DISEASE MARKERS, VOL. 13, 275–287 (1998)

COLLECTED ABSTRACTS

FIRST INTERNATIONAL WORKSHOP ON THE FUNCTION OF BRCA1 AND BRCA2

Supported by the Mary-Jean Mitchell Green Cancer Foundation
11 and 12 September, 1997
The Moller Centre, Churchill College, Cambridge, UK.

GENETICS AND GENETIC EPIDEMIOLOGY OF BRCA2

M.R. STRATTON
Section of Molecular Pathology and Epidemiology, Institute of Cancer Research, Haddow Laboratories, Cotswold Road, Sutton, Surrey SM2 5NG, UK

Since BRCA2 was identified a large number of disease associated mutations have been discovered. The risks of breast and ovarian cancer associated with BRCA2 mutations and the risks of other cancer types have been clarified. The relationship between particular mutations and disease risk have been investigated in more detail with indications that different BRCA2 mutations are associated with different risks of disease. Moreover it have become apparent that the modifying gene effects are detectable in the manifestations of BRCA2 mutations. Geographical variations in mutation diversity and prevalence have also been reported. The histological appearances of cancers due to BRCA1 and BRCA2 have been investigated and have revealed that cancers due to BRCA1 and BRCA2 differ from sporadic breast cancer and from each other. Finally exhaustive mutational and linkage analyses of large numbers of breast cancer families provide strong evidence that a substantial proportion are not due to either gene and that further breast cancer susceptibility genes are likely to exist.

MOLECULAR CHANGES IN BRCA-ASSOCIATED BREAST CANCER

T.R. CROOK*, P. OSIN*, S. CROSSLAND*, M. CROMPTON*, L. KNEE*, J. WALLER*, L. BROOKS#, B.A. GUSTERSON*
**Section of Cell Biology and Molecular Pathology, Institute of Cancer Research, Haddow Laboratories, Cotswold Road, Sutton, Surrey SM2 5NG, UK*
#Division of Clinical Sciences, London School of Hygiene and Tropical Medicine, Kepple Street, London WC1, UK

Other than inheritance of mutant alleles of BRCA1 and BRCA2, changes in the structure and expression of other cellular genes which correlate with development and progression of familial breast cancer remain largely unknown. We have investigated the structure and expression of a number of cellular genes, including tumour suppressor genes and steroid hormone receptors, in a series of BRCA1- and BRCA2-associated tumours and in a control series of grade 3 sporadic breast carcinomas. We have identified

 Received September, 1997

a number of changes in familial breast cancer which differ markedly from sporadic breast cancers, including high frequency of p53 mutation with novel "hot spot" codons for mutation, high frequency of expression of $p27^{Kip\ 1}$ with infrequent expression of cyclin D1, and abnormalities in expression of oestrogen and progesterone receptors. An outline of these findings and their possible role in BRCA-associated tumourigenesis will be presented.

EXPRESSION AND LOCALIZATION OF BRCA1 ISOFORMS IN BREAST AND OVARIAN CANCER CELL LINES

C. AFSHARI*, P.L. CABLE*, C. WILSON#, F. CALZONE†, D.E. HILL§
*National Institute of Environmental Health Sciences, Research Triangle Park, NC, USA
#Amgen, Inc., Thousand Oaks, CA, USA
†UCLA, Los Angeles, CA, USA
§Oncogene Research Products, Cambridge, MA, USA

Individuals with inherited mutations in either BRCA1 and BRCA2 genes have a predisposition to early onset breast cancer. Although the precise roles of BRCA1 and BRCA2 remain to be determined, both proteins are essential for embryogenesis and appear to be involved in DNA repair and recombination pathways, possibly as part of a complex that includes Rad51. The BRCA1 gene is comprised of 22 exons encoding the complete the full length p220BRCA1 but alternative splicing is also observed with the predominant splice variant, BRCA1-Δ11b, lacking most of exon 11. Using a series of monoclonal antibodies directed against epitopes in the aminoterminal (monoclonals MS110 and MS13) and carboxyterminal (monoclonal antibody SG11) portions of full length BRCA1, two discrete protein species are routinely observed. The major protein detected in immunoblots and immunoprecipitates migrates with a mobility of 220 kDa. A second, smaller protein, migrating with a mobility of 123 kDa, is also consistently observed by immunoblotting and immunoprecipitation. Both proteins are also observed when an aminoterminal antibody, MS110, is used to first immunoprecipitate BRCA1 and the precipitated proteins are then detected by immunoblotting with MS13 or SG11 suggesting that the epitopes for all three monoclonals are present in the two proteins. One explanation for the observed results is that there are two primary translation products. Previous work had demonstrated that multiple alternatively spliced mRNAs are expressed in normal and tumor cells. One alternatively spliced message, which utilizes a cryptic splice donor site within exon 11 and the normal splice acceptor site of exon 12 and therefore is lacking most of exon 11, is predicted to encode a protein of 759 amino acids compared to the 1863 amino acid full length BRCA1 protein. The calculated molecular weight of the conceptual translation product from the alternatively spliced mRNA is approximately 83,000 which is significantly less than the 123 kDA protein detected in immunoblots and immunoprecipitates. Transfection into Cos-7 cells of a cDNA derived from the alternatively spliced mRNA leads to expression of a protein with mobility and antibody reactivity identical to that of the endogenous 123 kDa protein detected in immunoblots.

Although we occasionally detect proteins smaller than 83 kDa in immunoblots with all three monoclonals, we have not observed any proteins with apparent molecular weights in the 83,000 ~ 100,000 range. This suggests that the 123 kDa protein is the

primary translation product of the alternatively spliced message. Calculation of the isoelectric point of both BRCA1 translation products shows them to be acidic; p220BRCA1 has a pI of 5.13 while the 123 kDa BRCA1-Δ11b protein is even more acidic with a pI of 4.78. On SDS/PAGE, acidic proteins migrate slower than normal which leads to an apparent molecular weight based on mobility that is larger than predicted.

The 123 kDa protein can be detected in both normal cells and tumor cells. However, similar to the reduced levels of p220BRCA1 typically seen in most tumor cells, p123 BRCA1-Δ11b is also detected in reduced amounts in breast and ovarian tumor cells. In the tumor cell lines examined, the reduction of p123 is greater than that seen for p220 relative to the amounts seen in normal cells. While the mechanism of this reduction is unclear, the process of cell immortalization may affect BRCA1 expression since chemically immortalized normal mammary epithelial cells also show reduced levels of p220 and p123 relative to the normal parent cells.

The p220BRCA1 protein contains three repeats of a canonical nuclear localization signal all within exon 11. Fractionation studies and immunohistochemical analysis demonstrate that p220BRCA1 is almost exclusively nuclear. In contrast to p220BRCA1, p123BRCA1-Δ11b is lacking all of the nuclear localization signals. Nevertheless, p123BRCA1-Δ11b is also found in the nuclear compartment. The mechanism by which p123BRCA1-Δ11b enters the nucleus is unknown. One hypothesis is that full length BRCA1 and BRCA1-Δ11b may form a complex upon synthesis which would allow BRCA1-Δ11b to be transported to the nucleus. Preliminary data from glycerol gradient sedimentation of nuclear lysates shows that p220BRCA1 and p1234BRCA1-Δ11b co-sediment which supports the idea of a BRCA1/BRCA1-Δ11b complex. The function of the various BRCA1 macromolecular complexes in regulating cell growth remains to be determined, however.

THE SIGNIFICANCE OF BRCA1 NUCLEAR FOCI IN BREAST CANCER

C.A. WILSON*, L. RAMOS*, K. ANDERS#, S. PEKAR†, E. CAJULIS†, R. VILLASENOR*, K. CLARKE#, D.J. SLAMON*, F.J. CALZONE†

*Department of Medicine, Division of Hematology-Oncology, UCLA School of Medicine, Los Angeles, California 90095, USA

#Department of Pathology, Kaiser Permanente Hospital, 5601 De Soto Ave., Woodland Hills, California 91365, USA

†Molecular Biology, Amgen Inc., Amgen Center, Thousand Oaks, California, 91320, USA.

Currently, the literature presents a somewhat confused picture of the primary structure of BRCA1 proteins, their expression level and their subcellular localization[1-5]. Conflicting models have been developed to explain the role of BRCA1 in tumor susceptibility and, these models have been the subject of considerable debate. We have suggested that much of the controversy is the result of BRCA1 antibody cross reactions with other proteins, such as the epidermal growth factor receptor and HER2, low BRCA1 protein abundance, the existence of BRCA1 splice variants and difficulties associated with BRCA1 overexpression[6,7]. In an attempt to further address these issues, we have characterized the specificity and usefulness of 17 different anti-BRCA1 antibodies from academic and commercial sources. These reagents consistently detected a 220 kDa

protein in nuclear fractions from several breast and ovarian cell lines using immunoprecipitation and western blotting techniques. In addition a 120 kDa protein that appears to be the translated product of the BRCA1 exon 11 minus splice variant (BRCA1-Δ11b) was identified. Discrete nuclear foci were observed using enzymatic immunocytochemistry with 15 different antibodies in a variety of breast and ovarian cell lines derived from both normal and malignant tissue. This result confirms similar findings obtained with immunofluorescent techniques[3,8]. The expression of BRCA1 in normal breast and ovarian tissues as well as in breast and ovarian cancers was examined using the Oncogene Ab-1, N-terminal monoclonal antibody and an immunohistochemistry protocol developed for formalin-fixed, paraffin embedded material. Here also, the punctate nuclear staining characteristic of cell lines was observed. Invasive lobular breast cancers and low grade ductal carcinomas consistently showed high levels of BRCA1 reactivity. In contrast, BRCA1 expression was dramatically reduced in high-grade, poorly-differentiated, invasive ductal breast carcinomas. These data suggest that a significant proportion of spontaneous breast cancers have lost the BRCA1 protein by some epigenetic phenomenon.

We reported previously[7] that transient overexpression of the full-length (1863 amino acids) BRCA1 protein in 293 and Cos-7 cells using CMV promoter constructs results in nuclear fragmentation, high levels of cytoplasmic BRCA1 accumulation and cell death. To evaluate the significance of this apparent BRCA1 cellular toxicity, we measured the natural abundance of BRCA1 proteins using SDS-PAGE immunoblots of total cell proteins and a bacterially expressed protein standard. The data indicate that the concentration of the BRCA1 and BRCA1-Δ11b protein is remarkably low; 3000-8000 molecules per HBL-100 cell or, roughly 150-400 molecules per dot and, 5–10 fold less in most other cells examined. Given this information, we question the physiological relevance of BRCA1 cell killing or growth suppression obtained with CMV and other highly active promoter constructs. We have obtained no evidence to suggest that the cellular toxicity associated with overexpression of the full-length BRCA1 is cell type specific. In contrast to the full-length protein, we have found that nuclear overexpression of isolated domains of BRCA1 or BRCA1-Δ11b, is well tolerated by transiently transfected cells. A nuclear targeted GFP tag was used to demonstrate that $BRCA1_{1\text{-}222}$ (amino acid residues 1–222) assembles into nuclear dots in a manner that recapitulates most aspects of the phenomenon observed for the endogenous protein. Mutation of critical cysteines in the ring motif did not prevent focus assembly. The BRCT domain (1651–1863) exhibited a distinctly different pattern of nuclear compartmentalization. The appearance of nuclear targeted, $BRCA1_{760\text{-}1060}$ (RAD51 interacting domain) and $BRCA1_{1142\text{-}1646}$ (transcriptional activation domain) was diffuse, homogeneous, and non-nucleolar in all cells. Interestingly, the incidence of mitotic cells or cells with two nuclei was increased 2–3 fold in cell cultures that overexpressed the transactivation domain, however, the relevance of this result with respect to the normal function of BRCA1 is not clear.

Taken together our data suggests that the analysis of BRCA1 nuclear dots or foci is likely to provide an important insight into the function of BRCA1. In our view, the evidence obtained to date for BRCA1 growth suppressive activity using highly active, artificial promoter systems is not likely to be physiologically significant. We have no evidence that aberrant subcellular localization of BRCA1 in breast and ovarian cancers occurs, nor have we obtained any evidence of a secreted 190 kDa BRCA1 protein.

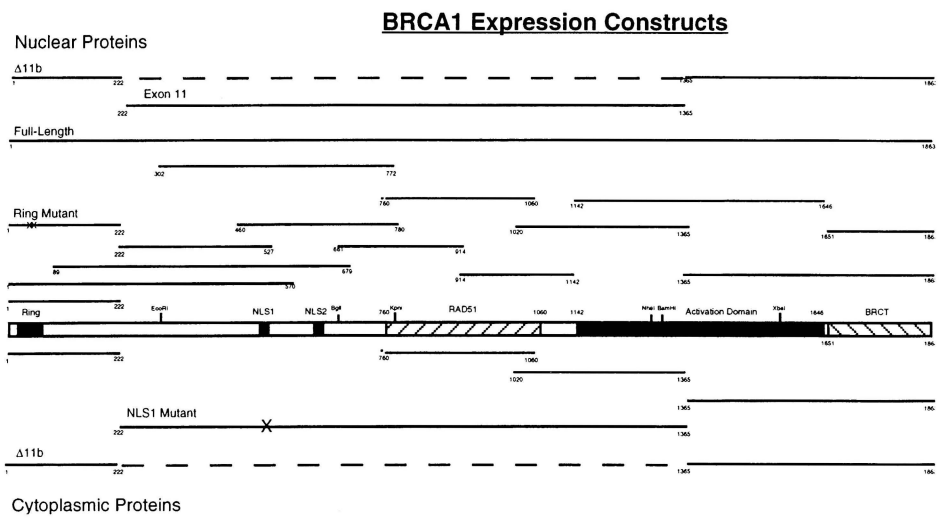

Figure 1. GFP tagged BRCA1 expression constructs. Subdomains of BRCA1 and BRCA1-11b were expressed with an N-terminal GFP tag in Cos-7 cells using a CMV promoter construct. The BRCA1 regions above the schematic representation of the full-length protein were targeted to the nucleus using the natural BRCA1 NLS (when present) or an SV40 NLS located between the GFP tag and the BRCA1 sequence. The BRCA1 subdomains indicated below the map did not include the BRCA1 or SV40NLS.

REFERENCES

1. Chen, Y.O., Chen, C.F., Riley, D.J., Allred, D.C., Chen, P.L., Von Hoff, D., Osborne, C.K., Lee, W.H. (1995). *Science,* **270,** 789–791.
2. Jensen, R.A., Thompson, M.E., Jetton, T.L., Szabo, C.I., Meer, R., Helou, B., Tronick, S.R., Page, D.L., King, M.C., Holt, J.T. (1996). *Nature Genet.,* **12,** 303–308.
3. Scully, R., Chen, J., Ochs, R.L., Keegan, K., Hoelstra, M., Feunteun, J., Livingston, D.M. (1997). *Cell,* **90,** 1–20.
4. Holt, T.H., Thompson, M.E., Szabo, C., Robinson-Benion, C., Arteaga, C.L., King, M.C., Jenson, R.A. (1996). *Nature Genet.,* **12,** 298–302.
5. Coene, E., Van Oostveldt, P., Willems, K., van Emmelo, J., De Potter, C.R. (1997). *Nature Genet.,* **16,** 122–124.
6. Wilson, C.A., Payton, M.N., Pekar, S.K., Zhang, K., Pacifici, R.E., Gudas, J.L., Thukral, S., Calzone, F.J., Reese, D.M., Slamon, D.J. (1996). *Nature Genet.,* **13,** 264–265.
7. Wilson, C.A., Payton, M.N., Elliott, G.S., Buaas, F.B., Cajulis, E.E., Grosshans, D., Ramos, L., Reese, D.M., Slamon, D.J., Calzone, F.J. (1997). *Oncogene,* **14,** 1–16.
8. Thomas, J.E., Smith, M., Tonkinson, J.L., Rubinfeld, B., Polakis, P. (1997). *Cell Growth and Diff.,* **8,** 801–809.

A BRCA2 MUTANT ALTERS THE GROWTH AND CELL CYCLE PATTERN OF HUMAN BREAST EPITHELIAL CELLS

J.S. LARSON, J.L. TONKINSON, M.T. LAI
Cancer Research Division, Lilly Research Laboratories, Indianapolis 46285, USA

Mutations in BRCA1 increase the risk of breast and ovarian cancer. Although it is not clear how different mutation alter growth regulation, the carboxy terminus of the gene appears to play a critical role: (1) The majority of mutations result in loss in the carboxy terminus. (2) The carboxy terminus is highly conserved and contains a potential transcription activation domain. (3) The region is required for the growth suppression. In order to examine the role played by the carboxy terminus, we introduce a vector expressing the carboxy residues 1293-1863 (CT-BRCA1) into the human mammary epithelial cell line 184A1 – an immortalized line derived from a tumor-free tissue. Overexpression of CT-BRCA1 resulted in altered cell morphology, accelerated growth and decreased dependence on growth factors. CT-BRCA1 also induced alterations in cell cycle control, mainly in G_2-M, including loss of G_2-M block by colchicine. These data suggest BRCA1 may be involved in the control of checkpoint(s) between DNA replication and mitosis. We also demonstrated that CT-BRCA1 was phoshorylated, indicating the presence of potential site(s) for kinases in this region. The mechanism by which CT-BRCA1 may function as a dominant negative mutant will be discussed.

GROWTH INHIBITION AND TUMOR SUPPRESSION BY BRCA1

J.T. HOLT, D.L. TAIT, P.S. OBERMILLER, R.A. JENSEN, A.M. GOWN, D.L. PAGE, M.-C. KING, D.L. JOHNSON.
Vanderbilt Cancer Center, University of Washington Medical School, USA.

We have demonstrated that overexpression of BRCA1 inhibits the growth of breast and ovarian cells *in vitro*, and that injection of retroviral vectors expressing BRCA1 or a BRCA1 splice variant inhibits the growth of established tumors in nude mouse xenograft models. Structure-function studies demonstrate that N-terminally truncated BRCA1 genes lacking the ring finger still inhibit growth and suppress tumors, but that C-terminally truncated BRCA1 genes inhibit ovarian cancer cells but not breast cancer cells. Required domains for growth inhibition and/or tumor suppression are presently being defined. Although the BRCA1 gene is rarely mutated in sporadic breast or ovarian cancer our studies show that tumors from microdissected sporadic breast and ovarian cancers have markedly decreased BRCA1 mRNA and protein.

As an initial step towards gene therapy for sporadic ovarian cancer we have completed a Phase I trial to assess pharmacokinetics and toxicity, and have recently begun a Phase II trial to assess efficacy. DNA and RNA studies of treated patients show that the vector is reasonable stable following peritoneal administration, and that the BRCA1 vector mRNA is expressed in patient tissues which show that up to 10% of tumor cells are transduced. 3/12 patients developed an acute sterile peritonitis that spontaneously resolved and one patient has shown a durable objective response to date, although completion of Phase II studies will be necessary to truly evaluate efficacy.

BIOLOGICAL FUNCTION OF BRCA GENE PRODUCTS

W.-H. LEE*, Y. CHEN*, P.-L. CHEN*, C.-Y. LIU*, C.-F. CHEN*, D. JONES*, A. FARMER*, A. NIKITEN*, Z.D. SHARP*, C.K. OSBORNE*, D.C. ALLRED#

**The Institute of Biotechnology, Department of Medicine, The University of Texas Health Science Center, San Antonio, Texas 78245, USA.*

#The Institute of Biotechnology, Department of Pathology, The University of Texas Health Science Center, San Antonio, Texas 78245, USA.

Mutations in the BRCA1 and BRCA2 genes are known to predispose members of affected families to breast cancer. Unlike typical tumor suppressor genes, mutations in BRCA1 and BRCA2 are rarely observed in sporadic tumors. To address this issue, we identified BRCA1 as a 220 kDa nuclear phosphoprotein in normal cells, one which is aberrantly located in the cytoplasm of most breast and some ovarian tumor cells. Immunostaining with a new, highly specific mAb conjugated with FITC, and expression of tagged BRCA1 confirms its mislocation in breast cancer cells. Using recently developed cDNA and immunological reagents, the product of BRCA2 gene was identified as a nuclear 380 kDa protein. Consistently, human RAD51 was identified as a BRCA2-interacting protein using yeast two-hybrid screens. *In vitro* and *in vivo* assays demonstrate cellular interactions between BRCA2 and RAD51. Sucrose gradient sedimentation analysis of cellular lysates shows that BRCA2 and RAD51 belong to a large complex possibly involved in DNA double strand break repair. Interestingly, the same assays show that BRCA1 interacts with RAD50, but sediments in a complex distinct from that containing BRCA2 and RAD51. These data suggest that both BRCA1 and BRCA2 interact with members of the RAD51 epistasis group but in separate cellular complexes. The working model is that BRCA1 and BRCA2 have similar but distinct roles in DNA repair and/or recombination. The potential biological significance of these data will be discussed.

ACKNOWLEDGMENTS

This work was supported by a grant from the NCI, SPORE CA58183

MOLECULAR CONTROLS ON CELL PROLIFERATION

R.A. WEINBERG

Whitehead Institute for Biomedical Research, Department of Biology, Massachusetts Institute of Technology, Cambridge, MA, USA.

Normal cells are responsive to both positive and negative controls on their proliferation. Thus, serum-associated mitogens are required to drive normal cells through their growth cycle; conversely, anti-mitogens such as TGF-β are potent in inhibiting the proliferation of normal cells. Conversely, cancer cells acquire a profoundly different responsiveness to and dependence on environmental signalling. They become refractory to the actions of certain anti-mitogens and independent of stimulation by exogenous mitogens. Much of this can be rationalized in terms of the circuitry that ties together components of the cell cycle clock apparatus, some of which will be described.

Cancer cells also acquire an additional change that represents a strong determinant of their proliferative abilities. This other alteration – immortalization – appears to represent an alteration in an exclusively intrinsic mechanism within the somatic cell that counts the number of doublings through which the cell's lineage has passed since embryogenesis. This counting mechanism ensures that when the lineage has exhausted its allotment of allowed doublings, the phenotypes of senescence and crisis ensue, blocking further proliferation. This generational counting mechanism appears to depend on the progressive shortening of telomeres in normal cell lineages. In cancer cells, this counting mechanism is usually subverted through the resurrection of the expression of the telomerase enzyme, expression of which is repressed in most normal, post-embryonic cell lineages. The cloning of the gene specifying the human telomerase will be described.

COMPLEX REGULATION OF THE BRCA1 GENE

C.-F. XU*, J. CHAMBERS*#, M. BROWN*, H. NICOLAI*#, E. SOLOMON*
*Division of Medical and Molecular Genetics United Medical and Dental Schools, Guy's Hospital, London SE1 9RT, UK.
#Imperial Cancer Research Fund, Lincoln's Inn Fields, London WC1, UK

We have analysed the promoter region of the human BRCA1 gene in detail and demonstrate the expression of the gene is under complex regulation. The human gene is under the control of two promoters generating two distinct transcripts *a* and *b*. In addition the *a* promoter is shared with the adjacent NBR2 gene and is bi-directional. The genomic organisation of the promoter regions and immediate cis control elements differ considerably between human and mouse. The human region contains a large duplication of part of BRCA1 and NBR2. The mouse region does not contain this duplication and Brca1 lies adjacent to a different gene, NBR1, which shares some regions of homology with NBR2. These regions shall be described in detail and data presented on expression of reporter constructs containing a series of deletions within the bi-directional promoter between BRCA1 and NBR2.

BRCA1 AND BRCA2 PROTEINS: ATTEMPTS TO STUDY THEIR FUNCTION

I.M. VERMA
Laboratory of Genetics, The Salk Institute, La Jolla, CA. 92037, USA.

Breast cancer is one of the most common malignancies affecting women and is diagnosed in approximately 180,000 women each year in the United States. About 5–10% of all cases are estimated to be familial of which mutation in BRCA1 and BRCA2 genes account for roughly equal proportions. We have characterized the BRCA1 gene product by using four polyclonal antibodies raised against peptides from four different regions of the protein. The antibodies specifically recognize a 220kDA BRCA1 protein that is predominantly expressed in the nucleus of both normal and neoplastic breast cancer cells. BRCA1 gene product is a serine phosphoprotein that undergoes hyperphosphorylation during late G1 and S phases of the cell cycle and is

transiently dephosphorylated early after M phase. We are trying to identify the sites of phosphorylation and the possible kinase(s) involved. We have also shown that the C-terminus of BRCA1 has the potential to act as a transcriptional transactivator. More recently we have observed that BRCA1 protein physically interacts with other transcriptional co-activators.

We have also raised a number of antibodies against peptides representing various portions of the BRCA2 protein. We show that a 380kDa protein can be identified from *in vitro* transcription-translation of BRCA2 cDNA, which appears to have the gel mobility similar to endogenous or overexpressed BRCA2 proteins. The BRCA2 protein also appears to be nuclear: a phosphoprotein, and likely to be involved in some aspect of the transcriptional; machinery. We will discuss the nature of the cellular proteins that may react with BRCA2 protein.

BRCA2 EXON 3 IS A REGULATED TRANSCRIPTIONAL ACTIVATION DOMAIN

J. MILNER*, L. HUGHES-DAVIES*#, M. SELTMANN*#, B. PONDER#, T. KOUZARIDES*
*Wellcome/CRC Institute, University of Cambridge, Cambridge, UK
#Institute and Department of Clinical Oncology, University of Cambridge, Cambridge, UK.

The BRCA2 gene is found mutated in 45% of familial breast cancers yet its function remains unknown. We have found that exon 3 of BRCA2 contains sequence similarity to the activation domain of the c-jun transcription factor. Consistent with this similarity, BRCA2 exon 3 is found to possess transcriptional activation functions both in yeast and in two different mammalian cell lines. Within exon 3 lies a tyr to cys mutation found in familial breast cancer. Introduction of this mutation into exon 3 sequences severely compromises the activation potential of BRCA2, suggesting that disruption of this activity is an important step in the generation of familial breast cancers. The activation potential of BRCA2 exon 3 is found to be under the negative control of adjacent sequences which mask its activation potential. Two-hybrid screens in yeast are currently underway to identify protein(s) which bind and regulate the activity of BRCA2 exon 3.

PROTEIN-PROTEIN INTERACTIONS INVOLVING THE BRCA1 GENE PRODUCT

R. BAER
Department of Microbiology, UT Southwestern Medical Center, 6000 Harri Hines Blvd., Dallas, Texas 75235, USA

Germline mutations of the BRCA1 gene predispose women to early-onset breast and ovarian cancer by compromising its presumptive function as a tumor suppressor. The polypeptides encoded by BRCA1 bear several recognizable amino acid motifs, including an amino-terminal RING domain and two carboxy-terminal BRCT domains. Findings from other laboratories suggest a role for BRCA1 in cell cycle regulation. In particular, the steady-state levels of BRCA1 peak during S phase of the cell cycle, at which time

BRCA1 polypeptides become hyperphosphorylated and accumulate into discrete subnuclear foci (termed "BRCA1 nuclear dots"). We have shown that BRCA1 interacts *in vivo* with the BRCA1-associated RING domain (BARD1) protein. BARD1 resembles BRCA1 in that it contains an amino-terminal RING domain and two carboxy-terminal BRCT motifs. Significantly, the interaction between these proteins is abolished by BRCA1 missense mutations that segregate with breast cancer susceptibility, indicating that BARD1 may be involved in mediating tumor suppression by BRCA1. The steady-state levels of BARD1, unlike those of BRCA1, remain relatively constant during cell cycle progression. However, immunostaining revealed that BARD1 resides within BRCA1 nuclear dots during S phase of the cell cycle, but not during the G1 phase. Therefore, progression to the S phase is accompanied by aggregation of BARD1 polypeptides into the BRCA1 nuclear dots. This cell cycle-dependent co-localization of BARD1 and BRCA1 supports a role for BARD1 in BRCA1 mediated tumor suppression.

BRCA1 PARTICIPATES IN A DNA DAMAGE RESPONSE PATHWAY

R. SCULLY
Harvard Medical School, Boston, MA 02115, USA.

Inherited loss-of-function BRCA1 mutations are linked to variably penetrant breast and/or ovarian cancer. Certain BRCA1 families are also marked by excess prostate cancer. BRCA1 is an 1863 nuclear, RING domain polypeptide with a C-terminal (BRCT) domain characteristic of several proteins active in DNA repair and the maintenance of genome stability. As shown by the Verma and Hanafusa laboratories, BRCT possesses transactivating function. Moreover, BRCA1 co-purifies with RNA polymerase II holoenzyme, except when this unit of structure is appropriately mutated, suggesting again that BRCA1 participates in transcription control.

BRCA1 localizes to subnuclear foci in S and G2 and not in G1. BRCA1 dots also contain the RecA-like mammalian equivalent, Rad51, with which BRCA1 interacts, as shown by co-IP of crude cell extracts. They also contain a second BRCA1 associated protein, BARD1, as shown by R. Baer's laboratory. Like Rad51, BRCA1 also interacts stably with development synaptonemal complexes in a periodic manner. Taken together, these results imply that, like Rad51, BRCA1 is active in the maintenance of genome stability. They also lead to the speculation that its loss predisposes to the accelerated development of tumor inducing mutations. Recent evidence from A. Bradley's laboratory on another inherited breast cancer gene product, BRCA2, imply that it operates in a similar manner.

In an effort to probe for a potential role for BRCA1 in genome stability control, we examined the gel migration and cytological properties of BRCA1 before and after DNA damage. Our results point to BRCA1 as a specific substrate of one more kinases activated by DNA damage. They also suggest that, after DNA damage, multiprotein complexes containing BRCA1, Rad51, and BARD1 are dispersed from nuclear dots and appear on damaged, replicating DNA. Taken together, the data suggest that BRCA1 is a component of a complex, DNA-damage-initiated signalling pathway, in which the nuclear dots play a dynamic role.

INDUCTION OF PHOSPHORYLATION ON BRCA1

J.E. THOMAS, M. SMITH, K.R. STAYROOK, J.L. TONKINSON.
Department of Cancer Research, Lilly Research Laboratories, Eli Lilly and Co., Indianapolis, IN 46285, USA.

We have found that the protein product of the breast cancer susceptibility gene, BRCA1, undergoes rapid phosphorylation during the cell cycle and following exposure to DNA damaging agents. Regulation of phosphorylation on BRCA1 during the cell cycle occurs as cells move through S-phase. Phosphorylation of Ser/Thr residues of BRCA1 also occurs rapidly when cell are exposed to DNA damaging agents including UV irradiation (50J/m^2) and hydrogen peroxide. Interestingly, following exposure to UV irradiation we observed that the punctate nuclear staining pattern for BRCA1 previously demonstrated in MC7 cells became diffuse. Since UV irradiation at low levels was toxic to the cells, we examined the effects of gamma-irradiation (^{137}Cs), BRCA1 shifted from the mostly hypophosphorylated state to a higher molecular weight, hyperphosphorylated form of the protein. This hyperphosphorylated form was concurrent with the induction of p53 and p21/CIP and detectable for several hours after irradiation. The relationship between phosphorylation on BRCA1 and the cellular response to DNA damaging agents will be presented.

TRANSCRIPTIONAL ACTIVATION OF THE P21$^{WAF1/CIP1}$ GENE BY BRCA1 INHIBITS CELL CYCLE PROGRESSION

H. ZHANG*, K. SOMASUNDARAM*, Y.X. ZENG* Y. HOUVRAS#, Y. PENG*, H. ZHANG*, J.D. LICHT#, W.S. EL-DEIRY*, B.L. WEBER*
*University of Pennsylvania School of Medicine, Philadelphia, PA, USA
#Mount Sinai School of Medicine, New York, NY, USA

BRCA1 is a nuclear protein with properties of a transcription factor, and can interact with the recombination and repair protein RAD51. We found that BRCA1 acted in a promoter and activator specific manner to activate transcription. Most prominently, both the murine and human promoters of the gene encoding CDK-inhibitor p21$^{waf1/cip1}$ were activated 5–20 fold in a number of different cell lines. A BRCA1-responsive element mapped to a proximal portion within the human promoter and did not overlap with p53 binding sites, suggesting that activation occurs in a p53-independent manner. Intriguingly this site is not conserved in the murine promoter. BRCA1 activated expression of the endogenous p21 gene and inhibited S-phase cell cycle progression following its transfection into human cancer cells. However, BRCA1 did not inhibit S-phase progression in p21 null HCT116 colon carcinoma cells. Tumor-associated mutants of BRCA1 were defective in both transactivation of p21 and cell cycle inhibition. These data suggest that one mechanism by which BRCA1 contributes to cell cycle arrest and growth suppression is through induction of p21 $^{WAF1/CIP1}$.

TUMOR SUPPRESSOR GENES BRCA1 AND BRCA2 ARE REQUIRED FOR EMBRYONIC CELLULAR PROLIFERATION

R. HAKEM, A. SUZUKI, J. DE LA POMPA, T.W. MAK.
Amgen Institute, Ontario Cancer Institute, Departments of Medical Biophysics and Immunology, University of Toronto, Ontario, Canada M5G 2C1

Mutations of the BRCA1 or BRCA2 gene in human are associated with predisposition to breast and ovarian cancer. To investigate the biological functions of these genes *in vivo* and to create mouse models to study breast and ovarian cancer, we regenerated BRCA1 or BRCA2 mutated mice by homologous recombination embryonic stem cells. We showed that Brca1 or Brca2 heterozygous mutants are normal and fertile and lack tumours. Homozygous Brca1 and Brca2 mutant mice, however, died before day 7.5 and 8.5 of embryogenesis, respectively. Although apoptosis is normal, cellular proliferation is impaired in these mutants both *in vitro* and *in vivo*. In addition, the expression of the cyclin-dependent kinase inhibitor p21 is dramatically increased in the mutant embryos. Buttressing the hypothesis that the embryos of Brca1 and Brca2 mutants may die because of impaired cellular proliferation due to G1 cell cycle arrest caused by increased p21 levels, we demonstrated that p53 or p21 null mutation can partially rescue the early embryonic lethality of the Brca1 mutant. Thus, we can postulate that the death of these mutant embryos prior to gastrulation may be due to a failure of the proliferative burst (partially due to G1 cell cycle arrest) required for the development of the different germlayers. The fact that mutation of neither p53 or p21 completely rescued Brca1 embryos suggests that their lethality is likely due to a multi-factorial process.

PLEIOTROPIC EFFECTS OF A HYPOMORPHIC MUTATION IN THE MOUSE *Brca2* GENE

A. ASHWORTH
Section of Cell and Molecular Biology, Institute of Cancer Research, Chester Beatty Laboratories, Fulham Road, London SW3 6JB, UK.

Germ line mutation of the BRCA2 carries a high risk of developing breast cancer. We have been studying the structure, function and evolution of this gene. We have shown that the gene encodes a 384kD protein that is regulated through the cell cycle and induced around G1/S. We have also defined proteins that interact with BRCA2.

To study the function of the gene we have created a hypomorphic mutation in the *Brca2* gene in mice. Some homozygous mutant animals are viable but we have a wide range of defects. These animals could provide insights into the mechanisms of tumour formation on loss of the BRCA2 gene.

TARGETED DISRUPTION OF Brca2 EXON II GIVES RISE TO VIABLE MICE WITH EMBRYONIC GROWTH RETARDATION, DEVELOPMENTAL DEFECTS AND THYMIC LYMPHOMA

L.S. FRIEDMAN[*], F. THISTLETHWAITE[#], K.J. PATEL[+], V.P.C.C. YU[+], A.R. VENKITARAMAN[+], M. CARLTON[#], S.M. HUNTER[#], K.J. ABEL[$], W.H. COLLEDGE[&], B.A.J. PONDER[$], M.J. EVANS[#].

[*]CRC Human Cancer Genetics Research Group, Addenbrooke's Hospital, Box 238, Hills Road, Cambridge CB2 2QQ, UK.

[#]Wellcome/CRC Institute of Cancer and Developmental Biology and Department of Genetics, University of Cambridge, Tennis Court Road, Cambridge CB2 1QR, UK.

[+]Medical Research Council, Laboratory of Molecular Biology, Hills Road, Cambridge, CB2 2QH, UK.

[$]Sequana Therapeutics, 11099 No. Torrey Pines Road, Suite 160, La Jolla, CA 92037, USA.

[&]Physiological Laboratory, University of Cambridge, Downing Street, Cambridge, CV2 3EG, UK.

Germline mutations in the BRCA2 gene cause inherited predisposition to breast and ovarian cancer. We created a mutation in the mouse Brca2 gene which terminated translation in exon 11 at 45% of the normal length. The resulting phenotype was less severe than in the other Brca2 knockout mice reported to date. Most Brca2 homozygous mutant mice died as embryos or as neonates, but some neonates were viable. Brca2 homozygotes were growth retarded, had prominent developmental defects of the skeleton, were infertile due to lack of spermatogonia, and died with thymic lymphomas between 3 and 4 months of age. The Brca2 gene product plays an essential role in a diverse group of cellular processes, including embryonic development, cell proliferation and tumor suppression.