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13. ABSTRACT ***(Maximum 200 Words)***

Germline mutations in *BRCA1* account for about 45% of all hereditary cases of breast cancer. However, the role of BRCA1 in mammary tumorigenesis is not established. My proposal outlines two approaches to extend our knowledge of BRCA1 function in normal development and malignant transformation. First, we used the *cre-lox* system to generate a mouse model in which loss of BRCA1 is limited to mammary epithelium. Four different mouse lines expressing the Cre recombinase in mammary epithelium were generated, and we are currently working towards generating a *lox P-Brca1-lox P* mouse. Secondly, we examined synergistic effects between BRCA1 and p53 in tumorigenesis. We found that survival of both $p53^{-/-}$ and $p53^{+/-}$ mice is not altered by BRCA1-deficiency. This suggests that BRCA1 does not play a role in the formation of most tumors. Similarly, the survival of the $p53^{+/-}$ mice after exposure to gamma-irradiation was unaltered by BRCA1-deficiency on a mixed genetic background. However, gamma-irradiated $p53^{+/-}$ mice on a BALB/c inbred genetic background showed a significant increase in mammary tumorigenesis as compared to mice on either a DBA/2J or mixed background. These results suggest that genetic factors in BALB/c mice along with environmental stimulus, like ionizing irradiation, play a role in mammary epithelial transformation, in particular, when cells are hemizygous for loss of *p53*.

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Michael G. Backlund 7/13/00

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INTRODUCTION

Breast cancer is a common and important disease affecting women. It is estimated that one in eight American women who reach age 95 will develop breast cancer. An inherited component to breast cancer has long been suspected. It is now estimated that genetic factors contribute to about 5% of all cases of breast cancer and approximately 25% of cases diagnosed before age 30 [1]. Mutation of one gene, *BRCA1*, is thought to account for approximately 45% of families with significantly high breast cancer incidence and at least 80% of families with increased incidence of both early-onset breast cancer and ovarian cancer [2-4].

To understand how the inheritance of a mutant allele of *BRCA1* results in a predisposition to breast and ovarian cancer, a mouse model was generated in our laboratory by introducing a mutation in the murine homologue to *BRCA1* [5]. The generation of these mice would also allow us to examine the role of BRCA1 in normal tissues. The $Brca1^{+/-}$ mice were found to be fertile and developmentally normal. Interestingly, unlike women with a single germline mutation in *BRCA1*, $Brca1^{+/-}$ mice did not have an increased risk of tumor formation of any type. Litters produced by mating these animals did not contain mice homozygous for the mutated allele ($Brca1^{-/-}$ mice). Analysis revealed that $Brca1^{-/-}$ embryos were resorbed between days 10 and 13 due to neural tube defects. While this discovery revealed an as yet unknown crucial function of BRCA1, it limited our ability to use this model to understand the function of BRCA1 in normal growth and development of mammary tissue.

In my proposal, I outlined two approaches to study the role of BRCA1 in mammary tumorigenesis. In the first technical aim, we proposed the generation of mice in which loss of BRCA1 is limited to the mammary gland epithelial cells. It was anticipated that this approach would circumvent the embryonic lethality associated with the loss of BRCA1 function and thereby allow us to examine the role of BRCA1 in the mammary epithelium. To accomplish this aim, a *cre-lox* targeting system was used to produce mammary-specific BRCA1-deficiency. As stated in the 1998 annual report, I had generated eight MMTV-*cre* transgenic lines, and identified four lines which express the Cre recombinase in the mammary epithelium of pregnant females. I am currently using a transgenic mouse line which contains the β-galactosidase gene (*lacZ*) as marker for Cre expression to determine the variegation of Cre expression in the mammary epithelium in these four lines.

In the second technical aim, I proposed to examine the possible cooperative effects between BRCA1 and another tumor suppressor, p53. By combining *Brca1* mutations with *p53* mutations, we hoped to initiate tumorigenesis in organs in which malignancy was absent in $Brca1^{+/-}$ mice[6,7]. Both p53 and BRCA1 are each thought to play a significant role in the progression of tumorigenesis in human mammary tissue. In this objective we were testing the hypothesis that BRCA1 and p53 display a synergistic interaction in promoting tumor formation, particularly in mammary epithelium. As of the 1998 annual report, each of the proposed experiments in the second technical aim had been accomplished, the results of which were published in *Cell Growth and Differentiation* in January 1999 (see Appendix D). Briefly, our studies revealed that the overall survival of both $p53^{-/-}$ and $p53^{+/-}$ mice was not altered by the presence of a mutant *Brca1* allele. Although no major changes in the tumor type were noted in either the $Brca1^{+/-}p53^{-/-}$ or $Brca1^{+/-}p53^{+/-}$ populations, four mammary tumors were seen in the $Brca1^{+/-}p53^{-/-}$ group while only one such tumor was seen among the control $p53^{-/-}$ group. We also found that neither the overall survival rate nor the overall tumor spectrum of $p53^{+/-}$ mice after exposure to gamma radiation was altered by the presence of a mutant *Brca1* allele.

However while none of the 14 *irradiated p53*$^{+/-}$ mice developed mammary tumors, five of the 23 tumors isolated from the BRCA1-deficient mice were of mammary epithelial origin and of these three had lost expression of the wild type *Brca1* gene. Although the number of mammary tumors obtained was small, these results are suggestive of a role for BRCA1 in mammary tumor formation after exposure to specific DNA damaging agents. The generation of the mice described in this study required extensive breeding. Surprisingly, during the course of these experiments, three *Brca1*$^{-/-}$*p53*$^{-/-}$ mice were generated which survived to adulthood. At that time, no group had reported being able to generate *Brca1*$^{-/-}$ mice which survived to birth. The survival of these mice allowed us to study the effects of the loss of BRCA1 on normal growth and development. In addition, we generated *Brca1*$^{-/-}$*p53*$^{-/-}$ skin fibroblasts from each of these three mice. Prior attempts to generate *Brca1*$^{-/-}$ embryonic fibroblasts had been unsuccessful due to the early embryonic lethality seen with loss of BRCA1. These cell lines therefore provided us the unique opportunity to examine the normal cellular role of BRCA1. This work has been published in *Molecular and Cellular Biology* in October 1999 (see Appendix E).

Additionally, we examined whether environmental factors, specifically ionizing radiation, in conjunction with genetic background alter the incidence of mammary tumors in mice deficient in either p53 and/or BRCA1. Our observation that a small percentage of mice deficient in both p53 and BRCA1 develop mammary gland tumors after exposure to ionizing radiation supports the hypothesis that environmental and genetic factors are required for mammary tumor formation. Although a systematic analysis has not been carried out, examination of mammary tumorigenesis in DBA/2J and BALB/c congenic mice heterozygous for *Brca1*or *p53* or homozygous for *p53* mutations suggests that these strains do not carry modifying alleles that alter their risk for development of mammary tumors. However, when exposed to ionizing radiation, a high percentage of BALB/c mice heterozygous for the mutant *p53* allele developed mammary tumors. A similar increase in formation of mammary tumors after exposure to gamma irradiation was not observed in the DBA/2J or mice of mixed genetic background. This unpublished observation suggests that the mammary epithelia of BALB/c mice is susceptible to DNA damage caused by ionizing radiation, in particular, when cells are hemizygous for loss of *p53*.

In this review, I summarize the results I have obtained during the course of this grant. A discussion of these results will complete my review. I have subdivided each section into the two technical objectives I originally proposed: (1) Tissue-specific inactivation of BRCA1 and (2) Interactions with tumor suppressor genes.

RESULTS

Technical Objective 1: Tissue-specific Inactivation of BRCA1.

Generation of an MMTV-*cre* mouse. Both MMTV-*cre* constructs were electroporated into ES cells and two positive clones from each construct was injected into C57BL/6 blastocysts. In addition, through pronuclear injection of the MMTV-*cre* transgene into fertilized oocytes, six mice carrying the MMTV-*cre* transgene were generated.

Generation of a *lox-Brca1-lox* mouse. Following the identification and cloning of exons 11 and 12 of *Brca1*, a construct was designed with *lox* sites surrounding exon 11 of *Brca1*. As described in the experimental methods section, an *Hprt* gene was also inserted into intron 10. *Lox* sites were also introduced into the construct to flank the *Hprt* gene. Following electroporation of this construct and HPRT selection, positive clones were identified. Transient expression of the Cre recombinase was accomplished by electroporation into the positive clones

to generate cells which had deleted the *Hprt* gene through Cre-mediated recombination, but had left exon 11 of *Brca1* intact. Despite multiple attempts, selective Cre-mediated recombination could not be achieved. In each case, both the *Hprt* gene and exon 11 of *Brca1* were deleted.
Localization of Cre expression. MMTV-*cre* transgenic mice were bred to mice containing the targeted *Nr1* allele flanked by *lox* sites. The resulting MMTV-*cre*(+), *lox-Nr1-lox*(+) females from each MMTV-*cre* transgenic line were euthanized as virgin females or pregnant females. DNA was generated from the mammary tissue, salivary gland, spleen, liver, and kidney. PCR analysis revealed that in four of the transgenic lines, Cre was expressed in the mammary tissue of pregnant females. However, in each of the transgenic lines, Cre expression was seen in some but not all of the pregnant females. In addition, Cre expression was not seen in the mammary tissue of virgin females in any of the lines. The four MMTV-*cre* lines which expressed the Cre recombinase in mammary tissue are currently being bred to the *lox-lacZ-lox* mice to localize expression of Cre in the mammary gland.

While we are continuing to pursue this objective, the generation of three *Brca1*$^{-/-}$*p53*$^{-/-}$ mice in our colony provided us the unique opportunity to study the effects of the loss of BRCA1 in overall murine development. As these mice were BRCA1-deficient in all organs and cells, it allowed us to go beyond the effects of BRCA1-deficiency on mammary development and tumorigenesis and study the effect on development of all organs of the mouse. We have therefore focused our efforts this year on the characterization of these mice (see Technical Objective 2), and have specifically examined mammary gland development in these mice.

Technical Objective 2: Interactions of Tumor Suppressor Genes

Characterization of *Brca1*$^{-/-}$*p53*$^{-/-}$ mice. As previously reported, no *Brca1*$^{-/-}$*p53*$^{-/-}$ mice were identified on examination of 56 offspring obtained from 9 litters. Further expansion of this population, however, led to the identification of two *Brca1*$^{-/-}$*p53*$^{-/-}$ males and one *Brca1*$^{-/-}$*p53*$^{-/-}$ female. All three *Brca1*$^{-/-}$*p53*$^{-/-}$ mice were severely growth retarded in comparison to age-matched and sex-matched *p53*$^{-/-}$ controls. Examination of the kidney, liver, brain, lungs, pancreas, and gastrointestinal tract did not reveal gross or histological abnormalities. Interestingly, the affected organs included the testes and the epithelia of the parotid, mammary, and prostate glands. The acinar units of the parotid gland were notable affected, displaying diffuse atrophy, and throughout the acinar epithelia, cytomegaly and karyomegaly were also observed. Interestinly, karyomegaly was observed in the prostate and mammary gland as well. Whole mount analysis of the mammary glands of the *Brca1*$^{-/-}$*p53*$^{-/-}$ mouse displayed a reduction of end bud formation and secondary branching as compared to the mammary glands of *p53*$^{-/-}$ and wild-type controls.

Spermatogenesis was also severely affected by the loss of BRCA1. The testes of both *Brca1*$^{-/-}$*p53*$^{-/-}$ mice were smaller in size than those of *p53*$^{-/-}$ and wild-type controls. While Sertoli cells and spermatagonia could be detected upon histological examination of the testes of the *Brca1*$^{-/-}$*p53*$^{-/-}$ mice, both spermatids and spermatogonia were completely absent. Only two spermatocytes were observed on survey of the entire section. To further define the stage at which spermatogenesis is halted, the sections were stained for HSP70-2 whose expression is initiated as cells enter meiosis. A marked reduction in the number of spermatocytes was seen in the testes of the *Brca1*$^{-/-}$*p53*$^{-/-}$ males, further supporting the hypothesis that BRCA1 plays a crucial role in the meiotic stage of spermatogenesis.

All three mice died of lymphomas, the most common tumor seen in the p53-/- populations. In addition, a hemangiosarcoma was found in one of the *Brca1*$^{-/-}$*p53*$^{-/-}$ mice. Cell lines were generated from each of these tumors.

Generation and characterization of *Brca1*$^{-/-}$*p53*$^{-/-}$ fibroblast lines. The generation of cell lines deficient in tumor suppressors has been instrumental in understanding the function of these proteins. However, a similar approach to address the function of BRCA1 has not been possible due to the difficulty in generating *BRCA1*$^{-/-}$ (human) or *Brca1*$^{-/-}$ (murine) lines. The survival of three *Brca1*$^{-/-}$*p53*$^{-/-}$ mice allowed us the unique opportunity to generate and study fibroblasts lines homozygous for the mutant *Brca1* allele. Because these fibroblasts are also deficient in p53, which itself will have a major impact on cell growth and senescence, I isolated two *p53*$^{-/-}$ primary fibroblast lines, as well as a *Brca1*$^{+/+}$*p53*$^{+/+}$ line to use as controls.

We first focused on the normal growth of two of the *Brca1*$^{-/-}$*p53*$^{-/-}$ fibroblast lines. Interestingly, the proliferative rate of the *Brca1*$^{-/-}$*p53*$^{-/-}$ fibroblasts was decreased compared to the *p53*$^{-/-}$ fibroblast controls, and was similar to that of wild-type cells. This data suggests that loss of BRCA1 alters the growth advantage that a *p53* mutation confers on fibroblasts. Previous reports have shown that an increase in the expression of p21 may explain the growth retardation seen in the *Brca1*$^{-/-}$*p53*$^{-/-}$ embryos. However, p21 levels were not elevated in the *Brca1*$^{-/-}$*p53*$^{-/-}$ fibroblasts, suggesting that the reduced growth rate of the *Brca1*$^{-/-}$*p53*$^{-/-}$ fibroblasts could not be explained by p21-mediated cellular senescence. Interestingly, with increasing passages, the growth rate and saturation density of the *Brca1*$^{-/-}$*p53*$^{-/-}$ fibroblasts increased.

To determine if the loss of BRCA1 resulted in a reduction in the rate of passage through the cell cycle, asynchronous early passage fibroblasts were subjected to DNA content analysis, using BRDU incorporation and propidium iodide staining. Surprisingly, the percentage of *Brca1*$^{-/-}$*p53*$^{-/-}$ cells in S phase was similar to that of the *p53*$^{-/-}$ controls, indicating that the *Brca1*$^{-/-}$ *p53*$^{-/-}$ fibroblasts are cycling as quickly as the *p53*$^{-/-}$ controls. Both populations therefore have a greater number of cells in S phase than the wild-type fibroblasts. This apparent inconsistency was resolved following an analysis of cellular death. A higher rate of cellular death was detected in the *Brca1*$^{-/-}$*p53*$^{-/-}$ fibroblasts than in the *p53*$^{-/-}$ controls. Multiple assays were used to confirm this increase in cellular death.

A number of lines of evidence support a role for BRCA1 in the maintenance of genome integrity [8,9]. If increased cellular death in the *Brca1*$^{-/-}$*p53*$^{-/-}$ lines is directly related to the inability of these cells to repair DNA damage, it would be expected that the growth of these cells would be further compromised by exposure to DNA damaging agents. We therefore examined the survival rate of the *Brca1*$^{-/-}$*p53*$^{-/-}$ fibroblasts and compared them to *p53*$^{-/-}$ control lines following treatment with ionizing radiation, hydrogen peroxide, or ultraviolet light. A decrease in cellular survival was seen in cells of both genotypes following treatment with these DNA damaging agents. However, the decrease in survival was significantly greater in the *Brca1*$^{-/-}$*p53*$^{-/-}$ fibroblasts as compared to the *p53*$^{-/-}$ controls following treatment with any of the three DNA damaging agents.

It has been previously shown that the increased sensitivity of the *Brca1*$^{-/-}$ embryonic stem (ES) cell line to ionizing radiation and hydrogen peroxide was in part due to a defect in transcription coupled repair (TCR). However, as this BRCA1-deficient cell line was a single isolate and no additional lines have been identified in numerous additional experiments, it is possible that this phenotype is not directly related to the loss of BRCA1, but rather to the accumulation of other mutations in the ES cell line. We therefore examined the ability of the

Brca1$^{-/-}$*p53*$^{-/-}$ fibroblasts to utilize the transcription coupled repair pathway. Following treatment with ionizing radiation, hydrogen peroxide, or ultraviolet light, TCR was examined in two *Brca1*$^{-/-}$*p53*$^{-/-}$ lines and two *p53*$^{-/-}$ control lines. A defect in TCR was detected in both *Brca1*$^{-/-}$*p53*$^{-/-}$ fibroblast lines following exposure to ionizing radiation and hydrogen peroxide, but not ultraviolet light.

Characterization of *p53*$^{-/-}$ mice. *p53*$^{+/-}$ heterozygotes on a 129/Ola, C57BL/6, and DBA/2 mixed genetic background were backcrossed onto both the BALB/c and DBA/2J inbred backgrounds to obtain animals both wild type and nullizygous for *p53*. As previously reported, *p53*$^{-/-}$ female mice on either background were present in a lower frequency than *p53*$^{-/-}$ males[18-19]. Fifty-three *p53*$^{-/-}$ and 52 *p53*$^{+/+}$ BALB/c along with 46 *p53*$^{-/-}$ and 52 *p53*$^{+/+}$ DBA/2J female animals were monitored biweekly for the development of tumors and sacrificed when animals displayed visible tumors or appeared moribund. The survival curve data in Fig.1A show that the latency to tumor formation was similar for both BALB/c and DBA/2J *p53* nullizygous animals through three generations of backcrossing (Logrank test, χ^2 =1.407, P = 0.24). By 119 days of age, around 50% of p53-deficient BALB/c animals had been sacrificed because of tumor formation, as compared to 136 days of age for p53-deficient DBA/2J animals. In contrast the data in Fig. 1B show that the latency to tumor formation was significantly different between BALB/c and DBA/2J p53 nullizygous animals from backcross four to six (Logrank test, χ^2 =7.680, P =0.0056). The median survival age for p53-deficient BALB/c animals occurred at 109 days; however, the median survival age for p53-deficient DBA/2J animals was 154 days.

Tumors arising in both wild type and p53-nullizygous mice were classified both by observation of anatomical location and histological classification by a trained pathologist. Tumor analysis indicated that the tumor spectra arising in *p53*$^{-/-}$ mice did not display significant diversity between the BALB/c and DBA/2J inbred strains (Table 1). As previously reported, *p53*$^{-/-}$ mice display a high frequency of lymphomas, while only displaying a small frequency of mammary tumors[20-21]. *p53* homozygotes on either inbred strain did not alter the prevalence of lymphomas, and additional histological examination of the liver, spleen, and kidney from these mice did not reveal distinct differences in metastases to other organ systems. Additionally, neither the BALB/c nor DBA/2J genetic background in *p53*$^{-/-}$ animals altered the frequency of mammary tumors seen from that previously reported.

Characterization of gamma-irradiated *p53*$^{+/-}$ mice. Consistent with published work[20-21], our observations show that *p53*$^{-/-}$ mice display a high incidence of lymphomas, in particular thymic lymphomas, thus potentially hindering our ability to observe the role of p53 in mammary tumor formation. As *p53* heterozygous animals show an increased latency to tumorigenesis[22], we examined the role of how environmental agents together with mutations in known tumor suppressor genes effect tumor latency and spectra in BALB/c and DBA/2J inbred strains. Forty-seven *p53*$^{+/-}$ and 52 *p53*$^{+/+}$ BALB/c along with 55 *p53*$^{+/-}$ and 55 *p53*$^{+/+}$ DBA/2J female animals between 4 and 6 weeks of age were exposed to a single dose of whole body γ-irradiation. Mice were observed biweekly, sacrificed when moribund, and necropsied. Compared with our previous report of unirradiated *p53*$^{+/-}$ mice[23], the age of median tumor incidence and morbidity decreased in both *p53*$^{+/-}$ irradiated BALB/c and DBA/2J inbred strains (backcrosses 1 through 3) from 500 days to 236 and 200 days, respectively (Fig. 1C). Comparison of tumor latency between irradiated *p53*$^{+/-}$ BALB/c and DBA/2J animals at later backcrosses (4-6) shows an

additional decrease of the age of median tumor incidence and morbidity to 207 and 184 days, respectively (Fig. 1D).

Tumors arising from mice heterozygous and wild type for *p53* were classified both by observation of anatomical location and by histological examination of tumor biopsies by a trained veterinary pathologist. Analytical comparison of the different genetic cohorts indicate that distribution of mammary carcinomas arising from BALB/c mice carrying a *p53* mutation differed significantly from those observed in either $p53^{+/-}$ mice on the mixed or DBA/2J background (Table 2, Fig. 3, χ^2= 10.68, *P*= 0.0048). In contrast to previous reports of $p53^{+/-}$ mice on a mixed genetic background, *p53* heterozygotes on a BALB/c background display an increased incidence of mammary carcinomas[24].

Characterization of $p53^{-/-}$ mice. $p53^{+/-}$ heterozygotes on a 129/Ola, C57BL/6, and DBA/2 mixed genetic background were backcrossed onto both the BALB/c and DBA/2J inbred backgrounds to obtain animals both wild type and nullizygous for *p53*. As previously reported, $p53^{-/-}$ female mice on either background were present in a lower frequency than $p53^{-/-}$ males[18-19]. Fifty-three $p53^{-/-}$ and 52 $p53^{+/+}$ BALB/c along with 46 $p53^{-/-}$ and 52 $p53^{+/+}$ DBA/2J female animals were monitored biweekly for the development of tumors and sacrificed when animals displayed visible tumors or appeared moribund. The survival curve data in Fig.1C show that the latency to tumor formation was similar for both BALB/c and DBA/2J *p53* nullizygous animals through three generations of backcrossing (Logrank test, χ^2 =1.407, *P* = 0.24). By 119 days of age, around 50% of p53-deficient BALB/c animals had been sacrificed because of tumor formation, as compared to 136 days of age for p53-deficient DBA/2J animals. In contrast the data in Fig. 1D show that the latency to tumor formation was significantly different between BALB/c and DBA/2J p53 nullizygous animals from backcross four to six (Logrank test, χ^2 =7.680, *P* =0.0056). The median survival age for p53-deficient BALB/c animals occurred at 109 days; however, the median survival age for p53-deficient DBA/2J animals was 154 days.

Tumors arising in both wild type and p53-nullizygous mice were classified both by observation of anatomical location and histological classification by a trained pathologist. Tumor analysis indicated that the tumor spectra arising in $p53^{-/-}$ mice did not display significant diversity between the BALB/c and DBA/2J inbred strains (Table 2). As previously reported, $p53^{-/-}$ mice display a high frequency of lymphomas, while only displaying a small frequency of mammary tumors[20-21]. *p53* homozygotes on either inbred strain did not alter the prevalence of lymphomas, and additional histological examination of the liver, spleen, and kidney from these mice did not reveal distinct differences in metastases to other organ systems. Additionally, neither the BALB/c nor DBA/2J genetic background in $p53^{-/-}$ animals altered the frequency of mammary tumors seen from that previously reported.

KEY RESEARCH ACCOMPLISHMENTS

- Generation of three $Brca1^{-/-}p53^{-/-}$ mice
- Histological characterization of $Brca1^{-/-}p53^{-/-}$ mice
- Generation of $Brca1^{-/-}p53^{-/-}$ fibroblasts cell lines (and $p53^{-/-}$ and wild-type lines as controls)
- Analysis of cellular growth and death of the $Brca1^{-/-}p53^{-/-}$ fibroblast lines
- DNA repair analyses of $Brca1^{-/-}p53^{-/-}$ fibroblast lines

Analysis of survival and mammary tumorigenesis in $p53^{-/-}$ mice and gamma-irradiated $p53^{+/-}$ mice on BALB/c, DBA/2J, and mixed genetic background

REPORTABLE OUTCOMES

- Publication of two manuscripts

Manuscripts

Cressman, V.L., Backlund, D.C, Hicks, E.M., Gowen, L.C., Godfrey, V., Koller, B.H. Mammary tumor formation in p53- and BRCA1-deficient mice (1999). *Cell Growth and Differentiation*, 10: 1-10.

Cressman, V.L., Backlund, D.C., Avrutskaya, A.V., Leadon, S.A., Godfrey, V., Koller, B.H. Growth retardation, DNA repair defects, and lack of spermatogenesis in BRCA1-deficient mice (1999). *Molecular and Cellular Biology*, 19: 7061-7075.

- Poster presentations at three conferences

Abstracts

Cressman, V.L., Backlund, D.C., Avrutskaya, A.V., Leadon, S.A., Godfrey, V., Koller, B.H. Growth retardation, DNA repair defects, lack of spermatogenesis, and mammary tumorigenesis in BRCA1-deficient mice. Cancer Biology and the Mutant Mouse: New Methods, New models, New Insights. January 1999.

Lee, V.M., Backlund, D.B., Hicks, E., Godfrey, V., Gowen, L.C., Koller, B.H. Mutations in p53 and Brca1 do not act synergistically in tumor formation. 89th Annual Meeting of the American Association for Cancer Research. March 1998.

Backlund, M.B., Trasti, S.L., Backlund, D.C., Cressman, V.L., Godfrey, V. Koller, B.H. Impact of gamma-irradiation and genetic background on mammary tumorigenesis in p53 and BRCA1-deficient mice. Era of Hope: Department of Defense Breast Cancer Research Program Meeting. June 2000.

DISCUSSION/CONCLUSION

Technical Objective 1: Tissue-specific Inactivation of BRCA1. The original intent of this aim was to understand the function of BRCA1 in the development processes of the mouse. To accomplish this, we intended to circumvent the embryonic lethality resulting from BRCA1-deficiency by utilizing the cre-lox system. Because a high risk of breast cancer is strongly associated with the loss of heterozygosity of *BRCA1* in mammary epithelia, we first wanted to examine the effects of BRCA1-deficiency in the murine mammary gland. Through the unexpected generation of *Brca1*$^{-/-}$*p53*$^{-/-}$ mice, we have been able to go beyond the scope of this aim and examine the effects of BRCA1-deficiency in the entire mouse. While we are continuing to pursue the completion of this aim, we have focused primarily on the unique opportunity to examine the effects of the loss of BRCA1 in the entire animal (see Technical Objective 2).

Technical Objective 2: Interactions of Tumor Suppressor Genes

At least five mutations in *Brca1* have been introduced into the mouse genome, all of which have resulted in embryonic lethality when both alleles are mutant [5,10-13]. While loss of p53 has increased embryonic survival by about two days, the overall survival to birth of the *Brca1*$^{-/-}$ embryos has not increased with mutations in *p53*. Therefore, while it is likely that the loss of

p53 contributed to the survival of the *Brca1*$^{-/-}$*p53*$^{-/-}$ mice, clearly additional genetic or environmental factors are crucial as well. The survival to adulthood of these three *Brca1*$^{-/-}$*p53*$^{-/-}$ mice could be explained through a number of different mechanisms. The close relationship of the two initial *Brca1*$^{-/-}$*p53*$^{-/-}$ mice and the derivation of the third *Brca1*$^{-/-}$*p53*$^{-/-}$ animal from relatives of the two initial mice would suggest that genetic factors are involved. These genetic factors could include spontaneous mutations introduced into the colony which permit normal neural tube formation despite the loss of BRCA1 expression and allow the survival of the *Brca1*$^{-/-}$*p53*$^{-/-}$ embryos to birth. Alternatively, as these mice are on a mixed strain background, a specific combination of modifier genes from the different strains could be important to the survival past this crucial stage in embryogenesis. Currently, the small number of surviving *Brca1*$^{-/-}$*p53*$^{-/-}$ mice do not permit us to distinguish between these possibilities. Until additional *Brca1*$^{-/-}$*p53*$^{-/-}$ mice are generated, it will not be possible to determine the cause for the survival.

Previous studies have shown that expression of BRCA1 is very high in the testes, and is highest in the spermatocytes, the cells undergoing meiosis and meiotic recombination [14-16]. Specifically, BRCA1 is associated with the unsynapsed axial elements that forms between sister chromatids as pairing occurs [17]. The inability of the *Brca1*$^{-/-}$*p53*$^{-/-}$ testes to complete spermatogenesis combined with these reports suggest that BRCA1 is crucial in meiosis of male gamete formation.

Loss of BRCA1 also resulted in abnormalities in mammary gland development. Specifically, a reduction in branching and abnormalities in end bud formation were detected. This finding would suggest that BRCA1 plays an important role specifically in mammary gland development, which of interest since the loss of BRCA1 did not affect the development of the majority of the other organs in the BRCA1-deficient mice.

Comparison of growth of the normal and BRCA1-deficient fibroblasts, consistent with the phenotype of the BRCA1-deficient embryos, shows that loss of expression of BRCA1 greatly decreases the growth potential of normal cells. I have shown that this decrease in growth potential is not due to a decrease in the number of cells entering cell cycle but is rather a result of a decrease in the survival of these cells. If, as has been proposed, BRCA1 is required for DNA repair, this increased cellular death may result from the rapid accumulation of chromosomal abnormalities and other mutations incompatible with the survival of normal cells. This interpretation is supported by our observation that the growth of the primary BRCA1-deficient cells is compromised to a greater extent than the *p53*$^{-/-}$ control cell lines by exposure to ultraviolet light, ionizing radiation, and hydrogen peroxide. Loss of transcription coupled repair likely plays an important role in the sensitivity of the *Brca1*$^{-/-}$*p53*$^{-/-}$ cells to ionizing radiation and hydrogen peroxide. In contrast, other mechanisms are responsible for the increased sensitivity of these cells to ultraviolet light.

Comparison of gamma-irradiated *p53*$^{+/-}$ mice on a mixed, BALB/c, and DBA/2J background show that genetic background and environmental factors may play a increasing critical role in the initial events that lead to mammary epithelial transformation in these animals. Additionally, our results suggest that the tumor spectrum may differ depending on the *p53* mutation status within a cell, as *p53*$^{-/-}$ BALB/c mice developed no mammary tumors while irradiated *p53*$^{+/-}$ BALB/c mice showed an increased incidence of mammary carcinomas.

Together our analysis of the *Brca1*$^{-/-}$*p53*$^{-/-}$ mice and fibroblasts, along with our recent unpublished data on irradiated *p53*$^{+/-}$ mice support the following model for the role of BRCA1 and p53 in tumorigenesis. In tumor progression, loss of the wild-type *BRCA1* allele is not the first event in tumor progression. Cells must first accumulate mutations in multiple genes, such

as *p53*, as a possible result of exposure to environmental agents, like ionizing irradiation, such that these cellular mutations confer a growth advantage. Only after having acquired an increased growth potential will loss of BRCA1 function be compatible with cell survival. With loss of the wild-type BRCA1 function, the resulting cell populations display growth rates that approach those of normal cells. However, if as suggested by our results, BRCA1 plays a role in multiple DNA repair pathways, then loss of BRCA1 function would be expected to accelerate the accumulation of additional mutations leading to malignant transformation. While further studies will be required to test this model, the results described here suggest that in a number of normal cell populations, loss of BRCA1 results in a growth disadvantage. Only in combination with other mutations and potential genetic modifier alleles, under specific growth conditions, or in specific populations of cells, can loss of BRCA1 contribute to tumorigenesis.

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APPENDIX A: FIGURE LEGENDS

Figure 1. Tumor incidence in p53-deficient ($p53^{-/-}$ and $p53^{+/-}$) and wild-type ($p53^{+/+}$) mice of two different genetic backgrounds. Mice with either one, two or no disrupted *p53* alleles were backcrossed from mixed genetic background onto both the inbred BALB/c and DBA/2J backgrounds and monitored weekly for tumors. **(A)** Survival of $p53^{+/+}$ and $p53^{-/-}$ mice on either the BALB/c or DBA/2J genetic backgrounds through three generations of backcrossing. Survival of $p53^{+/+}$ and $p53^{+/-}$ mice on either the BALB/c or DBA/2J genetic backgrounds after exposure to 5 Gy whole-body ionizing radiation through three generations of backcrossing. **(B)** Survival of $p53^{+/+}$ and $p53^{-/-}$ mice on either the BALB/c or DBA/2J genetic backgrounds from generation three to generation six of backcrossing. **(C)** Survival of $p53^{+/+}$ and $p53^{+/-}$ mice on either the BALB/c or DBA/2J genetic backgrounds after exposure to 5 Gy whole-body ionizing radiation through three generations of backcrossing. **(D)** Survival of $p53^{+/+}$ and $p53^{+/-}$ mice on either the BALB/c or DBA/2J genetic backgrounds after exposure to 5 Gy whole-body ionizing radiation from generation three to generation six of backcrossing

APPENDIX B: FIGURES

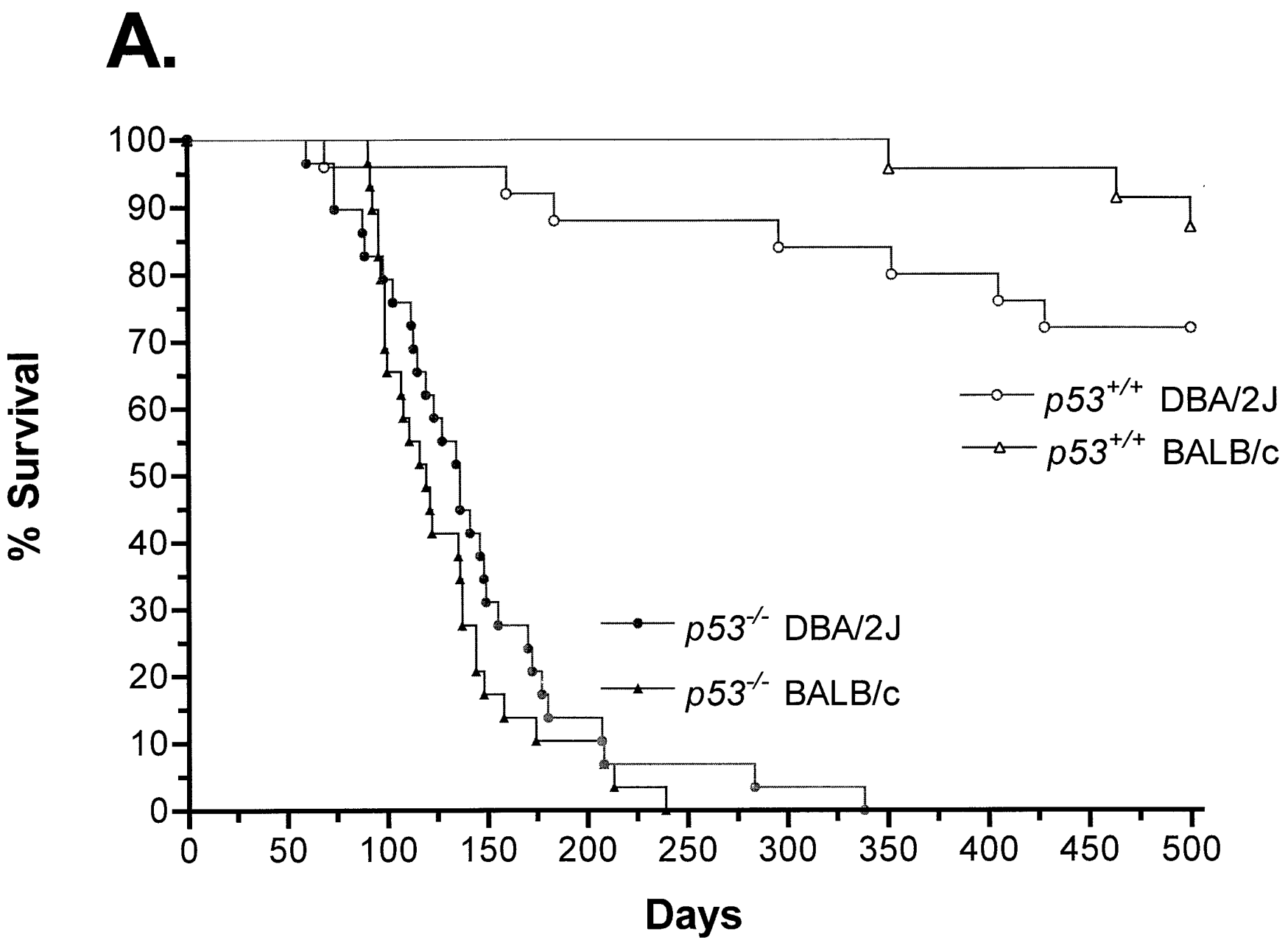

Figure 1A. Survival of $p53^{+/+}$ and $p53^{-/-}$ female mice on either a BALB/c or DBA/2J genetic background through three generations of backcrossing.

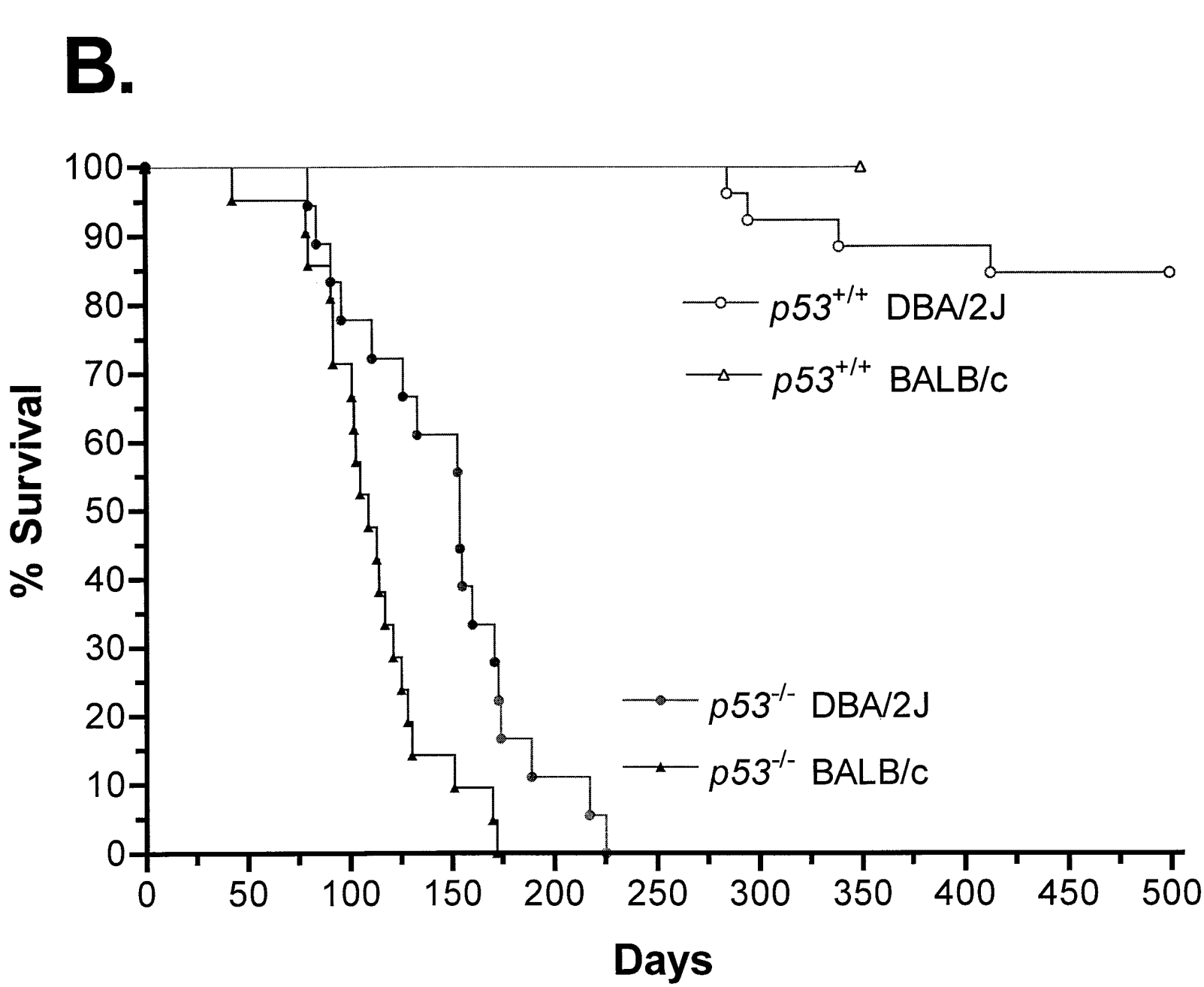

Figure 1B. Survival of $p53^{+/+}$ and $p53^{-/-}$ female mice on either a BALB/c or DBA/2J genetic background from generation four to six of backcrossing.

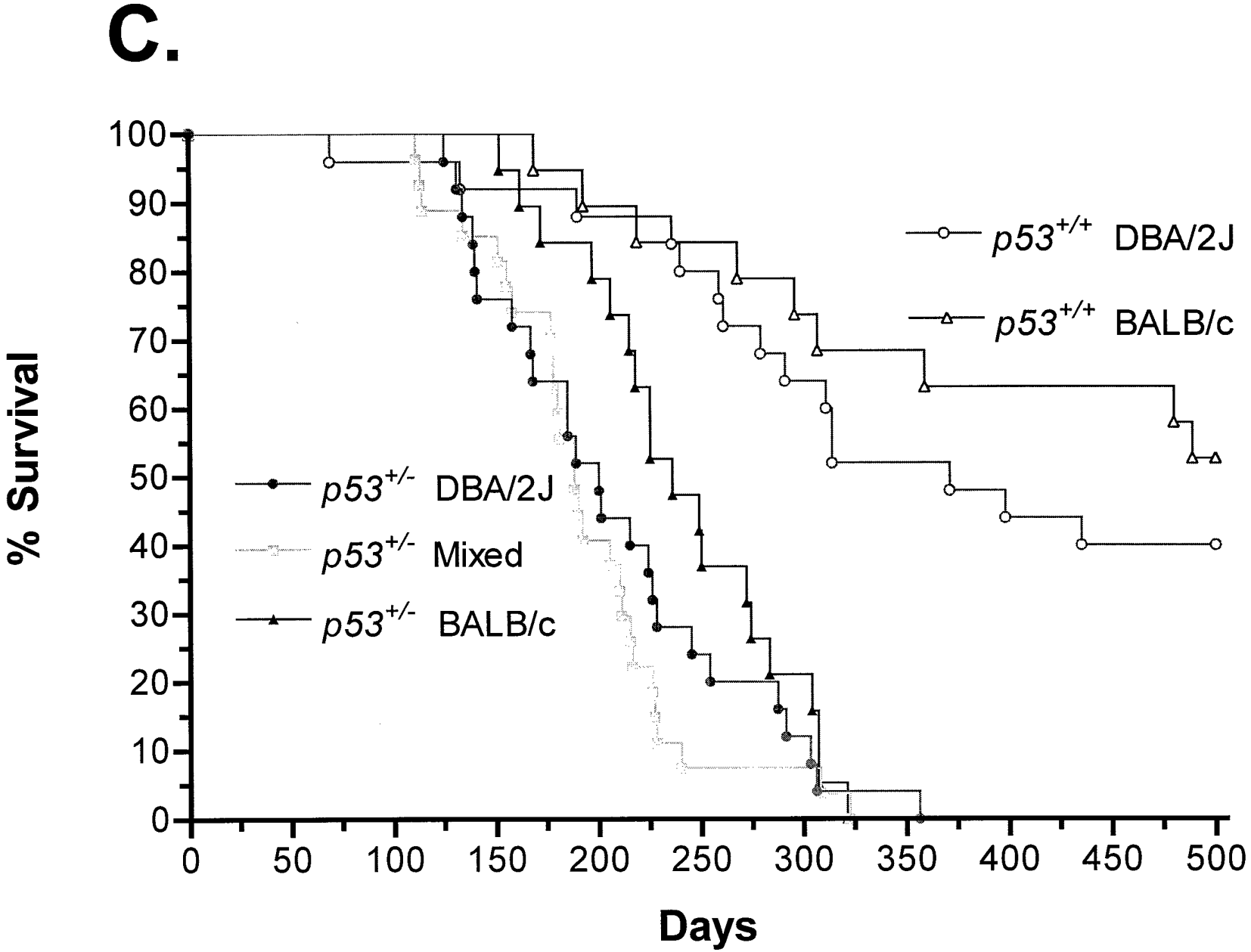

Figure 1C. Survival of $p53^{+/+}$ and $p53^{+/-}$ female mice on either a mixed, BALB/c, or DBA/2J genetic background after exposure to 5 Gy whole-body ionizing radiation through three generations of backcrossing.

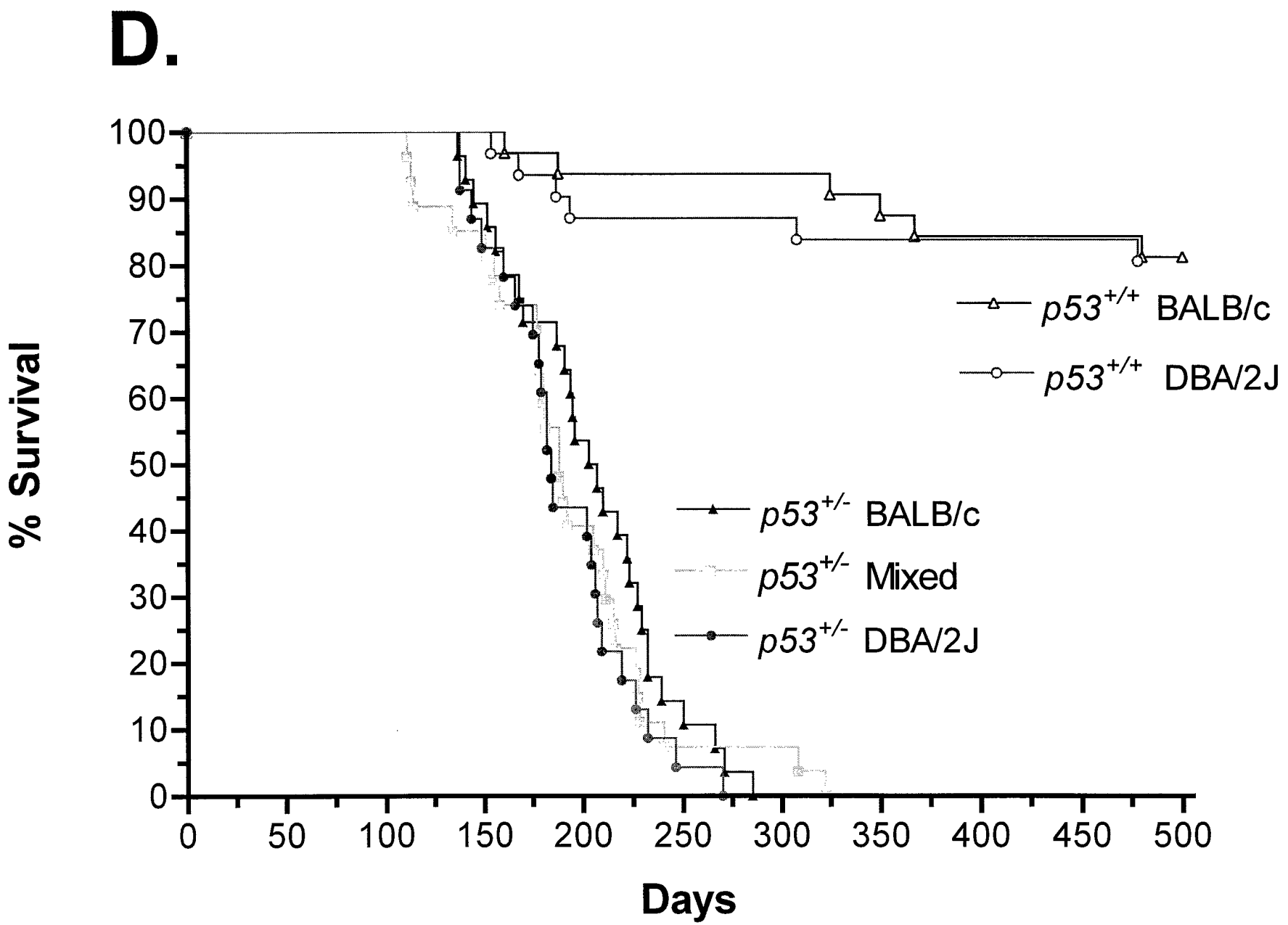

Figure 1D. Survival of $p53^{+/+}$ and $p53^{+/-}$ female mice on either a mixed, BALB/c or DBA/2J genetic background after exposure to 5 Gy whole-body ionizing radiation from generation four to generation six of backcrossing.

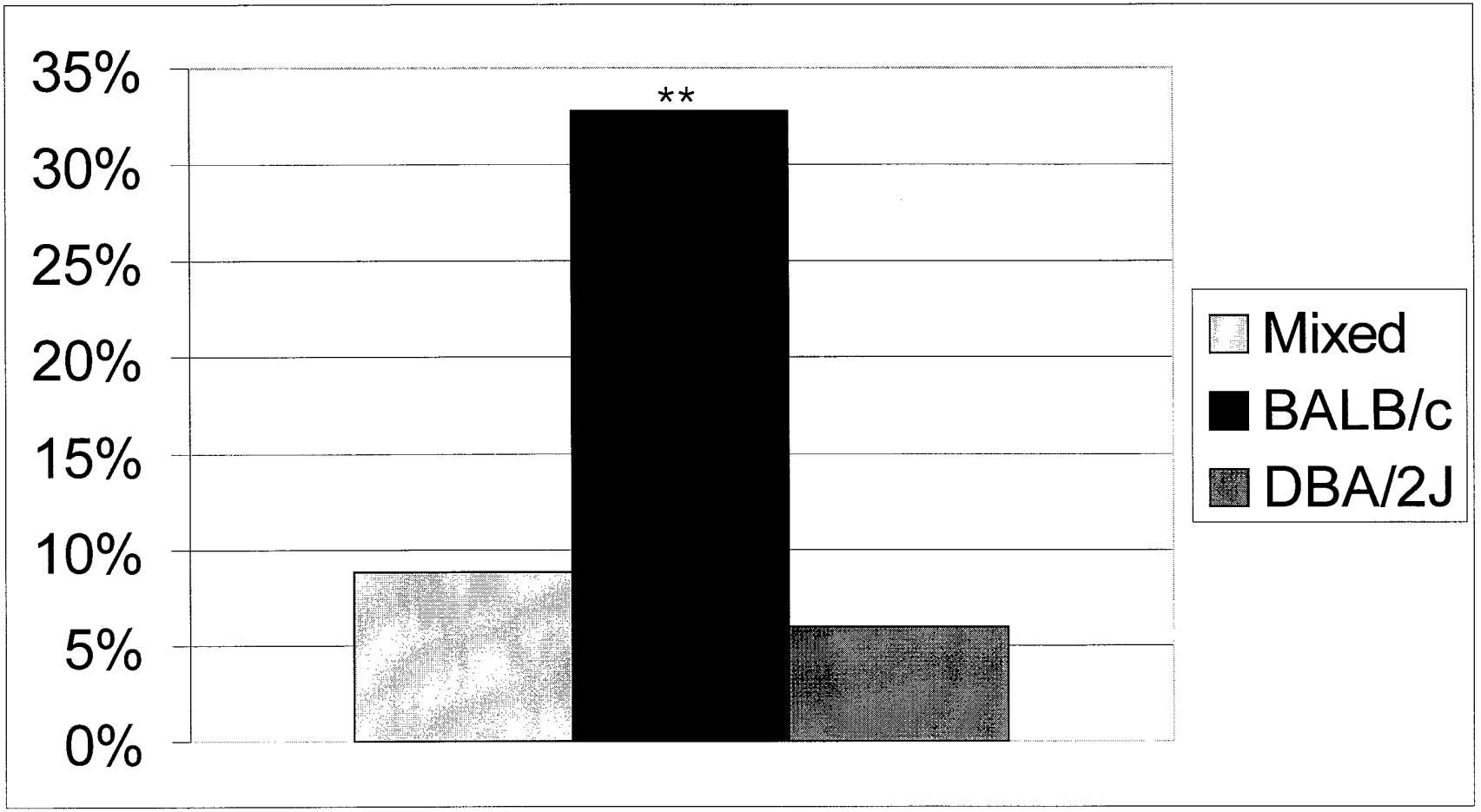

Figure 2. Mammary Gland Carcinomas in Irradiated *p53* Heterozygous Mice. Chi-Square= 10.68, *P*= 0.0048

APPENDIX C: TABLES

Table 1. Tumor Spectrum in p53-Deficient Mice

	BALB/c		DBA/2J	
	p53 $^{+/+}$	*p53* $^{-/-}$	*p53* $^{+/+}$	*p53* $^{-/-}$
	n	n	n	n
Lymphoma (NOS)		35 (59.3%)	2	40 (87.0%)
Hemangiosarcoma		15 (25.4%)		1 (2.2%)
Fibrosarcoma		1 (1.7%)		
Histiocytic sarcoma		1 (1.7%)		
Osteosarcoma				1 (2.2%)
Sarcoma (NOS)				1 (2.2%)
Carcinoma of unknown origin		1 (1.7%)		
Mammary Gland Carcinoma		0		0
No visible or metastic tumors at death		6 (10.2%)	8	3 (6.5%)
Total Number of Tumors	0	59	10	46
Total Number of Mice	52	53	52	46

The numbers in parentheses are the percentages of tumor types observed among all tumors from a given strain and genotype.

[a] NOS, not otherwise specified

Table 2. Tumor Spectrum in Irradiated p53-Deficient Mice

	Mixed		BALB/c		DBA/2J	
	p53 $^{+/+}$	*p53* $^{+/-}$	*p53* $^{+/+}$	*p53* $^{+/-}$	*p53* $^{+/+}$	*p53* $^{+/-}$
	n	n	n	n	n	n
Lymphoma (NOS)		23 (67.6%)	5	21 (36.2%)	8	36 (72.0%)
Mammary gland carcinoma		3 (8.8%)		19 (32.8%)		3 (6.0%)
Luteoma				2 (3.4%)		
Osteosarcoma		1 (2.9%)	1	3 (5.2%)		
Rhabdomyosarcoma				1 (1.7%)		
Pituitary gland adenocarcinoma				1 (1.7%)		
Adrenocortical adenocarcinoma				1 (1.7%)		
Fibrosarcoma				1 (1.7%)		
Basal cell carcinoma				1 (1.7%)		
Carcinoma (NOS)		1 (2.9%)		1 (1.7%)		
Sarcoma (NOS)						2 (4.0%)
Squamous cell carcinoma						1 (2.0%)
Teratoma		1 (2.9%)				
No visible or metastatic tumors at death		5 (14.5%)	5	7 (12.1%)	6	6 (12.0%)
Total Number of Tumors		34	10	58	14	50
Total Number of Mice	N/A	31	52	47	55	48

The numbers in parentheses are the percentages of tumor types observed among all tumors from a given strain and genotype. [a] NOS, Not Otherwise Specified

APPENDIX D: MANUSCRIPTS

Mammary Tumor Formation in p53- and BRCA1-deficient Mice[1]

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Abstract

The inheritance of a mutant copy of the *BRCA1* gene greatly increases a woman's lifetime risk for ovarian and breast cancer. While a homologous gene has been identified in mouse, mice carrying mutations in this gene do not display a detectable increase in tumor formation. To determine whether mutations in *p53* might increase the incidence of tumors associated with the loss of BRCA1 function in mice, we have generated mice carrying mutations at both of these loci. We report here that the presence of a mutant *Brca1* allele does not alter survival of either $p53^{-/-}$ or $p53^{+/-}$ mice. Although the tumor spectrum was not dramatically altered, an increased incidence of mammary tumors was observed in the $Brca1^{+/-}p53^{-/-}$ mice. Four mammary tumors were seen in the $Brca1^{+/-}p53^{-/-}$ group whereas only one such tumor was seen among the $p53^{-/-}$ control group. In addition, although the presence of a mutant *Brca1* allele did not alter the survival rate or the incidence of most tumor types in the $p53^{+/-}$ mice, 5 of the 23 tumors isolated from the $Brca1^{+/-}p53^{+/-}$ mice treated with ionizing radiation were of mammary epithelial origin, and 3 of these had lost expression of the wild-type *Brca1* gene. In contrast, no such tumors were observed in the irradiated $p53^{+/-}$ controls. Although the number of mammary tumors observed in these animals is small, these results are suggestive of a role for BRCA1 in mammary tumor formation after exposure to specific DNA damaging agents.

Introduction

Approximately 5% of breast cancer cases are believed to result from the inheritance of high penetrance risk factors (1). Mutations in the *BRCA1* gene have been identified as the risk factor responsible for disease in >90% of individuals from families with a high incidence of both breast and ovarian cancer as well as in approximately 45% of hereditary breast cancer cases (2–4).

Although the BRCA1 protein and its murine homologue display only 58% overall homology, in specific regions of the protein (such as the putative zinc finger domain and the COOH terminus) conservation of the protein sequence is >80% (5). This high degree of conservation suggests that not only do these regions represent important functional domains but at least some functions of this protein are conserved between mouse and man (6). It was, therefore, anticipated that the introduction of mutations similar to those seen in the human population into the murine homologue would result in a predisposition to tumor formation in these animals. Mice carrying a mutant copy of *Brca1* were generated from ES cells in which a portion of exon 11 of the *Brca1* gene was replaced with a marker gene by homologous recombination (7). $Brca1^{+/-}$ mice develop normally, are fertile, and, surprisingly, fail to show the increased susceptibility to tumors seen in humans carrying similar mutations. The failure of mutations in *Brca1* to lead to increased tumor susceptibility has been documented by several groups, each of which generated mouse lines carrying different *Brca1* mutations (7–11). Thus the mouse differs markedly from the human, in which the presence of a mutant *BRCA1* allele results in an estimated 80–90% overall lifetime risk for developing breast and/or ovarian cancer (3).

Differences between humans and mice in the phenotype resulting from mutations in tumor suppressor genes are not unique to BRCA1. For example, while mutations in the *WT-1* gene predispose to tumors in humans, no tumors are seen in the mouse (12, 13). Deletions and mutations in the *p53* gene are observed in 40–60% of all sporadic breast cancers, and Li-Fraumeni patients, who carry a mutant copy of *p53*, have an increased susceptibility to many types of tumors, especially mammary carcinomas (14–17). Mammary adenocarcinomas are rare in $p53^{+/-}$ and $p53^{-/-}$ mice and have been reported by only one group with a frequency of <2% of the tumors examined, whereas sarcomas are seen at a high frequency both in mice deficient in p53 and in Li-Fraumeni patients (18).

These observed differences between species seem less surprising when one considers that, despite the broad tissue distribution of a number of these tumor suppressor genes, inheritance of the mutated allele leads to very specific types of tumors. For example, although high levels of RB can be demonstrated in virtually all cell types, inheritance of a mutant *RB* allele in humans leads primarily to tumors of retinal neuronal and osteoblastic origin (19). Similarly, BRCA1 is expressed in a wide variety of actively dividing human cells, but mutations in BRCA1 are associated predominantly with an increased risk for breast, ovarian, and prostate cancer (20). The mechanisms that

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[2] To whom requests for reprints should be addressed, at University of North Carolina, 7007 Thurston-Bowles Building, CB#7248, Chapel Hill, NC 27599. Phone: (919) 962-2153; Fax: (919) 966-7524; E-mail: Treawouns@aol.com.

underlie the differential impact of the loss of function of a ubiquitously expressed gene on a cell's susceptibility to malignant transformation are poorly understood. However, a number of explanations for these findings have been proposed, including the theory that cell-to-cell and species-to-species variations reflect differences in lifetime exposure to environmental agents (such as ionizing radiation and carcinogens) or simply differences in the growth profiles of specific populations of cells (21, 22). Differences between species in the role of specific genes in mammary tumorigenesis may be related to the observation that the contribution of hormones to human and murine mammary tumor formation may be somewhat different. In mice, although prolonged exposure to high levels of prolactin and progesterone results in an increased incidence of mammary tumors, exposure of mice to high levels of estrogen itself does not alter the frequency with which these tumors are observed (23). It is also possible that parallel pathways and/or functional redundancy may be factors that contribute to both differences in tumor susceptibility of different cell types within a species as well as differences between species (24–26).

To generate better models of human disease and to begin to define the relationship between various oncogenes and tumor suppressor genes *in vivo*, investigators have intercrossed the mouse lines carrying mutant alleles of these genes. Often this results in either a change in the tumor types observed or in the latency of tumor formation (27, 28). We have, therefore, initiated a similar approach to examine tumor formation in mice carrying a mutant *Brca1* allele. Specifically, we have examined the impact of deficiency of p53 on mammary tumor formation in these animals.

Recent studies support multiple functions for p53 in protecting animals from tumor formation (24, 29). These studies demonstrate that p53 deficiency leads to increased chromosomal instability, the premature entry of cells with DNA damage into S phase, and, in certain circumstances, the loss of the apoptotic response. A number of lines of evidence suggest that p53 interacts with BRCA1. Embryos homozygous for mutations in the *Brca1* gene are severely growth retarded and die between embryonic days 7 and 13 (7, 9–11). Examination of these embryos suggested that there is a change in the expression of the p53-regulated genes *p21* and *mdm2* in cells deficient in BRCA1 (9). In addition, BRCA1-deficient embryos that are also deficient in either p53 or p21 survive several days longer (11, 30). A relationship between BRCA1 and the p53 pathway is further supported by studies demonstrating a striking coincidence between the cell cycle distribution of BRCA1 and p53 (31, 32). Recent reports demonstrate that BRCA1 and p53 directly interact and that BRCA1 requires the presence of p53 for the enhancement of transcriptional activation (33, 34). There is evidence to suggest that BRCA1 may play a critical role in DNA repair, especially after exposure to γ irradiation, UV light, or mitomycin C (35). It is, therefore, possible that the loss of BRCA1 impairs the ability of cells to efficiently repair DNA and that this in turn results in increased p53 activity, perhaps through alterations in MDM2 expression. If this interaction between the p53 pathway and BRCA1 is important in mammary epithelium, then the deficiency in p53 may increase the frequency of malignant transformation of cells that have impaired DNA repair pathways as a result of somatic loss of the wild-type *Brca1* allele.

To determine whether a deficiency in p53 and BRCA1 has a synergistic effect in tumor formation, particularly in mammary tumorigenesis, we have generated $Brca1^{+/-}$ mice that carry mutations in either one or both of their *p53* alleles. The p53 mutation introduced into these mice is a null allele, and tumor formation in $p53^{+/-}$ and $p53^{-/-}$ has been described in detail previously (36, 37). The *Brca1* mutation that these mice carry results in a complete loss of the expression of the native full length *Brca1* gene (7). However, a splice variant mRNA, 4 kb smaller than the wild-type transcript, can be seen in the ES cells heterozygous and homozygous for this mutation. We have designated this mutation $Brca1^{\Delta 223-763}$ to indicate that the DNA encoding amino acids 223 to 763 are deleted in this mutation. Because the splice acceptor site for exon 11 is also deleted, the splice variant mRNA found in cells carrying this mutation does not include any coding sequence from exon 11. Amino acids 223 through 1364 would be absent from any protein transcribed from this mRNA. This region of the protein has been shown to include two nuclear localization signals, a RAD51-binding domain, a p53-binding domain, and a phosphorylation site (34, 38–41). Domains outside exon 11 in BRCA1 include the RING finger domain located in the NH_2 terminus and the BRCT domains in the COOH terminus. The RING finger domain binds BARD1 and BAP1, while the BRCT domains contain three RNA helicase A binding domains, the minimal transcriptional activation domain, and another p53-binding domain. (33, 42–45). Similar to all of the other BRCA1-deficient lines generated to date, embryos homozygous for this mutation die early in gestation (7). However, although embryonic lethality in the majority of other homozygous embryos occurs between days 6 and 8 of gestation, those carrying the $Brca1^{\Delta 223-763}$ mutation survive until embryonic days 8 to 10 (7, 9–11). This increased survival is likely to reflect the more heterogeneous genetic background on which this mutation was studied. A similar impact of genetic background has been observed on analysis of mice carrying *Brca2* mutations (11, 46–48). However, we cannot at this point exclude the possibility that extended survival reflects some function imparted by protein encoded by the splice variant mRNA present in these embryos. Recently we have shown that BRCA1 has a role in transcription-coupled repair (49). No activity was seen in cells homozygous for the mutant allele despite high levels of expression of the splice variant. However, despite the severe developmental phenotype and the complete loss of transcription-coupled repair observed in mice and cells homozygous for the Δ223–763 mutation, it will not be possible to ascertain whether this mutation represents a null allele until all of the functions of BRCA1 have been defined. For simplicity, we will refer to mice carrying this mutation as $Brca1^{+/-}$ throughout the manuscript.

In this report, we compare the tumor type and rate of tumor formation in BRCA1- and p53-deficient mice to animals carrying *p53* mutations alone. We also examined the effect of ionizing radiation on tumor formation in these populations.

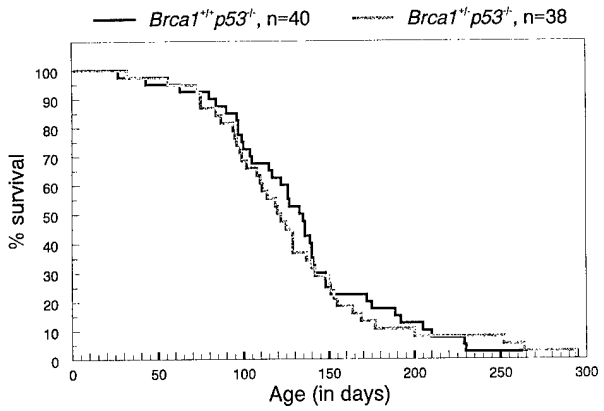

Fig. 1. Germline loss of *Brca1* does not affect the survival rate of $p53^{-/-}$ mice. $Brca1^{+/+}$ and $Brca1^{+/-}$ mice on a $p53^{-/-}$ background were carefully monitored for ill health. Moribund mice were killed, and necropsy was performed.

Results

Comparison of $Brca1^{+/+}$ and $Brca1^{+/-}$ Mice on a p53-deficient Background. $Brca1^{+/-}$ and $p53^{-/-}$ animals were intercrossed to obtain animals heterozygous for both alleles. These animals were again intercrossed and the genotype of the offspring determined. $Brca1^{+/+}$ and $Brca1^{+/-}$ mice homozygous for the mutant *p53* allele were observed at the expected frequency. As reported previously, female $p53^{-/-}$ mice were present in lower numbers than expected; however, this was true of both $Brca1^{+/+}$ and $Brca1^{+/-}$ female pups deficient in p53 (50, 51). Mouse embryos homozygous for the mutant *Brca1* allele fail to progress past day 10 of embryonic development (7). Deficiency in p53 has been reported to extend the survival of the embryos by several days (11, 30). However, consistent with results reported with other *Brca1* mutant lines, p53-deficiency did not rescue the embryonic lethality of the BRCA1-deficient animals. Of nine litters generated from $Brca1^{+/-}p53^{-/-}$ matings, no $Brca1^{-/-}$ offspring were observed. Genotyping of the pups identified 16 $Brca1^{+/+}$ and 40 $Brca1^{+/-}$ animals.

Thirty-eight $Brca1^{+/-}$ $p53^{-/-}$ and 40 $Brca1^{+/+}p53^{-/-}$ animals were monitored biweekly for the development of tumors and killed when behavior and appearance indicated that the death of the animals was imminent. As can be seen in Fig.1, latency to tumor formation was similar in both groups of animals. By 19 weeks of age, ~50% of both $Brca1^{+/+}$ and $Brca1^{+/-}$ mice on a $p53^{-/-}$ background had been killed because of tumor formation. Tumors were classified both by observation of anatomical location and by histological examination of tumor biopsies by a trained pathologist (V. G.). This analysis indicated that the distribution of tumors arising in the mice carrying a *Brca1* mutation did not differ significantly from those observed in the $Brca1^{+/+}p53^{-/-}$ animals (Table 1). As previously reported, the predominant tumor type observed in the $p53^{-/-}$ mice is the thymic lymphoma (36, 37). The presence of a mutant *Brca1* allele did not change the prevalence of this tumor in the $p53^{-/-}$ animals. Histological examination of lung, kidney, and liver from these mice also failed to reveal obvious differences between the two groups in the extent of metastases to other organ systems.

Mammary tumors were observed in both $Brca1^{+/+}$ and $Brca1^{+/-}$ mice with mutations in both *p53* alleles. Although only one mammary tumor was seen in the $Brca1^{+/+}$ mice (which is comparable to the frequency of such tumors seen in $p53^{-/-}$ mice by other investigators), 4 of the 23 $Brca1^{+/-}$ mice developed mammary tumors (18, 37). Two of these tumors were classified as papillary adenocarcinomas, and a third was classified as a tubular mammary carcinoma. Although histopathological examination established that a fourth tumor seen in this group of animals was of mammary gland origin, the characteristics of the tumor did not allow classification into established tumor subtypes. Although suggestive of an increased incidence of mammary tumor formation in mice heterozygous for a *Brca1* mutation on a $p53^{-/-}$ background, the overall low number of mammary tumors in $Brca1^{+/+}$ and $Brca1^{+/-}$ mice did not permit meaningful statistical evaluation (Fisher's exact test, $P = 0.17$).

Analysis of *Brca1* Gene Expression in $Brca1^{+/-}p53^{-/-}$ *Tumors.* Loss of the wild-type *BRCA1* allele is almost always observed on examination of DNA prepared from tumors arising in patients that have inherited a single mutant copy of the gene (52–54). The loss of heterozygosity of *BRCA1* in the cells of these patients is most likely an important event in mammary tumor formation. Although the similar tumor latency and spectrum in the $Brca1^{+/+}$ and the $Brca1^{+/-}$ mice did not suggest a general role for this gene in tumor formation in the mouse, we wished to verify this by examination of RNA and DNA from these tumors for the expression of the wild-type *Brca1* allele. The mutation introduced into the *Brca1* gene by homologous recombination results in a smaller RNA transcript than that produced from the wild-type allele. When the wild-type *Brca1* transcript was not seen, the presence of this mutant transcript served as an internal control and allowed us to distinguish between loss of transcription of *Brca1* and the loss of function of the wild-type gene. In tumors where analysis of RNA was not possible because of the postmortem changes in the tissue or in cases in which neither the wild-type nor mutant *Brca1* transcripts were seen by Northern blot analysis, DNA was prepared and analyzed by Southern blot for the presence of the wild-type allele. Analysis of 22 tumors from $Brca1^{+/-}p53^{-/-}$ mice identified only 2 tumors in which loss of the wild-type allele could be detected (Table 1 and Fig. 2*A*, *Lane 2*, and Fig. 2*C*). As can be seen in Fig. 2*A*, Northern analysis of mRNA prepared from the first of these two tumors failed to reveal a band corresponding to the wild-type *Brca1* transcript. In contrast, a transcript of approximately 4 kb from the mutated allele is present at high levels in the tumor RNA. Loss of heterozygosity in this tumor was confirmed by DNA analysis (Fig. 2*B*). In the second tumor, Northern analysis again failed to reveal expression of the native *Brca1* transcript, whereas the transcript from the targeted allele was easily detected. In addition, a novel transcript, smaller in size than the wild-type *Brca1* transcript, was also present in the RNA isolated from this tumor. Presumably this transcript originates from the wild-type allele that mutated during tumorigenesis (Fig. 2*C*). Both of the tumors that no longer expressed wild-type *Brca1* mRNA were thymic lymphomas (Table 1). None of the mam-

Table 1 Tumor spectrum in *Brca1*$^{+/+}$ *versus* *Brca1*$^{+/-}$ mice on a *p53*$^{-/-}$ background

	Brca1$^{+/+}$		*Brca1*$^{+/-}$		No. of *Brca1*$^{-/-}$ tumors
	n	%	*n*	%	No. of tumors examined for LOH[a]
Thymic lymphoma	28	54.9	19	45.2	2/9
Lymphoma	9	17.6	10	23.8	0/5
Osteosarcoma	1	2.0			
Hemangiosarcoma	3	5.9			
Cerebellar developmental defect	2	3.9	2	4.8	
Adenocarcinoma of the Hardarian gland	1	2.0			
Rhabdomyosarcoma	1	2.0			
Teratoma	2	3.9	2	4.8	0/2
Mammary carcinoma	1	2.0	4	9.5	0/3
Adenocarcinoma of *rete ovarii*			1	2.4	0/1
Carcinoma of unknown origin	1	2.0	3	7.1	0/3
No visible or metastatic tumors at death	2	3.9	1	2.4	
Total	51		41		

[a] LOH, loss of heterozygosity.

mary tumors examined had lost expression of the wild-type *Brca1* gene.

A cell line was established from the thymic lymphoma expressing *Brca1* mRNA derived only from the targeted allele. RNA and DNA derived from this line confirmed the loss of the wild-type *Brca1* allele in this tumor (Fig. 2*A*, *Lane 1*). p53-deficient thymic lymphomas have been previously reported to be CD4+8+ "double positive" or a mixed population of CD8+/CD4+8+ T cells. Flow cytometry revealed a mixed population phenotype (CD4+/8+and CD8+) in this *Brca1*$^{-/-}$*p53*$^{-/-}$ thymic lymphoma cell line (data not shown).

Tumorigenesis in *Brca1*$^{+/+}$ and *Brca1*$^{+/-}$ Mice on a p53-heterozygous Background. We considered the possibility that the high incidence and early appearance of thymic lymphomas leading to early death of the majority of the *Brca1*$^{+/+}$*p53*$^{-/-}$ and *Brca1*$^{+/-}$*p53*$^{-/-}$ mice might obscure our ability to observe cooperativity between these two tumor suppressor genes in mammary tumorigenesis. Although *p53*$^{+/-}$ mice are also predisposed to tumors, the tumor latency and life span of *p53*$^{+/-}$ mice are longer (36, 37). We therefore also examined tumor formation in *Brca1*$^{+/+}$ and *Brca1*$^{+/-}$ littermates on a *p53*$^{+/-}$ background. Twenty-three mice heterozygous for both the *p53* and *Brca1* mutation were generated, and survival and tumor formation were compared with 23 mice carrying only a single copy of the mutant *p53* gene. Germ line inactivation of one copy of *Brca1* does not alter the survival or the types of tumors that arise in the *p53*$^{+/-}$ animals (Table 2 and Fig. 3).

RNA was isolated from six tumors that arose in the *Brca1*$^{+/-}$ *p53*$^{+/-}$ mice. In all of the cases, Northern analysis revealed the presence of both the wild-type *Brca1* transcript and the transcript originating from the targeted allele (Table 2).

Tumorigenesis in Irradiated BRCA1- and p53-deficient Mice. As it is possible that exposure to specific environmental agents together with the inheritance of mutations in tumor suppressor genes is essential for tumor formation in humans, we studied the effect of γ-irradiation on the tumor latency and tumor distribution in the BRCA1/p53-deficient animals. Consistent with published work (21), our observations showed that the irradiation of *p53*$^{-/-}$ mice causes the appearance of thymic lymphomas at a younger age (data not shown). The accelerated formation of thymic lymphomas would likely hinder our ability to observe the role of p53 and BRCA1 in mammary tumor formation. Therefore, this study was continued with BRCA1-deficient mice heterozygous for the *p53* mutation. Seventeen female *Brca1*$^{+/+}$ and 21 female *Brca1*$^{+/-}$ littermates heterozygous for the *p53* mutation between 4 and 6 weeks of age were exposed to a single dose of 5 Gy whole body γ-irradiation. Mice were observed biweekly, killed when moribund, and necropsied. Compared with the unirradiated *p53*$^{+/-}$ mice, the age of onset of tumor formation was decreased in both *Brca1*$^{+/+}$*p53*$^{+/-}$ and *Brca1*$^{+/-}$*p53*$^{+/-}$ irradiated mice (compare Fig. 3 and 4). However, as observed with the unirradiated populations, the loss of one *Brca1* allele did not affect the survival rate of the *p53* heterozygotes (Fig. 4). Consistent with published reports, the tumor spectrum of the irradiated *p53*$^{+/-}$ animals differs from the untreated animals in that the predominant tumor seen is the thymic lymphoma (21). This finding was observed in both the irradiated *Brca1*$^{+/+}$*p53*$^{+/-}$ and the irradiated *Brca1*$^{+/-}$ *p53*$^{+/-}$ populations of mice (Table 3). However, although none of the irradiated *Brca1*$^{+/+}$*p53*$^{+/-}$ mice developed mammary tumors, five mammary tumors (verified by histopathology) were observed in irradiated *Brca1*$^{+/-}$*p53*$^{+/-}$ mice. Again, statistical significance was not achieved because of the small sample size of mammary tumors (Fisher's exact test, $P = 0.14$). Eleven wild-type and 11 *Brca1*$^{+/-}$ mice were also exposed to γ-irradiation. Five of the wild-type mice died as a result of tumors arising after treatment. Three of the *Brca1*$^{+/-}$ mice developed tumors; however, none of the tumors were of mammary or ovarian origin, and on DNA analysis, none had lost the wild-type *Brca1* allele.

BRCA1 Expression in Mammary Tumors Arising in Irradiated *Brca1*$^{+/-}$*p53*$^{+/-}$ *Animals.* Five tumors arising in the irradiated *Brca1*$^{+/-}$ *p53*$^{+/-}$ population were examined for loss of heterozygosity at both the *p53* and *Brca1* locus. By Southern blot analysis, the genomic fragment corresponding to the wild-type *p53* allele was absent in all of the five tumors (data not shown).

Loss of heterozygosity at the *Brca1* locus was examined as described above using RNA isolated from tumors. North-

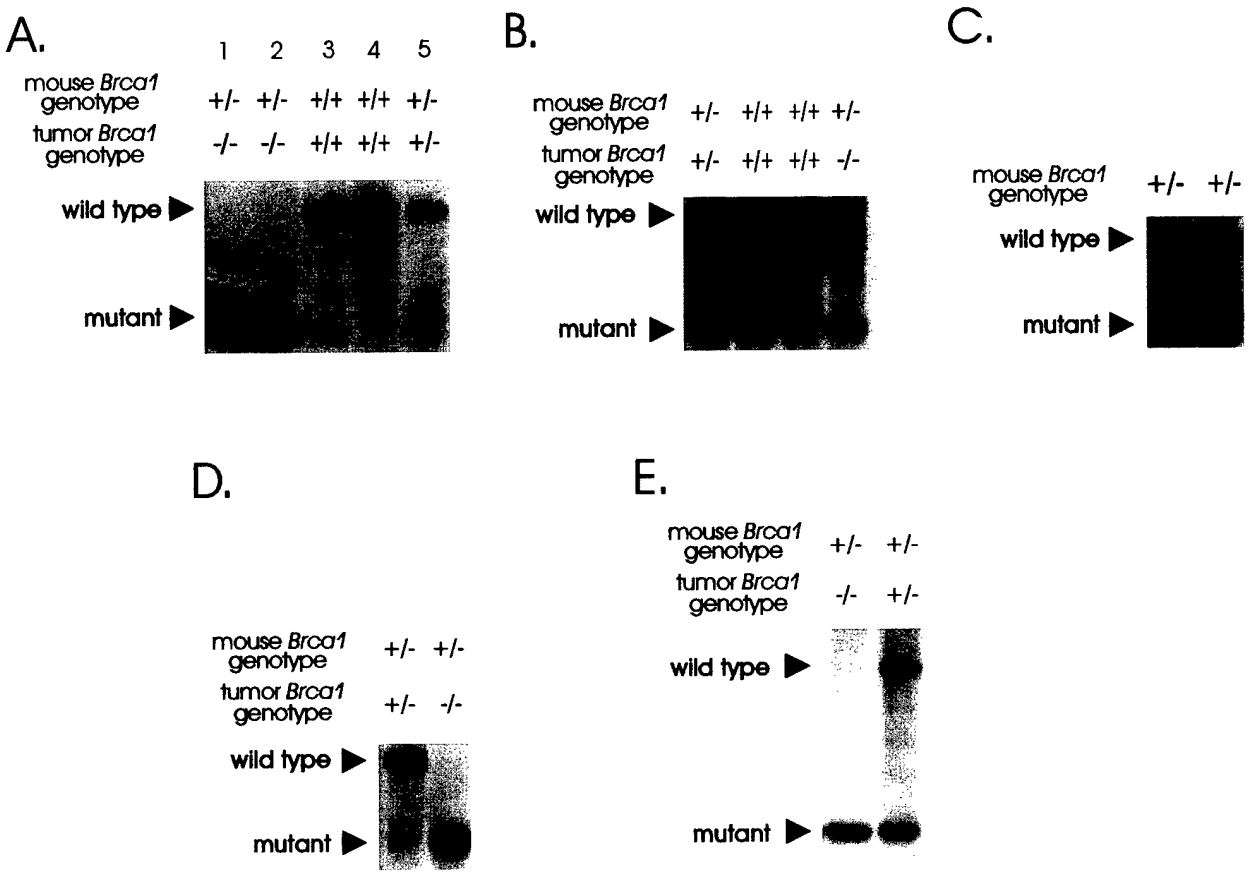

Fig. 2. Loss of *Brca1* heterozygosity in p53-deficient lymphomas. RNA was collected from tumor samples and cell lines derived from tumors and was examined for loss of *Brca1* expression by Northern blot analysis. The lower band (*mutant*) is the RNA derived from the targeted allele. Its presence acts as an internal control, verifying that the transcriptional machinery required to produce *Brca1* mRNA is present in the cell. *A*, RNA from one thymic lymphoma reveals the deletion of the wild-type copy of *Brca1* (*Lane 2*). RNA from the cell line derived from this tumor revealed that it was also BRCA1-deficient (*Lane 1*). *B*, DNA derived from this tumor confirms the loss of the *Brca1* allele. *C*, Northern analysis reveals that another thymic lymphoma line deleted a portion of *Brca1*. *D*, RNA made from one of three mammary tumors from irradiated $Brca1^{+/-}p53^{+/-}$ mice that had lost the wild-type *Brca1* allele. *E*, Southern analysis confirmed the loss of heterozygosity of *Brca1* in this tumor.

ern analysis of RNA prepared from three ovarian tumors, a single histiocytoma, a pilomatrixoma, and a hemangiosarcoma showed that expression of the *Brca1* wild-type allele was unaltered in these tumors (Table 3). Two of the mammary carcinomas also showed expression of both the mutant and the wild-type *Brca1* genes in the tumor tissue (data not shown). Analysis of a third mammary tumor, classified as a papillary adenocarcinoma, indicated that the wild-type *Brca1* allele was no longer expressed, whereas transcripts originating from the targeted allele were easily visible (Fig. 2*D*). Loss of heterozygosity was demonstrated by analysis of DNA obtained from the tumor, as only the band corresponding to the mutant allele was observed on Southern analysis (Fig. 2*E*). RNA analysis revealed that the loss of the wild-type allele of *Brca1* had also occurred in the remaining two mammary tumors. Histological analysis revealed that one tumor was a mammary intraductal carcinoma and the second was an anaplastic tumor of unknown origin. The high levels of cytokeratin 18 mRNA in the anaplastic tumor (data not shown), taken together with the s.c. location in the mammary gland region, suggest that this tumor is of mammary origin.

The loss of wild-type BRCA1 expression in three of the five mammary tumors in the irradiated $Brca1^{+/-}p53^{+/-}$ mice and the lack of mammary tumors in the $Brca1^{+/+}p53^{+/-}$ mice suggests a correlation between *Brca1* status and the formation of mammary tumors.

Discussion

We have generated mice that are carrying mutations in both *Brca1* and *p53* to determine whether mutations in both of these genes have a cooperative effect on tumorigenesis. These cooperative effects could result in a change in the latency of tumor formation already observed in the p53-deficient animals, or in the appearance of new tumor types arising in animals carrying mutations at both loci. We report that there is no alteration in the age at which $p53^{+/-}$ or $p53^{-/-}$ animals heterozygous for the mutant *Brca1* allele succumb to tumors. Consistent with this observation, only 2 of 23 tumors examined from the $Brca1^{+/-}p53^{-/-}$ population had lost expression of the wild-type *Brca1* gene. These two $Brca1^{-/-}$ thymic lymphomas did not differ from the other tumors in any

Table 2 Tumor spectrum in *Brca1*$^{+/+}$ *versus* *Brca1*$^{+/-}$ mice on a *p53*$^{+/-}$ background

	Brca1$^{+/+}$		*Brca1*$^{+/-}$		No. of *Brca1*$^{-/-}$ tumors
	n	%	*n*	%	No. of tumors examined for LOH[a]
Osteosarcoma	7	36.8	5	21.7	
Leiomyosarcoma	2	10.5			
Reticulum cell sarcoma	2	10.5	1	4.3	0/1
Bronchoalveolar carcinoma	2	10.5	2	8.7	
Hepatocellular carcinoma	1	5.3	2	8.7	
Mammary carcinoma	1	5.3			
Keratoacanthoma	1	5.3			
Basal cell carcinoma			2	8.7	0/2
Fibrosarcoma			4	17.4	0/2
Hemangiosarcoma			2	8.7	
Squamous cell carcinoma			1	4.3	
Benign spindle cell tumor			1	4.3	
No visible or metastatic tumors at death	3	15.8	3	13.0	
Total	19		23		

[a] LOH, loss of heterozygosity.

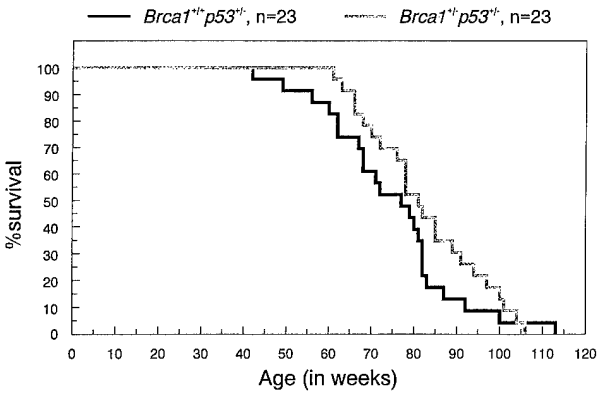

Fig. 3. Germline loss of Brca1 does not affect the survival rate of *p53*$^{+/-}$ mice. *Brca1*$^{+/+}$ and *Brca1*$^{+/-}$ mice on a *p53*$^{+/-}$ background were carefully monitored for ill health. Moribund mice were killed and necropsy was performed.

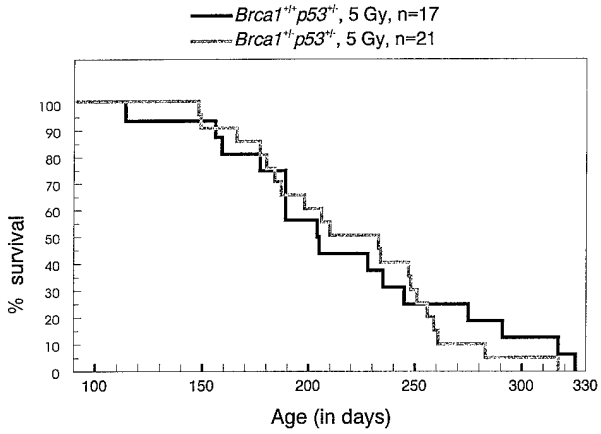

Fig. 4. γ irradiation of mice alters the overall tumor spectrum and latency of *p53*$^{+/-}$ mice but affects *Brca1*$^{+/+}$ and *Brca1*$^{+/-}$ mice similarly. Female *Brca1*$^{+/+}$ and *Brca1*$^{+/-}$ mice on a *p53*$^{+/-}$ background were irradiated with 5 Gy between 4 and 6 weeks of age. Moribund mice were killed and necropsied. Although the overall survival was decreased in both groups of *p53*$^{+/-}$ mice, the viability of mice was similar in the *Brca1*$^{+/+}$ and *Brca1*$^{+/-}$ mice.

of the parameters examined, including latency of tumor formation, histopathology, or the stage of T cell development from which they arose. These results are perhaps not surprising when one considers the results obtained in other model systems. For example, mice carrying a *Wnt-1* oncogene driven by the MMTV-LTR and homozygous for a null *p53* allele developed tumors earlier than mice carrying the transgene on a wild-type background (28). However, the failure to see an alteration in tumor latency in the *p53*$^{+/-}$ animals in this same model system raised the possibility that the decrease in tumor latency in the *Wnt-1* transgenic, *p53*$^{-/-}$ mice was caused by non-cell-autonomous factors. These authors proposed that the inhibitory influence of normal cells on the growth of tumor cells may be compromised in the *p53*$^{-/-}$ animals. The latency of tumor formation in mice carrying mutations in both the *p53* gene and the *Rb* gene was not substantially different from animals carrying mutations in only one of the genes (27). The age at which pituitary tumors formed in the *Rb*$^{+/-}$*p53*$^{+/-}$ animals was not greatly altered in comparison to mice with the *Rb* mutation alone, and loss of the wild-type *p53* allele in these tumors was rare.

In most model systems, the strongest evidence for a cooperative effect between mutations in two different loci in tumorigenesis comes from the appearance of novel tumors in mice carrying both mutations but not in mice carrying only one of the two mutations. For example, mice heterozygous for both *Rb* and *p53* mutations present with novel tumors of endocrine origin, such as islet cell carcinomas and medullary thyroid carcinomas, which are not seen in either the Rb- or the p53-deficient animals (27). Examination of DNA from these tumors from *Rb*$^{+/-}$*p53*$^{+/-}$ mice revealed that in most cases loss of the wild-type alleles of *Rb* and *p53* had occurred. Novel tumors seen in *Rb*$^{+/-}$*p53*$^{-/-}$ animals also resulted from the loss of the wild-type *Rb* allele. In contrast to these findings, we report that the tumor spectrum of *Brca1*$^{+/-}$ *p53*$^{+/-}$ mice is similar to that seen in *p53*$^{+/-}$ animals. A survey of RNA from tumors arising in these animals also failed to show any loss of the wild-type *Brca1* allele. Thus, analysis of

Table 3 Tumor spectrum in irradiated *Brca1*$^{+/+}$ *versus* *Brca1*$^{+/-}$ mice on a *p53*$^{+/-}$ background

	Brca1$^{+/+}$		*Brca1*$^{+/-}$		No. of *Brca1*$^{-/-}$ tumors
	n	%	*n*	%	No. of tumors examined for LOH[a]
Thymic lymphoma	8	53.3	10	41.7	
Lymphoma	1	6.7			
Brain tumor	1	6.7			
Ovarian granulosa/thecal cell tumor	2	14.3	3	12.5	0/3
Sarcoma	1	6.7	2	8.3	
Mammary carcinoma			5	20.8	3/5
Fibrous histiocytoma			1	4.2	0/1
Pilomatrixoma			1	4.2	0/1
Hemangiosarcoma			1	4.2	0/1
No visible or metastatic tumors at death	1	6.7	1	4.2	
Total	14		24		

[a] LOH, loss of heterozygosity.

this population of mice did not provide evidence for a cooperative effect between p53 and BRCA1 in the formation of these tumors.

The failure to observe cooperative effects between p53 and BRCA1 in the initial populations of animals examined is perhaps even more surprising in light of the recent publication demonstrating mutations in *p53* in all of the eight tumors examined from *BRCA1*$^{+/-}$ patients (55). The phenotype of the mouse embryos suggests that BRCA1 is essential for normal cell growth. It might, therefore, be expected that permissive mutations such as the loss of *p53*$^{-/-}$ would be important events in tumorigenesis before the loss of BRCA1 expression. However, in contrast to the results obtained with the *Brca1*$^{+/-}$*p53*$^{+/-}$ mice, results obtained on the analysis of tumors arising in the *Brca1*$^{+/-}$ *p53*$^{-/-}$ mice were suggestive of a role for cooperative effects between the two loci. Four mammary tumors were observed in this population of mice, whereas only one mammary tumor was observed in the *p53*$^{-/-}$ population. Mammary tumors, although frequent in Li-Fraumeni patients, are uncommon in mice carrying similar *p53* mutations. Jacks *et al.* (37) did not observe mammary tumors on analysis of 56 *p53*$^{-/-}$ mice, while Harvey *et al.* (18) reported one in 56 *p53*$^{-/-}$ mice analyzed, In contrast, we observed more mammary tumors in mice heterozygous for *Brca1* compared with control *Brca1*$^{+/+}$ mice on a *p53*$^{-/-}$ background. Differences between the number of mammary tumors in the *Brca1*$^{+/-}$*p53*$^{-/-}$ and the *p53*$^{-/-}$ populations did not achieve statistical significance because of the small overall proportion of mammary tumors found in the *Brca1*$^{+/-}$*p53*$^{-/-}$ animals. A study with larger groups of animals of each genotype would be necessary to establish whether a statistically significant difference exists in the development of mammary tumors in *Brca1*$^{+/-}$ animals. Examination of the mammary tumors for the wild-type allele of *Brca1* did not support a role for this gene in tumorigenesis, inasmuch as RNA transcripts corresponding to both the mutant and wild-type *Brca1* allele were present in all of the tumors. However, this may reflect the fact that point mutations occur more frequently than mutations that result in loss of the transcription of the *Brca1* gene.

BRCA1 has been found to colocalize with RAD51 in discrete nuclear foci during S phase (40). DNA damaging agents, such as ionizing radiation, result in the dispersal of these nuclear foci (35). If BRCA1 is involved in the response of cells to DNA damaging agents, it might be expected that a role for this gene in tumorigenesis may be dependent on exposure of the organism to these agents. The short life span of mice and the controlled environment in which they are raised may not result in a sufficient lifetime exposure to DNA damaging agents to allow BRCA1-deficiency to have a significant impact on tumor formation. We, therefore, examined the effect of BRCA1-deficiency on tumor latency in *p53*$^{+/-}$ mice after exposure to γ-irradiation. We found that in general the tumor spectrum was not altered on comparison of the irradiated *Brca1*$^{+/+}$*p53*$^{+/-}$ and *Brca1*$^{+/-}$*p53*$^{+/-}$ mice. However, when we specifically examined the formation of mammary tumors in the two groups, interesting differences in the populations emerged. If BRCA1 is involved in cellular pathways such as DNA repair, it is possible that a 50% decrease in BRCA1 expression in cells heterozygous of the mutant allele results directly in an increased tumor formation rate in the *Brca1*$^{+/-}$*p53*$^{-/-}$ mice.

Consistent with the previous reports, no mammary tumors were seen in the irradiated *p53*$^{+/-}$ mice (21). However, five mammary tumors were isolated from γ-irradiated double heterozygous animals. The low incidence of this tumor type did not allow determination of the significance of this finding by Fisher's exact test. However, in contrast to the mammary tumors seen in the *Brca1*$^{+/-}$*p53*$^{-/-}$ populations, loss of heterozygosity of the *Brca1* allele was observed on analysis of three of these tumors. Loss of heterozygosity of the *Brca1* allele was not seen in other tumors examined from this population of animals. The contribution of loss of p53 function in tumor formation was supported by the finding that the wild-type allele was absent in all of the tumors examined, including mammary tumors. Concomitantly, we studied the effect of irradiation on mice heterozygous only for the *Brca1* allele and wild-type at the *p53* locus. These studies further support a role for p53 in the formation of mammary tumors seen after irradiation, inasmuch as mammary tumors did not arise in these animals (data not shown). The observed loss of p53 in the *Brca1*$^{-/-}$ tumors is consistent with the observed

loss of expression of p53 in tumors from *BRCA1*$^{+/-}$ patients (55).

If studies with a larger group of animals confirm these results, it would suggest that cooperativity between BRCA1-deficiency and p53-deficiency can occur in mammary tumorigenesis but only with the exposure of the mammary gland to DNA damaging agents. This could reflect a direct role for BRCA1 in the response to DNA damage in mammary epithelial cells and would be consistent with the finding that BRCA1 is associated with RAD51 in the nucleus (35, 40). It is also possible that BRCA1 is itself a target for radiation-induced mutations. Exposure to radiation would then simply result in an increased frequency of loss of the wild-type *Brca1* allele. Other investigators have shown that the loss of the wild-type allele and duplication of the mutant locus are more likely to occur in tumors from irradiated *p53*$^{+/-}$ mice than in tumors arising spontaneously in this population of animals (56). The genetic mechanisms leading to loss of heterozygosity are poorly understood, and it remains unclear whether the frequency of this event will differ between different loci and at the same loci in different species. Some investigators have suggested that the high numbers of *Alu* repeats present in the human *BRCA1* gene may contribute to loss of heterozygosity at this locus during tumorigenesis (57). Differences in the structure and the number of repetitive elements in the mouse genome may make this a rare event unless animals are exposed to ionizing radiation. Alternatively, exposure to radiation may simply result in additional mutations that synergistically act with mutations in *Brca1* and *p53* in tumorigenesis. Finally, radiation damage may lead to initiating events critical for tumor development.

While the low frequency of mammary tumors precludes the establishment of the statistical significance of this finding, this is the first report of loss of the wild-type *Brca1* gene during mammary tumorigenesis in mouse. With the caveat that a large scale loss of heterozygosity that is not specifically targeted at *Brca1* may underlie the observed loss of BRCA1 expression, these studies suggest that a combination of genetic and environmental factors may allow the development of a mouse model to study the role of BRCA1 in tumorigenesis in the mouse.

Materials and Methods

Mice. p53 heterozygotes were obtained from Jackson Labs and were bred to *Brca1* heterozygotes generated in our colony (7, 37). Genomic DNA was recovered from tail biopsy, and genotypes were determined by PCR amplification, as described previously (7, 37). A subset of *Brca1*$^{+/+}$ and *Brca1*$^{+/-}$ female mice on a *p53*$^{+/-}$ background were treated with 5 Gy of ionizing radiation at 4–6 weeks of age. Mice killed because of health were necropsied, and tumor samples were frozen at −80°C for DNA extraction or fixed in 10% phosphate-buffered neutral formalin (pH 7.0) for histological examination. In most cases, cell lines were successfully derived from tumor samples. Briefly, nonnecrotic portions of each tumor were rinsed in PBS and then dispersed in DMEM-H media containing 15% fetal bovine serum, penicillin, streptomycin and gentamicin. The homogenized tumor samples were then plated directly on plastic 100-mm plates or on plates containing irradiated fibroblasts. Growth was monitored daily and nonadherent cells were separated from adherent cells when these two populations arose from the same tumor.

The Fisher's exact test was used for all of the statistical analyses.

Tumor Analysis/Histopathology. Tumor samples fixed in 10% phosphate-buffered neutral formalin were dehydrated and embedded in paraffin. Sections (3 μm) were processed for H&E staining. Classification was done in a blinded fashion by a veterinary pathologist (V. G.). Cytokeratin 18 levels were examined by Northern blot analysis. Total cellular RNA was isolated from frozen tumor samples with RNAzol B (TelTest, Inc., Friendswood, TX), according to the manufacturer's instructions. Twenty μg of RNA were electrophoresed in a 1.2% agarose formaldehyde gel, blotted to a Immobilon-NC transfer membrane (Millipore, Bedford, MA) and UV-cross-linked. The membranes were hybridized with a ^{32}P-labeled cytokeratin 18 cDNA for 1 h at 68°C using Quikhyb reagent (Stratagene, La Jolla, CA) and blots were washed twice with 2× SSC-0.1% SDS and once with 0.2× SSC-0.1%SDS at 42°C for 15 min each. The cytokeratin 18 cDNA was isolated through reverse-transcription followed by PCR of total RNA isolated from the mammary carcinomas (Invitrogen, Carlsbad, CA), using the primers 5′-ATGCCCCCAAATCTCAGGAC-3′ and 5′-AAGCGGCGGTAGGTGGCAATCTCT-3′. The PCR fragment was subcloned into the pCR 2.1 vector (Invitrogen, Carlsbad, CA) and sequenced. A ^{32}P-labeled probe was made and hybridized to a Northern blot containing total RNA from mammary tumors.

Loss of Heterozygosity. Total cellular RNA was isolated from frozen tumor samples as described above and transferred to nitrocellulose membranes. The membranes were probed with a ^{32}P-labeled *Brca1* cDNA containing exons 2 through 10 of *Brca1* (7) and hybridized as described above. Genomic DNA was prepared from additional tumor samples using DNAzol (Life Technologies, Grand Island, NY) according to the manufacturer's instructions. DNA was digested with *Eco*RV and analyzed by Southern blot with a probe containing a portion of intron 9 and exon 10 of the *Brca1* gene.

Southern blot analysis was used to examine loss of heterozygosity of p53. Genomic DNA from the tumors was digested with *Stu*I, electrophoresed in a 0.8% agarose gel, and transferred to Hybond-N nylon membrane (Amersham, Arlington Heights, Illinois). Hybridization was done using a ^{32}P-labeled probe specific for exons 7 through 9 of *p53* genomic sequence. This probe was prepared from murine genomic DNA using the primers 5′-GCCGGCTCTGAGTATACCACCATC-3′ and 5′-CTTTTGCGGGGGAGAGG-3′.

Flow Cytometry. Cells derived from a BRCA1-deficient, p53-deficient thymic lymphoma cell line and a p53-deficient thymic lymphoma control cell line were stained with phycoerthrin-conjugated anti-CD4 and fluorescein isothyocyanate-conjugated anti-CD8 antibodies (PharMigen, San Diego, CA) and analyzed using a FAScan cell sorter (San Jose, CA) and Cytomation, Inc. (Fort Collins, CO) data acquisition software.

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Growth Retardation, DNA Repair Defects, and Lack of Spermatogenesis in BRCA1-Deficient Mice

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BRCA1 is a nuclear phosphoprotein expressed in a broad spectrum of tissues during cell division. The inheritance of a mutant *BRCA1* allele dramatically increases a woman's lifetime risk for developing both breast and ovarian cancers. A number of mouse lines carrying mutations in the *Brca1* gene have been generated, and mice homozygous for these mutations generally die before day 10 of embryonic development. We report here the survival of a small number of mice homozygous for mutations in both the *p53* and *Brca1* genes. The survival of these mice is likely due to additional unknown mutations or epigenetic effects. Analysis of the $Brca1^{-/-}$ $p53^{-/-}$ animals indicates that BRCA1 is not required for the development of most organ systems. However, these mice are growth retarded, males are infertile due to meiotic failure, and the mammary gland of the female mouse is underdeveloped. Growth deficiency due to loss of BRCA1 was more thoroughly examined in an analysis of primary fibroblast lines obtained from these animals. Like $p53^{-/-}$ fibroblasts, $Brca1^{-/-}$ $p53^{-/-}$ cells proliferate more rapidly than wild-type cells; however, a high level of cellular death in these cultures results in reduced overall growth rates in comparison to $p53^{-/-}$ fibroblasts. $Brca1^{-/-}$ $p53^{-/-}$ fibroblasts are also defective in transcription-coupled repair and display increased sensitivity to DNA-damaging agents. We show, however, that after continued culture, and perhaps accelerated by the loss of BRCA1 repair functions, populations of $Brca1^{-/-}$ $p53^{-/-}$ fibroblasts with increased growth rates can be isolated. The increased survival of BRCA1-deficient fibroblasts in the absence of p53, and with the subsequent accumulation of additional growth-promoting changes, may mimic the events that occur during malignant transformation of BRCA1-deficient epithelia.

Germ line mutations in *BRCA1* account for 45% of all hereditary cases of breast cancer (35). Inheritance of one defective copy of *BRCA1* confers an estimated 80 to 90% overall lifetime risk for breast or ovarian cancer (15). Analysis of the primary structure of BRCA1 identified a number of potential functional motifs, including a RING finger domain in the N terminus and two putative nuclear localization sequences (5, 50, 52). In addition, two BRCT domains, characteristic of proteins involved in DNA repair, were identified in the C terminus (3). Expression of BRCA1 is detected early in embryonic development and continues to be found in a broad spectrum of tissues in the adult animal (27, 34). It has now been established that BRCA1 is a nuclear phosphoprotein expressed in dividing cells in a cell-cycle-specific manner, with maximal expression of BRCA1 occurring in the S phase (6, 42). Phosphorylation levels of BRCA1 also change throughout the cell cycle, peaking in late S phase (6, 42).

Despite extensive study, the function(s) of BRCA1 has not yet been clearly defined. The expression and phosphorylation of BRCA1 in a cell-cycle-specific manner suggest that this protein may be involved in the regulation of cell cycle transition. BRCA1 has been shown to induce expression of p21 and, more recently, BRCA1 has been shown to act as a p53 coactivator (38, 48, 54). In addition, a potential role as a transcriptional activator has been shown by the fusion of the C terminus of BRCA1 with a GAL4 DNA-binding domain (4, 36).

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The hyperphosphorylation and relocalization of BRCA1 with RAD51 to PCNA-containing foci after exposure to DNA-damaging agents suggest an additional or alternate function for this protein (43). RAD51 has been implicated in DNA repair, as demonstrated by the high sensitivity to DNA-damaging agents of *Saccharomyces cerevisiae rad51* mutants (17, 46). This increased sensitivity is thought to be due to a defect in recombinational repair of double-strand breaks. The recent demonstration that embryonic stem (ES) cells deficient in BRCA1 are more sensitive to some DNA-damaging agents provides direct evidence supporting a role for BRCA1 in maintaining genomic integrity (19). These BRCA1-deficient cells are unable to carry out transcription-coupled repair (TCR) after exposure to DNA-damaging agents, demonstrating the participation of this protein in at least one cellular repair pathway. This repair system, which preferentially repairs the transcribed strand of active genes, is important for the removal of lesions for which the global repair process is too slow.

Several mouse lines carrying mutations in the *Brca1* gene have been generated (18, 21, 32, 33). Unlike humans, mice carrying a mutant *Brca1* allele do not display an increased risk for tumor formation. This observation and the early embryonic lethality of mice homozygous for the mutant allele have to date limited the contribution of these models to understanding the role of BRCA1 in tumorigenesis. However, we have recently reported the generation of five mammary tumors from mice heterozygous for both a mutant *Brca1* allele and *p53* allele after exposure to ionizing radiation (9). Furthermore, loss of heterozygosity of both *Brca1* and *p53* could be demonstrated in tumor tissue obtained from three of these tumors. This suggests that exposure of $Brca1^{+/-}$ mice to specific environmental

risk factors may be necessary for the development of mammary tumors during the short lifespan of the mouse. It also suggests that mutations in genes, such as *p53*, which confer a growth advantage and in particular allow the continued growth of cells having incurred DNA damage may be critical to the development of these tumors.

Evidence for a relationship between p53 and BRCA1 is further suggested by the demonstration that survival of BRCA1-deficient embryos is extended in the absence of p53 expression (22, 33). However, the *Brca1*$^{-/-}$ *p53*$^{-/-}$ embryos still die early in embryogenesis, precluding examination of the loss of BRCA1 in later stages of development and in tumorigenesis. The early lethality of the *Brca1*$^{-/-}$ *p53*$^{-/-}$ embryos did not permit the generation of *Brca1*$^{-/-}$ *p53*$^{-/-}$ embryonic fibroblast cell lines.

We report here the survival and characterization of a small number of *Brca1*$^{-/-}$ *p53*$^{-/-}$ mice after extensive breeding of *Brca1*$^{+/-}$ *p53*$^{-/-}$ animals. Although growth retarded, the development of the somatic tissues of these mice is largely normal. Male mice are infertile due to a defect in spermatogenesis, thus defining a role for BRCA1 in meiosis. *Brca1*$^{-/-}$ *p53*$^{-/-}$ animals die of tumors typical of *p53*$^{-/-}$ mice; however, tumor latency is reduced compared to that observed in the *p53*$^{-/-}$ population in our colony. Fibroblast lines were generated from the *Brca1*$^{-/-}$ *p53*$^{-/-}$ mice and compared to the fibroblast lines from both wild-type and *p53*$^{-/-}$ animals. While, as expected, *p53*$^{-/-}$ fibroblasts grow rapidly, the growth of early passage *Brca1*$^{-/-}$ *p53*$^{-/-}$ fibroblasts is similar to that of wild-type cells. This decreased growth rate is largely the result of a decrease in the survival of the BRCA1-deficient cells. We also show here that the *Brca1*$^{-/-}$ *p53*$^{-/-}$ cells are more sensitive than *p53*$^{-/-}$ cells to DNA-damaging agents and that the ability of the cells to carry out transcription-coupled repair in response to DNA damage is compromised. These observations support the hypothesis that BRCA1 plays an important role in DNA repair pathways. In summary, our results suggest that loss of heterozygosity of BRCA1 is not an initiating event in tumorigenesis. Cells must instead attain a growth advantage, by mechanisms such as loss of cell cycle checkpoint control or responsiveness to trophic factors, for loss of BRCA1 function to predispose towards tumorigenesis.

MATERIALS AND METHODS

Animal husbandry. *p53*$^{+/-}$ mice were obtained from Jackson Labs and bred to *Brca1*$^{+/-}$ mice generated in our colony (18, 26). Genomic DNA was recovered from tail biopsy, and genotypes were determined by PCR amplification, as described elsewhere (18, 26). All three *Brca1*$^{-/-}$ *p53*$^{-/-}$ mice were generated from matings of *Brca1*$^{+/-}$ *p53*$^{-/-}$ females with a *Brca1*$^{+/-}$ *p53*$^{-/-}$ male. The *Brca1* genotype was verified by Southern blotting analysis. Genomic DNA was digested with *Eco*RV and analyzed by Southern blot with a probe containing a portion of intron 9 and exon 10 of the *Brca1* gene. Each mouse was euthanized when moribund and then necropsied. Tissues were fixed in 4% paraformaldehyde, dehydrated, and embedded in paraffin. Sections were cut (5 μm) and stained with hematoxylin and eosin. Testes sections were also stained with toluidine blue. The TUNEL (terminal deoxynucleotidytransferase-mediated dUTP-biotin nick end labeling) assay was performed on testes sections to determine the level of apoptosis, as per the manufacturer's instructions (Trevigen, Gaithersburg, Md.). Additional testes sections were immunostained with a rabbit polyclonal antibody for HSP70-2 (13) and staining was detected by a Peroxidase Elite ABC Kit (Vector Laboratories, Burlingame, Calif.) as per the manufacturer's instructions. Whole-mount preparations were done as described elsewhere (37a). Briefly, mammary epithelium was fixed in 10% phosphate-buffered neutral formalin and then rehydrated. The mammary glands were stained overnight with carmine alum, dehydrated, cleared in xylene, and mounted in Permount.

Generation of cell lines. Primary fibroblasts were obtained from the skin and ears of all three *Brca1*$^{-/-}$ *p53*$^{-/-}$ mice. These tissues were washed repeatedly with phosphate-buffered saline (PBS), finely minced, and digested overnight with collagenase (Life Technologies, Grand Island, N.Y.). The fibroblasts released were cultured in Dulbecco modified Eagle medium (DMEM) with 10% fetal bovine serum (FBS) and supplemented with L-glutamine, penicillin, streptomycin, and gentamicin at 37°C with 5% CO_2. Cells were passaged before they reached 100% confluence. Fibroblast lines were also generated from two *Brca1*$^{+/-}$ *p53*$^{-/-}$ mice and a *Brca1*$^{+/+}$ *p53*$^{+/+}$ mouse.

Analysis of cellular growth. Determination of cellular growth was performed as described elsewhere (12). Briefly, asynchronous fibroblasts from each cell line were plated onto a series of 35-mm dishes. Each of the *Brca1*$^{-/-}$ *p53*$^{-/-}$ lines was plated at a density of 4×10^4 cells per well, while 2×10^4 cells of each of the *Brca1*$^{+/-}$ *p53*$^{-/-}$ lines per well were plated. These values were determined based on the slower growth of the *Brca1*$^{-/-}$ *p53*$^{-/-}$ cells. Cells from two wells per line were counted daily by using trypan blue exclusion. The medium was changed daily for the remaining cells. For cell cycle analysis, asynchronous fibroblasts from each cell line were harvested after a 4-h incubation with 10 μM bromodeoxyuridine (BrdU; Boehringer Mannheim, Indianapolis, Ind.) and then fixed in cold 70% ethanol. Fixed cells were stored at 4°C until analysis. Nuclei were released by treatment with 0.08% pepsin in 0.1 N HCl at 37°C for 20 min. Nuclei were then treated with 2 N HCl at 37°C for 20 min, followed by neutralization with 0.1 M sodium borate. Cells were then incubated with the fluorescein isothiocyanate-conjugated anti-BrdU antibody (Becton Dickinson, San Jose, Calif.) and counterstained with propidium iodide (50 μg/ml) containing RNase (5 μg/ml) overnight at 4°C. Cell cycle analysis was performed on a FACScan cell sorter (San Jose, Calif.) with Cytomation data acquisition software (Fort Collins, Colo.). WinMDI software was used to determine the percentage of cells in each cell cycle phase.

Analysis of cellular death. To determine the ratio of dead to live cells, asynchronous cells from each line were plated onto six 100-mm dishes and grown to 70 to 80% confluence. The medium was removed, and the cells were incubated with 8 ml of fresh medium for 7 h. For quantitation of live cells, each plate was trypsinized, and cells were counted by trypan blue exclusion with a hemocytometer. For the dead cell count, the medium from each plate was centrifuged in separate tubes. Pelleted cells were resuspended in 10% FBS in PBS and cytospun onto a microscope slide. The dead cells were stained with Diff-Quik stain (Dade Diagnostics of P.R., Inc., Miami, Fla.), and photographs from random areas of each slide were taken to facilitate counting. Only cells containing nuclei were counted.

To confirm the decrease in cell viability, two additional assays were performed. For the first assay, nonadherent cells from two *Brca1*$^{-/-}$ *p53*$^{-/-}$ cell lines and one *Brca1*$^{+/-}$ *p53*$^{-/-}$ cell line were harvested as described above. The nonadherent cells were counted and plated in 1 ml of medium in 24-well dishes. Adherent cells were then trypsinized and plated at an equivalent density. Live adherent cells (as determined by trypan blue exclusion) were counted from each well 24 h later. The second assay utilized the MTT assay (Roche Molecular Biochemicals, Indianapolis, Ind.) with the following modifications. Nonadherent cells from two *Brca1*$^{-/-}$ *p53*$^{-/-}$ lines and one *Brca1*$^{+/-}$ *p53*$^{-/-}$ line were harvested as described above, resuspended in <1 ml of medium, and counted. Adherent cells were trypsinized and counted, and 5,000, 10,000, 20,000, and 40,000 cells (nonadherent and adherent) were plated in duplicate. All cells were immediately incubated with the MTT labeling reagent for 4 h and then incubated with the solubilization solution for 24 h. Conversion to formazan dye was detected at 595 nm.

To quantitate apoptosis in this dead cell population, the TUNEL assay was performed on the nonadherent cells immobilized on a microscope slide (as described above) as per the manufacturer's directions (Trevigen). To determine the percentage of apoptotic cells, photographs from random areas of each slide were taken.

For p21 protein analysis, asynchronous *Brca1*$^{+/-}$ *p53*$^{-/-}$, *Brca1*$^{-/-}$ *p53*$^{-/-}$, and wild-type fibroblasts were washed three times with PBS and mechanically lysed for 15 min at 4°C in 1% CHAPS {3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate} buffer (containing 20 mM Tris, 143 mM KCl, 5 mM EDTA, 10 mM dithiothreitol, 20 mM NaCl) supplemented with a Complete Mini Protease Inhibitor Cocktail Tablet (Roche Molecular Biochemicals, Indianapolis, Ind.). Supernatants of the cell lysates were collected following centrifugation (15,000 × *g* at 4°C for 15 min). Five micrograms of protein was resolved on a sodium dodecyl sulfate–12% polyacrylamide gel and then transferred to nitrocellulose. p21 was detected by a monoclonal antibody (F-5, 1:1,000 dilution; Santa Cruz Biotechnology, Inc., Santa Cruz, Calif.), followed by incubation with a horseradish peroxidase-conjugated secondary antibody and visualized with chemiluminescence regents (Pierce, Rockford, Ill.).

DNA damage analysis. The survival of the fibroblasts following treatment with DNA-damaging agents was determined as described elsewhere (40). In brief, to determine survival after ionizing radiation, asynchronous cells were irradiated in suspension at room temperature by using a ^{137}Cs source. The medium was changed immediately after irradiation. *Brca1*$^{-/-}$ *p53*$^{-/-}$ cells were then plated at a density of 4×10^4 cells per 35-mm dish and *Brca1*$^{+/-}$ *p53*$^{-/-}$ cells were plated at a density of 2×10^4 cells per dish. The surviving cells were counted 4 days later by trypan blue exclusion and expressed as a percentage, by using untreated, nonconfluent cells as the 100% level. To measure survival after hydrogen peroxide treatment, *Brca1*$^{-/-}$ *p53*$^{-/-}$ cells were plated at a density of 4×10^4 cells per 35-mm dish, and *Brca1*$^{+/-}$ *p53*$^{-/-}$ cells were plated at a density of 2×10^4 cells per dish. When cells were adherent 10 h later, cells were incubated with hydrogen peroxide for 15 min at 37°C. Cells were then washed twice with medium. Quantitation of cells was done 4 days later by trypan blue exclusion, and this is expressed as a percentage, using untreated, nonconfluent cells as the 100%

value. To determine survival after UV irradiation, cells were plated as described above for the hydrogen peroxide treatment. The cells were incubated for 10 h to allow adherence. The medium was then aspirated, and cells were irradiated by using a Stratalinker (Stratagene, La Jolla, Calif.). Medium was then immediately added. Quantitation was done 4 days later by trypan blue exclusion and expressed as a percentage, by using untreated, nonconfluent cells as the 100% level. Each of these experiments was conducted in duplicate, and in each replicate, the cell survival was determined four times at each experimental point.

Transcription-coupled repair analysis was performed as previously described (29). For labeling of parental DNA, cells were grown for 3 days in medium containing 1 μCi of [^{3}H]thymidine (TdR; Amersham) per ml in the presence of 5 μg of unlabelled TdR per ml. The medium was removed, and the cells were grown for an additional 2 days in nonradioactive medium. Prior to irradiation, cells were incubated for 1 h in medium containing 10 μM BrdU and 1 μM fluorodeoxyuridine (FdUrd). Cultures were washed with PBS and were irradiated with either a ^{60}Co gamma source at dose rates of 1.3 to 1.6 Gy/min or at 254 nm by using a germicidal lamp at an incident dose rate of 0.63 J/m^2/s. After irradiation, the cultures were either harvested immediately or incubated for various lengths of time in medium containing 10 μM BrdU and 1 μM FdUrd. For measurements of thymine glycol production and repair, cell cultures were exposed to 10 mM H_2O_2 for 15 min at 37°C. The cells were then incubated for various lengths of time in this medium. After incubation, the cultures were washed twice with PBS and harvested by lysis in 10 mM Tris–10 mM EDTA–0.5% sodium dodecyl sulfate.

Repair analysis of UV and ionizing-radiation-induced DNA damage was carried out as previously described (28) by using a monoclonal antibody against BrUra. Briefly, purified DNA, digested with *Bam*HI, was centrifuged to equilibrium in a CsCl gradient to separate parental density DNA (containing BrUra-substituted repair patches) from hybrid density DNA (synthesized by semiconservative replication). Unreplicated, parental-density DNA was then reacted with the monoclonal antibody against BrUra. The DNA bound by the antibody was separated by centrifugation. Aliquots of the supernatant and pellet were assayed for radioactivity by liquid scintillation counting to determine the relative amount of DNA bound by the antibody.

Repair analysis of thymine glycols was carried out as previously described (30) with a monoclonal antibody that recognizes thymine glycols in DNA. Heat-denatured DNA (50 to 100 μg) was incubated with the antibody (1:1,000 dilution) in PBS containing 0.1% bovine serum albumin for 1 h at 37°C, followed by an overnight incubation at 4°C. An equal volume of ice-cold saturated ammonium sulfate in PBS was added, and the mixture was incubated at 4°C for 15 min. The DNA bound by the antibody was collected as a pellet by centrifugation. Aliquots of the supernatant and pellet were assayed for radioactivity by liquid scintillation counting to determine the relative amount of DNA bound by the antibody.

Equal amounts of DNA, based on the ^{3}H prelabel, from the supernatant and pellet were electrophoresed on 0.7% neutral agarose gels. After electrophoresis, the DNA was transferred to a GeneScreen Plus (NEN) membrane. RNA probes were prepared as previously described (29). After hybridization, the membranes were washed and exposed to Kodak XAR-5 X-ray film. The intensity of hybridization to the fragments of interest was measured by using a Bio-Rad phosphorimager. The value for the density of each fragment was multiplied by the amount of DNA in the bound or free fractions to obtain the total amount of each gene in both fractions. The percentage of the dihydrofolate reductase gene (*DHFR*) gene bound by the antibody was then calculated from the total amount of the gene in the bound fraction divided by the total amount of the gene in the bound plus free fractions. For the studies on the time course of repair of thymine glycols, the percentage of the genes containing thymine glycols immediately after treatment was set at 100%.

RESULTS

Identification of *Brca1*$^{-/-}$ *p53*$^{-/-}$ mice. Mice heterozygous for the *Brca1* mutation, *Brca1*$^{\Delta 223-763}$, were intercrossed with mice carrying a mutant *p53* allele to obtain animals heterozygous for both mutations (18, 26). While litters obtained from the intercrossing of the derived double-heterozygous animals yielded *p53*$^{-/-}$ animals at reported frequencies, no *Brca1*$^{-/-}$ animals were identified among either the *p53*$^{-/-}$ or *p53*$^{+/-}$ offspring. *Brca1*$^{+/-}$ *p53*$^{-/-}$ mice were further intercrossed and again no double-homozygous animals were obtained on examination of 56 offspring obtained from nine different litters (9). Further expansion of this mouse population, however, led to the identification of three *Brca1*$^{-/-}$ mice, two males and a single female, all also homozygous for the *p53* mutant allele. Southern blot analysis revealed that only the targeted *Brca1* allele was present (data not shown). The absence of the normal *Brca1* mRNA transcript was further verified by Northern blot analysis (data not shown). Interestingly, two of these animals not only shared one parent but were further related in that the dam of the second *Brca1*$^{-/-}$ *p53*$^{-/-}$ mouse was the sibling of the first *Brca1*$^{-/-}$ *p53*$^{-/-}$ animal identified.

All three double-homozygous animals were severely growth retarded. The female mouse reached a maximum weight of 14 g at 12 weeks of age compared to the average weight of 21 g seen in the p53-deficient females in our colony. Similarly, the two *Brca1*$^{-/-}$ *p53*$^{-/-}$ males weighed only 16.3 and 14.5 g compared to an average weight of 27 g seen in *p53*$^{-/-}$ males at this age.

Similar to the majority of the *p53*$^{-/-}$ mice, the *Brca1*$^{-/-}$ *p53*$^{-/-}$ mice developed tumors and were killed when moribund. All three mice died of lymphomas, the most common tumor seen in the *p53*$^{-/-}$ populations (14, 26). Necropsy of one of the male mice also revealed the presence of a hemangiosarcoma. Lymphomas and hemangiosarcomas are seen in 59 and 18%, respectively, of the *p53*$^{-/-}$ population (23). While the tumor types observed in the *Brca1*$^{-/-}$ *p53*$^{-/-}$ mice are consistent with the tumors seen with p53 deficiency, the survival age of the double-homozygous animals was substantially less than the average 19 weeks of survival for *p53*$^{-/-}$ animals in our colony. All three *Brca1*$^{-/-}$ *p53*$^{-/-}$ mice died at between 10 and 12 weeks of age.

Alopecia (generalized loss of fur), as well as an unusually high percentage of white hairs dispersed through their coats, was noted in two of the three double-homozygous animals. On gross observation the skin of all the double homozygous animals appeared thinner and more transparent than that of the p53-deficient mice. Histological analysis revealed atrophy of follicles and adnexa with a reduction in the size of both the sebaceous glands and follicles. Follicles were also reduced in number in comparison to *p53*$^{-/-}$ mice.

Loss of BRCA1 did not result in morphological or histological changes in the kidney, liver, brain, lungs, pancreas, or gastrointestinal tract, other than those secondary to metastatic hematopoietic neoplasms. Histopathologic lesions were primarily confined to the epithelia of the parotid, mammary, and prostate glands. Parotid salivary glands exhibited diffuse acinar atrophy that is usually observed only in geriatric animals. Mild and multifocal cytomegaly and karyomegaly suggestive of polypoidy were seen in the acinar epithelium (Fig. 2E and F). Parotid ductal cells were normal, as was the histology of the submandibular and sublingual glands. While the coagulated and vesicular glands of the male *Brca1*$^{-/-}$ *p53*$^{-/-}$ did not differ from control mice, a rare benign granular cell tumor, usually found in older animals, was observed in the prostate glands. Cytomegaly and karyomegaly were also observed in the prostate glands.

BRCA1 is expressed in the mouse mammary gland during puberty, pregnancy, and regression after discontinuation of lactation (34). The mammary gland in the female *Brca1*$^{-/-}$ *p53*$^{-/-}$ mouse was examined by whole mount. While the primary ducts were easily identified, a substantial reduction in the branching is seen in comparison to whole mounts prepared from an age-matched *p53*$^{-/-}$ virgin mouse (compare Fig. 1A and B). In addition, the end buds of the BRCA1-deficient female appeared underdeveloped (compare Fig. 1C and D), and the fat pad in the region of the end buds contains finely dispersed cellular material. To further examine the structure of the mammary gland, the fat pad was embedded for histological analysis (Fig. 2A through D). Primary ducts in mammary glands from *Brca1*$^{-/-}$ *p53*$^{-/-}$ mice were fewer and more dilated than those in the mammary gland of the *p53*$^{-/-}$ mice. In the *Brca1*$^{-/-}$ *p53*$^{-/-}$ mice (Fig. 2B through D), the ducts were surrounded by loose, concentric aggregates of individualized

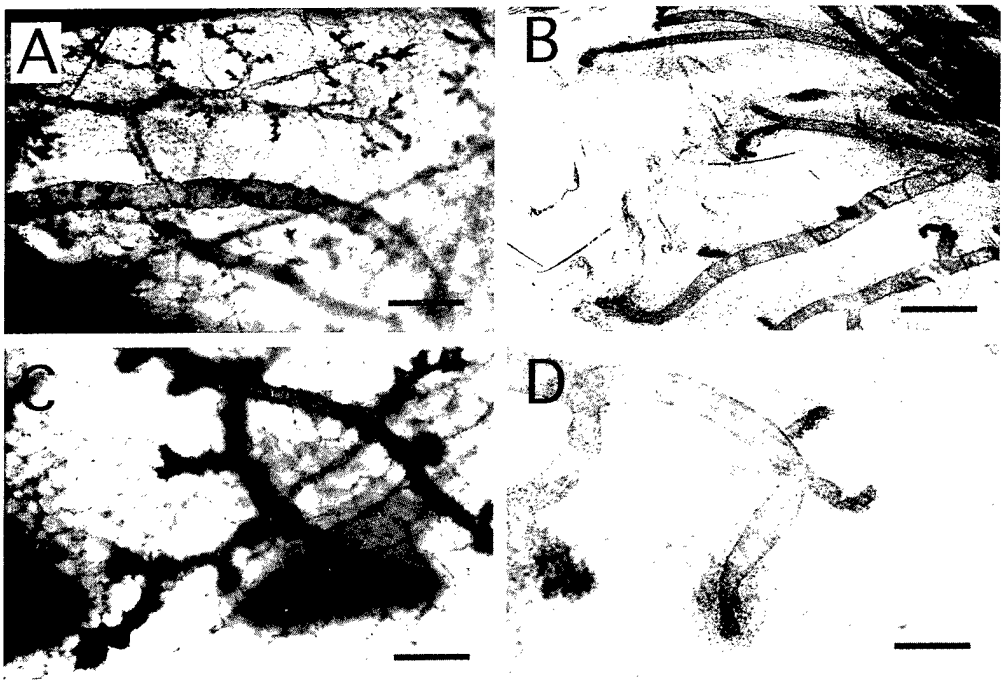


FIG. 1. Abnormal end bud formation and decreased ductal branching in BRCA1-deficient mice. (A) Mammary epithelium of a 12-week-old $p53^{-/-}$ female mouse displays extensive branching and numerous end buds. (B) In contrast, very few branches from the primary ducts are seen in the mammary gland of the $Brca1^{-/-}$ $p53^{-/-}$ female mouse. At a higher magnification, many end buds can be visualized in the $p53^{-/-}$ female (C), while only a few underdeveloped end buds are seen in the $Brca1^{-/-}$ $p53^{-/-}$ mouse (D). Magnification bars: A and B, 500 μm; C and D, 200 μm.

oval cells. These cells had variably sized round nuclei and scant-to-modest eosinophilic cytoplasm. Some of the periductal cells had pyknotic nuclei suggestive of cell death. These cells may be myoepithelial cells or ductal cells that failed to migrate and differentiate normally. We cannot, however, exclude the presence of inflammatory cells in these cellular aggregates on the basis of morphology.

Spermatogenesis is disrupted in the $Brca1^{-/-}$ $p53^{-/-}$ mice. Male $Brca1^{-/-}$ $p53^{-/-}$ mice failed to impregnate female mice. This deficiency was not the result of a failure of the mice to copulate, since copulation plugs were observed in females following mating with a $Brca1^{-/-}$ $p53^{-/-}$ male. On gross examination, the testes of both of the double-homozygous males were smaller in size than those of wild-type males of similar weight. We next compared the histological development of the seminiferous tubules of these testes to those from $p53^{-/-}$ control animals of similar age. Sertoli cells, the supporting cells within the tubules, were seen throughout the testes of the $Brca1^{-/-}$ $p53^{-/-}$ males, suggesting that spermatogenesis was not aborted due to loss of this cell population.

As expected, the seminiferous tubules of 10-week-old $p53^{-/-}$ male mice contain cells in all stages of spermatogenesis, including spermatogonia, spermatocytes and round and fully elongated spermatids (Fig. 3A and C). The spermatogonia, the stem cells of the testes, are located at the periphery of a seminiferous tubule. The developing spermatocytes, located just interior to the spermatogonia, undergo meiosis I and II to form the haploid round spermatids, which are easily identified by their light-staining nuclei. Fully elongated spermatids with tails extending into the lumen form from these round spermatids after spermiogenesis. The seminiferous tubules of the $Brca1^{-/-}$ $p53^{-/-}$ males appeared smaller in diameter in comparison to those of an age-matched $p53^{-/-}$ male and contained fewer cells (Fig. 3B). While the spermatogonia appeared relatively normal in both $Brca1^{-/-}$ $p53^{-/-}$ males, spermatids and spermatozoa were not observed (Fig. 3D). In addition, only two pachytene spermatocytes, identified by their distinct chromatin structure, were observed on survey of the entire section. To further define the defect in spermatogenesis of the double-homozygous animals, sections were stained with an antibody to HSP70-2 (13). This antibody is specific for a heat shock protein whose expression is initiated as cells enter into meiosis. As expected, the peripheral layer of cells, the spermatogonia, of both the $p53^{-/-}$ (Fig. 3G) and $Brca1^{-/-}$ $p53^{-/-}$ (Fig. 3H) mice failed to stain with this antibody. Cells luminal to this single layer of spermatogonia present in some of the seminiferous tubules of the testes of the $Brca1^{-/-}$ $p53^{-/-}$ males stained brightly with this antibody, indicating that these cells had likely entered into meiosis. Together with the absence of pachytene spermatocytes, this suggests that meiotic failure occurs during prophase I of meiosis in the $Brca1^{-/-}$ $p53^{-/-}$- mice.

To determine whether meiotic failure observed in the $Brca1^{-/-}$ $p53^{-/-}$ mice was paralleled by increased levels of apoptosis of spermatocytes, tissue sections obtained from the testes of both control $p53^{-/-}$ mice and $Brca1^{-/-}$ $p53^{-/-}$ animals were analyzed by using the TUNEL assay. Only a modest increase in the number of stained cells was observed (Fig. 3E and F). In 25 randomly examined seminiferous tubules, 13 apoptotic cells were seen in the testes of the $p53^{-/-}$ control mice compared to 37 apoptotic cells per 25 tubules in the testes of the $Brca1^{-/-}$ $p53^{-/-}$ mouse. Examination of other tissues from the double-homozygous animals also failed to reveal an increased level of apoptosis.

Growth properties of primary skin fibroblasts. Skin fibroblasts were prepared from $Brca1^{-/-}$ $p53^{-/-}$ mice, $Brca1^{+/-}$ $p53^{-/-}$ mice, and wild-type mice, and the growth properties of these primary cultures were examined. $Brca1^{+/-}$ $p53^{-/-}$ fibroblasts had growth characteristics similar to $Brca1^{+/+}$ $p53^{-/-}$ lines. For simplicity we will refer to the $Brca1^{+/-}$ $p53^{-/-}$ lines as $p53^{-/-}$ lines throughout the peper. Before these experi-

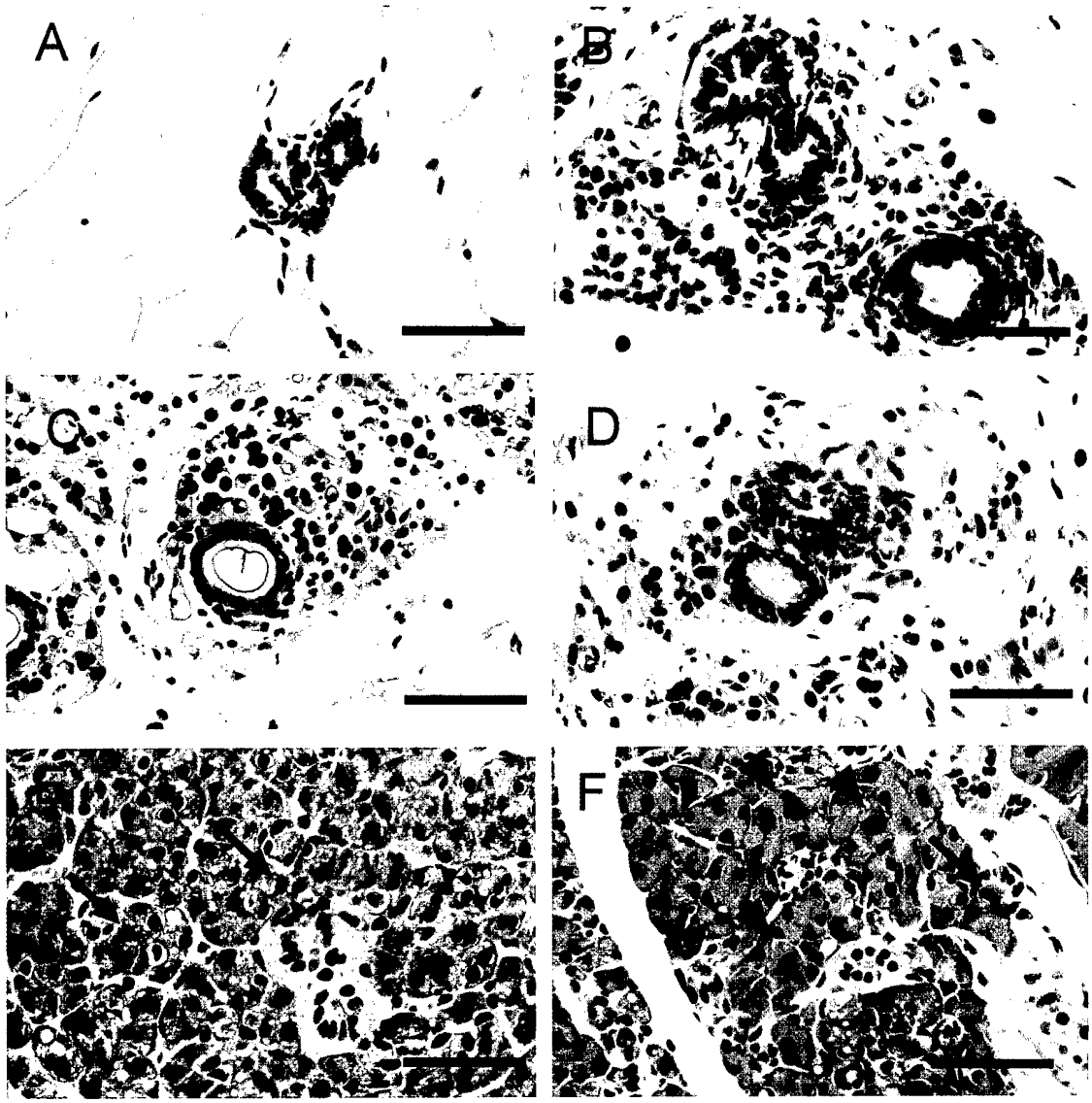


FIG. 2. Cellular abnormalities within the mammary and salivary glands of the *Brca1*$^{-/-}$ *p53*$^{-/-}$ mice. (A) Histological analysis of the mammary gland ducts from a *p53*$^{-/-}$ mouse shows a duct lined by one to two layers of low cuboidal epithelia. Mammary ducts of the *Brca1*$^{-/-}$ *p53*$^{-/-}$ female are dilated and lined by a single layer of flattened-to-low cuboidal cells. (B to D) In some regions, the ducts are surrounded by an array of individual cells. While acinar cells in the *p53*$^{-/-}$ salivary gland appear to be uniform (E), cytomegaly and karyomegaly (arrows) are seen sporadically throughout the acinar units of the salivary glands of the *Brca1*$^{-/-}$ *p53*$^{-/-}$ animals (F). Staining was done with hematoxylin and eosin. Magnification bar, 50 μm.

ments were begun, the plating efficiency of the fibroblasts of each genotype was established. This was done by plating known numbers of cells, allowing the cells to adhere, and then determining the number of viable surviving cells 7 h later. At this time point, cell numbers reflect the plating efficiency of the cells rather than differences in the growth rates of the various lines. No differences between the plating efficiencies of the *Brca1*$^{-/-}$ *p53*$^{-/-}$, *p53*$^{-/-}$, and wild-type lines were observed (data not shown).

Proliferation rates and saturation densities of fibroblast cultures prepared from wild-type mice and from two *p53*$^{-/-}$ and two *Brca1*$^{-/-}$ *p53*$^{-/-}$ mice were determined at both low and high passage points (Fig. 4A and B, respectively). Early-passage *p53*$^{-/-}$ fibroblasts grew more quickly and reached higher saturation densities than cells obtained from wild-type control animals. Both the growth rate and the saturation density of the early passage *Brca1*$^{-/-}$ *p53*$^{-/-}$ fibroblasts resembled more closely those of the wild-type cells than those observed for the *p53*$^{-/-}$ cells.

As an increase in the expression of *p21* in BRCA1-deficient embryos (embryonic days 6 to 8) was previously reported (21), it was of interest to determine whether a similar increase might underlie the decreased growth rate of the *Brca1*$^{-/-}$ *p53*$^{-/-}$ cells. Because these cells are deficient in p53, such an increase if observed would be dependent on stimulation of p21 expression by p53-independent pathways (1, 39, 55). Protein levels of p21 were determined by Western blot analysis in asynchronous, proliferating *Brca1*$^{-/-}$ *p53*$^{-/-}$, *p53*$^{-/-}$, and wild-type fibroblasts. While high levels of p21 were observed in the wild-type fibroblasts, a finding consistent with the rapid senescence of these cells, only very low levels of p21 were detected in the *Brca1*$^{-/-}$ *p53*$^{-/-}$ and *p53*$^{-/-}$ fibroblasts. These results fail to support a role of p21 in the decreased growth rate of the *Brca1*$^{-/-}$ *p53*$^{-/-}$ cells (Fig. 5A).

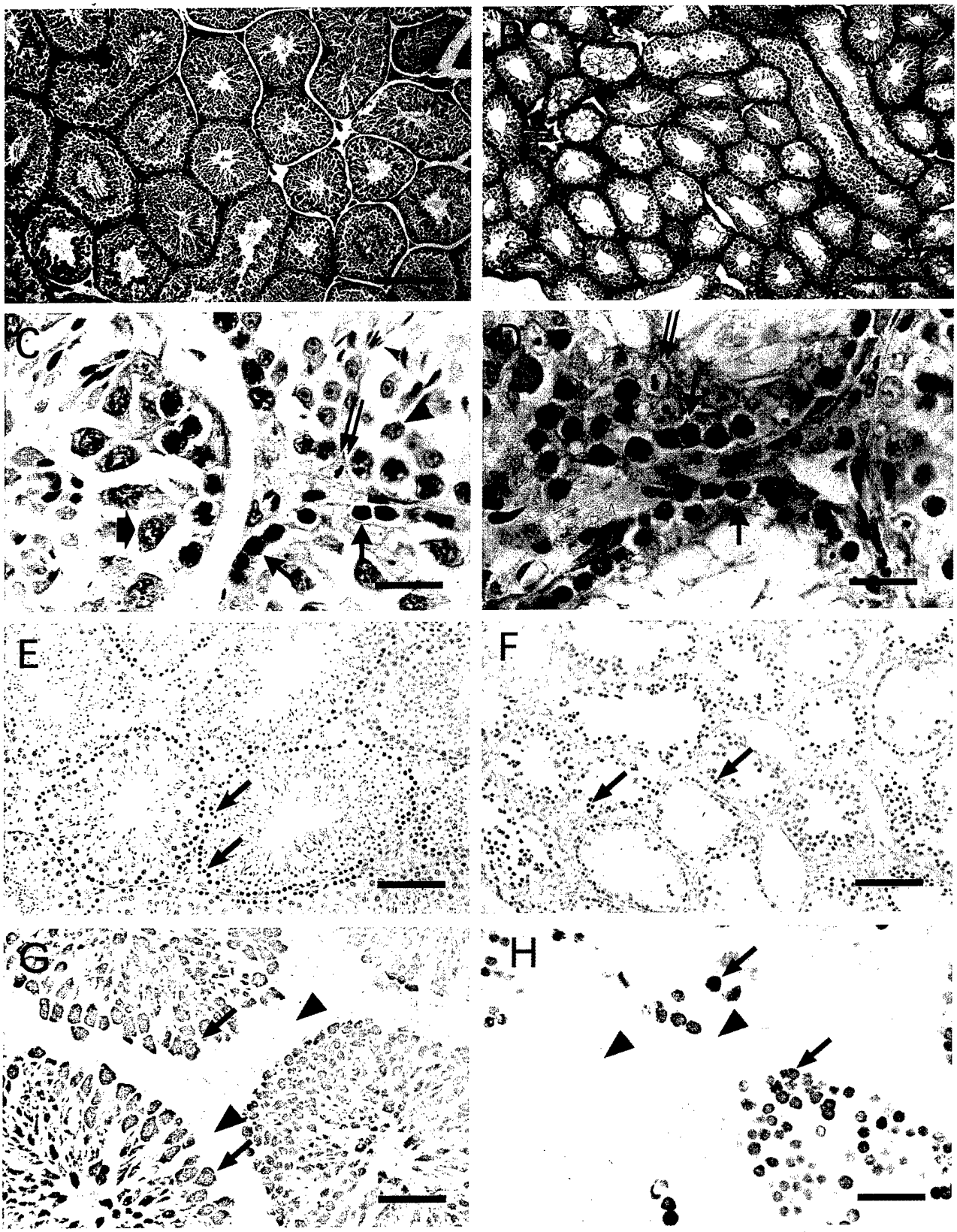


FIG. 3. Absence of spermatids and spermiogenesis in a BRCA1-deficient male. The testes and seminiferous tubules of a *Brca1*$^{-/-}$ *p53*$^{-/-}$ male (B) are smaller than those of an age-matched *p53*$^{-/-}$ control male (A). While the seminiferous tubules of the *p53*$^{-/-}$ control male mouse (C) contained all of the cells involved in spermatogenesis (spermatogonia, thin arrows; spermatocyte, wide arrow; spermatid, large arrowhead; spermatozoa, small arrowhead), only spermatogonia (solid arrows) can be seen in the testes of the *Brca1*$^{-/-}$ *p53*$^{-/-}$ male (D). Sertoli cells (double arrow) were seen in abundance in the seminiferous tubules of the *Brca1*$^{-/-}$ *p53*$^{-/-}$ males and the *p53*$^{-/-}$ males (C and D). (E) Apoptosis as determined by the TUNEL assay (apoptotic cells, arrow) occurs rarely in spermatogenesis in *p53*$^{-/-}$ males. Apoptotic

We next wished to determine whether the growth differences between the cell lines would be maintained after extended in vitro culture (passage 15 to 25). Senescence of the wild-type cells by passage 8 precluded their inclusion in this experiment. While a small increase in the growth rate and saturation density of one of the $p53^{-/-}$ lines was observed on comparison of early- and late-passage cell cultures, a dramatic increase in both parameters was seen in both late passage $Brca1^{-/-}$ $p53^{-/-}$ lines (Fig. 4B).

Cell cycle kinetics of $Brca1^{-/-}$ $p53^{-/-}$ fibroblasts. Previous studies of $p53^{-/-}$ and wild-type embryonic fibroblasts indicated that the increased proliferation rate of the $p53^{-/-}$ cells correlated with an increase in the percentage of cells in S phase and a decrease in the number of cells in G_0/G_1 (24). These alterations are believed to stem largely from a loss of the G_1/S checkpoint in the $p53^{-/-}$ lines. To determine whether further alterations in the cell cycle kinetics underlie the decreased growth rate of the $Brca1^{-/-}$ $p53^{-/-}$ lines, asynchronous early-passage fibroblasts of each of the three genotypes were labeled with BrdU, stained with propidium iodide, and subjected to flow cytometry analysis (Fig. 4C). As reported previously and consistent with observed growth rates, a higher percentage of $p53^{-/-}$ cells are observed in S phase than seen in similarly labeled wild-type cells (24). In addition, loss of the spindle checkpoint due to p53 deficiency results in an increased percentage of tetraploid cells (11). Surprisingly, in light of the growth properties of the $Brca1^{-/-}$ $p53^{-/-}$ cells, the percentage of these cells in S phase was similar to that observed in the $p53^{-/-}$ fibroblast cultures. Thus, while at low passage the growth rate of the $Brca1^{-/-}$ $p53^{-/-}$ lines is similar to wild-type lines, the percentage of cells in S phase is approximately two to three times greater than seen in this control population.

To determine whether the increased proliferation rate seen in the late-passage $Brca1^{-/-}$ $p53^{-/-}$ cells was a result of an increased rate through the cell cycle, BrdU and DNA content analyses were performed on asynchronous cells from a $p53^{-/-}$ control line and two $Brca1^{-/-}$ $p53^{-/-}$ lines. Again, the percentage of cells in S phase was similar in all three lines ($p53^{-/-}$ control, 58.2%; $Brca1^{-/-}$ $p53^{-/-}$ line 1, 64.2%; $Brca1^{-/-}$ $p53^{-/-}$ line 2, 57.6%).

Decreased growth rate of $Brca1^{-/-}$ $p53^{-/-}$ cells is due to an increased rate of cellular death. In order to resolve the discrepancy between the observed growth rate of the $Brca1^{-/-}$ $p53^{-/-}$ cells and the percentage of the cells in S phase, we next determined the rate of cellular death in the cultures of $Brca1^{-/-}$ $p53^{-/-}$, $p53^{-/-}$, and wild-type fibroblasts. Dead cells can be collected and enumerated easily in these cultures because after cellular death, the fibroblasts become nonadherent, lift off from the surface of the culture dishes, and can be harvested by aspiration and centrifugation of the tissue culture medium. Collection of these cells from cultures of all three genotypes and analysis with trypan blue exclusion dye confirmed that virtually all of the cells harvested in this manner were dead and that the collection procedure had not resulted in disturbance of the loosely attached mitotic cells (Fig. 5B).

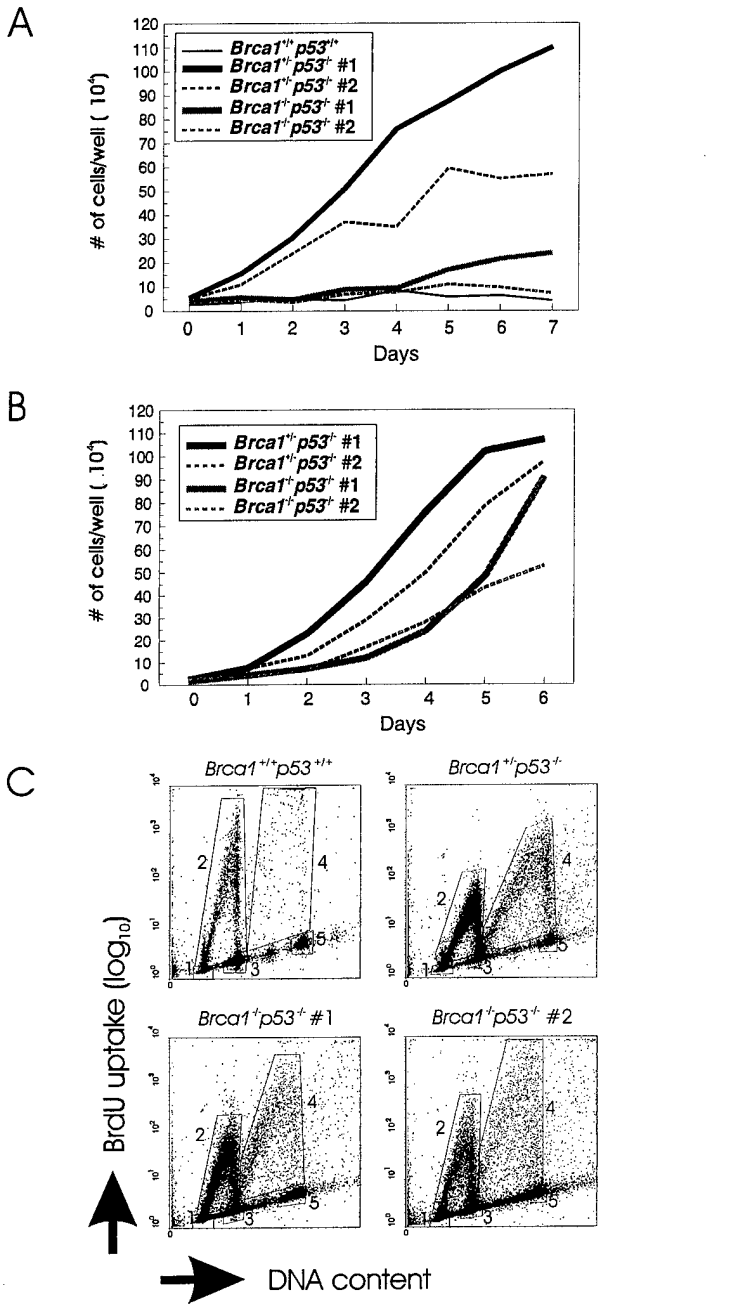
To confirm that this nonadherent population of cells was in fact dead and did not simply reflect a change in anchorage dependence of the $Brca1^{-/-}$ $p53^{-/-}$ fibroblasts during various stages of the cell cycle, we examined the plating efficiency of these cells. $Brca1^{-/-}$ $p53^{-/-}$ nonadherent cells were collected and plated in 24-well plates at a density similar to that used in passage of adherent cells. After 24 h, the numbers of adherent and nonadherent cells were determined. A decrease in the total number of cells present had occurred, and only 3% of the nonadherent cells plated had attached. Analysis of these cells and the nonadherent population indicated that only the attached cells excluded the dye. The viability of the $Brca1^{-/-}$ $p53^{-/-}$ cells was further examined by using a colorimetric MTT-based assay capable of reliably detecting small numbers of viable cells (37). Between 5×10^3 and 4×10^4 adherent or nonadherent cells were collected, and the conversion of MTT to formazan dye was examined quantitatively. In two $Brca1^{-/-}$ $p53^{-/-}$ fibroblast lines and one $p53^{-/-}$ line, fewer than 15% of the nonadherent cells examined were viable.

To determine whether cellular death was occurring through an apoptotic pathway, the TUNEL assay was performed on the nonadherent cells of two $Brca1^{-/-}$ $p53^{-/-}$ lines and two $Brca1^{+/-}$ $p53^{-/-}$ lines. Although nonadherent cells are not viable as determined by trypan blue exclusion, fewer than 25% stained positively for apoptosis in the four lines examined (data not shown).

$Brca1^{-/-}$ $p53^{-/-}$ fibroblasts show increased sensitivity to DNA-damaging agents. A number of lines of evidence support a role for BRCA1 in the maintenance of genome integrity (19, 43). If increased cellular death in the $Brca1^{-/-}$ $p53^{-/-}$ lines is directly related to the inability of these cells to repair DNA damage, it would be expected that the growth of these cells would be further compromised by exposure to DNA-damaging agents. We therefore determined the survival rate of the two $Brca1^{-/-}$ $p53^{-/-}$ lines and two $p53^{-/-}$ lines after treatment with ionizing radiation, hydrogen peroxide, and UV light. A dose-dependent decrease in cellular survival was seen in cells of both genotypes after exposure to 2.5, 5.0, and 7.5 Gy of ionizing radiation (Fig. 6A). However, the decrease in survival after treatment was significantly greater in the double-homozygous lines. A similar increase in sensitivity to DNA-damaging agents was observed after exposure of the cells to hydrogen peroxide (Fig. 6B). Again, while hydrogen peroxide treatment decreased cellular survival of both $Brca1^{-/-}$ $p53^{-/-}$ and $p53^{-/-}$ fibroblasts, the impairment of survival of the double-homozygous cells was significantly greater. The difference between the $Brca1^{-/-}$ $p53^{-/-}$ and $p53^{-/-}$ cells was most pronounced after exposure to 500 μM hydrogen peroxide. Although differences in the survival of the $Brca1^{-/-}$ $p53^{-/-}$ and $p53^{-/-}$ cells were not as dramatic after exposure of these lines to ultraviolet light (Fig. 6C), survival impairment of the double-homozygous lines was greater than that observed in the $p53^{-/-}$ cultures. These differences achieved statistical significance in two of the UV radiation doses examined.

Transcription-coupled repair in $p53^{-/-}$ and $Brca1^{-/-}$ $p53^{-/-}$ cells. We have previously demonstrated an increased sensitivity to ionizing radiation and hydrogen peroxide in an ES cell line that is BRCA1 deficient and that this increased sensitivity paralleled a defect in TCR (19). However, as this BRCA1-deficient ES cell line was a single isolate and as no additional lines could be identified in numerous additional

cells (arrows in panels E and F) appear to be more numerous in the testes of the $Brca1^{-/-}$ $p53^{-/-}$ male. However, to correct for the decreased size of the seminiferous tubules of the $Brca1^{-/-}$ $p53^{-/-}$ male in a given area, we counted the number of apoptotic cells present in 25 randomly chosen seminiferous tubules from the two animals. (F) TUNEL-positive cells were only increased approximately threefold in testes of the $Brca1^{-/-}$ $p53^{-/-}$ male. (G) Spermatocytes (arrows), spermatids, and spermatozoa can be detected in the testes of a $p53^{-/-}$ control male stained with HSP70-2 antibody. Incubation with the HSP70-2 antibody revealed the presence of spermatocytes (arrows) in the testes of a $Brca1^{-/-}$ $p53^{-/-}$ male. Staining: A and B, hematoxylin and eosin; C and D, toluidine blue; E and F, diaminobenzidine and methyl green; G and H, diaminobenzidine. Magnification bars: A and B, 200 μm; C and D, 20 μm; E and F, 100 μm; G and H, 67 μm.



	G0/G1 (diploid)	S (diploid)	G2[diploid]/ G1[tetraploid]	S (tetraploid)	G2 (tetraploid)	S high passage
	1	2	3	4	5	
Brca1$^{+/+}$*p53*$^{+/+}$	67.3%	7.3%	21.8%	0.9%	2.7%	x
Brca1$^{+/-}$*p53*$^{-/-}$	42.5%	20.9%	23.6%	8.9%	4.1%	58.2%
Brca1$^{-/-}$*p53*$^{-/-}$ #1	47.2%	19.1%	24.2%	6.0%	3.5%	64.2%
Brca1$^{-/-}$*p53*$^{-/-}$ #2	45.9%	12.1%	27.6%	7.1%	7.3%	57.6%

FIG. 4. Growth rate of *Brca1*$^{-/-}$ *p53*$^{-/-}$ fibroblasts. (A) At early passages, *Brca1*$^{-/-}$ *p53*$^{-/-}$ and *p53*$^{-/-}$ control lines and a wild-type fibroblast line were plated at a low density and were counted daily. *Brca1*$^{-/-}$ *p53*$^{-/-}$ fibroblasts have a growth rate similar to that of the wild-type fibroblasts. In contrast, the *Brca1*$^{+/-}$ *p53*$^{-/-}$ control fibroblast lines have a higher growth rate, and contact inhibition occurs at a higher density. (B) However, at later passages, the growth rate of the *Brca1*$^{-/-}$ *p53*$^{-/-}$ fibroblasts has increased, and contact inhibition occurs at a higher cell density. (C) *Brca1*$^{-/-}$ *p53*$^{-/-}$ fibroblasts were incubated with BrdU and stained with anti-BrdU antibody and propidium iodide. Quantitation of the percentage of cells in each cell cycle stage was performed by flow cytometry. One *Brca1*$^{+/-}$ *p53*$^{-/-}$ fibroblast line and one wild-type fibroblast line were used as controls. Cells at passage numbers 3 and 4 were considered early-passage cells, while cells at passage numbers 10 through 14 were considered late-passage cells.

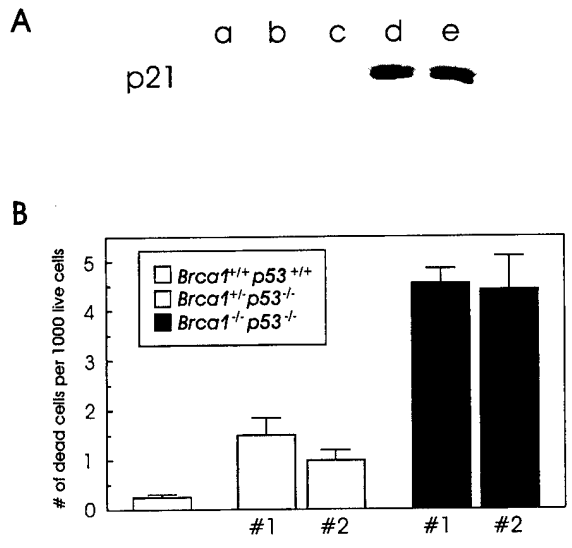


FIG. 5. A higher rate of cellular death occurs in $Brca1^{-/-}$ $p53^{-/-}$ primary fibroblast cultures. (A) p21 protein levels are not elevated in the $Brca1^{-/-}$ $p53^{-/-}$ fibroblasts (lanes a and b) or the $Brca1^{+/-}$ $p53^{-/-}$ fibroblasts (lane c). In contrast, high levels of p21 were detected in wild-type fibroblasts (lanes d and e). (B) Nonadherent $Brca1^{-/-}$ $p53^{-/-}$ fibroblasts were collected and counted. This number was normalized to the number of adherent live cells in each fibroblast population. Nonadherent and adherent counts were obtained from six plates for each of the cell lines. The bars represent the standard errors.

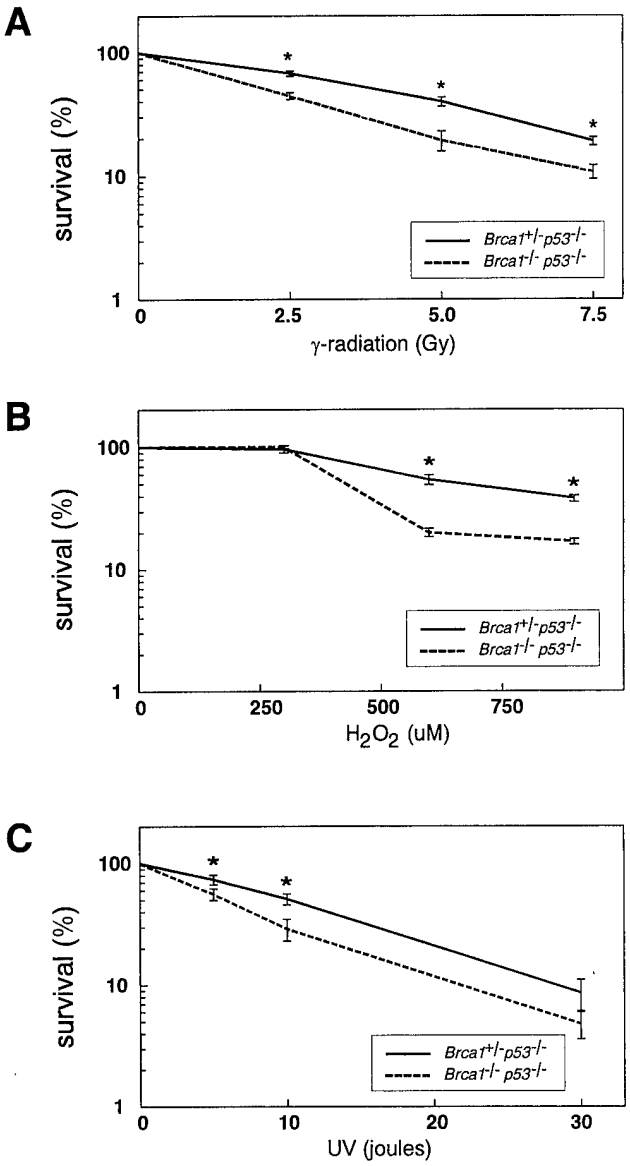


FIG. 6. $Brca1^{-/-}$ $p53^{-/-}$ fibroblasts are sensitive to gamma radiation, hydrogen peroxide, and UV radiation. Fibroblasts from two $Brca1^{-/-}$ $p53^{-/-}$ cell lines and two $Brca1^{+/-}$ $p53^{-/-}$ cell lines were exposed to 0, 2.5, 5.0, or 7.5 Gy of gamma radiation (A); 0, 250, 500, or 750 μM hydrogen peroxide (B); or 0, 5, 10, or 30 J of UV radiation (C). Survival was determined in each cell line by normalizing surviving cells following DNA damage to the number of cells which were untreated. An asterisk indicates statistical significance ($P < 0.05$).

experiments, it is possible that this phenotype was not directly related to the loss of BRCA1 but rather to the accumulation of other mutations in the cell line. To determine if TCR is dependent on normal BRCA1 function, a $Brca1^{-/-}$ $p53^{-/-}$ fibroblast line and a $Brca1^{+/-}$ $p53^{-/-}$ control line were exposed to ionizing radiation, UV light, or hydrogen peroxide, and the rate of repair on the *DHFR* gene was measured. After exposure to 10 Gy of ionizing radiation, a rapid repair of the transcribed strand of the *DHFR* gene was found in the $Brca1^{+/-}$ $p53^{-/-}$ cells (Fig. 7A). In contrast, the rate of repair on the transcribed strand of the *DHFR* gene was similar to that of the nontranscribed strand and the genome overall in the $Brca1^{-/-}$ $p53^{-/-}$ cells, similar to what was observed in the BRCA1-deficient ES cells. Similarly, when cells were exposed to 10 mM H_2O_2 and the TCR of thymine glycols was examined, a deficiency in the rapid removal of this oxidized base from the transcribed strand of the *DHFR* gene was also observed in $Brca1^{-/-}$ $p53^{-/-}$ cells (Fig. 7B). However, when cells were exposed to 10 J of UV per m^2, no deficiency in TCR was observed in the $Brca1^{-/-}$ $p53^{-/-}$ cells compared to the $Brca1^{+/-}$ $p53^{-/-}$ control line (Fig. 7C). These results indicate that TCR of oxidative DNA damage is absent in cells defective in BRCA1.

DISCUSSION

We report here the survival to adulthood of a small number of mice homozygous for a mutation in the *Brca1* gene. BRCA1-deficient males are infertile, and the paucity of spermatocytes is consistent with a role for BRCA1 in meiosis. Surprisingly, the presence of growing follicles in the ovary of the BRCA1-deficient female suggests that BRCA1 is not required for the early meiotic events in female germ line cells. Examination of these BRCA1-deficient mice and primary cells derived from these mice indicates that loss of normal BRCA1 expression results in growth retardation: mice homozygous for the mutation are smaller than littermates, and the growth rate of the $Brca1^{-/-}$ $p53^{-/-}$ cells is reduced in comparison to $p53^{-/-}$ control lines. Furthermore, we show that the mechanism underlying this growth deficit is largely due to an increase in the percentage of cells in these cultures that die and not to a decrease in the transition rate of the cell cycle. This cellular death does not occur by apoptosis. After extensive passage, the growth rate of the $Brca1^{-/-}$ $p53^{-/-}$ cultures increases to approach that of the $p53^{-/-}$ fibroblasts. The rate of transition through the cell cycle increases in the $Brca1^{-/-}$ $p53^{-/-}$ fibroblasts; however, this is also true of the $p53^{-/-}$ fibroblasts maintained in culture over a similar length of time. We believe that it is primarily the decreased rate of cellular death of the

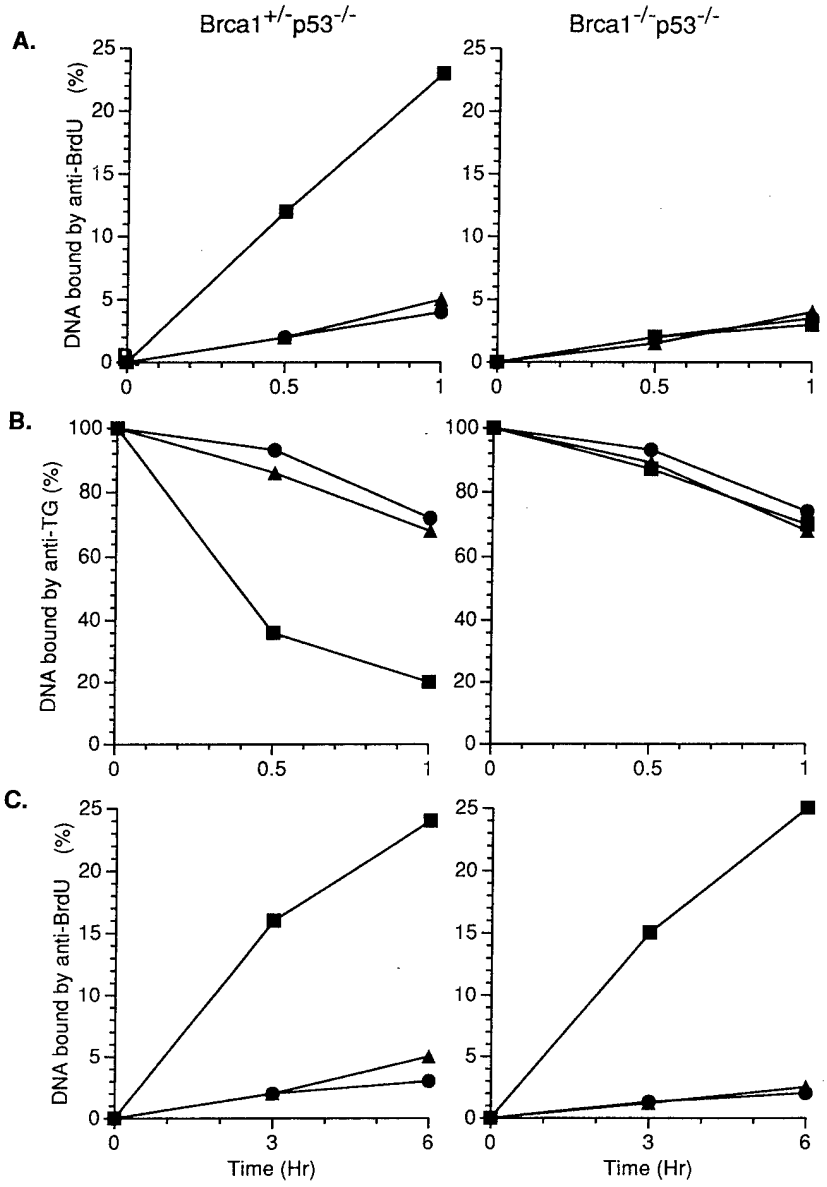


FIG. 7. TCR is defective in the *DHFR* gene of *Brca1*$^{-/-}$ *p53*$^{-/-}$ fibroblasts after exposure to ionizing radiation and hydrogen peroxide but not after exposure to UV light. Cells were exposed to 10 Gy of gamma rays (A) or 10 J of UV radiation per m^2 (C) and allowed to repair in the presence of 10 μM BrdU. Genomic DNA, digested with *Bam*HI, was reacted with an antibody to BrdU. DNAs from the bound and free fractions were electrophoresed and transferred to a GeneScreen Plus membrane. The percentage of total DNA (▲) bound by the antibody was determined from the ^3H prelabel. The percentage of the transcribed strand (■) and nontranscribed strand (●) of the *DHFR* gene was analyzed with a Bio-Rad phosphorimager. (B) Cells were exposed to 10 mM H_2O_2 for 15 min at 37°C. Purified DNA was digested with *Bam*HI and incubated with an antibody against thymine glycols, and the extent of removal was determined as described above. The percentage of total DNA (▲) bound by the antibody was determined from the ^3H prelabel. The percentage of the transcribed strand (■) and nontranscribed strand (●) of the *DHFR* gene was analyzed with a Bio-Rad phosphorimager.

Brca1$^{-/-}$ *p53*$^{-/-}$ fibroblasts that leads to a decrease in the difference in growth rates observed on comparison of the later passage *Brca1*$^{-/-}$ *p53*$^{-/-}$ and *p53*$^{-/-}$ fibroblasts.

Mechanisms for survival of BRCA1-deficient, p53-deficient mice. We and others have reported that loss of normal BRCA1 expression results in death early in mouse embryogenesis and that BRCA1-deficient embryos are severely growth retarded (21, 32, 33, 47). Heterogeneity has been observed in the embryonic stage at which death occurs, both within a mouse line and between lines carrying different mutations. The embryos homozygous for the *Brca1*$^{\Delta 223-763}$ mutation (the mutation carried by the animals described in this paper) on average survived the longest of all of the *Brca1*$^{-/-}$ embryos described to date. While in general embryos homozygous for other *Brca1* mutations do not survive past embryonic day 8, the majority of the *Brca1*$^{-/-}$ embryos in our colony survive to embryonic day 9 or 10. There are a number of possible reasons for these findings. The *Brca1*$^{-/-}$ embryos generated in our mouse colony were maintained on a mixed genetic background consisting of 129, C57BL/6, and DBA/2. In contrast, other BRCA1-deficient embryos examined were on a mixed genetic background consisting of only 129 and C57BL/6. It is possible that the

additional vigor of the more heterogeneous background, or a specific modifier gene(s) present in the DBA/2 strain, contributed to this extended survival. Interestingly, mice homozygous for the *Brca2* truncation mutation were reported to survive only when on a mixed genetic background consisting of 129, C57BL/6, and DBA/2 (7, 16). No adult animals were obtained on the 129, C57BL/6 background (33, 45, 49).

It is also possible that the difference in the survival of the various BRCA1-deficient lines is related to the mutation carried by these animals. A neomycin resistance gene replaces the 3′ portion of intron 10 and the 5′ portion of exon 11 of the *Brca1* gene in our BRCA1-deficient mouse line. Analysis of RNA from cell lines heterozygous and homozygous for the mutant allele shows that a *Brca1* mRNA, 4 kb smaller than the wild-type message, is transcribed from the mutant allele. Sequence analysis of this message indicates that it is a splice variant deficient in all exon 11-derived sequences (9). Exon 11 encodes the two nuclear localization motifs and a region of the gene believed to be essential for binding with RAD51 (5, 44, 50). In multiple cases, mutations resulting in deficiencies only in exon 11 are associated with increased risk for mammary tumorigenesis in $BRCA1^{+/-}$ patients (8, 20). In addition, this mutation has recently been shown to represent a null allele for at least some functions of BRCA1, since TCR is undetected in the ES cell line homozygous for this mutation (19). It is possible, however, that this splice variant can produce a protein with some activity and that this activity extends the survival of the embryos.

The survival of BRCA1-deficient embryos on a p53 null background has been examined by introduction of a *p53* mutation into two different BRCA1-deficient lines (22, 33). In both cases, a modest extension of the survival of the embryos was reported, although no embryos survived beyond 14 days. Similarly, p53 deficiency has not resulted, in general, in the survival of embryos homozygous for the $Brca1^{\Delta 223-763}$ mutation (9). Therefore, while it is likely that lack of p53 expression contributed to the survival to adulthood of the mice reported here, it is also apparent that genetic factors in addition to loss of p53 are required for the survival of mice without normal BRCA1 function. The close relationship of the two initial $Brca1^{-/-}$ animals identified and the derivation of the third $Brca1^{-/-}$ animal from mice related to these two initial survivors suggest that this survival is dependent on additional genetic factors. The increased survival of $Brca2^{-/-}$ mice carrying DBA/2 loci would suggest that alleles specific to the DBA/2 strain may not only be responsible for the extended survival of our $Brca1^{-/-}$ embryos (7, 16) but also play a role in the survival of the three $Brca1^{-/-}$ $p53^{-/-}$ mice described here. However, until larger numbers of surviving mice are identified and the relationship between these animals is examined, it will not be possible to determine whether alleles of a single gene or a number of modifier loci confer this survival advantage. While the number of BRCA1-deficient mice is small, the phenotype observed in these animals and the fibroblasts isolated from these animals was never seen on examination of siblings or related mice in this colony. Therefore, while the phenotype is undoubtedly modified by the *p53*-null background and the presence of a modifier gene, it is most likely dependent on homozygosity for the mutant *Brca1* allele. However, we cannot rule out the possibility that the $Brca1^{-/-}$ $p53^{-/-}$ mice independently acquired spontaneous somatic mutations that enabled embryonic survival and that these mutations contribute in part to the observed phenotype.

Characterization of BRCA1-deficient, p53-deficient mice. BRCA1 expression can be detected at high levels in the adult testes, and in situ analysis indicates that expression is highest in spermatocytes, in particular those in the late pachytene and diplotene stages of the first meiotic prophase (2, 27, 35, 53). In contrast, Sertoli cells and Leydig interstitial cells do not express BRCA1. Immunohistological studies have further localized BRCA1 within the meiotic cells (44). Extensive morphological changes accompany the pairing or synapsis of homologous chromosomes during the first meiotic prophase. A proteinaceous axis forms between sister chromatids and, as pairing continues, the axes of homologues adhere to each other, thereby initiating the formation of the synaptonemal complex. Lateral elements and additional proteins associate with the paired chromosomal region, completing the formation of a synaptonemal complex. BRCA1 is associated with both unsynapsed axial elements and the axes in the process of synapsing. BRCA1 was also detected on unsynapsed centromeric heterochromatin, on remaining univalent chromosomes, and at pairing forks. The identification of cells which express HSP70-2 (a 70-kDa heat shock protein) indicates that spermatogonia develop into spermatocytes and initiate meiosis in the double-homozygous animals. However, the virtual absence of pachytene spermatocytes in the BRCA1-deficient males suggests that the association of BRCA1 with the prophase chromosomes is essential for normal completion of this process.

The comparable expression pattern of BRCA1 and BRCA2, the association of loss of both genes with mammary tumors, and the related structure of the two genes suggest that these proteins carry out similar functions. Interestingly, however, the effect of the loss of each gene on gamete formation is very different. In contrast to BRCA1-deficient mice, no germ cells were detected in mice homozygous for a *Brca2* truncation mutation (7). However, we cannot rule out the possibility that the expressed *Brca1* mRNA from the mutant allele can confer functions of BRCA1 in these mitotic cells but cannot carry out the function of BRCA1 during meiosis. Alternatively, it is possible that the absence of p53 contributes to the survival of the mitotic spermatogonia but cannot rescue the meiotic cells deficient in BRCA1.

Although BRCA1 expression has been demonstrated in the rapidly dividing thecal cells of the ovary, expression during the formation of female gametes has not been established (2, 41). Histological examination of the female BRCA1-deficient animal revealed the presence of both primary and growing follicles. In normal mice, growing follicles have completed the pachytene stage of prophase of meiosis I, the stage of highest expression of BRCA1 in spermatogenesis (25, 53). The presence of these follicles in the $Brca1^{-/-}$ $p53^{-/-}$ female suggests that BRCA1 is not required for completion of these stages of meiosis I.

Despite the growth retardation of the $Brca1^{-/-}$ $p53^{-/-}$ mice, with the exception of the testes, mammary gland, and skin, the organ systems of the BRCA1-deficient mice appeared in general to develop normally. In the female mouse, the branching of the mammary epithelium was reduced and the end buds were smaller. Histological analysis showed individual cells arrayed around the duct. One interpretation of these observations is that these cells represent epithelial cells that fail to differentiate into normal gland structures and instead migrate away from the developing end bud into the surrounding fat pad. The observed alterations in the development of the mammary gland are of particular interest given that both in vivo and in vitro loss of BRCA1 function are associated primarily with mammary and ovarian tumors in humans. These observations will require confirmation by analysis of additional animals or by the generation of animals in which the loss of BRCA1 function is limited to mammary epithelium. However, they do suggest that the impact of the loss of BRCA1 on

growth and differentiation is more severe than that seen in other cell lineages. While these changes were not observed in the analysis of a number of wild-type and p53-deficient animals, we cannot rule out the possibility that the developmental change in the mammary gland of the *Brca1*$^{-/-}$ *p53*$^{-/-}$ female is in whole or in part dependent on the loss of both BRCA1 and p53. Cytomegaly and karyomegaly were noted in the parotid gland of all of the double-homozygous animals and in the prostate glands of the male animals. This is of particular interest in light of evidence suggesting that BRCA1 is associated with centrosomes and may be important in segregation of the chromosome during mitosis (13).

BRCA1 in DNA damage repair. Consistent with the observed growth retardation of *Brca1*$^{-/-}$ *p53*$^{-/-}$ mice and the phenotype of BRCA1-deficient embryos, primary cell cultures obtained from the *Brca1*$^{-/-}$ *p53*$^{-/-}$ mice grow more slowly than those obtained from *p53*$^{-/-}$ animals. The overall growth rate of a cell culture is determined by both the rate of cellular division and the rate of cellular death. Differences in the growth of the *p53*$^{-/-}$ and *Brca1*$^{-/-}$ *p53*$^{-/-}$ cultures appear to stem largely from an increased frequency of cellular death in the absence of normal BRCA1 expression. If, as has been proposed, BRCA1 is required for DNA repair, this increased rate of cellular death may result from the rapid accumulation of chromosomal abnormalities and other mutations incompatible with the survival of normal cells. A similar mechanism may underlie the growth retardation of BRCA1-deficient embryos. Variability in the stage to which BRCA1-deficient embryos develop, even when carrying identical mutations, may reflect the fact that environmental factors influence the rate at which mutations accumulate, with embryos surviving longer if a number of cell divisions occur before accumulation of mutations leads to cell death or senescence.

This interpretation is supported by our observation that the growth of the primary BRCA1-deficient cells is compromised to a greater extent than that of the p53-deficient control cells by exposure to UV light, gamma irradiation, and H_2O_2. *Brca1*$^{-/-}$ ES cells have also been reported to display increased sensitivity to gamma irradiation and H_2O_2 in comparison to the parental *Brca1*$^{+/+}$ lines (19). However, unlike the *Brca1*$^{-/-}$ *p53*$^{-/-}$ fibroblasts, no difference in the response of the *Brca1*$^{-/-}$ ES cells to UV radiation was observed. This may reflect differences in the dependence of various cell lineages on a particular DNA repair pathway after exposure to UV light. The loss of one repair pathway due to BRCA1 deficiency may result in cellular death in fibroblasts but may be compensated adequately by another repair pathway in ES cells. It is also possible that the contribution of BRCA1 in the response to UV damage is detected only when p53-dependent repair pathways are disabled.

The *Brca1*$^{-/-}$ ES cell line was shown to be deficient in TCR. However, this interpretation was dependent on results obtained from a single ES cell line isolate. Attempts to isolate similar lines have failed, raising the possibility that the cell line carries other mutations that have accumulated, allowing it to survive without normal BRCA1 function, and that these alterations, not the loss of BRCA1, lead to the altered TCR. A role for BRCA1 in TCR is confirmed here by the demonstration that *Brca1*$^{-/-}$ *p53*$^{-/-}$ primary fibroblasts are also deficient in TCR. Again, this deficiency was observed after exposure to H_2O_2 and gamma irradiation but not to UV light. In contrast to the *Brca1*$^{-/-}$ ES cell line, the growth sensitivity of the *Brca1*$^{-/-}$ *p53*$^{-/-}$ fibroblasts to DNA-damaging agents did not correlate directly with TCR deficiency. This suggests that defects in TCR repair may not account for all of the growth deficiency observed in BRCA1-deficient cells. The fact that TCR is unlikely to be the only function of BRCA1 is supported by the comparison of the phenotype of mice homozygous for a mutant allele of the CSB gene, which results in a loss of TCR (51). These mice survive, appear normal, and are fertile. Our results, therefore, support the hypothesis that BRCA1 is a multifunctional protein either as a result of the presence of multiple functional domains or due to the ability of BRCA1 to activate a number of different cellular pathways.

As discussed above, loss of p53 extends the survival of the embryos carrying other *Brca1* mutations and is likely to play a role together with other as-yet-unidentified genetic factors in the survival to adulthood of the mice described here. p53 is involved in multiple cellular pathways, and we cannot at present determine whether the inactivation of one specific pathway or a number of these pathways contributes to survival of the BRCA1-deficient embryos and cells (31). For example, it is possible that in the absence of p53-mediated apoptosis, BRCA1-deficient cells that have accumulated DNA damage survive. However, our data, as well as the observations made on examination of histological sections from BRCA1-deficient embryos stained for apoptotic cells by TUNEL, do not support this hypothesis. In *Brca1*$^{-/-}$ embryos, in which the p53-mediated apoptotic pathway is intact, no increase in the number of apoptotic cells was observed (21, 32). Furthermore, the high percentage of dead cells in primary *Brca1*$^{-/-}$ *p53*$^{-/-}$ cultures indicates that p53-independent pathways can lead to the death of BRCA1-deficient cells.

p53 has also been implicated in numerous cell cycle checkpoints, and it is possible that it is the disruption of these pathways that contributes to the survival of the *Brca1*$^{-/-}$ *p53*$^{-/-}$ animals (31). It is possible that, in normal cells, accumulation of genetic alterations as a result of loss of BRCA1 leads to growth arrest via p53-dependent pathways. Analysis of growth-arrested BRCA1-deficient embryos indicated increased expression of p21 (22). This and the finding that p21 deficiency also extends the survival of the BRCA1-deficient embryos support the hypothesis that the loss of the p53-dependent G_1/S checkpoint contributes to the extended survival of the BRCA1-deficient cells (21). p53 has also been implicated in other cell cycle checkpoints, including the spindle checkpoint (11). Deficiency in the spindle checkpoint contributes to the increased chromosomal aberrations in cells lacking normal p53 expression. It is not possible to discern at this time whether deficiencies in this and other checkpoints also contribute to the survival of the *Brca1*$^{-/-}$ *p53*$^{-/-}$ mice.

Contribution of mutations in *BRCA1* to tumorigenesis. The studies reported here indicate that loss of p53, in combination with other genetic modifiers, can extend the survival of BRCA1-deficient mice and cells. Even then, BRCA1-deficient cells grow slowly, making it difficult to reconcile the BRCA1-deficient phenotype with the observation of increased tumor risk of individuals heterozygous for mutant *BRCA1* alleles. Examination of the growth of the primary BRCA1-deficient cells after extended passage in cell culture provides a possible explanation: once a number of mutational events have occurred that allow survival of BRCA1-deficient cells, the loss of BRCA1 may accelerate the accumulation of additional mutations necessary for malignant transformation. Thus, after extended passage in tissue culture, clones of *Brca1*$^{-/-}$ *p53*$^{-/-}$ cells can be identified that have an increased percentage of cells in the S phase. The percentage of dead cells in these cultures also decreases, resulting in a growth rate that equals that of the p53-deficient primary fibroblasts.

Together, our analysis of the *Brca1*$^{-/-}$ *p53*$^{-/-}$ mice and fibroblasts supports the following models for the role of BRCA1 in tumorigenesis (Fig. 8). In the first model the loss of

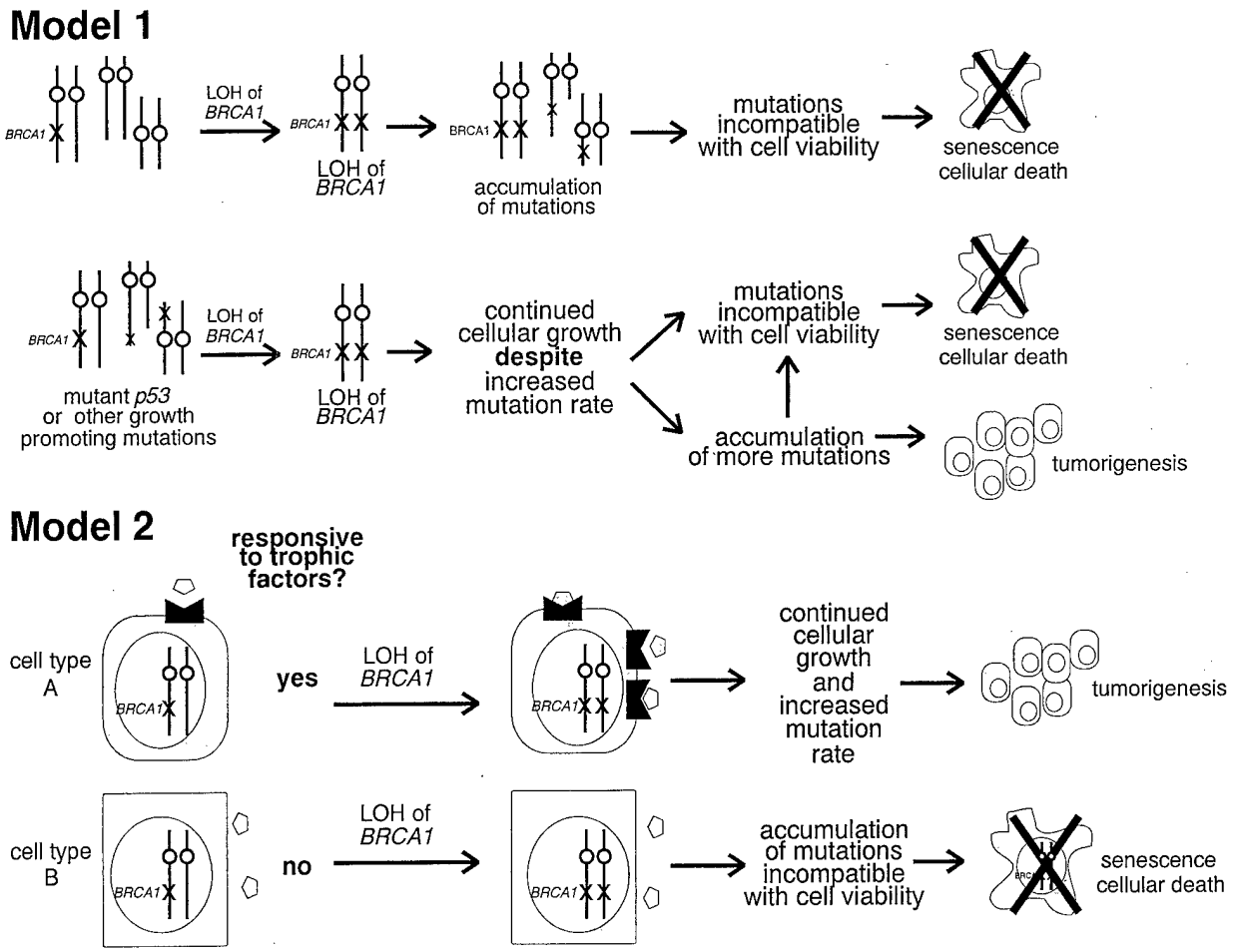


FIG. 8. Models for the role of mutations in *BRCA1* in tumorigenesis. Experiments described in this study suggest that the loss of BRCA1 results in deficits in cellular growth. However, loss of BRCA1 is consistently seen in rapidly proliferating tumors from patients carrying a germ line mutation in *BRCA1*. Two models attempt to resolve these two conflicting concepts. In model 1, mutations in genes resulting in a growth advantage are necessary for the survival of cells which have lost BRCA1 function. The loss of BRCA1, as well as the growth-promoting mutations, results in an increased mutation rate, thus accelerating tumorigenesis. Therefore, in order for loss of BRCA1 to promote tumorigenesis, other growth promoting mutations must first occur. In model 2, cellular growth or death after the loss of BRCA1 function is dependent on the ability of the cell type to compensate by utilizing trophic factors. This model accounts for the specificity of tumor types resulting from the loss of BRCA1 in humans.

the wild-type *BRCA1* allele is not the first event in tumor progression. Cells must first accumulate mutations in multiple genes, such as *p53*, that confer a growth advantage. Only after having acquired an increased growth potential will loss of BRCA1 function be compatible with cell survival. With loss of wild-type BRCA1 function, the resulting cell populations display growth rates that approach those of normal cells. However, if as suggested by our results BRCA1 plays a role in multiple DNA repair pathways, loss of BRCA1 function would be expected to accelerate the accumulation of additional mutations leading to malignant transformation. This model is supported by the recent demonstration that virtually all *BRCA1*$^{-/-}$ tumors examined to date have lost p53 function (10). In addition, we have identified mammary tumors in *Brca1*$^{+/-}$ *p53*$^{+/-}$ mice after exposure to irradiation (9). A number of these tumors have lost the wild-type *p53* and *Brca1* alleles.

In a second model, the impact of loss of BRCA1 on cellular survival depends on the cell type, the growth state of the cell, and the presence of trophic factors capable of overriding signals that would, under other growth conditions, lead to the death of cells with damaged DNA. For example, it is possible that during the growth of the mammary epithelia, high levels of hormones and growth factors can take the place of growth-enhancing mutations such as loss of p53, allowing *BRCA1* heterozygous cells that lose the wild-type allele to survive. This differential sensitivity of cells to loss of BRCA1 would lead to the observed tumor types associated with inheritance of *BRCA1* mutations. This model is not supported by our observation that the mammary epithelium of the *Brca1*$^{-/-}$ *p53*$^{-/-}$ mice was underdeveloped, suggesting that mammary epithelia may in fact have increased sensitivity to loss of BRCA1 function. It is possible, however, that this dependency on BRCA1 function reflects an underlying difference between mice and humans in the growth and responsiveness of mammary epithelium to hormones and that this characteristic contributes to the differential effect that heterozygosity of *BRCA1* has on mammary tumorigenesis in the two species. While further studies will be required to test these and additional models, the results presented here suggest that in a number of normal cell populations, the loss of BRCA1 results in a growth disadvantage. Only in combination with other mutations, under specific

growth conditions or in specific populations of cells, can a loss of BRCA1 contribute to tumorigenesis.

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