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

Chia-Yang Liu, Andrea Flesken-Nikitin, Shang Li, Yingying Zeng, and Wen-Hwa Lee

Center for Molecular Medicine/Institute of Biotechnology, The University of Texas Health Science Center at San Antonio, San Antonio, Texas 78245 USA

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
established mutant Brcal(+/-) mouse lines using gene targeting in embryonic stem (ES) cells. Mice lacking one wild-type allele of the Brcal appear normal and are fertile until 10 months of age. However, Brcal(-/-) mutants die during early embryogenesis. Histological examinations of early concepti and genotyping after fixation revealed that Brcal(-/-) mutant embryos appear normal and are fer­tile. This result is consistent with the wild-type allele of the Brcal gene in early mouse development.

**Results**

**Targeted mutation of the mouse BRCAL homolog**

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

The targeting vector was constructed by deleting a 184-bp EcoRI fragment corresponding to mouse Brcal 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 Brcal-ko(s) and Brcal-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 Brcal 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 Brcal genomic DNA lying 3‘ external to the targeting vector as a hybridization probe [Figs. 1C and 2A]. The use of Brcal-ko(s) and Brcal-ko(o) vectors resulted in a total of 6 and 13 homologous recombinant clones, respectively. Brcal-ko(o) #3 and Brcal-ko(s) #291 were analyzed further by Southern blot analysis using different probes to confirm the targeted gene disruption [Fig. 2B].

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

ES cells from clone Brcal-ko(o) #3 and Brcal-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 Brcal-ko(o) #3. Of the 120 offspring from male chimera/female C57BL/6J crosses, 53% were heterozygous (Brcal +/-) and 47% homozygous wild type (Brcal +/+), 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 Brcal alleles leads to embryonic lethality**

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

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**Figure 1. Strategy for the generation of a targeted mutation in the mouse Brcal gene.** [A] Restriction map of the mouse Brcal fragment, encompassing exon 11 and flanking DNA. An 8.0-kb HindIII–BamHI fragment was used to create the replacement targeting vector. [B] Restriction map of the targeting constructs Brcal–ko(s) and Brcal–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 Brcal 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] BamHI; [H] HindIII; [S] SalI; [X] XhoI.
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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,0), 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,0).

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

| Age (DNA source) | Litter | Number | +/+ | +/− | −/− | Resorbed |
|------------------|--------|--------|-----|-----|-----|---------|
| 10 days (toes)  | 19     | 97     | 36  | 61  | 0   | 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-μm sections were prepared as described in Materials and methods. All uterine decidua 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 μm.

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 μg/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 deoxynucleotidyl 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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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-
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 fugl(−/−) 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 fugl(−/−) 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 fugl, Brca1 may be required for the organized development of the embryo at the egg cylinder stage, or for subsequent gastrulation. Whether Brca1 interacts with fugl 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 pgkneoA 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 as Brcal-ko(s) and Brcal-ko(o), respectively.

A 184-bp EcoRI fragment, corresponding to amino acid residues 10-exon 11 junction yielded the restriction map 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-BamHI fragment of the mouse Brcal gene containing exon 11 was subcloned into the pBluescript SK vector (Stratagene). Restriction mapping and DNA sequencing vector (Stratagene). Restriction mapping and DNA sequencing were performed by ligation with a HindIII-BamHI fragment of the mouse Brcal gene, yielding the plasmid pHB-8. The HindIII site was then opened and changed to BamHI by ligation with a BamHI linker. A 184-bp EcoRI fragment, corresponding to amino acid residues 300–361, was deleted and replaced with a pgkneoA 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 pAlt-linearized targeting vector was electroporated into a suspension of trypanosome cells [7x10^6] in Dulbecco’s modified Eagle medium [DMEM] (GIBCO/BRL) using a Bio-Rad gene pulser (250 V, 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 418 [250 µg/ml] and FIAU [1 µM]. 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 allowed 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 µg] was digested in 30 µl of a restriction enzyme mixture [1× restriction buffer, 100 mg/ml of bovine serum albumin [BSA], 50 µg/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 32P-labeled 1.1-kb BamHI fragment, which lies 3’ of the genomic sequence in the targeting vector, identified bands of 10.0 and 3.0 kb, and 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 BamHI and SalI 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×C57BL/6] 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 µl of lysis buffer [10 mM Tris-Cl [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 µg/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 µl of PCR cocktail solution containing 50 mM KCl, 1.5 mM MgCl2, 10 mM Tris-Cl [pH 8.3], 0.001% gelatin, 200 µM each of the four dNTPs, 0.4 µM of each primer, and 0.5 unit of recombinant Taq polymerase [AmpliTaq, Perkin-Elmer]. The mixture was then overlaid with 20 µl 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 54°C for 30 sec; 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′-TGTATTGCTGAAAGGCCTGGCCGC-3′ and an antisense oligonucleotide, 5′-TGGGATGTCGCACCTTCAGGTCTCA-3′, within the pgkneoA cassette. To detect the wild-type allele,
a 150-bp product was generated using a sense oligonucleotide, 5'-AACAGCCTGCATAGCAAGC-3', and antisense oligonucleotide, 5'-TTGCCGGTGATCCACTTCTCTCTA-3' within exon 11 of Bical. 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 Paraffin X-tra (Polysciences). Sections (4 |xm) were collected on Superfrost/Plus microscope slides [Fisher Scientific], stained with Mayer's hematoxylin/eosin, and mounted in Canada Balsam (Fisher Scientific). Images were 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 |xm diam.) mounted on a micromanipulator [Leitz], under 400-fold magnification, and transferred into a 0.5-ml Eppendorf tube containing 7 |JL1 of lysis buffer under 20 |JL1 of light mineral oil. Cells were lysed at 55°C for 1 hr. The proteinase K 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 |g/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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