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TITLE: Role of the Wnt-4 Protein in the Mouse Mammary Gland

PRINCIPAL INVESTIGATOR: Louise J. Huber, Ph.D.
Lewis A. Chodosh, Ph.D.

CONTRACTING ORGANIZATION: University of Pennsylvania
Philadelphia, PA 19104-3246

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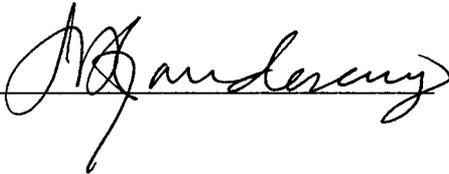
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13. ABSTRACT (Maximum 200 Words) The subject of the research described within this report is the mouse BRCA1 protein. The primary purpose of the experiments outlined below is to further the understanding of the role the mouse BRCA1 protein in cell proliferation and DNA damage. The scope of this research encompasses experiments that are designed to determine if the mouse and human BRCA1 proteins govern similar cellular functions and aims to identify novel regulatory components implicated in BRCA1-derived cancers through the use of a genetic model system in which mouse BRCA1 is mutated. An immortalized mouse embryonic fibroblast cell line that harbors an exon 11 mutant form of Brca1 has been generated. We have generated several murine Brca1 antibodies and have characterized the expression of the exon 11-deleted Brca1 protein in these cells. Significantly, this protein localizes to nuclear foci during S phase. We have also demonstrated that the exon 11 region of Brca1 is required for efficient Rad51 foci formation in response to gamma radiation.				
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(4) Introduction

The subject of the research described within this report is the mouse BRCA1 protein. The primary purpose of the experiments outlined below is to further the understanding of the role the mouse BRCA1 protein in cell proliferation and DNA damage. Current evidence indicates that the human BRCA1 protein functions in biochemical pathways that are relevant to these processes. The emphasis of the research that involves the human version of the BRCA1 gene product conducted elsewhere is limited by a lack of a genetic system which can be manipulated with facility. The scope of this research encompasses experiments that are designed to determine if the mouse and human BRCA1 proteins govern similar cellular functions and aims to identify novel regulatory components implicated in BRCA1-derived cancers through the use of a genetic model system in which mouse BRCA1 is mutated.

(6) BODY:

Specific Aim 1. Generation of a transgenic mouse that expresses human BRCA1.

This aim involved generating a transgenic mouse model in order to determine if human BRCA1 can rescue the embryonic lethality of a mouse Brca1 knockout model. As described in the report submitted last year, mRNA expression of the transgene would be determined and lines that express human BRCA1 mRNA would be crossed into Brca1 knockouts. Bitransgenic animals would be further crossed and females with embryos that are Brca1 knockouts harboring the transgene would be sacrificed. The entire litter of embryos would be removed and analyzed by PCR analysis at day 9.5 of gestation. Since all knockout embryos die no later than day 6.5 of embryogenesis, embryos that are genotyped as knockouts and that are positive for the transgene would be deemed as rescued animals. As outlined in the Statement of Work we have constructed a transgenic construct for microinjection and generated 13 founder lines. These lines have been analyzed by RNase protection assays to determine if human BRCA1 is expressed. Fig.1 demonstrates expression of human BRCA1 in four independent lines. These lines have been bred with Brca1 heterozygous animals followed by breeding with bitransgenic animals heterozygous for Brca1. Embryos obtained from these crosses have been analyzed by PCR analysis to determine if rescue has occurred. According to the Statement of Work we are currently breeding bitransgenic animals with Brca1 heterozygous mice.

Fig.1

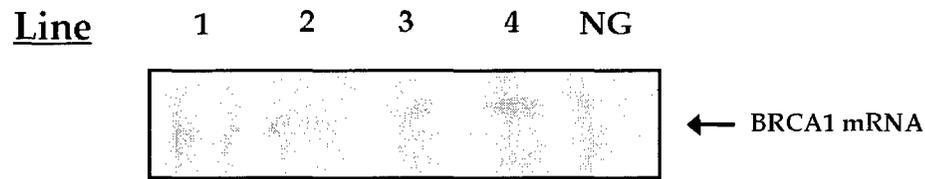


Figure 1. Embryos derived from matings of transgenic mice were PCR-genotyped for the presence of the transgene. RNA was generated from embryos from four independently derived transgenic lines. A human BRCA1 probe was employed in RNase protection assays. NG indicates a sample derived from an embryo that was negative by the genotyping assay.

Specific Aim 2. Identification of genes regulated by mouse Brca1 expression.

As outlined in the Statement of Work, mRNA from wild type and $Brca1^{\Delta-11/\Delta-11}$ MEFS has been harvested for Affymetrix chip analysis. Cells were harvested one hour following dosing with 3 grays of gamma radiation since it was determined that a cell cycle defect can be detected under these conditions. According to the manufacturers instructions cDNA was generated followed by *in vitro* transcription reactions to generate product to be used in the hybridization analysis. The

resulting mRNA has been generated successfully as determined by the length of the products which the results of hybridization to test chips has determined as well. According to the Statement of Work, the current plan is to hybridize these samples to the arrays followed by Northern analysis with candidate genes.

Specific Aim 3. Characterization of Brca1 exon 11-deficient fibroblasts.

In order to determine the molecular mechanisms that may contribute to defective DNA repair and cell cycle checkpoints in Brca1 exon 11-deleted fibroblasts, a thorough characterization of the Brca1 protein in these cells was necessary. This obviated the need to generate murine Brca1 antisera of which none are commercially available. Regions encompassing nucleotides 231-834 (mAb1), 3102-3850 (mAb2), 2541-3298 (mAb3), and 4214-4939 (mAb4) of the murine Brca1 cDNA were subcloned into pGEX-6P-1 (Pharmacia). Lysates from *E. coli* transformed with these constructs were passed over a glutathione-Sepharose column and recombinant Brca1 protein was cleaved from the GST polypeptide with PreScission Protease according to the manufacturers instructions (Pharmacia). Antisera to purified Brca1 polypeptides were raised in rabbits (Cocalico Biologicals) and were affinity purified according to published methods [Harlow, 1999 #1552]. Immunoblotting analysis of HC11 murine mammary epithelial cell extracts using murine Brca1 antibodies mAb1, mAb2, mAb3, and mAb4, identified a specific band that migrated at a predicted molecular weight of 210 kDa not recognized by preimmune sera (data not shown). To confirm that the antibodies recognize bona fide mouse Brca1, 293T cells were transfected with a mouse *Brca1* cDNA and lysates were prepared for immunoblotting and immunoprecipitation analysis. As shown in Figure 2A, mAb1 recognizes a specific band at the predicted molecular weight for mouse Brca1 in extracts of *Brca1*-transfected 293T cells that were subjected to

immunoprecipitation with mAb1 antisera. Similar results were obtained with mAb2, mAb3, and mAb4 (data not shown).

To determine if mAb1 could specifically recognize endogenous mouse Brca1, extracts from wild-type and from fibroblasts derived from mice harboring a germline deletion of the exon 11 region of Brca1 were analyzed by immunoblotting [1]. Extracts prepared from wild type MEFs revealed the presence of p210 Brca1 whereas extracts prepared from Brca1^{Δ11/Δ11} MEFs did not, confirming that the 210 kDa polypeptide recognized by mAb1 is indeed Brca1 (Fig. 2B).

Brca1^{Δ11/Δ11} MEFs are predicted to express a murine isoform of Brca1 analogous to the naturally occurring human BRCA1 variant encoding p97BRCA1. Northern hybridization was performed as described using PCR probes encompassing nucleotides 2541-3298 within exon 11 and nucleotides 4827-5354 within the carboxyl terminus of Brca1 [2]. Northern analysis with a probe that encompasses nucleotides 4827-5354 predicted to recognize both the full-length and exon 11-deleted Brca1 transcripts was performed. As expected, a 3.9 kB transcript was detected in Brca1^{Δ11/Δ11} cells and a 7.2 kB transcript was detected in cells that express p210Brca1 (Fig.2C, left panel). A similar analysis with a probe encompassing nucleotides 2541-3298 within exon 11 detected only the full length Brca1 transcript (Fig.2C, right panel). In order to determine if the exon 11-deleted transcript is expressed, immunoblotting analysis was performed on extracts derived from Brca1^{Δ11/Δ11} MEFs. Cell lysates for immunoblotting were prepared in 50 mM Tris pH 8.0, 120 mM NaCl, 0.5% Nonidet P-40 (NP-40) with 100 μg/ml Pefabloc (BMB), 20 μg/ml aprotinin, 10 μg/ml leupeptin, 0.1 mM β-glycerophosphate, 50 mM NaF, and 1 mM sodium orthovanadate. Samples were routinely loaded onto 7% SDS-PAGE gels with the exception of experiments designed to detect changes in the mobility of Brca1, for which 5 or 6% gels were run for extended periods. Wet transfer to nitrocellulose was performed overnight in buffer containing

192 mM glycine, 25 mM Tris base, and 20% methanol. Membranes were blocked for 1 hr in phosphate buffered saline containing 5% nonfat dried milk and 0.5% NP-40. A major band of the predicted molecular weight was recognized by mAb1 antisera designated as p92Brca1 (Fig. 2D). This Brca1 isoform was also detected in embryonic brain extracts prepared from embryos heterozygous for the wt and exon 11-deleted alleles of Brca1, as well as in extracts of testis derived from wild type mice indicating that p92Brca1 is a naturally occurring isoform of Brca1 (Fig 2E).

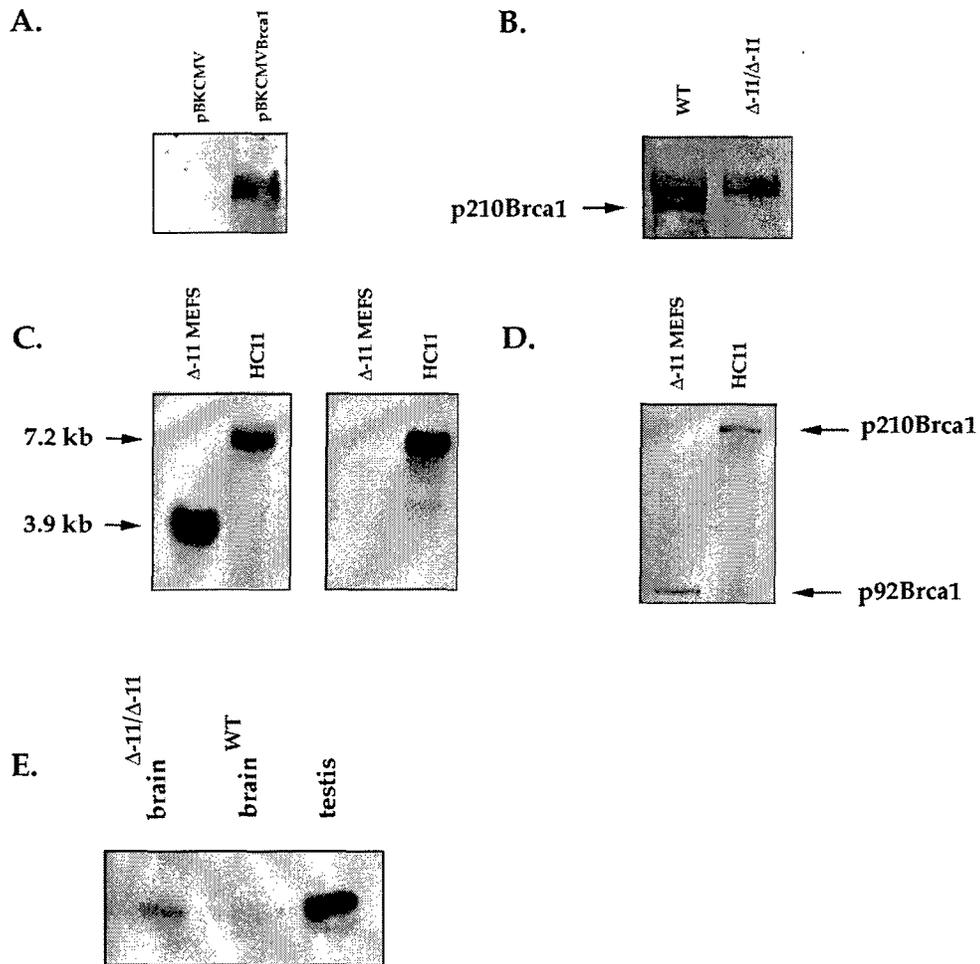


Fig. 2. Detection of mouse Brca1 exon 11-deleted isoform. A) Immunoprecipitation analysis demonstrating that mAb1 recognizes mouse Brca1. 10 μ g of either empty vector or pBK.B1 was introduced into 293T cells by calcium phosphate transfection. Cell extracts were prepared 48 hrs following transfection and 1 mg of lysate was used for immunoprecipitation. 10 μ l of purified mAb1 IgG were used for per sample. B) Immunoblot analysis of p210 Brca1 expression in wild-type and Brca1 Δ -11/ Δ -11 MEFS. 50 μ grams of cell extract per lane was probed with affinity purified mAb1 at 1 μ g/ml. C) Northern analysis demonstrating the absence of full length Brca1 transcripts in Brca1 Δ -11/ Δ -11 MEFS. 10 μ gms of poly A mRNA was loaded per lane. Probes encompass exon 11-specific sequences (right panel and Materials and Methods) and C-terminal nucleotides (left panel and Materials and Methods) were derived by PCR amplification using the mouse Brca1 cDNA as template. D) mAb1 recognizes a predominant gene product of ~ 92kDA in Brca1 Δ -11/ Δ -11 MEFS and the p210 kDA full length mouse protein in HC11 cells. E) p92Brca1 is expressed in testis of wild-type mice. 150 μ gs of lysate per sample was subjected to PAGE on an 8% gel.

Previous experiments have shown that murine *Brca1* mRNA expression is regulated in a cell cycle-dependent manner with maximal levels of *Brca1* occurring during S phase of the cell cycle [2]. Human *BRCA1* mRNA and protein share this cell cycle-dependent pattern of expression suggesting that the human and mouse proteins have a conserved S phase-specific function [3-6]. In order to determine if the protein expression pattern of Brca1 parallels that of its mRNA, synchronization experiments were performed using HC11 murine mammary epithelial cells. HC11 cells were grown in RPMI medium containing 10% bovine calf serum, 5 μ g/ml insulin (Sigma), 10 ng/ml epidermal growth factor (Sigma), 2 mM L-glutamine, 100 units/ml penicillin, and 100 μ g/ml streptomycin. Mouse embryo fibroblasts were grown in DMEM containing 15% fetal bovine serum supplemented with 2 mM L-glutamine, 100 units/ml penicillin, and 100 μ g/ml streptomycin. 293T cells were grown in DMEM containing 10% bovine calf serum supplemented with 2 mM L-glutamine, 100 units/ml penicillin, and 100 μ g/ml streptomycin. HC11 cells and fibroblasts were serum starved at 75% confluency and refed with regular growth media 48 hours later. As shown in Fig. 3A, the mouse Brca1 protein, p210Brca1, is undetectable in serum starved cells and becomes apparent when cells have progressed into the G1 phase of the cell

cycle. As shown in Fig. 3B, in a similar experiment performed on $Brca1^{\Delta-11/\Delta-11}$ fibroblasts, the expression of p92Brca1 closely mimics that of p210Brca1. . RAD51 (Ab-1, Calbiochem), RAD50 (Clone 13, Transduction Laboratories), Cyclin A (H-432, Santa-Cruz Biotechnology) antibodies were each used at a 1:1000 dilution in blocking buffer for 1 hr. The expression of p92 and p210 Brca1 is similar to the expression profile of Rad51 and cyclinA with peak levels during S phase, and in contrast to Rad50 which is expressed at constant levels throughout the cell cycle.

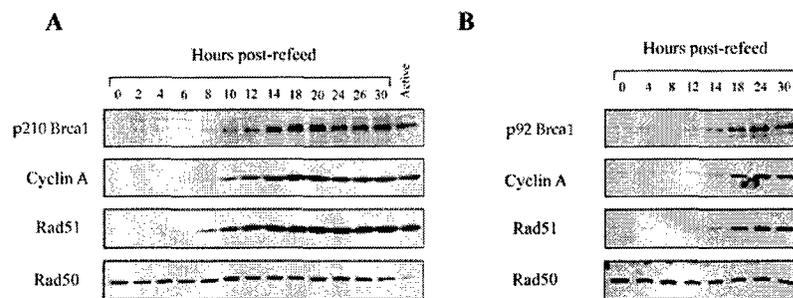


Fig.3 A) Immunoblot analysis of cell cycle regulation of p210^{Brca1}, cyclin A, and Rad51 in serum-starved HC11 cells. B) Immunoblot analysis of cell cycle regulation of p92^{Brca1- Δ 11}, cyclin A, and Rad51 in serum-starved mouse embryonic fibroblasts homozygous for the targeted deletion of exon 11. Cells were starved as described in Materials and Methods. Cells stimulated to reenter the cell cycle by refeeding were harvested at the time points indicated. Active refers to exponentially growing cells. Cell extracts were prepared as described in Materials and Methods and 10 μ g of lysate was loaded per lane. Antibodies mAb1 and mAb2 revealed identical results in HC11 cells whereas only mAb1 recognized a cell cycle regulated band in $Brca1^{\Delta-11/\Delta-11}$ MEFS.

The phosphorylation of p220BRCA1 following treatment of cells with DNA damaging agents suggested that human BRCA1 is involved in a DNA damage response pathway. Ultraviolet (UV) doses were administered using a Stratalinker (Stratagene). Hydroxyurea (HU; Sigma) was used at a final concentration of 1 mM. Cells were lysed 1 hr following treatment with genotoxic agents. Immunoblotting analysis of lysates generated from HC11 cells one hour following

treatment with UV, γ -radiation, or HU revealed a dose-dependent shift in the migration of full length mouse Brca1 by SDS-PAGE (Fig. 4A, top panel). A complete shift of p210Brca1 occurred in cells treated with 5000 Rads similar to that described for human BRCA1.

^{32}P -orthophosphate labeling of HClI cells following treatment with 50 Rads demonstrated an increase in Brca1 labeling consistent with the supposition that, similar to human BRCA1, the observed mobility shift is due to phosphorylation (Fig. 4B). Gamma irradiation was administered using a CIS bio international (IBL 437c) source. For *in vivo* labeling experiments, irradiated cells were incubated with 5 mCi of ^{32}P orthophosphate in serum free medium immediately following dosing for 1 hr prior to lysis and immunoprecipitation. Exposure of cells to ^{32}P -orthophosphate has previously been shown to cause an increase in phosphorylation of human BRCA1 [7]. The basal levels of phosphorylation observed in un-irradiated cells may be due either to the activation of a DNA damage response pathway by ^{32}P , or to cell cycle-dependent phosphorylation of Brca1. Orthophosphate labeling experiments are currently underway to confirm the lack of phosphorylation of p92Brca1.

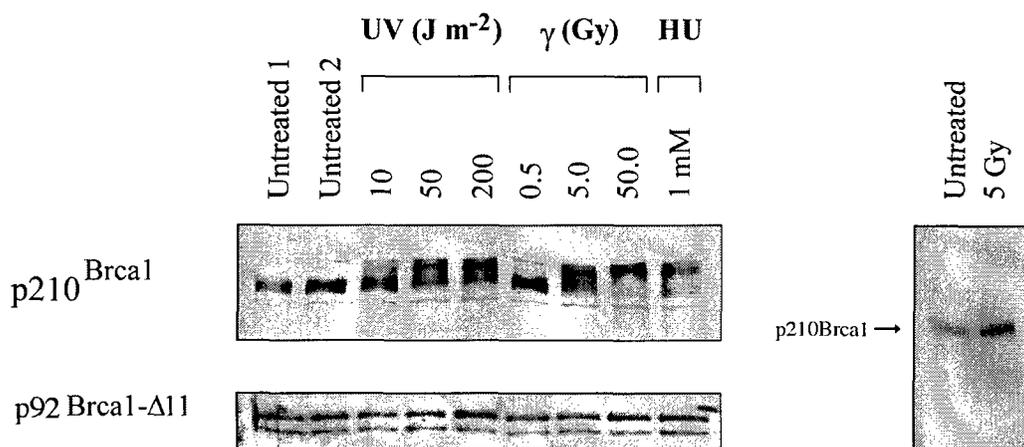


Fig. 4. (Left) Immunoblot analysis of p210^{Brca1} and p92^{Brca1-Δ11} in cells treated with UV, γ-irradiation, or HU. p210^{Brca1} demonstrates a dose-dependent shift in response to UV and gamma irradiation. HClI cells (top panel) or MEFs that express only the p92^{Brca1-Δ11} isoform (bottom panel) were subject to identical treatments with UV, gamma irradiation, or HU. 20 μg of lysate was loaded per lane and immunoblotted with antibody mB1. (Right) Analysis of p210^{Brca1} phosphorylation in ³²P-orthophosphate labeled HClI cells treated with γ-irradiation. Immediately following irradiation, HClI cells were incubated with 5 mCi of ³²P-orthophosphate for 1 hr. 3 mg of cell extract was used for immunoprecipitation with 10 μl of the IgG fraction of mB1 antibody. Resolution was not sufficient to detect a mobility shift of phosphorylated products.

In order to determine if the lack of phosphorylation of p92Brca1 is due to aberrant cellular localization, biochemical fractionation of exponentially growing Brca1^{Δ11/Δ11} fibroblasts was performed and nuclear and cytoplasmic extracts were analyzed by immunoblot analysis. To confirm the purity of these fractions, blots were probed with antisera specific either for β-tubulin or RAD50 as controls for cytoplasmic and nuclear proteins, respectively. To our surprise, p92Brca1 was present in the nuclear fraction.

Localization to nuclear foci during S phase is a cardinal feature of human BRCA1. In order to establish the subnuclear localization of murine p210 and p92Brca1 immunofluorescence analysis (IF) was performed (Fig. 5). Cells were fixed and permeabilized according to published protocols [8]. Affinity purified Brca1 antisera were used at a concentration of 2 μg/ml. RAD51 (Ab-1) antisera were used at a 1:1000 dilution. TRITC-conjugated secondary antibody (Jackson Immunoresearch) was used at a dilution of 1:250. All images were obtained by confocal microscopy.

Four independent antisera (mAb1-mAb4) revealed that p210Brca1 localizes to nuclear foci during S phase. Strikingly, when similar experiments were performed on Brca1^{Δ11/Δ11} fibroblasts, distinct nuclear foci were observed that were similar to those observed in HClI

mammary epithelial cells and in wild type mouse embryo fibroblasts (Fig. 5). Since these cells do not express p210Brca1, any specific signal is due to the p92Brca1. Notably, no signal was observed in the cytoplasm of these cells. Moreover, nuclear foci were not detected with exon 11-specific antisera mAb2 and mAb3.

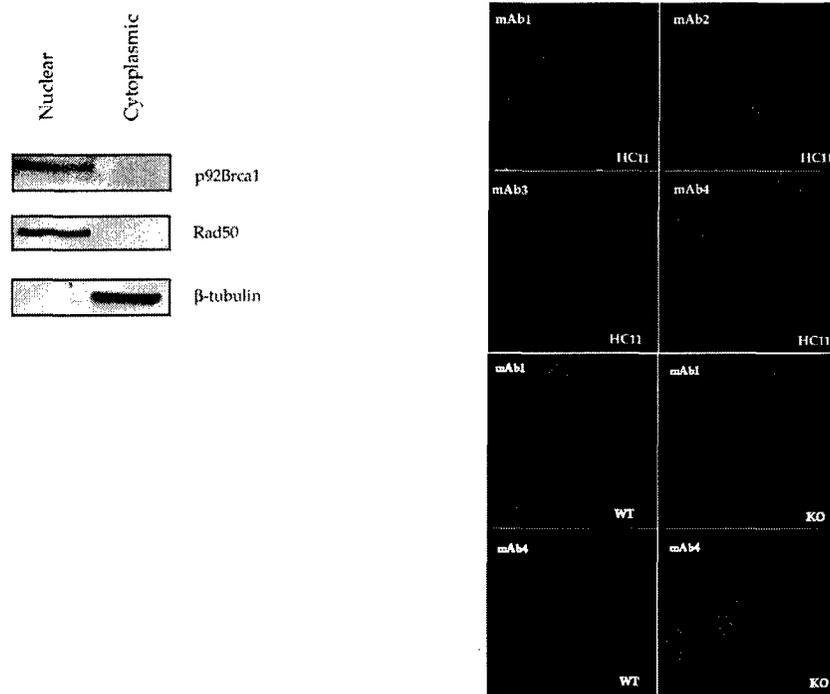


Fig. 5 Western analysis of biochemical fractionation of Brca1 ^{Δ -11/ Δ -11} MEFs (4A). Immunofluorescence analysis of Brca1 subcellular localization. HC11 cells, wild-type MEFs, and Brca1 ^{Δ -11/ Δ -11} MEFs were grown on microscope slides. Following permeabilization, S phase cells were incubated with affinity purified Brca1 antibodies at a concentration of 1 μ g/ml.

The experimental methodology employed to generate the data described herein have not been explicitly stated. In order to analyze the defects in repair in the cells we have analyzed it was necessary to characterize the cells as we have described here. We are currently in the process of putting together a manuscript which will include this data. The construction of plasmids for homologous recombination assays has been completed and the assays to determine if these cells are defective in transcription-coupled repair and homologous recombination are currently underway.

Statement of Work

Task 1: Generation of a transgenic mouse model that expresses human BRCA1: months 1-36

- Construction of transgenic construct for microinjection: months 1-6
- Breeding of transgenic lines for harvest of genomic DNA and screening for founder lines: months 6-12
- Breed transgenic mice into Brca1 heterozygous background: months 12-18
- Breed bitransgenic animals with Brca1 heterozygous mice: months 18-24
- Analyze complementation capacity of human BRCA1 in mouse cells by cell cycle analysis and DNA repair assays: months 24-36

Task 2: Identification of genes regulated by mouse Brca1 expression: months 1-36

- Generation of mRNA from mouse embryo fibroblasts: months 1-6
- Preparation of mRNA for hybridization and application to test chips: months 6-12
- Hybridization of samples to arrays and analysis of results: months 12-18
- Analysis of candidate genes by Northern blot analysis: months 18-24
- Experiments designed to demonstrate functional relevance of candidate genes with respect to Brca1 function: months 24-36.

Task 3: DNA damage and cell cycle checkpoint in exon 11 Brca1 knockout mouse embryo fibroblasts: months 12-36

- Construction of plasmids for homologous recombination assay: months 12-16
- Transfection of cell lines to assay for homologous recombination: months 16-24
- Transcription-coupled repair of thymine glycol lesions in MEFS: months 16-24
- Generation of retroviral constructs for infection of MEFS: months 18-24.
- Experiments to determine if Brca1 mutant phenotype is reversible: months 24-36.

(6) Key Research Accomplishments

- Generation of transgenic mouse lines that express human BRCA1.
- Generation of immortalized exon 11 mutant Brca1 mouse embryo fibroblasts for analysis of cell cycle and DNA damage.
- Generation of four specific mouse Brca1 antisera
- Demonstration that mouse Brca1, like human BRCA1 is cell cycle regulated and phosphorylated in response to DNA damage.
- Demonstration that unlike the human BRCA1 exon 11 deficient isoform, mouse exon 11-deficient Brca1 localizes to the nucleus.
- Demonstration that the exon 11 region of mouse Brca1 is critical for phosphorylation in response to DNA damaging agents.
- Demonstration that full length and the exon 11-deleted Brca1 isoform localize to nuclear foci.

(7) Reportable Outcomes

- Generation of four specific mouse Brca1 antisera
- Demonstration that mouse Brca1, like human BRCA1 is cell cycle regulated and phosphorylated in response to DNA damage.
- Demonstration that unlike the human BRCA1 exon 11 deficient isoform, mouse exon 11-deficient Brca1 localizes to the nucleus.
- Demonstration that the exon 11 region of mouse Brca1 is critical for phosphorylation in response to DNA damaging agents.
- Demonstration that full length and the exon 11-deleted Brca1 isoform localize to nuclear foci.

(8) Conclusions

The data presented in this report demonstrate that multiple features of the regulation, localization, and interactions of the mouse and human Brca1 proteins are conserved. Similar to its human homolog, mouse Brca1 is cell cycle regulated and localizes to nuclear foci during S phase. In addition, mouse Brca1 is phosphorylated in a dose-dependent manner in response to genotoxic agents suggesting that a similar kinase(s) exists in human and mouse cells that is upstream of Brca1 in a DNA damage response pathway. Like human BRCA1, mouse Brca1 also forms a complex with Rad51 consistent with experiments demonstrating that mouse Brca1 functions in the repair of double-stranded breaks by homologous recombination.

We have analyzed the expression of a Brca1 isoform predicted to arise in fibroblasts derived from mouse embryos in which the exon 11 region of Brca1 is specifically deleted from the mouse germline. Strikingly, we found that p92^{Brca1-Δ11} is localized to nuclear foci in a manner that is indistinguishable from that observed in wild-type cells. Our findings contrast with the cytoplasmic localization previously reported for human p97^{BRCA1-Δ11} and p110^{BRCA1-Δ11b}. Each of these isoforms lacks the nuclear localization sequences reported to be required for nuclear transport of BRCA1. Nevertheless, the partial nuclear localization of human p110^{BRCA1-Δ11b} suggests that sequences other than the canonical *BRCA1* nuclear localization sequences can be utilized for transport into the nucleus. Such cryptic nuclear localization sequences may also be responsible for the nuclear localization of p92^{Brca1-Δ11}. Alternatively, the difference in localization between the mouse and human isoforms may be due to either cell type-

specific differences, species-specific differences or the nature of the assays employed for these studies. We favor the latter possibility. Whereas studies in human cells have relied on exogenously expressed BRCA1 using transient transfection assays, we have determined the pattern of expression of the endogenous Brca1 protein. Nevertheless, we cannot rule out the possibility that p92^{Brca1-Δ11} may localize to the cytoplasm in cell types other than those examined here, or that human and mouse exon 11-deleted isoforms may localize differently.

Significantly, in response to DNA damage, p210^{Brca1} displays a mobility shift indicative of phosphorylation, whereas the p92^{Brca1-Δ11} isoform does not. The inability to detect a shift is not due to a conformational change in the p92^{Brca1} protein that would preclude altered mobility by SDS-PAGE since P32 labeling experiments failed to reveal an increase in phosphate incorporation in response to gamma radiation. An explanation for this result is that the region encoded by exon 11 of *Brca1* may be a target for phosphorylation. The Cds1 kinase has been shown to phosphorylate serine 988 of human BRCA1 in response to gamma radiation. A putative Cds1 phosphorylation site is present in mouse Brca1 within the exon 11 region suggesting that regulation of p92^{Brca1} function in response to activation of this kinase may be impaired. This suggestion is bolstered by experiments that have defined a region within exon 11 of human BRCA1 that is required for binding to Cds1.

Following high dose radiation serines 1423 and 1524 of human BRCA1 are targets of the ATM kinase. Although these potential ATM phosphorylation sites are present in p92^{Brca1}, our data indicates that they are not phosphorylated. ATM has been placed in a pathway upstream of Cds1. An interpretation that is suggested by our data is

that ATM phosphorylation of Brca1 is dependent on prior phosphorylation of serine 988.

This mechanism supports a model in which ATM-dependent Cds1 activity directly phosphorylates BRCA1 in response to lower doses of gamma radiation and in which BRCA1 becomes a direct target of ATM at high doses of gamma radiation.

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21 Feb 03

MEMORANDUM FOR Administrator, Defense Technical Information
Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir,
VA 22060-6218

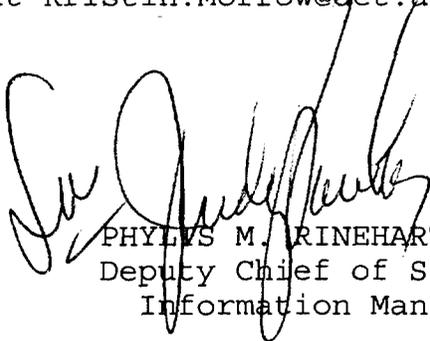
SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports written for this Command. Request the limited distribution statement for the enclosed accession numbers be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Kristin Morrow at DSN 343-7327 or by e-mail at Kristin.Morrow@det.amedd.army.mil.

FOR THE COMMANDER:

Encl


PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

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