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

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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/cBr1+/–/– mice) or a Brca2 genetic alteration on the 129/SvEv genetic background (129Br12+/–/– 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/cBr1+/–/–, 129Br12+/–/–, 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/c and C3H/HeNMMTV+/+ 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/cBr1+/–/– mice compared with similarly treated BALB/cBr1+/–/– littermates. In addition, the majority of BALB/cBr1+/–/– mice had atrophied ovaries, whereas wild-type littermates were largely diagnosed with arrested follicular development. The mammary ductal architecture in untreated 129Br12+/–/– mice revealed a subtle inhibited branching phenotype that was enhanced with DES treatment. However, no significant differences were observed in ovarian pathology between 129Br12+/–/– and 129Br12+/–/– mice. These data suggest that estrogenic compounds may modulate mammary gland or ovarian morphology in BALB/cBr1+/–/– and 129Br12+/–/– 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 DES4 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 growth and proliferation.

4 The abbreviations used are: DES, diethylstilbestrol; NTP, National Toxicology Program; C3H, C3H/HeNMMTV–/–; CL, corpora lutea.

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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 Mullerian 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/c mice that inherit a Brca1 mutation (BALB/cBrca1 neo), 129/SvEv mice heterozygous for a Brca2 mutation (129Brca2 neo), and their respective wild-type littermates, BALB/cBrca1 neo and 129Brca2 neo. 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/cBrca1 neo and 129Brca2 neo 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/cBrca1 neo mice have been described previously (49) and have been maintained by back-crossing to wild-type BALB/c mice (Jackson Laboratories, Bar Harbor, ME). The neo insertion in the BALB/cBrca1 neo 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 (129Brca2 neo) were established in our laboratory by replacing the 3' end of exon 10, intron 9, and the 5' end of exon 11 with a pgkNeo cassette (50). 129Brca2 neo mice are maintained by mating mutation carriers to wild-type 129/SvEv inbred mice (Taconic, Germantown, NY). The 129Brca2 neo 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 129Brca2 neo and 129Brca2 neo mice used in this experiment had an approximate contribution of 6–12% from the 129/Ola strain 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 129Brca1 neo, 129Brca2 neo, BALB/cBrca1 neo, BALB/cBrca2 neo, 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 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). Because the differences between 129Brca1 neo and 129Brca2 neo mice and their wild-type littermates were sacrificed at 6 months of age by CO2 asphyxiation. The C3H mice were sacrificed at 56 weeks of age because this was a point at which approximately 50% of the C3H mice used in a previous study had developed tumors (44). At the time of sacrifice CO2 levels were 0.5% and #4 mammary glands were collected for whole mount analysis (see below), and complete necropsies were performed. Three BALB/cBrca1 neo and two BALB/cBrca2 neo mice treated with DES died before the end of the experiment, and one BALB/cBrca1 neo 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.

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Treatment</th>
<th>n</th>
<th>Mean (SD)</th>
<th>n</th>
<th>Mean (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/cBrca1 neo</td>
<td>Control</td>
<td>5</td>
<td>1.17 (0.47)</td>
<td>15</td>
<td>22.4 (1.36)</td>
</tr>
<tr>
<td>DES</td>
<td>7</td>
<td>1.24 (0.29)</td>
<td>15</td>
<td>21.7 (1.43)</td>
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<tr>
<td>129Brca2 neo</td>
<td>Control</td>
<td>3</td>
<td>1.90 (0.57)</td>
<td>15</td>
<td>19.4 (1.50)</td>
</tr>
<tr>
<td>DES</td>
<td>5</td>
<td>1.90 (0.57)</td>
<td>15</td>
<td>19.4 (1.50)</td>
<td></td>
</tr>
<tr>
<td>C3H</td>
<td>Control</td>
<td>6</td>
<td>0.97 (0.16)</td>
<td>14</td>
<td>30.0 (4.59)</td>
</tr>
<tr>
<td>DES</td>
<td>5</td>
<td>1.60 (0.26)</td>
<td>11</td>
<td>23.7 (1.95)</td>
<td></td>
</tr>
</tbody>
</table>

* Reproductive tract fraction = reproductive tract weight (g)/total body weight (g) at 2 months of age.
* Total body weight in grams at 6 months of age.
* ND, not determined.

1. Cynthia Smith, personal communication.
2. Total body weight in grams at 6 months of age.
3. Reproductive tract fraction (Repro. tract fraction) = reproductive tract weight (g)/total body weight (g) at 2 months of age.

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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 pellets 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 129B2+/− and 129B2−/− 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 and treatments were analyzed by either Wilcoxon’s rank-sum test or the Mann-Whitney U test.

Because the grading among the eight pathologists was in excellent agreement, subsequent grading of mammary gland morphology for untreated BALB/c129+/− and BALB/c129−/− 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 129B2+/− and 129B2−/− 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/c129+/− and BALB/c129−/− genotypic classes were scored independently of the 129B2+/− and 129B2−/− 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 χ2 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 129B2+/− and 129B2−/− 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 129B2+/−, 129B2−/−, BALB/c129+/−, BALB/c129−/−, and C3H mice were evaluated (Fig. 1; Table 2; results for C3H mice are not shown). The DES-treated BALB/c129+/− and BALB/c129−/− uteri were characterized by a pau-
city of endometrial glands and diffuse severe chronic fibrosis (Fig. 1; Table 2). One BALB/c\textsuperscript{B1+/+} mouse developed a cervicovaginal squamous cell carcinoma, and more than half of the BALB/c\textsuperscript{B1+/+} and BALB/c\textsuperscript{B1+/−} mice displayed cystic endometrial hyperplasia (Table 2). The uteri of the DES-treated 129\textsuperscript{B2+/−} and 129\textsuperscript{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\textsuperscript{B2+/−} and one 129\textsuperscript{B2+/+} mouse, and a uterine carcinoma was observed in a 129\textsuperscript{B2+/−} female. Six of 14 (43%) 129\textsuperscript{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\textsuperscript{B2+/+}, 129\textsuperscript{B2+/−}, BALB/c\textsuperscript{B1+/+}, and BALB/c\textsuperscript{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\textsuperscript{B2+/+}, BALB/c\textsuperscript{B1+/+}, and BALB/c\textsuperscript{B1+/−} mice. The ducts of untreated 129\textsuperscript{B2+/−} mice were compared with their wild-type littermates and generally appeared less complex than those of 129\textsuperscript{B2+/+} mice (Fig. 3). The 129\textsuperscript{B2+/−} mammary ducts were elongated with less lateral and side branching and showed decreased alveolar bud formation compared with wild-type ducts. Mammary glands isolated from the heterozygous 129\textsuperscript{B2+/−} mice had ductal branching patterns that ranged from simple to moderately complex. In comparison, 4 of 14 129\textsuperscript{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\textsuperscript{B2+/−} group. Four 129\textsuperscript{B2+/+} mice had ductal branching patterns that were as well developed as those of 129\textsuperscript{B2+/−} mice, with side branching and alveolar buds. Despite subtle differences that appeared to exist between untreated 129\textsuperscript{B2+/−} and 129\textsuperscript{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 Duct 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 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\textsuperscript{B1+/−} mice and their wild-type littermates. Mammary ductal branching in untreated BALB/c\textsuperscript{B1+/+} and BALB/c\textsuperscript{B1+/−} mice was essentially identical between these genotypic classes (Fig. 2). The mammary arborization complexity values for the BALB/c\textsuperscript{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\textsuperscript{B1+/−} mice, respectively (Table 3).

The ducts of untreated 129\textsuperscript{B2+/−} mice were compared with their wild-type littermates and generally appeared less complex than those of 129\textsuperscript{B2+/+} mice (Fig. 3). The 129\textsuperscript{B2+/−} mammary ducts were elongated with less lateral and side branching and showed decreased alveolar bud formation compared with wild-type ducts. Mammary glands isolated from the heterozygous 129\textsuperscript{B2+/−} mice had ductal branching patterns that ranged from simple to moderately complex. In comparison, 4 of 14 129\textsuperscript{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\textsuperscript{B2+/−} group. Four 129\textsuperscript{B2+/+} mice had ductal branching patterns that were as well developed as those of 129\textsuperscript{B2+/−} mice, with side branching and alveolar buds. Despite subtle differences that appeared to exist between untreated 129\textsuperscript{B2+/−} and 129\textsuperscript{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 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

| Reproductive pathology in DES-exposed Brca1- and Brca2-heterozygous and wild-type mice at 6 months of age |
|---------------------------------|-----------------|-----------------|-----------------|-----------------|
| BALB/c\textsuperscript{B1+/+}   | BALB/c\textsuperscript{B1+/−} | 129\textsuperscript{B2+/+} | 129\textsuperscript{B2+/−} |
| Uterus                          |                  |                  |                  |
| Cystic endometrial hyperplasia  | 7 (58%)*         | 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 a            | 12               | 13               | 14              | 14              |
| Vagina/cervix                   |                  |                  |                  |                |
| Inflammation                    | 4 (36%)          | 3 (23%)          |                  | 2 (14%)         |
| Hyperplasia                     |                  | 5 (38%)          | 5 (36%)          | 4 (29%)         |
| Squamous hyperplasia            | 1 (9%)           |                  |                  |                |
| Squamous cell carcinoma         |                  | 1 (7%)           |                  | 2 (14%)         |
| Total observations b            | 11               | 13               | 14              | 15              |

* Percentage of total.

Fisher’s exact test, P = 0.05 versus corresponding wild-type genotype.

Total number of animals for which samples were available.
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 Brca1+/− mice were compared with those in their wild-type littermates. Both the BALB/c Brca1+/− and BALB/c Brca2+/− 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 Brca1+/− and 129 Brca2+/− mice. There was a statistically significant difference in branching phenotype between the BALB/c Brca1+/− and BALB/c Brca2+/− 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 Brca1+/− mice and 5.8 ± 0.60 for BALB/c Brca2+/− mice (P, 0.01).

The DES-treated mammary glands from 129 Brca2+/− and wild-type mice were also examined. In general, 129 Brca2+/− and 129 Brca2+/− 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

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>No. of mice</th>
<th>Treatment</th>
<th>Arborization complexitya</th>
</tr>
</thead>
<tbody>
<tr>
<td>BALB/c Brca1+/−</td>
<td>15</td>
<td>Control</td>
<td>2.4 (0.91)</td>
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<tr>
<td>BALB/c Brca1+−</td>
<td>14</td>
<td>Control</td>
<td>2.4 (0.93)</td>
</tr>
<tr>
<td>BALB/c Brca1+/−</td>
<td>10</td>
<td>DES</td>
<td>6.7 (0.48)</td>
</tr>
<tr>
<td>BALB/c Brca1+/−</td>
<td>13</td>
<td>DES</td>
<td>5.8 (0.60)</td>
</tr>
<tr>
<td>129 Brca2+/−</td>
<td>14</td>
<td>Control</td>
<td>2.9 (0.69)</td>
</tr>
<tr>
<td>129 Brca2+/−</td>
<td>14</td>
<td>Control</td>
<td>2.5 (0.87)</td>
</tr>
<tr>
<td>129 Brca2+/−</td>
<td>15</td>
<td>DES</td>
<td>6.9 (0.88)</td>
</tr>
<tr>
<td>129 Brca2+/−</td>
<td>13</td>
<td>DES</td>
<td>6.4 (0.65)</td>
</tr>
</tbody>
</table>

a Values are mean (SD).

Two-tailed Mann-Whitney U test P < 0.01 versus the corresponding wild-type genotype.

Two-tailed Mann-Whitney U test P = 0.07 versus the corresponding wild-type genotype.
DES-treated 129\textsuperscript{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\textsuperscript{B2/+} and 129\textsuperscript{B2/-} mice was of marginal statistical significance \( (P = 0.07); \) Table 3).

**Comparative Ovarian Pathology among Wild-Type and BALB/\textit{c}\textsuperscript{B1/+} and 129\textsuperscript{B2/-} Mice.** The ovarian pathology from DES-treated BALB/\textit{c}\textsuperscript{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/\textit{c}\textsuperscript{B1/+} and BALB/\textit{c}\textsuperscript{B1/-} mice, but the distribution between genotypic classes was distinct. Seven of 13 (59\%) DES-treated BALB/\textit{c}\textsuperscript{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/\textit{c}\textsuperscript{B1/+} females, respectively, as compared with 6 of 13 (46\%) BALB/\textit{c}\textsuperscript{B1/-} mice \( (P = 0.03); \) Fig. 4). Six of 13 (46\%) BALB/\textit{c}\textsuperscript{B1/-} mice examined developed follicular cysts as compared with only 1 of 11 (9\%) wild-type animals \( (P = 0.06). \) In addition, five BALB/\textit{c}\textsuperscript{B1/-} females were diagnosed with ovarian inflammation.

The ovaries of DES-treated 129\textsuperscript{B2/+} and wild-type littermates were also compared. Some pathology observed in the DES-treated 129\textsuperscript{B2/+} and 129\textsuperscript{B2/-} mice was similar to that described for the BALB/\textit{c}\textsuperscript{B1/-} mice and their wild-type littermates. Eight of 10 (80\%) DES-treated 129\textsuperscript{B2/+} mice and 5 of 10 (50\%) 129\textsuperscript{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\textsuperscript{B2/+} female diagnosed with ovarian atrophy, and neither 129\textsuperscript{B2/+} nor 129\textsuperscript{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/\textit{c}\textsuperscript{B1/+} and 129\textsuperscript{B2/-} mice developed distinct reproductive tract and mammary gland pathology from DES treatment that was influenced by both genetic background and the inherited \textit{Brca1} or \textit{Brca2} alteration. Apparent differences in the ductal branching phenotype between the 129\textsuperscript{B2/+} and 129\textsuperscript{B2/-} genotypic classes were observed in the mammary gland whole mounts. Hemizygous \textit{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 \textit{Brca1} in the mouse mammary gland results in inhibited ductal morphogenesis (53, 54). We predict that homozygous disruption of \textit{Brca2} in the mouse mammary gland, as done for \textit{Brca1}, will result in a more dramatic inhibition of ductal branching than was observed in the \textit{Brca2} hemizygous mice. A phenotypic consequence of hemizygous gene expression is compatible with reports that \textit{Brca1} and \textit{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 \textit{BRCA1} and \textit{BRCA2} are critical for normal growth control in the human breast (59).

Untreated virgin BALB/\textit{c}\textsuperscript{B1/+} and BALB/\textit{c}\textsuperscript{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/\textit{c}\textsuperscript{B1/+} mice than in their wild-type littermates. Specifically, differences were observed in the branching pattern of mammary ductal...
epithelium in BALB/c<sup>B1</sup>+/- mice treated with DES. We hypothesize that the inhibited ductal development in the BALB/c<sup>B1</sup>+/- and 129<sup>B2</sup>+/- 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<sup>B1</sup>+/- and 129<sup>B2</sup>+/- 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<sup>B1</sup>+/- and 129<sup>B2</sup>+/- 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<sup>B1</sup>+/- and 129<sup>B2</sup>+/- 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<sup>MMTV+/-</sup> 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<sup>B1</sup>+/- and 129<sup>B2</sup>+/- 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<sup>B1</sup>+/- 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<sup>B1</sup>+/- and 129<sup>B2</sup>+/- 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<sup>MMTV+/-</sup> 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<sup>B1</sup>+/- mice. Whereas DES exposure resulted in a large proportion of BALB/c<sup>B1</sup>+/- and 129<sup>B2</sup>+/- animals developing hypoplastic ovaries, the modulation of ovarian development by DES treatment was clearly different in the BALB/c<sup>B1</sup>+/- 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<sup>B1</sup>+/- mice. It is also conceivable that the ovaries of BALB/c<sup>B1</sup>+/- mice had fewer follicles at birth than their BALB/c<sup>B1</sup>+/- 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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