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13. ABSTRACT ***(Maximum 200 Words)*** Women who inherit a BRCA1 or BRCA2 mutation have an 80-90% chance of developing breast cancer. Gene targeting techniques were used to create a Brca2-deficient mouse. A portion of exon 10 was replaced with the Neo gene resulting in premature truncation of the protein product. Examination of normal mammary ductal development in $129^{(+/+)}$ and $129^{(+/Brca2-)}$ mice revealed phenotypic differences between the two genotypic classes. $129^{(+/+)}$ and $129^{(+/Brca2-)}$ mice and several inbred mouse strains were used to study the effect of genetic background on radiation induced mammary tumor induction. BALB/c and SWR mice are susceptible to radiation induced mammary tumorigenesis and C57BL/6, FVB/N and C3H mice are relatively resistant. While FVB/N mice do not develop tumors, radiation treatment has a dramatic effect on ductal morphogenesis. To date, no mammary tumors have been observed in the $129^{(+/+)}$ and $129^{(+/Brca2-)}$ mice. Brca2-deficient mice were crossed to p53 mutant mice to generate four genotypic classes of offspring. A study is in progress to assess the consequences of harboring mutation in the two tumor suppressor genes with and without radiation exposure. Mouse strains more and less sensitive to mammary tumor induction are being used to make mice congenic for the Brca2 mutation.

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N/A In the conduct of research involving hazardous organisms, the investigator(s) adhered to the CDC-NIH Guide for Biosafety in Microbiological and Biomedical Laboratories.

L Michelle Bennett 8/18/2000

PI - Signature Date

Table of Contents

Introduction

Approximately 174,000 women will be diagnosed with breast cancer this year and 40,000 women will die from the disease. It is the most commonly diagnosed cancer and the second leading cause of cancer deaths among women. Approximately 7% of breast cancers are attributed to the inheritance of BRCA mutations. Women who inherit a mutated copy of the BRCA2 gene have a 28% chance of developing breast cancer before the age of 50 and a lifetime risk that has been determined to be as high as 85%. In order to better understand the consequences of inheriting an alteration in the BRCA2 gene I proposed to develop a mouse model using gene targeting technology. $129^{(+/Brca2-)}$ deficient mice were created with a targeted mutation in exon 10 of the endogenous mouse Brca2 gene. The study objectives for the use of these mice were to: (a) assess differences in normal and neoplastic growth control in mice carrying one or two defective copies of the BRCA2 gene, (b) determine the cancer risks of radiation exposure in mice with BRCA2 defects, and (c) study the effect of carrying defects in two tumor suppressor genes by mating BRCA2-deficient mice with transgenic mice that carry a mutant copy (Ala135Val) of the p53 tumor suppressor gene. These studies were designed to understand potential gene-environment and gene-gene interactions. It was of particular interest to study germline Brca2 mutations in association with radiation, a known breast carcinogen, and a mutated p53 gene, which when inherited also predisposes women to breast cancer development.

Body

Specific Aim 1:

Development of a Brca-2 deficient mouse

As described in the 1999 Annual Report, this aim of the proposal has been completed. Gene targeting and homologous recombination techniques were used to generate mice with a heritable mutation in the endogenous Brca2 gene. The Brca2 mutation has been backcrossed to the C57BL/6NCI strain for 13 generations (>99% C57BL/6NCI) and the 129/SvEv strain for 9 generations (>99% 129/SvEv). In addition, as explained under Specific Aim 4 of the year 1999 Annual Report, the BALB/c genetic background was also made congenic for the Brca2 mutation. The Brca2 mutation has been backcrossed to the BALB/c inbred mouse strain for 10 generations (>99% BALB/c). The development of 129/SvEv, C57BL/6, and BALB/c mice with a Brca2 mutation has been described and is currently in press with Molecular Carcinogenesis (Appendix; Bennett *et al.*).

These mice have been used to address the objectives proposed in the originally submitted grant application, as well as other studies.

Specific Aim 2:

Information Described in the 1999 Annual Report

1. Brca2-deficient mice on the 129 genetic background ($129^{(+/Brca2-)}$) are not more susceptible to spontaneous mammary tumor induction than their wild-type littermates.
2. Brca2-KO mice are embryonic lethal.
3. Radiation Induced Mammary Tumorigenesis in Inbred Mouse Strains.
4. Radiation exposure of $129^{(+/Brca2-)}$ mice does not increase their risk for mammary tumor induction compared to wild type littermates.

Current and Extended Results

The day of embryonic lethality is prolonged by two days on the BALB/cJ genetic background.

As described in the 1999 Annual Report (point 2 above), mice that inherit two copies of the Brca2 mutation die during embryonic development. The breeding of the mutation onto different genetic backgrounds did not prolong the survivability of the mice to birth. The day of embryonic lethality was determined to be E8.5 for the homozygous-mutants on the 129/SvEv genetic background. In addition to evaluating the timing of embryonic lethality on the 129/SvEv inbred genetic background, the timing of embryonic lethality was evaluated on the BALB/c genetic background. This work was done in collaboration with Pamela Blackshear and Barb Davis (NIEHS). During the collection of Brca2(-/-) embryos from different background strains, it was observed that the day of embryonic lethality was prolonged on the BALB/cJ genetic background. This observation was followed up and it was determined that Brca2-null mice on the BALB/c genetic background die at embryonic day 10.5, two days later than on the 129/SvEv background. These observations are in press with Molecular Carcinogenesis (Appendix; Bennett ..).

Radiation induced mammary tumorigenesis in inbred mouse strains

The two-year study to evaluate the response of several inbred mouse strains to mammary tumor induction by low-level radiation has been completed. The C57BL/6NCI, BALB/cJ, C3H/HeNCI, SWR/J, and FVB/N inbred mouse strains were evaluated. The BALB/cJ and SWR/J strains are among the most sensitive to radiation-induced mammary tumor induction when treated with 0.3 Gy at 5 weeks of age. In collaboration with Dr. Barbara Davis (NIEHS) and John Seely (Pathco, RTP, NC) all the tissues that were taken from the treated and control inbred mice at interim sacrifice timepoints, when moribund, or at the two year sacrifice are in the process of being examined for histological alterations and diagnosed. Once the data has been collected and evaluated, a manuscript will be prepared to describe the effects of whole body radiation in the C3H/HeNCI, C57BL/6NCI, FVB/N, BALB/cJ and SWR/J inbred mouse strains.

Does radiation exposure of $129^{(+/Brca2-)}$ mice increase their risk for mammary tumor induction?

As described in the 1999 Annual Report, $129^{(+/+)}$ and $129^{(+/Brca2-)}$ mice were generated by crossing Brca2-heterozygous with wild-type 129/SvEv mice. One hundred twenty mice of each genotypic class were divided into two groups to be treated with 0.3 Gy at 5 weeks of age or to be observed as untreated controls. Animals were or are being held until moribund or until they reach two years of age. The two year final sacrifices began in the fall of 1999 and there are less than 10 mice left to be sacrificed. Three mice from each group were sacrificed at 3, 6, 9, and 12 months of age to assess mammary gland morphology and determine if preneoplastic lesions were forming. No gross lesions were observed in any of the mice analyzed. Analysis of the gross pathology collected at necropsy does not suggest that 129 mice with a Brca2 mutation are more susceptible to mammary tumor induction, or the induction of tumors at any other site. However, no conclusions can be made until all the collected tissues have been evaluated histologically. The tissues are currently being prepared for histologic evaluation by the Study Pathologist John Seely (Pathco). Once all the slides have been prepared they will be evaluated and diagnosed by John Seely and Barb Davis (NIEHS). From there we will be able to perform statistical analyses and determine if there are any differences among the treated and control groups with and without a Brca2 mutation.

It was reported last year that the analysis of 6 month mammary glands from control $129^{(+/+)}$ and $129^{(+/Brca2-)}$ mice revealed an apparent difference in mammary gland morphology between the two genotypic classes. In spite of this observation being made by a panel of six independent pathologists (at or associated with NIEHS) the difference in morphology between the genotypic classes did not achieve statistical significance. The results for these control animals have been written up in association with a study funded in part by the FCCBC in which the effect of DES-exposure in combination with mutations in Brca1 or Brca2 in mouse models were evaluated. This study is currently "in press" with Cancer Research and is expected to be published in their July 1, 2000 issue (Appendix; Bennett *et al.*).

Specific Aim 3:

Reported in the 1999 Annual Report

1. $FVB129F1^{(p53mut/Brca2-)}$ females do not display increased tumor development compared to wild-type, $FVB129F1^{(p53mut)}$, or $FVB129F1^{(Brca2-)}$ mice.

2. Complications due to mouse hepatitis virus infection.

Current Status

A study was designed to examine potential gene-environment effects. In this experiment the $129^{(+/Brca2-)}$ mice were mated to $FVB^{(p53mut)}$ mice. This cross resulted in four genotypic classes of animals. Forty female mice of each genotypic class were placed into treatment and control groups. Treated animals were exposed to 0.3 Gy at 5 weeks of age. The animals from this study began reaching the two year final sacrifice timepoint in the fall of 1999. At the final sacrifice, full necropsies are performed. To date, we have not observed an increased incidence of mammary tumors in the $FVB129F1^{(+/Brca2-)}$, $FVB129F1^{(p53mut/Brca2-)}$, or $FVB129F1^{(p53mut/+)}$ compared to one another or the wild type $FVB129F1^{(+/+)}$ littermates. In addition, no other gross pathology, apparent at necropsy, distinguishes the genotypic classes. Partial or full necropsies were performed on the mice that became moribund during the course of the experiment. All the tissues from the mice are being processed for routine histology under the direction of John Seely (Pathco). Once tissues from all the mice have been collected, they will be analyzed, and diagnosed. Once the data has been compiled it will be evaluated statistically. The fourth abdominal mammary glands have been or will be stained and mounted to slides for morphologic assessment.

Specific Aim 4:

Modification of Specific Aim 4 from original proposal

Specific Aim 4 proposed to study the influence of genetic background on modifying the outcome of BRCA2 loss of function. I had proposed to set up five crosses between Brca2-heterozygous mice and the BALB/c, SWR, C3H, FVB and C7BL/6 mouse strains and test the progeny for mammary tumor induction. Since no overt phenotype was observed in the $129^{(+/Brca2-)}$ mice, I decided that a different approach should be taken to study the potential modifying effects of genetic background on the susceptibility of mice harboring a Brca2 mutation. The Brca2 mutation was made congenic on the BALB/c genetic background that is susceptible to radiation-induced mammary tumorigenesis.

Since the MHV outbreak we had in the Spring of 1999, the 129/SvEv, C57BL/6, and BALB/c strains with a Brca2 mutation have been rederived and newly established mouse colonies are being maintained at our facility. The SWR/Brca2 congenic mice we had initiated before the MHV outbreak were not established enough to maintain. We have not reinitiated this backcross.

Key Research Accomplishments

• Development of Brca2-deficient mouse strains on the 129/SvEv, C57BL/6NCI, and BALB/c genetic backgrounds.

• The timing of embryonic lethality in Brca2(-/-) mice is delayed two days on the BALB/c genetic background compared to the 129/SvEv genetic background.

• BALB/cJ and SWR/J inbred mouse strains are relatively susceptible to radiation-induced mammary tumorigenesis, whereas C57BL/6NCI, C3H/HeNCI and FVB/N mouse strains are relatively resistant.

• Brca2-deficient radiation study is in progress – all of the sacrifices are near completion and once all tissues are collected they will be prepared for histological evaluation and diagnoses.

• Brca2 x p53-mut study is in progress – all of the sacrifices are near completion and once all tissues are collected they will be prepared for histological evaluation and diagnoses.

List of Reportable Outcomes

Papers

1. *Brca2*-Null Embryonic Survival is Prolonged on the BALB/c Genetic Background. L. Michelle Bennett, Kimberly A. McAllister, Pamela E. Blackshear, Jason Malphurs, Gina Goulding, N. Keith Collins, Toni Ward, Donna O. Bunch, Mitch E. Eddy, Barbara J. Davis, and Roger W. Wiseman. In press, Molecular Carcinogenesis.
2. Mice heterozygous for a *Brca1* or *Brca2* mutation display distinct mammary gland and ovarian phenotypes in response to diethylstilbestrol. L. Michelle Bennett, Kimberly A. McAllister, Jason Malphurs, Toni Ward, N. Keith Collins, John C. Seely, Lori C. Gowen, Beverly H. Koller, Barbara J. Davis, and Roger W. Wiseman. In press, Cancer Research. (Expected publication date: July 1, 2000.)

Abstracts

3. Brca1 and Brca2 heterozygous mice display distinct mammary gland and ovarian phenotypes in response to diethylstilbestrol. Bennett, L.M., McAllister, K.A., Ward, T., Malphurs, J., Collins, N.K., Seely, J.C, Gowen, L.C, Koller, B.H., Davis, B.J., and

Wiseman, R.W. Era of Hope II, Atlanta, GA, 2000.

Presentations and Outreach

7. Faculty member for the National Breast Cancer Coalition Fund's Project LEAD; An Innovative Science Program for Breast Cancer Activists. 1998- present.
8. "Development of Mouse Models in which to study Germline BRCA2 Defects" Midwest Regional Society of Toxicology, Lincolnshire, Illinois, 2000.
9 "Development of a Mouse Model for BRCA-2 Defects", Breast and Prostate Faculty Seminar, NIEHS, RTP, NC, 2000.

Animal Models

10. Development of Brca2-heterozygous mice on the 129, C57BL/6 and BALB/c genetic backgrounds.

Funding Applications

11. L. Michelle Bennett applied for an was awarded an NIH Transition to Independent Position (TIP) Award through an RFA mechanism by NIEHS. The grant will be awarded once I start my independent position.

Employment Applications That Led to Interviews

12. National Institute of Environmental Health Sciences, National Toxicology Program, Research Triangle Park, North Carolina.
13. University of Texas, MD Anderson Cancer Center, Science Park, Smithville, Texas.

Employment Application that Led to an Accepted Job Offer

14. Lawrence Livermore National Laboratory, Livermore, California.

Conclusions

A mouse that is heterozygous for a Brca2 mutation has been developed. This mutation has been crossed back to the 129/SvEv, C57BL/6, and BALB/c inbred backgrounds. Strain 129 mice that inherit a mutated copy of Brca2 are not predisposed to an increased incidence of spontaneous or radiation-induced mammary tumors compared to wild type controls. When two copies of the Brca2 mutation are inherited on the129 or a mixed 129 and C57BL/6 background the embryos die at d8.5 gestation. BALB/c mice that have inherited two mutant copies of Brca2 are also embryonic lethal. However, we have determined that the day of death is prolonged to d10.5 on the BALB/c background. The apparent alterations in ductal morphology between 129 mice with and without a Brca2 mutation, presented in the 1999 Annual Report, did not hold up to statistical analysis. There are two possibilities to explain this. One, it may be there is no difference. Alternatively, because there is intrastrain variation in ductal morphology, it may be that the difference is subtle and may require more animals for analysis. The analysis of a large number of animals might provide evidence to make a firm conclusion. However, the emphasis of the

laboratory at the present time is more focused on other animal models and such an investigation is not likely to occur at this time.

References

Brca2-Null Embryonic Survival is Prolonged on the BALB/c Genetic Background. L. Michelle Bennett, Kimberly A. McAllister, Pamela E. Blackshear, Jason Malphurs, Gina Goulding, N. Keith Collins, Toni Ward, Donna O. Bunch, Edward M. Eddy, Barbara J. Davis, and Roger W. Wiseman. In press, Molecular Carcinogenesis.

Mice heterozygous for a *Brca1* or *Brca2* mutation display distinct mammary gland and ovarian phenotypes in response to diethylstilbestrol. L. Michelle Bennett, Kimberly A. McAllister, Jason Malphurs, Toni Ward, N. Keith Collins, John C. Seely, Lori C. Gowen, Beverly H. Koller, Barbara J. Davis, and Roger W. Wiseman. In press, Cancer Research. (Expected publication date: July 1, 2000.)

Appendix:

Brca2-Null Embryonic Survival is Prolonged on the BALB/c Genetic Background

L. Michelle Bennett, Kimberly A. McAllister, Pamela E. Blackshear, Jason Malphurs, Gina Goulding, N. Keith Collins, Toni Ward, Donna O. Bunch, Edward M. Eddy, Barbara J. Davis, and Roger W. Wiseman. Molecular Carcinogenesis, 28:174-183, 2000.

Mice heterozygous for a _Brca1_ or _Brca2_ mutation display distinct mammary gland and ovarian phenotypes in response to diethylstilbestrol

L. Michelle Bennett, Kimberly A. McAllister, Jason Malphurs, Toni Ward, N. Keith Collins, John C. Seely, Lori C. Gowen, Beverly H. Koller, Barbara J. Davis, and Roger W. Wiseman. Cancer Research, 60: 3461-3469, 2000.

MOLECULAR CARCINOGENESIS 28:174–183 (2000)

Brca2-Null Embryonic Survival is Prolonged on the BALB/c Genetic Background

L. Michelle Bennett,[1]* Kimberly A. McAllister,[1]* Pamela E. Blackshear,[2] Jason Malphurs,[1] Gina Goulding,[3] N. Keith Collins,[1] Toni Ward,[1] Donna O. Bunch,[3] Edward M. Eddy,[3] Barbara J. Davis,[2] and Roger W. Wiseman[1]

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Women who inherit mutations in the BRCA2 cancer susceptibility gene have an 85% chance of developing breast cancer. The function of the BRCA2 gene remains elusive, but there is evidence to support its role in transcriptional transactivation, tumor suppression, and the maintenance of genomic integrity. Individuals with identical BRCA2 mutations display a different distribution of cancers, suggesting that there are low-penetrance genes that can modify disease outcome. We hypothesized that genetic background could influence embryonic survival of a Brca2 mutation in mice. Brca2-null embryos with a 129/SvEv genetic background ($129^{B2-/-}$) died before embryonic day 8.5. Transfer of this Brca2 mutation onto the BALB/cJ genetic background ($BALB/c^{B2-/-}$) extended survival to embryonic day 10.5. These results indicate that the BALB/c background harbors genetic modifiers that can prolong Brca2-null embryonic survival. The extended survival of $BALB/c^{B2-/-}$ embryos enabled us to ask whether transcriptional regulation of the Brca1 and Brca2 genes is interdependent. The interdependence of Brca1 and Brca2 was evaluated by studying Brca2 gene expression in $BALB/c^{B1-/-}$ embryos and Brca1 gene expression in $BALB/c^{B2-/-}$ embryos. Nonisotopic in situ hybridization demonstrated that Brca2 transcript levels were comparable in $BALB/c^{B1-/-}$ embryos and wild-type littermates. Likewise, reverse transcriptase–polymerase chain reactions confirmed Brca1 mRNA expression in embryonic day 8.5 $BALB/c^{B2-/-}$ embryos that was comparable to *Brca2*-heterozygous littermates. Thus, the Brca1 and Brca2 transcripts are expressed independently of one another in Brca1- and Brca2-null embryos. *Mol. Carcinog. 28:174–183, 2000.* © 2000 Wiley-Liss, Inc.

Key words: Brca2, BALB/c, gene targeting, embryonic lethality

INTRODUCTION

Genetic inheritance represents a major risk factor for breast-cancer development. Alterations in a number of genes have been linked to hereditary breast cancer including *BRCA1*, *BRCA2*, *p53*, Ataxia telangiectasia mutated, PTEN, and Adenomatous polyposis coli. Inherited mutations in the breast cancer susceptibility genes, BRCA1 and BRCA2, are implicated in 40–50% of women with a familial history of breast cancer and have been reported to increase a woman's lifetime breast-cancer risk to as high as 85% [1,2]. BRCA2 mutations have been associated with a wide spectrum of disease outcomes including male breast, ovarian, prostatic, colon, pancreatic, and stomach cancers [3]. Studies of the BRCA2 999del5 mutation in the Icelandic population suggest the presence of modifier genes that can influence tumor phenotype [4]. However, the inheritance of a BRCA2 mutation in combination with a germline mutation in the breast-cancer–susceptibility gene BRCA1 has not been associated with more severe phenotypes [5,6].

Although the functions of the BRCA1 and BRCA2 genes have yet to be elucidated, there is evidence that they play a role in responses to DNA damage and the maintenance of genomic stability [7–13]. BRCA1 and BRCA2 interact directly in a complex with RAD51, suggesting interdependence in at least one DNA damage-repair pathway [13]. We and others have demonstrated that Brca1 and Brca2 expression patterns throughout embryonic and

L. Michelle Bennett and Kimberly A. McAllister contributed equally to the work presented in this report.

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Abbreviations: E, embryonic day; 129, 129/SvEv; BALB/c, BALB/cJ; ES cell, embryonic stem cell; PCR, polymerase chain reaction; neo, neomycin resistance; RT, reverse transcription.

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mammary-gland development and in adult tissues are very similar, although not identical [14,15]. These similar expression patterns may or may not be the consequence of direct interactions between factors that control the expression of these two genes.

Most targeted genetic mutations described to date in the Brca1 and Brca2 loci cause embryonic lethality when present in the homozygous state [16]. The age of embryonic lethality in several Brca1- and Brca2-null embryos is dependent on which portion of the gene is targeted for disruption [17]. Targeted disruption of Brca1 in the 5′ region of the gene causes embryonic lethality from embryonic day (E) 4.5 to E7.5 [18,19]. A Brca1 mutation causing an in-frame exon 11 deletion can extend survival beyond E10.5 [20]. Similar observations have been made with Brca2 mice that target the 5′ end of exon 11. Targeted disruption 5′ of the well-conserved BRC repeats in exon 11 results in embryonic lethality when two mutant alleles are inherited [21–23]. However, retention of three or more of the BRC repeats, which bind Rad51, appears to be sufficient for survival of a small subgroup of homozygous-null animals to at least several months of age and is influenced by genetic background [24,25].

The targeted disruption of mouse genes has made it possible to study the modification of specific gene mutations in a controlled environment [17]. Several studies have demonstrated that combining a Brca1 or Brca2 alteration with *p53* or *p21* mutations extends embryonic survival. Brca1 and p53 or Brca1 and *p21* double-null embryos have survived to E9.5 [19,23], whereas Brca2/p53 double nullizygotes, on a mixed 129/SvEv (129) and C57BL/6 background, appear in most cases to be developmentally similar to or slightly more advanced than Brca2 mutants alone [23]. It is unclear how much of the phenotypic rescue described by introducing p53 or p21 in these studies is attributable to these specific targeted alterations as opposed to other genetic influences introduced by inbred strain backgrounds.

We introduced a single Brca2 gene mutation on the 129/SvEv (129) and BALB/cJ (BALB/c) genetic backgrounds. Although embryonic survival in Brca1- and Brca2-null mice can be influenced by an additional tumor suppressor gene alteration, the effects of genetic background alone have not been carefully investigated. We developed Brca2-deficient mice on a 129 background and established BALB/c inbred mice congenic for this alteration. The $129^{B2-/-}$ embryos express an embryonic lethal phenotype similar to previously reported Brca2-deficient mice with mutations introduced 5′ of the BRC repeats [21–23]. However, we demonstrate that the BALB/c inbred genetic background can prolong survival of homozygous-null embryos. The early embryonic death of Brca2-null embryos limits their use for studying loss of Brca1 and Brca2 function during neoplastic transformation. The extension of embryonic survivability enabled us to assess the interdependence of Brca1 and Brca2 by examining the expression pattern of Brca2 in $BALB/c^{B1-/-}$ embryos and Brca1 in $BALB/c^{B2-/-}$ embryos. We report that the BALB/c genetic background could enhance embryonic survivability in Brca2-null embryos and that Brca1 and Brca2 transcripts were expressed independently of one another.

MATERIALS AND METHODS

Development of Brca2-Deficient Mice

Brca2-deficient mice were generated by disrupting exons 10 and 11 with a pgkNEO cassette (Figure 1A). The pgkNEO gene replaced a portion of exon 10, intron 10, and the 5′ region of exon 11, extending from nucleotide 1745 to 2033 (accession number U89652) [26]. The targeting vector was linearized with SalI and electroporated (125 mFd, 0.36 kV; Bio-Rad gene pulser; Bio-Rad, Richmond, CA) into 129/Ola-derived BK-4 embryonic stem (ES) cells [27]. After positive and negative selection with geneticin (250 μg/mL; Gibco/BRL, Rockville, MD) and gancyclovir (2 μM; Roche, Hertfordshire, UK), three of 40 geneticin-resistant and gancyclovir-sensitive colonies were identified as correctly targeted by polymerase chain reaction (PCR), Southern analysis, and direct sequencing. One ES cell clone, 2S2, was used to generate several chimeric males by well-established techniques [28]. Initially, male chimeras were bred to wild-type C57BL/6 female mice, and DNA isolated from agouti tail biopsies was analyzed by PCR to identify offspring that carry the mutant Brca2 allele. The Brca2 mutation has been established on the C57BL/6N (Charles River Laboratory, Raleigh, NC) background by successive back crossing. Chimeric male mice were also bred directly to 129/SvEv (Taconic, Germantown, NY) and BALB/c (Jackson Laboratories, Bar Harbor, ME) females and back–crossed repeatedly to create inbred mouse strains congenic for the Brca2 mutation. The $BALB/c^{B2+/-}$ mice used in this experiment were from the seventh back-cross generation (> 99% contribution from the BALB/c genetic background).

Mice

$BALB/c^{B1+/-}$ mutant mice were maintained by back crossing to wild-type BALB/c mice and have been described previously as $Brca1^{\Delta 223-763}$ mice [20]. The neomycin-resistance (*neo*) insertion in the $BALB/c^{B1+/-}$ mutant mice results in an alternatively spliced transcript that encodes an in-frame deleted Brca1 protein lacking amino acids 223–763 from exon 11. DBA/2J mice were purchased from Jackson Laboratories. All mice were group housed in plastic cages with pressed wood-chip bedding.

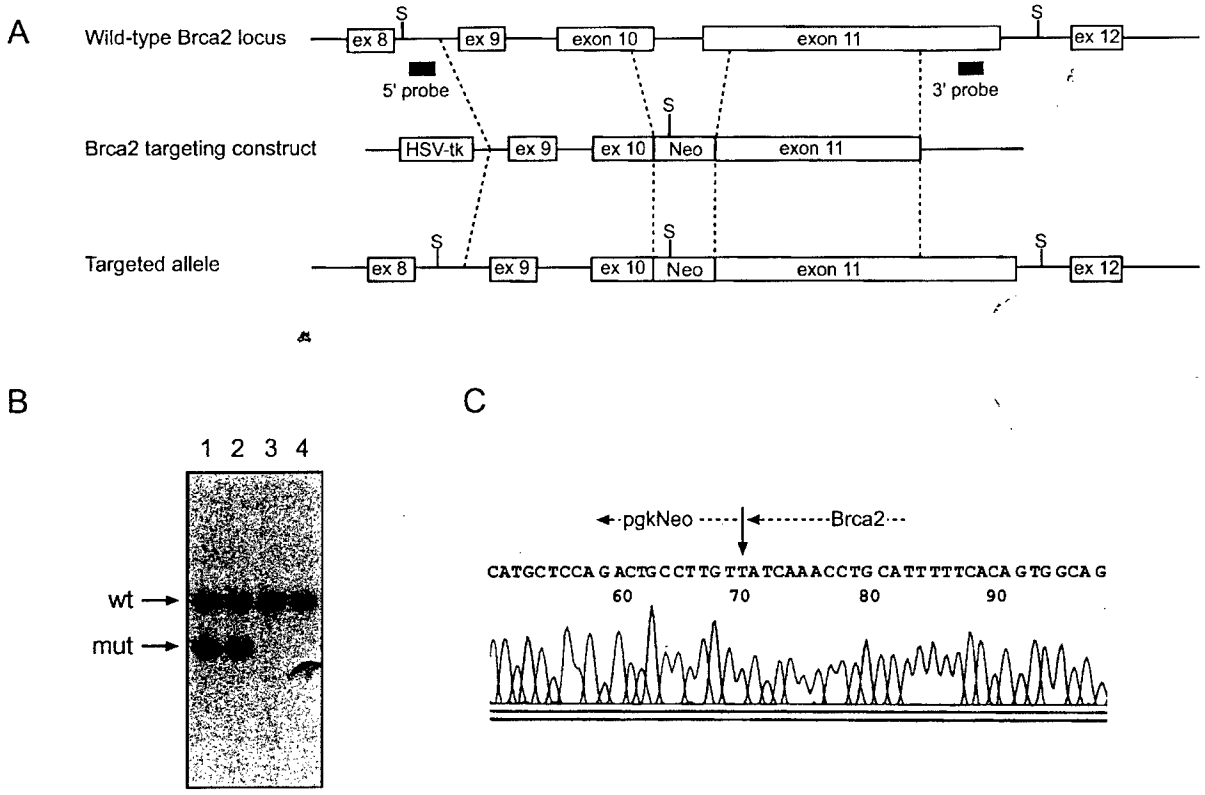

Figure 1. (A) Brca2 targeting strategy. The exon 10 and 11 region of the mouse Brca2 gene was disrupted by homologous recombination by using a targeting vector containing the selectable pgkNeo and HSV-tk genes. The pgkNeo gene replaced a portion of exon 10/11, causing a shift in the *Spe* I restriction enzyme pattern from 8.5 kb for the wild-type to 6 kb and 2 kb because of the presence of an additional SpeI site in the Neo gene. Homologous recombination was detected by both PCR and Southern blot analysis by using the SpeI restriction enzyme digest and the 3′ and 5′ probes. (B) Southern blot analysis of the DNA isolated from four agouti offspring of a chimeric Brca2-mutant male. Digestion with the restriction enzyme SpeI permits detection of an altered DNA digest pattern in two offspring (lanes 1 and 2) that have inherited the targeted Brca2 allele and two wild-type offspring (lanes 3 and 4) when hybridized with a 3′ exon 11 probe. (C) Direct sequencing of an RT-PCR product from BALB/$c^{B2-/-}$ mouse testis RNA. Arrow indicates *Brca2/neo* junction.

Animals had access to an NIH-31 diet (18% protein, 4% fat, and 5% fiber; Zeigler Bros., Gardeners, PA) and water ad libitum.

Genotyping by PCR and Southern Analysis

DNA from ES cells, tail biopsies, whole embryos, or day-old pups was isolated after overnight digestion with proteinase K at 55°C by phenol/chloroform extraction using serum separation tubes (Becton-Dickinson, Franklin Lakes, NJ). The wild-type Brca2 allele is detected by using the exon 11–specific primers KMF10 (5′-CTGAAGAGCCATCCTTGACC) and KM8R (5′-TCACTGTTCCCATCTGATTTC), which yield a 980-bp PCR product. The Brca2 Δ10/11 mutant allele is identified by PCR by using primers TkNeoF3 (5′-GAAGGATTGGAGCTACGGG) and JMR2 (5′-CTGAAGGAAGACATGCTGAAA), which yield a 300-bp product. Both Brca2 genotyping reactions are performed under the following conditions: denature at 94°C for 2 min followed by 30 cycles: 94°C for 30 s, 55°C for 30 s, 72°C for 30 s, and 72° for a 7-min extension.

The wild-type Brca1 allele is identified by using primers MBF3 (5′-GCACATTTATTACAGAACCAC) and MBR4 (5′-ACTTCCTCCTCAGCCTATTTTT) from exon 11 that yield a PCR product of 390 bp. The targeted Brca1 allele is identified by using the primers TkNeoF3 and MBR11 (5′-TTAAGCGCGTGTCTCAAGG), which yield a product of 550 bp. Both Brca1 primer pairs are used under the following conditions: denature at 94°C for 2 min, followed by 35 cycles: 94°C for 1 min, 55°C for 1 min, 72°C for 1 min, with a final 8-min extension at 72°C.

The Brca2 Δ10/11 mutant allele was also detected by Southern blot analysis. Isolated DNA was digested with SpeI (New England Biolabs, Beverly, MA), resolved on a 0.8% agarose gel, and transferred to GeneScreen Plus (NEN Life Sciences Products, Boston, MA). Filters were hybridized with a 5′ mouse probe outside the targeting construct (Figure 1A) and generated by using the primers KMF22 (5′-CCCCAGCTAGCCTGAATTTT) and KMR9 (5′-CTTCTTGCTGGTTTTTGTTTTC).

Embryo Collection

Sexually mature adult female $129^{B2+/-}$ and BALB/$c^{B2+/-}$ mice were superovulated with 5 IU of

pregnant mare serum gonadotropin (Sigma, St. Louis, MO) followed by 5 IU of human chorionic gonadotropin (Sigma) 48 h later and were then mated with $129^{B2+/-}$ and BALB/$c^{B2+/-}$ males, respectively. E8.5, E10.5, and E11.5 embryos or deciduas were collected from timed-pregnant mice and fixed in 10% neutral buffered formalin for 2–24 h or frozen at −70°C for genotypic analysis. Sufficient tissue was not always available for the extraction of DNA and RNA. Female BALB/$c^{B1+/-}$ mice were also superovulated and mated with BALB/$c^{B1+/-}$ males to generate Brca1 embryos of all genotypes. E10.5 embryos or deciduas were subsequently collected from these timed-pregnant BALB/$c^{B1+/-}$ females and fixed as described earlier.

Reverse Transcription (RT)–PCR

RNA was extracted from mouse testes or whole embryos with Tri-Reagent (Molecular Research Center, Inc., Cincinnati, OH) as directed. Frozen tissue samples were finely ground with mortar and pestle in liquid nitrogen. One milliliter of Tri-Reagent was added per 50–100 mg of tissue, the samples were homogenized, and RNA was isolated. The cDNA was generated by RT from the mRNA using Superscript II-RT (Boerhinger Mannheim, Indianapolis, IN). The Brca2 cDNA was amplified by an exon 10–specific forward primer KCF1 (5′-AGGACAGCATTTTGAATCACT) and a neo-specific reverse primer KCR1 (5′-GGTGGATGTGGAATGTGT). This RT-PCR product was gel isolated and sequenced directly with an internal reverse *neo* primer (5′-AGACGTGCTACTTCCATTTGT). Brca1 cDNA from Brca2-intercross progeny was amplified by RT-PCR with the following primer pair that spanned from exon 6 to exon 7 of Brca1: MBF29 (5′-GGGAAGCACAAGGTTTAGTCA) and MBR10 (5′-GGTGGCATTTCCAGGTTC).

In Situ Hybridization

Brca1 and Brca2 digoxigenin-labeled probes were generated as described by Blackshear et al. [14] except that the transcription reactions were performed with the Maxiscript T3/T7 kit (Ambion, Austin, TX). Paraffin-embedded sections were hybridized and washed at 60°C. Whole-embryo in situ hybridization was performed essentially as described by Wilkinson [29]. After fixation, the embryos were prehybridized for 1 h at 63°C and then hybridized overnight to either a Brca2-antisense or a Brca2-sense digoxigenin-labeled probe. After hybridization, the embryos were taken through a series of stringent 63°C washes (50% formamide, 0.75 M sodium chloride, and 0.075 M sodium citrate). The embryos were then treated with ribonuclease A to remove unbound single-stranded probe. The embryos were incubated with 10% sheep serum for 60–90 min and then incubated overnight at 4°C with antidigoxigenin antibody conjugated to alkaline phosphatase. The embryos were incubated with nitroblue tertrazolium/5-bromo-4-chromo-3-indolyl phosphate (Boehringer Mannheim) in the dark to develop the alkaline phosphatase histochemical reaction.

RESULTS

Genotype and Transcript Analysis in Brca2-Deficient Mice

The genotypes of agouti offspring resulting from crosses between chimeric males and C57BL/6 females were determined by Southern analysis (Figure 1B) and PCR (data not shown). RT-PCR analysis was performed on BALB/$c^{B2+/-}$ testis RNA because Brca2 RNA is abundantly expressed in this tissue [14,15,26]. A 550-bp fragment was generated from the BALB/$c^{B2+/-}$ samples with primers specific for Brca2 exon 10 and the *neo* gene. Direct sequencing of this PCR product indicated that the *Brca2/neo* fusion transcript was expressed (Figure 1C). The putative Brca2 protein is truncated at codon 569, with 75 additional amino acids contributed by the pgkNeo cassette. Repeated attempts to visualize a truncated transcript by northern blot analysis were unsuccessful, presumably due to unstable mRNA.

Phenotypic Evaluation of Brca2-Deficient and *Brca2*-Null Mice

Mendelian inheritance of the Brca2 mutation was studied by intercrossing male and female Brca2 heterozygous mice, with a mixed 129 × C57BL/6 background, to generate Brca2-null mice. Genotypic analyses by Southern blotting and PCR amplification failed to identify a single Brca2-null animal from 142 intercross offspring examined at weaning (Table 1). Mice heterozygous for a germline Brca2 mutation did not display any overt phenotypic abnormalities compared with their wild-type littermates at birth, weaning, or as adults. Female and male Brca2-heterozygous mice were able to breed normally. Females carried their pups to term and did not exhibit difficulty with lactation. We did not observe an increased incidence of spontaneous tumors in $129^{B2+/-}$ virgin females versus wild-type littermates up to 2 yr of age.

Genetic Modification of Embryonic Survivability

Genetic background has been determined to influence phenotype and survival of mutants in several gene knockout mouse models [30]. Brca1-null offspring on a mixed 129 × DBA/2 × C57BL/6 genetic background survive up to E13.5 [20], which is several days beyond that described for other Brca1-null mice. This altered phenotype may be the consequence of this specific Brca1 mutation that yields an in-frame deletion of exon 11 (Δ223–763) or, alternatively, may be enhanced by the presence

Table 1. Genotypes of Brca2-Intercross Progeny on Different Inbred Genetic Backgrounds

Strain background	Time point	Brca2 genotype		
		+/+	+/−	−/−
($C57^{B2+/-} \times 129^{B2+/-}$) F2	3 wk	65	77	0
(DBA/2 × $C57^{B2+/-}$) F2	1 d	6	19	0
(DBA/2 × $129^{B2+/-}$) F2	1 d	4	17	0
(DBA/2 × $C57^{B2+/-}$) F1 × $129^{B2+/-}$	1 d	13	12	0

of the DBA/2 genetic background. Thus, we evaluated survival to birth of Brca2-null mice by using several combinations of the DBA/2 with 129 and/or C57BL/6 genetic backgrounds. Seventy-one offspring from the following crosses were genotyped at 1 d of age: (DBA/2 × 129 $^{B2+/-}$)F2, (DBA/2 × C57BL/$6^{B2+/-}$)F2, and (DBA/2 × C57BL/$6^{B2+/-}$)F1 × $129^{B2+/-}$. As shown in Table 1, the incorporation of the DBA/2 genetic background did not prolong the survival of Brca2-null offspring to birth.

The embryonic lethal phenotype was studied in Brca2-null mice on the 129 genetic background. Intercrosses between male and female $129^{B2+/-}$ mice generated 22 E8.5 embryos, but genotypic analysis of the embryos was not completed because of the large number of reabsorbed fetuses observed at this time point. The $129^{B2+/-}$ intercrosses generated 35 E10.5 embryos, 11 of which were dissected out of the decidua or the yolk sacs and genotyped. Only heterozygotes and wild types were present at E10.5. Thus, $129^{B2-/-}$ embryos die and are largely reabsorbed before E8.5, which is consistent with previous reports of Brca2-deficient mice on a mixed C57BL/6 and 129 genetic background [21–23].

In contrast, when the Brca2 mutation was placed on a BALB/c genetic background, the day of embryonic lethality in the Brca2-null mice was extended to approximately E10.5. Ten E8.5 embryos were generated from BALB/$c^{B2+/-}$ intercross matings. Seven of 10 E8.5 embryos were genotyped, and the remaining three were either partly or completely reabsorbed. PCR analysis conclusively identified three BALB/$c^{B2-/-}$ embryos and four BALB/$c^{B2+/-}$ embryos (Figure 2). Genotyping was performed on one sample that was partly reabsorbed, but the PCR product was probably due to maternal contamination (Figure 2, lane 4). BALB/$c^{B2-/-}$ E8.5 embryos were grossly smaller, developmentally delayed, and resembled E6–7 embryos (Figure 3). Twenty-six yolk sacs and/or embryos were isolated from intercrosses

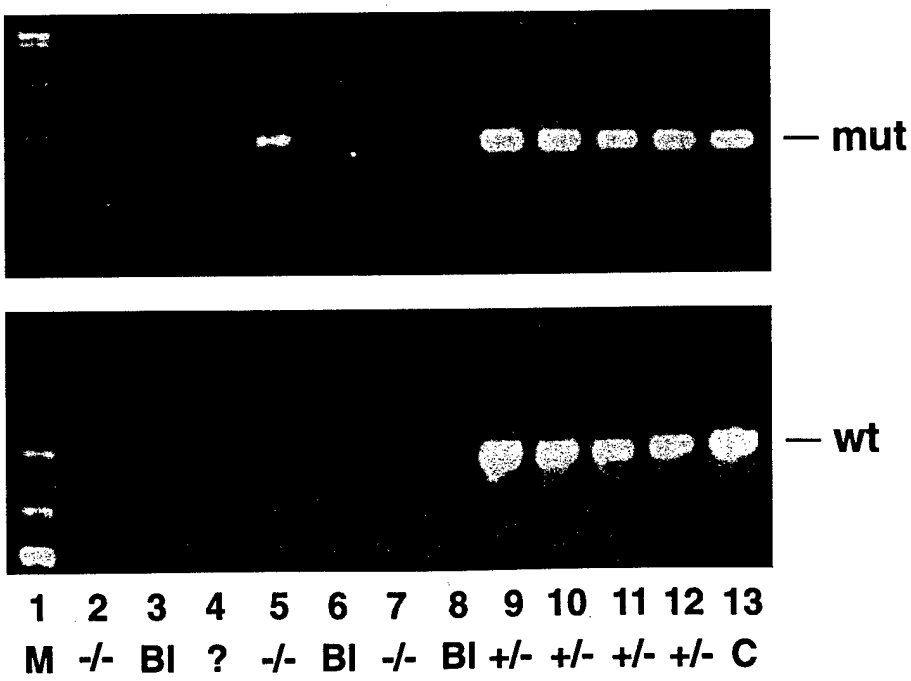

Figure 2. Genotypic analysis of DNA extracted from eight intercross E8.5 BALB/c^{B2} embryos. The upper gel contains PCR products using primers that amplify the mutant allele. The lower gel contains PCR products generated with wild-type–specific primers. Each lane represents an individual embryo. No samples were loaded in lanes 3, 6, and 8. Lane 1: DNA MW marker. Lanes 2, 5 and 7: BALB/$c^{B2-/-}$ embryo. Lane 4: inconclusive result. Lanes 9–12: BALB/$c^{B2+/-}$ embryos. Lane 13: control adult BALB/$c^{B2+/-}$ DNA.

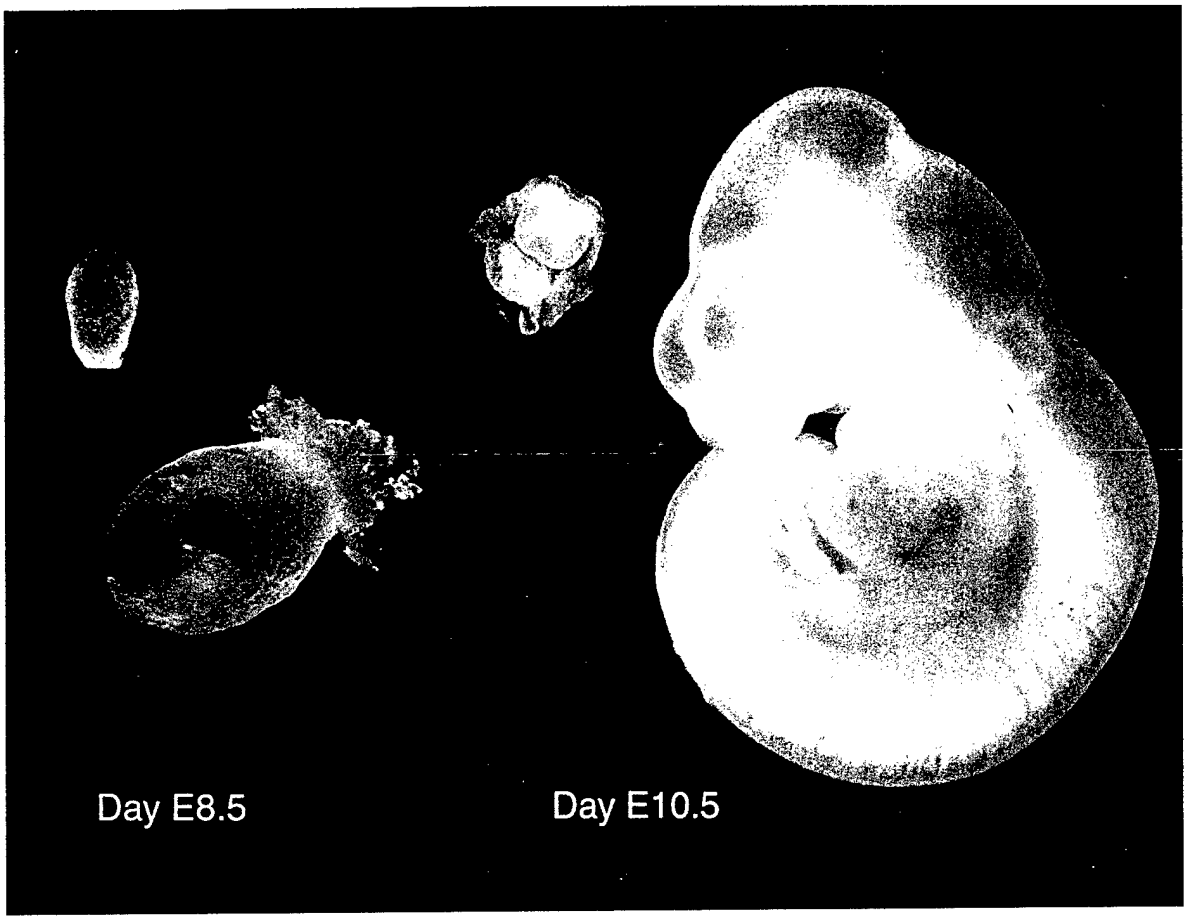

Figure 3. Growth inhibition of BALB/$c^{B2-/-}$ embryos at E8.5 and E10.5. The E8.5 growth-retarded BALB/$c^{B2-/-}$ embryo is compared with a normal BALB/$c^{B2+/-}$ littermate. The Brca2 (−/−) embryo is still in an egg-cylinder stage. The Brca2 (+/−) embryo has approximately four to five somites and well-developed ectoplacental cone and yolk sac. The E10.5 growth-retarded BALB/$c^{B2-/-}$ embryo is compared with a morphologically normal wild-type littermate. The Brca2 (−/−) embryo is developmentally delayed, with an open cranial neural tube characteristic of a normal E8.5 embryo.

at E10.5. The yolk sacs and/or embryos were genotyped by PCR and confirmed that all morphologically normal embryos were of the heterozygous or wild-type genotypes. The one confirmed BALB/$c^{B2-/-}$ embryo that survived until E10.5 was developmentally delayed and smaller than its heterozygous littermates. The cranial neural tube remained open, characteristic of an E8.5 embryo. However, the turning process was completed and the second brachial arch and the heart were prominent, indicating some development beyond the E8.5 time point. Three additional embryos displayed similar developmental delays, but confirmation of genotype was hindered by maternal tissue contamination. No embryonic tissue was found in five reabsorption sites. At the E11.5 time point, 10 morphologically normal and two developmentally delayed yolk sacs were dissected. There were three completely reabsorbed sites that could not be dissected. The 10 morphologically normal embryos were genotyped as wild types or heterozygotes. The confirmation of genotype for the two developmentally delayed embryos was not possible because of contaminating maternal tissue. Developmentally, these presumed mutant embryos were characteristic of normal E8.5 embryos, similar to that observed at the E10.5 time point.

The extension of Brca2-null embryo survival on the BALB/c genetic background enabled us to address whether transcriptional regulation of the Brca1 and Brca2 genes was interdependent. Brca1 expression was evaluated by RT-PCR analysis of mRNA isolated from E8.5 BALB/$c^{B2-/-}$ embryos. These studies demonstrated that Brca1 expression in BALB/$c^{B2-/-}$ E8.5 embryos was comparable to age-matched wild-type BALB/$c^{B2+/-}$ embryos (Figure 4).

In addition, we examined the timing of embryonic lethality and the expression of Brca2 in Brca1-null embryos on the BALB/c genetic background. Forty-two embryos were generated from BALB/$c^{B1-/-}$ intercrosses, and 19 viable progeny were examined. Genotypic analysis showed two BALB/$c^{B1-/-}$, seven BALB/$c^{B1+/+}$, and 10 BALB/$c^{B1+/-}$ offspring. The BALB/$c^{B1-/-}$ embryos were grossly

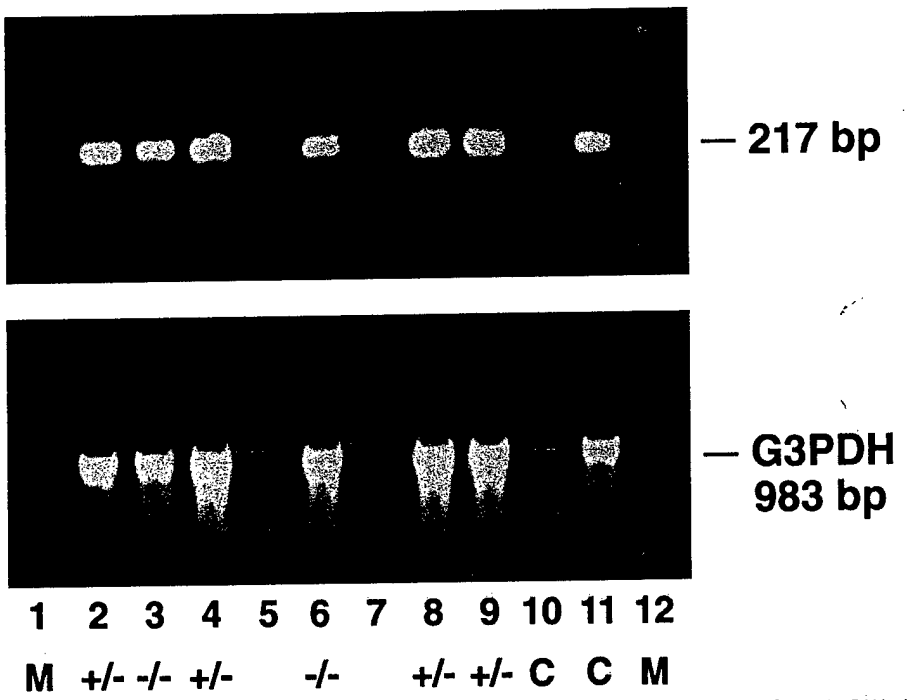

Figure 4. Analysis of Brca1 expression in the E8.5 BALB/c^{B2} embryos from Figure 3 by RT-PCR. The genotypes are indicated. The upper gel shows Brca1 cDNA (217 bp) amplified with a primer pair that spans exons 6 and 7. G3PDH, used as an internal control, is shown on the lower gel. Lanes 1 and 12: DNA MW markers. Lanes 2–4, 6, 8, and 9: amplification of Brca1 cDNA. Lane 11: positive control. Lanes 5 and 7: no Brca1 cDNA was amplified because of the minimal amount of embryo tissue available. Lane 10: negative control.

smaller and developmentally delayed. The E10.5 Brca1-null embryos had completed the turning process characteristic at this stage of development. However, the open cranial neural tube was characteristic of E8–8.5 embryos. These observations are comparable to results reported by Gowen et al. [20] with Brca1-null mice on a mixed 129, DBA/2, and C57BL/6 genetic background. Thus, we did not observe a dramatic alteration in the timing of embryonic lethality in BALB/$c^{B1-/-}$ embryos.

Brca2 expression was examined in E10.5 BALB/$c^{B1-/-}$ embryos. In situ hybridization analysis detected widespread Brca2 expression in Brca1-null embryos that was qualitatively comparable to the levels of Brca2 expression in age-matched BALB/$c^{B1+/+}$ embryos (Figure 5). The most intense localization of Brca2 mRNA was in the neuroepithelial cells lining the neural tube and was similar between wild-type and null embryos (data not shown). Neuroepithelial cells comprised the most active proliferating population, correlating with rapid brain development, in E8–10 embryos.

DISCUSSION

This study demonstrates that the BALB/c genetic background can modify survival of *Brca2*-null mouse embryos. Previous reports have associated embryonic survival with the location of mutations within the gene but have not shown an effect due to variation of genetic background alone. The 129$^{B2-/-}$ embryos were presumed to have died before E8.5 because 129$^{B2+/-}$ intercrosses had few viable progeny, as indicated by the large percentage of reabsorbed fetuses. This observation is consistent with other reports of Brca2 mutant mice in which the targeted alteration was 5′ of the BRC repeat region [21–23]. In contrast, matings between BALB/$c^{B2+/-}$ mice generated BALB/$c^{B2-/-}$ embryos that survived to E10.5. Thus, the BALB/c genetic background extends survival of *Brca2*-null embryos that inherit an alteration in exons 10 and 11 in Brca2.

Genetic background has been shown to influence phenotype and survival of mutants in a variety of gene knockout animal models [30]. For example, the BALB/c genetic background has a dramatic effect on the normal development of *p130* knockout mice [31]. Likewise, the timing of embryonic lethality of PTEN knockout embryos is modified by the CD-1 genetic background [32,33]. Epidermal growth factor receptor knockout mice with a 129 inbred background die during mid-gestation but survive to birth or longer with mixed inbred or outbred genetic backgrounds [34,35]. Thus, embryonic lethality for a variety of gene defects can be modified by allelic differences contributed by various inbred strain backgrounds.

We have demonstrated that modifiers in the BALB/c genetic background can prolong the survival of Brca2-null embryos. Variation of disease onset and severity has been reported for women with

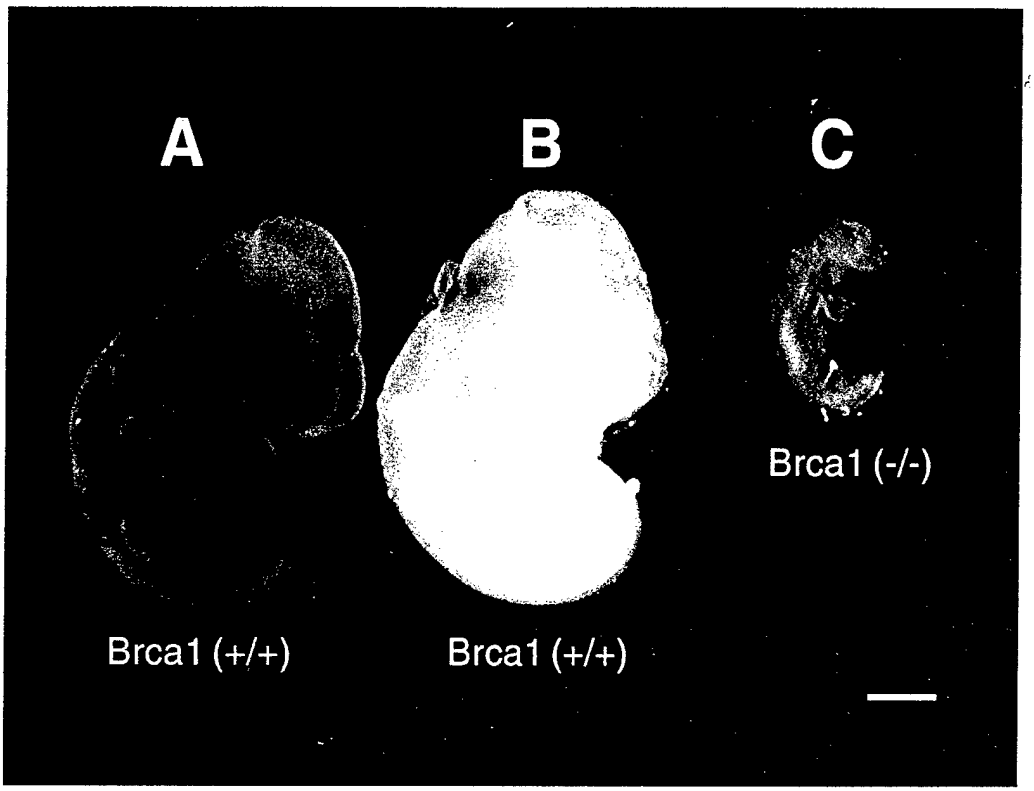

Figure 5. In situ hybridization analysis of Brca2 expression in E10.5 BALB/c$^{B1+/+}$ and BALB/c$^{B1-/-}$ embryos: BALB/c$^{B1+/+}$ whole embryos were hybridized with cRNA digoxigenin-labeled Brca2 antisense (A) and sense (B) probes. (C) BALB/c$^{B1-/-}$ embryo hybridized with cRNA digoxigenin-labeled antisense probe. Bar = 1 mm.

various mutations in BRCA1 and BRCA2 [4,36]. Penetrance variations in different populations suggest that genetic modifier loci or environmental factors must modulate the effect of BRCA1 or BRCA2 inactivation [1]. The inheritance of mutations in both BRCA1 and BRCA2 does not appear to affect severity or latency of disease outcome in humans [5,6], as might be expected if the genes directly interacted. However, modifier genes are strongly implicated by the various disease phenotypes that have been observed in individuals with identical BRCA2 mutations [4]. Certainly, there may be highly prevalent low-penetrance susceptibility genes that have a more profound effect on breast-cancer development in the general population than BRCA1 or BRCA2 mutations do [37,38]. Such genes may also modify breast-cancer risk in BRCA1 and BRCA2 mutation carriers, but the limitations of human linkage analysis makes identifying those genes very challenging.

Radiation-induced DNA damage is repaired less efficiently in cells from BALB/c than in cells from C57BL/6 mice. Chromosomal aberrations persist for up to 28 population doublings in irradiated BALB/c mammary epithelial cells, whereas similarly treated C57BL/6 cells repair damage within six population doublings [39]. Perhaps this increased tolerance for DNA damage in BALB/c mice is related to the prolonged embryonic survival of BALB/c$^{B2-/-}$ mice compared with other strains. In addition, a Brca2 mutation on the BALB/c genetic background may result in more dramatic phenotypic outcomes in the adult mammary gland as a result of inefficient DNA damage repair and a predicted tissue specificity.

This study also demonstrated that Brca1 mRNA was expressed in Brca2-null embryos and Brca2 mRNA was expressed in Brca1-null embryos. Expression levels of the null embryos were qualitatively comparable to those of wild-type and heterozygous littermates, although small quantitative differences in expression cannot be excluded. Thus, Brca1 and Brca2 transcripts are expressed independently of each other. If the regulation of either of these genes were dependent on the other, dysregulated expression would be expected in the gene-deficient embryos. Our results support those of a report by Suzuki et al. [22] who used *Brca1*-null mice with a targeted disruption of exons 5 and 6 and *Brca2*-null mice with disruption of exons 10 and 11 to study Brca2 and Brca1 gene expression, respectively. In the mouse embryo, adequate proliferation to sustain normal development appears to require the expression of both Brca1 and Brca2. Mice that inherit mutations in both Brca1 and Brca2 genes do not display more severe embryonic lethality, which might be expected if the effect of the losses were

additive [23]. Our investigation was limited to mRNA expression in embryos and did not address potential interactions between the Brca1 and Brca2 proteins. Because these gene products form a complex in vitro [13], it is possible that the functions of these proteins require a mutual interaction that permits normal cellular proliferation such that dysregulation of either Brca1 or Brca2 can contribute to neoplastic development.

We have described a substantial extension in the survival time of Brca2-null embryos on the BALB/c genetic background. These results suggest the presence of modifier loci that could be mapped in these mice. Future studies will evaluate the influence of the BALB/c genetic background on mammary-gland morphogenesis and neoplastic development in Brca2-deficient mice. Identification of such genetic modifiers in mice may permit the identification of human orthologues that could be evaluated for their role in breast-cancer susceptibility.

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Mice Heterozygous for a *Brca1* or *Brca2* Mutation Display Distinct Mammary Gland and Ovarian Phenotypes in Response to Diethylstilbestrol[1]

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ABSTRACT

Women who inherit mutations in the breast cancer susceptibility genes, *BRCA1* and *BRCA2*, are predisposed to the development of breast and ovarian cancer. We used mice with a *Brca1* mutation on a BALB/cJ inbred background (BALB/c$^{B1+/-}$ mice) or a *Brca2* genetic alteration on the 129/SvEv genetic background (129$^{B2+/-}$ mice) to investigate potential gene-environment interactions between defects in these genes and treatment with the highly estrogenic compound diethylstilbestrol (DES). Beginning at 3 weeks of age, BALB/c$^{B1+/-}$, 129$^{B2+/-}$, and wild-type female mice were fed a control diet or a diet containing 640 ppb DES for 26 weeks. DES treatment caused vaginal epithelial hyperplasia and hyperkeratosis, uterine inflammation, adenomyosis, and fibrosis, as well as oviductal smooth muscle hypertrophy. The severity of the DES response was mouse strain specific. The estrogen-responsive 129/SvEv strain exhibited an extreme response in the reproductive tract, whereas the effect in BALB/cJ and C3H/HeN$^{(MMTV-)}$ mice was less severe. The *Brca1* and *Brca2* genetic alterations influenced the phenotypic response of BALB/cJ and 129/SvEv inbred strains, respectively, to DES in the mammary gland and ovary. The mammary duct branching morphology was inhibited in DES-treated BALB/c$^{B1+/-}$ mice compared with similarly treated BALB/c$^{B1+/+}$ littermates. In addition, the majority of BALB/c$^{B1+/-}$ mice had atrophied ovaries, whereas wild-type littermates were largely diagnosed with arrested follicular development. The mammary ductal architecture in untreated 129$^{B2+/-}$ mice revealed a subtle inhibited branching phenotype that was enhanced with DES treatment. However, no significant differences were observed in ovarian pathology between 129$^{B2+/+}$ and 129$^{B2+/-}$ mice. These data suggest that estrogenic compounds may modulate mammary gland or ovarian morphology in BALB/c$^{B1+/-}$ and 129$^{B2+/-}$ mice. These observations are consistent with the hypothesis that compromised DNA repair processes in cells harboring *Brca1* or *Brca2* mutations lead to inhibited growth and differentiation compared with the proliferative response of wild-type cells to DES treatment.

INTRODUCTION

Breast cancer is a major health problem in the United States, with more than 170,000 cases diagnosed annually. The inheritance of mutations in the breast cancer susceptibility genes *BRCA1* and *BRCA2* has been reported to increase a woman's lifetime risk for breast cancer development from the 12% observed in the general population to as high as 85% (1, 2). In addition, mutations in these genes have been associated with ovarian cancer risks as high as 60% and 27% in *BRCA1* and *BRCA2* mutation carriers, respectively (1). The inactivation of both alleles of either *BRCA1* or *BRCA2* is very frequent during tumor development in women carrying germ-line mutations, resulting in the characterization of these genes as tumor suppressors. Whereas the functions of the *BRCA1* and *BRCA2* gene products have yet to be fully elucidated, there is evidence that they play key roles in DNA repair pathways (3–8) and cell cycle regulation (9–12) and may inhibit estrogen receptor signaling (13). In addition, *BRCA1* and *BRCA2* have been shown to interact with each other as well as with DNA repair genes such as *Rad51*, *Rad50*, and *Bard1* (3, 8, 14–17). Expression of *BRCA1* and *BRCA2* is induced during cell proliferation, but this induction does not appear to be directly regulated by estrogen (13, 18–23).

Although mutations in *BRCA1* and *BRCA2* have been clearly associated with breast and ovarian cancer development in women, the effect of the environment on individuals who have inherited mutations is not well established. Investigations have begun to evaluate the consequences of environmental exposure in *BRCA1* and *BRCA2* mutation carriers predisposed to breast and ovarian cancer. For example, smoking is associated with reduced breast cancer risk in *BRCA1* mutation carriers (24). Oral contraceptive use may increase the risk for breast cancer in *BRCA1* and *BRCA2* mutation carriers (25), whereas it appears to reduce the risk of ovarian cancer development (26). Likewise, prophylactic oophorectomy significantly reduces the risk for breast cancer in *BRCA1* mutation carriers (27). Thus, as for the general population, hormonal modulation can influence breast and ovarian cancer risk in genetically predisposed populations.

Between 1940 and 1970, approximately 10% of pregnant women received the estrogenic compound DES[4] to prevent spontaneous abortion and other pregnancy-associated indications (28). DES has proved to be a transplacental carcinogen, as demonstrated by its ability to induce vaginal clear cell adenocarcinomas in the daughters of exposed women (28–30). DES-exposed women developed reproductive tract abnormalities including vaginal adenosis, transverse fibrous ridges in the vagina or on the cervix, and cervical ectropion (28). In addition, the breast cancer risk for women prescribed DES during pregnancy has been evaluated in several epidemiological studies (31–35). Whereas the results of the individual studies varied as to whether or not there was a statistically increased risk for breast cancer in women given DES, when evaluated together, the data provide enough evidence to classify DES as a human breast carcinogen (30). An increased risk for breast cancer has not been firmly established for daughters exposed transplacentally (36, 37).

DES may mediate its carcinogenic effects in estrogen-responsive tissues, such as the breast and reproductive tract, through several mechanisms. DES is a potent estrogenic compound that binds the estrogen receptor with 2–3-fold greater affinity than 17β-estradiol (38) and stimulates cell proliferation (39). DES can be metabolized to catechol and quinone compounds that can disrupt mitosis, form free radicals, and induce damage by directly binding DNA or proteins (40). Thus, DES has the potential to both initiate and promote tumor

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[4] The abbreviations used are: DES, diethylstilbestrol; NTP, National Toxicology Program; C3H, C3H/HeN$^{(MMTV-)}$; CL, corpora lutea.

Table 1 *Mean reproductive and body weights for untreated and DES-treated Brca1-deficient, Brca2-deficient, and C3H mice*

Strain	Treatment group	Repro. tract fraction[a]		Final body weight (g)[b]	
		n	Mean (SD)	*n*	Mean (SD)
BALB/$c^{B1+/+}$	Control		ND[c]	15	22.4 (1.87)
BALB/$c^{B1+/-}$	Control		ND	15	23.5 (2.14)
BALB/$c^{B1+/+}$	DES		ND	12	20.8 (1.15)
BALB/$c^{B1+/-}$	DES		ND	12	21.1 (1.36)
$129^{B2+/+}$	Control	5	1.17 (0.47)	15	22.4 (1.36)
$129^{B2+/-}$	Control	7	1.24 (0.29)	15	21.7 (1.43)
$129^{B2+/+}$	DES	2	1.70 (0)	15	19.4 (1.50)
$129^{B2+/-}$	DES	3	1.90 (0.57)	15	19.6 (0.97)
C3H	Control	6	0.97 (0.16)	14	30.0 (4.59)
C3H	DES	5	1.60 (0.26)	11	23.7 (1.95)

[a] Reproductive tract fraction (Repro. tract fraction) = reproductive tract weight (g)/total body weight (g) at 2 months of age.
[b] Total body weight in grams at 6 months of age.
[c] ND, not determined.

development (40). DES has been shown to cause reproductive tract abnormalities during mouse development by inducing epidermal growth factor and altering the *Wnt* signaling pathway in the Müllerian duct system and uterus (41, 42). Similarly, DES causes mammary gland abnormalities during development. For example, newborn BALB/cCrgl mice treated with daily injections of 0.1–2 μg of DES on days 1–5 after birth displayed an immediate inhibition of mammary ductal branching that persisted 4 weeks later (43).

In addition to altered mammary ductal morphology, prolonged exposure of mice to dietary DES induces mammary tumors in dose- and age-dependent manners (44, 45). C3H mice fed DES beginning at 3 weeks of age developed tumors earlier than those treated at 5 weeks of age or at later time points (45). A linear dose-response curve, from 25 to 500 ppb DES, was observed for mammary tumor induction in mice given DES-containing feed between 4 and 6 weeks of age (44), a time during which the mammary gland terminal end buds are plentiful, and the ductal epithelium has been hypothesized to be particularly susceptible to carcinogenic insults.

The NTP, which studies compounds for their potential carcinogenicity, is evaluating alternatives to 2-year bioassays for suspected carcinogen testing. *p53*-deficient and Tg.AC (carriers of an activated Ha-*ras* oncogene) transgenic mice, both with cancer-predisposing mutations, are currently being evaluated as a rapid bioassay systems (46–48). These genetically predisposed mice are being exposed to a series of previously tested compounds in 6-month assays for comparison with the results from the 2-year NTP studies (47, 48). *p53*-deficient and Tg.AC mice were treated with DES by s.c. injection and topical application, respectively, for 26 weeks. DES-exposed *p53*-deficient mice did not develop any tumors by 6 months of age but did display ovarian degeneration and uterine hydrometra. In contrast, 53% of the Tg.AC mice developed squamous cell papillomas. Uterine hyperplasia and pituitary hyperplasia were also observed, as was atrophy of the seminal vesicles and thymus (48).

We investigated potential interactions between DES treatment and defects in the *Brca1* and *Brca2* genes. We used female BALB/cJ mice that inherit a *Brca1* mutation (BALB/$c^{B1+/-}$), 129/SvEv mice heterozygous for a *Brca2* mutation ($129^{B2+/-}$), and their respective wild-type littermates, BALB/$c^{B1+/+}$ and $129^{B2+/+}$. Because the inheritance of *BRCA1* and *BRCA2* mutations is associated with increased human breast and ovarian cancer susceptibility, we chose to target the mouse mammary gland and reproductive tissues by administering DES orally to female mice. We report here the effects of DES exposure on the growth and development of the mammary glands and reproductive tracts, as well as nonneoplastic morphological alterations, and the potential induction of neoplasias in BALB/$c^{B1+/-}$ and $129^{B2+/-}$ mice.

MATERIALS AND METHODS

Mice. C3H mice were obtained from the National Cancer Institute-Frederick Cancer Research & Development Center /Animal Production Area (Bethesda, MD). BALB/$c^{B1+/-}$ mutant mice have been described previously (49) and have been maintained by back-crossing to wild-type BALB/cJ mice (Jackson Laboratories, Bar Harbor, ME). The *neo* insertion in the BALB/$c^{B1+/-}$ mutant mice results in an alternatively spliced transcript that encodes an in-frame-deleted Brca1 protein lacking exon 11 amino acids 223–763. Mice that inherit a *Brca2* mutation on a 129/SvEv genetic background ($129^{B2+/-}$) were established in our laboratory by replacing the 3′ end of exon 10, intron 10, and the 5′ end of exon 11 with a *pgkNeo* cassette (50). $129^{B2+/-}$ mice are maintained by mating mutation carriers to wild-type 129/SvEv inbred mice (Taconic, Germantown, NY). The $129^{B2+/-}$ mice were generated from chimeric mice, derived from BK4 ES cells (129/Ola), and back-crossed for three or four generations to the 129/SvEv inbred mouse strain. Thus, the $129^{B2+/-}$ and $129^{B2+/-}$ mice used in this experiment had an approximate contribution of 6–12% from the 129/Ola substrain genetic background. Mice were housed (five mice/cage) in a temperature- and humidity-controlled room with a 12-h dark/light cycle and had access to food and water *ad libitum*.

Chemical Treatment. DES was administered to the treated animals in their feed. Five sets of 30 mice each were separated into treated and untreated groups. Fifteen $129^{B2+/+}$, $129^{B2+/-}$, BALB/$c^{B1+/+}$, BALB/$c^{B1+/-}$, and C3H mice received control NTP2000 diet (13% protein, 8% fat, and 12% fiber; Zeigler Bros., Gardeners, PA), and 15 mice from each strain received NTP2000 diet supplemented with 640 ppb DES (CAS:56-53-1) that was quality-assured for purity and shelf life (Research Triangle Institute, Research Triangle Park, NC). The mice were given control diet or DES-containing diet when weaned at 21 ± 2 days of age until they were sacrificed. Food consumption was not measured directly for this study. Close estimations depend on the age of the animal and other factors. The consumption of approximately 5 g of feed per day is a reasonable estimate[5] that would result in an average daily dose of 3.2 μg of DES for mice in the treated groups. Because food consumption was not measured in this study, it is possible that palatability played a role in the weight reduction of the DES-treated animals (Table 1).

The BALB/$c^{B1+/-}$ and $129^{B2+/-}$ mice and their wild-type littermates were sacrificed at 6 months of age by CO_2 asphyxiation. The C3H mice were sacrificed at 56 weeks of age because this was a time point at which approximately 50% of the C3H mice used in a previous study had developed tumors (44). At the time of sacrifice the #2, #3, and #4 mammary glands were collected for whole mount analysis (see below), and complete necropsies were performed. Three BALB/$c^{B1+/+}$ and two BALB/$c^{B1+/-}$ mice treated with DES died before the end of the experiment, and one BALB/$c^{B1+/-}$ mouse became moribund and was sacrificed 1 month early with bladder pathology. Two C3H mice on the DES diet were sacrificed at 9 or 10 months of age because of the development of palpable mammary masses. Two additional C3H mice, one on the DES-containing diet and one on the control diet, died before the terminal sacrifice.

[5] Cynthia Smith, personal communication.

Histology and Mammary Gland Whole Mounts. After complete necropsy, all tissues were fixed in 10% neutral buffered formalin, processed for routine histology, and evaluated for pathology. The mammary glands were fixed on the pelts in 10% neutral buffered formalin for 18–24 h and then stained essentially as described by Russo *et al.* (51). Three #4 mammary glands from each genotypic class that had been previously mounted whole were selected at random for histological analysis. The slides were soaked in xylene to release the glands from the permount, hydrated with incubations through graded alcohols, processed for routine histology, and evaluated microscopically.

Statistical Analyses. The mammary glands from $129^{B2+/+}$ and $129^{B2+/-}$ untreated mice were coded and graded for the extent of overall branching complexity on a scale of 1 (minimal complexity; simple) to 4 (maximal complexity; highly complex) by eight pathologists. The criteria for grades included the extent of growth into the fat pad and the complexity of side-branching and the degree of epithelial density, which were reflected in the relative number of terminal end buds, lateral buds, and/or alveolar buds penetrating the surrounding stroma. Overall comparisons of severity grades among the eight pathologists were carried out by Friedman's two-way ANOVA (52). Correlations between each pair of pathologists were assessed by Kendall's τ. There was strong correlation among each pair of pathologists in terms of relative grading. The nonparametric correlation coefficients (Kendall's τ) among the 28 possible pairs of pathologists ranged from 0.37–0.80, and all were statistically significant. Because there was excellent agreement among the pathologists as to which mammary glands were more complex than others, the final analysis for the phenotypes was based on the pooled severity grade from the eight pathologists. Differences in genotypes were analyzed by either Wilcoxon's rank-sum test or the Mann-Whitney U test (52).

Because the grading among the eight pathologists was in excellent agreement, subsequent grading of mammary gland morphology for untreated BALB/$c^{B1+/+}$ and BALB/$c^{B1+/-}$ mice was performed by the primary study pathologist (B. J. D.). As described above, the extent of overall branching was graded for complexity on a scale of 1 (minimal complexity; simple) to 4 (maximal complexity; highly complex) and was graded independently from the $129^{B2+/+}$ and $129^{B2+/-}$ mice because the inbred genetic background contributes to the ductal branching phenotype.

DES treatment had a dramatic proliferative effect on the ductal epithelium, resulting in ductal branching structures distinct from those of the untreated animals. The mammary glands from DES-treated mice were coded and graded for the extent of overall branching complexity on a scale of 5 (minimal complexity; simple) to 8 (maximal complexity; highly complex) to reflect the proliferative effect by DES treatment. The DES-treated mammary glands were graded by the primary study pathologist. The treated BALB/$c^{B1+/+}$ and BALB/$c^{B1+/-}$ genotypic classes were scored independently of the $129^{B2+/+}$ and $129^{B2+/-}$ mice. Differences in genotypes and treatments were analyzed by either Wilcoxon's rank-sum test or the Mann-Whitney U test.

Overall differences among the groups in reproductive organ responses were evaluated using χ^2 analysis. Pairwise comparisons were made by using Fisher's exact test (52).

RESULTS

DES-treated BALB/c and 129 mice displayed a number of phenotypes distinct from their corresponding untreated controls. Reproductive weights were determined for a subset of DES-treated and control mice at 8 weeks of age (Table 1). Animals consuming the DES-containing diet had greater relative mean reproductive tract weights than untreated controls (Table 1). The relative reproductive tract weights for $129^{B2+/+}$ and $129^{B2+/-}$ mice were approximately 65% greater than those of their untreated littermates. Likewise, the reproductive tracts of C3H mice were 60% heavier than those of the untreated animals. All DES-treated mice gained weight more slowly than did controls (data not shown). Mean body weights for the DES-treated BALB/c and 129 mice were approximately 10% less than in untreated controls at 6 months of age (Table 1). Similarly, untreated C3H mice were 21% heavier than DES-treated mice at the 1 year time point.

DES treatment caused uterine and cervicovaginal pathology in all mouse strains. All DES-treated mice were diagnosed with uterine hyperkeratosis, cervical epithelial hyperkeratosis, and oviductal smooth muscle hypertrophy (data not shown). The uterus and cervicovaginal area of untreated and DES-treated $129^{B2+/+}$, $129^{B2+/-}$, BALB/$c^{B1+/+}$, BALB/$c^{B1+/-}$, and C3H mice were evaluated (Fig. 1; Table 2; results for C3H mice are not shown). The DES-treated BALB/$c^{B1+/+}$ and BALB/$c^{B1+/-}$ uteri were characterized by a pau-

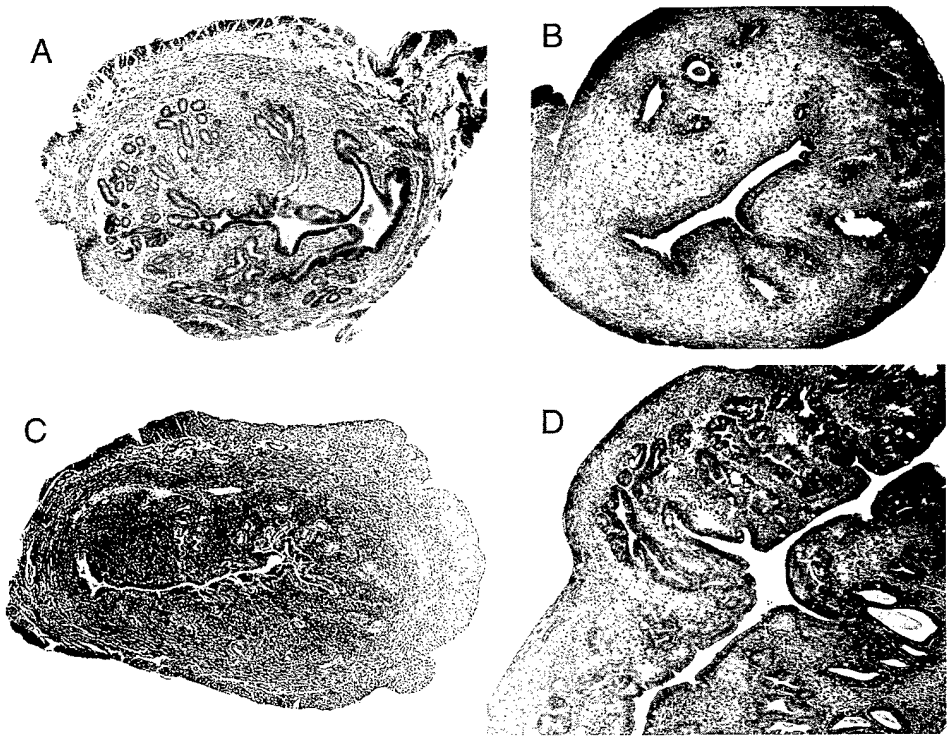

Fig. 1. Photomicrograph of uteri from untreated and DES-treated BALB/c and 129 mice. *A*, characteristically normal uterus from a vehicle control wild-type BALB/c mouse. *B*, DES-treated BALB/c wild-type uterus demonstrating a paucity of endometrial glands and chronic fibrosis. *C*, characteristically normal uterus from a vehicle control wild-type 129 mouse. *D*, DES-treated 129 wild-type mouse uterus characterized by severe active inflammation and marked endometrial and glandular hyperplasia and dysplasia. Magnification: *A–C*, ×4; *D*, ×10.

Table 2 *Reproductive pathology in DES-exposed Brca1- and Brca2-heterozygous and wild-type mice at 6 months of age*

	BALB/c$^{B1+/+}$	BALB/c$^{B1+/-}$	129$^{B2+/+}$	129$^{B2+/-}$
Uterus				
Cystic endometrial hyperplasia	7 (58%)[a]	10 (77%)	2 (14%)	4 (29%)
Fibrosis	12 (100%)	13 (100%)	2 (14%)	1 (7%)
Inflammation	5 (42%)	5 (38%)	9 (64%)	10 (71%)
Hyperplasia	1 (8%)	3 (23%)	6 (43%)	5 (36%)
Dysplasia	1 (8%)		5 (36%)	5 (36%)
Metaplasia			1 (7%)	1 (7%)
Squamous metaplasia			2 (14%)	2 (14%)
Adenocarcinoma			2 (14%)	
Squamous cell carcinoma			1 (7%)	1 (7%)
Adenomyosis			1 (7%)	6 (43%)[b]
Carcinoma			1 (7%)	1 (7%)
Total observations[c]	12	13	14	14
Vagina/cervix				
Inflammation		3 (23%)		2 (14%)
Hyperplasia	4 (36%)	5 (38%)	5 (36%)	4 (29%)
Squamous hyperplasia			1 (7%)	2 (14%)
Squamous cell carcinoma	1 (9%)			
Total observations	11	13	14	15

[a] Percentage of total.
[b] Fisher's exact test, $P = 0.05$ *versus* corresponding wild-type genotype.
[c] Total number of animals for which samples were available.

city of endometrial glands and diffuse severe chronic fibrosis (Fig. 1; Table 2). One BALB/c$^{B1+/+}$ mouse developed a cervicovaginal squamous cell carcinoma, and more than half of the BALB/c$^{B1+/+}$ and BALB/c$^{B1+/-}$ mice displayed cystic endometrial hyperplasia (Table 2). The uteri of the DES-treated 129$^{B2+/+}$ and 129$^{B2+/-}$ mice were characterized by diffuse active inflammation and marked endometrial and glandular hyperplasia and dysplasia (Fig. 1; Table 2). A uterine squamous cell carcinoma was diagnosed in one 129$^{B2+/+}$ and one 129$^{B2+/-}$ mouse, and a uterine carcinoma was observed in a 129$^{B2+/-}$ female. Six of 14 (43%) 129$^{B2+/-}$ mice developed adenomyosis compared with only 1 of 14 (7%) wild-type littermates ($P = 0.05$). Of 14 DES-treated C3H mice examined at 56 weeks of age, 7 developed uterine adenocarcinomas, and 1 developed a cervicovaginal squamous cell carcinoma; none of the untreated animals developed uterine adenocarcinomas or cervicovaginal squamous cell carcinomas (data not shown).

Mammary ductal morphogenesis was examined in stained whole mount preparations from 129$^{B2+/+}$, 129$^{B2+/-}$, BALB/c$^{B1+/+}$, and BALB/c$^{B1+/-}$ DES-treated and untreated mice sacrificed at 6 months of age. No tumors were observed in the mammary glands of DES-treated or untreated 129$^{B2+/+}$, 129$^{B2+/-}$, BALB/c$^{B1+/+}$, or BALB/c$^{B1+/-}$ mice.

The mammary glands isolated from the treated and untreated C3H mice were studied grossly by mammary gland whole mount. Previous studies showed that C3H mice develop mammary tumors and reproductive tract lesions after oral exposure to DES (45). In our study, 2 of 14 (14%) DES treated C3H females developed mammary tumors; 1 was classified as a cystic papillary adenocarcinoma, and the other was classified as an adenocarcinoma. This tumor incidence was unexpectedly low compared with the approximately 50% incidence previously reported in C3H/HeN–MTV–/Nctr mice fed 640 ppm DES at 1 year of age (45). However, the analysis of mammary gland whole mount preparations revealed a profound phenotypic response to 1 year of DES treatment. The mammary epithelium had formed a dense network of ducts, ductules, alveoli, and alveolar buds in the gland, and the ducts were often dilated and filled with homogeneous material (data not shown).

Mammary Gland Morphology in Untreated Mice. Mammary ductal morphogenesis was studied, and comparisons were made between the BALB/c and 129 inbred mouse strains. The ductal morphology in untreated wild-type 129 and BALB/c animals was typically well developed with complete growth into the fat pad and lateral and side branches emanating from elongated ducts. Low to moderate numbers of alveolar buds branched from the lateral ducts.

Mammary ductal structures were compared in the BALB/c$^{B1+/-}$ mice and their wild-type littermates. Mammary ductal branching in untreated BALB/c$^{B1+/+}$ and BALB/c$^{B1+/-}$ mice was essentially identical between these genotypic classes (Fig. 2). The mammary arborization complexity values for the BALB/c$^{B1+/-}$ mice and their wild-type littermates ranged from simple to moderately complex, with the exception of one animal in each genotypic class that was diagnosed as having a highly complex branching structure. The mean grade values were 2.4 ± 0.91 and 2.4 ± 0.93 for the wild-type and BALB/c$^{B1+/-}$ mice, respectively (Table 3).

The ducts of untreated 129$^{B2+/-}$ mice were compared with their wild-type littermates and generally appeared less complex than those of 129$^{B2+/+}$ mice (Fig. 3). The 129$^{B2+/-}$ mammary ducts were elongated with less lateral and side branching and showed decreased alveolar bud formation compared with wild-type littermates. Mammary glands isolated from the heterozygous 129$^{B2+/-}$ mice had ductal branching patterns that ranged from simple to moderately complex. In comparison, 4 of 14 129$^{B2+/+}$ mice had mammary ductal structures that were slightly less mature than the rest of the wild-type animals but were not blunted as those seen in the 129$^{B2+/-}$ group. Four 129$^{B2+/-}$ mice had ductal branching patterns that were as well developed as those of 129$^{B2+/+}$ mice, with side branching and alveolar buds. Despite subtle differences that appeared to exist between untreated 129$^{B2+/+}$ and 129$^{B2+/-}$ littermates, the mean mammary arborization complexity values were 2.9 ± 0.69 and 2.5 ± 0.87, respectively, and were not significantly different (Table 3).

Mammary Gland Ductal Morphogenesis in DES-treated Animals. DES treatment of all mice caused mammary ductal proliferation. In general, the mammary ducts from DES-treated animals were grossly visible, beige, and dilated within the mammary fat pad when the mice were sacrificed. The 129 and BALB/c inbred mouse strains responded to DES with extensive and complex filling of the mammary fat pad with branching ducts, ductules, alveolar lobules, and alveoli, all greatly distended with copious amounts of homogenous material (Figs. 2 and 3). Histologically, the branching ductules and alveoli appeared typically ordered or flattened by the accumulated material, but occasionally cells piled together, forming irregular nodules with ill-defined lumens. In all cases, inflammatory cells including neutrophils, lymphocytes, and macrophages and, to a lesser extent, mast cells surrounded and

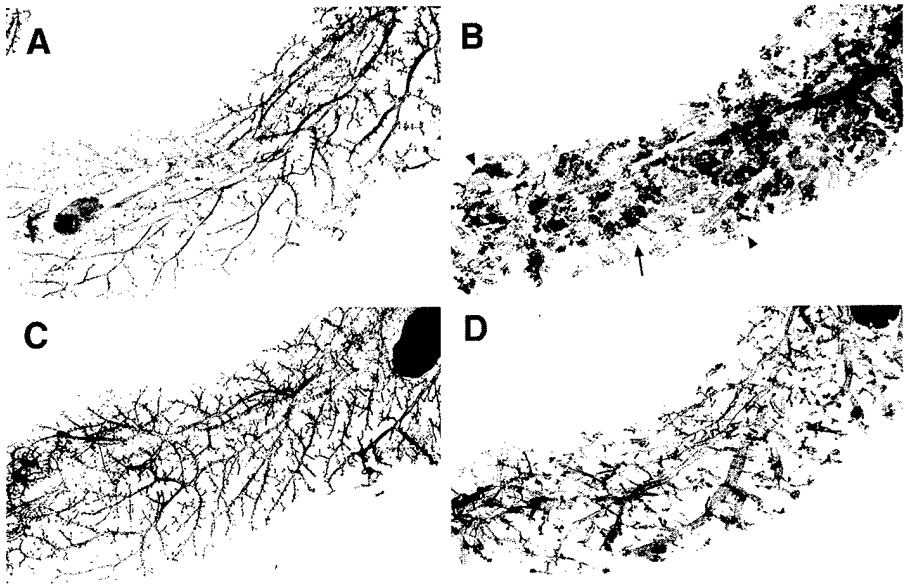

Fig. 2. Photomicrograph of mammary gland whole mount from control and DES-exposed BALB/$c^{B1+/+}$ and BALB/$c^{B1+/-}$ mice. Ductal branching pattern in a representative (*A*) BALB/$c^{B1+/+}$ untreated mouse, (*B*) BALB/$c^{B1+/+}$ DES-exposed mouse with dilated ducts (*arrow*), marked glandular density, and multiple cystic alveoli (*arrowheads*), (*C*) BALB/$c^{B1+/-}$ untreated mouse, and (*D*) BALB/$c^{B1+/-}$ DES-exposed mouse with a paucity of ductal branches and cystic alveoli as compared with similarly treated wild-type littermates.

infiltrated the periductular stroma and glandular epithelium (data not shown). Much of the ductal lumen contained calcified plaques and cellular debris.

DES treatment effects in the BALB/$c^{B1+/-}$ mice were compared with those in their wild-type littermates. Both the BALB/$c^{B1+/+}$ and BALB/$c^{B1+/-}$ mice responded to DES exposure with ductal dilation, resulting in moderate to severe ectasia (Fig. 2). The ducts were occasionally distended into 1–3-mm-diameter cysts filled with the material characteristic of galactoceles. Although the ducts exhibited some ductule branching and alveolar bud formation, both were seen to a lesser extent than that observed in $129^{B2+/+}$ and $129^{B2+/-}$ mice. There was a statistically significant difference in branching phenotype between the BALB/$c^{B1+/+}$ and BALB/$c^{B1+/-}$ genotypic classes after DES treatment (Table 3). Analysis of the mammary ductal branching patterns in response to DES treatment yielded average complexity grades of 6.7 ± 0.48 for the BALB/$c^{B1+/+}$ mice and 5.8 ± 0.60 for BALB/$c^{B1+/-}$ mice ($P < 0.01$).

Table 3 *Mean mammary gland ductal arborization complexity grades for Brca1- and Brca2-deficient mice*

Strain	No. of mice	Treatment	Arborization complexity[a]
BALB/$c^{B1+/+}$	15	Control	2.4 (0.91)
BALB/$c^{B1+/-}$	14	Control	2.4 (0.93)
BALB/$c^{B1+/+}$	10	DES	6.7 (0.48)
BALB/$c^{B1+/-}$	13	DES	5.8[b] (0.60)
$129^{B2+/+}$	14	Control	2.9 (0.69)
$129^{B2+/-}$	14	Control	2.5 (0.87)
$129^{B2+/+}$	15	DES	6.9 (0.88)
$129^{B2+/-}$	13	DES	6.4[c] (0.65)

[a] Values are mean (SD).
[b] Two-tailed Mann-Whitney *U* test $P < 0.01$ *versus* the corresponding wild-type genotype.
[c] Two-tailed Mann-Whitney *U* test $P = 0.07$ *versus* the corresponding wild-type genotype.

The DES-treated mammary glands from $129^{B2+/-}$ and wild-type mice were also examined. In general, $129^{B2+/+}$ and $129^{B2+/-}$ mammary whole mount preparations displayed proliferation characterized by increased ductular formation and branching as well as the formation of alveolar lobules and alveoli after DES treatment (Fig. 3). In addition, the subtle inhibition of ductular branching and alveolar-lobular formation observed in untreated females persisted in the

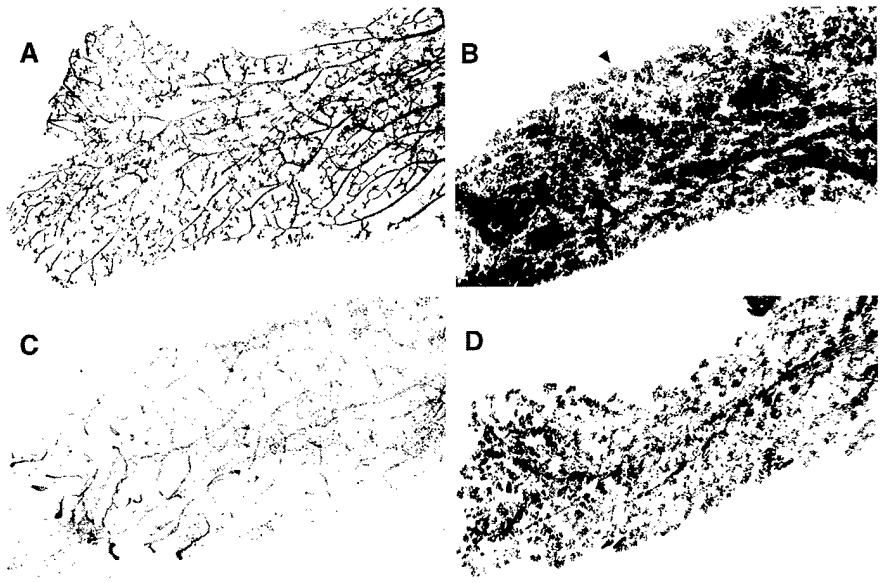

Fig. 3. Photomicrograph of the mammary epithelial ductal pattern in control and DES-exposed $129^{B2+/+}$ and $129^{B2+/-}$ mice. Ductal branching pattern in a representative (*A*) $129^{B2+/+}$ untreated mouse, (*B*) $129^{B2+/+}$ DES-exposed mouse with complex filling of the white adipose tissue with branching ducts, ductules, alveolar lobules, and multiple cystic alveoli (*arrowhead*), (*C*) $129^{B2+/-}$ untreated mouse with a subtle inhibited branching structure compared with the wild-type littermate, and (*D*) $129^{B2+/-}$ DES-exposed mouse with an inhibited ductal branching complexity as compared with the DES-treated wild-type littermate.

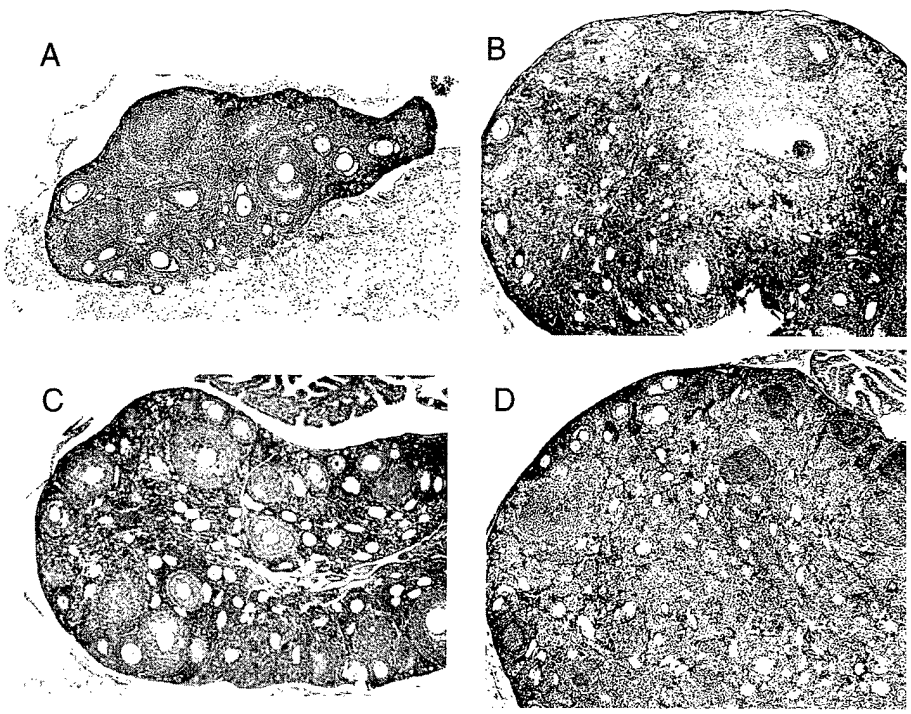

Fig. 4. Photomicrograph of ovarian pathology from DES-treated mice. *A*, ovary from a DES-treated BALB/$c^{B1+/+}$ mouse demonstrating arrested follicular development. *B*, ovary from a DES-treated BALB/$c^{B1+/-}$ mouse characteristic of aged or atrophied ovaries. *C*, arrested follicular development in a DES-treated $129^{B2+/+}$ mouse ovary. *D*, ovary from a DES-treated $129^{B2+/-}$ mouse with arrested follicular development. Magnification, ×4.

DES-treated $129^{B2+/-}$ mice. There was overlap between the genotypic classes, as observed in the untreated animals. The difference between the mammary ductal morphology of DES-treated $129^{B2+/+}$ and $129^{B2+/-}$ mice was of marginal statistical significance ($P = 0.07$; Table 3).

Comparative Ovarian Pathology among Wild-Type and BALB/$c^{B1+/-}$ and $129^{B2+/-}$ Mice. The ovarian pathology from DES-treated BALB/$c^{B1+/-}$ mice was compared with that of similarly treated wild-type littermates. In general, a similar spectrum of ovarian pathologies was observed in DES-treated BALB/$c^{B1+/+}$ and BALB/$c^{B1+/-}$ mice, but the distribution between genotypic classes was distinct. Seven of 13 (59%) DES-treated BALB/$c^{B1+/-}$ mice were diagnosed with ovarian atrophy, characterized by loss of follicles, a paucity of CL, and increased interstitial tissue, as compared with only 1 of 11 (9%) wild-type mice ($P = 0.03$; Fig. 4). Follicular arrest was observed in 10 of 11 (91%) DES-treated BALB/$c^{B1+/+}$ females, respectively, as compared with 6 of 13 (46%) BALB/$c^{B1+/-}$ mice ($P = 0.03$; Fig. 4). Six of 13 (46%) BALB/$c^{B1+/-}$ mice examined developed follicular cysts as compared with only 1 of 11 (9%) wild-type animals ($P = 0.06$). In addition, five BALB/$c^{B1+/-}$ females were diagnosed with ovarian inflammation.

The ovaries of DES-treated $129^{B2+/-}$ and wild-type littermates were also compared. Some pathology observed in the DES-treated $129^{B2+/+}$ and $129^{B2+/-}$ mice was similar to that described for the BALB/$c^{B1+/-}$ mice and their wild-type littermates. Eight of 10 (80%) DES-treated $129^{B2+/+}$ mice and 5 of 10 (50%) $129^{B2+/-}$ mice had ovaries characterized by arrested follicular development ($P = 0.18$; Fig. 4). Twenty percent of the animals from each genotypic class developed follicular cysts. There was one $129^{B2+/+}$ female diagnosed with ovarian atrophy, and neither $129^{B2+/+}$ nor $129^{B2+/-}$ mice displayed ovarian inflammation.

C3H mouse ovaries were also evaluated for pathology. Nine of 12 (75%) DES-exposed C3H mice had ovaries that displayed arrested follicular development at 56 weeks of age. Of the nine mice with arrested follicular development, two mice (15%) and one mouse (8%) arrested with antral and leutenized follicles, respectively, and the six remaining females arrested with small to mid-sized follicles. These results are consistent with the absence of CL reported for 96% of C3H mice in a lifetime DES treatment study (45).

Discussion

BALB/$c^{B1+/-}$ and $129^{B2+/-}$ mice developed distinct reproductive tract and mammary gland pathology from DES treatment that was influenced by both genetic background and the inherited *Brca1* or *Brca2* alteration. Apparent differences in the ductal branching phenotype between the $129^{B2+/-}$ and $129^{B2+/+}$ genotypic classes were observed in the mammary gland whole mounts. Hemizygous *Brca2* expression resulted in an apparent decrease in the ability of the mammary ductal epithelium to proliferate and densely fill out the mammary fat pad compared with mice with two functional copies of the gene. Treatment of the mice with DES resulted in an exaggeration of this subtle difference. This observation is likely to be biologically relevant and is consistent with reports describing that homozygous deletion of *Brca1* in the mouse mammary gland results in inhibited ductal morphogenesis (53, 54). We predict that homozygous disruption of *Brca2* in the mouse mammary gland, as done for *Brca1*, will result in a more dramatic inhibition of ductal branching than was observed in the *Brca2* hemizygous mice. A phenotypic consequence of hemizygous gene expression is compatible with reports that *Brca1* and *Brca2* are highly expressed in the mouse mammary gland during ductal morphogenesis, are associated with proliferation (19, 55, 56), and are implicated in mitotic and meiotic DNA repair processes required for genomic stability (11, 57, 58) and with a proposal that *BRCA1* and *BRCA2* are critical for normal growth control in the human breast (59).

Untreated virgin BALB/$c^{B1+/+}$ and BALB/$c^{B1+/-}$ mice had essentially identical patterns of ductal morphogenesis at 6 months of age but responded quite differently to the chronic DES treatment. DES induced proliferation of the mammary ducts in both genotypic classes, but the overall response was significantly less dramatic in BALB/$c^{B1+/-}$ mice than in their wild-type littermates. Specifically, differences were observed in the branching pattern of mammary ductal

epithelium in BALB/$c^{B1+/-}$ mice treated with DES. We hypothesize that the inhibited ductal development in the BALB/$c^{B1+/-}$ and $129^{B2+/-}$ mice may result in increased susceptibility to mammary tumor formation either at later time points or in combination with additional carcinogenic exposures or genetic alterations. It has been suggested that a less complex ductal branching structure and the large population of terminal ductal lobule units in the nulliparous human breast correlate with increased susceptibility to carcinogenic insults (60). In addition, mammary ductal branching in women with family histories of breast cancer has been described to be inhibited and immature compared with that of women who do not have a family history of breast cancer (60). The correlation between morphology and susceptibility is consistent with the observation that the terminal ductal lobule units in the human are the predominant site of tumor development in the breast (61).

The treatment of BALB/$c^{B1+/-}$ and $129^{B2+/-}$ mice with DES resulted in a less complex ductal phenotype compared with wild-type littermates at 6 months. Although it is not clear from these experiments whether ductal morphogenesis in the DES-treated BALB/$c^{B1+/-}$ and $129^{B2+/-}$ mice is delayed or permanently inhibited compared with their wild-type littermates, it is possible that this less complex ductal branching pattern provides a prolonged window of susceptibility to DNA-damaging agents. The severe inhibition of ductal phenotypes observed by Xu *et al.* (53) in mice with conditional deletions of *Brca1* in the mammary gland correlated well with subsequent tumor formation. Complete inactivation of *Brca1* contributed to genomic instability in the mammary gland, resulting in tumor formation (53). In our case, it is possible that reduced levels of *Brca1* or *Brca2* gene product in the mammary gland contribute to genomic instability and result in the inhibition of complex ductal branching in the mammary glands of the BALB/$c^{B1+/-}$ and $129^{B2+/-}$ mice. Taken together, one could speculate that the morphology of the mammary ductal epithelium might serve as an early biological marker for cancer susceptibility. Whether or not this is significant for women who have inherited mutations in the *BRCA1* or *BRCA2* genes and were given DES during pregnancy or gestation is unknown and deserves further investigation.

The mammary glands from the DES-treated animals, in particular, the BALB/c mice, displayed dilated ductal epithelium and galactoceles. Ductal ectasia, dilation, and dysplasia have been reported in BALB/c mice treated with progesterone neonatally for 5 days, beginning at 36 h after birth (62). The galactoceles and extent of differentiation are characteristic of a prolactin-stimulated state, and prolactin is known to contribute to mammary tumorigenesis in the mouse (63). Increased levels of prolactin have previously been implicated in the pathogenesis of preneoplastic, nonneoplastic, and neoplastic mammary gland lesions in C3H/HeN$^{(MMTV+)}$ mice chronically treated with DES in feed at concentrations at or below those used in the current study, although serum levels were not measured in either study (64). All doses of DES that influenced nonneoplastic mammary changes also increased mammary gland tumorigenesis (64).

The chronic dietary treatment of virgin female mice with DES for 26 weeks did not result in mammary tumor development in the BALB/$c^{B1+/-}$ and $129^{B2+/-}$ mice or in their wild-type littermates. Consequently, these animals may not be useful as a rapid model system for the testing of putative endocrine-disrupting carcinogens by the NTP with an end point of solid tumor development. Future long-term studies will address the possibility that the early nonneoplastic changes described in the current study may indeed represent a biomarker for neoplastic development. In addition, the influence of hormonal stimulation, which causes nonneoplastic phenotypes in target tissues, on tumor development will be considered.

Brca1 and *Brca2* have been classified as tumor suppressor genes, yet it is unlikely that their inactivation alone is sufficient for tumorigenesis. Instead of acting as "gatekeepers" by regulating cellular proliferation, it is more likely that Brca1 and Brca2 function in a "caretaker" role by maintaining genomic stability (65). Evidence to support this model comes from both human studies and experiments using mice as models (52, 66, 67). *P53* mutations are commonly found in breast tumors from women who have inherited *BRCA1* or *BRCA2* gene alterations (68). BALB/$c^{B1+/-}$ mice crossed onto a *p53*-deficient background developed a few mammary tumors after high-dose radiation exposure (67). Conditional targeting of a *Brca1* mutation to mouse mammary epithelial cells in combination with a *p53* mutation resulted in 73% of the females developing tumors by 8 months of age (53). If *Brca1* and *Brca2* heterozygous mutant mice had functionally inactivated the second allele of the *Brca1* or *Brca2* gene as a consequence of DES treatment, one might predict the appearance of mammary tumors at time points later than 6 months of age. Based on the caretaker model, such tumor development would likely be in combination with multiple genetic mutations that relax cell cycle checkpoints and permit cells with DNA damage to survive and proliferate. Similar to BALB/$c^{B1+/-}$ and $129^{B2+/-}$ mice, *p53*-deficient mice receiving DES by s.c. injection do not develop neoplasms in a 6-month time period (48). It is possible that the combined inactivation of *p53* and *Brca1* or *Brca2* in mice would enhance the carcinogenic response to DES.

The spectrum of DES-induced reproductive tract lesions observed in this study is similar to that reported previously (69). The organs that were affected by DES in mice included the ovary, uterus, cervix, vagina, and mammary gland and represent the spectrum observed in humans who took the hormone or were exposed to the drug *in utero* (70). Exposure of adult C3H and C3H/HeN$^{(MMTV+)}$ mice to a range of DES doses in their diet resulted in the inhibition of CL formation in the ovaries (45, 64), which is consistent with our diagnosis of arrested follicular development.

DES had a notable effect on the ovaries of BALB/$c^{B1+/-}$ mice. Whereas DES exposure resulted in a large proportion of BALB/$c^{B1+/+}$ and $129^{B2+/+}$ animals developing hypoplastic ovaries, the modulation of ovarian development by DES treatment was clearly different in the BALB/$c^{B1+/-}$ mice. DES induced atrophy in the ovaries of many heterozygous animals, suggesting that *Brca1* haploinsufficiency may contribute to premature follicular failure in a highly estrogenic environment. However, we cannot exclude the possibility that DES is acting indirectly to modulate endogenous circulating hormones in the BALB/$c^{B1+/-}$ mice. It is also conceivable that the ovaries of BALB/$c^{B1+/-}$ mice had fewer follicles at birth than their BALB/$c^{B1+/+}$ littermates or that hemizygous expression of *Brca1* could affect oocyte proliferation. In humans, the loss of ovarian follicles has been associated with infertility as well as early menopause, which can contribute to osteoporosis and heart disease. Alterations of both the human *BRCA1* and *BRCA2* genes are clearly linked to an increased incidence of ovarian tumors (1), but the occurrence of premature ovarian failure in this population of women has not been reported.

DES has been shown, in various systems, to induce sister chromatid exchange, unscheduled DNA synthesis, chromosomal aberrations, and mitotic spindle disruption and may be able to act as an initiating agent (28). In addition, DES treatment clearly resulted in massive proliferation of the mammary ductal epithelium of exposed animals in this study. If the wild-type allele of the *Brca1* or *Brca2* genes had been mutated, DES may have been an effective promoter of carcinogenesis in the mammary gland. The relevance of these findings to the human population has yet to be determined.

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