cultured for another 72 h in fresh media followed by TUNEL (**c**) and PARP cleavage (**d**) assays. (**e**) 236BRCA2 and Capan-1/neo cells were treated with or without DMBA (1 μg/ml), and subsequently analysed for apoptosis by flow cytometry

observation is consistent with the previous findings that increased BRCA2 expression inhibits cell growth (Wang *et al*., 2002) and increased cell proliferation was observed in *BRCA2* hereditary tumors, as compared with that in sporadic tumors (Levine *et al*., 2002). Thus, this result indicates that the growth rate cannot simply account for the delayed tumorigenesis of *Brca2*$^{+/-}$ mice. To test the sensitivity of these cell lines to DMBA-induced apoptosis, we treated these cell lines with or without DMBA (1 μg/ml), followed by apoptosis assays such as TUNEL (Figure 3c) and PARP cleavage (Figure 3d). Our results clearly show both *Brca2*$^{+/-}$ cell lines are highly sensitive to apoptosis induced by DMBA. In contrast, little or no apoptosis was detected in the DMBA-treated *Brca2*$^{+/+}$ cell line. These results suggested that *Brca2*$^{+/-}$ cells were more sensitive to DMBA-induced apoptosis, presumably caused by the reduced expression of Brca2 in *Brca2*$^{+/-}$ cells. To further test this hypothesis, we performed a similar experiment using derivatives of a human pancreatic cancer cell line, Capan-1, which expresses only a truncated BRCA2 protein (Goggins *et al*., 1996; Abbott *et al*., 1998; Su *et al*., 1998). These derivatives of Capan-1 either expressed a moderate level of the wild-type BRCA2 protein (236BRCA2) or carried only the empty vector (Capan-1/neo) (Wang *et al*., 2002). Consistent with our hypothesis, we found that 236BRCA2 were less susceptible to DMBA-induced apoptosis, than were Capan-1/neo (Figure 3e). This result indicates that expression of the wild-type BRCA2 attenuates the DMBA-induced apoptosis in the *BRCA2*-null Capan-1 cells, and supports the notion that the enhanced apoptosis in mammary epithelium of the DMBA-treated *Brca2*$^{+/-}$ mice resulting from reduced DNA repair activity contributes to the delayed tumorigenesis of these mice (Figure 4).

Although Brca2 plays a role in DNA double-strand break repair (Zheng *et al*., 2000; Venkitaraman, 2002), the degree of its importance in repairing DNA double-strand break may depend on cell type. For instance, while *Brca2* mutant MEFs were more sensitive to the apoptosis induced by ionizing radiation (e.g., X-rays) than were the wild-type MEFs, the repair of double-strand DNA break during V(D)J recombination in *Brca2* mutant lymphoid cells remained intact (Patel *et al*., 1998). It is interesting to note *Brca2*$^{+/-}$ MEFs were slightly but consistently more sensitive to apoptosis induced by UV, X-rays, and MMS, than were *Brca2*$^{+/+}$ MEFs (Patel *et al*., 1998). Similar to that observation, our *Brca2*$^{+/-}$ tumor cell lines are more sensitive to DMBA-induced apoptosis than are *Brca2*$^{+/+}$ tumor cells (Figure 3c, d). Together, these observations suggest that the role of Brca2 in DNA double-strand break repair may be cell type-specific, and supports a potential role of Brca2 in removing DNA adducts induced by alkylating carcinogens such as DMBA.

BRCA2 has an essential function in DNA repair through binding to Rad51 (Zheng *et al*., 2000; Venkitaraman, 2002). Thus, a decreased level of BRCA2 may render the cancer cells to be sensitive to certain chemotherapy. Consistent with that prediction, it has been shown that women with BRCA2-associated hereditary tumors have an improved recurrence-free interval following initial chemotherapy (Boyd *et al*., 2000). Furthermore, a lower level of BRCA2 appears to be associated with a better response to chemotherapy (Egawa *et al*., 2001). Here we report that *BRCA2* mutant cells exhibited an increased sensitivity to apoptosis when exposed to the DNA damage agent DMBA, and propose a model to explain these observations (Figure 4). We assume that the number of cells with different degrees of DNA damage caused by environmental carcinogens such as DMBA follows a Poisson distribution. The data shown in Figure 3 suggest that *Brca2*$^{+/-}$ cells could not efficiently repair DNA damages and, thus, would cause the distribution shift to the right in relation to that of *Brca2*$^{+/+}$ cells. As shown in Figure 4, the hypothetical degree of DNA damage beyond which tumorigenesis occurs is designated as critical point 1 (C-1). Critical point 2 (C-2) is a hypothetical point beyond which apoptosis takes place due to severe DNA damage. Thus, cells with the degree of DNA damage range between C-1 and C-2 will have a high probability to develop tumors, whereas cells with the degree of DNA damage beyond C-2 will likely undergo apoptosis. Since the distribution patterns of cells with various degrees of DNA damage differ between *Brca2*$^{+/-}$ and wild-type mice, this model provides a plausible interpretation for the delayed tumorigenesis observed in the DMBA-treated *Brca2*$^{+/-}$ mice. This model can also be used to explain the intriguing finding that heavy smoking was correlated with a reduced risk of breast cancer in *BRCA1* or *BRCA2* mutant carriers (Brunet *et al*., 1998). Like DMBA, cigarette smoking induces DNA damages, such as adduct formation, and that can lead to tumorigenesis (e.g. lung cancer) and apoptosis (Hecht, 2002). Based on this model, heavy smoking predominantly causes apoptosis in the breast epithelial cells of *BRCA2* mutant carriers, thus eliminating some of the premalignant breast epithelial cells. One of the consequences would be

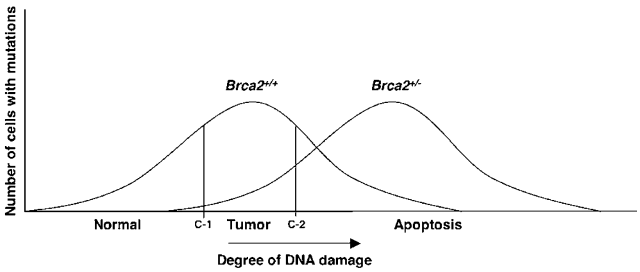

Figure 4 A model for the delayed DMBA-induced tumorigenesis in *Brca2* mutant mice. We propose that the number of cells with different degrees of DNA damage caused by environmental carcinogens such as DMBA or cigarette smoking follow a Poisson distribution. The fact that *Brca2*$^{+/-}$ cells could not efficiently repair DNA damage would cause the curve shift to the right in relation to that of *Brca2*$^{+/+}$ cells. Cells with the degree of DNA damage beyond which tumorigenesis occurs is designated as critical point 1 (C-1). Critical point 2 (C-2) is a hypothetical degree of DNA damage beyond which apoptosis takes place due to severe DNA damage

the apparent reduced risk of breast cancer. Our working model also raises a possibility of using the noncarcinogenic agents that induce apoptosis in breast epithelial cells to prevent breast cancer for BRCA2 mutant carriers. Further systematic investigation will be required to address these interesting and critical issues.

Materials and methods

Isolation of a mouse genomic Brca2 clone

A mouse embryonic stem (ES) cell genomic library in Lambda DASHII (Stratagene, La Jolla, CA, USA) was screened by hybridizing with human *BRCA2* exon 11 probes under a low stringent condition. Out of 107 clones screened, five appeared to be positive after five runs of screening. A 14-kb mouse *Brca2* genomic DNA fragment was excised from one of these clones by *Not*I, and then subcloned into pBlueScript vector (Stratagene). Sequencing analysis revealed that this genomic clone spanned from exon 9 to exon 14. A restriction map was constructed by restriction enzyme digestions, and the relevant enzyme sites are shown in Figure 1.

Construction of Brca2 knockout (KO) plasmid

Southern blot analysis identified a 2.0-kb *Spe*I/*Kpn*I fragment, which contained the 3′ one-third of *Brca2* exon 11. We chose this region to be deleted to abolish the Brca2 function. A 1.3-kb *Kpn*I/*Bam*HI fragment 5′ to this region and a 1.9-kb *Bgl*II/*Spe*I fragment 3′ to this region were selected to be homologous recombination regions. These fragments were subcloned into a KO vector, pLG1, which harbors two selection markers, a neomycin phosphotransferase (neo) gene and a thymidine kinase (tk) gene (Figure 1a). Insertion of a *Bgl*II linker abolishes an endogenous *Eco*RV site in the 3′ region.

Generation of Brca2 KO mice

The targeting construct was first linearized by *Not*I digestion and then electroporated into the AB-1 ES cell line derived from 129SvEv (129S) mice (McMahon and Bradley, 1990). Out of 576 ES clones that survived G418 and 1-[2′-deoxy-2′-fluoro-β-D-arabinofuranosyl]-5-iodouracil (FIAU) double selection, we obtained four $Brca2^{+/-}$ ES clones (0.67%) using Southern blot genotyping method. The $Brca2^{+/-}$ ES cells were expanded and microinjected into C57BL/6J blastocysts. The chimera embryos were then transferred into the uterus of pseudo-pregnant Swiss Webster females. Taking advantage of the coat color difference between 129SvEv mice (agouti) and C57BL/6J (albino), the resulting mice with higher chimerism were backcrossed with C57BL/6J. The germ line transmission of the *Brca2* KO genotype was revealed by Southern blot analysis of mice with agouti coat color in the F1 offspring.

Genotyping of Brca2

Southern blot was performed using a standard protocol (Maniatis *et al*., 1982). DNA extracted from ES clones or mouse tails was digested with *Eco*RV and subsequently hybridized with either probe A or probe B. The mutant *Brca2* allele would be revealed as a 9-kb band when either probe A or probe B was used, whereas the wild-type *Brca2* allele would be revealed as a 5- or a 4-kb band when probe A or probe B was used, respectively (Figure 1a). Genotyping of *Brca2* by PCR was performed using three primers: P1: 5′-CTCTAAGGAGACTGAAATGC-3′, P2: 5′-TGTTCCAAGCTTATCACCC -3′, and P3: 5′-GAAAGCGAAGGAGCAAAGC-3′. P1 and P2 are specific for the *Brca2* allele and P3 is specific for the exogenous neomycin-resistant gene (Figure 1a). The wild-type *Brca2* allele was revealed as a ~280-bp fragment (P1/P2 PCR product), whereas the mutant allele was revealed as a ~470-bp fragment (P1/P3 PCR product).

Chemically induced tumorigenesis

We used a chemically induced tumorigenesis protocol that predominantly caused mammary tumors (Aldaz *et al*., 1996a, b). Briefly, 6-week-old female virgin mice received subcutaneous interscapular implants of two MPA pellets (40 mg/pellet) (Hormone Pellet Press, Leawood, KS, USA). The mice were then treated intragastrically with 100 μl of DMBA (Sigma) in vegetable oil at 1 mg/dose at 9, 10, 12, and 13 weeks of age. The tumor incidence was monitored weekly.

Generation of $Brca2^{+/+}$ and $Brca2^{+/-}$ mammary tumor epithelial cell lines

Mouse mammary tumors were collected under sterile condition and chopped into small pieces using surgical scissors under sterile condition. The samples were washed with PBS followed by trypsin digestion (1.25% Trypsin in PBS) at 37°C for 20–30 min. After trypsin was removed, the tissues were placed in a complete medium (DMEM/F12 with 10% FBS) and then pipetted through the pipette tip multiple times. The tissue/cell mixture was filtered through a six-layer sterile gauze to remove tissue debris. The cell density was determined by using a hemocytometer, and cells were plated on a 100 mm tissue culture dish in 20 ml complete medium at a density of 5×10^6. The fibroblast cells were removed mechanically by physical scraping and repeated washing.

Apoptosis assays

The TUNEL assay was performed as described previously (Gavrieli *et al*., 1992). Briefly, cells were harvested into a cytospin chamber. TUNEL assay was performed to score the apoptotic cells with or without DMBA (1 μg/ml) treatment under $\times 40$ magnification, from 10 random fields with total more than 300 cells. Each sample was counted twice. The percentage of apoptotic cells per field was calculated. The PARP cleavage assay was performed as described previously (Ding *et al*., 2002). For measuring sub-G1 apoptotic cell population using flow cytometry, cells treated with or without DMBA (1 μg/ml) were washed with PBS and suspended in 0.5 ml of PBS containing 0.1% (v/v) Triton X-100 for nuclei preparation. The nuclei were re-suspended in PBS containing 0.1% (w/v) RNase and propidium iodide (50 μg/ml), followed by flow cytometry using a FACScan cytometer.

Abbreviations

DMBA, 7,12-dimethylbenz[a]anthracene; MPA, medroxyprogesterone acetate; FIAU, 1-[2′-deoxy-2′-fluoro-b-D-arabinofuranosyl]-5-iodouracil.

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