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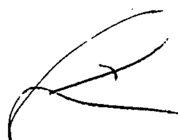

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INTRODUCTION:

Mutations in the BRCA1 locus account for approximately 45% of hereditary breast cancers in the United States (5). While expectations that mutant forms of this gene would also be observed in sporadic breast cancers have not been met, studies have found that the BRCA1 RNA is underexpressed, or the protein mislocalized, in sporadic disease (1, 18). These studies indicate that BRCA1 is a potential target in both hereditary and non hereditary breast cancer syndromes.

Since the identification of the BRCA1 gene in 1994 (13), a great deal of research has been focused on elucidating its function. BRCA1 has been shown to be a nuclear phosphoprotein (2, 15, 17, 20) and that it interacts with a number of gene products, including E2A, BARD1, and RAD51 (9, 16, 19).

By noting colocalization with RAD51, and stabilization of this interaction following ionizing radiation, Scully et al suggested that BRCA1 could function in double strand DNA break repair (16). Further evidence that BRCA1 mutant cells have a defects in DNA repair have come from mice that lack BRCA1 (BRKO). These mice die early in embryogenesis and blastocysts are sensitive to ionizing radiation (6, 8, 11, 12). Furthermore, embryonic lethality is partially alleviated by loss of p53 or p21, indicating that the absence of BRCA1 leads to a requirement for these gene products. (7).

In our original application, we proposed to investigate the following specific aims:

1. Characterize of the effects of *Brca1* expression on the proliferation and differentiation of breast cancer cells of known genotype.
2. Establish mice that lack functional *Brca1* by targeted disruption.
3. Genetic complementation of *Brca1* deficient mice with strains that express oncogenes known to contribute to the development of breast cancer.

To date we have made significant progress on all three aims. In particular, we have studied the effect of overexpressing BRCA1 in mouse mammary epithelial cells and have studied the subcellular localization of the murine Brca1 gene product (Aim 1). We have created a line of transgenic mice that overexpress human BRCA1 (MBR) (aim 1) and have created a second group of mice that overexpress an antisense construct of mouse Brca1(BAS) (aim 1). We have created a line of mice that carry an inactivated Brca1 locus (BRKO) (aim 2) and have crossed the BRKO mice with mice predisposed to cancer, including ($p53^{+/-}$, $p21^{+/-}$, and MMTV-myc). In the course of these experiments, we experienced several technical difficulties which initially hindered our progress. However, we have now established systems which appear to be working effectively in order to address the Aims proposed.

BODY:

Plasmid generation: As proposed, we generated BRCA1 expression vectors based on a full length cDNA for human BRCA1. After finding mutations in BRCA1 cDNA's from several sources (by direct sequencing), we eventually settled on a cDNA (KEE69) provided by E. Solomon (Guy's hospital, London). The cDNA insert was to large to allow effective viral packaging, so we went on to make the constructs in standard plasmid vectors using both beta actin-promoter and the MMTV promoter described in Aim 1. We have also inserted the 5' end of the mouse Brca1 cDNA (980bp) in both the sense and anti sense orientation into the MMTV promoter as described in Aim 1.

Generation of stable transfected cell lines: We made several attempts to introduce plasmids expressing BRCA1 into mammary cell lines using both beta-actin and MMTV-promoters. Stable cells expressing BRCA1 were never recovered indicating that these constructs were incompatible with growth in culture. In transient transfection (see below) assays, we observed apoptotic figures and the appearance of large senescent cells indicative of growth arrest. These results indicate that overexpression of BRCA1 leads to rapid growth arrest, and/or apoptosis, in mammary epithelial cells.

Analysis of subcellular location of Brca1 protein: Since full length Brca1 could not be stably expressed in cells, we used transient transfection to introduce epitope tagged full length and partial clones and analyzed the subcellular location of the fragments. Consistent with published reports on the human BRCA1 protein, we observed that the mouse protein is present in the nucleus of cells and that a two part nuclear localization domain is encoded by exon 11 of the gene (Figures 1 and 2).

A) CMV-LTR Cloning Site SV-40 intron + polyA

B)

		Subcellular distribution
B1.myc	Exon 11, MYC-tag	nuclear
B1.A		cytoplasmic
B1.B		nuclear

200 400 600 800 1000 1200 1400 1600 1800

Figure 1: Mouse Brca1 protein is present in the nucleus of cells and the N-terminus is not required for this activity. Panel A) A map of the expression vector used to express myc-tagged Brca1 protein in cells. Panel B) A summary of the localization of myc tagged Brca1 fragments when expressed in COS or 293 cells.

A

ZF N1 N2 GD

NH_2 Exon 11 COOH

200 400 600 800 1000 1200 1400 1600 1800

B

10 20

S S S E A T A D S Q H S T P P K K K R - K V E D P — SV40 NLS
\- - - - - - - - - - - - - - - N K L K R - K R R P — NLS#1_Hu
\- - - - - - - - - - - - - - - N K L K R - K R — NLS#1_mo
S N M E L E L N I H N S K A P K K N R L R R K S S — NLS#2_Hu
S D L E V E L N V H S S K A P K K N R L R R K S S — NLS#2_mo

CKII NLS

Figure 2: Panel A: A map of mouse Brca1 protein showing the location of the putative functional domains. ZF, zinc binding domain, N1 and N2, potential nuclear localization signals (NLS) identified by sequence analysis, GD, potential granin homology (10). Panel B) Alignment of two potential nuclear localization signals N1 and N2 in human and mouse BRCA1 genes. CKII, conserved CKII phosphorylation site 14 amino acids upstream of the NLS in SV40 T-antigen and in BRCA1 NLS #2.

Generation of Transgenic mice expressing MMTV-Brca1 Antisense and MMTV-BRCA1:

A) MMTV-Brca1 antisense (BAS) mice: We have now established 8 independent lines of MMTV-Brca1 antisense (BAS) mice (see Table 1). These mice are now 1 to 1.4 years old and have a number of interesting phenotypes. The mice express a mammary hyperplasia characterized by precocious branching and ductal development, difficulty in lactation, and reduced mammary regression following weaning. Histological examination of these glands indicates that cells have a normal cytological appearance. In addition, there is no evidence of inflammation. Recently, one of the older females (14 months of age) developed a large mammary adenocarcinoma (breast cancer). We are in the process of studying this line extensively. Cells from the adenocarcinoma have been adapted to culture and are being passaged in syngeneic mice. We are in the process of analyzing expression Brca1 protein in these glands to assess reduced synthesis.

B) MMTV-BRCA1 (MBR) mice: We experienced difficulty establishing BRCA1 transgenic mice. Thus far, we have injected over 300 embryos with the beta-actin BRCA1 transgene (zero founders) and 350 embryos with the MMTV-BRCA1 transgene (2 founders), (see Table 1). The single founder that has passed the MMTV-BRCA1 transgene was born on 8/17/98 and his first offspring are just now reaching sexual maturity. They will be bred then tested for BRCA1 expression. Because of the poor survivability associated with BRCA1 overexpression, we are starting a new project (not within the scope of the current proposal) to use the enhancer/promoter of the BRCA1 gene to direct expression of transgenic constructs.

Table 1 Transgenic mice (new lines)

Construct/transgene	Name	# of days injected[1]	# of founders[2]	# of lines[3]
MMTV-Brca1 antisense	BAS	2	8	8
MMTV-BRCA1	MBR	4	2	1
beta-actin-BRCA1		4	0[4]	0

[1]Number of days injected provides an estimate of the number of embryos that received DNA. Typically, 90 embryos (60-150) are injected per day. Only embryos that look healthy after injection (60-80%) are implanted into pseudo pregnant foster mothers.
[2]Number of founders represent the number of mice generated that contain integrated transgenic DNA.
[3]Number of lines represents the number of founders that transmitted transgenic DNA to offspring.
[4]None recovered.

Generation of Mice lacking Brca1 by Homologous Recombination: As described in the proposal, Dr. Chuxia Deng (now at the NIH, Bethesda, Md), when he was in the lab, introduced a neo selection marker into exon 11 (Figure 3) of the mouse Brca1 gene and introduced this new Brca1 allele into mice via homologous recombination in embryonic stem cells. The mice have been in the lab since 1996 and we have been characterizing the phenotype. In all respects, the phenotype of these mice appears similar to that described by other groups who have knocked out the Brca1 gene (6, 8, 11, 12).

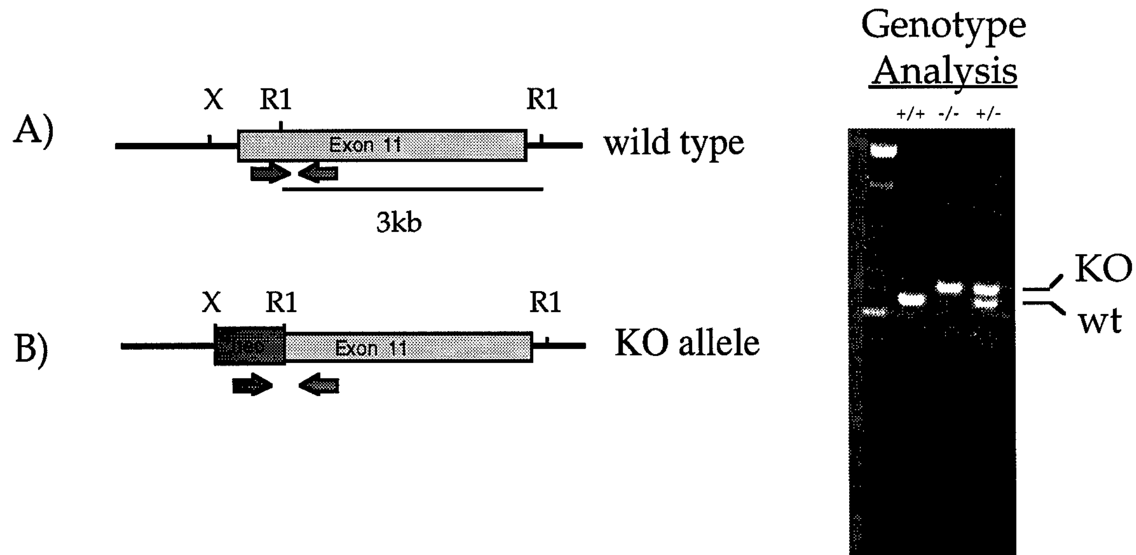

Figure 3: Panel A and B: A map of mouse Brca1 gene and the targeting construct used to for homologous recombination in embryonic stem cells. Arrows indicate the location of primers used to genotype embryos from a cross between two heterozygous BRKO mice. Panel C, Results of a polymerase chain reaction experiment to genotype 5.5d embryonic mice using primers described in Panel A and B. The upper band is represents the product of a primer from the neo cassette and a reverse primer from exon 11. The lower band represents the binding of a primer to the 5' end of exon 11 (which is absent in the KO allele).

We observe no phenotype in heterozygous mice. To date, we have followed over 100 heterozygous mice for 1.5 years and have not observed a single tumor. Homozygous mice die at between day 6 and 7 (table 2).

Table 2 Offspring from Heterozygous crosses of Brca1 knockout mice (BRKO)

	Genotype[1]		
Age	+/+	+/-	-/-
d5.5	7	16	8
d8.5	14	31	1
term	44	79	0

[1]Genotype was assessed by PCR as described in Figure 3 for embryos, or Southern blotting of EcoR1 digested tail DNA from animals after birth. The expected ratio of 1:2:1 is observed in d5.5 embryos, but the -/- animals are lost in older litters.

Genetic complementation of *Brca1* deficient mice: We have been crossing the BRKO mice generated from Aim 2 with p53 null mice (4), p21 null mice (3), and with MMTV-c-myc transgenic mice (14). To date, none of these mice have developed cancers at a rate different from the parental line. In addition, tumors that do develop in bigenic mice heterozygous for the BRKO allele retain the wild-type Brca1 allele.

Because of the hyperplasias observed in the BAS transgenic lines generated for Aim 1, we have crossed BAS mice with BRKO to generate BAS mice with only one functional Brca1 allele. The oldest bigenic mice from this cross are 6 months old and remain tumor free. We will continue to monitor these mice and will also begin treating BAS animals with dexamethasone, a steroid which stimulates expression from MMTV promoter constructs.

Conclusions:

At the end of year 1, we have made progress on all 3 Aims. We have shown that mouse Brca1 is a nuclear protein that blocks cell proliferation when overexpressed. We have also shown that Brca1 is an essential gene. Loss of the gene in BRKO mice results in early embryonic lethality. Heterozygous BRKO animals are health and do not appear to show increased susceptibility to breast cancers, or to any other disease states. While the lack of disease in BRKO mice has been disappointing, the Brca1 antisense (BAS) approach appears to be working. Specifically, we appear to be able to reduce Brca1 protein levels to the point were we can observe increased proliferation (hyperplasias) without inducing cellular lethality. We will continue characterizing these mice. In particular, we will test all 8 lines of BAS mice for diminished Brca1 protein expression, dexamethasone responsiveness, and sensitivity to DNA damage mediated by ionizing radiation. We will present these results at the end of year 2. If promising, we will attempt to carry out the complementation experiments originally proposed in Aim 3 with the BAS transgenics instead of the BRKO mice.

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