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13. ABSTRACT (Maximum 200 Words)

Understanding molecular mechanisms of normal mammary gland development is essential for elucidating the relationship between normal, hormonally driven developmental events and carcinogenesis. We have studied a molecule, Brca2, which is highly expressed early in murine mammary gland development and report that this protein shares significant properties (upregulation at G1/S boundary, localization to "nuclear dots", interaction with Brca1) with its human homologue despite relatively low sequence identity (~57%). Additionally, we have undertaken a DNA microarray-based survey of gene expression during murine mammary gland development. Expression of ~6000 genes has been ascertained at each of 13 developmental time points. We have developed informatics tools which allow us to manipulate and interpret patterns of gene expression within this data set, thus allowing us to look at mammary gland development in a more detailed way than has been possible heretofore.

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



PI - Signature

11/3/99

Date

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Introduction

As originally submitted, this training grant undertook a molecular characterization of normal murine mammary gland development during puberty. A related goal of this research was the identification of pathways active during puberty which might influence the susceptibility of the gland to subsequent carcinogenic events. As detailed in previous annual reports, "Year One" focused on the generation of non-cross-hybridizing libraries specific to mid-pubertal (5 week) and adult (15 week) murine mammary gland as well as additional studies which attempted to elucidate lineage relationships between cell types in the developing mammary gland. Technical difficulties with this latter project, coupled with the low percentage of clones from the stage-specific libraries which showed detectable differential expression on Northern analysis, led to a shift in emphasis during year two to a molecule (Brca2) that is known to be upregulated in early development and which exhibits differential regulation in 5-week vs. 15-week mammary gland RNA. The "Year Two" update reported the generation and characterization of reagents appropriate for the study of murine Brca2. Additionally, we reported that murine Brca2 appears to be a nuclear phosphoprotein which is regulated at the protein level in cell culture during a serum starve/refeed experiment.

This final report will begin by presenting additional data describing murine Brca2 (relationship of starve/refeed data to cell cycle progression, presence in "nuclear dots", co-immunoprecipitation of murine Brca1 with murine Brca2) and summarize progress in developing cell lines which inducibly downregulate mBrca2 levels. This portion of the report thus completes the description of experimental results germane to the updated statement of work implicit in last year's report. Additionally, however, we have obtained access to oligonucleotide microarray technology which allows us to revisit fundamental questions posed in our initial grant proposal in a manner which circumvents technical difficulties which we previously encountered. We will thus conclude by describing preliminary expression data gathered over murine mammary gland development as well as informatics tools for the analysis of these data that were developed during the period supported by this training grant.

Body

Specific Aim I. Determine the role of BRCA2 in the response to DNA damage

Task 1: Generate a full-length clone of BRCA2 (months 13-18)

See "Year 2" report.

Task 2: Generate/test antibodies which detect mouse and human BRCA2 (months 13-24)

See "Year 2" report.

Task 3: Determine the subcellular localization of Brca2 (months 19-24)

See "Year 2" report.

Additional data:

We previously demonstrated that Brca2 is localized to the nucleus. We have subsequently demonstrated that a subset of NmuMG cells localize Brca2 to "nuclear dots", a subnuclear pattern which has been associated with Brca1/Rad51/BARD expression (data not shown; Scully *et al* 1997a; Scully *et al* 1997b). These data suggest that the murine protein has a similar subcellular distribution to human BRCA2. For the significance of this point, see below (Task 5).

In addition, some of our preliminary data suggests that mBrca2 may colocalize with mitotic centrosomes (data not shown). This result, if confirmed by further work, would be of particular interest given a recent report of centrosomal abnormalities in Brca2 knockout cells. (Tutt *et al*, 1999); additionally, it has previously been reported (Hsu *et al* 1998) that BRCA1 also associates with the mitotic centrosome.

Task 4: Determine whether Brca2 is a phosphoprotein (months 19-24)

See "Year 2" report.

Task 5: Determine whether BRCA1 interacts with BRCA2 (months 25-36)

Work within these last 12 months by multiple members of our lab, one of whom has generated antisera which detect and immunoprecipitate mBrca1, has demonstrated that mBrca1 can be co-immunoprecipitated with mBrca2. This has previously been reported for human BRCA1 and BRCA2 (Chen *et al* 1998), and our results are important in providing the first evidence that the murine protein behaves in a similar manner to the human protein. This point should not be overlooked, since the relatively low conservation between hBRCA2 and mBrca2 (~57% identity) has led some to question whether knockouts of the murine gene provide relevant indications of the function of BRCA2 in human disease. These data (conserved interaction, presence in nuclear dots, and similar regulation on serum starve/refeed), taken together, make a strong case that mBrca2 function is likely to be a good model for human BRCA2 function in development and carcinogenesis.

Task 6: Determine the effect of altered BRCA2 levels on the ability to repair DNA damage. (months 25-36)

Final resolution of this aim has depended on a subtask aimed at generating cell lines which inducibly downregulate Brca2 levels. As previously reported ("Year 2" report), we have a murine mammary epithelial cell line which expresses the reverse tetracycline transactivator (rtTA); thus, stable transfection of this cell line with a construct containing a gene of interest downstream of TetO should provide the ability to inducibly turn on gene expression upon addition of tetracycline or doxocycline to culture medium. We designed hammerhead ribozymes which recognize four different regions of mBrca2,

placed them under the control of TetO, and stably transfected our rtTA-containing cell line (HC11.C1). Clones representing each construct (as well as a control construct) have been analyzed, and several clones containing a ribozyme directed against the 5' end of *mBrca2* appear to show dox-dependent regulation of Brca2 levels (as measured by Western blot). However, characterization of these clones is ongoing and direct experiments measuring DNA repair depend on the successful resolution of this phase.

An additional strategy to determine the effect of altered Brca2 levels in vivo has been undertaken in collaboration with other members of the lab. A full-length human BRCA2 clone (generated as part of Specific Aim I, Task 1) was placed under the control of TetO and used to generate transgenic mice. These mice have been crossed with other transgenic mice containing rtTA under the control of a breast-specific promoter (MMTV), and analysis of these bitransgenic mice is ongoing.

Specific Aim II: Determine the role of BRCA2 in Cell Cycle progression

Task 1: Generate a full-length clone of BRCA2 (months 13-18)

See "Year 2" report.

Task 2: Generate/test antibodies for detecting BRCA2 (months 13-24)

See "Year 2" report.

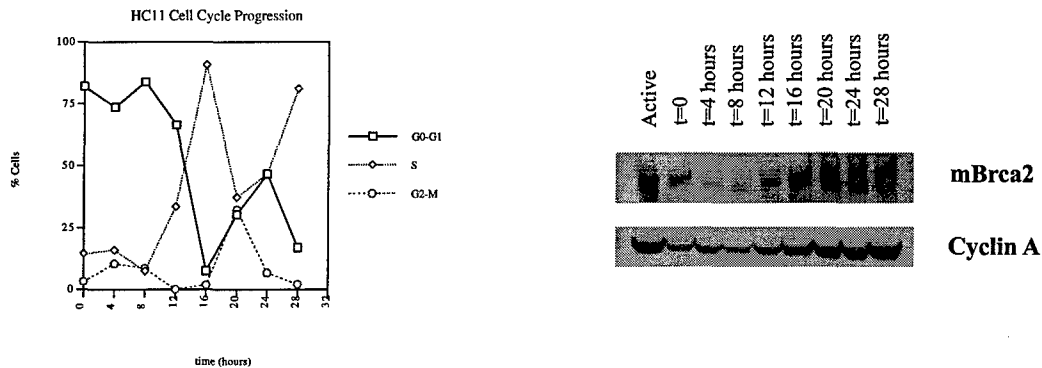
Additional data: An additional anti-mBrca2 antisera (Ab 1102) suitable for Western blotting and immunoprecipitation (but not immunofluorescence) has now been obtained by a member of the lab. Additionally, a chicken anti-Brca2 antibody has shown preliminary promise for immunofluorescence.

Task 3: Determine whether Brca2 levels are regulated as a function of cell cycle progression (months 19-24).

See "Year 2" report.

Additional data: We previously reported that mBrca2 protein levels are regulated during a serum starve/refeed experiment in multiple murine mammary epithelial cell lines. To further characterized the timing of this upregulation, flow cytometry was used to measure progression of HC11 cells through the cell cycle after refeeding with 20% serum. Additionally, cyclin A protein levels were measured in order to correlate the expression of mBrca2 with a protein of known cell-cycle distribution. Taken together, these data clearly demonstrate an upregulation of Brca2 protein levels near the G1/S transition in a pattern very similar to cyclin A.

HC11 serum starve/refeed



HC11 FACS: cells were stained with propidium iodide and counted. Gaussian curve-fitting was used to estimate the proportion of cells in each phase.

HC11 Western blots: extracts were harvested at each time point following refeed and analyzed by Western blot.

In order to determine whether mBrca2 is similarly regulated at the G1/S transition in freely cycling cells, we attempted to simultaneously measure DNA content (using propidium iodide) and Brca2 levels (using anti-Brca2 antisera Ab945 and a FITC-conjugated anti-rabbit secondary antibody) in actively growing cell cultures. While we have been able to demonstrate cell-cycle-regulated expression of cyclin A and cyclin E using appropriate antisera, we were unable to detect cell-cycle-related differences in Brca2 levels. Whether this is due to low levels of Brca2 protein, an inability of Ab945 to robustly detect Brca2 in cells fixed with ethanol, or a *bona fide* lack of regulation outside the context of prior serum starvation is unknown.

Task 4: Determine the effect of altered BRCA2 levels on cell cycle progression (months 25-36)

See Specific Aim I, Task 6.

Supplementary Aim: Additional work addressing original statement of work.

As originally submitted, this training grant proposed the use of differential screening and subtractive hybridization in order to identify genes which are up- or down-regulated in the pubertal (versus adult) murine mammary gland. The emergence of high-density oligonucleotide microarrays for high-throughput expression analysis has allowed us to readdress this problem in a technically superior way. We used Affymetrix GeneChip arrays (see, e.g. Fambrough *et al* 1999; Alon *et al* 1999 for recent uses of this technology) capable of detecting the expression levels of ~6000 murine genes to measure mammary gland RNA levels at 13 developmental time points (2 week G0P0, 5 week G0P0, 10 week G0P0, 15 week G0P0, day 6 pregnancy, day 12 pregnancy, day 18

pregnancy, hour 18 lactation, day 9 lactation, day 2 post-lactational regression, day 7 regression, day 28 regression, and 9.5 week Male). Additionally, we obtained multiple samples at several time points (15 week GPO, d28 regression) in order to ensure that we would be able to assess the reproducibility of this technique.

To handle the >78,000 data points gathered over murine mammary gland development, we have developed a number of informatics tools designed to aid in our analysis. All data was first transferred to a relational database (MySQL engine) prior to scaling. Sample-to-sample scaling was accomplished by rank-ordering on a per-chip basis and discarding the top and bottom ~2% of samples prior to scaling to the mean of remaining levels. The maximum per-chip noise value (reported by Affymetrix software) across development was used to set a minimum expression threshold below which levels were clipped. In order to allow easy browsing of expression profiles, a Java-based program ("ExpBrowser") was written which allows graphical access to the expression database.

Additional routines were constructed which allow the use of clustering software from other labs (e.g. hierarchical clustering, Eisen *et al* 1998; self-organizing maps, Tamayo *et al* 1999) in the analysis of our expression data. To further aid in the interpretation of these results, we determined the need for systematic categorization of the ~6000 genes on each chip. In light of the size of this task and its eventual extension to chips of higher density (11K and 19K chips anticipated from Affymetrix), the optimal solution to this problem would leverage existing public database information. We therefore wrote a series of programs which compare chip sequences to existing SwissProt entries and identify keywords from identical or similar sequences. A database of categories was thus constructed and Java-based front end ("Gene-In-A-Box") which allows easy manipulation of this database and seamless linking to external data sources (GenBank, GeneCards, Entrez, etc.) was written. Once cluster analysis had been performed, categories were cross-referenced by cluster in order to determine disproportionate distributions of gene classes among specific expression patterns during mammary gland development. In order to determine the statistical significance of these results, a Monte Carlo analysis program was subsequently written.

The approach described above has allowed us to identify underlying biological processes during mammary gland development. Trivially, it allowed us to fulfill our initial goal of identifying genes differentially expressed during puberty (57 genes 3-fold upregulated in puberty versus adult; 59 genes 3-fold upregulated in adult versus puberty). More significantly, we have been able to identify broader patterns of gene expression during murine mammary gland development. Details of this analysis will be reported (see "Papers in preparation").

Training

In addition to the results discussed above, other training aspects of activity during the last year should once again be highlighted. Interaction of the trainee (SRM) with his advisor (LAC) and other members of the Chodosh lab is facilitated by regularly scheduled meetings with his advisor, by biweekly meetings of the BRCA1/BRCA2 working group

in the lab (which consists of three graduate students and two postdoctoral fellows), and by weekly lab meetings at which members alternate presenting data and strategy for critical analysis by other members of the lab . In addition, within the past year the PI (SRM) has delivered a talk in the Institute for Human Gene Therapy graduate student/postdoc seminar series and presented posters at the 1999 Gordon Research Conference on Mammary Gland Biology as well as the 1999 University of Pennsylvania School of Medicine Combined Degree Retreat. In addition, SRM was able to attend the recent Jackson Laboratories conference ("Modeling Human Mammary Cancer in Mice") at which LAC presented some of the preliminary Affymetrix data described above. Once again, a wide variety of relevant seminars are have been available at the University of Pennsylvania and have contributed significantly to SRM's predoctoral training. Upon completion of the PhD thesis (projected April, 2000 defense), SRM is scheduled to return to clinical training for the completion of his MD/PhD program.

REFERENCES

- Alon U, Barkai N, Notterman DA, Gish K, Ybarra S, Mack D, and Levine AJ. (1999). Broad patterns of gene expression revealed by clustering analysis of tumor and normal colon tissues probed by oligonucleotide arrays. *Proc Natl Acad Sci USA* 96(12):6745-50.
- Bertwistle D, Swift S, Marston NJ, Jackson LE, Crossland S, Crompton MR, Marshall CJ, and Ashworth A. (1997). Nuclear location and cell cycle regulation of the BRCA2 protein. *Cancer Res* 57:5485-8.
- Chen J, Silver DP, Walpita D, Cantor SB, Gazdar AF, Tomlinson G, Couch FJ, Weber BL, Ashley T, Livingston DM, and Scully R. (1998). Stable interaction between the products of the BRCA1 and BRCA2 tumor suppressor genes in mitotic and meiotic cells. *Mol Cell* 2(3):317-28.
- Eisen MB, Spellman PT, Brown PO, and Botstein D. (1998). Cluster analysis and display of genome-wide expression patterns. *Proc Natl Acad Sci USA* 95(25):14863-8.
- Fambrough D, McClure K, Kazlauskas A, and Lander ES. (1999). Diverse signaling pathways activated by growth factor receptors induce broadly overlapping, rather than independent, sets of genes. *Cell* 97(6):727-41.
- Hsu LC, and White RL. (1998). BRCA1 is associated with the centrosome during mitosis. *Proc Natl Acad Sci USA* 95(22):12983-8.
- Scully R, Chen J, Plug A, Xiao Y, Weaver D, Feunteun J, Ashley T, and Livingston DM. (1997a). Association of BRCA1 with Rad51 in mitotic and meiotic cells. *Cell* 88(2):265-75.
- Scully R, Chen J, Ochs RL, Keegan K, Hoekstra M, Feunteun J, and Livingston DM. (1997b). Dynamic changes of BRCA1 subnuclear location and phosphorylation state are initiated by DNA damage. *Cell* 90(3):425-35.
- Tamayo P, Slonim D, Mesirov J, Zhu Q, Kitareewan S, Dmitrovsky E, Lander ES, and Golub TR. (1999). Interpreting patterns of gene expression with self-organizing maps: methods and application to hematopoietic differentiation. *Proc Natl Acad Sci USA* 96(6):2907-12.
- Tavazoie S, Hughes JD, Campbell MJ, Cho RJ, and Church GM. (1999). Systematic determination of genetic network architecture. *Nature Genetics* 22(3):281-5.
- Tutt A, Gabriel A, Bertwistle D, Connor F, Paterson H, Peacock J, Ross G, and Ashworth A. (1999). Absence of *brca2* causes genome instability by chromosome breakage and loss associated with centrosome amplification. *Current Biology* 9(19):1107-10.

APPENDIX

This Department of Defense predoctoral training grant, which expired in September 1999, was used to fund PhD thesis work for Stephen R. Master. In addition to work described in the annual report to which this is appended, the following accomplishments supported by this grant should be noted:

Key research accomplishments:

- Generation of non-cross-hybridizing cDNA libraries from pubertal and adult virgin murine mammary gland.
- Generation of a full-length, epitope-tagged BRCA2 clone.
- Demonstration that mBrca2 protein levels are upregulated near the G1/S transition upon serum starvation/refeeding.
- Demonstration that mBrca2 localizes to "nuclear dots" in murine mammary epithelial cell lines.
- Measurement and analysis of the expression of ~6000 genes over 13 timepoints during murine mammary gland development.
- Development of a library of software tools to aid in the analysis of gene expression profiles.

Reportable outcomes:

Ph.D. degree, expected April, 2000:

Cell and Molecular Biology Graduate Group, Biomedical Graduate Studies,
University of Pennsylvania School of Medicine, Philadelphia, PA
Thesis Advisor: Lewis A. Chodosh, M.D., Ph.D.

Ph.D. Thesis: A Molecular View of Mammary Gland Development

Poster, "Regulation and Localization of mBrca2 in Mammary Epithelial Cells." 1999
Gordon Research Conference on Mammary Gland Biology, New Hampshire, June 1999.
(see attachment).

Anticipated Publications/Manuscripts in preparation:

Master SR., Hartman JL, D'Cruz CM, Moody SE, and Chodosh LA. Global patterns of gene expression during mammary gland development: microarray analysis in a complex organ.

Master SR., Hartman JL, D'Cruz CM, Moody SE, and Chodosh LA. Oligonucleotide microarray analysis of postlactational mammary gland regression reveals multiple waves of coordinate gene expression.

Master SR^{*}, Sarkisian CJ^{*}, Huber LJ, Yang T, and Chodosh LA. Murine and human BRCA2 have conserved regulatory and functional properties.

Huber LJ, Sarkisian CJ, Master SR, Yang T, Deng CX, and Chodosh LA. Murine Brca1 exon 11-deleted isoform shares properties of full length Brca1 protein.

D'Cruz CM, Moody SE, Hartman JL, Master SR, Cox JD, Ha SI, Wang JY, and Chodosh LA. Reproductive history results in a permanent change in the expression of specific genes in the murine breast.

Software:

Gene Expression Relational Database
ExpBrowser
Gene-In-A-Box
Swiss-Prot Mining Suite
Cluster/Category Analysis Suite
(additional informatics infrastructure)

^{*} Both authors contributed equally to this work.

Abstract – 1999 Gordon Research Conference on Mammary Gland Biology

Regulation and Localization of mBrca2 in Mammary Epithelial Cell Lines

Stephen R. Master, Christopher J. Sarkisian, L. Julie Huber, Thomas Yang,
Seung I. Ha, and Lewis A. Chodosh

Department of Molecular and Cellular Engineering,
University of Pennsylvania School of Medicine,
Philadelphia, Pennsylvania

Inherited mutations in the tumor suppressor gene *BRCA2* have been reported to account for >30% of familial breast cancer cases. A number of groups has suggested that the murine homologue (mBrca2) mediates a cellular response to DNA damage; additionally, data from our laboratory has shown that *Brca2* mRNA is upregulated in actively proliferating and differentiating compartments during murine development. The use of animal models is essential for addressing the biological relevance of *BRCA2* function to breast development and carcinogenesis; however, the relatively low sequence conservation between the mouse and human homologues (~57% identity) necessitates confirmation that these proteins behave in similar ways. To address this issue, we have raised antisera which detect mBrca2 in multiple murine cell lines. We have confirmed that these antisera detect *bona fide* mBrca2 by demonstrating detection of a band that comigrates with both endogenous simian (and human) *BRCA2* and with an exogenously expressed, epitope-tagged h*BRCA2*. Like the human homologue, mBrca2 levels are regulated in a cell cycle-dependent fashion with the greatest increase near the G1/S boundary. Additionally, mBrca2 can be found in "nuclear dots" similar to the human structures known to contain both *BRCA2* and *BRCA1*. These data suggest that both the subcellular localization and regulation of mBrca2 are conserved, lending further weight to the idea that murine models may accurately reflect the role of *BRCA2* in human tumorigenesis. We further describe systems which will allow us to inducibly up- and down-regulate *Brca2* expression in order to more directly address functional questions.

Appendix 2:

Referenced portions of Year 2 Report

Body

Specific Aim I. Determine the role of BRCA2 in the response to DNA damage

Task 1: Generate a full-length clone of BRCA2 (months 13-18)

Long-range PCR was used to amplify overlapping pieces of human *BRCA2* cDNA, and the resulting clone was confirmed by sequencing and comparison to published *BRCA2* sequences. Mutations resulting from this PCR cloning strategy were corrected (MORPH, 5'-3'), and the relevant fragments were resequenced to ensure that no further mutations were introduced during this correction process. A single new mutation was corrected, and the resulting wild-type clone was assembled (Figure 1). In addition, mutations were introduced in order to create clones containing either the 999del5 (common in the Icelandic population—Thorlacius *et al*, 1996) or 6174delT (common in the Ashkenazi Jewish population—Oddoux *et al*, 1996) mutations. Thus, full-length wild-type and mutant *BRCA2* clones were successfully constructed.

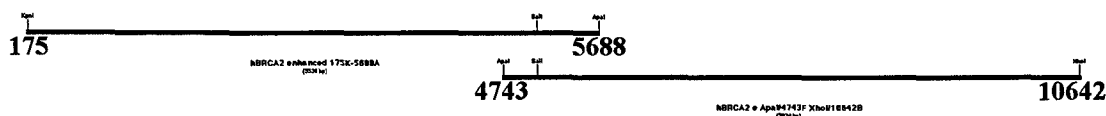


Figure 1: Construction of full-length human *BRCA2* clone

After long-range PCR had been performed to generate two overlapping fragments of human *BRCA2* cDNA, the clones were sequenced. Mutations (defined as nonsilent deviations from the published *BRCA2* sequences or common polymorphisms) were corrected and the clones were resequenced to confirm that no further mutations had been introduced. In addition, clones containing common mutations (999del5, 6174delT) were generated.

In order to facilitate further manipulation of *BRCA2* despite the lack of single-cutting enzymes in its (large) coding sequence, we designed primers which would introduce silent mutations spaced across *BRCA2* and which create unique restriction sites. This results in the construct which we have designated B2R (Figure 2).

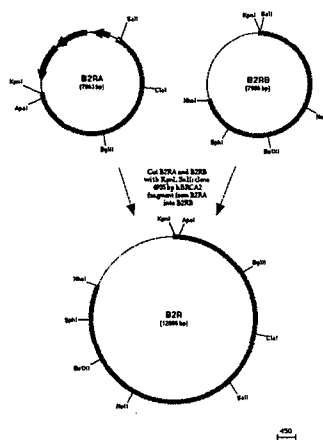


Figure 2: Construction of B2R

Primers were designed which incorporate silent mutations into *BRCA2* and which create unique restriction sites in order to allow manipulation of *BRCA2* sequence. This full-length construct (in pSP73 backbone) is shown at the bottom with unique sites marked.

Additionally, a version of B2R incorporating an appropriate restriction site immediately 5' to the initiating ATG was used to create full-length constructs in the pcDNA3 expression vector (Invitrogen) which contain a Kozak sequence followed by an N-terminal epitope tag (FLAG or HA). Transient transfection of COS7 cells with either FLAG- or HA-tagged B2R produces a large protein consistent with a predicted size of 384 kD protein which migrates above a similarly tagged BRCA1 (Figure 4). Thus, we have created full-length, epitope-tagged BRCA2 constructs which can be transiently expressed and detected on Western blot.

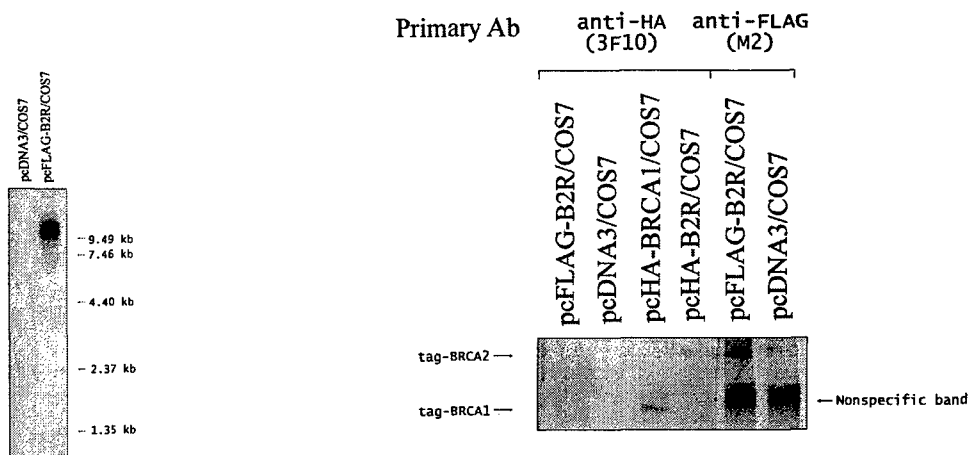


Figure 3: Constructs (pcFLAG-B2R or a control) were transiently transfected into COS7 cells, and RNA was harvested. The Northern blot was probed with a fragment of *BRCA2* (N.B. longer exposures detect endogenous *BRCA2* in control-transfected cells)

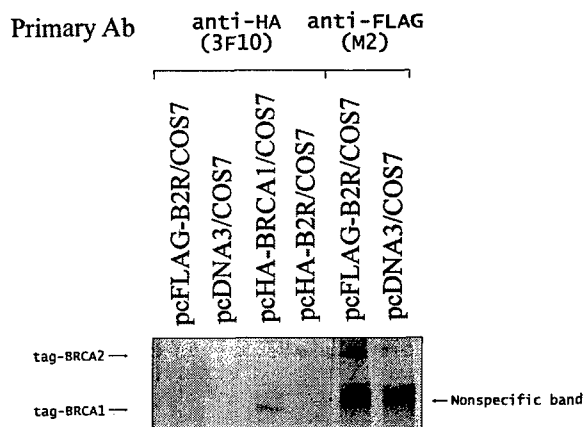


Figure 4: Constructs (FLAG- or HA-tagged BRCA1 or BRCA2) were transiently transfected into COS7 cells, and protein was harvested. A Western blot of these lysates was probed with either anti-HA (3F10, Boehringer Mannheim) or anti-FLAG (M2, Kodak) antibodies. Note the presence of a nonspecific band detected by the anti-mouse secondary antibody used to visualize M2.

Task 2: Generate/test antibodies which detect mouse and human BRCA2 (months 13-24)

A rabbit polyclonal antiserum raised against the carboxy terminus of human BRCA2 is now commercially available from Oncogene Research (BRCA2 Ab-2). In order to confirm the identity of the band representing full-length BRCA2, we compared the migration of our full-length FLAG-tagged clone with the prominent band detected by Ab-2 in lysates of the human mammary epithelial cell line MCF7 (data not shown) and the simian SV40-large-T-transformed line COS7 (Figure 5).

Although a commercially available antiserum exists for the detection of human BRCA2 (BRCA2 Ab-2), no reagents have been described which detect murine Brca2. Our lab therefore generated a GST fusion protein containing an amino-terminal fragment of mBrca2 and had purified protein injected into rabbits. The resulting rabbit serum, hereafter designated Ab945, was affinity-purified by covalently binding the original fragment (against which it was raised) to Sepharose beads and performing column purification. Ab945 detects a prominent band in lysates from murine mammary epithelial cell lines NMuMG and HC11, and this band comigrates with the band detected by Ab-2 in COS7 cells (Figure 6). Thus, our evidence suggests that Ab945 is able to detect bona-fide murine Brca2. We further demonstrated that this antiserum is suitable for immunoprecipitation (Figure 7), thus establishing that this reagent has the properties required for crucial aspects of our further experimental design (see below). We thus conclude that we have successfully characterized reagents which allow us to detect either human or murine Brca2.

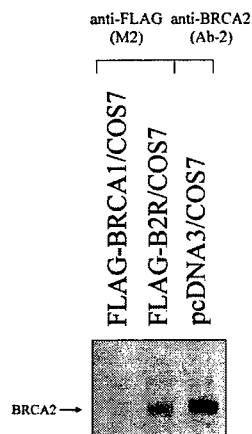


Figure 5: Epitope-tagged BRCA2 comigrates with endogenous BRCA2

Constructs (FLAG-tagged BRCA1 or BRCA2, control vector) were transiently transfected into COS7 cells, and protein was harvested. Protein was separated on an SDS-PAGE gel and blotted onto nitrocellulose. The resulting Western blots were probed with either an anti-FLAG monoclonal antibody or anti-BRCA2 polyclonal antiserum.

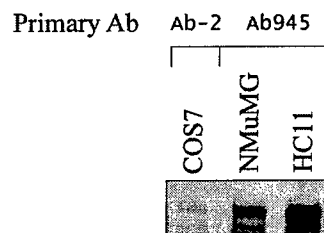


Figure 6: Ab945 detects an band which comigrates with simian BRCA2

Lysate from simian (COS7) or murine (NmuMG, HC11) cell lines were separated by SDS-PAGE and blotted onto nitrocellulose. The resulting Western blots were probed with either anti-human BRCA2 (Ab-2, Oncogene research) or anti-mouse Brca2 (Ab945, see text) antiserum.

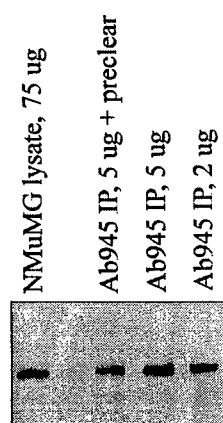


Figure 7: Ab945 Immunoprecipitates mBrca2

Ab945 (2 or 5 μ g, with or without preclearing with Protein-A Sepharose) was used to immunoprecipitate from 1 mg NmuMG lysate, and $\sim 2/3$ of the resulting material was loaded on an SDS-PAGE gel. A Western blot probed with Ab945 demonstrates that Ab945 can immunoprecipitate a protein which comigrates with the mBrca2 band which is detected in NmuMG lysate.

Task 3: Determine the subcellular localization of Brca2 (months 19-24)

If Brca2 is genuinely involved in the response to DNA damage, it is reasonable to hypothesize that it should be localized to the nucleus. To test this hypothesis, we used affinity-purified Ab945 to examine the subcellular localization of mBrca2 in the murine mammary epithelial cell line NMuMG. Briefly, cells were plated on slides several days before analysis and then fixed in 3% paraformaldehyde/2% sucrose (protocol from Ralph Scully, personal communication). Following permeabilization with Triton X-100, cells were incubated with Ab945 (2 μ g/mL in 3% BSA/1X PBS) @37°C x 20'. Following several additional PBS washes, FITC-conjugated anti-rabbit secondary antibodies (Jackson ImmunoResearch) were added to enable visualization. Confocal fluorescence microscopy demonstrates that Ab945 stains the nucleus of NMuMG cells in a nonuniform pattern with prominent exclusion of the nucleoli (Figure 8). Further, it should be noted that this staining is variable from cell to cell, reflecting significant differences in the localization and/or absolute levels of mBrca2; this may reflect regulation through cell cycle progression (see below) or responsiveness to some other, as yet undefined, signal. We conclude from these experiments that mBrca2 is indeed localized to the NMuMG nucleus, as we hypothesized.

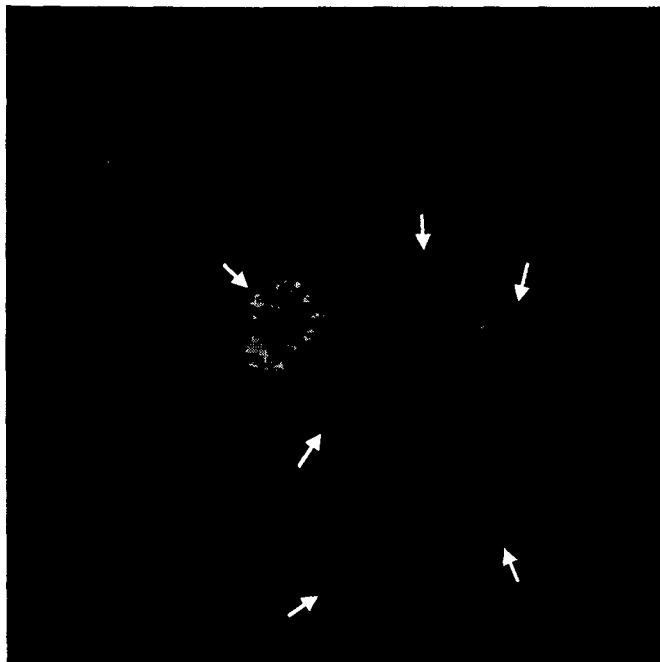


Figure 8: mBrca2 is a nuclear protein which is nonuniformly distributed within the nucleus and which shows significant variation in absolute levels from cell to cell.

NMuMG (a mouse mammary epithelial cell line) cells were plated on slides, fixed, and stained with our anti-mBrca2 antiserum (Ab945). Cells were further stained with a FITC-conjugated anti-rabbit secondary antibody and visualized by confocal microscopy. White arrows indicate the positions of nuclei.

Task 4: Determine whether Brca2 is a phosphoprotein (months 19-24)

Published literature (Scully *et al*, 1997b) suggested a relationship between BRCA1 phosphorylation and the response to DNA damage. We therefore reasoned that determining whether Brca2 is a phosphoprotein would be an important precursor to determining if this posttranslational modification might play a regulatory role in the response to DNA damage. We first incubated one NMuMG plate with ^{32}P -labelled orthophosphate (5 mCi/100 mm plate) for 2 hours prior to lysis in EBC. Following preclearing of the lysate with Protein A-Sepharose, immunoprecipitations were performed with either anti-mBrca2 (Ab945) or control (anti-JAK3, Santa Cruz) rabbit antiserum. In order to identify the expected location of radiolabelled mBrca2, unlabelled NMuMG lysate was loaded onto the same gel. The portion of the gel containing unlabelled lysate was subsequently blotted onto nitrocellulose using semi-dry transfer and probed with Ab945. The results (Figure 9) demonstrate the presence of a phosphoprotein which appears to comigrate with mBrca2 and which is immunoprecipitated by Ab945, but not control, antiserum. We believe that this provides evidence that mBrca2 is in fact a phosphoprotein.

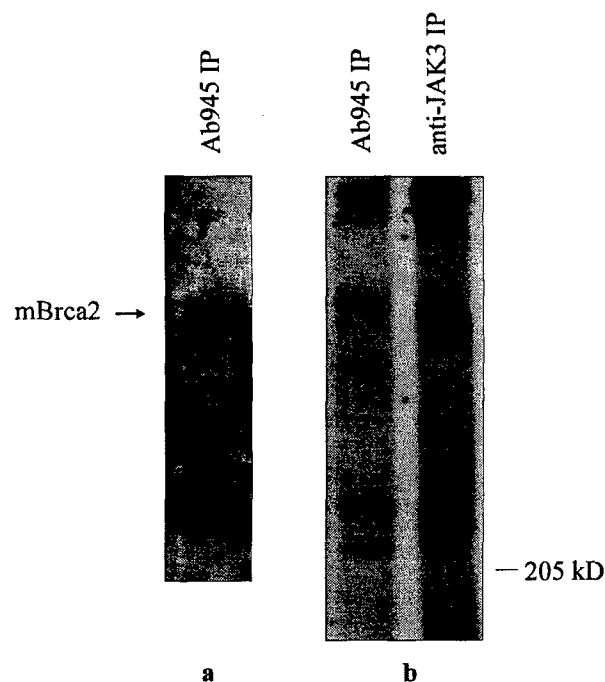


Figure 9: mBrca2 is a phosphoprotein

NmuMG cells were labelled with ^{32}P orthophosphate and lysed. Immunoprecipitations were performed with anti-mBrca2 (Ab945) or control (anti-JAK3) antiserum, and the resulting gel was visualized by autoradiography (figure 9b). To determine the expected migration of mBrca2, a portion of the gel was removed immediately after running and blotted onto nitrocellulose. This Western blot was then probed with Ab945 (figure 9a) and aligned with the autoradiograph.

Task 5: Determine whether BRCA1 interacts with BRCA2 (months 25-36)

We are currently pursuing this question, and it should be noted that the recent first report of an interaction between human BRCA1 and BRCA2 (Chen *et al*, 1998) does not affect our ability to pursue questions related to the murine protein or our ability to use the modular nature of the B2R clone to address the portion of BRCA2 which mediates this interaction.

Task 6: Determine the effect of altered BRCA2 levels on the ability to repair DNA damage. (months 25-36)

Subtask 6.1 Create stable clones which inducibly upregulate BRCA2 expression (months 25-30)

We are currently pursuing the creation of tetracycline-inducible full-length human BRCA2 clones (wild type, 999del5, 6174delT). These clones will be transfected into MCF7 cells containing either the tetracycline transactivator (tTA, cells obtained from

Clontech) or the reverse tetracycline transactivator (rtTA, MCF7 clone transfected with CMV-rtTA construct—data not shown).

Subtask 6.2 Create stable clones which inducibly downregulate Brca2 expression (months 25-30)

Similarly, it is important that we have the ability to downregulate Brca2 levels. To this end, we are continuing to screen HC11 clones which inducibly express antisense Brca2 RNA in the presence of doxocycline (Figure 10). As these clones have not shown the desired degree of downregulation, however (although see Figure 10, clone AS6-5-97 for possible modest regulation), we have designed ribozymes which recognize human and mouse BRCA2 sequence, and we intend to create tet-inducible, stable clones based on these catalytic RNA sequences (see, e.g Hua *et al*, 1996).

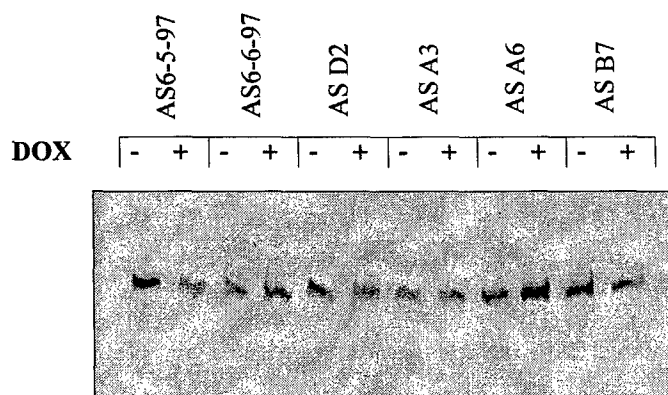


Figure 10: Screening of inducible antisense *Brca2* clones

An HC11 clone which inducibly expressed the reverse tetracycline transactivator (rtTA) was transfected with constructs which contain a segment of antisense *mBrca2* (spanning the translational initiation site) downstream of the tetracycline operator. Stable clones were induced with doxocycline, and Brca2 levels were assayed by Western blot probed with Ab945.

Subtask 6.3 Determine the effect of up- or down-regulating Brca2 on the ability of the cell to respond to DNA damage (months 31-36).

It is anticipated that this analysis will consist of assays for cell survival following gamma- and UV-irradiation, as well as assays for double-stranded break repair (comet assay) and nucleotide excision repair (unscheduled DNA synthesis assay).

Specific Aim II: Determine the role of BRCA2 in Cell Cycle progression

Task 1: Generate a full-length clone of BRCA2 (months 13-18)

See above, under Specific Aim I.

Task 2: Generate/test antibodies for detecting BRCA2 (months 13-24)

See above, under Specific Aim I.

Task 3: Determine whether Brca2 levels are regulated as a function of cell cycle progression (months 19-24).

Our lab has previously demonstrated that *mBrca2* mRNA levels are regulated as a function of cell cycle progression (Rajan *et al*, 1996), and thus we hypothesized that protein levels would be similarly regulated. Although a single report has appeared in the literature during the past year indicating that human BRCA2 protein behaves in this way, to date there has been no report describing the regulation of murine Brca2 protein levels. We therefore serum-starved the murine mammary epithelial cell lines NMuMG and HC11 for >47 hours prior to refeeding in high-serum (20%) growth medium and harvested at various times post-refeed. mBRCA2 protein levels were assayed by probing a Western blot probed with Ab945, and the results are shown in figure 11. These data demonstrate that the murine protein is regulated in a cell-cycle-dependent fashion, thus confirming our hypothesis. In view of the recent report that >60% of cell-cycle-regulated genes in *S. cerevisiae* have identified roles in cell cycle progression (Cho *et al*, 1998), we believe that this evidence suggests the possibility of a direct (as opposed to incidental) role for Brca2 in cell cycle progression.

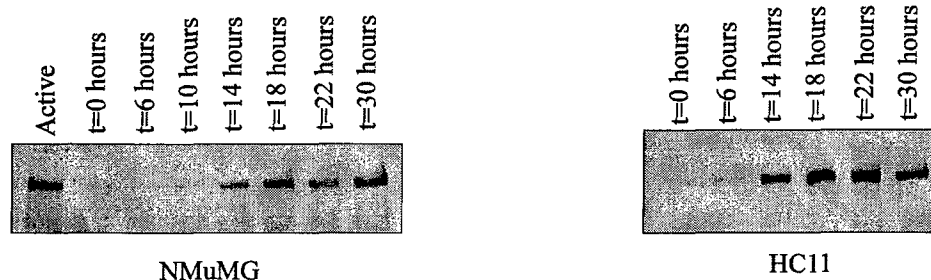


Figure 11: Brca2 is cell-cycle regulated in murine mammary epithelial cell lines

Cells were serum-starved to synchronize them in G0 prior to refeeding. Upon refeeding with 20% serum, cells were harvested at the indicated time points and lysates were analyzed on Western blots (probed with anti-mBrca2 Ab945).

Task 4: Determine the effect of altered BRCA2 levels on cell cycle progression (months 25-36)

Subtask 4.1 Create stable clones which inducibly upregulate BRCA2 expression (months 25-30)

See above, under Specific Aim I.

627 BRB II/III
421 Curie Blvd.
Philadelphia, PA 19104

U.S. Army Medical Research and Materiel Command
ATTN: MCMR-RMI-S
504 Scott St.
Fort Detrick, MD 21702-5012

November 3, 1999

RE: DAMD17-96-1-6111

Puberty, Differentiation and Breast Cancer Risk

To Whom It May Concern:

Enclosed is the final report for grant DAMD17-96-1-6111. This report contains proprietary data, and as such we request that distribution statement be changed to limit the report to Government agencies only. Please note that several locations in the body of this report refer to results previously discussed in the "Year Two" annual report. In the event that this report is not readily available, the relevant portions have been added as "Appendix 2". If you require anything further, please contact me at the above address or via email (srmaster@mail.med.upenn.edu).

Sincerely,



Stephen R. Master



DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

23 Aug 01

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 the technical reports listed at enclosure. Request the limited distribution statement for these reports 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. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl


PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

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