Brca2 is required for embryonic cellular proliferation in the mouse

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Mutations of the tumor suppressor gene BRCA2 are associated with predisposition to breast and other cancers. Homozygous mutant mice in which exons 10 and 11 of the Brca2 gene were deleted by gene targeting (Brca2 I°-11) die before day 9.5 of embryogenesis. Mutant phenotypes range from severely developmentally retarded embryos that do not gastrulate to embryos with reduced size that make mesoderm and survive until 8.5 days of development. Although apoptosis is normal, cellular proliferation is impaired in Brca2 I°-H mutants, both in vivo and in vitro. In addition, the expression of the cyclin-dependent kinase inhibitor p21 is increased. Thus, Brca2 I°-H mutants are similar in phenotype to Brca1 I°-H mutants but less severely affected. Expression of either of these two genes was unaffected in mutant embryos of the other. This study shows that Brca2, like Brca1, is required for cellular proliferation during embryogenesis. The similarity in phenotype between Brca1 and Brca2 mutants suggests that these genes may have cooperative roles or convergent functions during embryogenesis.

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The control of cell proliferation is achieved through a balance between negative and positive regulators of growth (for review, see Hunter 1997). Tumor suppressors are negative regulators of growth, and genetic lesions that inactivate them release the cells from normal growth constraints, causing the deregulated proliferation of cancer cells. Loss of growth control is accompanied by alterations in normal pathways of differentiation and development. Tumor suppressor genes have been shown to encode proteins involved in growth control and DNA repair processes. Germ-line mutations in tumor suppressor genes such as p53 (Malkin et al. 1990; Srivastava et al. 1990), Wilms’ tumor [WT1] [Gessler et al. 1990], and retinoblastoma [RB] [Hansen et al. 1985] are associated with inherited predispositions to cancer. Targeted mutation of these genes in mice causes cancer susceptibility but also results in abnormalities in cellular proliferation and differentiation and embryonic development (Dougherty et al. 1992; Lee et al. 1992; Kreidberg et al. 1993).

In the breast, the observation that endocrine factors that control breast development [Kleinberg and Newman 1986] also influence breast cancer risk [Siiteri et al. 1986] suggests that mammary gland development and carcinogenesis are processes that are fundamentally related. BRCA2 is a breast cancer susceptibility gene recently isolated by positional cloning [Wooster et al. 1995]. Mutations in BRCA2 are thought to account for as many as 35% of all inherited breast cancers, as well as a proportion of inherited ovarian cancers [Wooster et al. 1995; Tavtigian et al. 1996]. Like BRCA1, BRCA2 appears to be a tumor suppressor gene, because the loss of the wild-type BRCA2 allele in heterozygous carriers can result in the development of tumors [Collins et al. 1995; Gudmundsson et al. 1995]. Few somatic mutations in either BRCA1 [Futreal et al. 1994] or BRCA2 [Miki et al. 1996] have been identified in sporadic breast tumors, but germ-line mutations in both BRCA1 [Miki et al. 1994] and BRCA2 [Phelan et al. 1986] predispose carriers to breast adenocarcinoma. However, unlike BRCA1, germ-line mutations in BRCA2 predispose both males and females to breast cancer, and female carriers of BRCA2 germ-line mutations show a lower incidence of ovarian cancer than do BRCA1 carriers [Thorlacius et al. 1995; Wooster et al. 1995].
The **BRCA2** gene consists of 27 exons that generate an 11-kb mRNA transcript predicted to encode a novel protein of 3418 amino acids (Tavtigian et al. 1996). Interestingly, the **BRCA1** and **BRCA2** genes are similar in several respects, despite a marked lack of nucleotide or amino acid sequence homology. Both **BRCA1** and **BRCA2** are large AT-rich genes, which include an exon of unusually large size. Neither gene shows homology to any known protein. The numerous germ-line mutations that have been identified in both **BRCA1** and **BRCA2** are distributed throughout their lengths, and most of them result in protein truncation (Gayther et al. 1997). In addition, the spatial and temporal pattern of **BRCA2** mRNA expression in the mouse is strikingly similar to that of **Brca1** during fetal development, and in adult tissues in vivo and in mammary epithelial cells in vitro (Rajan et al. 1996). Both **Brca1** and **Brca2** are expressed in a cell cycle-dependent manner, peaking at the G_{1}/S boundary. Nevertheless, because there are no obvious extended regions of nucleotide or amino acid homology between **BRCA1** and **BRCA2**, whether these similarities reflect an underlying structural or functional homology remains to be determined. Taken together, the available data suggest that **Brca1** and **Brca2** may function in overlapping regulatory pathways involved in the control of cell proliferation and differentiation (Rajan et al. 1996, and unpubl.; Vaughn et al. 1996).

To determine the role of **Brca2** in embryonic development and adult cellular physiology, we have generated a **Brca2**-deficient mouse by targeted deletion of exons 10–11 of the **Brca2** gene. Heterozygous **Brca2** mutation mice are viable and normal, but homozygous **Brca2** mutation embryos die during early postimplantation development. Analysis of **Brca2** mutation embryos in vivo and in vitro indicates that **Brca2** is essential for the control of the proliferative process that occurs in early embryonic development. The similarities and differences in phenotype between the **Brca1** and **Brca2** mutants, and their possible functional relationship, are discussed.

**Results**

**Generation of Brca2^{10–11} mutant mice**

The **Brca2** gene was disrupted in embryonic stem (ES) cells using a targeting vector that deleted the 3' half of exon 10 and the 5' half of exon 11 of **Brca2**, leading to the introduction of two termination codons in-frame. A cassette containing the neomycin resistance (**neo**) gene with the phosphoglycerokinase (PGK) promoter and a poly(A) addition signal was inserted in the sense orientation in the targeting vector, so that the short arm consisted of 516 bp of exon 10 and the long arm contained 5.0 kb of exon 11 (Fig. 1A). The targeting construct was electroporated into ES cells. PCR and Southern blot analyses showed that 3 of 1960 **G418**-resistant colonies were heterozygous for the **Brca2** locus, with no random integrations. These three ES clones were used to generate chimeric mice, two of which successfully contributed to germ line. Chimeras were backcrossed to C57BL/6J or CD1 mice, and heterozygotes were intercrossed to produce homozygous mutant offspring. The genotypes of the mice were confirmed by Southern blot analysis.

**Phenotype of heterozygous Brca2^{10–11} mice**

Mice heterozygous for the **Brca2** deletion were phenotypically normal and fertile. Heterozygous **Brca2** mice did not develop any type of cancer up to 7 months of age. It remains possible, however, that **Brca2** heterozygotes could develop tumors at a more advanced age.

**Brca2^{10–11} mutation results in embryonic lethality**

No viable **Brca2**^{10–11} pups were identified among 51 offspring born from heterozygous intercrosses, indicating that homozygosity for the **Brca2**^{10–11} mutation causes embryonic lethality (Fig. 1B; Table 1). To assess the consequences of the **Brca2**^{10–11} 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–d (Fig. 1A,C). At embryonic day 6.5 (E6.5) ~25% of all embryos were morphologically abnormal; these were genotyped as mutants. E6.5 **Brca2**^{10–11} mutant embryos were half the size of their wild-type E6.5 littermates and showed a poorly defined boundary between the embryonic and the extraembryonic regions (Fig. 2A). At E7.5, the difference in size between wild-type and mutant embryos was even more dramatic (Fig. 2B), and some mutant embryos had commenced resorption. At E8.5, most mutant embryos were in resorption (Table 1), although some did develop a head fold, a primordial neural tube, allantois, and expanded yolk sac but no somites (Fig. 2C). All mutant embryos were dead or in resorption by E9.5.

Mutant mice generated from the two independent targeted ES cell clones showed identical phenotypes. In addition, the analyses were performed in both an inbred (C57BL/6J) and an outbred (CD1) background that gave comparable phenotypes. These results demonstrate that homozygosity for the **Brca2**^{10–11} allele results in embryonic lethality before E9.5 and indicate that **Brca2** is essential for postimplantation development.

**Histological analysis of the Brca2^{10–11} mutant embryos**

The structural organization of **Brca2**^{10–11} mutant embryos was characterized in detail by histological analysis of serially sectioned E5.5–E7.5 embryos obtained from heterozygous crosses (Fig. 3A–H). Differences in size between wild-type and mutant embryos were apparent at the E5.5 egg cylinder stage (Fig. 3A). At E6.5, phenotypic differences between wild-type and mutant embryos were more obvious (Fig. 3C–E). Wild-type embryos exhibited a well-organized ectoderm and endoderm, and a nascent mesoderm (Fig. 3C). **Brca2**^{10–11} embryos could be grouped into two phenotypically mutant classes. The less severely affected mutant embryos had ectoderm and
endoderm, and formed a primitive streak (Fig. 3D). Embryos in the more severe class showed only epiblast, no detectable visceral and parietal endoderm, and a proamniotic cavity (Fig. 3E). At E7.5, only some of the Brca2 mut-ant embryos were organized in three embryonic layers (Fig. 3G). Interestingly, cellular debris was observed frequently in the proamniotic cavity of E7.5 mutant embryos (Fig. 3G,H), reminiscent of the debris observed in earlier wild-type embryos that is generated during the process of cavitation (Coucovanis and Martin 1995). In addition, the amnion was very thin or absent in mutant embryos (Fig. 3G,H). Because of the increasing severity of the Brca2 mut-ant phenotype at later stages of development, we focused our phenotypic analyses on days E6.5–E7.5 of embryogenesis.

Brca2 expression during early embryogenesis

Northern blot analysis using a probe for Brca2 exons 9–11 revealed the expression of a Brca2 transcript of 11 kb in ES cells, and in E7.5, E11.5, E15.5, and E17.5 wild-type embryos (Fig. 4) The spatial expression of Brca2 in E6.5 wild-type and mutant embryos was analyzed by in situ hybridization in tissue sections, using an antisense probe for Brca2 exons 12–16. Brca2 transcripts could be detected at low levels throughout the epiblast, developing mesoderm, visceral endoderm, and extraembryonic ectoderm and endoderm (Fig. 5 A,B). Mutant em-bryos failed to show any Brca2 transcripts (Fig. 5 C,D), indicating that the Brca2 mut-ant is a null mutation.

Trophoblast and mesoderm development in Brca2 mut-ant embryos

At E6.5, Brca2 mut-ant mutant embryos were poorly de-veloped, showing a drastically reduced embryonic region, although the extraembryonic region appeared normal (Fig. 6E). The latter was confirmed by analyzing the ex-

![Figure 1. Targeted disruption of the Brca2 locus. (A)(Top) A portion of the mouse Brca2 wild-type locus showing exons 10–11 and HindIII sites (H). The 4.7-kb HindIII fragment present in the wild-type allele and the PCR primers c and d are shown. (Middle) Targeting vector and the neo gene positioned in the sense orientation to Brca2 gene transcription. The targeting vector was designed such that the neo cassette replaced exons 10 and 11 of the Brca2 gene. Two stop codons were inserted at the 3' end of the short arm in-frame. (Bottom) The mutated Brca2 locus showing the 2.7-kb HindIII DNA fragment present in the recombinant Brca2 allele. The position of the 5'-flanking probe used for Southern blot analysis and the PCR primers a and b used to identify the mutated allele are shown. (B) Southern blot analysis of representative genomic tail DNA from one litter of Brca2 mut-ant heterozygous intercrosses. DNA was digested with HindIII and hybridized with the 5' flanking probe. The 4.7-kb band representative of the wild-type allele and the 2.7-kb band representative of the mutated allele are indicated. (C) Representative genotypic analysis of E6.5 embryos from a Brca2 heterozygote breeding. DNA samples were subjected to PCR using the primer pairs a/b or c/d (see Materials and Methods for sequences). PCR amplification of the mutated Brca2 allele by primer pair c/d produces a 602-bp DNA fragment (top), and PCR amplification of the wild-type Brca2 allele by primer pair a/b produces a 433-bp DNA fragment (bottom).]
Table 1. Genotypic and phenotypic analyses of neonates and embryos from Brca2<sup>10-11</sup> heterozygous intercrosses

| Stage | Brca2<sup>+/+</sup> | Brca2<sup>-/-</sup> | Brca2<sup>+/−</sup> | Total |
|-------|------------------|------------------|------------------|-------|
| E6.5  | 18 (0)           | 36 (0)           | 11 (11)          | 65 (11)|
| E7.5  | 5 (0)            | 15 (0)           | 8 (8)            | 28 (8) |
| E8.5  | 12 (0)           | 26 (0)           | 21 (21)          | 59 (21)|
| E9.5  | 1 (0)            | 5 (0)            | 2 (2)            | 8 (2)  |
| Neonate | 16 (0)          | 35 (0)           | 0                | 51 (0) |

Neonates were genotyped by Southern blot analysis using the 5' flanking probe shown in Fig. 1B. The embryos were collected on days 6.5, 7.5, 8.5, and 9.5 of pregnancy from Brca2<sup>10-11</sup> heterozygous intercrosses. The embryo genotypes were determined by PCR amplification using DNA extracted from the whole embryo or from the yolk sac, using primers a, b, c, and d. Figures in parentheses indicate the number of phenotypically abnormal embryos. In comparison to their normal-looking control littermates, all homozygous mutant embryos exhibited an abnormal phenotype, characterized by poor embryonic organization and a reduced size.

<sup>a</sup>Includes embryos in resorption [30%-40]%.

expression of the early trophoblast lineage marker Mash-2, a gene expressed in the wild-type embryo throughout preimplantation development. In wild-type E6.5 embryos, Mash-2 is expressed in the diploid trophoblast lineage (Fig. 5E,F) but not in the trophoblast giant cells. In Brca2<sup>10-11</sup> mutant embryos Mash-2 expression was normal [Fig. 5G].

To determine at the molecular level whether mesoderm was formed in mutant embryos, we examined the expression of the Brachyury (T) protein by immunohistochemistry in tissue sections. In wild-type E6.5 embryos, the Brachyury protein was expressed in the primitive streak in six of six embryos analyzed (Fig. 5I). However, Brca2<sup>10-11</sup> embryos at E6.5 could be grouped into two classes: The less severely affected embryos, although disorganized, did express Brachyury (Fig. 5I). The more severely affected mutants did not show any Brachyury expression (three of four embryos analyzed; Fig. 5K). These phenotypic differences in Brachyury expression were entirely consistent with the histological analysis and indicated that, unlike Brca1<sup>+/−</sup> mutant embryos, some Brca2<sup>10-11</sup> embryos were able to make mesoderm.

Brca2<sup>10-11</sup> mutation decreases embryonic growth in vitro and in vivo

Hakem et al. [1996] and Liu et al. [1996] showed that the growth deficit of Brca1 mutant embryos was correlated with decreased cell proliferation. The reduced size at E6.5 of Brca2<sup>10-11</sup> mutant embryos was likely not attributable to an excess of apoptosis, as judged by Hoechst dye staining of wild-type and mutant embryos (data not shown). To determine whether the proliferative capability of Brca2<sup>10-11</sup> mutant embryos was intrinsically impaired, the growth of the inner cell mass (ICM) was examined. Blastocysts from heterozygous matings (E3.5) were collected and cultured individually in vitro. Mutant blastocysts showed a normal phenotype, indicating that the Brca2<sup>10-11</sup> mutation does not affect preimplantation development (Fig. 6A,B). However, after 7 days in culture, the ICM did not grow in 90% of the mutant blastocysts, compared to failure in only ~10% of wild-type and heterozygous blastocysts (Fig. 6C,D; Table 2). Only trophoblast giant cells developed in cultured mutant embryos (Fig. 6D). In addition, numerous attempts to generate Brca2<sup>10-11</sup> homozygous mutant ES cells by increasing the G418 concentration, or by using a second targeting vector with a hygromycin resistance cassette, were unsuccessful (data not shown).

To directly study the effects of the Brca2<sup>10-11</sup> 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 and E7.5, the times at which the highest mitotic activity has been observed in the mouse embryo [Snow 1977]. Whole litters were serially sectioned and embryo phenotypes were classified as wild-type or mutant. A proliferative index based on the ratio of proliferating cells (BrdU-positive nuclei) to total cell number was cal-
Figure 3. Histological analysis of Brca2\textsuperscript{10-11} mutant embryos. Sagittal sections are shown. (A,B) E5.5; (C-E) E6.5; (F-H) E7.5. (A) E5.5 wild-type embryo (early egg cylinder), (B) E5.5 Brca2\textsuperscript{10-11} mutant embryo. The embryonic region (arrow) is very reduced. (C) E6.5 wild-type embryo (egg-cylinder stage). Both the embryonic and extraembryonic regions are well organized. (D) E6.5 Brca2\textsuperscript{10-11} mutant embryo. The large arrow points to the separation between the embryonic and extraembryonic region. The embryonic region is poorly developed, although the proamniotic cavity is apparent. (E) E6.5 severe Brca2\textsuperscript{10-11} mutant embryo. The embryonic region is very reduced. (F) E7.5 wild-type embryo. The three germ layers are apparent. (G) E7.5 Brca2\textsuperscript{10-11} mutant embryo. The embryonic region is reduced. Compare the difference in size with the wild-type embryo shown in F. Cellular debris can be observed in the proamniotic cavity (arrow). Mesoderm (m) has been generated. The amnion is absent. (H) Severe Brca2\textsuperscript{10-11} mutant embryo. The embryonic region (arrow) is drastically reduced. (al) Allantois, (am) amnion, (ch) chorion, (ee) embryonic ectoderm, (eee) extraembryonic ectoderm, (epc) ectoplacental cone, (m) mesoderm, (pa) proamniotic cavity, (ve) visceral endoderm. Bar, 60 \mu m in A–E; 150 \mu m in F–H.

Figure 4. Brca2 is expressed throughout embryogenesis. Northern blot analysis of poly(A)\ RNAs prepared from ES cells and from wild-type embryos at 7.5, 11.5, 15.5, and 17.5 days of gestation. Hybridization with \textsuperscript{32}P-labeled exon 11 Brca2 probe, and detected expression of the Brca2 gene in ES cells and in all analyzed stages of embryonic development. A probe to \textit{\beta}-actin was used as a control for sample loading.
Figure 5. Spatial expression of Brca2, Mash-2, and Brachyury in E6.5 Brca2<sup>1°-11</sup> mutant embryos. (A,C,E,G) In situ hybridization, bright-field view; (B,D,F,H) dark-field view. (A–D) Brca2 expression, (E–H) Mash-2 expression, (I,J) Brachyury expression. Sagittal sections are shown (A,C,E,G). The arrow points to the separation between the embryonic and extraembryonic regions. (A,B) Brca1 expression in an E6.5 wild-type embryo. Sections were hybridized with an antisense Brca2 exon 12-16 probe. Brca2 is expressed ubiquitously in the embryo. (C,D) Brca2<sup>1°-11</sup> mutant embryo. No Brca2 expression is detected. (E,F) Mash-2 expression in an E6.5 wild-type embryo. Strong expression is detected in the diploid trophoblast of the extraembryonic region of the embryo. (G,H) Mash-2 expression in a Brca2<sup>1°-~</sup> mutant embryo. Expression is normal. (I,J,H) Immunohistochemical analysis of Brachyury expression. (I) Wild-type embryo. Branchyury-positive cells can be observed in the nascent streak. (J) Brca2<sup>1°-11</sup> mutant embryo. Weak Brachyury expression is detected. The arrows in I and J point to the Brachyury-positive cells. (K) Severe Brca2<sup>1°-~</sup> mutant embryo. No Brachyury expression is observed. The arrow points to the embryonic region. Bar, 60 μm.

ous observations with Brca<sup>15-6</sup> mutant embryos [Hakem et al. 1996].

The Brca2<sup>10-11</sup> mutation does not affect Brca1 and Rad51 expression

Brca2<sup>10-11</sup> and Brca1<sup>15-6</sup> mutant embryos share many phenotypic features, suggesting that these genes may be functionally related. As an initial step toward determining whether Brca1 and Brca2 are indeed related, we used RT–PCR and in situ hybridization to examine the expression of Brca1 in Brca2<sup>10-11</sup> mutant embryos and that of Brca2 in Brca1<sup>15-6</sup> mutant embryos. No differences in Brca1 expression were detected in Brca2<sup>10-11</sup> mutants [Fig. 7; data not shown]. Similarly, no effect on Brca2 expression was observed in Brca1<sup>15-6</sup> mutant embryos [data not shown]. The recently reported association between hRad51, a molecule involved in DNA recombination repair, and BRCA1 [Scully et al. 1997] prompted us to examine whether the expression of mRad51 was affected in Brca2<sup>10-11</sup> mutants. No alteration of Rad51 expression in Brca2<sup>10-11</sup> mutants was detected by RT–PCR [Fig. 7].

Discussion

The Brca2<sup>10-11</sup> mutant phenotype

We have generated a null mutation of the Brca2 gene by homologous recombination in mouse ES cells. The Brca2<sup>10-11</sup> mutation affects embryonic growth and caused lethality before day 9.5 of gestation. Mutant embryos are abnormal as early as E5.5, although some embryos gastrulate and organize an anterior-posterior pattern before dying at around E8.5. We and others have shown that mutation of the Brca1 gene causes embryonic lethality in mice [Gowen et al. 1996; Hakem et al. 1996; Liu et al. 1996]. In particular, Brca1<sup>15-6</sup> mutant embryos show a very severe growth retardation and die before E7.5 [Hakem et al. 1996]. The similarities in phenotype between Brca2<sup>10-11</sup> and Brca1<sup>15-6</sup> mutant embryos suggest that these genes may be involved in the same or related processes.

Brca2 and cellular proliferation

At E7.5, BrdU incorporation was markedly reduced in Brca2<sup>10-11</sup> mutant embryos, indicating a slow cell cycle
progression that ultimately culminated in cell cycle arrest. Unlike Brca1<sup>−/−</sup> mutant embryos, apparently normal cyclin E expression was observed in Brca2<sup>10−11</sup> mutants. This is consistent with the observation that the developmental arrest and death of Brca2<sup>10−11</sup> mutant embryos occurs almost 2 days later than in Brca1<sup>−/−</sup> mutant embryos. The temporal difference in the onset of detrimental effects caused by these mutations is also reflected in the development of outgrowths of cultured blastocysts. E3.5 Brca2<sup>10−11</sup> and Brca1<sup>−/−</sup> blastocysts appeared normal, although their proliferative abilities in vitro were reduced. In Brca2<sup>10−11</sup> mutant blastocysts, growth retardation was apparent after 3 days of culture, but a fully penetrant growth defect was observed only after 7 days. In the case of Brca1<sup>−/−</sup> mutant blastocysts, poor growth or absence of ICM was apparent after 3 days of culture (Hakem et al. 1996). In Brca2<sup>10−11</sup> mutant blastocysts, as in Brca1<sup>−/−</sup> blastocysts (Hakem et al. 1996), only giant cells survived after a period of culture, suggesting that this lineage is more resistant to the absence of either the Brca1 or Brca2 protein. Interestingly, neither Brca1<sup>−/−</sup>, Brca2<sup>10−11</sup>, nor Rad51 mutant embryos [Lim and Hasty 1996] grow in vitro, and only trophoblast giant cells, in which the DNA endoreplicates, are observed to undergo transient proliferation. It is still formally possible that the observed differences in proliferation between wild-type and Brca2<sup>10−11</sup> mutant embryos could arise as a secondary effect of the mutation.

Gastrulation in the mouse embryo occurs at ~6.5 days of gestation, at which time the mesoderm is generated from the epiblast. This process is associated with rapid epiblast cell proliferation, resulting in a 100-fold increase in cell number between E5.5 and E7.5 (Snow 1977; Power and Tam 1993). Three-quarters of the Brca2<sup>10−11</sup> mutant embryos analyzed did not develop mesoderm at E6.5, as revealed by the absence of Brachyury expression. Considering that the cardinal feature of Brca2<sup>10−11</sup> mutant embryos is their reduced size, the most likely interpretation of this observation is that defective mesoderm development in Brca2<sup>10−11</sup> mutant embryos arises as a consequence of a primary proliferative defect.

Brca1, Brca2, and genetic integrity in the early embryo

We have shown that the Brca2<sup>10−11</sup> mutation causes an increase in the expression of p21. p21 is a universal cdk inhibitor that impedes the cell cycle at the G<sub>1</sub> transition (Gu et al. 1993; Harper et al. 1993). The transcription of p21 is directly regulated by p53 and other factors [El-Deiry et al. 1993]. As the expression of p53 and mdm-2, whose protein product regulates p53 activity, appeared to be unaffected in Brca2<sup>10−11</sup> embryos, this would suggest that other regulatory signals downstream of Brca2

### Table 2. Genotypic and phenotypic analysis of Brca2<sup>10−11</sup> mutant blastocysts cultured in vitro

| Genotype | Total no. | normal | abnormal |
|----------|-----------|--------|----------|
| Brca2<sup>+/−</sup> | 11 | 10 (90%) | 1 (10%) |
| Brca2<sup>−/−</sup> | 22 | 20 (91%) | 2 (9%) |
| Brca2<sup>+/−</sup> | 8 | 1 (12%) | 7 (88%) |

Blastocysts from Brca2<sup>10−11</sup> heterozygous intercrosses were collected at day 3.5 of gestation and individually cultured in ES media without LIF. Embryos grown in vitro for 7 days were photographed daily and genotyped by PCR amplification using primers a, b, c, and d as shown in Fig. 1. A and C. Embryo phenotype was scored as abnormal if the ICM did not develop after 7 days of culture. Numbers in parentheses indicate percentages of cultured blastocysts with normal and abnormal phenotypes.
Brca2 might participate in the same process or be present in the same complex as Rad51 and Brca1. In this scenario, the lethality of Brca2^{10-11} mutant embryos would be attributable to cell cycle arrest caused by genome errors occurring in early postimplantation development, the time at which the rates of mitotic division are the highest and the stresses exerted on the maintenance of genetic integrity are greatest. Subsequent activation of checkpoint genome guardian functions would result in cell cycle arrest. It has been shown that p53 is the key cell cycle regulator responding to DNA damage [Cox and Lane 1995; Levine 1997]. Although p53 expression was unaffected in Brca2^{10-11} mutant embryos, the expression of its downstream transcriptional target p21 was increased. Because p21 is the most pleiotropic mediator of p53-mediated cell cycle arrest, the enhanced expression of p21 is consistent with the growth arrest observed in Brca2^{10-11} mutant embryos. Interestingly, the survival of Rad51 mutant embryos was increased in a p53 mutant background [Lim and Hasty 1996].

The possible functional relationship among Brca2, Brca1, and Rad51 may perhaps be dissected by genetic means, that is, by analyzing the phenotypes of mice mutated for combinations of these genes. In the simplest case, if these three proteins belong to the same complex or are involved in the same process, a synergistic interaction would be expected in any pairwise combination of mutations, resulting in a more severe phenotype in the double mutant. However, the extreme severity of the Brca1^{5-8} and Rad51 mutant phenotypes would make the interpretation of such double mutants very difficult. Biochemical approaches or tissue-specific knockout mutants will likely prove more fruitful for analyzing the function of Brca1 and Brca2, and their relation to Rad51.

Materials and methods

Generation of Brca2 mutant ES cells and mutant mice

The targeting vector was designed to replace a 5-kb genomic fragment containing part of Brca2 exons 10 and 11 with the PGK-neo resistance expression cassette containing a poly[A] addition site in sense orientation to Brca2 transcription. The short arm was engineered by PCR amplification to include two stop codons in-frame at the 3' end. The construct was linearized by digestion with SacI, and 50 pg of DNA was electroporated into 1 x 10^7 E14K ES cells (Bio-Rad Gene Pulser, 0.34 V, 0.25 mF). E14K ES cells from 129/ola mice were maintained on a layer of mitomycin C-treated embryonic fibroblasts in Dulbecco's modified Eagle's medium (DMEM) supplemented with leukaemia inhibitory factor (LIF), 15% fetal calf serum (FCS), l-glutamine, and b-mercaptoethanol. ES cell colonies resistant to G418 (250 μg/ml, Sigma) were screened for homologous recombination by PCR using primers specific for sequences in the Brca2 gene and neo resistance genes (sense primer a, 5'-CTGCCTTACTAGCGGTAG-3'; antisense primer b, 5'-GCGCCTCGAAGGGCCATAC-3'). Recombinant colonies were confirmed by Southern blotting using a Brca2-specific probe.

Chimeric mice were produced by microinjection of targeted ES cells into 3.5-day C57BL/6] blastocysts. Chimeras were bred to C57BL/6 mice (Jackson Laboratories), and germ-line trans-
mission of the mutant allele was confirmed by genomic Southern blot analysis of tail DNA from F1 offspring with agouti coat color. Two injected ES cells showed successful germ-line transmission. F2 offspring from heterozygous intercrosses were genotyped by PCR or Southern blot analysis. The heterozygous males were also crossed with CD1 mice. Mutants derived from both mouse backgrounds showed the same phenotype.

PCR analysis of Brca2<sup>10-11</sup> genotypes

Genomic DNA from ES cells and neonate tail was isolated and used in PCR amplification. Yolk sacs, E6.5-8.5 embryos, or blastocystcs cultured in vitro were incubated overnight at 37°C in 100 μl of lysis buffer [50 mM KCl, 10 mM Tris (pH 8.3), 2 mM MgCl2, 0.1 mg/ml of gelatin, 0.45% NP-40, 0.45% Tween-20] containing 100 μg/ml of proteinase K. After 10 min of boiling, 1-5 μl of the samples was subjected to PCR amplification. Primers c (5'-CCAGGGTGAACGCGGAGAAG-3') and d (5'-GTCTGTCGTAATGCGCTCTC-3'), specific for the deleted portion of the Brca2 gene, were used to detect the wild-type allele, and primers a and b (see above) were used to detect the recombinant allele. Temperature cycling conditions were one initial cycle at 94°C for 7 min, 62°C for 5 min, 72°C for 1 min, followed by 40-50 cycles at 94°C for 1 min, 62°C for 1 min, and 72°C for 1 min and 30 sec. [DNA thermal cycler, Perkin Elmer Cetus]. Half of each reaction mixture was electrophoresed on a 1.7% agarose gel and stained with ethidium bromide. Primer pair c/d amplified a 433-bp fragment in both heterozygote and homozygous mutant DNA.

RNA analysis

Total RNA was extracted from ES cells or E7.5, E11.5, E15.5, and E17.5 embryos using Trizol [Life Technologies]. For Northern blot analysis, 1 μg of poly[A] RNA was electrophoresed in a 1% agarose/formaldehyde gel and transferred to a nylon membrane (Hybond N+, Amersham). RNA blots were hybridized overnight at 65°C in Church and Gilbert buffer (Church and Gilbert 1984). The filters were hybridized with a cDNA probe specific for the deleted portion of the Brca2 gene, whereas the primer pair a/b amplified a 602-bp fragment in both heterozygote and homozygous mutant DNA.

In vitro culture of preimplantation embryos

Brcα<sub>2</sub> heterozygous 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 LIF, in 5% CO2 at 37°C. Photographs of the cultured embryos were taken every 24 hr. After 7 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 PBS at E5.5-E8.5, fixed overnight in 4% paraformaldehyde at 4°C, dehydrated, and embedded in paraffin. Sections 6 μm thick were cut and stained with hematoxylin and eosin.

In situ hybridization

Uteri were isolated in ice-cold PBS at E6.5 and processed as for histological analysis. The probes used were Brca2 (exons 12-16), Mash-2 (Guillomet et al. 1994), and full-length p53, mdm-2, and p21 cDNAs. Probes were labeled with [α-32P]UTP and processed according to protocols described previously [Hui and Joyner 1993].

Immunohistochemistry

Uteri were isolated in ice-cold PBS at E6.5, fixed in 4% paraformaldehyde for 4 hr, dehydrated, embedded in wax, and sectioned at 6 μm. The following polyclonal antisera were used: anti-Brachyury [1:500 dilution] recognizes amino acids 328-420 of the carboxy-terminal part of the T protein; Kispert and Hermann (1993); anti-cyclin A (1:200); and anti-cyclin E (1:200). For immunostaining, the protocol described in Hakem et al. (1996) was used.

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/gram of body weight) was injected intra-peritoneally into pregnant females at E6.5 and E7.5. 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).

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