Inactivation of the mouse *Brca1* gene leads to failure in the morphogenesis of the egg cylinder in early postimplantation development

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BRCA1 is proposed to be a tumor suppressor gene. To explore the biological function of **BRCA1**, a partial deletion (amino acids 300–361) of mouse *Brca1* exon 11 was introduced into the genome of embryonic stem (ES) cells by homologous recombination. Mice carrying one mutated allele of *Brca1* appear normal and are fertile up to 10 months of age without any sign of illness. However, no viable progeny homozygous for the *Brca1* mutant allele were obtained. Detailed analysis of large numbers of embryos at different stages of development indicated that the homozygous mutant concepti are severely retarded in growth as early as embryonic day 4.5 (E4.5) and are resorbed completely by E8.5. Although the homozygotes at E5.5–E6.5 are able to synthesize DNA and display distinguishable embryonic and extraembryonic structures, they fail to differentiate and form egg cylinders. Consequently, they were unable to form primitive streaks and undergo gastrulation. Consistent with these *in vivo* results, blastocysts homozygous for mutated *Brca1* alleles are at a considerable disadvantage when grown *in vitro*. These observations suggest that *Brca1* has an important role in the early development of mouse embryos.

[Key Words: *Brca1* gene; gene targeting; mouse embryogenesis]

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Hereditary breast cancer is characterized as an early-onset, bilateral disease and is associated with other tumors of ovarian, endometrial, and prostate origins (Tulinius et al. 1992; Anderson and Badzioch 1993; Sellers et al. 1994). The heterogeneity in breast cancer suggests that the manifestation of multiple genetic factors intertwines with environmental factors, such as hormones and diet, that modify expression of the phenotype. At the molecular level, breast tumor development is thought to result from mutations of several growth regulatory genes. The **BRCA1** gene is mutated in the germ line of a subset of families with inherited breast cancer, and loss of the remaining wild-type allele is often found in tumor tissues (Hall et al. 1990; Smith et al. 1992; Miki et al. 1994). This recessive nature of **BRCA1** fits with the properties of human tumor suppressor genes (Riley et al. 1994).

BRCA1 mutations are associated with 45% of familial breast cancer, which accounts for ~10% of the total number of these neoplasms. However, they are rarely linked with sporadic cases (Futreal et al. 1994). Therefore, the role of **BRCA1** in the pathogenesis of 95% of breast cancer is unclear. Interestingly, the **BRCA1** gene

product, which is normally a nuclear protein, is aberrantly mislocated in the cytoplasm of most breast tumor cell lines (Chen et al. 1995). Although the molecular mechanism for the failure of nuclear transport is not known, these findings suggest that **BRCA1** may be involved in many breast cancers, sporadic as well as familial.

BRCA1 is a large gene spread over ~100 kb of the genome. It consists of 24 exons and encodes a nuclear phosphoprotein of 1863 amino acids with a molecular weight of ~220 kD (Chen et al. 1995, 1996). **BRCA1** is a novel protein with an amino-terminal ring finger motif similar to those found in other proteins that interact with DNA, RNA, or proteins (Miki et al. 1994). The mouse **BRCA1** homolog, *Brca1*, was characterized recently. The predicted *Brca1* gene product shares ~58% amino-acid identity with the human protein, and the mRNA expression patterns in mice and humans are also quite similar. Interestingly, *Brca1* mRNA expression in mouse mammary gland involution parallels its differentiation, suggesting an important regulatory role for *Brca1* in tissue proliferation and differentiation (Lane et al. 1995; Marquis et al. 1995).

To understand the physiological function of *Brca1* and study its role in breast and ovarian carcinogenesis, we

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established mutant *Brca1*(+/-) mouse lines using gene targeting in embryonic stem (ES) cells. Mice lacking one wild-type allele of the *Brca1* appear normal and are fertile until 10 months of age. However, *Brca1*(-/-) mutants die during early embryogenesis. Histological examinations of early concepti and genotyping after fixation by microdissection-polymerase chain reaction (MD-PCR) demonstrated that *Brca1*(-/-) mutant embryos are growth-retarded as early as embryonic day (E4.5). They form embryonic and extraembryonic tissues but develop into severely disorganized masses incapable of forming egg cylinders at E5.5-E6.5. The embryos are resorbed completely by E8.5. These results reveal an important role for the *Brca1* gene in early mouse development.

Results

Targeted mutation of the mouse *BRCA1* homolog

For genetic ablation of mouse *Brca1*, exon 11 was chosen as the target because its counterpart in the human *BRCA1* is the largest coding exon (3425 bp) and is frequently mutated in families with histories of breast and ovarian cancer. Mouse *Brca1* was obtained by screening a 129/Sv-derived genomic library with PCR-amplified DNA from exon 11 of the human *BRCA1*.

The targeting vector was constructed by deleting a 184-bp *EcoRI* fragment corresponding to mouse *Brca1* amino acids 300-361 (Fig. 1A) and replacing it with a *pgkneopA* cassette in either the sense (s) or antisense (o) orientation. These two constructs were then subcloned into the p2TK vector to produce two final targeting vectors designated as *Brca1*-ko(s) and *Brca1*-ko(o), respectively (Fig. 1B). Both vectors were individually transfected into ES cells, and 384 colonies doubly resistant to G418 and FIAU were isolated. DNA from these colonies

was analyzed by Southern blotting to identify clones containing a disruption of the *Brca1* gene resulting from a targeted homologous recombination event. These were identified by the appearance of a novel *HindIII* fragment of the predicted size using a fragment of *Brca1* genomic DNA lying 3' external to the targeting vector as a hybridization probe (Figs. 1C and 2A). The use of *Brca1*-ko(s) and *Brca1*-ko(o) vectors resulted in a total of 6 and 13 homologous recombinant clones, respectively. *Brca1*-ko(o) #3 and *Brca1*-ko(s) #291 were analyzed further by Southern blot analysis using different probes to confirm the targeted gene disruption (Fig. 2B).

Production of *Brca1* heterozygote mutants by germ-line transmission

ES cells from clone *Brca1*-ko(o) #3 and *Brca1*-ko(s) #291 were injected independently into C57BL/6J blastocysts, which were then implanted into the uteri of pseudopregnant CBA female foster mice. We generated 5 germ-line chimeras from a total of 16 male chimeras, all derived from clone *Brca1*-ko(o) #3. Of the 120 offspring from male chimera/female C57BL/6J crosses, 53% were heterozygous (*Brca1* +/-) and 47% homozygous wild type (*Brca1* ++), as shown by PCR analysis of toe DNA samples. The heterozygous mice were further confirmed by Southern blot analysis of tail DNA samples (data not shown). Heterozygous animals appear normal, healthy, and are fertile for at least 10 months after birth.

Absence of wild-type *Brca1* alleles leads to embryonic lethality

To investigate the in vivo effect of the homozygous mutations, *Brca1*(+/-) mice were interbred. The genotypes of offspring were determined at 1 week of age by PCR

Figure 1. Strategy for the generation of a targeted mutation in the mouse *Brca1* gene. (A) Restriction map of the mouse *Brca1* fragment, encompassing exon 11 and flanking DNA. An 8.0-kb *HindIII*-*Bam*HI fragment was used to create the replacement targeting vector. (B) Restriction map of the targeting constructs *Brca1*-ko(s) and *Brca1*-ko(o). A 184-bp *EcoRI* fragment from the 5' end of exon 11 was deleted and replaced with a *pgkneopA* cassette in the sense and antisense orientation with respect to the *Brca1* gene. In addition, the genomic fragment was flanked by two pMC1-tk cassettes. (C) The predicted structure of a mutant allele after homologous recombination. The probes used for identification of allele-specific recombination are also shown. (D) The expected sizes of various restriction fragments detected by flanking and *neo* probes. Abbreviations: (RI) *EcoRI*; (RV) *EcoRV*; (B) *Bam*HI; (H) *Hind*III; (S) *Sall*; (X) *Xho*I.

Figure 2. Targeted disruption of the *Brca1* gene in mouse ES cells. (A) Initial screen of the targeted *Brca1* clones by Southern blotting analysis. The *HindIII* 4.6- or 3.0-kb restriction fragment indicated the expected recombinant allele by *Brca1*-ko(s) or *Brca1*-ko(o) vectors, respectively. (B) Confirmation of the targeted disruption. DNA samples from parental E14.1 cells and two candidate recombinant clones [*Brca1*-ko(o) 3 and *Brca1*-ko(s) 291] were digested with *HindIII* or *Sall*-*Bam*HI and probed with either of the flanking probes or the *neo* probe. An additional fragment of the expected size was found in each of the recombinant clones.

analysis of toe DNA samples. Of the 97 animals tested, 63% were heterozygous and 37% were wild type. No

mice homozygous for the mutation were detected, indicating that the *Brca1*(-/-) mutants died in utero (Table 1). To determine the time of death, pregnant females from *Brca1*(+/-) intercrosses were sacrificed, and the fetuses at different gestation times from E8.5 to E12.5 were examined. Of the 81 decidua tested, 58 (72%) contained morphologically normal fetuses, of which 39 (48%) were heterozygous, 19 (24%) were wild type, and none were homozygous. The remaining 23 (28%) decidua were much smaller and contained completely resorbed embryos (Table 1; Fig. 3J,O), indicating that the *Brca1*(-/-) mutant concepti die before E8.5.

Developmental deficiency in Brca1(-/-) mutant embryos appears shortly after postimplantation

To precisely pinpoint the differences between wild-type and *Brca1*(-/-) mutant embryos, we next examined the histology of embryos between implantation and gastrulation. Intact decidual swellings of litters from *Brca1*(+/-) intercrosses obtained between E4.5 and E7.5 (Table 1) were fixed, sectioned, and stained with hematoxylin/eosin. Following implantation (E4.5-E5.5), abnormalities that distinguished normal concepti from *Brca1*(-/-) concepti could be readily observed. Both wild-type (Fig. 3A,B) and heterozygous embryos (data not shown) show normal growth and elongation of the egg cylinder, which contains both embryonic and extraembryonic ectoderm and distinct proamniotic cavities. In striking contrast, *Brca1*(-/-) embryos are at least 50% smaller than the wild type and fail to form egg cylinders (Fig. 3F,G,K,L), although they do display embryonic and extraembryonic tissues at this stage (Fig. 3G,L). By E6.5, wild-type embryos are almost ready for gastrulation, with the egg cylinders nearly filling the yolk sac cavities. Elongated proamniotic and distinct exocoelomic cavities are also well developed (Fig. 3C). By comparison, *Brca1*(-/-) embryos show increasing cellular disorganization and start to degenerate (Fig. 3H,M). By the time wild-type embryos undergo gastrulation (E7.5) and the mesoderm develops (Fig. 3D), the *Brca1*(-/-) embryos are significantly developmentally retarded and there is no sign of mesoderm differentiation (Fig. 3I,N). Of the 26 decidua examined at E8.5, 8 (30%) contained no embryo, indicating complete resorption (Table 1; Fig. 3J,O).

Table 1. Genotype analysis of the progeny from *Brca1*(+/-) heterozygous intercrosses

Age (DNA source)	Litter	Number	Genotype			Resorbed
			+/+	+/-	-/-	
10 days (toes)	19	97	36	61	0	
E9.5-12.5 (yolk sac)	7	55	11	29	0	15
E8.5 (yolk sac)	1	9	3	5	0	1
E8.5 (paraffin sections)	2	17	5	5	0	7
E7.5 (paraffin sections)	3	23	6	14	3	
E6.5 (paraffin sections)	5	37	10	20	7	
E5.5 (paraffin sections)	3	21	10	6	5	
E4.5 (paraffin sections)	1	7	3	2	2	
E3.5 (outgrowth)	6	37	10	19	8	

Figure 3. Histological sections of wild-type and *Brca1*($-/-$) mutant embryos grown in utero. The uteri of *Brca1*($+/-$) females were dissected between 4.5 and 8.5 days after intercross matings, and 4- μ m sections were prepared as described in Materials and methods. All uterine decidia were sectioned transversely according to the nomenclature of Smith (1985), and the mesometrial to anti-mesometrial axis is left to right. (A–E) Wild-type embryos; (F–O) *Brca1*($-/-$) embryos; (A,F,K) E4.5 egg cylinders; (B,G,L) E5.5 egg cylinders; (C,H,M) E6.5 embryos; (D,I,N) E7.5 embryos; (E,J,O) E8.5 embryos. Note the appearance of the proamniotic cavity and the clearly differentiated embryonic and extraembryonic ectoderm in a wild-type E4.5 egg cylinder (A), an elongated egg cylinder ready for gastrulation in E6.5 embryo (C), and a gastrulating embryo with primitive streak and mesoderm differentiation in an E7.5 embryo (D). Abbreviations: (eee) Extra-embryonic ectoderm; (pac) proamniotic cavity; (al) allantois; (am) amnion; (ch) chorion; (ec) exocoelomic cavity; (ee) embryonic ectoderm; (em) embryo mass; (hf) head fold; (ht) heart; (ne) neuroepithelium; (pe) parietal endoderm; (ve) visceral endoderm. (Insets in F and K) enlarged twofold. Bar, 100 μ m.

The morphologically abnormal phenotype is inferred to be the consequence of the homozygous mutant genotype. To confirm that the smaller malformed embryos do result from the loss of the wild-type *Brca1* gene, sectioned embryonic tissues are collected by microdissection and subjected to PCR genotyping analysis. PCR analysis of E4.5 littermates representative of *Brca1* heterozygote matings shows that the developmentally impaired embryos are homozygous for the mutated *Brca1* allele (Fig. 4A, right, and B). These observations demonstrate that *Brca1*($-/-$) embryos have growth and morphogenetic defects before the onset of gastrulation and die before E8.5. To test whether the *Brca1*($-/-$) embryos between E5.5 and E6.5 remain alive, we injected 5-bromo-2'-deoxyuridine (BrdU) (100 μ g/gram body weight) intraperitoneally into heterozygous pregnant females 1 hr before sacrifice. The embryos were fixed, sectioned, and subjected to immunostaining with anti-BrdU antibody. The *Brca1*($-/-$) embryos were found to incorporate BrdU, indicating that the mutant embryos synthesize DNA (Fig. 5C,D). These results suggest that the cells are still alive, although the embryos are underdeveloped. When we compared the percentage of BrdU-labeled cells of three genotypes ($+/+$, $+/-$, and $-/-$) at E5.5–E6.5 (Fig. 5E), the wild-type and heterozygote embryos had ~82%–85% of their nuclei labeled, whereas the homozygous mutant embryos had only 61%–63%. On the other hand, when we compared apoptotic index

obtained by TUNEL [terminal deoxynucleotide transferase TdT]-mediated dUTP-biotin nick end labeling] assays of wild-type and mutant embryos, no significant difference was found (data not shown). These results indicate that the overall growth and morphogenetic defect in the *Brca1*($-/-$) mutant embryos are, at least in part, attributable to a decrease in the proliferation capability, not an increase in apoptosis of embryonic cells.

Brca1($-/-$) blastocysts have a growth disadvantage in culture

Blastocyst outgrowth in an in vitro culture offers an alternative method to study early postimplantational development. Blastocysts (E3.5) from intercrosses between *Brca1*($+/-$) mice were isolated by uterine flushing, and photographed individually before and after in vitro culture. All 37 blastocysts examined were indistinguishable (Fig. 6A,C,E,H), indicating that embryos homozygous for the targeted mutation of *Brca1* were morphologically normal before implantation. However, a disadvantage in the outgrowth of homozygous embryos was noted after 5 days in culture. Whereas cultured blastocysts of each genotype ($+/+$, $+/-$, and $-/-$) gave rise to adherent sheets of trophoblastic giant cells, the *Brca1*($-/-$) blastocysts showed impaired outgrowth of the inner cell mass (Fig. 6F,G,I,J). These results are consistent with the

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Figure 4. MD-PCR genotype analysis of littermate E4.5 embryos from a *Brca1* heterozygote intercross. (A) Representative images before (top) and after (bottom) microdissection. Arrowheads point to the area from which the embryo's cells had been dissected out for DNA isolation and PCR analysis. (B) Design of primers for detection of exon 11 in the wild-type *Brca1* gene (solid arrow), and those for the *pgkneopA* cassette (open arrow) integrated into mouse genome by homologous recombination. PCR products amplified from the DNA of microdissected embryonic samples were resolved on a 4% NuSieve/SeaKem (3:1) agarose gel in TAE buffer. Abbreviations: (pgk) phosphoglycerate kinase I promoter; (neo) gene for neomycin resistance; (pA) polyadenylation sequence of the bovine growth hormone gene.

in vivo observations of growth retardation in homozygous embryos as described above.

Discussion

In humans, mutations of the *BRCA1* gene are strongly implicated in familial breast, ovarian, and perhaps other types of cancer. One of the purposes in generating *Brca1* mutant mice was to establish an in vivo model system to study the genetic and/or environmental factors responsible for the pathogenesis of these cancers. In concert with Knudson's "two-hit" theory of carcinogenesis (Knudson 1971), germ-line mutation of one allele of the *BRCA1* gene followed by loss of the other allele in somatic cells is currently a favored explanation for familial breast and ovarian tumorigenesis. If human *BRCA1* and mouse *Brca1* are functionally equivalent, *Brca1*(+/-) mice should produce breast, ovarian, and/or other type of cancers. Consistent with the results published previ-

Figure 5. Epiblast cells proliferation is retarded in *Brca1*(-/-) mutant embryos. In vivo BrdU incorporation was described in Materials and methods. (A-D) Transverse sections of uterine decidua were immunostained with an anti-BrdU monoclonal antibody. (A,B) Wild-type embryos; (C,D) *Brca1*(-/-) embryos; (A,C) E5.5 egg cylinders; (B,D) E6.5 egg cylinders. Arrows point to BrdU-labeled cells (dark brown). Unlabeled nuclei appear blue as a result of counterstaining with hematoxylin. (E) Histogram summary of the percentage of BrdU-labeled cells of different genotypes. (Solid bar) *Brca1*(+/+); (shaded bar) *Brca1*(+/-); (open bar) *Brca1*(-/-). Numbers in parenthesis indicate the number of embryos analyzed. Two-tailed *P* value (homozygote mutant vs. wild-type/heterozygote) is <0.0001. Bar, 50 μ m.

Figure 6. Outgrowths of wild-type and *Brca1*($-/-$) blastocysts in vitro. Wild-type blastocysts (A,C) and *Brca1*($-/-$) blastocysts (E,H) appear to be morphologically normal (arrows point to inner cell mass). Representative example of wild-type (B,D) and *Brca1*($-/-$) (F,I) blastocysts cultured for 5 days in vitro. G and J are higher magnifications of F and I, respectively. Abbreviations: (en) endoderm; (icm) inner cell mass; (tg) trophoblast giant cells. Bar, 10 μ m.

of these steps in implantation. After implantation (E4.5), but before gastrulation (E7.5), the inner cell mass of wild-type embryos undergoes rapid proliferation that extends into the blastocoel cavity to form a structure known as the egg cylinder (Fig. 3A–C). The egg cylinder is initially a double-layered structure that encloses a narrow lumen termed the proamniotic cavity. The two layers consist of an inner layer of ectoderm and an outer layer of endoderm cells (Kaufman 1992). In *Brca1*($-/-$) mutant embryos, the embryonic and extraembryonic tissues are present but are severely retarded in growth. Although the cells remain alive, the embryos are incapable of proceeding toward gastrulation (Fig. 3F–I,K–N). By E8.5, the *Brca1*($-/-$) concepti are resorbed completely. Because maternal RNAs are typically degraded (Sawicki et al. 1981) and at least some embryonic de novo gene expression is required (Johnson 1981) after implantation, it is not clear whether maternal *Brca1* can maintain the cell viability until E7.5. Consistent with in vivo observations, the in vitro blastocyst outgrowth experiments also showed a disadvantage in the growth of the *Brca1*($-/-$) inner cell mass (Fig. 6), suggesting that the *Brca1* is important for cell growth at this early embryonic stage.

The phenotype of *Brca1*($-/-$) embryos described here is similar to those of other mutant mice. The murine *evx1*($-/-$) mutant leads to embryonic malformations after implantation but before gastrulation (Spyropoulos and Capecchi 1994). Unlike *Brca1*($-/-$) mutants, *evx1*($-/-$) mutants failed to differentiate into distinct embryonic and extraembryonic tissues. Cells in *evx1*($-/-$) embryos do not establish proper communication between embryonic and extraembryonic tissues, which is

critical for their further differentiation. The *fgfr-1*($-/-$) mouse mutant is also similar in terms of the timing of embryonic demise. Unlike *Brca1*($-/-$) mutants, however, *fgfr-1*($-/-$) concepti do form egg cylinders, undergo gastrulation, and generate mesoderm. In this instance, embryonic lethality may be caused by the aberrant mesodermal patterning (Deng et al. 1994; Yamaguchi et al. 1994). The embryos of the *fug1* (failure to undergo gastrulation) mouse mutant seem to be the closest to the *Brca1*($-/-$) homozygotes with regard to timing and the high degree of disorganization within the embryo. Homozygous *fug1* mutant embryos also arrest growth at the egg cylinder stage (E6.0) and are mostly resorbed by E8.5 (DeGregori et al. 1994). Little is known about the genes required for the differentiation of primitive ectoderm and endoderm, the formation of the proamniotic cavity, or the organization of the ectoderm into an organized epithelium. The genetic evidence from our results demonstrated that like *fug1*, *Brca1* may be required for the organized development of the embryo at the egg cylinder stage, or for subsequent gastrulation. Whether *Brca1* interacts with *fug1* or other cellular factors in the control of early morphogenesis is unknown.

The developmental stage of embryonic death of the *Brca1*($-/-$) mutant in our experiments was different from that published by Gowen et al. (1996). In their report, mice lacking a functional *Brca1* gene exhibited neural tube defects at E9.5. These neural defects were proposed to be the major cause of embryonic lethality between E10 and E13, which is later than what we observed here. To confirm that the mutant phenotype was concurrent with loss of the wild-type *Brca1* gene, we genotyped all sectioned decidua by MD-PCR and obtained a complete correlation between the mutant genotype and the phenotype. Although the discrepancy between these two observations remains to be resolved, the phenotypic variation of the two *Brca1* mutations is probably not attributable to different genetic backgrounds because both mutations were analyzed on the hybrid 129/C57BL backgrounds.

Interestingly, the phenotypes of three *MRF4* homozygous mutants varied from perinatal death to viable with only minor abnormalities (Braun and Arnold 1995; Patapoutian et al. 1995; Zhang et al. 1995). One potential explanation for these phenotypic variations is that the regulation of a neighboring gene, named *Myf5*, was interfered with by two of the constructs but not by the other (Olson et al. 1996). In our targeting construct, a 184-bp *EcoRI* fragment within the 5' end of exon 11 was deleted and replaced with the *pgkneopA* cassette. In their construct, Gowen et al. (1996) deleted a 1.7-kb *XhoI*–*KpnI* genomic fragment that contains 0.1 kb of intron 10 in addition to 1.6 kb of exon 11. Although we do not know whether either of the insertion/deletion manipulations of *Brca1* affects the expression of other genes surrounding exon 11 of *Brca1*, removal of a splicing acceptor site in the latter construct may generate different splicing variants of *Brca1*. Nevertheless, the evidence together strongly supports the notion that *Brca1* is needed for mouse early embryonic development.

Recently, we isolated two *Brcal*-interacting proteins that are structurally similar to transcription factors. One is a LIM-type homeo box-containing protein and the other is a zinc-finger motif-containing protein (S. Li and W.-H. Lee, unpubl.). Both classes of proteins are known to participate in the control of development. Our hypothesis is that *Brcal*, at least in the mouse, somehow participates in regulating the spatial-temporal organization of the developing embryo through interactions with these, and perhaps other, developmental control proteins. This exciting possibility is currently under investigation.

Materials and methods

Construction of targeting vectors

The mouse *Brcal* gene was isolated by screening a λ DASH mouse genomic library derived from the 129/Sv mouse strain (provided by Dr. Tom Doetschman, University of Cincinnati, OH), using a 3.5-kb fragment of human *BRCA1* exon 11 as a probe. Positive clones were subcloned into the pBluescript SK vector (Stratagene). Restriction mapping and DNA sequencing of the intron 10–exon 11 junction yielded the restriction map shown in Figure 1A. To generate a targeting vector, an 8.0-kb *HindIII*–*Bam*HI fragment of the mouse *Brcal* gene containing exon 11 was subcloned into the pBluescript SK vector (Stratagene), yielding the plasmid pHB-8. The *HindIII* site was then opened and changed to *Bam*HI by ligation with a *Bam*HI linker. A 184-bp *Eco*RI fragment, corresponding to amino acid residues 300–361, was deleted and replaced with a *pgkneopA* cassette (Soriano et al. 1991) in both the sense and antisense orientation [designated as *Brcal*–neo(s) and *Brcal*–neo(o), respectively]. These two constructs were then subcloned into the p2TK vector (Lee et al. 1992) to produce two final targeting vectors designated as *Brcal*–ko(s) and *Brcal*–ko(o), respectively.

Electroporation and selection of ES cells

E14.1 ES (Handyside et al. 1989) cells derived from mouse strain 129/Ola were maintained on a monolayer of mitomycin C-inactivated, neomycin-resistant, fibroblast feeder cells, as described previously (Robertson 1987). Thirty micrograms of *Sall*-linearized targeting vector was electroporated into a suspension of trypsinized cells (7×10^6) in Dulbecco's modified Eagle medium (DMEM) (GIBCO/BRL) using a Bio-Rad gene pulser (250 μ F, 800 V). Cells were then incubated at room temperature for 5 min, plated, and allowed to recover for 24 hr before selection in medium containing G418 (250 μ g/ml) and FIAU (1 μ M). Cells were fed daily, and after 8 days the resulting double-resistant ES clones were individually picked and transferred onto 24-well plates with feeders. On the following day, each clone was trypsinized and divided in half. One half was frozen (-80°C), whereas the other half was plated into a 12-well plate without feeder cells and used to prepare DNA.

Analysis of targeted ES cell clones

The analysis of DNA from ES cell clones was described previously (Laird et al. 1991). In brief, cells in a 12-well plate were washed with PBS, lysed in 0.5 ml of lysis buffer containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 10 mM EDTA, 0.5% SDS, and 0.1 mg/ml of proteinase K, and transferred into a 1.5-ml Eppendorf tube. After 5 hr of incubation at 55°C with shaking, an equal volume of isopropanol was added, and DNA was al-

lowed to precipitate by gentle inversion of the tube several times. The supernatant was discarded, and the DNA was washed with 70% ethanol, air-dried, and resuspended in distilled water. DNA (15 μ g) was digested in 30 μ l of a restriction enzyme mixture ($1 \times$ restriction buffer, 100 mg/ml of bovine serum albumin (BSA), 50 μ g/ml of RNaseA, and 15 units of *HindIII*) overnight at 37°C . Electrophoresis and Southern blotting of the digested DNA was performed as described previously (Sambrook et al. 1989). A ^{32}P -labeled 1.1-kb *Bam*HI fragment, which lies 3' of the genomic sequence in the targeting vector, identified bands of 10.0 and 3.0 kb, or 10.0 and 4.6 kb, corresponding to germ-line wild-type *Brcal* and homologous recombinant bands. Targeted clones were recovered from the 24-well plates and expanded in 6-well plates. DNA from these clones was then digested with *Bam*HI and *Sall* and probed with a 5' flanking probe. In addition, a *neo* probe was used to confirm that only homologous recombination had occurred in targeted clones rather than a random integration event.

Generation of mice carrying the disrupted *Brcal* allele

Two different targeted ES clones, *Brcal*–ko(o) 3 and *Brcal*–ko(s) 291, were used to generate chimeric mice according to procedures described previously (Bradley 1987). C57BL/6J blastocysts injected with 10–12 ES cells were implanted into pseudopregnant F₁ (CBA $\times$ C57BL/6J) foster mothers (The Jackson Laboratory, Bar Harbor, ME). Chimeric mice, identified by agouti coat color, were mated with C57BL/6J mice. Offspring with agouti coat color were tested for the presence of the targeted locus by PCR and Southern blotting analysis. Heterozygotes were interbred, and PCR analysis was used to distinguish between offspring with zero, one, or two copies of the mutant gene.

DNA isolation, PCR genotyping, and histology of embryos from *Brcal* +/– intercrosses

F₁ mice heterozygous for the *Brcal* mutant allele were mated, and toes were cut from the F₂ progeny for genotyping analysis. For timed pregnancies, the day on which a vaginal plug was detected was considered to be E0.5. At desired time points, the embryos were dissected from maternal decidua for further analysis. For embryos older than E8.5, the visceral yolk sac was collected and subjected to genotype determination by PCR. DNA from toes, yolk sacs, and blastocysts was prepared and analyzed by PCR as follows. Tissues were lysed at 55°C in 40 μ l of lysis buffer [10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl₂, 0.1 mg/ml of gelatin, 0.45% NP-40, 0.45% Tween 20, and 60 μ g/ml of proteinase K] for 1 hr. Samples were then boiled for 10 min and cooled on ice. Seven microliters of proteinase K-digested cell lysate was mixed with 18 μ l of PCR cocktail solution containing 50 mM KCl, 1.5 mM MgCl₂, 10 mM Tris-HCl (pH 8.3), 0.001% gelatin, 200 μ M each of the four dNTPs, 0.4 μ M of each primer, and 0.5 unit of recombinant *Taq* polymerase (AmpliTaq; Perkin-Elmer). The mixture was then overlaid with 20 μ l of light mineral oil. PCR was performed for 35 cycles using a pTC-100 thermal controller (MJ Research, Inc.) using the following reaction conditions: Denaturing temperature of 94°C for 30 sec; annealing temperature of 65°C for 1 min; and elongation temperature of 72°C for 1 min. The resulting PCR products were resolved on 4% NuSieve/SeaKem (3:1; FMC) composite gel in TAE buffer at 80 V for 30 min and visualized with UV light after staining with ethidium bromide. For the targeted allele, a 236-bp product was generated using a sense oligonucleotide, 5'-TGATATTGCTGAAGAGCTTGGCGGC-3' and an antisense oligonucleotide, 5'-TGGGAGTGGCACCTTCCAGGGTCAA-3', within the *pgkneopA* cassette. To detect the wild-type allele,

a 150-bp product was generated using a sense oligonucleotide, 5'-AACAGCCTGGCATAGCAGTGAGCCA-3', and antisense oligonucleotide, 5'-TTGCGGGTGAGTCCACTTCTCTCTA-3' within exon 11 of *Brca1*. For embryos between E4.5 and E7.5, entire uteri were fixed in 4% paraformaldehyde overnight at 4°C. Uterine horns were excised and dehydrated through a graded ethanol series, cleared in chloroform, and then infiltrated and embedded in Paraplast X-tra (Polysciences). Sections (4 µm) were collected on Superfrost/Plus microscope slides (Fisher Scientific), stained with Mayer's hematoxylin/eosin, and mounted in Canada Balsam (Fisher Scientific). Images were recorded using a C5810 Color Camera (Hamamatsu) and cropped using a Macintosh Power PC and Adobe Photoshop Software. The slides with sections were demounted in xylene followed by soaking in 100% ethanol and air-dried. Embryonic tissues were microdissected and collected into glass capillaries (~50 µm diam.) mounted on a micromanipulator (Leitz), under 400-fold magnification, and transferred into a 0.5-ml Eppendorf tube containing 7 µl of lysis buffer under 20 µl of light mineral oil. Cells were lysed at 55°C for 1 hr. The proteinase K was then inactivated by incubation at 95°C for 10 min, and the mixture was then cooled on ice. The PCR cocktail solution (18 µl) was then added into the tube and mixed with the DNA sample by centrifugation at 12,000 rpm for 1 min. PCR was performed for 50 cycles.

In vitro blastocyst culture

Blastocyst cultures followed those described previously (Hsu 1979), with some modifications. Briefly, blastocysts were isolated from females at E3.5 and cultured for 5 days on tissue culture plates in DMEM plus 20% fetal bovine serum, supplemented with BSA (4 mg/ml), glutamine, antibiotics, and 2-mercaptoethanol (0.1 mM). Blastocyst outgrowths were inspected daily and photographed to monitor their development. Finally, they were lysed and genotyped by PCR.

Detection of BrdU incorporation by immunohistochemistry

At the desired time points, BrdU (100 µg/gram body weight) (Sigma) was injected intraperitoneally into pregnant females. One hour later, the entire uterus was dissected and fixed in 4% paraformaldehyde overnight at 4°C. The individual decidua were embedded, sectioned, and subjected to further analysis. Immunohistochemical detection of BrdU incorporation was performed as described previously (Lee et al. 1994). The percentage of cells incorporating BrdU in each embryo was determined by counting >50 cells in representative histological sections. Statistical comparisons were performed by two-tailed ANOVA using In Stat software (Graph Pad, San Diego, CA).

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