Cell, Vol. 85, 1009–1023, June 28, 1996, Copyright ©1996 by Cell Press

# The Tumor Suppressor Gene *Brca1* Is Required for Embryonic Cellular Proliferation in the Mouse

**Razqallah Hakem,[1,2] José Luis de la Pompa,[1,2] Christian Sirard,[1,2] Rong Mo,[3,5] Minna Woo,[1,2] Anne Hakem,[1,2] Andrew Wakeham,[1,2] Julia Potter,[1,2] Armin Reitmair,[1,2] Filio Billia,[2] Eduardo Firpo,[6] Chi Chung Hui,[3,5] Jim Roberts,[6] Janet Rossant,[3,4,7] and Tak W. Mak[1,2]**

[1]Amgen Institute
[2]Ontario Cancer Institute
Department of Medical Biophysics and Immunology
University of Toronto
Toronto, Ontario M5G 2C1
Canada
[3]Department of Molecular and Medical Genetics
[4]Department of Obstetrics and Gynecology
University of Toronto
[5]Program in Developmental Biology
Division of Endocrinology Research Institute
The Hospital for Sick Children
Toronto, Ontario M5G 1X8
Canada
[6]Department of Basic Sciences
Fred Hutchinson Cancer Research Center
1124 Columbia Street
Seattle, Washington 98104
[7]Samuel Lunenfeld Research Institute
Mount Sinai Hospital
Toronto, Ontario M5G 1X5
Canada

## Summary

**Mutations of the *BRCA1* gene in humans are associated with predisposition to breast and ovarian cancers. We show here that *Brca1*$^{+/-}$ mice are normal and fertile and lack tumors by age eleven months. Homozygous *Brca1*$^{5-6}$ mutant mice die before day 7.5 of embryogenesis. Mutant embryos are poorly developed, with no evidence of mesoderm formation. The extraembryonic region is abnormal, but aggregation with wild-type tetraploid embryos does not rescue the lethality. In vivo, mutant embryos do not exhibit increased apoptosis but show reduced cell proliferation accompanied by decreased expression of cyclin E and *mdm-2*, a regulator of p53 activity. The expression of cyclin-dependent kinase inhibitor *p21* is dramatically increased in the mutant embryos. Buttressing these in vivo observations is the fact that mutant blastocyst growth is grossly impaired in vitro. Thus, the death of *Brca1*$^{5-6}$ mutant embryos prior to gastrulation may be due to a failure of the proliferative burst required for the development of the different germ layers.**

## Introduction

Breast carcinoma is the most common female cancer. In western societies, one in ten women will develop the disease in her lifetime. Although reproductive, hormonal, and dietary factors are known to contribute to breast cancer risk, family history is also an important factor. Breast cancer susceptibility genes such as *BRCA1* (Hall et al., 1990; Miki et al., 1994) and *BRCA2* (Wooster et al., 1994, 1995; Tavtigian et al., 1996) have been recently identified and cloned.

A large number of mutations in the *BRCA1* gene have been reported in families with predisposition to both inherited and sporadic breast and ovarian cancers (Castilla et al., 1994; Friedman et al., 1994; Futreal et al., 1994; Miki et al., 1994; Merajver et al., 1995a; Hosking et al., 1995). Manifestation of the disease is associated with the loss of the wild-type *BRCA1* allele (Futreal et al., 1994; Cornelis et al., 1995; Merajver et al., 1995b), suggesting that *BRCA1* is a tumor suppressor gene.

The *BRCA1* gene contains 22 coding exons and encodes a 1863 amino acid protein with an amino-terminal $Cys_3$-His-$Cys_4$ zinc finger and a carboxy-terminal acidic domain (Miki et al., 1994). Missense mutations in the *BRCA1* zinc finger domain at cysteines 61 and 64 have been reported in inherited breast cancer and sporadic ovarian cancer (Castilla et al. 1994; Friedman et al., 1994; Miki et al., 1994; Merajver et al., 1995a), suggesting a critical role for the zinc finger domain in the BRCA1 function. The nuclear localization of BRCA1 as reported in two studies (Chen et al., 1995; Rao et al., 1996) and the presence of a zinc finger domain and a carboxy-terminal acidic domain suggest that this tumor suppressor protein may act as a transcriptional regulator. Paradoxically, however, a recent report suggests the possibility that the BRCA1 protein might be secreted (Jensen et al., 1996).

*Brca1*, the mouse homolog of *BRCA1* located on chromosome 11, encodes a 1812 amino acid protein (Lane et al., 1995). The Brca1 protein shows an overall 58% amino-acid identity to the human homolog, and the zinc finger domain is conserved. *Brca1* mRNA is expressed in several adult tissues, including breast, ovary, thymus, and testis (Lane et al., 1995; Marquis et al., 1995). In situ hybridization analysis has shown that *Brca1* is expressed at 9.5 days and later stages of embryogenesis (Lane et al., 1995; Marquis et al., 1995). Little is known about *Brca1* expression during earlier embryonic development, and its normal function in the adult, as well as during embryogenesis, remains to be defined.

To investigate the biological function of *Brca1* in vivo and to create a mouse model to study breast and ovarian cancer, we generated a *Brca1* mutant mouse by deleting exons 5 and 6 of the *Brca1* coding region. Analysis of *Brca1*$^{5-6}$ mutant embryos in vivo and in vitro suggests that *Brca1* may be involved in the control of the proliferative process that occurs in early embryonic development.

## Results

### Generation of *Brca1*$^{5-6}$ Mutant Mice

The *Brca1* gene in embryonic stem (ES) cells was disrupted using a targeting vector in which exons 5 and 6

of *Brca1* were deleted, leading to the introduction of termination codons in all three reading frames and the removal of the zinc finger domain (see Experimental Procedures). The neomycin resistance (*neo*) gene was inserted in the targeting vector in the opposite orientation to *Brca1*, such that *neo* was flanked by 0.6 kb of genomic DNA 5′ of *Brca1* exon 5 and 7 kb of genomic DNA 3′ of *Brca1* exon 6 (Figure 1A). When this targeting construct was electroporated into ES cells, 4 out of 1152 G418-resistant colonies analyzed by polymerase chain reaction (PCR) and Southern blotting were heterozygous for the *Brca1* locus. Two of these ES clones were used to generate chimeric mice, and both successfully contributed to the germ line.

The chimeras were backcrossed to C57BL/6J, CD1, or C3H/HeJ mice, and heterozygotes were crossed to produce homozygous mutant offspring. The genotypes of the mice were confirmed by Southern blot analysis.

### Phenotype of Heterozygote *Brca1*$^{5-6}$ Mice

Humans heterozygous for the *BRCA1* mutation are estimated to have, by age 60, a penetrance of ~50% for breast cancer and ~30% for ovarian cancer (Easton et al., 1993; Ford et al., 1994). Mice heterozygous for the *Brca1*$^{5-6}$ deletion were phenotypically normal and fertile. By eleven months of age, heterozygote *Brca1*$^{5-6}$ females did not show any type of cancer. However, it remains possible that these *Brca1*$^{5-6}$ heterozygote mice may develop tumors at a more advanced age.

### *Brca1*$^{5-6}$ Mutation Results in Early Embryonic Lethality

No viable *Brca1*$^{5-6}$ pups were identified among 88 offspring born from heterozygous intercrosses, indicating that homozygosity for the *Brca1*$^{5-6}$ mutation causes embryonic lethality (Figure 1B; Table 1).

To assess the consequences of the *Brca1*$^{5-6}$ mutation on embryonic development, we analyzed embryos from heterozygote intercrosses at different days of gestation (Table 1). Genomic DNA was isolated from yolk sacs or from whole embryos, and genotyping was performed by PCR amplification using primers a, b, c, and d (Figures 1A and 1C). Degenerating or resorbed embryos were observed at E7.5 and E8.5 and were genotyped as *Brca1*$^{5-6}$ homozygotes. At E6.5, ~25% of all embryos were morphologically abnormal; these were also genotyped as mutants. Mutant E6.5 embryos could be categorized into two phenotypic classes according to the severity of their phenotype (Figures 2A–2D). Embryos in the less severely affected class were less than half the size of their wild-type E6.5 littermates and showed no clear boundary between the embryonic and the extraembryonic regions (Figures 2A–2B). The more severely affected class contained embryos that resembled egg-cylinders, with no morphological organization (Figures 2C and 2D). By E7.5, mutant embryos showed no increase in size and were beginning to be resorbed (Figures 2E and 2F).

Mutant mice generated from the two independent targeted ES cell clones showed identical phenotypes. These results demonstrate that homozygosity for the *Brca1*$^{5-6}$ allele results in embryonic lethality before E7.5 and indicate that *Brca1* is essential for postimplantation development at the time of initiation of gastrulation.

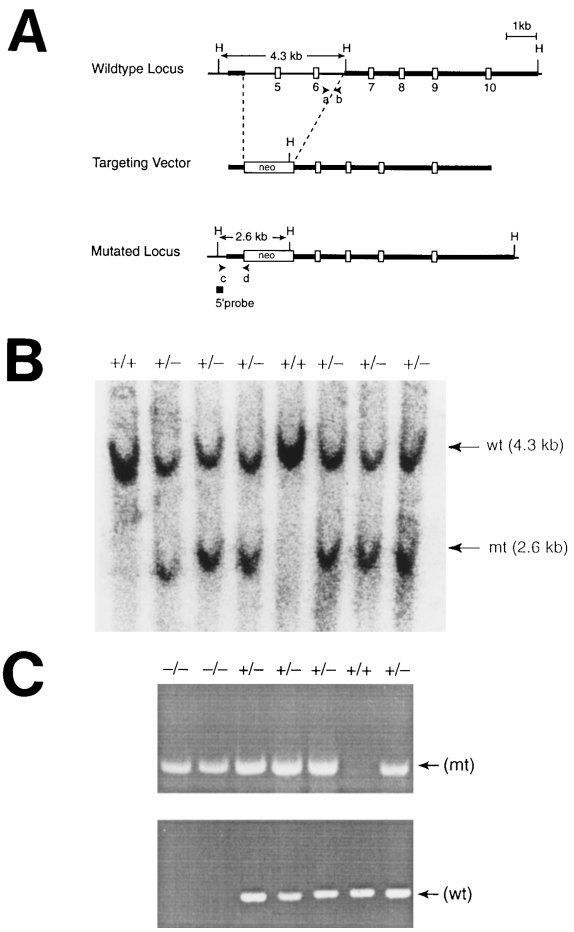


Figure 1. Targeted Disruption of the *Brca1* Locus

(A) A portion of the mouse *Brca1* wild-type locus (top) showing exons 5–10 (open boxes) and HindIII sites. Closed boxes represent the 5′ arm and the 3′ arm of the targeting construct. The 4.3 kb HindIII fragment present in the wild-type allele and the PCR primers "a" and "b" used to identify the wild-type allele are shown. The targeting vector (middle) and the position of the neomycin resistance gene (*neo*), oriented in the opposite orientation to *Brca1* gene transcription. The targeting vector was designed such that the *neo* cassette replaced exon 5 and exon 6 of the *Brca1* gene. The mutated *Brca1* locus (bottom) showing the 2.6 kb HindIII DNA fragment present in the recombinant *Brca1* allele. The position of the 5′ flanking probe used for Southern blot analysis and the PCR primers "c" and "d" used to identify the mutated allele are shown.

(B) Southern blot analysis of representative genomic tail DNA from one litter of *Brca1*$^{5-6}$ heterozygous intercrosses. DNA was digested with HindIII and hybridized with 5′ external probe. The 4.3 kb band representative of the wild-type allele (wt) and the 2.6 kb band representative of the mutated allele (mt) are indicated by arrows.

(C) Representative genotypic analysis of E6.5 embryos from a *Brca1* heterozygous breeding. DNA samples were subjected to PCR using the primer pairs a/b or c/d (see Experimental Procedures for sequences). PCR amplification of the mutated *Brca1* gene allele by primer pair c/d produced a 616 bp DNA fragment (upper panel), while PCR amplification of the wild-type *Brca1* gene allele by primer pair a/b produced a 353 bp DNA fragment (lower panel).

Table 1. Genotypic and Phenotypic Analysis of Neonates and Embryos from *Brca1*$^{5-6}$ Heterozygous Intercrosses

| | Phenotype | | Genotype | | | |
|---|---|---|---|---|---|---|
| Stage | Normal | Abnormal | *Brca1*$^{+/+}$ | *Brca1*$^{+/-}$ | *Brca1*$^{-/-}$ | Total |
| Neonate | 88 | 0 | 27 | 61 | 0 | 88 |
| E8.5 | 11 | 6 | 2 | 9 | 6 | 17 |
| E7.5 | 13 | 2 | 2 | 11 | 2 | 15 |
| E6.5[a] | 25 | 9 | 6 | 19 | 9 | 34 |

Neonates were genotyped by Southern blot analysis using the 5′ flanking probe as shown in Figure 1B. The embryos were collected on days 8.5, 7.5, and 6.5 of pregnancy from *Brca1*$^{5-6}$ heterozygous females crossed to *Brca1*$^{5-6}$ heterozygous males. The embryo genotypes were determined by PCR amplification using DNA extracted from the whole embryo or from the yolk sac, and primers a, b, c, and d as shown in Figure 1C. Homozygous mutant embryos exhibited an abnormal phenotype, characterized by poor embryonic organization and a reduced size in comparison with normal-looking control littermates.

[a] In the histological analysis, whole litters were analyzed. E6.5 embryos were classified phenotypically into two categories, wild type (126 embryos) and mutant (46).

### Histological Analysis of the *Brca1*$^{5-6}$ Mutant Embryos

The structural organization of *Brca1*$^{5-6}$ mutant embryos was characterized in greater detail by histological analysis of serially sectioned E5.5–E6.5 embryos obtained from heterozygous crosses (Figures 3A–3E). Upon examination of E5.5 egg cylinder–stage embryos (Figures 3A and 3B), two phenotypes were obvious, one normal and the other abnormal. Smaller, poorly organized, abnormal embryos could be readily distinguished (Figure 3B) among their wild-type littermates (Figure 3A). At E6.5, phenotypic differences between wild-type and mutant embryos became even more apparent (Figures 3C–3E). Wild-type embryos exhibited a well-organized ectoderm and endoderm (Figure 3C) while the mutant embryos (~25% of all embryos) showed an abnormal organization and could be grouped into two phenotypically mutant classes. Embryos in the less severe class showed epiblast, no detectable primitive streak, a very thin visceral and parietal endoderm, and a proamniotic cavity. In addition, the extraembryonic region was poorly organized with no compact diploid core of trophoblast cells (Figure 3D). Embryos with the more severe mutant phenotype exhibited few ectoderm cells and no detectable extraembryonic region (Figure 3E). The most developed E6.5 mutant embryos did not appear to progress significantly beyond E5.5. Interestingly, the time of the apparent developmental block of *Brca1*$^{5-6}$ mutant embryos coincides with the dramatic increase in size that occurs at E5.5–E6.5 of mouse development (Snow, 1977).

### Alternative Splicing of *Brca1* Exon 11

Northern blot analysis using a *Brca1* Exon 11 probe showed the expression of a *Brca1* transcript (over 7 kb) in ES cells and in E7.5, E8.5, E9.5, and E10.5 embryos (Figure 4A). Northern blot experiments using a probe for *Brca1* exons 7–9 indicated the presence, in ES cells and in E7.5, E8.5, E9.5, and E10.5, of a *Brca1* exon 11 positive transcript (7.2 kb) together with a smaller *Brca1* transcript (~2 kb) (Figure 4B). The small *Brca1* transcript was not detected with the exon 11 probe, indicating the existence of *Brca1* exon 11 negative transcripts. PCR experiments using cDNA from ES and E7.5 cells confirmed the presence of the *Brca1* exon 11 negative transcript. Primers specific for *Brca1* exons 9 and 13 could amplify the 352 bp product expected if *Brca1* exon 11 is spliced out. Southern blot hybridization showed that the 352 bp PCR product contains both *Brca1* exons 10 and 12 (Figure 4C). The existence of a transcript in which *Brca1* exons 5 and 6 were spliced out was also addressed by PCR amplification of cDNAs from ES and E7.5 cells, using *Brca1* primers specific for exons 3 and 7. A 328 bp product was amplified, the size expected if exons 5 and 6 were not spliced out. The specificity of this product and the presence of *Brca1* exons 5 and 6 in all *Brca1* transcripts was further confirmed by PCR Southern blot analysis using primers specific for exon 5 and exon 6 (Figure 4C).

Thus we show, in ES cells and during embryogenesis, the coexistence of *Brca1* exon 11 negative and *Brca1* exon 11 positive transcripts. However, more importantly we show that no *Brca1* exon 5 and exon 6 spliced form is detected.

Two approaches were used to investigate whether the gene targeting of the *Brca1* locus resulted in a null mutation. First, individual preimplantantion embryos at E2.5 (B2.5) and E4 (B4) were genotyped and their total cDNAs amplified by PCR in an unbiased fashion (see Experimental Procedures). Two of the ten B2.5 and one of the eight B4 PCR genotyped embryos examined were homozygous for the *Brca1*$^{5-6}$ mutation (data not shown). Southern blot analysis of the amplified cDNAs using 3′ untranslated (3′ UTR) probes showed that *Brca1* was not expressed in the homozygous mutant blastocysts (Figure 4D). However, a ribosomal housekeeping gene, *L32*, was strongly expressed in the wild type, as well as in the homozygous mutant.

In the second approach, the spatial expression of *Brca1* in E6.5 wild-type and mutant embryos was analyzed by in situ hybridization in tissue sections, using an antisense *Brca1* exon 11 probe. *Brca1* transcripts could be detected homogeneously throughout the epiblast, developing mesoderm and extraembryonic regions (Figures 5A and 5B). Mutant embryos failed to show any expression of *Brca1* transcript (Figures 5C and 5D).

These results indicate that the *Brca1*$^{5-6}$ mutation is a

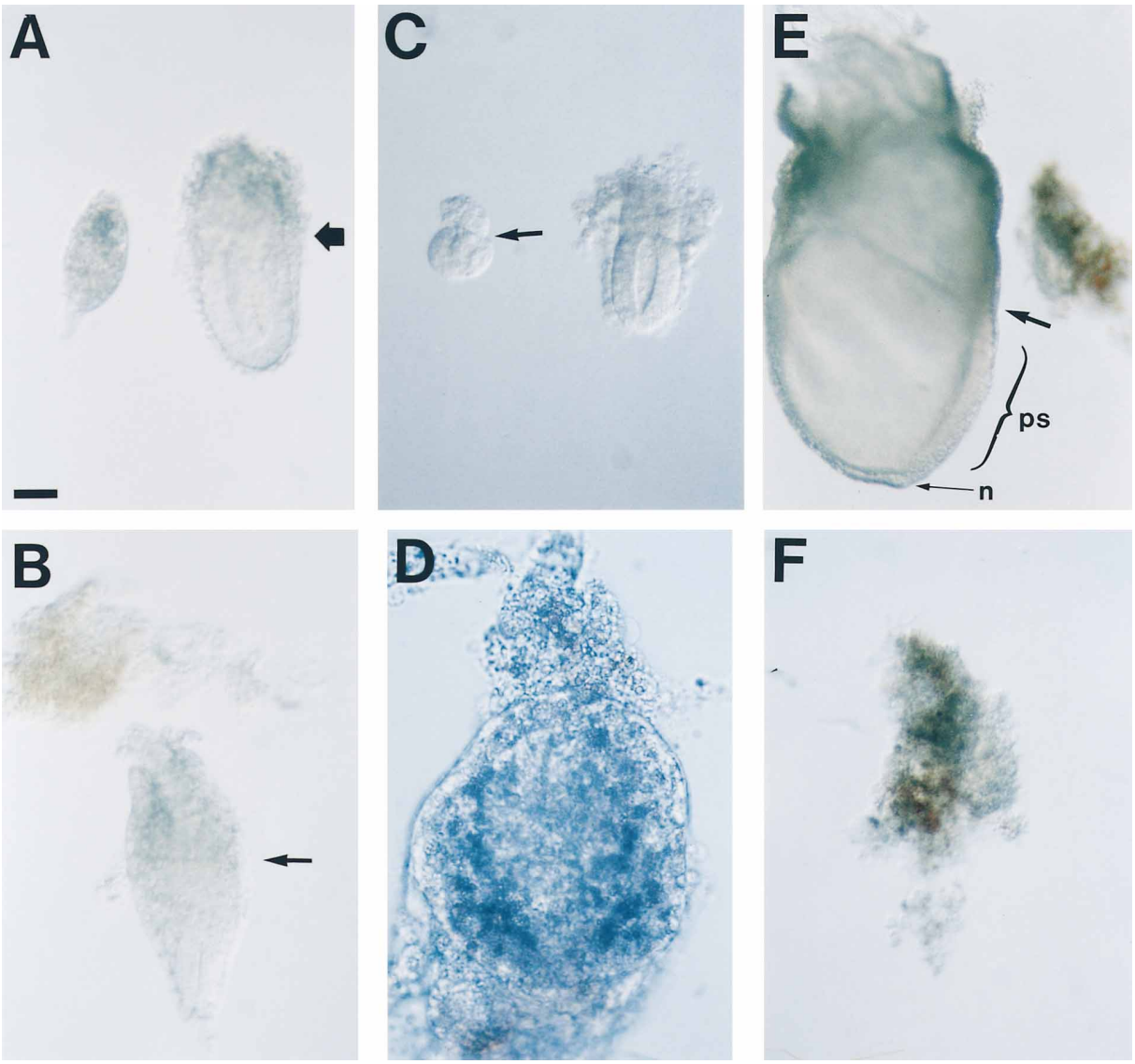


Figure 2. Morphology of *Brca1*$^{5-6}$ Mutant Embryos

Whole-mount preparations at E6.5, egg-cylinder (A–D), and E7.5, late streak (E and F) stages.

(A and B) At E6.5, two mutant classes can be distinguished. The less severe class is represented by the embryo shown in the left part of (A). This embryo is smaller than its wild-type littermate, although it is relatively well organized (B).

(C and D) The left part of (C) shows a severely affected mutant embryo. It is a compacted egg cylinder, much smaller than the wild-type embryo, with no signs of organization (D).

(E and F) At E7.5, the wild-type embryo is well advanced in gastrulation. In (E) the size difference between wild-type (left) and mutant (right) embryos can be appreciated. High magnification (F) of E7.5 *Brca1*$^{5-6}$ mutant. At this stage, mutant embryos are being resorbed and appear surrounded by maternal tissues.

In (A), (B), (C), and (E), the arrows point to the separation between embryonic and extraembryonic regions. n, node; ps, primitive streak. Bar, 70 μm in (A) and (C); 140 μm in (B); 20 μm in (D); 100 μm in (E); and 50 μm in (F).

null mutation. Furthermore, *Brca1* appears to be expressed throughout early embryogenesis.

### *Brca1*$^{5-6}$ Mutant Embryos Show Defective Trophoblast Development and Do Not Form Mesoderm

Death at early postimplantation stages in *Brca1*$^{5-6}$ mutant embryos could be explained either by some generalized cellular defect resulting in growth failure, or by lineage-specific defects. *Brca1*$^{5-6}$ mutants showed particularly abnormal development of the extraembryonic region, with absence of diploid trophoblast and apparent abundance of giant cells. The expression analysis of the early trophoblast lineage marker *Mash-2* confirmed this phenotype. This gene is expressed in the wild-type embryo throughout preimplantation develop-

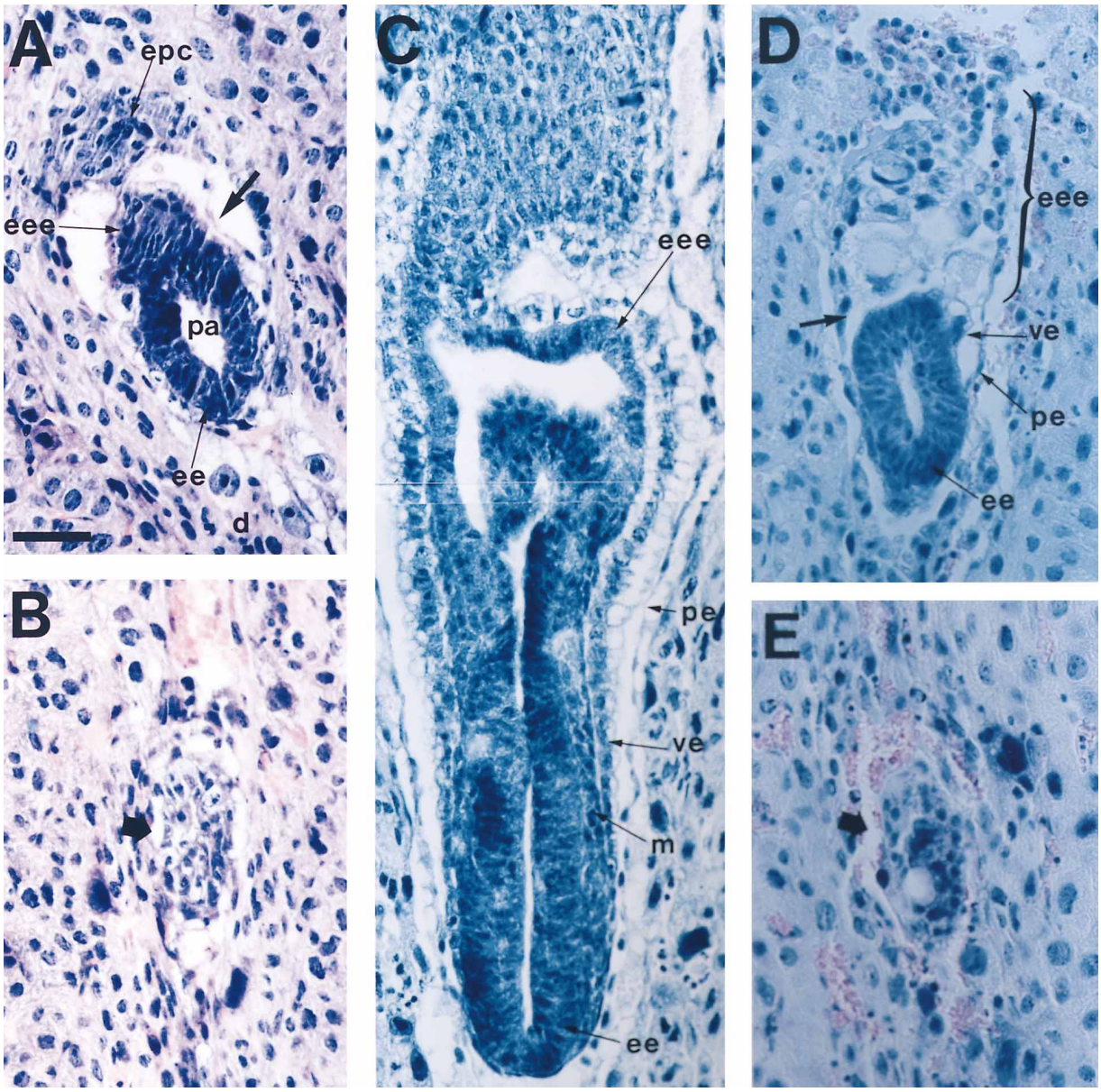


Figure 3. Histological Analysis of *Brca1*$^{5-6}$ Mutant Embryos at E5.5 and E6.5

Sagittal sections. (A and C) Wild-type embryos; (B, D, and E) mutant embryos.

(A) E5.5 wild-type embryo. Early egg cylinder.

(B) Mutant embryo at E5.5 (arrow). The embryo is small and disorganized.

(C) E6.5 wild-type embryo. Egg-cylinder stage.

(D) Less severe mutant phenotype at E6.5. The embryo is small, with a thick ectoderm, a very thin endoderm, and an abnormal extraembryonic region.

(E) More severe mutant phenotype at E6.5. The embryo (arrow) is completely disorganized.

In (A) and (D), the arrow points to the separation between embryonic and extraembryonic regions. d, decidua; ee, embryonic ectoderm; eee, extraembryonic ectoderm; epc, ectoplacental cone; m, mesoderm; pa, proamniotic cavity; pe, parietal endoderm; ve, visceral endoderm. Bar, 60 μm.

ment. In wild-type E6.5 embryos, *Mash-2* is expressed in the diploid trophoblast lineage (Figures 5E and 5F) but not in the trophoblast giant cells. No *Mash-2* transcript was detected in *Brca1*$^{5-6}$ E6.5 mutant embryos after in situ hybridization (Figures 5G and 5H), suggesting the absence of diploid trophoblast cells.

Morphological and histological criteria suggested that mesoderm was also missing in *Brca1*$^{5-6}$ mutant embryos. To determine at the molecular level if mesoderm was being formed in mutant embryos, we examined the expression of the Brachyury (T) protein by immunohistochemistry in tissue sections. In wild-type E6.5 embryos,

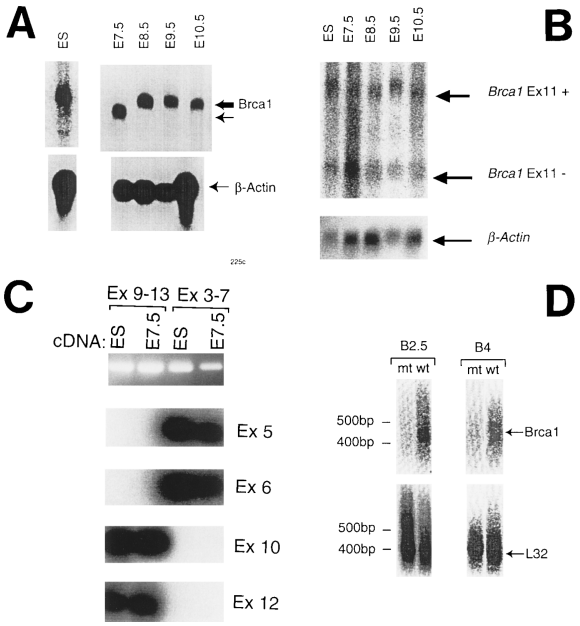


Figure 4. Expression of *Brca1* Gene in Wild-Type and Mutant *Brca1*$^{5-6}$ Embryos

(A and B) Northern blot analysis of total RNA prepared from ES cells and from wild-type embryos at 7.5, 8.5, 9.5, and 10.5 days of gestation. Hybridization with $^{32}$P-labeled exon 11 *Brca1* probe (A) detected strong expression of the *Brca1* gene in ES cells and in all analyzed stages of embryonic development. (B) Hybridization with $^{32}$P-labeled *Brca1* exon 7–9 probe showed two forms of *Brca1* transcripts (*Brca1* exon 11$^{+}$ and *Brca1* exon 11$^{-}$ transcripts). A probe to β-*actin* was used as a control for sample loading.

(C) *Brca1* alternatively spliced transcripts during embryogenesis. PCR amplification of *Brca1* exons 3–7 and exons 9–13 was performed using ES cells and E7.5 cDNA as described in Experimental Procedures. The ethidium bromide–stained agarose gel shows the 352 bp and 328 bp PCR products resulting from the amplification of exons 9–13 and exons 3–7, respectively. The 328 bp exons 3–7 PCR product hybridized specifically with the *Brca1* exon 5 and exon 6 probes, while the 352 bp exons 9–13 PCR product hybridized with the *Brca1* exon 10 and exon 12 specific probes, indicating the existence of exon 11 spliced transcript forms in both ES and E7.5 cells.

(D) Detection of *Brca1* expression in PCR-amplified total cDNAs from wild-type and mutant preimplantation embryos. Blots of PCR-amplified cDNA from E2.5 and E4.0 embryos were hybridized with a 3′ UTR probe from the *Brca1* gene. In contrast to the wild-type, no *Brca1* expression was detected in the E2.5 and E4.0 mutant embryos. A probe containing the 3′ UTR of the ribosomal housekeeping gene L32 was used as a control for the cDNA amplification.

the Brachyury protein is expressed in the primitive streak (Figure 5I). No signal was detected in mutant embryos (Figure 5J), further confirming that *Brca1*$^{5-6}$ mutant embryos fail to form mesoderm.

### Wild-Type Extraembryonic Tissue Does Not Rescue the *Brca1*$^{5-6}$ Mutant Phenotype

Since the most dramatic early defect in *Brca1*$^{5-6}$ mutant embryos occurred in their trophoblast lineages, it was determined whether later embryonic deficiencies were secondary to a failure of trophoblast–uterine interaction. Morulas from heterozygous intercrosses were aggregated with 4-cell stage wild-type CD1 tetraploid embryos. In such aggregates, the wild-type tetraploid cells are able to produce trophoblast and primitive endoderm lineages. Embryos from the aggregates were genotyped by PCR at day 10.5 of gestation because this time point was appropriate to determine if wild-type extraembryonic tissues rescued the lethality of *Brca1*$^{5-6}$ mutant embryos. From a total of 19 embryos, 15 embryos with normal morphology were genotyped as wild-type or as heterozygous, and four severely abnormal, degenerating embryos were found to be homozygous for the *Brca1*$^{5-6}$ mutation. These results suggest that the extraembryonic defect observed in the *Brca1*$^{5-6}$ homozygous embryos is not the sole cause of their early lethality.

### *Brca1*$^{5-6}$ Mutation Does Not Affect Apoptosis

To determine if the growth deficit of *Brca1*$^{5-6}$ mutant embryos could be accounted for by an excess of programmed cell death, serially sectioned E6.5 embryos were analyzed by Hoechst staining and TUNEL assay (Gavrieli et al., 1992). At this stage of development, morphological abnormalities were obvious but the mutant embryos were not yet degenerating. Staining with Hoescht dye revealed only a few apoptotic nuclei in the proamniotic cavity of mutant embryos (data not shown). Coucouvanis and Martin (1995) have suggested that these apoptotic nuclei are the cellular debris generated in the process of cavitation.

The results obtained by Hoechst staining were confirmed using a TUNEL assay. A few TUNEL-positive nuclei close to the proamniotic cavity could be detected in both wild-type and mutant embryos (data not shown), and no significant differences were detected. These results indicate that no excess of apoptosis occurs in *Brca1*$^{5-6}$ mutant embryos.

### *Brca1*$^{5-6}$ Mutation Decreases Embryonic Cell Growth

To study directly the effects of the *Brca1*$^{5-6}$ mutation on cellular proliferation, we examined the incorporation of 5-bromo-2′-deoxyuridine (BrdU) into DNA during the S phase of the cell cycle. Analysis was performed at E6.5, the time at which gastrulation initiates and when the highest mitotic activity has been observed in the mouse embryo (Snow, 1977). Whole litters were serially sectioned and phenotypes classified as wild type or mutant. A mitotic index showing the ratio between proliferating cells (BrdU-positive nuclei) and total cell number was made. Wild-type embryos show an index of 0.7–0.8, while mutant embryos show an index between 0.4 and 0.5. As exemplified in Figure 6A, nuclei in 70%–80% of epiblast cells from 57 phenotypically wild-type embryos were strongly positive for BrdU staining after 1 hr exposure. However, all 15 mutant embryos analyzed showed ~50% nuclear staining. Furthermore, the level of BrdU incorporation in the mutants was significantly weaker than in wild-type embryos (Figures 6B and 6C). Thus, BrdU incorporation is quantitatively and qualitatively affected in the mutant embryos. These results indicate

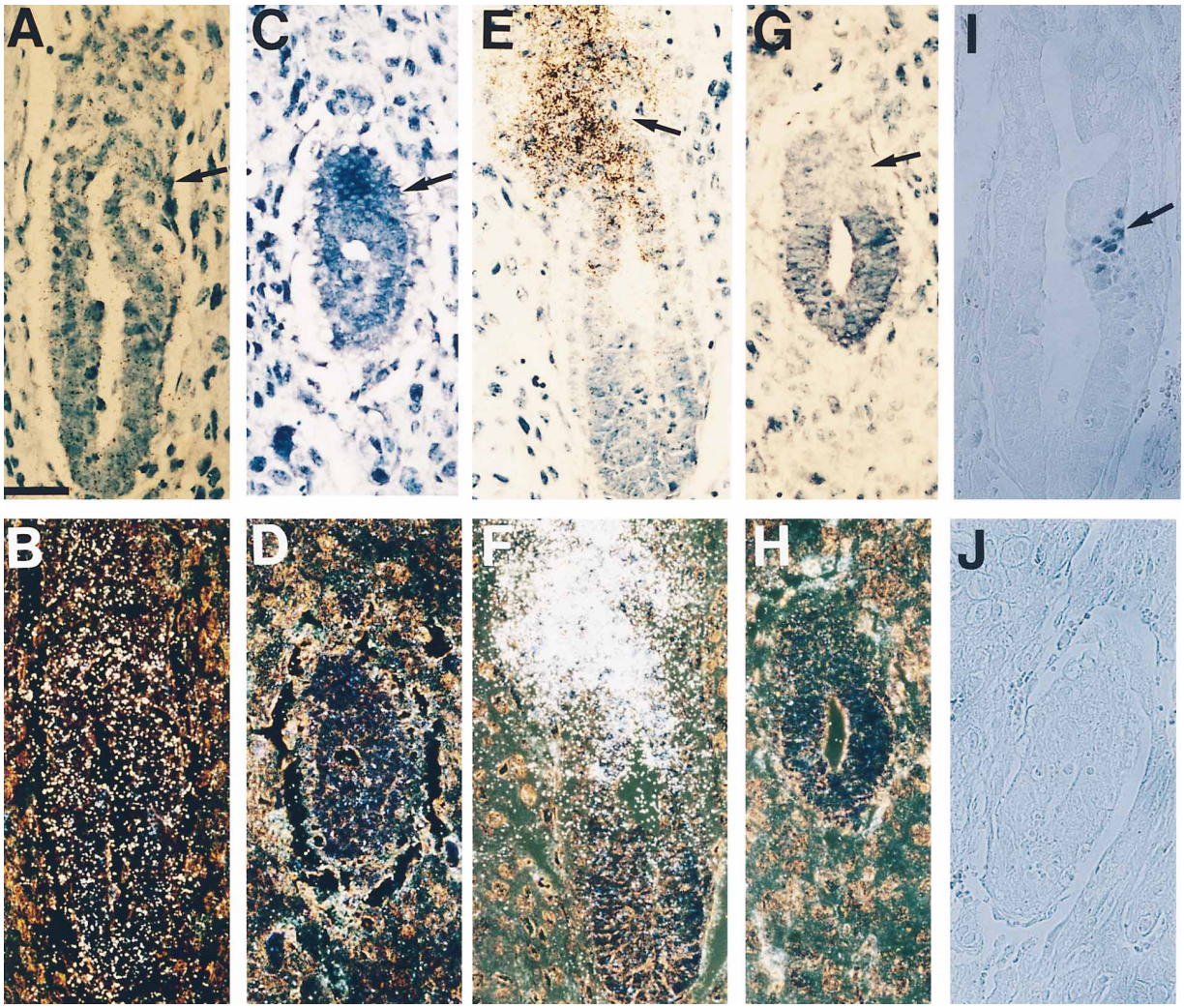


Figure 5. Spatial Expression of *Brca1*, *Mash-2*, and Brachyury in E6.5 *Brca1*$^{5-6}$ Mutant Embryos

In situ hybridization, bright-field (A, C, E, and G) and dark-field views (B, D, F, and H). Sagittal sections showing *Brca1* (A–D), *Mash-2* (E–H), and Brachyury (I and J) expression.

(A and B) *Brca1* expression in a E6.5 wild-type embryo. Sections were hybridized with an antisense *Brca1* exon 11 probe. *Brca1* is expressed ubiquituosly. No signal was detected with a sense-strand probe on an adjacent section to A (data not shown).

(C and D) Mutant embryo. No *Brca1* expression is detected. In (A) and (C), the arrow points to the separation between embryonic and extraembryonic regions.

(E and F) *Mash-2* expression in a E6.5 wild-type embryo. Strong expression is detected in the diploid trophoblast of the extraembryonic region of the embryo (arrow in [E]).

(G and H) *Mash-2* expression in a *Brca1*$^{5-6}$ mutant embryo. No signal is detected in the extraembryonic region (arrow in [G]).

(I and J) Immunohistochemical analysis of Brachyury expression. (I) Wild-type embryo. Arrow points to the Brachyury-positive cells in the nascent streak. (J) Mutant embryo. No expression is detected.

Bar, 60 μm.

that proliferative capabilities of *Brca1*$^{5-6}$ mutant embryos are severely impaired at E6.5.

To confirm this finding, we characterized the expression of regulatory proteins of cell-cycle progression in the developing embryo. Cyclin E and cyclin A were chosen, because the levels of these molecules change dramatically at specific points in the cell cycle. Cyclin E expression peaks at the G1/S transition (Koff et al., 1992) and has been shown to control the ability of cells to enter the S phase (Ohtsubo and Roberts 1993; Ohtsubo et al., 1995). Cyclin A expression peaks in late G2 phase and decreases toward mitosis. Affinity-purified antisera against cyclin E and cyclin A were used to stain serially sectioned E6.5 embryos.

Cyclin E was expressed in a large proportion (80%) of epiblast cells from 28 phenotypically wild-type embryos. The expression of cyclin E was markedly pronounced in the extraembryonic region, particularly in the trophoblast cells (Figure 6D), correlating with the results of the BrdU analyses. However, in the 10 mutant embryos analyzed, a lower proportion (50%–60%) of epiblast cells were positive for cyclin E. The signal was also weak, although staining of the trophoblast giant cells was comparable to that of their wild-type littermates

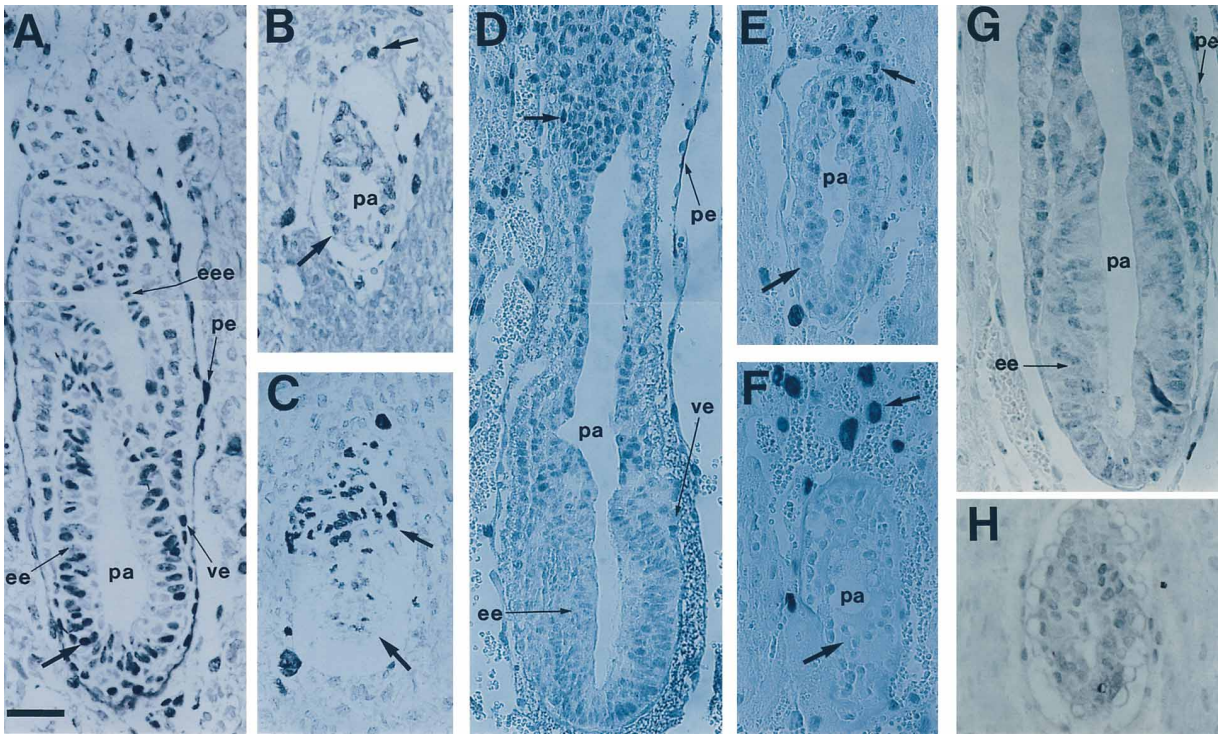


Figure 6. In Vivo Proliferation and Cell Cycle Alterations in E6.5 *Brca1*$^{5-6}$ Mutant Embryos

(A–C) BrdU incorporation; (D–F) Cyclin E expression. Sagittal sections. Positive nuclei are stained darkly. Unlabeled nuclei appear pale or white.

(A) Wild-type E6.5 embryo. Strongly BrdU-positive nuclei can be seen throughout the embryo. Thick arrow points to strongly positive cell in the ectoderm.

(B) Mutant *Brca1*$^{5-6}$ embryo. The large arrow points to weakly positive cells in the embryo. The small arrow points to strongly positive giant cells.

(C) More severe mutant *Brca1*$^{5-6}$ embryo. The small arrow points to the positive cells in the extraembryonic region. Few positive cells are detected in the embryo proper (large arrow).

(D) E6.5 wild-type embryo. Cyclin E expression is detected throughout the embryo and is particularly strong in the extraembryonic region (thick arrow).

The severity of the *Brca1*$^{5-6}$ mutant phenotype is paralleled by the levels of cyclin E expression:

(E) Less severe mutant *Brca1*$^{5-6}$ embryo. Positive cells are seen in the embryo (large arrow). In the extraembryonic region, giant cells (small arrow) are strongly positive.

(F) More severe *Brca1*$^{5-6}$ phenotype. Weakly positive cells are seen in the embryo (large arrow) and strongly positive cells in the trophoblast giant cells (small arrow).

(G) E6.5 wild-type embryo. Cyclin A expression is ubiquitous throughout the embryo.

(H) Mutant *Brca1*$^{5-6}$ embryo. No major changes in cyclin A expression compared with the wild type are observed.

Abbreviations as in Figure 3. Bar, 60 μm.

(Figures 6E and 6F). It should be noted that trophoblast cells do not divide, only endoreduplicate their DNA.

No apparent differences in the level of cyclin A expression were detected between wild-type (Figure 6G) and *Brca1*$^{5-6}$ mutant embryos (Figure 6H), indicating that the mutant embryos do not have a specific block in cell cycle progression during the G2 phase.

The decreased *Brca1*$^{5-6}$ embryo size apparent at E6.5 is consistent with the impaired proliferative capability observed in mutant epiblast cells. Gastrulation initiates at about E6.5, and a dramatic increase in cell proliferation occurs in normal embryos. It is possible that one of the causes of mutant embryo demise at about E7.5 is the inability of *Brca1*$^{5-6}$ mutants to proliferate at the required rate.

### Expression of *mdm-2*, *p53*, and *p21* in the Mutant Embryos

The reduced BrdU incorporation and cyclin E expression observed in *Brca1*$^{5-6}$ mutant embryos was consistent with an increase in the length of the mutant cell cycle, possibly due to a defect in the G1/S transition. Mutation of the *mdm-2* proto-oncogene, a negative regulator of the tumor suppressor p53 (Momand et al., 1992), leads to embryonic lethality before gastrulation with a phenotype reminiscent of *Brca1*$^{5-6}$ mutants (Jones et al., 1995; Montes de Oca Luna et al., 1995). The p53 protein blocks cell cycle progression at the G1 phase, and thus, mutation of *mdm-2* results in a block of cell proliferation that causes early embryonic lethality. The absence of p53 activity rescues the lethality of the *mdm-2* mutant, since mice

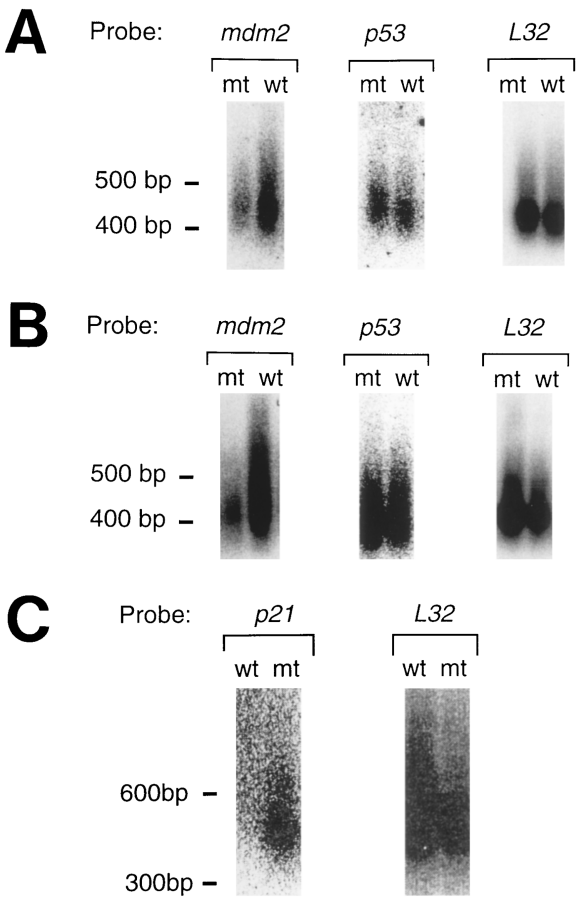


Figure 7. *Brca1*$^{5-6}$ Mutant Embryos Show Decreased *mdm-2* Expression and Increased *p21* Expression

The expression of *p53* and its negative regulator *mdm-2* was analyzed in *Brca1*$^{5-6}$ mutants using blots of total cDNA made from E2.5 (A) and E4 (B) single embryos (see Experimental Procedures). The blots were hybridized to 3′ UTR probes specific for *mdm-2*, *p53* or *L32* (control). While *p53* expression was not affected, the expression of *mdm-2* was reduced in *Brca1*$^{5-6}$ mutant embryos. The *L32* gene was expressed equally in wild-type and mutant blastocysts. (C) The expression of *p21*, weakly expressed in the E4 wild type, was dramatically increased in the E4 *Brca1*$^{5-6}$ mutant.

that are double mutants for *mdm-2* and *p53* are viable (Jones et al., 1995; Montes de Oca Luna et al., 1995).

Based on the phenotypic similarity between *Brca1*$^{5-6}$ and *mdm-2* mutant mice and the effect of the *Brca1*$^{5-6}$ mutation on cellular proliferation, we determined whether the *Brca1*$^{5-6}$ lethality could be due to changes in *mdm-2* or *p53* expression. *mdm-2* and *p53* expression in *Brca1*$^{5-6}$ embryos was examined using 3′ UTR probes in a Southern blot analysis of the total cDNA generated from E2.5 and E4 wild-type and mutant embryos. *mdm-2* expression was found to be decreased in mutant embryos, whereas the level of *p53* appeared to be unaffected (Figures 7A and 7B).

Interestingly, the expression of a downstream effector and transcription activation target for p53, the cyclin-dependent kinase (cdk) inhibitor *p21* (Gu et al., 1993; Harper et al., 1993), was found to be dramatically increased in the mutant E4 blastocyst cDNA (Figure 7C), while the very weak expression of *p27* (another cdk inhibitor) was not affected (data not shown). In situ experiments confirmed the highly significant increase in *p21* expression in the mutant E6.5 compared with the wild-type (data not shown).

### *Brca1*$^{5-6}$ Mutation Affects Inner Cell Mass Proliferation In Vitro

To directly determine the growth capability of mutant *Brca1*$^{5-6}$ embryos, E3.5 blastocysts from heterozygous matings were collected and cultured individually in vitro. Mutant blastocysts showed a normal phenotype, indicating that the *Brca1*$^{5-6}$ mutation does not affect preimplantation development (Figures 8A and 8C). After 3 days in culture, both mutant and wild-type blastocysts produced trophoblast giant cell outgrowths (Figures 8B and 8D). However, the inner cell mass (ICM) did not grow in 41% of the mutant blastocysts, compared with failure in only ~15% of wild-type and heterozygous blastocysts (Figure 8; Table 2). Longer periods of blastocyst culture confirmed the inability of homozygous *Brca1*$^{5-6}$ mutant cells to grow in vitro. After 10 days in culture, only one of 24 recovered blastocyst outgrowths was found to be homozygous for the *Brca1*$^{5-6}$ mutation. This mutant had not developed any ICM, only trophoblast giant cells (data not shown). In addition, numerous attempts to generate *Brca1*$^{5-6}$ homozygous mutant ES cells by increasing G418 concentration, or by using a second targeting vector with a hygromycin resistance cassette, were unsuccessful (data not shown). These results from in vitro experiments directly support a generalized failure in cellular proliferation in *Brca1*$^{5-6}$ mutants.

## Discussion

### The *Brca1*$^{5-6}$ Mutant Phenotype

In this study, we have generated a null mutation of the *Brca1* gene by homologous recombination in mouse ES cells. The *Brca1*$^{5-6}$ mutation drastically affects embryonic growth and the differentiation of the diploid trophoblast tissue, resulting in lethality before day 7.5 of gestation.

Our results contrast with the recent analysis of *Brca1* mutant mice obtained by deletion of exon 11 (Gowen et al., 1996). These *Brca1* exon 11 mutant mice survived until midgestation, dying in utero between days 10 and 13 of development. The primary site of anomaly was the neural tube, with mutants presenting varying degrees of spina bifida and anencephaly. In addition, these mutant embryos showed both increased cellular proliferation and cell death in the hindbrain neuroepithelium.

Previous studies have demonstrated the presence of several alternatively spliced forms of both human and murine breast cancer 1 gene transcripts (Miki et al., 1994; Lane et al., 1995; Marquis et al., 1995). A splice variant of *Brca1* that is missing exon 11 is coexpressed at similar levels to the full-length *Brca1* transcript in most cells and tissues (Frank Calzone, personal communication). We show here, in ES cells and during embryogenesis, the coexpression of *Brca1* exon 11 positive and exon 11 negative transcripts. However, *Brca1* exons

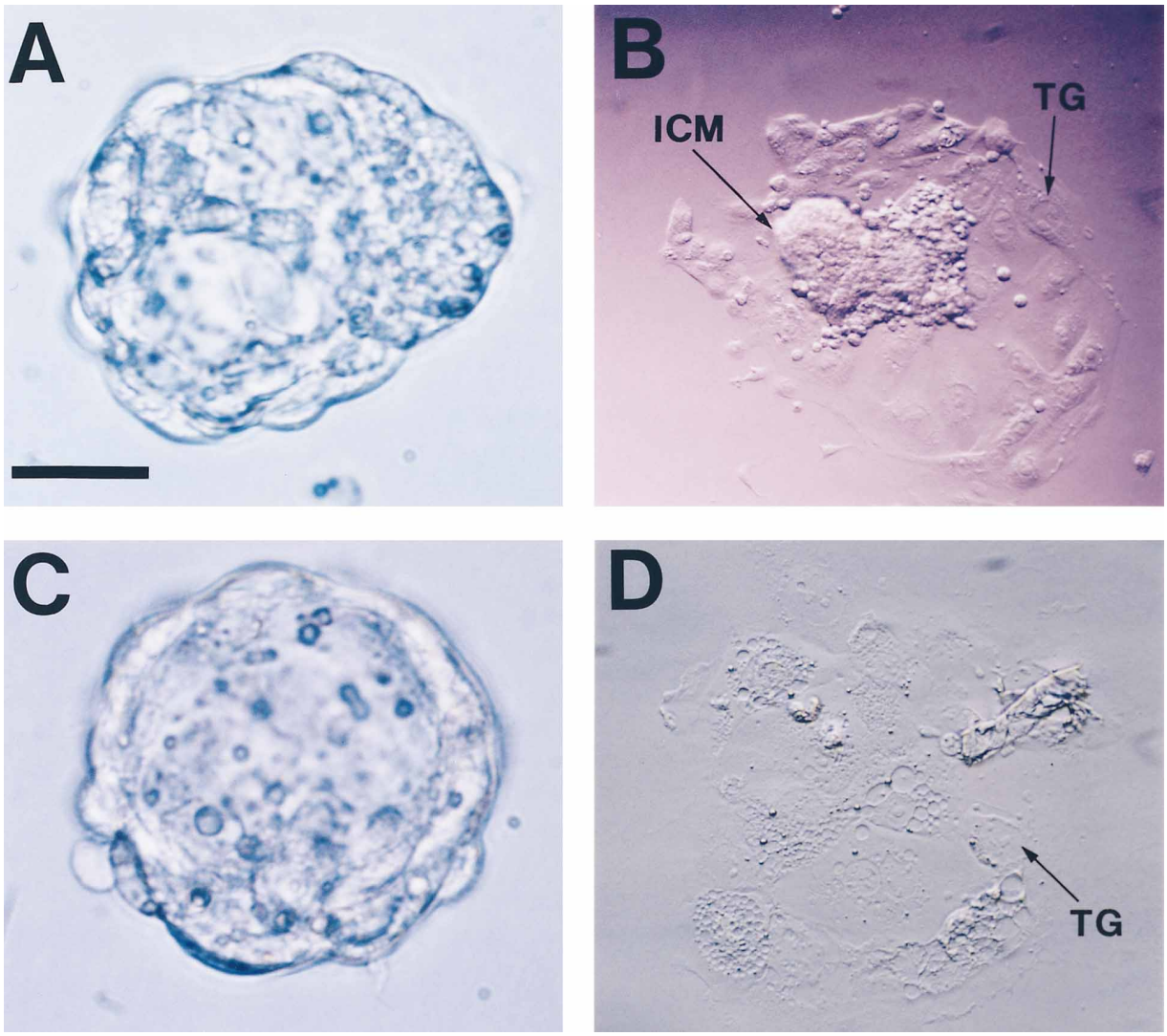


Figure 8. Defective Growth of Mutant *Brca1*$^{5-6}$ Blastocysts In Vitro

(A) Wild-type E3.5 blastocyst. (B) Outgrowth after three days of culture. The inner cell mass (ICM) is surrounded by trophoblast giant cells (TG). (C) Mutant E3.5 blastocyst. (D) Outgrowth after three days of culture. Only trophoblast giant cells have grown (no ICM growth). Bar, 20 μm in (A) and (C); 40 μm in (B) and (D).

Table 2. Genotypic and Phenotypic Analysis of *Brca1*$^{5-6}$ Mutant Blastocysts Cultured In Vitro

| | | Phenotype | |
|---|---|---|---|
| Genotype | Total Number | Normal | Abnormal |
| *Brca1*$^{+/+}$ | 13 | 11 (85%) | 2 (15%) |
| *Brca1*$^{+/-}$ | 35 | 30 (86%) | 5 (14%) |
| *Brca1*$^{-/-}$ | 12 | 7 (59%) | 5 (41%) |

Blastocysts at day 3.5 of gestation from *Brca1*$^{5-6}$ heterozygous intercrosses were collected and individually cultured in ES media without LIF. Embryos grown in vitro for three days were photographed and genotyped by PCR amplification using primers a, b, c, and d as shown in Figure 1. The embryo phenotype was scored as abnormal when the ICM did not develop after three days in culture. Numbers in parentheses indicate percentages of cultured blastocysts with normal and abnormal phenotypes. Twenty-four blastocyst outgrowths were recovered after 10 days in culture; only one was *Brca1*$^{5-6}$, and it had not developed any ICM.

5 and 6 are always present during embryogenesis, suggesting that they are essential for Brca1 function at these stages. Thus, deletion of exons 5 and 6 results in the inactivation of *Brca1* gene function. The weaker phenotype associated with the deletion of *Brca1* exon 11 could therefore result from incomplete inactivation of the *Brca1* gene due to alternative splicing of this exon during early embryogenesis. Alternative splicing variants of other tumor suppressor genes have previously been reported to have different functions (Englert et al., 1995).

### *Brca1* and Trophoblast Differentiation

Extraembryonic tissues play an important role in development from the very early stages, allowing the embryo to attach to the uterus during implantation, and later participating in the formation of the chorioallantoic placenta. Histological analysis of *Brca1*$^{5-6}$ mutant embryos

at E6.5 revealed a poorly organized extraembryonic region. In addition, *Mash-2*, a transcription factor expressed at high levels in the diploid component of the trophoblast, was not expressed in *Brca1*$^{5-6}$ mutant embryos. *Mash-2* mutant mice die at day 10 of gestation but can be rescued by providing wild-type extraembryonic tissues (Guillemot et al., 1994). It was possible that the early death of the *Brca1*$^{5-6}$ mutant embryos could have been due to abnormal development of the trophoblast. However, aggregation of *Brca1*$^{5-6}$ morulas with wild-type tetraploid embryos did not rescue their lethality. Therefore, abnormal trophoblast development is likely to be a factor in, but not the sole cause of, lethality in *Brca1*$^{5-6}$ mutant embryos.

Interestingly, the polyploid trophoblast giant cells (which do not normally express *Mash-2*) appear to be unaffected in *Brca1*$^{5-6}$ mutant embryos. The normal development of these cells suggests that *Brca1* might be exclusively involved in the differentiation of the diploid lineages of the trophoblast. Alternatively, the giant cells that survive may be the primary giant cells that derive from the blastocysts (Varmuza et al., 1988). It is possible that a maternal supply of Brca1 protein or RNA is sufficient to allow the generation of these early trophoblast cells. Survival of the trophoblast giant cells has been observed in mice mutant for the *Min* allele of the tumor suppressor gene *Apc*. *Min* mutant embryos die at about E7.5 and show a phenotype similar to that of *Brca1*$^{5-6}$ embryos, in that epiblast development is impaired and only trophoblast giant cells survive after E7.5 (Moser et al., 1995).

### *Brca1* and Cellular Proliferation and Gastrulation

Gastrulation in the mouse embryo takes place at about day 6.5 of gestation, when the mesoderm is generated from the epiblast. Gastrulation is associated with rapid epiblast cell proliferation. It has been reported that 1400–1500 cells must accumulate in the epiblast to initiate this process (Power and Tam., 1993). A wild-type embryo at E5.5 contains 120 epiblast cells, whereas at E6.5 and E7.5, this number increases to 660 and 14,290 cells, respectively (Snow, 1977). This dramatic increase in cell number correlates with a decrease in cell cycle length from 11.5 hr at E5.5 to 4.4 hr at E6.5 of gestation.

A defect in *Brca1*$^{5-6}$ epiblast cell proliferation was clearly defined by the BrdU incorporation analysis in E6.5 *Brca1*$^{5-6}$ mutants. Our results indicate that cellular proliferation was impaired quantitatively and qualitatively. In addition, cyclin E expression was decreased in *Brca1*$^{5-6}$ mutant embryos, further suggesting a defect in the ability of these cells to enter the S phase. If *Brca1* plays a role in the control of cellular proliferation, its inactivation should cause lethality at earlier developmental stages. Nevertheless, E3.5 *Brca1*$^{5-6}$ blastocysts appeared normal, although their proliferative abilities in vitro were dramatically reduced. Only giant cells survived after culture, suggesting that this lineage may be less dependent on Brca1 protein. The in vivo survival of *Brca1*$^{5-6}$ mutant embryos until E6.5 could be due to persistence of maternal Brca1 protein or mRNA that permits the development of the embryo until early gastrulation stages.

Lack of mesoderm formation has been previously observed in other mutant embryos having defects in epiblast proliferation. Mice mutant for *Bmpr*, a gene coding for a protein member of the TGF-β superfamily receptor, showed decreased epiblast cell proliferation at E6.5 and no mesoderm formation (Mishina et al., 1995). At the time of gastrulation, E6.5 *Brca1*$^{5-6}$ mutant embryos showed a severely abnormal phenotype, with no obvious mesoderm formation, and died shortly thereafter. The lack of morphological differentiation of mesoderm in E6.5 *Brca1*$^{5-6}$ mutants was confirmed by their failure to express Brachyury. Our data are consistent with an indirect requirement for *Brca1* in mesoderm development, related to its role in epiblast proliferation prior to the stage of gastrulation.

### Novel Role for *Brca1*

Cancers result from genetic lesions leading to uncontrolled cell growth. Several tumor suppressor genes have been cloned, and mutations of these genes have been shown to be responsible for tumor development. Inherited mutations and loss of heterozygosity of *BRCA1* have been shown in patients from families with early onset of breast and ovarian cancer. Transfection of the wild-type *BRCA1* gene inhibits breast and ovarian cancer cell growth but does not affect the proliferation of lung and colon cancer cells (Holt et al., 1996). Thus, in the adult, *BRCA1* has a tissue-specific growth inhibitory function and acts as a tumor suppressor gene.

*Brca1* is highly expressed in proliferating mouse ES cells and in activated proliferating splenocytes (data not shown), suggesting that *Brca1* might be acting in these cells as a positive regulator of cell growth. Wild-type E6.5 epiblast cells, which express *Brca1*, are actively proliferating cells, whereas the *Brca1*$^{5-6}$ mutation causes a decrease in cellular proliferation. In addition, we were unsuccessful in our attempts to obtain viable ES cells homozygous for the *Brca1*$^{5-6}$ mutation, supporting the defect in cell growth identified in vivo and in vitro. Thus, the *Brca1* gene may play, at some developmental stages and in specific tissues, a positive role in control of cell growth.

We provide evidence suggesting that *Brca1* might affect cell cycle regulation. *p53* expression levels were unaffected in *Brca1*$^{5-6}$ mutants, although expression of *mdm-2*, a gene coding for a negative regulator of p53 activity, was reduced. Furthermore, the expression of *p21*, a G1 cell cycle transition inhibitor, was increased in E4 and E6.5 *Brca1*$^{5-6}$ mutants. P21 is a universal cdk inhibitor (Gu et al., 1993; Harper et al., 1993), and its expression is directly regulated by p53 protein (El-Deiry et al., 1993). Although the expression of *p21* can be induced by other regulatory signals, the decrease in *mdm-2* expression observed in *Brca1*$^{5-6}$ embryos suggests that there could be an increase in p53 activity which could, in turn, increase *p21* expression, causing the impaired growth of the *Brca1*$^{5-6}$ mutants.

Cyclin cdk complexes containing more than one p21 subunit are not catalytically active, suggesting that the stoichiometry of p21 and cdk's is important for the function of these complexes (Zhang et al., 1994). Transgenic mice overexpressing *p21* in their hepatocytes showed

a dramatic block in hepatocyte proliferation (Wu et al., 1996). The in vivo effect of *p21* overexpression in these transgenic mice was cell autonomous and did not lead to an increase in apoptosis. As mentioned above, no defect in apoptosis was observed in *Brca1*$^{5-6}$ mutant mice. Previous studies have suggested a role for p21 both in differentiation and in the inhibition of cellular proliferation. However, *p21* mutant mice were found to develop normally, suggesting that p21 is not essential for cell differentiation (Brugarolas et al., 1995; Deng et al., 1995). Thus, the increased *p21* expression in the *Brca1*$^{5-6}$ mutant embryos is more likely to have affected cellular proliferation rather than differentiation.

The high level of p21, the low BrdU incorporation, and persistent expression of cyclins in *Brca1*$^{5-6}$ mutant embryos are all strongly suggestive of a slowed progression through the cell cycle (or even a cell cycle block). In addition to these defects observed in vivo, growth of the mutant embryos was also grossly retarded in vitro. Though unlikely, it remains a possibility that *Brca1* mutation results in the expansion of a particular cell type that in turn exerts a negative growth effect on the embryos, leading to lethality.

In summary, our results demonstrate that *Brca1* may be a positive regulator of cellular proliferation in early embryogenesis. We also provide evidence supporting the requirement of *Brca1* for the cell cycle progression of embryonic cells, suggesting that Brca1 may be involved directly or indirectly in its regulation. Establishment of tissue-specific *Brca1* mutations will help define the precise function of *Brca1* during oncogenesis and normal development.

## Experimental Procedures

### Cells

E14K embryonic stem cells from 129/Ola mice were maintained on a layer of mitomycin C–treated embryonic fibroblasts in Dulbecco's modified Eagle's culture medium, supplemented with leukemia inhibitory factor, 15% fetal calf serum, L-glutamine, and β-mercaptoethanol.

### Generation of *Brca1*$^{5-6}$ Mutant Mice

A 129/J mouse genomic library was screened with an exon 11 *BRCA1* human probe made by PCR amplification using primers at positions 1161 and 1981 (5′-TGT GAG AGA AAA GAA TGG AAT-3′ and 5′-TGA ATA TGC CTG GTA GAA GAC-3′). Five overlapping phage genomic clones containing exons 5–13 were isolated. A targeting vector was designed to replace a 3.3 kb genomic fragment containing exons 5 and 6 with the PGKneo resistance expression cassette in reverse orientation to *Brca1* transcription. The targeting vector (20 μg) was linearized with KpnI and electroporated into ($5 \times 10^6$) E14K ES cells (Bio-Rad Gene Pulser, 0.34 kV, 0.25 mF). The cells were subsequently cultured in the presence of 300 μg/ml of G418 (Sigma) for 10 days. Homologous recombinants were identified by PCR. An external primer "c" specific for the *Brca1* gene upstream of the targeting construct (5′-TCC TTT GGT TTA GCA CCT ACT-3′) and a primer "d" specific for the neomycin resistance gene cassette (5′-CCA GCT CAT TCC TCC CAC TCA T-3′) were used in the PCR analysis. Colonies positive by PCR were genotyped by Southern blotting of HindIII digested DNA and hybridized with random hexamer $^{32}$P-dCTP-labeled DNA probes (Amersham). A HindIII–KpnI 378 bp *Brca1* external probe and a neomycin–specific probe were used for hybridization. Four correct targeted colonies were identified.

Chimeric mice were produced by microinjection of targeted ES cells into 3.5-day C57BL/6J blastocysts that were transferred to CD1 pseudopregnant foster mothers. Chimeric males were mated with C57BL/6J females (Jackson Laboratories), and germline transmission of the mutant allele was verified by PCR and Southern blot analysis of tail DNA from F1 offspring with agouti coat color. Two injected ES colonies contributed to the germline of mice. F2 offspring from heterozygous intercrosses were genotyped by Southern blotting. The heterozygous males were also crossed with CD1 and C3H/HeJ females (Jackson Laboratories). Mutant mice derived from the two targeted ES cells showed the same phenotype.

### PCR Analysis of *Brca1*$^{5-6}$ Genotypes

Genomic DNA from ES cells and neonate tails was isolated and used in PCR amplification. Yolk sacs, E6.5, E7.5, E8.5 embryos, or in vitro cultured blastocysts were incubated overnight at 37°C in 100 μl of lysis buffer (50 mM KCL, 10 mM Tris (pH 8.3), 2 mM $MgCl_2$, 0.1 mg/ml gelatin, 0.45% NP-40, and 0.45% Tween-20) containing 10 μg/ml of proteinase K. After 10 min boiling, 1–5 μl of the samples was subjected to PCR amplification. Primers "a" (5′-GAT TTG GTC TTT TTC CTC ACA-3′) and "b" (5′-CTT ATT GCA CTT ATT TAT TGG-3′), specific for the targeted intronic sequence downstream of *Brca1* exon 6, were used to detect the wild-type allele, while the primers "c" and "d" (see above) were used to detect the recombinant allele. Temperature cycling conditions were: one initial cycle at 94°C for 5 min, 59°C for 5 min, and 72°C for 2 min, followed by 40 cycles at 94°C for 30 s, 59°C for 1 min, and 72°C for 1 min. One-fifth of each reaction mixture was electrophoresed on a 2% agarose gel and stained with ethidium bromide. Primer pair a/b amplified a 353 bp fragment in the heterozygote and wild-type samples, whereas the primer pair c/d amplified a 616 bp fragment in both heterozygous and homozygous DNA.

### RNA Analysis

Total RNA was extracted from ES cells or E7.5, E8.5, E9.5, and E10.5 using Trizol (Life Technologies). For Northern blot analysis, 10 μg RNA were electrophoresed in a 1% agarose/formaldelhyde gel and transferred to a nylon membrane (Hybond N$^+$, Amersham). RNA blots were hybridized overnight at 65°C in Church and Gilbert buffer. The filters were initially hybridized with either a 243 bp exon 11, or a 248 bp exons 7–9, *Brca1*-specific probes, and subsequently stripped and rehybridized with a mouse β-*actin* probe. *Brca1* exon 11 probe was amplified by PCR using an upstream primer at position 1270 (5′-AAG TGG ATG GGG GTT TTA GTT-3′) and a downstream primer at position 1513 (5′-TTA TTT GTG AAG GGC TGC TCT-3′). *Brca1* exons 7–9 probe was amplified using an upstream primer at position 360 (5′-AAT AAT TCT TGT GAG CGT TTG-3′) and a downstream primer at position 587 (5′-CCT GGC TTA GTT ACT GCT TCT-3′). RNA blots were washed in 2× SSC at room temperature for 15 min, followed by two washes in 2× SCC 0.1% SDS at 65°C for 30 min, and finally with 2 washes in 0.2× SCC 0.1% SDS at 65°C for 30 min.

cDNAs from ES and E7.5 cells were generated using random primers and reagents from Amersham. PCR amplification of *Brca1* exons 3–7 was performed using an exon 3 upstream primer at position 111 (5′-GAA CTG ATC AAA GAA CCT GTT-3′′) and an exon 7 downstream primer at position 418 (5′-GAA GCC TTC TGA CAC GGT TCC-3′). PCR amplification of *Brca1* exons 9–13 was performed using an exon 9 upstream primer at position 593 (5′-AGT AAC TAA GCC AGG TGA TTG-3′) and an exon 13 downstream primer at position 4225 (5′-CTG ACA TGT TTG GTT CCA GAT-3′). Oligonucleotides used for the *Brca1* PCR Southern blot hybridization were: an exon 5 specific primer (5′-TTT GTA TGC TGA AAC TTC TTA-3′), an exon 6 specific primer (5′-CAA GCT CAA AAG CAG CCA TTA-3′), an exon 10 specific primer (5′-TGT TAC AGA CCG CCC CTC AAG-3′), and an exon 12 specific primer (5′-GCG AGC AGT CTT CAG AAA GGT-3′).

### Amplification of Total cDNA in the Preimplantation Embryos

Embryos at day 2.5 and day 4 of gestation were collected from heterozygous crosses, and total cDNA was amplified following a protocol previously described for single cell PCR (Brady et al., 1990). In brief, individual embryos were lysed at room temperature for 20 min in 6 μl of first strand buffer (5 mM Tris–HCL [pH 8.3], 75 mM KCL, 3 mM $MgCl_2$, 2 μM of each dNTP, 100 ng/ml (dT)24, 100 U/ml Inhibit Ace [5′-3′ Inc.], 2000 U/ml RNAguard [Pharmacia], and 0.5%

NP-40). The nuclei were recovered by centrifugation at 12,000 g for 50 s, and 4 μl of the supernatant were used to generate the total cDNA. The nuclei were lysed in 10 μl of water by heating the samples at 95°C for 2 min, and 1/5 of the sample was used to genotype the embryos by PCR using the primers a/b and c/d described above. For most embryos, a second round of PCR was required to detect a PCR product using nested primers. The remaining supernatant was heated at 65°C for 1 min, cooled at 22°C for 3 min, and returned to ice. The poly(A) mRNA present in the supernatant was then oligo(dT)-primed and converted to cDNA with 100 U Moloney (GIBCO–BRL) and 2 U avian reverse transcriptase (Boehringer/Mannheim) by incubating the samples at 37°C for 15 min. The reaction was terminated by heat inactivation of the enzymes at 65°C for 10 min, and returned to ice. These conditions limit the length of the cDNAs generated to between 300 to 700 bases. Total cDNA was tailed with oligo(dA) using terminal transferase followed by PCR amplification using an oligo(dT) primer. This process generates an almost unlimited source of cDNA and results in the unbiased amplification of cDNAs representing all polyadenylated RNAs present in the sample. The expression of any gene can be assessed by Southern blotting of the PCR reaction using probes prepared by PCR amplification of the 3′ untranslated region of the gene tested.

### Generation of Tetraploid Aggregates

E1.5 embryos at the 2-cell stage were flushed from the oviduct of CD1 females and subjected to electrofusion (Nagy and Rossant, 1993). Successfully fused embryos were cultured overnight in embryo culture medium in 5% $CO_2$ at 37°C. E2.5 embryos at the 8-cell stage were flushed from the oviduct of a *Brca1*$^{5-6}$ heterozygote female who had been mated to a *Brca1*$^{5-6}$ heterozygote male. Two 4-cell tetraploid embryos were sandwiched around one diploid 8-cell stage embryo from the heterozygous cross and aggregated overnight. Successfully aggregated embryos formed single blastocysts, which were transferred to E2.5 pseudopregnant recipients. Recipients were sacrificed at E10.5 and embryonic morphology was scored. Genotypes in samples of embryonic tissue were determined by PCR analysis.

### In Vitro Culture of Preimplantation Embryos

*Brca1*$^{5-6}$ heterozygote males and females were intercrossed for 2 hr, and E3.5 embryos were collected by flushing them from the uterus of the plugged females. Blastocysts were individually cultured in 24-well plates in ES cell media without leukemia inhibitory factor, in 5% $CO_2$ at 37°C. Photographs of the cultured embryos were taken every 12 hr. After 3 days in culture, the morphology of the embryos was noted and their genotype was determined by PCR.

### Histological Analysis

Uteri from females plugged in a 2-hr mating period were isolated in ice-cold phosphate-buffered saline at E5.5 and E6.5, fixed overnight in 4% paraformaldehyde at 4°C, dehydrated, and embedded in paraffin. Sections 5 μm thick were cut and stained with hematoxylin and eosin.

### In Situ Hybridization

Uteri were isolated in ice-cold phosphate-buffered saline at E6.5 and processed as for histological analysis. The probes used were *Brca1* (exon 11, 243 bp; see RNA Analysis, above), *Mash-2* (Guillemot et al., 1994), and full-length *p21*, *p27*, and *cdk2* cDNAs. Probes were labeled with $^{33}$P-UTP and processed according to described protocols (Hui and Joyner, 1993).

### Immunohistochemistry

Uteri were isolated in ice-cold phosphate-buffered saline at E6.5, fixed in 4% paraformaldehyde for 3 hr, dehydrated, embedded in wax, and sectioned at 5 μm. The following polyclonal antisera were used: anti-T (1:500 dilution) (recognizes amino acids 328–420 of the carboxy-terminal part of the T protein; Kispert and Herrmann, 1993); anti–cyclin A (1:200) and anti–cyclin E (1:200). A biotinylated secondary antibody against rabbit IgG and avidin-conjugated peroxidase (Vector Laboratories) were used for immunostainings. A protocol described by Trumpp et al. (1992) was used with the following modifications: after rehydration, endogenous peroxidases were quenched by incubating the sections for 40 min in methanol containing 1% $H_2O_2$. Sections were incubated for 5 hrs at room temperature with the primary antibody, 1 hr with goat anti-rabbit IgG, and 1 hr with avidin–peroxidase complex, at room temperature. The signal was visualized in 30–60 min by an horseradish peroxidase reaction (Vector Laboratories) using diaminobenzidine (DAB, 1 mg/ml in 0.1 M Tris–HCl [pH 7.5]) and hydrogen peroxide (0.03% final) as substrates. To enhance the signal, $NiCl_2$ (0.04% final) was used in the developer cocktail.

### BrdU Labeling of Embryos

BrdU labeling of cells in the S phase of the cell cycle was performed according to the protocol described by Hayashi et al. (1988). BrdU (100 μg per gram of body weight) was injected intraperitoneally into pregnant females at E6.5. The females were sacrificed 1 hr after injection, the uteri were removed, and the decidual swellings were fixed in 4% paraformaldehyde at 4°C overnight and processed for immunohistochemistry. The sections were incubated with an anti-BrdU monoclonal antibody (Boehringer Mannheim) at a 1:10 dilution. Staining was performed according to the protocol described by Mishina et al. (1995).

### Acknowledgments

Correspondence should be addressed to T. W. Mak. We thank Sam Benchimol for providing us with several probes; Bernhard Herrmann for the Brachyury antiserum; Arda Shahinian and Christine Brunaud for helping with the cell culture; Yuji Mishina and Richard Behringer for providing and giving us helpful advice about their excellent BrdU protocol; and Stephan Gasca for critical reading of the manuscript. Janet Rossant is a Terry Fox Cancer Research Scientist of the National Cancer Institute of Canada and a Howard Hughes International Scholar, supported by a grant from the Medical Research Council of Canada.

Received April 8, 1996; revised May 31, 1996.

### References

Brady, G., Barbara, M., and Iscove, N.N. (1990). Representative in vitro cDNA amplification from individual hematopoietic cells and colonies. Meth. Mol. Cell. Biol. *2*, 17–25.

Brugarolas, J., Chandrasekaran, C., Gordon, J.I., Beach, D., Jacks, T., and Hannon, G.J. (1995). Radiation-induced cell cycle arrest compromised by p21 deficiency. Nature *377*, 552–557.

Castilla, L.H., Cough, F.J., Erdos, M.R., Hoskins, K.F., Calzone, K., Garber, J.E., Boyd, J., Lubin, M.B., Deshano, M.L., Brody, L.C., et al. (1994). Mutations in the *BRCA1* gene families with early-onset breast and ovarian cancer. Nature Genet. *8*, 387–391.

Chen, Y.M., Chen, C.F., Riley, D.J., Allred, D.C., Chen, P.L., Von Hoff, D., Osborne, C.K., and Lee, W.H. (1995). Aberrant subcellular-localization of BRCA1 in breast cancer. Science *270*, 789–791.

Cornelis, R.S., Neuhausen, S.L., Johansson, O., Arason, A., Kelsell, D., Ponder, B.A.J., Tonin, P., Hamann, U., Lindblom, A., Lalle, P., et al. (1995). High allele loss rates at 17q12-q21 in breast and ovarian tumors from BRCA1-linked families. Genes Chrom. Cancer *13*, 203–210.

Coucovanis, E., and Martin, G.R. (1995). Signals for death and survival: a two-step mechanism for cavitation in the vertebrate embryo. Cell *83*, 279–287.

Deng, C., Zhang, P., Harper, J.W., Elledge, S.J., and Leder, P. (1995). Mice Lacking p21*CIP1/WAF1* undergo normal development, but are defective in G1 checkpoint control. Cell *82*, 675–684.

Easton, D.F., Bishop, D.T., Ford, D., and Crockford, G.P. (1993). Genetic linkage analysis in familial breast and ovarian cancer: results from 214 families. Am. J. Hum. Genet. *52*, 678–701.

El-Deiry, W.S., Tokino, T., Velculescu, V.E., Levy, D.B., Parsons, R., Trent, J.M., Lin, D., Mercer, E., Kinzler, K.W., and Vogelstein, B. (1993). WAF1, a potential mediator of p53 tumor suppression. Cell *75*, 817–825.

Englert, C., Hou, X., Maheswaran, S., Bennett, P., Ngwu, C., Re, G.G.,

Garvin, A.J., Rosner, M.R., and Haber, D.A. (1995). WT1 suppresses synthesis of the epidermal growth factor receptor and induces apoptosis. EMBO J. *14*, 4662–4675.

Ford, D., Easton, D.F., Bishop, D.T., Narod, S.A., and Goldgar, D.E. (1994). Risks of cancer in BRCA1-mutation carriers. Breast cancer linkage consortium. Lancet *343*, 692–695.

Friedman, L.S., Ostermeyer, E.A., Szabo, C.I., Dowd, P., Lynch, E.D., Rowell, S.E., and King, M.C. (1994). Confirmation of BRCA1 by analysis of germline mutations linked to breast and ovarian-cancer in 10 families. Nature Genet. *8*, 399–404.

Futreal, P.A., Liu, Q.Y., Shattuck Eidens, D., Cochran, C., Harshman, K., Tavtigian, G., Bennett, L.M., Haugenstrano, A., Swensen, J., Miki, Y.K.E., et al. (1994). *BRCA1* mutations in primary breast and ovarian carcinomas. Science *266*, 120–122.

Gavrieli, Y., Sherman, Y., and Ben-Sasson, S.A. (1992). Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. J. Cell Biol. *119*, 493–501.

Gowen, L.C., Johnson, B.L., Latour, A.M., Sulik, K.K., and Koller, B.H. (1996). BRCA1 deficiency results in early embryonic lethality characterized by neuroepithelial abnormalities. Nature Genet. *12*, 191–194.

Gu, Y., Turek, C.W., and Morgan, D.O. (1993). Inhibition of CDK2 activity in vivo by an associated 20k regulatory subunit. Nature *366*, 707–710.

Guillemot, F., Nagy, A., Auerbach, A., Rossant, J., and Joyner-A, L. (1994). Essential role of Mash-2 in extraembryonic development. Nature *371*, 333–336.

Hall, J., Lee, M.K., Newman, B., Morrow, J.E., Anderson, L.A., Huey, B., and King, M.C. (1990). Linkage of early on-set familial breast cancer to chromosome 17q21. Science *250*, 1684–1689.

Harper, J.W., Adami, G.R., Wei, N., Keyomarsi, K., and Elledge, S.J. (1993). The p21-cdk interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell *75*, 805–816.

Hayashi, Y., Koike, M., Matsunami, M., and Hoshino, T. (1988). Effects of fixation time and enzymatic digestion on immunohistochemical demonstration of bromodeoxyuridine in formalin-fixed, paraffin-embedded tissue. J. Histochem. Cytochem. *36*, 511–514.

Holt, J.T., Thompson, M.E., Szabo, C., Robinsonbenion, C., Arteaga, C.L., King, M.C., and Jensen, R.A. (1996). Growth-retardation and tumor-inhibition by BRCA1. Nature Genet. *12*, 298–302.

Hosking, L., Tronsdale, J., Nicolai, H., Solomon, E., Foulkes, W., Stamp, G., Signer, E., and Jeffreys, A. (1995). A somatic *BRCA1* mutation in an ovarian tumor. Nature Genet. *9*, 343–344.

Hui, C.C., and Joyner, A. (1993). A mouse model for the Greig cephalopolysyndactyly syndrome: The *extra-toes*$^{J}$ mutation contains an intragenic deletion of the *Gli3* gene. Nature Genet. *3*, 241–246.

Jensen, R.A., Thompson, M.E., Jetton, T.L., Szabo, C.I., Vandermeer, R., Helou, B., Tronick, S.R., Page, D.L., King, M.C., and Holt, J.T. (1996). BRCA1 is secreted and exhibits properties of a granin. Nature Genet. *12*, 303–308.

Jones, S.N., Roe, A.E., Donehower, L.A., and Bradley, A. (1995). Rescue of embryonic lethality in Mdm2-deficient mice by absence of p53. Nature *378*, 206–208.

Kispert, A., and Herrmann, B.G. (1993). The *brachyury* gene encodes a novel DNA binding protein. EMBO J. *12*, 3211–3220.

Koff, A., Giordano, A., Desai, D., Yamashita, K., Harper, J.W., Elledge, S., Nishimoto, T., Morgan, D.O., Franza, R., and Roberts, J.M. (1992). Formation and activation of cyclin E/CDK2 complex during the G1 phase of the human cell cycle. Science *257*, 1689–1694.

Lane, T.F., Deng, C.X., Elson, A., Lyu, M.S., Kozak, C.A., and Leder, P. (1995). Expression of BRCA1 is associated with terminal differentiation of ectodermally and mesodermally derived tissues in mice. Genes Dev. *9*, 2712–2722.

Marquis, S.T., Rajan, J.V., Wynshaw-Boris, A., Xu, T.J., Yin, G.Y., Abel, K.J., Weber, B.L., and Chodosh, L.A. (1995). The developmental pattern of BRCA1 expression implies a role in differentiation of the breast and other tissues. Nature Genet. *11*, 17–26.

Merajver, S.D., Pham, T.M., Caduff, R.F., Chen, M., Poy, E.L., Cooney, K.A., Weber, B.L., Collins, F.S., Johnston, C., and Frank, T.S. (1995a). Somatic mutations in the *BRCA1* gene in sporadic ovarian-tumor. Nature Genet. *9*, 439–443.

Merajver, S.D., Frank, T.S., Xu, J.Z., Pham, T.M., Calzone, K.A., Bennettbaker, P., Chamberlain, J., Boyd, J., Garber, J.E., Collins, F.S., and Weber, B.L. (1995b). Germline *BRCA1* mutations and loss of the wildtype allele in tumors from families with early-onset breast and ovarian -cancer. Clin. Cancer Res. *1*, 539–544.

Miki, Y., Swensen, J., Shattuckeidens, D., Futreal, P.A., Harshman, K., Tavtigian, S., Liu, Q.Y., Cochran, C., Bennett, L.M., Ding, W., et al. (1994). A strong candidate for the breast and ovarian-cancer susceptibility gene. Science *266*, 66–71.

Mishina, Y., Suzuki, A., Veno, N., and Behringer, R.R. (1995). *BMPR* encodes a type I bone morphogenetic protein-receptor that is essential for gastrulation during mouse embryogenesis. Genes Dev. *9*, 3027–3037.

Momand, J., Zambetti, G.P., Olson, D.C., George, D., and Levine, A.J. (1992). The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53 mediated transactivation. Cell *69*, 1237–1245.

Montes de Oca Luna, R., Wagner, D.S., and Lozano, G. (1995). Rescue of early embryonic lethality in MDM2-deficient mice by deletion of p53. Nature *378*, 203–206.

Moser, A.R., Shoemaker, A.R., Connelly, C.S., Clipson, L., Gould, K.A., Luongo, C., Dove, W.F., Siggers, P.H., and Gardner, R.L. (1995). Homozygosity for the *min* allele of APC results in disruption of mouse development prior to gastrulation. Dev. Dyn. *203*, 422–433.

Nagy. A., and Rossant, J. (1993). Production of completely ES cell-derived fetuses. In Gene Targeting: A Practical Approach, A.L. Joyner, ed. (Oxford: IRL Press), pp.147–179.

Ohtsubo, M., and Roberts, J.M. (1993). Cyclin dependent regulation of G1 in mammalian fibroblasts. Science *59*, 1908–1912.

Ohtsubo, M., Theodoras, A.M., Schumacher, J., Roberts, J.M., and Pagano, M. (1995). Human cyclin E, a nuclear protein essential for the G1 to S phase transition. Mol. Cell. Biol *15*, 2612–2624.

Power, M.-A., and Tam, P.P.L. (1993). Onset of gastrulation, morphogenesis and somitogenesis in mouse embryos displaying compensatory growth. Anat. Embryol. *187*, 493–504.

Rao, V.N., Shao, N.S., Ahmad, M., and Reddy, E.S.P. (1996). Antisense RNA to the putative tumor-suppressor gene *BRCA1* transforms mouse fibroblasts. Oncogene *12*, 523–528.

Snow, M.H.L. (1977). Gastrulation in the mouse: growth and regionalization of the epiblast. J. Embryol. Exp. Morph. *42*, 293–303.

Tavtigian, S.V., Simard, J., Rommens, J., Couch, F., Shattuckeidens, D., Neuhausen, S., Merajver, S., Thorlacius, S., Offit, K., Stoppalyonnet, D., et al. (1996). The complete *BRCA2* gene and mutations in chromosome 13q-linked kindreds. Nature Genet. *12*, 333–337.

Trumpp, A., Blundell, P.A., de la Pompa, J.L., and Zeller, R. (1992). The chicken limb deformity gene encodes nuclear proteins expressed in specific cell types during morphogenesis. Genes Dev. *6*, 14–28.

Varmuza, S., Prideaux, V., Kothary, R., and Rossant, J. (1988). Polytene chromosomes in mouse trophoblast giant cells. Development *102*, 127–134.

Wooster, R., Neuhausen, S.L., Mangion, J., Quirk, Y., Ford, D., Collins, N., Nguyen, K., Seal, S., Tran, T., Averill, D., et al. (1994). Localization of a breast-cancer susceptibility gene, *BRCA2*, to chromosome 13q12-13. Science *265*, 2088–2090.

Wooster, R., Bignell, G., Lancaster, J., Swift, S., Seal, S., Mangion, J., Collins, N., Gregory, S., Gumbs, C., Micklem, G., et al. (1995). Identification of the breast cancer susceptibility gene *BRCA2*. Nature *378*, 789–792.

Wu, H., Wade, M., Krall, L., Grisham, J., Xiong, Y., and Van Dyke, T. (1996). Targeted in vivo expression of the cyclin-dependent kinase

inhibitor p21 halts hepatocyte cell-cycle progression, postnatal liver development, and regeneration. Genes Dev. *10*, 245–260.

Zhang, H., Hannon, G.J., and Beach, D. (1994). p21-containing cyclin kinases exists in both active and inactive states. Genes Dev. *8*, 1750–1758.