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Role of the tumor suppressor gene *Brca1* in genetic stability and mammary gland tumor formation

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Germline mutations in the tumor suppressor *BRCA1* predispose women to breast and ovarian cancers. Current evidence demonstrates that mutations in BRCA1 do not directly result in tumor formation, but instead cause genetic instability, subjecting cells to high risks of malignant transformation. In an animal model in which *Brca1* is mutated specifically in mammary epithelium, tumorigenesis occurs in mutant glands at low frequency after a long latency. Notably, introduction of a p53-null allele significantly enhanced mammary gland tumor formation in *Brca1* conditional mutant mice. These results are consistent with a model that *Brca1* is a caretaker gene, whose absence causes genetic instability and triggers further alterations, including inactivation of tumor suppressor genes and/or activation of oncogenes, leading to tumor formation. *Oncogene* (2000) **19,** 1059–1064.

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Introduction

Breast cancer is the most common cancer and the second leading cause of cancer mortality in women, with approximately one in nine being affected over their lifetime. An estimated 175 000 women will be diagnosed with breast cancer this year, and approximately 43 000 will die. About 5–10% of breast cancers are heritable, which may be caused by mutations of tumor suppressor genes (reviewed in Alberg and Helzlsouer, 1997; Brody and Biesecker, 1998; Ellisen and Haber, 1998; Paterson, 1998). The first gene found to be associated with increased susceptibility to familial breast and ovarian cancer is *BRCA1*, which was mapped in 1990 (Hall *et al*., 1990) and was subsequently cloned 4 years later (Miki *et al*., 1994). Germline mutations in *BRCA1* have been detected in approximately 90% of familial breast/ovarian cancers and about 50% of familial cases with breast cancer alone (reviewed in Alberg and Helzlsouer, 1997; Paterson, 1998). Despite strong evidence to support the view that BRCA1 is a tumor suppressor in humans, the precise mechanism through which BRCA1 mutations cause tumor formation is not clear.

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The *BRCA1* gene contains 24 exons, which encode a large protein of 1863 amino acids in humans and 1812 amino acids in mice (Bennett *et al*., 1995; Lane *et al*., 1995; Marquis *et al*., 1995; Miki *et al*., 1994). It also contains a number of motifs that may have distinct functions [reviewed in (Paterson, 1998), and Figure 1]. The zinc finger domain in the amino terminus interacts with DNA directly or indirectly through protein–protein interactions (Jin *et al*., 1997; Wu *et al*., 1996). The carboxyl terminus contains a BRCT domain, which is a relatively common feature of proteins involved in DNA damage response checkpoints (Bork *et al*., 1997). The BRCT domain interacts with p53 and functions as a transactivator in both p53 dependent and independent fashions (Chai *et al*., 1999; Chapman and Verma, 1996; Ouchi *et al*., 1998; Zhang *et al*., 1998). Some of the known downstream target genes of BRCA1 include p21 (Somasundaram *et al*., 1997), ER (Fan *et al*., 1999) and Gadd45 (Harkin *et al*., 1999). Exon 11 of the *BRCA1* gene constitutes over 60% of the protein and encodes two putative nuclear localization signals (Chen *et al*., 1996a,b). It also contains a domain that interacts with RAD51, a homolog of *E. Coli* RecA involved in DNA damage repair (Scully *et al*., 1997a,b; Shinohara *et al*., 1992).

In the past 4 years, at least five groups have disrupted *Brca1* in the mouse using gene targeting (Gowen *et al*., 1996; Hakem *et al*., 1996; Liu *et al*., 1996; Ludwig *et al*., 1997; Shen *et al*., 1998). Embryos homozygous for these mutations exhibited phenotypic variations and died between embryonic days (E)5.5–13.5 (Table 1), displaying severe developmental delay and cellular defects. Although the molecular basis for the phenotypic variations is not well understood, it has been shown that the *Brca1* locus exhibits a complex pattern of RNA splicing since its initial cloning (Cui *et al*., 1998a,b; Lu *et al*., 1996; Miki *et al*., 1994; Thakur *et al*., 1997; Wilson *et al*., 1997; Xu *et al*., 1995). It has been demonstrated recently that alternative splicing of *Brca1* exon 11 generates two major transcripts, a 7.2 kb full length transcripts and a 3.9 kb Δ-exon 11 transcripts (Figure 1 and Xu *et al*., 1999b). Thus, it is conceivable that residual activities of undisrupted splicing variants could partially compensate for the loss of the full length Brca1 transcripts, resulting in the extended survival of mutant embryos. Indeed, a recent report showed that embryonic stem cells homozygous for a targeted disruption of *Brca1* exon 11 (Gowen *et al*., 1996) did contain the Δ-exon 11 transcripts (Cressman *et al*., 1999). Nonetheless, the proliferation defects associated with the targeted disruption of *Brca1* seem incompatible with the tumor suppressor function assigned to this gene and raise questions about the

mechanism by which BRCA1 mutations cause tumorigenesis. Moreover, in contrast to humans heterozygous for *BRCA1* mutations, who have a greatly increased risk for breast cancer, the heterozygous mice for the targeted mutations are tumor free. Apparently, the early lethality associated with the loss of Brca1 in the mouse obscures functions of this gene in later stages of mammalian development, cell cycle regulation, and especially in tumor suppression.

Much of our understanding of Brca1 functions comes from several recent studies analysing cultured cells which lack Brca1, in addition to a specifically engineered mouse, which carries a *Brca1* mutation that is specifically introduced into mammary epithelial cells (Gowen *et al*., 1998; Hagmann, 1999; Shen *et al*., 1998; Xu *et al*., 1999a,b). These studies uncovered multiple roles for Brca1 in DNA damage repair, G_2-M and centrosome checkpoints, and mammary gland tumor formation. The present review will focus on these recent findings regarding Brca1 functions in genetic stability and tumorigenesis.

BRCA1 and DNA damage repair

Increasing evidence suggests that BRCA1 is involved in DNA damage repair. The first hint of such a function of BRCA1 came from observations that BRCA1 colocalizes with RAD51 (Scully *et al*., 1997a), and is relocated to intranuclear structures where DNA replicates after treatment with DNA damaging reagents (Scully *et al*., 1997b). RAD51 is involved in ATP-dependent DNA strand exchange reactions and is known to be a homolog of yeast RecA, which functions in homologous recombination and DNA damage repair (Ogawa *et al*., 1993). More evidence connecting Brca1 to DNA damage repair comes from two recent findings. The first study used cultured embryonic stem cells and demonstrated that Brca1-deficient cells were hypersensitive to oxidative reagents, including γ-irradiation and hydrogen peroxide, and were defective in transcription-coupled repair (Gowen *et al*., 1998). Studying blastocysts and embryos, Shen *et al*. (1998) found that Brca1-deficient embryos were not only hypersensitive to γ-irradiation but also displayed numerical and structural chromosomal aberrations, which may be a direct consequence of unrepaired DNA damage.

Notably, embryos carrying a targeted disruption of Rad51 or Brca2, another tumor suppressor involved in breast tumor formation (Tavtigian *et al*., 1996; Wooster *et al*., 1995), exhibited similar phenotypes to the Brca1 – null embryos. They were all hypersensitive to γ-irradiation and displayed chromosomal abnormalities, and exhibited early embryonic lethality, which could be partially rescued by a p53 mutation (Lim and Hasty, 1996; Ludwig *et al*., 1997; Patel *et al*., 1998; Sharan *et al*., 1997; Shen *et al*., 1998). These striking similarities of RAD51, BRCA1, and BRCA2 mutants suggested a functional link between BRCA1 and BRCA2 in the RAD51-mediated DNA damage repair process. Disruption in any one of these genes impairs the function of the proposed BRCA1/BRCA2/RAD51 complex (Brugarolas and Jacks, 1997; Chen *et al*., 1998) and leads to genetic instability.

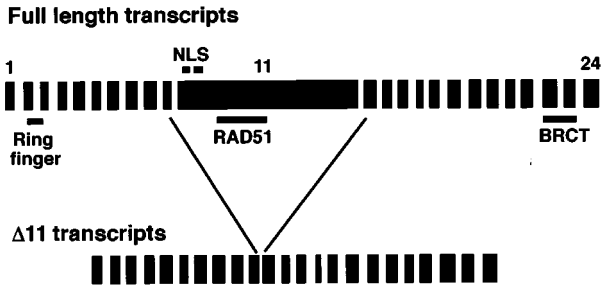

Figure 1 Structure of Brca1 and its major functional domains. Brca1 contains 24 exons (black boxes), which encode several major functional domains (as indicated). Alternative splicing of *Brca1* exon 11 generates two major transcripts, i.e. the full length (7.2 kb) and the Δ-exon 11 (3.9 kb) transcripts

Brca1 and cell cycle checkpoints

Ample evidence suggests that BRCA1 is involved in cell cycle control, which monitors the physical integrity of DNA and coordinates cell cycle transitions (reviewed in Morgan and Kastan, 1997; Weinert, 1998). It has been shown that BRCA1 is associated with many cell cycle proteins, including E2F, cdc2 and cyclins (Wang *et al*., 1997). BRCA1 levels vary at different phases of the cell cycle. The protein become hyperphosphorylated during late G_1 and S phases, and is transiently dephosphorylated early after M phase

Table 1 Brca1 mutations and phenotypes

Mutations of Brca1	*Phenotypes of homozygous embryos/mammary glands*	*Authors*
Deletion of exons 5 and 6	Die between E6-7, proliferation defects	Hakem *et al*. (1996)
Deletion of exon 2	Similar to above	Ludwig *et al*. (1997)
Deletion of 184 bp within 5′ portion of exon 11	Similar to above	Liu *et al*. (1996)
Deletion of 330 bp of intron 10 and 1.5 kb of exon 11	Die between E8.5 – 13.5, neuroepithelial abnormalities	Gowen *et al*. (1996)
Deletion of 330 bp of intron 10 and 440 bp of exon 11	Die at E8, growth retardation, hypersensitive to γ-irradiation, chromosomal abnormalities	Shen *et al*. (1998)
Cre-mediated deletion of exon 11 in mammary epithelium	Blunted ductal development of mammary gland. Increased apoptosis and tumor formation	Xu *et al*. (1999a)
Deletion of exon 11 (*neo* gene was removed through a cre-mediated recombination)	Die between E12.5 – 18.5, widespread apoptosis. Genetic instability, G_2-m and centrosome checkpoints defects	Xu *et al*. (1999b), and our unpublished observations

(Ruffner and Verma, 1997; Vaughn *et al*., 1996). In addition, overexpression of wild-type BRCA1 induces G_1-S arrest in cultured cells (Somasundaram *et al*., 1997), whereas expression of a mutant form of BRCA1 attenuates the G_2-M checkpoint (Larson *et al*., 1997). Because these results were obtained from studies that overexpress BRCA1 in normal cells, it is necessary to carry out further analyses using cells lacking BRCA1. However, it is not possible to study the cell cycle in Brca1-deficient cells because mouse embryonic fibroblasts (MEFs) that lack Brca1 failed to grow, even when *p53* gene was disrupted (Shen *et al*., 1998).

In an attempt to extend the embryonic survival of Brca1 mutant embryos, Xu *et al*. (1999b) created a hypomorphic mutation, which specifically deletes exon 11 of *Brca1* from the mouse germline. Embryos homozygous for this mutation ($Brca1^{\Delta 11/\Delta 11}$) lack full-length *Brca1* transcripts, but still express the shorter Δ11-transcripts, which are transcription products generated normally by direct splicing from exon 10 and 12 (Figure 1). These mutant embryos died at E12–18.5, displaying chromosomal abnormalities and increased apoptosis (X Xu and C Deng, unpublished observation). The extended survival made it possible to derive MEFs and assess Brca1 function in cell cycle checkpoints (Xu *et al*., 1999b). The data of Xu *et al*. indicated that $Brca1^{\Delta 11/\Delta 11}$ MEFs maintained an intact G_1-S checkpoint, since the cells showed similar arrest upon γ-irradiation compared to wild-type controls. However, $Brca1^{\Delta 11/\Delta 11}$ MEFs were found to be defective in a γ-irradiation induced G_2-M checkpoint. An essential function of this checkpoint is to arrest cells containing damaged DNA in the G_2 phase. Loss of the G_2-M checkpoint allows mutant cells to enter the mitotic (M) phase and pass unrepaired DNA to their daughter cells, leading to genetic instability.

Brca1 and centrosome amplification

Using $Brca1^{\Delta 11/\Delta 11}$ MEFs, Xu *et al*. (1999b) also demonstrated that Brca1 plays an essential role in regulating centrosome duplication. Cells normally contain one or two centrosomes at interphase and at various phases of mitosis. Centrosomes duplication is under precise control and only occurs once during the normal mammalian cell cycle. The replication process is initiated in late G_1 and continues into S phase. Before mitosis, the centrosome divides and forms bipolar spindles, which are essential for equal chromosome segregation (Pihan *et al*., 1998; Rudner and Murray, 1996; Winey, 1996). Xu *et al*. (1999b) found that 30% of $Brca1^{\Delta 11/\Delta 11}$ cells contained multiple centrosomes. These centrosomes appeared to be functional, since most of them were connected to the spindle as revealed by double labeling using antibodies to α- and β-tubulin. The multipolar spindles pull chromosomes in different directions and lead to unequal chromosomal segregation and micronuclei formation. It was recently shown that BRCA1 is physically associated with the centrosome (Hsu and White, 1998). Considering that BRCA1 has both protein–protein interaction and transactivation activities (Chapman and Verma, 1996; Wu *et al*., 1996), it is possible that BRCA1 regulates the centrosome duplication process through transactivation of centrosome-specific genes.

Increasing evidence suggests that dysregulation of centrosome duplication can cause malignant transformation. Many human tumors, including high grade breast tumors, contain abnormal centrosomes (Carroll *et al*., 1999; Lingle *et al*., 1998; Pihan *et al*., 1998). Interestingly, overexpression of a breast tumor associated kinase (STK15/BTAK, also known as

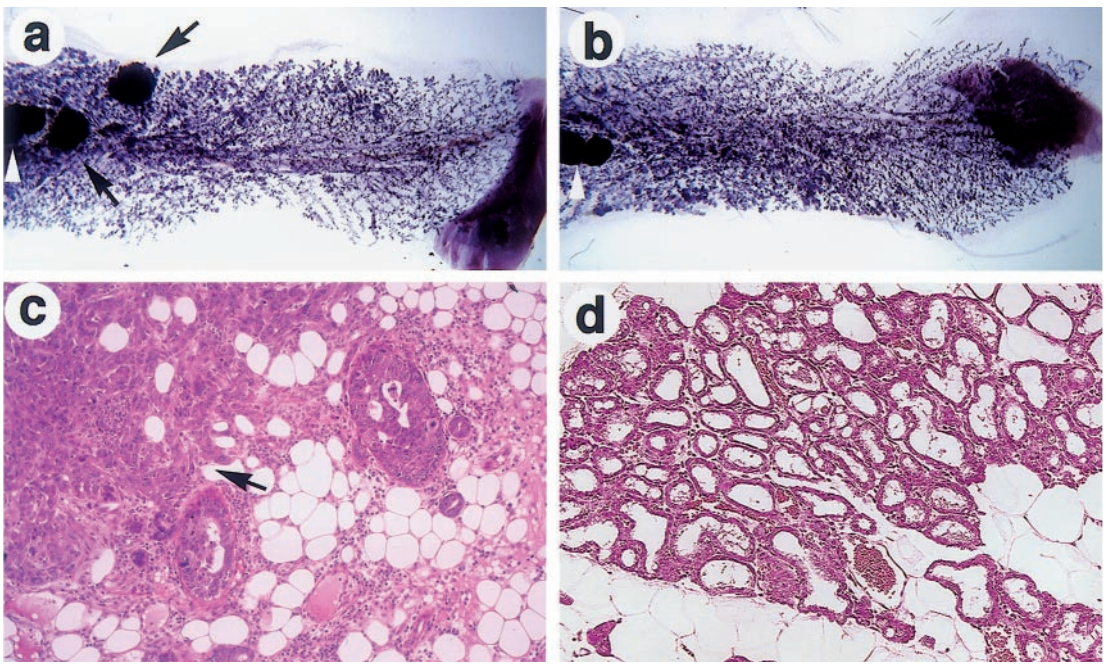

Figure 2 Cre-mediated disruption of *Brca1* in mammary epithelial cells results in tumor formation. (**a** and **b**) Involution day 11 mammary glands isolated from a $Brca1^{Ko/Co}$;*MMTV-Cre*;$p53^{+/-}$ mouse. White arrowheads point to lymph nodes. One gland developed two small tumors (**a**) black arrows, while the other gland (**b**) appeared tumor free. (**c**) Histology of an adenocarcinoma from a 13 month old $Brca1^{Ko/Co}$;*WAP-Cre* female mouse. There is no clear boundary between the tumor and surrounding mammary tissues (arrow), suggesting the highly invasive nature of the adenocarcinoma. (**d**) Histology section of a normal gland serving as a control

AIK1(3)/aurora2) in near-diploid human breast epithelial cells revealed centrosome abnormalities and induction of aneuploidy (Zhou *et al*., 1998). *STK15/BTAK* was recently identified as an oncogene that is overexpressed in about 94% of invasive ductal breast adenocarcinomas (Tanaka *et al*., 1999) and in other types of primary tumors and cell lines at a lower frequency (Zhou *et al*., 1998). On the other hand, loss of tumor suppressor genes, such as p53, can also result in amplification of centrosomes (Carroll *et al*., 1999; Fukasawa *et al*., 1996, 1997). It will be important to determine if there are any functional interactions among these genes during the centrosome duplication process.

Conditional mutation of Brca1 results in mammary tumor formation

Due to the embryonic lethality associated with targeted disruption of Brca1, it remained unknown whether or not mutation of Brca1 in the mouse results in tumor formation. To address this issue and to create a mouse model for Brca1 associated tumorigenesis, Xu *et al*. (1999a) used a Cre-loxP approach to mutate Brca1 specifically in mouse mammary epithelial cells. Three different types of mice were used in this study, a *Brca1* knockout (*Brca1*^{Ko}, Shen *et al*., 1998), a conditional knockout (*Brca1*^{Co}, Xu *et al*., 1999a), and *MMTV-Cre* or *WAP-Cre* transgenic mice (Wagner *et al*., 1997). Since both the *MMTV-Cre* and the *WAP-Cre* transgenes are expressed predominantly in mammary epithelium (Wagner *et al*., 1997), Cre-mediated disruption of *Brca1* was confined to mammary epithelium. Indeed, Northern blot analysis indicated that the *Brca1* transcripts were dramatically reduced in mammary tissues from *Brca*$^{Ko/Co}$*;WAP-Cre* and *Brca1*$^{Ko/Co}$*;MMTV-Cre* mice (Xu *et al*., 1999a).

However, the route to uncover the tumor suppressor function of Brca1 has never been straightforward. The researchers found that a prominent feature associated with the inactivation of Brca1 in mammary epithelium was abnormal gland development and increased apoptosis (Xu *et al*., 1999a). These Brca1 associated cellular proliferation defects may have been caused by the activation of the G_1-S cell cycle checkpoint, which is mainly controlled by p53. Despite these abnormalities, females could nurse their young and were tumor free when they were examined between 2–10 months of age. By 10–13 months, five out of 23 *MMTV-Cre* and *WAP-Cre* females developed diverse mammary tumors (Figure 2). The entire genome seemed intrinsically unstable in tumor cells, showing massive chromosomal abnormalities. Because disease onset was not universal and occurred relatively late in life, it is conceivable that the genetic instability triggered mutations of other factors, which in turn resulted in tumorigenesis. Surprisingly, p53 transcription was found to be changed in two of three tumors analysed (Xu *et al*., 1999a).

While this finding suggested a link between p53, Brca1, and mammary tumor formation, the researchers began to seek more proof. It has been reported in the literature that human BRCA1 familial breast tumors frequently contain p53 mutations (Crook *et al*., 1998, 1997; de Cremoux *et al*., 1999; Eisinger *et al*., 1997). However, it is not clear whether such mutations are simply a consequence of genetic instability associated with tumor progression, or if they are promoting factors that accelerate tumor formation, since p53 mutations have been found in about 55% of all human tumors. It was also recently shown that mice heterozygous for both Brca1 and p53 mutations (*Brca1*$^{+/-}$;p53$^{+/-}$) developed mammary gland tumors at a low frequency after γ-irradiation (Cressman *et al*., 1999).

To directly test the role of p53 in Brca1 associated tumor formation, Brca1 conditional mice were bred with mice heterozygous for a targeted mutation in p53 (Donehower *et al*., 1992), yielding animals that have only one functional copy of p53 (Xu *et al*., 1999a). If p53 loss could accelerate tumor formation, these mice would suffer increased tumorigenesis, as the cells would need to inactivate only one copy of p53 as opposed to the two copies which must be altered in p53 wild-type cells. The researchers showed that removal of one p53 allele indeed dramatically accelerated tumor formation in both frequency and timing of onset (Xu *et al*., 1999a). Southern blot analysis revealed that 80% of tumors had lost their wild-type p53 allele. Taken together, these findings indicate that loss of Brca1 itself does not directly cause cancer, but rather destabilizes the genome. This theoretically increases the mutation rates of genes including tumor suppressors and oncogenes, ultimately leading to tumor formation. However, this process, which involves mutations in multiple genes, in addition to the loss of heterozygosity of wild-type *Brca1* allele, needs times. This may account for the relatively long latency of cancer incidence in people carrying *Brca1* mutations and the rare incidence of somatic *Brca1* mutations in sporadic cancers (Easton, 1997; Futreal *et al*., 1994; Struewing *et al*., 1997; Xu and Solomon, 1996).

Conclusion and future directions

It was recently proposed that *Brca1* is in a class of caretaker genes, which function in maintaining genetic stability (Kinzler and Vogelstein, 1997). The observation that mutations of Brca1, whether null or hypomorphic, result in massive chromosomal alterations supports this hypothesis (Shen *et al*., 1998; Xu *et al*., 1999b). It is now clear, as summarized in Figure 3, that Brca1 maintains genome integrity through at least three functions, its ability to repair DNA damage (Gowen *et al*., 1998), G_2-M checkpoint control (Xu *et al*., 1999b), and regulation of centrosome duplication (Xu *et al*., 1999b). Loss of these important functions due to Brca1 mutations results in genetic instability, which in turn, activates cell cycle checkpoints, including a p53 dependent G_1-S checkpoint since the loss of p53 could partially rescue the early embryonic lethality caused by *Brca1* mutations (Hakem *et al*., 1997; Ludwig *et al*., 1997; Shen *et al*., 1998). The activation of cell cycle checkpoints subjects cells to growth arrest and apoptosis, which result in embryonic lethality. In contrast, in the *Brca1* conditional knockout mice, these abnormalities only occurred in the mammary glands, and the animals have a normal life span. This allows further genetic alterations, including the inactivation of p53, to occur, which eventually lead to tumorigenesis (Figure 3).

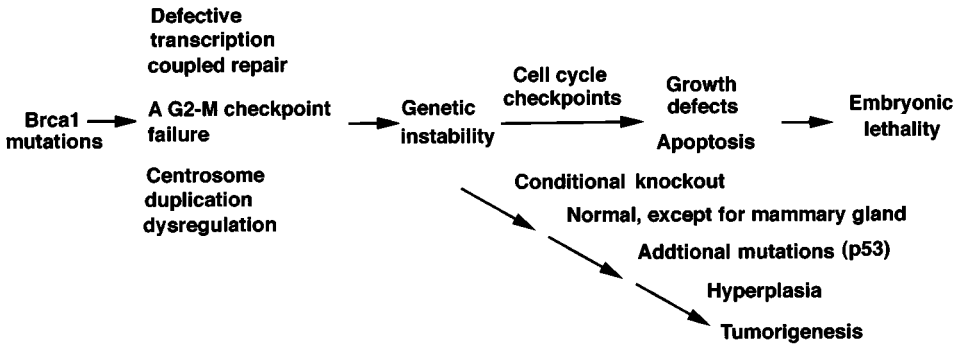

Figure 3 A diagram summarizing Brca1 functions and tumorigenesis in Brca1-conditional mutant mice. Brca1 plays an essential role in transcription-coupled repair, G2-M checkpoint and centrosome duplication (Gowen *et al*., 1998; Xu *et al*., 1999b). Loss of these functions results in genetic instability, which causes growth defects and increased apoptosis through activation of cell cycle checkpoints, leading to embryonic lethality. Conditional mutation of *Brca1* in the mammary epithelium overcomes the embryonic lethality and allows further genetic alterations, including the inactivation of p53, to occur, which eventually lead to mammary gland tumor formation

The generation of this important animal model provides opportunities to study the molecular aberrations arising from Brca1 deficiency and identify exogenous factors that influence the onset of tumor formation (Dennis, 1999; Hagmann, 1999). It also provides an experimental system to study the sensitivity of tumors to hormones and drugs that are associated with breast cancers, such as estrogen, tamoxifen, adriamycin and others, and to perform therapeutic studies for this devastating disease.

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