Targeted mutations of breast cancer susceptibility gene homologs in mice: lethal phenotypes of *Brca1*, *Brca2*, *Brca1/Brca2*, *Brca1/p53*, and *Brca2/p53* nullizygous embryos

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Mutations of the human *BRCA1* and *BRCA2* genes encoding tumor suppressors have been implicated in inherited predisposition to breast and other cancers. Disruption of the homologous mouse genes *Brca1* and *Brca2* by targeting showed that they both have indispensable roles during embryogenesis, because nullizygous embryos become developmentally retarded and disorganized, and die early in development. In *Brca1* mutants, the onset of abnormalities is earlier by one day and their phenotypic features and time of death are highly variable, whereas the phenotype of *Brca2* null embryos is more uniform, and they all survive for at least 8.5 embryonic days. Observations with *Brca1/Brca2* double nullizygotes raise the possibility that the two developmental pathways could be linked. Interestingly, the impact of the *Brca1* or *Brca2* null mutation is less severe in a *p53* null background.

[Key Words: *Brca1*; *Brca2*; *p53*; mouse mutant; embryonic lethality]

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Two genes of unknown biochemical function, *BRCA1* (Miki et al. 1994) and *BRCA2* (Wooster et al. 1995; Tavtigian et al. 1996), have been implicated in inherited predisposition to female breast cancer that is a causal factor for the disease in ~5%–10% of all patients [for review, see Claus et al. 1991; Easton et al. 1993; Szabo and King 1995; Cannon-Albright and Skolnick 1996; Marcus et al. 1996; Stratton and Wooster 1996]. Mutations of *BRCA1* also predispose to ovarian cancer [see, e.g., Easton et al. 1995; Gayther et al. 1995; Johannsson et al. 1996; Serova et al. 1996], whereas mutations of *BRCA2* are involved in male breast cancer and apparently in pancreatic cancer (Schutte et al. 1995; Goggins et al. 1996; Teng et al. 1996). Loss of heterozygosity (LOH) consistently affecting the wild-type allele in tumors from patients carrying *BRCA1* or *BRCA2* mutations (Smith et al. 1992; Neuhausen and Marshall 1994; Collins et al. 1995) indicated that these genes encode tumor suppressors. Moreover, *BRCA1* antisense strategies using cultured cells resulted either in acceleration of proliferation (Thompson et al. 1995) or cell transformation (Rao et al. 1996). Most of the mutations affecting the *BRCA1* gene (Szabo and King

1995; Cannon-Albright and Skolnick 1996; Feunteun and Lenoir 1996; Stratton and Wooster 1996; Xu and Solomon 1996) or the *BRCA2* gene (Wooster et al. 1995; Couch et al. 1996; Neuhausen et al. 1996; Phelan et al. 1996; Tavtigian et al. 1996; Teng et al. 1996; Thorlacius et al. 1996) result in truncated protein products. In sporadic tumors, somatic *BRCA1* or *BRCA2* mutations occur infrequently (Lancaster et al. 1996; Miki et al. 1996; Stratton and Wooster 1996; Teng et al. 1996).

The major *BRCA1* mRNA (Miki et al. 1994; Xu et al. 1995; Brown et al. 1996; Smith et al. 1996) encodes a nuclear phosphoprotein (X. Chen et al. 1996; C.F. Chen et al. 1996) of 1863 amino acid residues, but differential splicing events generate additional mRNAs that are only partially characterized (C.F. Chen et al. 1996; Thakur et al. 1997; Wilson et al. 1997). The amino-terminal portion of the *BRCA1* protein contains a RING finger domain (Freemont 1993; Saurin et al. 1996) that should be important for function, because it is invariant between the human and mouse genes, otherwise exhibiting only 58% sequence identity at the amino acid level (Abel et al. 1995; Bennett et al. 1995; Lane et al. 1995; Sharan et al. 1995). The carboxy-terminal region of *BRCA1*, which acts as a transcriptional transactivator when fused with the GAL4 DNA-binding domain (Chapman and Verma 1996; Monteiro et al. 1996), contains two copies of a

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domain (BRCT; Koonin et al. 1996) exhibiting weak similarity with the mammalian p53-binding protein 53BP1; with the yeast RAD9 protein that is involved in cell cycle arrest at checkpoints on DNA damage; and with other proteins (Koonin et al. 1996; Bork et al. 1997; Callebaut and Mornon 1997), including BARD1 (Wu et al. 1996) that interacts with BRCA1 in vivo. The highly charged protein of 3418 residues encoded by *BRCA2* is novel and, like BRCA1, not well conserved between humans and mice (59% sequence identity at the amino acid level; Connor et al. 1997; Sharan and Bradley 1997). Eight variable internal repeats of unknown significance have been identified in the region encoded by *BRCA2* exon 11 (Bork et al. 1996), which are also present in the homologous proteins of six other mammals, including mouse (Bingell et al. 1997).

To provide clues about the normal function of *Brca1* and *Brca2*, we have disrupted these genes in mice by targeted mutagenesis. Our results demonstrate that either of these mutations results in early embryonic lethality in homozygous mutants, while heterozygous and double heterozygous mice are indistinguishable from wild type. We have also assessed the combined effects of the two mutations in double nullizygous embryos, and have examined potential relationships between the *Brca1* or *Brca2* and *p53* functions. While this work was in progress, three reports appeared (Gowen et al. 1996; Hakem et al. 1996; Liu et al. 1996) describing *Brca1* targeted mutations. Similarities and differences between these studies and our results are discussed.

Results

Targeted disruption of the mouse Brca1 and Brca2 genes

To generate a null mutation of the *Brca1* gene, we used a replacement vector that would delete a DNA fragment consisting of exon 2, encoding a part of the conserved RING finger motif, and portions of its flanking introns (see Materials and Methods). The targeted allele was designated *Brca1^{ex2}*. Similarly, we disrupted the *Brca2* gene by replacing a segment of exon 11 with a *neo* cassette. Because of a frame-shift in the targeted allele (*Brca2^{ex11}*), a stop codon is encountered in the *neo* sequence. Therefore, any processed *Brca2* transcript would generate a truncated protein product that should be inactive.

Genotyping of progeny (129/Sv × C57BL/6J) heterozygous for either the *Brca1* or the *Brca2* mutation showed that these mice were indistinguishable from their wild-type littermates. *Brca1* heterozygous females are still tumor-free at the age of 15 months, whereas of the *Brca2* heterozygotes, the oldest of which are eight months old, only a single four-month-old female developed a squamous cell carcinoma of the skin in the thoracic region (data not shown). However, the appearance of this carcinoma cannot be formally attributed to loss of *Brca2* tumor suppressing activity, as PCR analysis of DNA ex-

tracted from histological sections and corresponding exclusively to tumor tissue indicated that *Brca2* heterozygosity was maintained, and sequence analysis was not performed to determine whether the nontargeted allele was mutated.

Embryonic lethality of nullizygous Brca1 and Brca2 mutants: comparative analysis

Genotyping of 183 progeny derived from intercrosses of heterozygous *Brca1*(+/-) mice showed that 59 were wild type, whereas the remaining 124 were heterozygous for the mutation. In analogous crosses between *Brca2*(+/-) heterozygotes, 160 pups were analyzed, 58 of which were wild type and 102 were heterozygous. In both cases, the complete absence of nullizygous mutants among these offspring, whereas heterozygous and wild-type progeny were obtained at the expected ratio, indicated that in the homozygous state the *Brca1* and *Brca2* null mutations result in embryonic lethality.

To analyze the lethal phenotypes, pregnant females from matings between heterozygous *Brca1* or *Brca2* mutants were sacrificed at different times post coitum, and embryos were examined in histological sections of dissected decidua and then genotyped. The gross morphology of additional embryos, some of which were genotyped, was also examined. The data are summarized in Table 1. In both the histological and the gross morphological analyses, we observed a high incidence of empty decidua in the *Brca1* series. Although genotyping was precluded, it is likely that most, if not all, of these empty decidua corresponded to *Brca1* null mutants, because their occurrence in the *Brca2* series was very rare (Table 1). Moreover, a number of decidua contained only giant cells and extraembryonic membranes, which on genotyping were found to be *Brca1* mutants (eight cases; five shown in Table 1). Therefore, the decidua with or without embryonic remnants are considered as one of the aspects of the variable *Brca1* null phenotype. In contrast, the phenotypic features of the *Brca2* nullizygotes are overall quite uniform, especially before embryonic day 9.5 (E9.5).

At the earliest age examined (E5.5), the wild-type and heterozygous embryos (hereafter referred to indiscriminately as "normal," for brevity) were small egg cylinders with distinct embryonic and extraembryonic regions, which had initiated the formation of a proamniotic cavity. Moreover, the visceral endoderm was differentiated into extraembryonic (columnar) and embryonic (squamous) layers (Fig. 1A). This characteristic regional difference in the appearance of endodermal cells was not evident in *Brca1* nullizygous mutants, which were recognizable morphologically as small and shorter-than-normal egg cylinders without a visible demarcation of embryonic and extraembryonic regions (Fig. 1B). A proamniotic cavity was present in only one of five examined mutant embryos (data not shown). The primary giant cells, Reichert's membrane and distal endoderm of the mutants did not differ from those of the controls.

Table 1. Embryonic phenotypes

Genotype →	Normal				Abnormal			Giant cells ± membranes only		Empty decidua (N.D.)
	+/+	+/-	-/-	N.D.	+/-	-/-	N.D.	-/-	N.D.	
A. Brca1										
Histology										
E5.5	3	5				5			1	1
E6.5	2	5				2				
E7.5	6	9		7		4	2	1	1	1
E8.5	2	5				4		1	1	1
Gross morphology										
E6.5				46			8			6
E7.5	4	15		60	1	3	15		1	5
E8.5				6			2	3		2
E9.5	6	10		23		6	3		2	6
B. Brca2										
Histology										
E5.5				15						
E6.5	4	9	2			2				
E7.5	6	11		4		8	2			
E8.5	7	11		2		5	2			1
Gross morphology										
E7.5				22			6			
E8.5				17			14			1
E9.5	3	11				5				

The genotyping data do not correspond to a random sample of embryos. However, if only a subset of the data from complete litters are considered, the expected Mendelian ratios are observed (not shown).

(N.D.) Genotype not determined.

At E6.5, the *Brca1* mutant embryos were two-layered cylinders that were about half of the normal size (Fig. 1, cf. C and D). Although the boundary between embryonic and extraembryonic regions was poorly demarcated, differentiation of the primary endoderm into columnar and squamous epithelium had occurred. The embryonic portion was disproportionately smaller (Fig. 1D) and, in contrast to the normal embryos, the mutants lacked amniotic folds. The primary giant cells associated with the mutant embryos were prominent and very large (at the upper size limit of the normal range). Of four *Brca2* mutant embryos examined at this age, two were indistinguishable from their normal littermates, whereas the other two were slightly shorter-than-normal egg cylinders with expanded proamniotic cavities (see Fig. 1E). This reduction in size was disproportionate for the extraembryonic and embryonic regions, the latter being affected more severely, as in the case of the *Brca1* nullizygotes.

The normal embryos that were examined at E7.5 had gastrulated and possessed a third (mesodermal) germ layer and, with one exception (1/22 in the *Brca1* series), fused amniotic folds (Fig. 1F). Some of the normal embryos in the *Brca1* series and all of the normal embryos in the *Brca2* series exhibited head-folds. The *Brca1* nullizygotes were small egg cylinders that lacked amniotic folds, whereas presence of mesoderm was not readily visible. However, large primary giant cells, a small ectoplacental cone, Reichert's membrane with distal endoderm,

extraembryonic ectoderm and columnar endoderm, and a meager epiblast and squamous endoderm surrounding a proamniotic cavity containing some pyknotic nuclei and cell debris were discernible (Fig. 1G). The phenotype of one of the mutants was more severe. It consisted of a loose network of giant cells, and a cluster of cells surrounded by a small sac with the appearance of distal endoderm (data not shown). In contrast to the *Brca1* mutants, the *Brca2* nullizygotes were clearly three-layered egg cylinders with mesoderm present in the amniotic folds and in the disproportionately reduced embryonic region (Fig. 1H). However, the amniotic folds were not fused and the proamniotic cavity, containing cellular debris and some pyknotic nuclei, communicated with the ectoplacental cavity. The embryos were surrounded by large giant cells and had a prominent ectoplacental cone and extraembryonic ectoderm. Despite the presence of pyknotic nuclei in the proamniotic cavities of some *Brca1* or *Brca2* embryos that occasionally exceeded the level seen in controls, signs of excessive cell death in embryonic regions were not observed in any of the mutants at this or any other age.

At E8.5, the normal embryos in the *Brca1* series had head-folds and up to eight somites, whereas five genotyped *Brca1* mutants were variable in shape and developmental stage (Fig. 1I–K). One of them consisted only of giant cells, whereas in the embryonic region of three other mutants, the columnar cells of the stubby epiblast were surrounded by squamous endoderm, but there was

Figure 1. Histological comparisons of normal and *Brca1* and *Brca2* nullizygous embryos. Sections of normal embryos (n) and *Brca1* (b1) and *Brca2* (b2) mutants are displayed in five columns corresponding to the embryonic ages indicated on top. (A) Normal (*Brca1* heterozygous) elongating egg cylinder with the beginning of a proamniotic cavity (pac). In this and subsequent panels, an arrowhead marks the boundary between the extraembryonic and embryonic regions. At this boundary, the endoderm changes from columnar to squamous epithelium. (B) *Brca1* nullizygous embryo. Endoderm surrounding the primitive ectoderm is evident, but the egg cylinder has not elongated and there is not yet any distinction between the extraembryonic and embryonic regions. (C) Wild-type egg cylinder with proamniotic cavity (pac); ectoplacental cone (epc). (D,E) *Brca1* and *Brca2* nullizygous egg cylinders with prominent proamniotic cavities. (F) *Brca1* heterozygous embryo with three distinct germ layers: (m) mesoderm; (am) amnion; (ch) chorion; and (ys) yolk sac. (G) *Brca1* nullizygous embryo. There are no amniotic folds and the proamniotic cavity, which contains cellular debris, does not extend to the proximal region of the extraembryonic ectoderm. (H) *Brca2* nullizygous embryo. The

amniotic folds (af) have formed, but not fused (*). Mesoderm (m) is evident in both the embryonic and extraembryonic regions. Cellular debris is present in the proamniotic cavity. (I) An arrow indicates the remains of a severely affected *Brca1* nullizygous embryo consisting of giant cells and a mass of undifferentiated tissue. A large eosinophilic mass (e) is present mesometrially. (J) A less severely affected *Brca1* nullizygous embryo. A loose network of giant cells (gc) surrounds Reichert's membrane and distal endoderm. The extraembryonic ectoderm (ee) has the appearance of a solid cellular mass, although a chorion (ch) has formed and extraembryonic mesoderm is present. In the embryonic region, the epiblast and endoderm surround a small amniotic cavity. (K) Sagittal section of the most advanced *Brca1* nullizygote observed in this study. The heart (h), head-fold (hf), posterior neural plate (*) and extraembryonic membranes are shown. An eosinophilic mass (e) is present antimesometrially. (L) *Brca2* nullizygous embryo showing development of an ectoplacental cone (epc) and amniotic folds (af), but with a persistent proamniotic canal (*). The embryonic region is underdeveloped, but contains mesoderm (m). (M) Transverse section of a *Brca2* nullizygous embryo with an allantois (al), a diffuse amnion (am), and three germ layers in the underdeveloped embryonic region. (N) Sagittal section of a wild-type embryo with head-folds (hf), heart (h), somites (s), neural plate (*), and the allantois (al) attached to the chorion (ch). Scale bar, 100 μ m (A,B); 180 μ m (C-E); 250 μ m (F-J,L); 400 μ m (K,M); and 500 μ m (N).

no visible mesoderm. However, extraembryonic mesoderm was detectable, whereas the extraembryonic ectoderm, instead of being hollow, had the appearance of a solid cellular mass (Fig. 1J). In two of these mutants a distinct chorion was formed, therefore creating a small ectoplacental cavity. The ectoplacental cone was very small with little or no placental development. The embryos were surrounded by a loose network of giant cells with large nuclei (Fig. 1J,K). The fifth *Brca1* mutant was more advanced than any other examined; it contained an expanded yolk sac with blood islands, an amnion and an allantois, whereas the embryonic region, although severely reduced in size, was quite organized and had head-folds, a neural tube closed in one region, and a heart (Fig. 1K). The normal embryos in the *Brca2* series had turned and contained somites (Fig. 1N), whereas the *Brca2* nullizygotes were much smaller and lacked somites (Fig.

1L,M). Despite their reduced size, all of these mutants possessed embryonic mesoderm and had an anterior/posterior axis. Several of them were forming head-folds and exhibited rudimentary heart differentiation. All mutant embryos had a yolk sac with blood islands, a prominent allantois, and a well-developed chorion. The development of the amnion, however, was poor, with a loose mesodermal layer (Fig. 1M), and, in one of the mutants (1/7), the proamniotic canal persisted (Fig. 1L). The embryos were surrounded by giant cells and had developing placentas with ectoplacental cones.

Gross morphological analysis was consistent with the previous observations and indicated that at E9.5 the range of phenotypes of *Brca1* mutants was similar to that observed at E8.5, except that the most advanced embryos had developed an axial embryonic region and possessed an allantois and an expanded yolk sac with blood islands

(Table 1). In contrast to this phenotypic range of *Brca1* mutants, the E9.5 *Brca2* nullizygotes were all similar, and exhibited a reduced embryonic region with head-folds, a large allantois, and an expanded yolk sac with visible blood. One of these mutants had three to four somites, whereas another had a beating heart.

Of 60 genotyped *Brca1* mutant embryos, nine (15%) were associated with eosinophilic masses present in the implantation crypt either on the mesometrial or on the antimesometrial side, which were infiltrated with maternal neutrophils and possibly with endothelial cells (see Fig. 1I,K). This material had the appearance of a fibrin-like deposit probably formed after hemorrhage of uterine vessels. Twenty additional eosinophilic masses were associated with nongenotyped abnormal embryos or with empty decidua. In the *Brca2* series, on the other hand, the appearance of these deposits was very infrequent, and observed in association with a single empty deciduum and only 1/32 (3%) genotyped *Brca2* mutant embryos.

Cell proliferation assay in *Brca2* mutants

Previously, the growth deficiency of *Brca1* nullizygotes was correlated with decreased cell proliferation (Hakem et al. 1996; Liu et al. 1996). To ascertain whether the same occurs in *Brca2* mutants, pregnant females from heterozygous crosses were injected with 5-bromo-2'-deoxyuridine (BrdU) 1 hr before sacrifice, and decidua recovered at E6.5 and E7.5 were processed for histology. Incorporation of BrdU into DNA was assayed by counting labeled and unlabeled nuclei in the ectoplacental cone, the extraembryonic region and the embryonic region in representative sagittal sections close to the midline, whereas other sections were used for genotyping. Statistical differences in the averaged percentages of BrdU-positive nuclei were not detected between the three embryonic regions examined either in mutants or in controls (Student's *t*-test; $P < 0.05$). Therefore, for comparison, overall averages for the embryos of each group were used. At E6.5, there was no difference between the mean values of the two groups; $67.5 \pm 2.7\%$ and $68.4 \pm 1.8\%$ of the nuclei were labeled in controls and mutants, respectively (three genotyped embryos in each group). The percentage of BrdU-positive cells was within the normal range even in one of the embryos of the latter group that was morphologically distinguishable as a mutant. At E7.5, however, the corresponding values were significantly different: $67.6 \pm 2.3\%$ and $52.3 \pm 2.0\%$ for controls and mutants, respectively (two embryos in each group). We conclude that, at the time when the *Brca2* phenotype becomes unequivocally detectable by morphology, growth retardation is correlated with relative hypoproliferation.

Expression of marker genes

Previous reports suggested that the *Brca1* mutants fail to form mesoderm (Hakem et al. 1996; Liu et al. 1996). In

our morphological analyses, however, extraembryonic mesoderm was detectable, whereas mesoderm in the embryonic region was present in the most advanced mutants (see Fig. 1K). To investigate further the differentiation of primitive streak and mesoderm, we used whole-mount in situ hybridization to examine E7.5 *Brca1* mutants and control littermates for the expression of the gene *Brachyury* (*T*), an early marker of mesodermal cells (Fig. 2A). At this stage, *T* is expressed in the primitive streak and the head process (see Wilkinson et al. 1990; Herrmann 1991), and all 10 wild-type embryos that were examined exhibited this pattern of labeling. In contrast, only 3/5 mutants examined were labeled with the *T* probe in a region corresponding to the primitive streak, exhibiting variable extent and intensity of signal that was commensurate with the degree of their development (see Fig. 2A). Parallel examination of E8.5 *Brca2* mutants, in which mesoderm is morphologically detectable, verified the expected expression of *T* in the primitive streak and the node (Fig. 2A).

We also examined E7.5 *Brca2* mutants and E8.5 *Brca1* and *Brca2* mutants for expression of *Oct-4* transcripts encoding a POU transcription factor (Fig. 2B). In postimplantation embryos, *Oct-4* is specifically expressed in

Figure 2. Expression of *Brachyury* (*T*) and *Oct-4* genes examined by whole-mount in situ hybridization. (A) Shown from left to right are: A normal (n) E7.5 embryo expressing *T* (brown stripe) in the primitive streak (ps) and the head process (hp); an E7.5 *Brca1* (b1) null mutant showing a posterior stripe of *T* expression (arrow); an E8.5 *Brca2* (b2) mutant embryo (side view) with yolk sac removed (expression of *T* is detectable in an axial stripe along the length of the embryo; the arrow indicates the allantois); and an E8.5 *Brca2* mutant embryo (posterior view) with a stripe of *T* expression (arrowhead). (B) Shown from left to right are: An E7.5 normal embryo with evident expression of *Oct-4*; an E8.5 normal embryo, in which the *Oct-4* expression has become undetectable; an E8.5 *Brca1* mutant embryo; and an E7.5 and two E8.5 *Brca2* mutant embryos. The level of *Oct-4* expression remains high in the epiblast of all mutant embryos. Scale bar, 350 μ m (A) and 600 μ m (B).

the epiblast at high levels, but, as development progresses, the level of expression declines, and after E8.5 becomes restricted to the primordial germ cells (Schöler et al. 1990; Yeom et al. 1996). All of the normal specimens examined showed this characteristic pattern of labeling, including confinement of signal to germ cells in the most advanced embryos. In the developmentally retarded mutant embryos of both types, however, *Oct-4* signal remained positive in the embryonic portion of the egg cylinders (Fig. 2B).

In vitro outgrowth of blastocysts

Our histopathological analyses indicated that the *Brca1* and *Brca2* null mutations are first manifested with developmental delays at E5.5 and E6.5, respectively, and subsequently result in dysmorphogenesis and embryonic death. To complement these data, we examined E3.5 blastocysts that were cultured *in vitro* for four or five days and subsequently genotyped. As shown in Table 2, the absence of *Brca1* is associated with a failure of the inner cell mass (ICM) to grow and survive in outgrowths, although an exception was noted in one of 10 developing mutant blastocysts. Abnormal ICM development became evident in some *Brca1* mutants after only two days in culture, when the ICM appeared smaller than normal. Subsequently, these small ICMs diminished further in size and many disappeared by four or five days in culture, leaving behind a monolayer of trophoblast cells with large nuclei (Fig. 3, cf. B with A). In contrast, only a small proportion of wild-type and heterozygous blastocysts exhibited small ICMs, and only one of these outgrowths lost its ICM entirely after four days of culture.

The absence of *Brca2* was also associated with abnormal ICM development evident in some mutants as early as two days in culture, but some of the nullizygous outgrowths appeared normal until day four or five. The most striking feature of the mutant phenotype was a loss of cohesiveness in the ICM, resulting in an irregular appearance and a reduction in size (Fig. 3D). In a few cases, the ICMs of mutants had disappeared by five days in culture. In contrast, the ICMs of wild-type and heterozygous outgrowths were generally large and cohesive

Figure 3. *In vitro* blastocyst outgrowths after five days in culture. (A) Wild-type outgrowth from the *Brca1* series with an ICM and a monolayer of trophoblast giant cells. (B) *Brca1* nullizygous outgrowth lacking an ICM. (C) Wild-type outgrowth from the *Brca2* series with a well-developed ICM and trophoblast giant cells. (D) *Brca2* nullizygous outgrowth showing a small and disorganized ICM. The difference in appearance between controls (A, C) reflects variation between experiments.

(Fig. 3C), with only two of them acquiring a disorganized appearance similar to that of the *Brca2* mutants.

It is apparent from these results that the abnormal development of outgrowths was more severe in *Brca1* than in *Brca2* nullizygotes. Although the earliest time of onset did not differ between the two cases, the average time of onset was earlier by one day for *Brca1*. Moreover, the ICM cells died and disappeared in the majority of the *Brca1* mutants by four to five days, whereas some ICM cells survived in most of the *Brca2* mutants. In both cases, however, there was an apparent paucity of small trophoblast cells, although large giant cells were present.

Phenotype of Brca1/Brca2 double nullizygotes

To examine the combined effect of the *Brca1* and *Brca2* null mutations, we intercrossed *Brca1*(+/-)/*Brca2*(+/-) double heterozygotes and recovered 51 decidual swellings at E9.5, which were dissected, examined morphologically, and then genotyped by Southern analysis (Table 3). Nine of the decidua were empty, whereas among the remaining 42, all nine expected genotypes were recovered at expected frequencies ($\chi^2 = 13.2$, 8 df, $P > 0.05$). Embryos with at least one wild-type allele of each gene were normal and had 25–30 somites, whereas embryos lacking only *Brca1* or only *Brca2* exhibited phenotypic features characteristic for each mutation (see Fig. 4A–C and Table 3). Two of the recovered decidua contained double nullizygous mutants. One of these was an embryo with an allantois and a severely reduced embryonic region without head-folds (Fig. 4C), whereas the other consisted of a yolk sac and giant cells only. Therefore, in comparison with the two classes of single nullizygotes in terms of the degree of development, embryos

Table 2. *In vitro* outgrowth of blastocysts after 4 days in culture

	Genotype					
	<i>Brca1</i>			<i>Brca2</i>		
	+/+ or +/-	-/-		+/+ or +/-	-/-	
Total number examined	31	10		36	10	
Normal	28	1		33	2	
Small ICM	2	4		1	3	
Disorganized ICM				2	5	
No ICM	1	5				

Combined data from two experiments in each case.

Table 3. Phenotypes of E9.5 embryos from intercrosses between *Brca1* (+/-)/*Brca2*(+/-) double heterozygotes

		Genotype								
		wild type				<i>Brca2</i> null		<i>Brca1</i> null		double null
		<i>Brca1</i> → <i>Brca2</i> →	+/+	+/-	+/-	+/-	+/-	-/-	-/-	-/-
Phenotype (most advanced feature)	Total number ^a		19			12		9		2
Normal (25–30 somites)			19							
Allantois, small head folds						4				
Reduced embryonic region (± allantois, no head folds)						7		4		1
Giant cells and yolk sac only								2		1
Giant cells only						1		3		

^aNine empty decidua were also recovered.

lacking both *Brca1* and *Brca2* fell within the range of *Brca1* null mutants.

Phenotype of *Brca1*/p53 and *Brca2*/p53 double nullizygotes

To ascertain genetically whether a relationship between *Brca1* and p53 exists, as inferred previously from molecular analyses of *Brca1*^{5–6} nullizygotes (Hakem et al. 1996), embryos from crosses producing litters that included

Brca1/p53 double nullizygotes were examined morphologically (Table 4). At E8.5, most of the embryos that possessed at least one wild-type allele of each gene or were lacking only p53 were normal and contained three to five somites, whereas one embryo in each category was less advanced. As expected, the embryos lacking only *Brca1* were morphologically typical for the mutation, exhibiting a greatly reduced embryonic region, an allantois, and blood islands in the yolk sac. Interestingly, all five *Brca1*/p53 double nullizygotes recovered at this

Figure 4. Gross morphology of E9.5 (A–D) and E10.5 (E) embryos derived from intercrosses between *Brca1*/*Brca2* double heterozygotes (A–C), between *Brca1*/p53 double heterozygotes (D), or between *Brca2*/p53 double heterozygotes and *Brca2*(+/-)/p53(-/-) mice (E). (A) Normal (double heterozygous) embryo (n) and *Brca1* (*b1*) nullizygous mutant (heterozygous for *Brca2*). (B) A *Brca2* (*b2*) nullizygous embryo (wild-type for *Brca1*) with a yolk sac containing blood islands. The yolk sac has been opened to reveal head-folds (arrowhead) within the expanded amnion. A well-developed allantois is indicated with an arrow. (C) A *Brca1*/*Brca2* (*b1/b2*) double nullizygous embryo is shown in the middle and is flanked by two *Brca1* nullizygotes (wild-type for *Brca2*) that exhibit a similar extent of development. (D) On the left are two p53 nullizygous embryos (wild-type and heterozygous for *Brca1*, respectively); the second embryo has open neural folds. These are compared with a *Brca1*/p53 (*b1/p53*) double nullizygous littermate, which has developed much further than any *Brca1* single mutant observed. Three *Brca1* single mutants are shown on the right. (E) The normal embryo on the left (*p53*) is a p53 nullizygote (heterozygous for *Brca2*). The two embryos in the middle (*b2*; variable phenotypes) are *Brca2* nullizygotes (heterozygous for p53). The embryo on the right is a double nullizygote (*b2/p53*) and exhibits exencephaly. Scale bar, 1200 μ m (A,D,E) and 600 μ m (B,C).

Table 4. Results of intercrosses between *Brca1* (+/-)/*p53*(+/-) double heterozygotes and of crosses between double heterozygotes and *Brca1*(+/-)/*p53*(-/-) mice

Phenotype (most advanced feature)	Genotype									
	wild type				<i>p53</i> null		<i>Brca1</i> null		double null	
	<i>Brca1</i> → <i>p53</i> →	+/+	+/+	+/-	+/-	+/+	+/-	-/-	-/-	-/-
A. E8.5										
Total number ^a		7				10		3		5
Normal (3–5 somites)		5				9				
Normal (head fold; 0–3 somites)		1								4
Retarded (egg cylinder)						1				1
Reduced embryonic region								3		
Yolk sac only		1								
B. E9.5										
Total number ^b		22				17		11		4
Normal (15–20 somites)		20				13				
Exencephalic ^c (15–20 somites)		1				3				
Retarded (5 somites)						1				
Abnormal head fold (1–8 somites)										4
Reduced embryonic region (allantois, streak)								7		
Reduced embryonic region (egg cylinder)								2		
Giant cells and yolk sac only								2		
Giant cells only		1								

^aAn additional embryo consisting of giant cells only was not genotyped. Three empty decidua were also recovered.

^bTwo empty decidua were also recovered.

^cExencephaly (i.e., protrusion of the brain above the skull) results from failure of neural tube closure. The term is used here for embryos exhibiting open midbrain and hindbrain.

age were more advanced than their *Brca1* null littermates. One of them was an egg cylinder with a constriction in the junction between the embryonic and extra-embryonic regions, whereas the remaining four, although developmentally retarded in comparison with their most advanced normal littermates, appeared to be normal and had reached the head-fold stage. Two of these four embryos possessed one to three somites. At E9.5 (see Fig. 4D and Table 4), most of the embryos possessing at least one wild-type allele of each gene or lacking only *p53* were normal with 15–20 somites, whereas a minority of these embryos were exencephalic (exhibiting an open and flattened midbrain and hindbrain). The occurrence of exencephaly in a subset of *p53* nullizygotes has been reported previously (Armstrong et al. 1995; Sah et al. 1995). We also observed a retarded *p53* nullizygote with only five somites, whereas only giant cells were recovered from another embryo (heterozygous for *Brca1* and wild-type for *p53*). All embryos lacking *Brca1* exhibited a range of typical abnormal phenotypes, whereas four *Brca1/p53* double nullizygous mutants were further advanced than any of the embryos lacking only *Brca1*, although they were overtly retarded and abnormal in comparison with the controls and the *p53* nullizygotes. The heart, a few somites, and large

head-folds had formed in the double mutants, whereas the neural folds had a zigzag or kinky appearance. In several of the embryos the allantois was fused with the chorion.

A similar analysis was performed by examining litters of embryos that included *Brca2/p53* double nullizygotes, to determine if there is also a relationship between *Brca2* and *p53* (Table 5). The majority of E9.5 embryos possessing at least one wild-type allele of each gene or lacking only *p53* were normal with 15–25 somites, although some of them were exencephalic. Of eight embryos lacking *Brca2* (all heterozygous for *p53*), two were smaller than their most advanced littermates, but otherwise normal with 12–20 somites, whereas the remaining six were abnormal with variable phenotypes. Two double nullizygous embryos recovered at this age were similar to the most advanced *Brca2* single mutants, except that they exhibited exencephaly. At E10.5, all of the embryos with at least one wild-type allele of each gene and most of the embryos lacking only *p53* were normal with more than 30 somites, whereas a few *p53* nullizygotes were exencephalic. All five embryos lacking only *Brca2* (heterozygous for *p53*) were abnormal, ranging from fairly well-formed embryos about one third the size of normal littermates to retarded, head-fold stage em-

Table 5. Results of crosses between *Brca2*(+/-)/*p53*(+/-) double heterozygotes and *Brca2*(+/-)/*p53*(-/-) mice

Phenotype (most advanced feature)	Genotype							
	wild type				<i>p53</i> null		<i>Brca2</i> null	double null
	<i>Brca2</i> → <i>p53</i> →	+/+	+/-	+/-	+/+	+/-	-/-	-/-
A. E9.5								
Total number ^a		27			24		8	2
Normal (15–25 somites)		23			14			
Exencephalic (15–25 somites)		1			9			
Small (12–20 somites)							2	
Small, exencephalic (10–20 somites)								2
Retarded, half-turned (8–12 somites)		2			1		1	
Retarded, head-fold (0–6 somites)							4	
Yolk sac with reduced embryo							1	
Giant cells only		1						
B. E10.5								
Total number ^b		15			16		5	5
Normal (30+ somites)		15			13			1
Exencephalic ^c (20–30+ somites)					3			
Retarded (10–20 somites)							2	
Retarded, exencephalic (10–20 somites)								4
Retarded, head-fold stage (turned/part turned)							3	

^aOne empty deciduum was also recovered.^bThree empty decidua were also recovered.^cThe frequency of exencephalic *p53* null embryos was higher in this study than observed previously (Armstrong et al. 1995; Sah et al. 1995), although the sex distortion (association of exencephaly predominantly with female embryos) was not as dramatic as reported (Armstrong et al. 1995).

bryos (see Fig. 4E and Table 5). Of five double nullizygous embryos, one was indistinguishable from wild type, whereas four were similar to or somewhat more developed than the most advanced *Brca2* single mutants, except that they exhibited exencephaly (see Fig. 4E and Table 5). All but one of the latter embryos had a heartbeat, one had a distended pericardium, and in two of them there was blood stasis.

Discussion

Comparison of the *Brca1* and *Brca2* null phenotypes

It was shown previously that *Brca1* has an indispensable role in early mouse embryogenesis (Hakem et al. 1996; Liu et al. 1996). While confirming this *Brca1* function with a different deletion mutation, we showed that *Brca2* is also an essential gene acting during the same period of development. Although both the *Brca1* and *Brca2* null mutations result in postimplantation embryonic lethality, the onset of growth and morphogenetic retardation from the lack of *Brca1* occurs one embryonic day earlier. Thus, all *Brca1* mutants are compromised at least as early as E5.5, whereas the *Brca2* nullizygotes are not distinguishable from wild type until E6.5 or later. This temporal difference in the onset of detrimental ef-

fects caused by these mutations is also reflected in the development of outgrowths of cultured blastocysts. Another difference in the manifestations of the two mutations is that the range of abnormal phenotypic features detected at a particular age is much wider for *Brca1* than for *Brca2*. In addition to their morphological variability at any given age, the *Brca1* mutants die at any time between E5.5 and E9.5 or shortly thereafter, assuming that most, if not all, of the observed empty decidua correspond to resorptions of mutant embryos. Nevertheless, particular *Brca1* null embryos morphologically very similar to *Brca2* mutants can be observed at all ages. The *Brca2* nullizygotes, on the other hand, are much more uniform in morphology until E8.5, becoming variable later. Moreover, there is no indication that these mutant embryos die before E8.5, and their placental development is not as severely compromised as in *Brca1* mutants.

The phenotypic characteristics of the *Brca1*^{ex2} mutation are overall similar to those reported for *Brca1*⁵⁻⁶ nullizygotes (Hakem et al. 1996) and also for nullizygotes carrying a deletion in the 5' terminal region of *Brca1* exon 11 (Liu et al. 1996). However, mesoderm formation, which does occur in a subset of *Brca1* nullizygotes according to our observations, was not detected in these less extensive morphological analyses, and the

high incidence of eosinophilic masses associated with the mutants was not reported. On the other hand, these data (Hakem et al. 1996; Liu et al. 1996; this study), all obtained with 129 × C57 hybrids, differ significantly from the results obtained with another *Brca1* gene knockout (Gowen et al. 1996). Lethality was also observed, but the phenotype was less severe, and abnormal embryos exhibiting neural tube defects developed up to E13.5. It remains to be seen whether this difference can be attributed to effects of a different genetic background that included a DBA strain component (Gowen et al. 1996). However, genetic components of the CD1 and C3H/HeJ strains (Hakem et al. 1996), and also of the MF1 strain (T. Ludwig, D.L. Chapman, V.E. Papaioannou, and A. Efstratiadis, unpubl.), did not alter the *Brca1* null phenotype.

Although embryonic lethality was observed in all of the *Brca1* mutants, a 32-year-old patient was described with inherited homozygosity for a *BRCA1* mutation analyzed only at the DNA level, which would result in mature mRNA encoding a truncated protein of 900 amino acids (Boyd et al. 1995). Whether this difference reflects *BRCA1* functional divergence in humans and mice remains an open question, because it is unknown whether the mutations are directly comparable (the longest peptide that could be synthesized in one of the mouse mutants would have only 300 *Brca1* residues; Liu et al. 1996). Nevertheless, a point of concern is that mammary tumors did not appear yet in *Brca1* heterozygotes that have reached the age of 15 months. This may mean that, as in the case of *Wt-1* (Kreidberg et al. 1993), absence of *Brca1* is not a tumor predisposing mutation in mice. On the other hand, very long latency, as that observed with *Nf1* null mice (17–27 months; Jacks et al. 1994a) cannot be excluded.

The *Brca1* and *Brca2* developmental pathways

The temporal overlap of *Brca1* and *Brca2* gene actions, the predominant features of growth retardation and dysmorphogenesis that the two phenotypes share, and the observations made with *Brca1/Brca2* double mutants raise the possibility of a relationship between the functions served by the two genes. Consistent with this hypothesis, the spatial and temporal pattern of *Brca2* expression during mouse embryonic development and in adult tissues is strikingly similar to that of *Brca1* (Connor et al. 1997; and data cited by Rajan et al. 1996). Moreover, *BRCA1* and *BRCA2* are similarly induced in a cell cycle-dependent fashion at the G₁/S boundary (Y. Chen et al. 1996; Gudas et al. 1996; Vaughn et al. 1996a,b; Rajan et al. 1996) and both mRNAs are coordinately elevated by estrogen in breast cancer cell lines (Spillman and Bowcock 1996). This apparently coordinate regulation suggests that similar control mechanisms may be involved. Interestingly, a sequence comparison between the *BRCA1* and *BRCA2* promoter regions led to the identification of two homologous segments with similar spacing upstream from the transcriptional start sites that

are conserved between human, mouse, and rat DNA (cited by Vaughn et al. 1996b). Finally, it seems hardly coincidental that both genes encode suppressors of mammary tumors, although the differences between *BRCA1* and *BRCA2* germ-line mutations in the manifestation of ovarian, pancreatic, and male breast cancers cannot be ignored.

The relatively low frequency of obtaining double mutants (1/16) allowed us to examine only two *Brca1/Brca2* nullizygotes thus far. Nevertheless, although interpreting these results with caution, we believe that at least some tentative conclusions can be reached by considering that detection of double nullizygotes was possible at the advanced age of E9.5, and that their phenotype fell within the range of *Brca1* single mutants. Thus, the *Brca1* mutation could be epistatic over the *Brca2* mutation. A mutation is defined as epistatic over another, when the phenotypes of the single mutants differ from each other and from the wild-type, but the double mutant resembles phenotypically one of the single mutants (see Avery and Wasserman 1992). Although this relationship does not imply an interaction between pathways, we favor a model postulating that the *Brca1* and *Brca2* pathways are linked and may be overlapping to some degree. The competing hypothesis that the two pathways are parallel and functionally completely independent cannot be excluded, but appears to be less likely. Had that been the case, the double nullizygotes should have exhibited additive effects of the mutations, as they are both associated with growth retardation.

Interestingly, examination of seven grade 3 infiltrating ductal breast carcinomas from members of a breast/ovarian cancer family known to carry a *BRCA1* mutation indicated that five of these tumors also exhibited LOH in the region of the *BRCA2* locus (Kelsell et al. 1996). An independent report described the case of a patient diagnosed with a grade 3 ductal breast carcinoma and ovarian adenocarcinoma that were associated with germ-line mutations in both *BRCA1* and *BRCA2* (Ramus et al. 1997). Concordant LOH in the regions of both the *BRCA1* and *BRCA2* loci was also observed in 21 of 45 tumors of similar grade from sporadic breast cancer cases, whereas such an association was absent from grade 1 and 2 ductal carcinomas (Kelsell et al. 1996). These observations suggest a combined role for *BRCA1* and *BRCA2* in the tumorigenic pathway of grade 3 breast tumors.

Functional relationships between *Brca1* or *Brca2* and *p53*

It was shown previously that *mdm2* null embryos lacking a negative regulator of *p53* activity die before gastrulation, but are completely rescued in a *p53* null background (Jones et al. 1995; Montes de Oca Luna et al. 1995). Because inhibition of cell-cycle progression and promotion of apoptosis are included among the many diverse functions of *p53* (for review, see Gottlieb and

Oren 1996; Bates and Vousden 1996; Levine 1997; Hansen and Oren 1997), the lethality of the *mdm2* single mutants, which temporally coincides with an abrupt increase in cell-cycle rate at E5.5–E6.0 (Snow 1977), was attributed to their inability to down-regulate p53, resulting in growth arrest (Jones et al. 1995; Montes de Oca Luna et al. 1995). This interpretation is consistent with observations indicating that excessive p53 activity causes severe abnormalities (see, e.g., Godley et al. 1996), in contrast to the lack of p53 that does not usually perturb development, with the exception of some mutants exhibiting exencephaly (Armstrong et al. 1995; Sah et al. 1995; this study).

Molecular analyses of *Brca1*⁵⁻⁶ nullizygous embryos (Hakem et al. 1996) indicated that the level of p53 transcripts remained unaffected. However, an increase in p53 activity was not excluded, because the expression of the gene encoding one of the downstream effectors of p53, the p21 cyclin-dependent kinase inhibitor, was increased significantly with parallel reduction of *mdm2* transcript levels. In addition, BrdU incorporation assays showed that the mutant-to-control ratio of the percentage of labeled nuclei in the epiblast was reduced (Hakem et al. 1996; Liu et al. 1996), suggesting slow progression through the cell cycle. The *Brca2* nullizygotes that we examined also exhibited a decreased proliferative ability as assayed by BrdU labeling.

However, the *Brca1* and *Brca2* mutants differ significantly in phenotypic features from *mdm2* nullizygotes according to our data, and as our genetic analysis demonstrated their development is shifted to more advanced stages when p53 is absent, but complete rescue of the mutations does not occur. The results were clear-cut in the case of *Brca1/p53* double mutants, which did not overlap phenotypically with the severely retarded *Brca1* single mutants, whereas the impact of the *Brca2* mutation in the absence of p53 was ameliorated to various degrees. Thus, one of the *Brca2/p53* double nullizygotes was indistinguishable from wild type, whereas others were similar to or further developed than the most advanced *Brca2* single mutants.

Interestingly, the early embryonic lethal phenotype of embryos nullizygous for the mouse *rad51* gene, which encodes a homolog of the *Escherichia coli* RecA and yeast ScRad51 proteins involved in recombinational repair of double-strand breaks in DNA, was also partially ameliorated in a p53 null background (Lim and Hasty 1996). Wild-type p53 forms complexes with human Rad51 (hRad51) in vivo and is able to inhibit RecA activities in vitro (Stürzbecher et al. 1996). hRad51, which apparently participates in a complex of RNA polymerase II (Maldonado et al. 1996), was shown recently to interact with BRCA1 in vitro and to co-localize with BRCA1 in nuclear foci of cultured cells during the S phase implying a functional relationship that could be related to the maintenance of genome integrity (Scully et al. 1997). It remains to be seen whether the Brca1 BRCT domain, which is present in several proteins with cell-cycle checkpoint functions (Bork et al. 1997; Callebaut and Mornon 1997), interacts directly with p53, like the simi-

lar motif used for p53-binding by 53BP1 (Iwabuchi et al. 1994; Bork et al. 1997).

Whatever the real mechanistic aspects of the Brca1 or Brca2 connections to p53 may be, they do not appear to be related to p53-mediated apoptosis, because increased cell death is not a feature of *Brca1* and *Brca2* mutants (Hakem et al. 1996; Liu et al. 1996; this study). On the other hand, a p53-mediated elongation or arrest of the cell cycle in the absence of Brca1 is consistent with the available evidence (Hakem et al. 1996), and a simple hypothesis postulating that Brca1 (or Brca2) and p53 have antagonistic functions cannot be excluded. However, considering the interactions of Rad51 with p53 and BRCA1, and the high incidence of spontaneous DNA damage in cells (see Carr and Hoekstra 1995), an alternative conjecture is that the *Brca1* null phenotype is the consequence of a failure of proposed surveillance functions (Scully et al. 1997). For example, if Brca1 is related to recombinational repair of double-strand breaks, which are known to initiate a p53 response (see Hansen and Oren 1997; Levine 1997), absence of Brca1 will result in posttranscriptional induction of p53 activity causing a cell-cycle block. Even if correct, this view is most probably an oversimplification. Nevertheless, it can account for the variability in growth retardation and dysmorphogenesis characterizing the *Brca1* null phenotype, considering that spontaneous DNA damage should be occurring stochastically within a range of severity. This would result in various degrees of proliferative arrest affecting differentially growth and also patterning by variable impairment of the orderly sequence of morphogenetic events. When both Brca1 and p53 are absent, however, damaged cells could continue to proliferate for some time, and embryonic death would be shifted to a later developmental stage.

The absence of functional BRCA1 or BRCA2 in tumors on one hand, and the *Brca1* and *Brca2* gene knockouts in mouse embryos on the other, appear paradoxically to exert opposite effects on cell proliferation. In particular, BRCA1-associated breast cancers were found to be highly proliferating tumors (Eisinger et al. 1996), whereas the level of BRCA1 mRNA appeared to be markedly decreased during the transition from sporadic breast carcinoma in situ to invasive cancer (Thompson et al. 1995). Moreover, overexpression of BRCA1 inhibited the proliferation of breast and ovarian cell lines, whereas transfer of BRCA1 into MCF-7 breast cancer cells inhibited their ability to form tumors in nude mice (Holt et al. 1996). Conversely, antisense oligonucleotide-mediated reduction in BRCA1 expression increased the proliferation of mammary epithelial cells (Thompson et al. 1995). Therefore, it was thought that BRCA1 is involved in negative regulation of tumor cell proliferation. In contrast, a positive role of Brca1 in cell growth control was proposed for mouse embryos (Hakem et al. 1996). However, from the point of view of the hypothetical scenario described above, this paradox may be more apparent than real and related to epiphenomena. The inability to monitor DNA damage would be manifested as hypoproliferation in *Brca1* mutant embryos, whereas in tu-

mors, it could allow accumulation of additional mutations promoting abnormal overgrowth, if the lack of BRCA1 is involved in tumor progression, rather than initiation, as proposed (Scully et al. 1997).

Comparison of the *Brca1* and *pnlf5* mutant phenotypes

Comparison of our data with the features of other mouse mutations resulting in postimplantation lethality (see Copp 1995) showed that the *Brca1* nullizygous embryos exhibit a striking resemblance with the early embryonic lethal phenotype of homozygotes carrying the radiation-induced deletion mutation *5RD300H* or *se¹* (*short-ear lethal*; Russell 1971; Dunn 1972) that includes the *se* (*short-ear*) locus encoding Bmp-5 (bone morphogenetic protein 5; Kingsley et al. 1992) on mouse chromosome 9. The locus associated with the early embryonic lethality identified by the *se¹* mutation is designated *pnlf5* (prenatal lethal function 5; Imai and Kingsley 1994). The histological appearance of the *pnlf5* nullizygotes at around E8.0, which exhibit large giant cells, a severely reduced embryonic region, and an ectoplacental cavity filled with a solid mass of extraembryonic ectodermal cells (Dunn 1972), is almost indistinguishable in morphology from *Brca1* mutants at E8.5. The segment of mouse chromosome 9 where *pnlf5* resides is syntenic with the q12-13 region of human chromosome 6 (DeBry and Seldin 1996). Interestingly, the presence of a tumor suppressor gene at 6q13 has been postulated because of a high incidence of

allelic imbalance in this region in malignant breast tumors (Orphanos et al. 1995).

Materials and methods

Construction of replacement vectors

To isolate mouse clones carrying portions of the *Brca1* and *Brca2* genes, we screened a genomic λ phage library from strain 129/Sv using probes of corresponding human *BRCA1* (exons 1–6) and *BRCA2* (exon 11) sequences that were synthesized, respectively, by reverse transcription (RT)-PCR from HeLa cell poly(A)⁺ RNA and by PCR from DNA of a *BRCA2* cosmid clone (see Fischer et al. 1996). Eight *Brca1* overlapping clones containing exons 1–11 and four *Brca2* overlapping clones containing exon 11 were identified and characterized by restriction mapping, Southern analysis and DNA sequencing. Southern analysis of mouse genomic DNA indicated that, as in humans, the *Brca2* gene is unique in mice (see also Connor et al. 1997; Sharan and Bradley 1997). From subcloned fragments of the isolated clones, targeting vectors (Fig. 5) were constructed in several steps. In each construct, we included a neomycin resistance gene (*neo*) cassette (1.1 kb from plasmid pMC1neo polyA), for positive selection using the antibiotic G418, and a thymidine kinase (*tk*) gene cassette (1.85 kb from plasmid pMC1tk), to enrich for targeted clones by negative selection with gancyclovir. Both of these cassettes were placed in the same transcriptional orientation as that of the gene promoters. The final *Brca1* product consisted of a 5' genomic fragment (2.1 kb); the *neo* cassette replacing a 0.7-kb genomic fragment containing exon 2; a downstream genomic fragment (7.2 kb); and the *tk* cassette. The final *Brca2* vector consisted of a 5' genomic fragment (2.5

Figure 5. Targeting of the *Brca1* and *Brca2* genes. Restriction maps of the first three *Brca1* exons (E1–E3; solid rectangles) (a), and in the region of *Brca2* exon 11 (E11) (d). The restriction sites are: *EcoRI* (E), *PstI* (P), *EcoRV* (V), *NotI* (N), and *BamHI* (B). Diagrams of the targeting constructs used for gene disruption by homologous recombination (denoted by large X symbols) are shown for *Brca1* (b) and *Brca2* (e). The transcriptional orientations of the *neo* and *tk* genes are indicated with arrows. The wavy line represents plasmid vector sequence. The structures of the *Brca1* and *Brca2* loci after targeting (large open arrow) are shown in c and f, respectively. Examples of genotyping of E9.5 embryo DNA by *PstI* digestion are shown in autoradiograms of Southern blots. The sizes of fragments corresponding to wild-type and mutated alleles that are hybridizing with the indicated probes are correlated with the diagrams. The size markers are *HindIII* fragments of phage λ DNA.

kb); the *neo* cassette replacing a 0.52-kb fragment within exon 11; a downstream genomic fragment (9.1 kb); and the *tk* cassette.

Gene targeting and blastocyst injections

Linearized vector DNA was introduced by electroporation into passage 12 129/Sv ES cells (CCE-33) grown on mitomycin C-treated, G418-resistant STO cell feeder layers, and after drug selection, DNA of resistant clones was analyzed by Southern blotting using a *neo* probe and also probes with sequences corresponding to genomic DNA upstream from the 5' region of homology in the vectors (Fig. 5).

To generate chimeras, cells of targeted clones were microinjected into host blastocysts from the C57BL/6J mouse strain that were transferred into the uteri of pseudopregnant females. Chimeras were mated with C57BL/6 females, and germ-line transmission was verified by Southern analysis. Three independent targeted clones for *Brca1* and two for *Brca2* yielded germ-line transmitters. F₁ heterozygous animals were intercrossed and F₂ offsprings were analyzed by Southern blotting. Heterozygous males for *Brca1* were also crossed with females heterozygous for *Brca2* or heterozygous for *p53*, to derive double heterozygotes, which were subsequently intercrossed. The *p53* mutant mice (Jacks et al. 1994b) were obtained from the Jackson Labs.

Genotyping

For genotyping by Southern analysis with 5' flanking and/or *neo* cassette probes, DNA was prepared from yolk sacs, whole E9.5 and E10.5 embryos or the tail tip of two-week-old mice. Embryos were sexed by Southern blotting using a Y chromosome-specific probe (Bishop and Hatat 1987). For genotyping of embryos from stained histological sections by PCR amplification, we used a protocol that we developed previously (see Zeitlin et al. 1995). To identify *Brca1* mutants we used the oligonucleotides: (1) *Brca1* forward primer 5'-GGACGGCAGATAAATCCATTTCTTCC-3' (near the 3' end of intron 1); (2) *Brca1* reverse primer 5'-GGTACAAAGCCAGTGTGGGTACATG-3' (within exon 2); (3) *neo* forward primer 5'-GGAATGTTTCACCCAATGTCGAGC-3'; and (4) *neo* reverse primer 5'-CATCAGAGCCGATTGTCTGTTG-3'. The *Brca1* primers generate a 169-bp amplification product from wild-type and heterozygous embryos, whereas the *neo* primers amplify a 266-bp fragment in heterozygous and homozygous embryos. To identify *Brca2* mutants we used the primers 5'-GGGAACAGTGATTGTTTCAGACAAATGGTC-3' (forward) and 5'-GCTGTGATCTGTCTGACCTACAGAGG-3' (reverse) flanking the segment of exon 11 that is deleted in the mutants. Amplification of the wild-type allele results in a 589-bp product, whereas the product of the targeted allele is 1.2 kb, because of the insertion of the *neo* cassette.

Histological analyses

Whole uteri at E5.5 and decidua dissected at E6.5, E7.5, and E8.5 were fixed overnight in 4% paraformaldehyde, 0.1 M phosphate buffer (pH 7.3), washed for 24 hr at 4°C in 0.25 M sucrose, 0.2 M glycine, 0.1 M phosphate buffer (pH 7.3), dehydrated, and embedded in paraffin. Paraffin blocks were sectioned at 6 µm and stained with hematoxylin and eosin.

BrdU labeling of embryos

Labeling of cells in the S phase with BrdU was performed as described (Hayashi et al. 1988). BrdU (100 µg/gram of body

weight) was injected intraperitoneally into pregnant females at E6.5 and E7.5, which were sacrificed 1 hr after the injection. The decidua were dissected, fixed in 4% paraformaldehyde and processed for immunohistochemistry. Sections were incubated with a monoclonal anti-BrdU antibody (Becton-Dickinson) at a 1:20 dilution, and staining was performed as described (Mishina et al. 1995).

In situ hybridization

Whole-mount in situ hybridization was performed as described (Wilkinson 1992), using digoxigenin-labeled antisense RNA probes for *Brachyury* and *Oct-4* that were synthesized, respectively, from linearized plasmids pME685 (Herrmann 1991) and Oct-4Stu462 (Schöler et al. 1990).

Culture of blastocyst outgrowths

Pregnant females from heterozygous intercrosses were sacrificed at E3.5, and embryos were collected by flushing the uteri. Following removal of the zona pellucida with acidified Tyrode's solution, blastocysts were cultured individually in 24-well plates in Dulbecco's modified Eagle medium (DMEM) supplemented with 20% fetal calf serum (FCS) at 37°C in 5% CO₂ in air, and examined and photographed daily for four to five days. At the end of the experiment, the tissue was scraped off the dish, and DNA was extracted for genotyping by PCR.

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