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PRINCIPAL INVESTIGATOR: Lisa K. Gilliam

CONTRACTING ORGANIZATION: Duke University Medical Center
Durham, North Carolina 27710

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Duke University Medical Center
Durham, North Carolina 27710

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Prevention of metastatic breast cancer is limited to early detection and surgical resection of a localized tumor. Treatment for advanced breast cancer is often unsuccessful due to micrometastasis of the tumor prior to therapy. Thus, new methods to treat and prevent metastatic breast cancer are sorely needed.

EGFRvIII, a variant form of the epidermal growth factor receptor, is found in a large percentage of breast tumors and thus may be a viable target for immunotherapy. We are developing a bispecific antibody against both EGFRvIII and a T-cell activation antigen to redirect the cytotoxic response of T-cells with a broad range of specificities against breast cancer cells that express EGFRvIII.

Prevention and treatment of breast cancer is also advanced via an improved understanding of breast cancer biology. We have developed immunological reagents against the inherited breast cancer susceptibility gene product, BRCA2, and have shown that this protein is expressed at high levels in thymic epithelium, skin, and squamous carcinoma, suggesting a potential role in differentiation. Elucidation of the role this protein plays in normal breast biology may help us to understand why tumorigenesis occurs in hereditary cases, and these findings may also be applicable to non-inherited breast cancer.

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Lisa Hillman 6/29/98

PI - Signature Date

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Introduction

Breast Cancer

Breast cancer is a devastating and often times incurable disease for women in whom metastasis or micro-metastasis has already occurred before therapy is instituted. High dose chemotherapy and bone marrow transplantation is currently the only potentially curative treatment for such women. Chemotherapy is toxic for dividing cells in the body; hence, its relative specificity for tumor cells lies in the fact that these cells are rapidly proliferating. However, this therapy is also toxic to normal dividing cells, which accounts for many of its serious and potentially life-threatening side effects, such as bone marrow suppression and gastrointestinal mucositis. Furthermore, although chemotherapy has a very high cure rate for some types of cancers including acute lymphoblastic leukemia, gestational trophoblastic disease, and testicular cancer, its usefulness has been limited in treating breast cancer. Therefore, new types of therapy to prevent or cure metastatic disease are sorely needed.

Immunotherapy

In 1970, Macfarlane Burnet coined the term "immunosurveillance" to describe the hypothesis that "an important and possibly primary function of immunological mechanisms is to eliminate cells which as a result of somatic mutation or some other inheritable change represent potential dangers to life." (Burnet, 1970) Proponents of the concept of immunosurveillance believe that this "policing" activity of the immune system plays a very important role in the day-to-day prevention of the development of cancer in an individual. It is clear that there is an interaction between tumors and the immune system, but the importance of this interaction in natural *in vivo* defense against cancer remains controversial. Regardless of whether the immune system *naturally* plays an important role in defense against tumor development, countless animal studies and preliminary clinical studies have suggested that manipulation of an individual's immune system may provide a specific, effective, and relatively non-toxic therapeutic modality for tumors (termed immunotherapy). Several different immunotherapeutic approaches to treating tumors are undergoing clinical trials, including the administration of a patient's own lymphocytes which have been activated *in vitro* (lymphokine activated killer cells, or LAK cells) as adjuvant therapy for metastatic or micrometastatic disease (reviewed in Sussman et al., 1994), the transfection of cytokines into the tumor cells themselves to form an autologous tumor vaccine (Gilboa et al., 1994), and the administration of tumor-specific antibodies conjugated to a toxin or radionuclide to enhance the specificity of an otherwise non-specific chemotherapeutic agent or radiation therapy (reviewed in Pai and Pastan, 1994). All of these modes of therapy require that the immune system be able to differentiate between tumor cells and normal cells, via antigens expressed solely or predominantly by the tumor cells (tumor-specific or -associated antigens).

EGFRvIII

The wild-type epidermal growth factor receptor (EGFR) is a 170kDa transmembrane protein which serves to transmit growth stimulatory signals from the surface of the cell to the cell's interior. Because of EGFR's role in the proliferative signaling pathway, it is capable of cellular transformation via two mechanisms: overexpression of the structurally normal protein, and activation of the molecule via a molecular event. Dr. Darell Bigner at Duke as well as others have demonstrated that rearranged variants of the EGFR are expressed in a variety of malignancies, including breast carcinomas (Wikstrand et al., 1995). The most frequently detected variant, type III (denoted EGFRvIII), is characterized by an 801 base pair deletion resulting in the loss of 267 amino acids spanning the first and second extracellular domains of the receptor, with generation of a glycine residue at the novel splice site. EGFRvIII binds EGF, but with markedly decreased affinity relative to structurally normal EGFR. Furthermore, the transforming potential of EGFRvIII is ligand-independent and unregulated *in vivo* and *in vitro* (Batra et al., 1995). Both of these observations suggest that naturally occurring EGFRvIII may play an oncogenic role in the

tumor cells in which it is expressed, since these abnormal proteins may be capable of functioning in the absence of EGF. Consequently, cells expressing EGFRvIII would have a growth advantage over cells expressing the structurally normal EGFR in the presence of low levels of EGF (Bigner et al., 1990).

The most significant finding with respect to EGFRvIII, however, is that this protein appears to be commonly found in particular types of malignancies in adult tissues. We have demonstrated the presence of EGFRvIII protein by immunohistochemistry in 3/11 breast carcinomas tested, with mRNA corresponding to EGFRvIII detectable in these 3 plus an additional 5 (total 8/11) by RT-PCR followed by ethidium bromide staining (Wikstrand et al., 1995). Furthermore, flow cytometric analysis demonstrated cell-surface reactivity of tumor cells with anti-EGFRvIII mAbs in 5 of 5 mechanically-dissociated primary breast carcinomas. Until recently, no normal adult tissues tested, including those from the peripheral and central nervous system, the lymphoid system, skin, breast, liver, lung, ovary, testes, kidney, and colon were found to express EGFRvIII by immunohistochemical and/or genetic analysis (Wikstrand et al., 1995). Although we have recently demonstrated low level expression of EGFRvIII in thymocytes, this protein is expressed at much higher levels in tumor tissues. **Thus, EGFRvIII is a tumor-associated antigen and may be a useful target for specific therapies including immunotherapy**.

Hypothesis and Technical Objectives: The goal of this project is to evaluate the feasibility of and develop new strategies for immune-based therapy for breast cancer, based on expression of the tumor-associated antigen EGFRvIII. My proposal includes the following technical objectives:

1) The expression pattern of the epidermal growth factor receptor variant III (EGFRvIII) tumor antigen, including tumor specificity and prevalence, will be determined for a large panel of specimens from breast cancer patients, for established breast cancer cell lines, and for a broad spectrum of normal adult and fetal tissues, using RT-PCR, flow cytometry, immunohistochemistry, and Western blot assays.

2) The existence of a naturally occurring anti-EGFRvIII immune response in breast cancer patients as well as the ability to generate a humoral or cellular immune response against EGFRvIII in these patients will be tested in vitro, using enzyme immunoassays of patients' serum and cytotoxicity assays of patient TILs and PBLs against antigen presenting cells loaded with EGFRvIII-specific peptides.

3) An antibody specific for both EGFRvIII and CD3ε will be constructed to investigate this bispecific construct as a means to bypass requirements for specific T-cell receptor/peptide-MHC matching and to redirect CTLs to kill tumor cells regardless of their natural specificity.

In the process of completing **technical objective #1**, evaluating the expression pattern of EGFRvIII in normal and tumor tissues, we noted expression of this variant protein in normal adult thymocytes, suggesting that this antigen is not entirely tumor specific and may thus not be an optimal candidate protein for active immunotherapy. However, as EGFRvIII is much more highly expressed in tumor tissues and the contribution of adult thymopoiesis to the T cell repertoire is likely minimal (Haynes and Hale, 1998), passive immunotherapy may still be a viable option. Thus, during the past two years, I have focused on **technical objective #3**, completing the work outlined in the Statement of Work for years 1 and 2. The construction of the bispecific antibody is very near completion. I should be generating protein products of my genetically engineered constructs in the next few months, and testing it functionally thereafter, with completion of the project as scheduled by the end of year #3.

Because of the unanticipated findings regarding EGFRvIII expression in normal adult tissues, I have pursued an alternate project in place of **technical objective #2**. In collaboration with Dr. Jeffrey Marks of the Duke University Specialized Program of Research Excellence (SPORE) in Breast Cancer, I have generated immunological reagents for use in studying the

inherited breast cancer gene, BRCA2. Enclosed is a manuscript based on that work, which has recently been submitted for publication (please see manuscript included in Appendices section of this report for background on BRCA2). Thus, my revised technical objective #2 is the following:

Revised Technical Objective #2:

Immunological reagents will be developed for the inherited breast cancer susceptibility gene, BRCA2. These will be used to study the expression pattern of the BRCA2 protein in various tissues as well as its subcellular localization, and to gain clues regarding the normal function of this gene product.

Thus, over years 1 and 2 of this project, I have made considerable progress toward completion of an immune-based prevention and/or treatment strategy for breast cancer based on the high level expression of EGFRvIII in tumor tissues. In addition, I have completed a project involving generation of immunological reagents for use in studying the BRCA2 inherited breast cancer susceptibility gene product. Thus, I have completed technical objecive #1 as well as revised technical objective #2, and I am on schedule to complete technical objective #3 within the allotted time.

Body

Bispecific Antibody Construction

A large portion of the work for this part of the project (Technical Objective #3) has already been accomplished. In brief, the strategy I have used in construction of the bispecific antibody is to link leucine zipper peptides derived from the Fos and Jun proteins to the Fab' portions of EGFRvIII and CD3ε mAbs by gene fusion using PCR.

My original plan was to clone the heavy and light chain variable regions, construct an Ig heavy chain-leucine zipper fusion gene, and insert these sequences into expression vectors designed specifically for expression of immunoglobulin heavy chain (pSV2DHgpt-HuG1) and light chain (pSV184DHneo-HuK). In year 1 of the grant, I cloned the heavy and light chain variable regions from the anti-CD3ε hybridoma cell line, OKT3 and from the anti-EGFRvIII hybridoma cell line, L8A4 by RT-PCR (Figure 1).

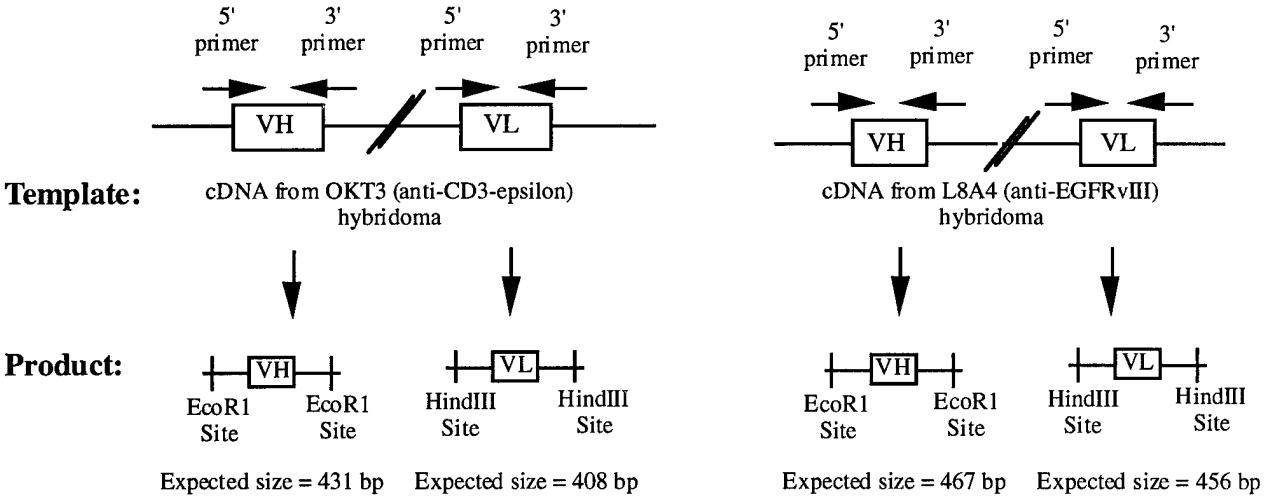


Figure 1: Strategy for PCR amplification and cloning of anti-EGFRvIII and anti-CD3ε variable regions.

For construction of the IgG-leucine zipper fusion gene, I initially planned to use a BstX1 site, unique in the CH1 region of the human IgG1 sequence, as a means of removing the unnecessary portions of the IgG1 constant region from the intended expression vector and replacing them with the PCR-generated IgG1-leucine zipper fusion gene. Then, I planned to insert the specific cloned heavy chain variable region (OKT3 or L8A4) into the variable region cloning site to complete the construct. Similarly, I planned to insert the specific cloned light chain variable region into the variable region cloning site in the Kappa expression construct. I successfully joined the fos or jun leucine zipper sequences to the first codon of the C_H2 exon of the human IgG1 gene (Figure 2), using the overlapping PCR method described by Yon and Fried (Yon and Fried, 1989). These IgG1/Fos and IgG1/Jun constructs were then inserted into the PCRII cloning vector. Unfortunately, when I tried to insert the completed construct into the pSV2DHgpt-HuG1 expression vector, I found that the vector had several previously unidentified BstX1 sites which cleaved preferentially with respect to the desired BstX1 cleavage and thus precluded specific insertion of the construct. Furthermore, both the heavy and light chain vectors were very large (>15,000 kB) and were poorly mapped, as their intended use was for the simple exchange of variable region "cassettes" to make chimeric antibodies with specific human isotypes.

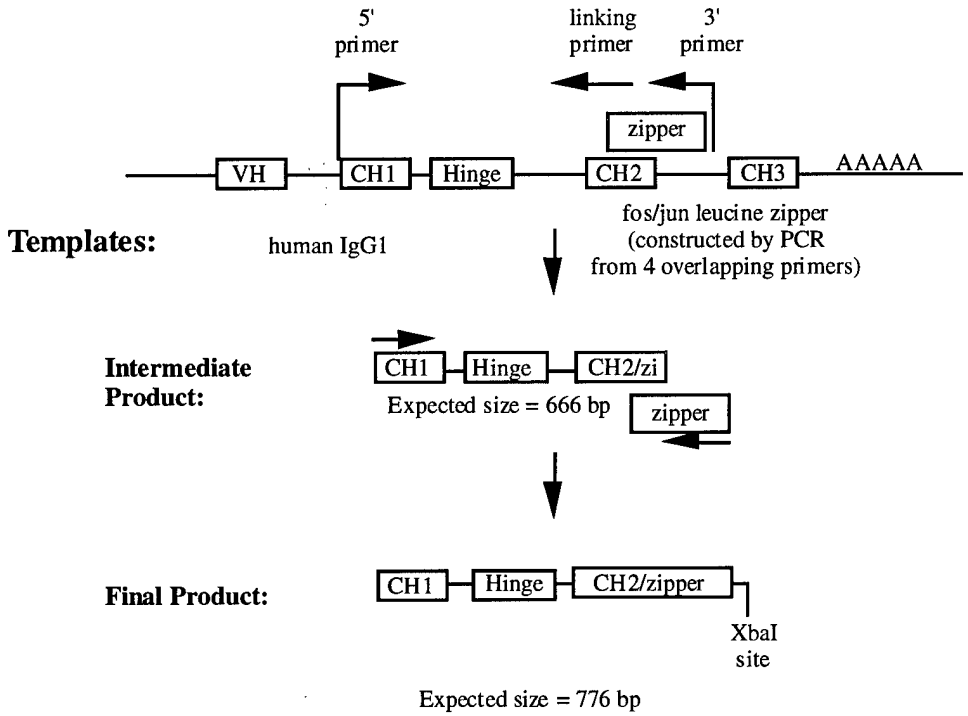


Figure 2: Construction of a fusion gene (antibody constant region + leucine zipper) by overlapping PCR.

Thus, I devised another strategy for construction of the bsAbs. First, I decided to use well characterized expression constructs for production of the heavy and light chain proteins *in vitro*; PC1-neo (Promega, Carlsbad, CA) was chosen for the heavy chain, and pcDNA3.1/Hygro (Invitrogen, Madison, WI) for the light chain. Again, I used the overlapping PCR method to join the jun or fos leucine zipper sequence to the first codon of the C_H2 exon of the human IgG1 gene, this time including the *entire* coding sequence of the C_H1 and hinge regions and the first codon of the C_H2 exon. The cloned OKT3 sequence was then directly joined (in a second fusion PCR step) to the beginning of the IgG1-fos fusion gene, with the L8A4 sequence joined to the IgG1-jun fusion gene in an analagous fashion (Figure 3a).

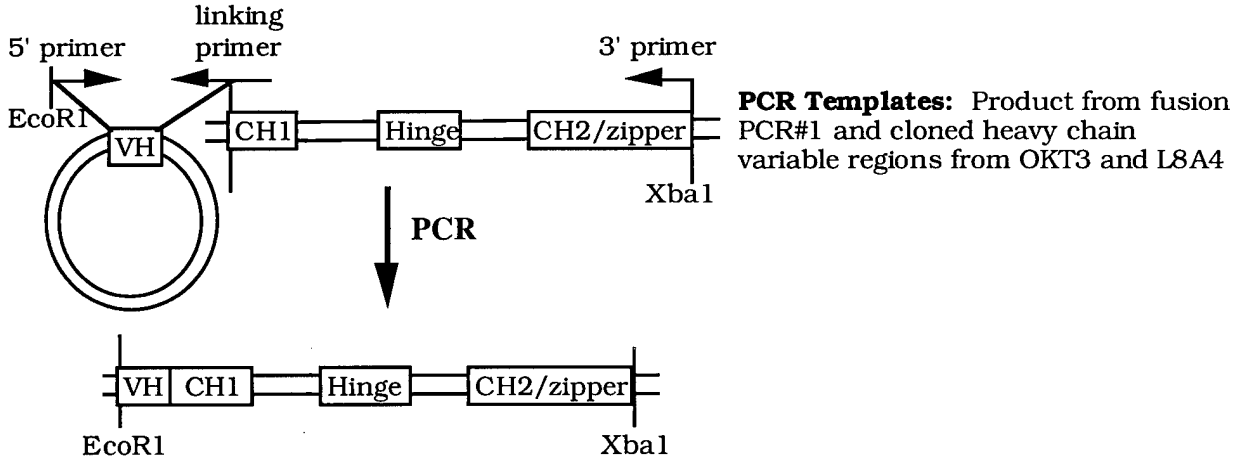
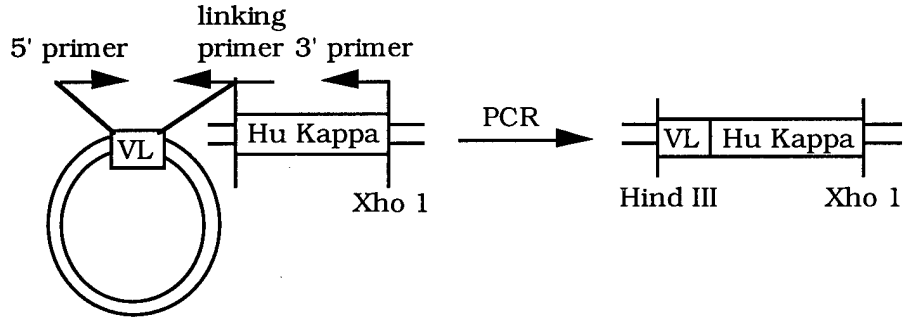


Figure 3a: Joining heavy chain variable region to IgG1/Fos or IgG1/Jun fusion gene by overlapping PCR.

Similarly, the OKT3 and L8A4 light chain variable regions were directly joined to the human kappa sequence by overlapping PCR (Figure 3b).



PCR Templates: Human kappa template and cloned light chain variable regions from OKT3 and L8A4

Figure 3b: Joining light chain variable region to human kappa gene by overlapping PCR.

The fusion junctions of these four constructs were sequenced to rule out PCR-generated mistakes. One fusion junction, where the heavy chain C_H2 region was joined to the jun sequence, was particularly PCR mistake-prone (likely due to a high GC content in that region), and no good clones were produced, even after several fusion PCR reactions. Thus, I performed site-directed mutagenesis on one clone which was correct at the other fusion junction (L8A4-IgG1) to correct the PCR-generated mistake at the C_H2-jun junction. After confirming that all of the fusion junctions were now correct, I removed the sequences from the PCR-II cloning vectors and inserted them into the appropriate expression vectors (Figures 4a and 4b).

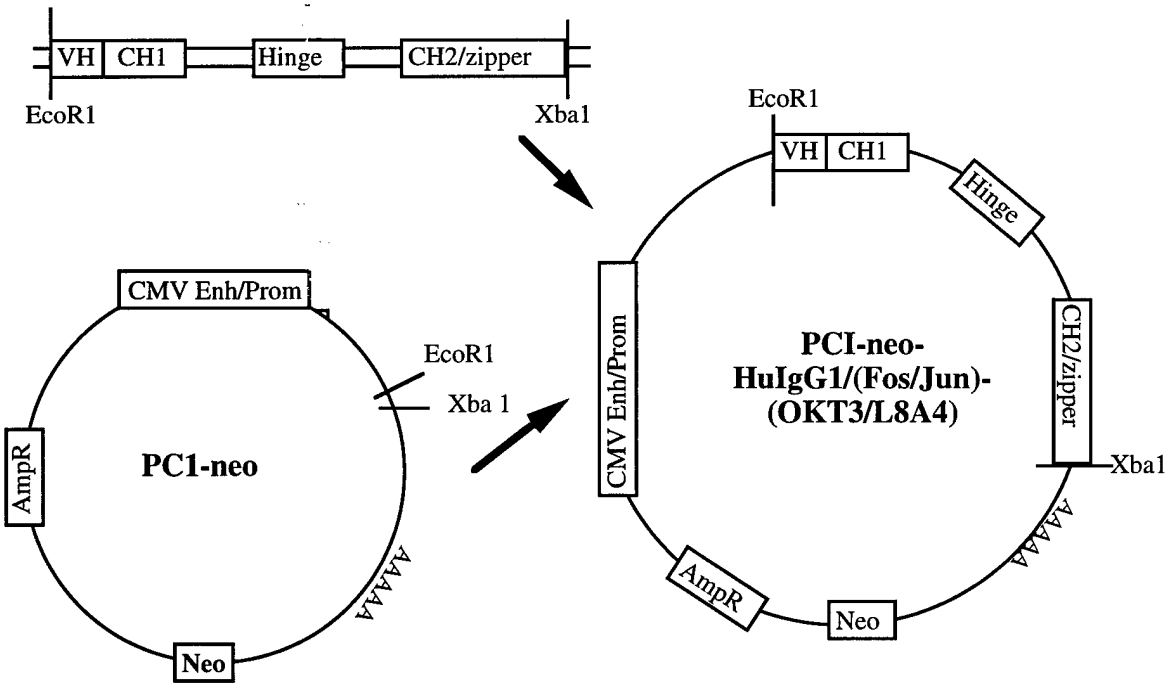


Figure 4a: Construction of heavy chain immunoglobulin expression vector.

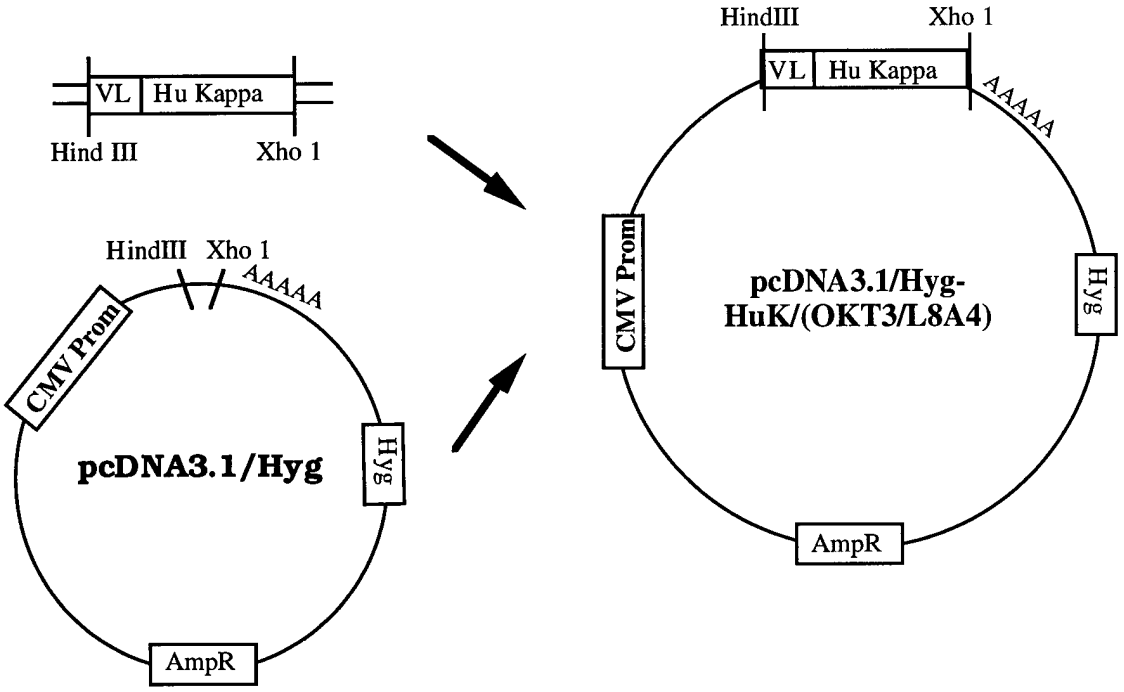


Figure 4b: Construction of light chain immunoglobulin expression vector.

To screen for clones that produced protein of the appropriate molecular weight for the heavy and light chain constructs, I transfected the constructs individually into 293 cells, in which we have had a very high transfection efficieny in our lab. Unfortunately, clones from only one of the four constructs produced protein (OKT3-kappa) that was detectable by Western blot (Figure 5).

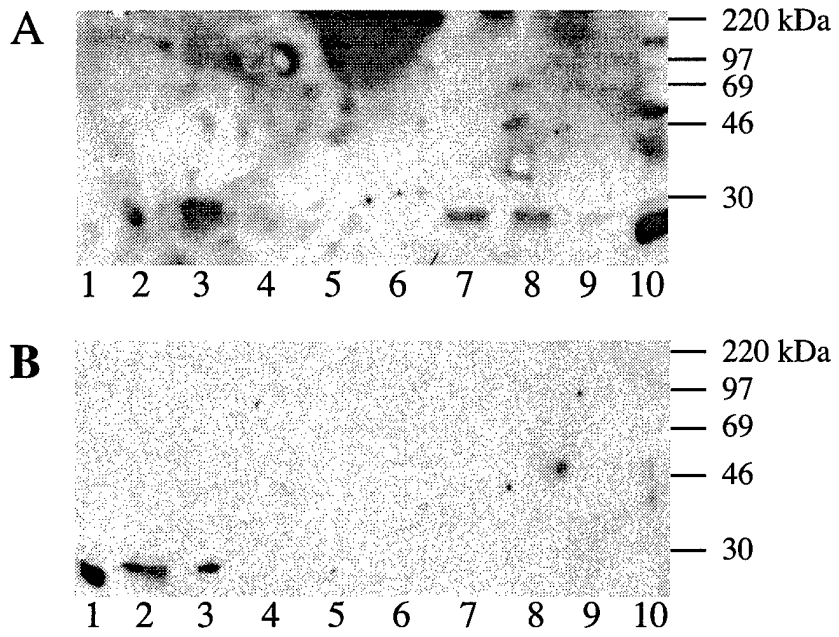


Figure 5: Screening L8A4-Jun, L8A4-Kappa, OKT3-Fos, and OKT3-Kappa clones for protein-producing constructs. Lysates from 293 cells transfected with various bispecific antibody clones were reacted with goat anti-human-F(ab)^2 Ig in Western blot assays. Lanes were loaded as follows: OKT3-Kappa #1-1A, 1-2A, 1-3A, 1-5A, 1-6A, 1-7A, 1-8A, 3-2B, 3-3A (lanes 1-9, panel A); OKT3-Kappa #3-7A, 3-8A (lanes 2 and 3, panel B); OKT3-Fos #1B, 2A, 7B (lanes 4-6, panel B); L8A4-Kappa #6B, 7B, 9B (lanes 7-9, panel B); L8A4-Jun #M7 (lane 10, panel B). Markers + positive control human Ig (reduced with dithiothreitol to separate heavy and light immunoglobulin chains) were loaded in lanes 10 (panel A) and 1 (panel B). Bands of the appropriate molecular weight for Ig light chain, 25 kDa, were seen in lanes containing OKT3-Kappa clones #1-2A, 1-3A, 1-8A, 3-2B, 3-7A, 3-8A (lanes 2,3,7, and 8 in panel A; lanes 2 and 3 in panel B) as well as in lanes containing human Ig control (lane 10, panel A; lane 1, panel B). No bands were seen in any lanes containing the L8A4-Kappa, L8A4-Jun, or OKT3-Kappa constructs. Clone **OKT3-Kappa #1-8A** (lane 7, panel A) was selected for DNA maxiprep for use in future experiments.

Furthermore, co-transfection of the L8A4-Jun construct #M7 (the sequence of which was corrected by site-directed mutagenesis) with each of the three L8A4-Kappa constructs (#6A, 7A, and 9A) into SP2/0 myeloma cells produced clones which were resistant to the selection agents encoded by the vectors used, but no immunoglobulin was detectable either in the supernatant or in the cells themselves by Western blot or ELISA. This suggests that the transfection was successful, but the constructs are not correct. Our conclusion from these results was that PCR-generated mistakes must exist in regions other than the fusion junctions. Thus, I will sequence the constructs which are not producing protein, correct any PCR-generated mistakes by site-directed mutagenesis, and repeat the transfection into 293 cells to confirm that the constructs are now correct. After these constructs have been produced and transfected into myeloma cells, culture supernatant will be screened for anti-EGFRvIII or anti-CD3ε activity followed by purification, heterodimerization, and characterization of the bsAb products (Figure 6) (Kostelny et al., 1992). Thus, despite the unanticipated difficulties which arose in preparation of these bsAb molecular constructs, I am on the schedule outlined in my original Statement of Work.

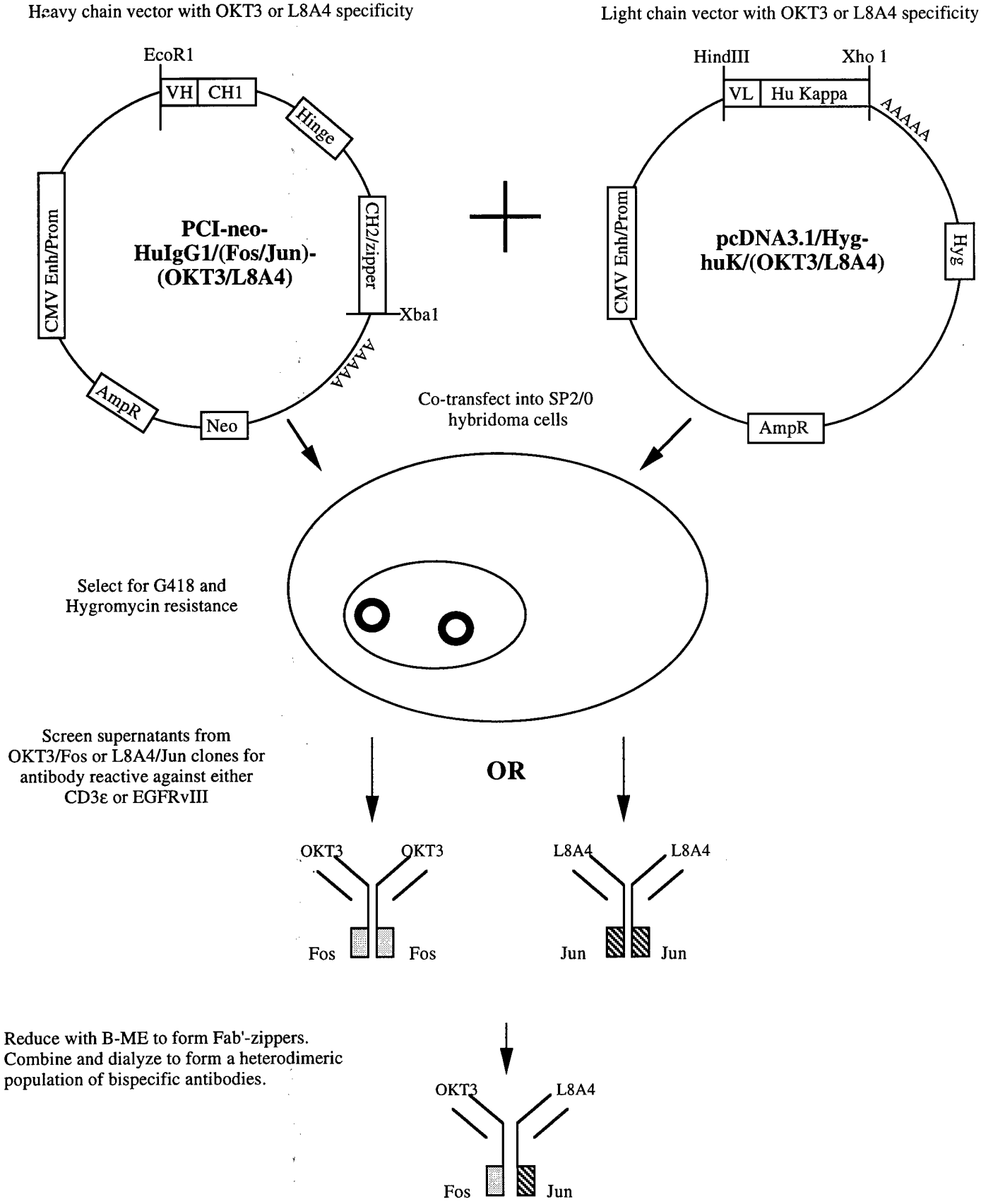


Figure 6: Transfection of immunoglobulin expression vectors into SP2/0 hybridoma cells and production of an antibody bispecific for EGFRvII and CD3-epsilon.

Production of an EGFRvIII Adenovirus Construct

Characterization of the antibodies bispecific for EGFRvIII and CD3ε will involve functional testing using cytotoxic T lymphocyte (CTL) assays in which naive effector cells are incubated with non-major histocompatibility complex (MHC) matched EGFRvIII-expressing target cells. Lysis of the target cells will suggest that we have, in fact, bypassed the specificity of the T-cells in generating this cytotoxic response. I have previously analyzed 4 available human breast cancer permanent cell lines (MCF-7, MDA-MA-231, SKBR3, and ZR75-1) for the presence of EGFRvIII by RT-PCR analysis and found that none express this EGFR variant. Therefore, it will be necessary to construct EGFRvIII expressing breast cancer target cells to test the efficacy of the bispecific antibody construct. Originally, I planned to do this by transfection of EGFRvIII cDNAs, a process we have successfully accomplished in murine cell lines. Attempts to obtain stable EGFRvIII transfectants in these breast cancer cell lines, using various transfection methods or commercially available transfection agents (calcium phosphate precipitation method, lipofectamine, transfectam, and DOTAP) were unsuccessful. Furthermore, production of stable EGFRvIII-expressing cell lines using retroviral EGFRvIII constructs, which has resulted in very high level expression in other cell types, was unsuccessful in breast cancer cells, as the cells lost expression of EGFRvIII rapidly with repeated passaging. Based on these numerous unsuccessful attempts, we conclude that EGFRvIII is likely to be detrimental to survival of human breast cancer cells *in vitro*. This conclusion is supported by the observation that expression of variant forms of EGFR by human gliomas is lost over time with *in vitro* culture, necessitating the propagation of tumors known to express these variant forms of EGFR as xenografts in immunodeficient mice (Bigner et al., 1990). Therefore, we decided to create an EGFRvIII-producing adenovirus for use in these experiments. Adenoviral constructs efficiently infect epithelial cells, transducing genes with close to 100% efficiency. These constructs can be used to infect target cell lines for CTL assays, creating cells expressing high levels of EGFRvIII from 24-48 hours after infection.

Construction of the EGFRvIII-producing adenovirus was done in collaboration with Dr. Andy Amalfitano at this institution (Figure 7).

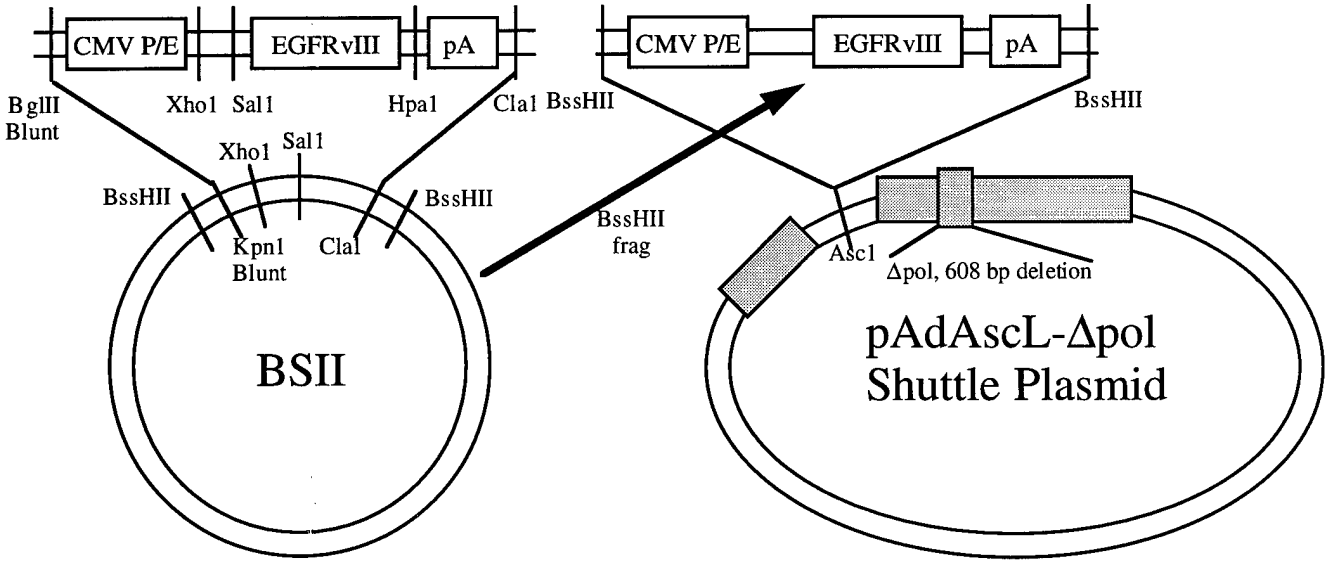


Figure 7: Construction of EGFRvIII-expressing adenovirus. After the EGFRvIII, CMV promoter and enhancer, and polyA sequences were inserted into pAdAscL-Δpol, the shuttle plasmid was cotransfected into adenovirus along with non-infectious viral genomic plasmid. Shaded regions of the shuttle plasmid represent viral sequences that undergo homologous recombination with the genomic plasmid, allowing insertion of the EGFRvIII sequence into the viral genome.

First, the EGFRvIII sequence was cut out of pHβApr-1-neo-EGFRvIII, an expression vector constructed in the laboratory of our collaborator, Dr. Darrell Bigner. A unique restriction site, Sal1, was present at the beginning of the sequence; however, due to lack of a unique site at the end of the sequence, I digested with Hpa1, which cut in the middle of the polyA sequence. I then inserted the end of the polyA sequence (derived from PC1-neo), the CMV I/E promoter and enhancer (also derived from PC1-neo), and the EGFRvIII sequence in a four piece ligation into the Bluescript-II cloning vector. The adenovirus shuttle plasmid, pAdAscL-Δpol, was designed so that digestion of the shuttle plasmid with Asc1 would allow insertion of a compatible BssHII fragment from Bluescript-II. After successful insertion of the EGFRvIII sequence into the adenovirus shuttle plasmid, the shuttle plasmid was cotransfected with non-infectious viral genomic plasmid, and recombinants were selected by their ability to form plaques on the E1- and Pol-complementing cell line B6. Five clones were obtained, and I screened these for presence of the EGFRvIII sequence by isolation and digestion of the genomic viral DNA, as well as by transient infection of cultured LP293 cells and Western blot analysis (Figure 8).

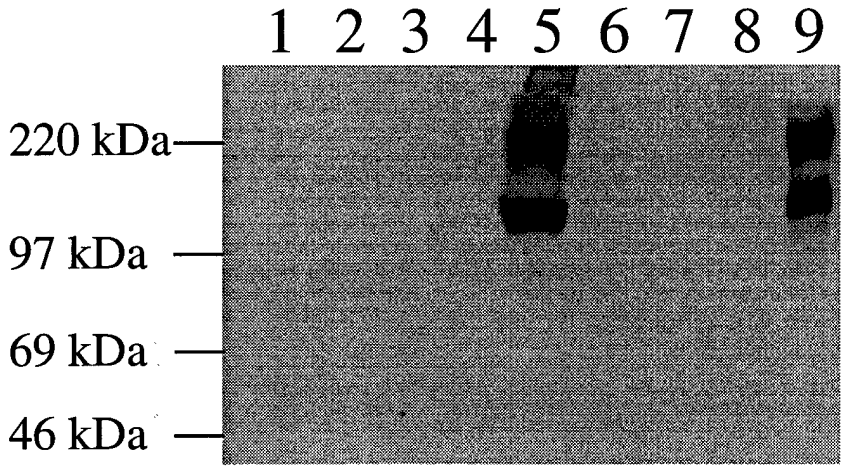


Figure 8: Screening AV-EGFRvIII clones for production of EGFRvIII protein. Lysates from LP293 cells infected with five AV-EGFRvIII clones (lanes 1-5) or uninfected (lane 6) were reacted with anti-EGFRvIII mAb L8A4 in Western blot assays. As a positive control, a protein lysate from the HC mouse cell line stably transfected with EGFRvIII, was loaded in lane 9. Lanes 7 and 8 contained molecular weight markers and no protein, respectively. Identical bands consistent with the 140 kDa molecular weight of EGFRvIII were detected by mAb L8A4 in both the positive control lane (9) as well as the lane containing AV-EGFRvIII clone #5 (lane 5). No bands were detected on an identical blot using negative control antibody P3x63 (not shown). Higher molecular weight bands represent glycosylated forms of EGFRvIII and are consistently seen, along with the 140 kDa band, on Western blot.

One clone of the five contained functional EGFRvIII sequence, and this clone was expanded in culture. We have recently demonstrated that infection of breast carcinoma cells with this EGFRvIII-producing adenovirus results in very high level expression in these cells, as assessed by Western blot (Figure 9), immunohistochemistry, and flow cytometry (data not shown). This construct is likely to be useful, not only for evaluation of the bispecific antibody construct, but for many other applications, including study of the EGFRvIII tumor antigen in breast cancer cells *in vitro*, as well as for applications *in vivo* studying the interaction between the immune system and the EGFRvIII tumor-associated antigen.

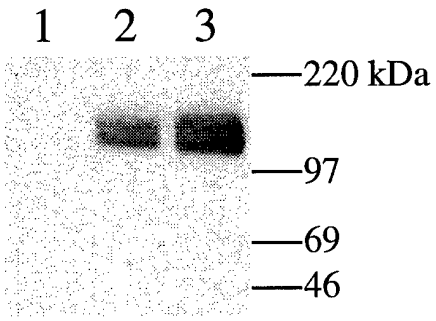


Figure 9: Infection of MCF-7 breast carcinoma cells with AV-EGFRvIII results in high level expression of the variant EGFR protein. MCF-7 cells were infected with 600 ul (lane 2) or 1 mL (lane 3) of AV-EGFRvIII clone #5. The cells were harvested 46 hours following infection, and lysates were reacted with anti-EGFRvIII mAb L8A4 in Western blot assays. Bands consistent with the 140 kDa protein EGFRvIII were specifically detected in lysates from AV-EGFRvIII-infected MCF7 cells, but not in lysate from uninfected MCF-7 cells (lane 1). No bands were detected on an identical blot using negative control antibody P3x63 (not shown). The two bands represent the 140 kDa EGFRvIII species and a higher molecular weight, glycosylated species. The pattern appears different here than in figure 8, as these samples were separated on a gradient gel, whereas the samples in figure 8 were separated on a 7.5% gel.

Conclusions

In conclusion, I have been investigating two approaches to breast cancer detection and treatment, utilizing immuno-based reagents. Both the development of a monoclonal antibody against BRCA2 and the evaluation of EGFRvIII as a target for passive immunotherapy hold promise for improved clinical treatment of patients who suffer from this terrible disease. Furthermore, the approaches used will be rapidly generalizable to other tumor types and other tumor antigens.

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Appendix: BRCA2 manuscript

BRCA2 mAbs React with Differentiating Epithelium

By

Lisa K. Gilliam[1], Edward K. Lobenhofer[1,5], Paula K. Greer[1], Richard M. Scearce[2], Frank D. Cirisano[3], Alexander Miro[4], Andrew Berchuck[3], Jeffrey R. Marks[4], and Laura P. Hale[1]

From the Departments of Pathology[1], Medicine[2], Obstetrics and Gynecology[3], and Surgery[4] and the Program of Cell and Molecular Biology[5]

Duke University School of Medicine,

Duke University Medical Center, Durham, NC 27701

Reprint requests and correspondence to: Dr. Laura P. Hale

Box 2608, DUMC

Durham, NC 27710

Phone: 919-684-4771

Fax: 919-681-8337

E-mail: Hale0002@mc.duke.edu

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BRCA2 mAbs React with Differentiating Epithelium

Introduction

Individuals with germline mutations in the BRCA2 breast cancer susceptibility gene are at increased risk for breast cancer (including male breast cancer), ovarian cancer, pancreatic cancer, prostate cancer, and potentially other malignancies (reviewed in Stratton, 1996a). Identified by positional cloning (Wooster *et al.*, 1994; Wooster *et al.*, 1995; Tavtigian *et al.*, 1996), the BRCA2 gene consists of 27 exons extending over 70 kB and encodes a protein with a predicted MW of 384 kDa. The amino acid sequence of BRCA2 shows no significant homologies to other known proteins. Although the BRCA2 gene is generally poorly conserved between species (human BRCA2 is 58% and 59% homologous to rat and mouse, respectively (McAllister *et al.*, 1997)), Bork et al described 8 copies of a 30-80 aa internally repeated domain within exon 11 (BRC repeats), four of which are highly conserved between rat, mouse, and human (Bork *et al.*, 1996; Bignell *et al.*, 1997; Connor *et al.*, 1997a). Aside from this structural clue, analysis of the BRCA2 gene and its predicted protein product has not contributed significantly to a better understanding of either the normal cellular functions of this protein or how mutation of the gene contributes to development of breast cancer.

The BRCA2 gene is widely transcribed but at relatively low levels in most tissues (Wooster *et al.*, 1995; Tavtigian *et al.*, 1996). Initial tissue screens demonstrated moderate expression of BRCA2 mRNA in normal breast tissue (Tavtigian *et al.*, 1996), with high levels found in thymus and testis, two organs characterized by normally high levels of proliferation and differentiation. High levels of BRCA2 mRNA were seen in murine breast only during development of terminal end buds during puberty and in differentiating alveoli during pregnancy (Rajan *et al.*, 1996). Other organs with significant BRCA2 mRNA expression include ovary, spleen, eye, and certain areas of the brain (Connor *et al.*, 1997a). BRCA2 mRNA expression is regulated by the cell cycle in breast and ovarian epithelial cell lines, with increased BRCA2 mRNA detected in late G1 and early S phases (Rajan *et al.*, 1996; Vaughn *et al.*, 1996). The kinetics of BRCA2 mRNA expression is similar to that observed for BRCA1, leading to the suggestion that these two proteins are regulated in a coordinate fashion (Rajan *et al.*, 1996; Vaughn *et al.*, 1996). BRCA2 mRNA is upregulated in cultured mammary epithelial cells in response to estrogen (Spillman and Bowcock, 1996), as well as in differentiating mammary epithelial cells in response to glucocorticoids (Rajan *et al.*, 1996).

Mice genetically homozygous for BRCA2 truncated prior to exon 11 ("BRCA2-negative") show growth retardation beginning at embryonic day 6.5 and die by embryonic day 8.5 (Ludwig *et al.*, 1997; Suzuki *et al.*, 1997; Sharan *et al.*, 1997b). Post-mortem histologic examination demonstrates lack of appropriate cell numbers and decreased proportion of proliferating cells, leading to the hypothesis that BRCA2 is important in cell proliferation. BRCA2-negative embryos have decreased levels of cyclin E, decreased levels of mdm2, and increased levels of p21 suggesting that BRCA2 protein interacts with the p53 pathway and may similarly function as a "guardian of the genome", to protect the integrity of DNA replication (Suzuki *et al.*, 1997). BRCA2-negative embryonic stem cells are extraordinarily sensitive to radiation exposure which results in (ds) DNA breaks (Sharan *et al.*, 1997b). More recent studies have demonstrated that viable mice can be generated using BRCA2 truncated at bp 6038, beyond the BRC repeat regions in exon 11 (Connor *et al.*, 1997b). These mice exhibit growth retardation and die by week 22, primarily of thymic lymphoma. Levels of p21 and p53 are consistently elevated in embryonic fibroblasts homozygous for the truncated BRCA2, however induction of p53 by ionizing radiation appears intact, suggesting that BRCA2 is not involved in sensing dsDNA breaks upstream of p53.

Cells homozygous for truncated BRCA2 require increased time to repair dsDNA breaks generated by ionizing radiation, suggesting that BRCA2 is necessary for efficient repair of dsDNA breaks. Studies using the yeast two-hybrid system report that, similar to BRCA1, BRCA2 protein interacts with Rad51, a component of the synaptonemal complexes important in DNA exchange processes during meiosis, and a homologue of the bacterial RecA protein (Shinohara *et al.*, 1992; Mizuta *et al.*, 1997; Scully *et al.*, 1997; Sharan *et al.*, 1997b). Furthermore, a recent report has demonstrated that Rad51 binds BRCA2 via the BRC repeat regions, and these domains are required for resistance to methyl methanesulfonate (MMS), which putatively induces double-stranded DNA breaks, in cells lacking full-length BRCA2 (Chen *et al.*, 1998). Taken together, these studies have led to the recent proposal of a "caretaker" role for BRCA2 protein in monitoring and/or repair of DNA double-strand breaks (Kinzler and Vogelstein, 1997; Sharan *et al.*, 1997b).

Recently, BRCA2 protein has been immunoprecipitated from nuclear fractions of breast and bladder carcinoma cells, and its expression was shown to be upregulated with the cell cycle, with induction at late G_1/early S phase (Bertwistle *et al.*, 1997; Chen *et al.*, 1998). However, no descriptions of the normal cellular and tissue distribution of this protein have been published to date. Here we report the distribution of BRCA2 protein expression using novel monoclonal and polyclonal antisera. We have found reactivity of BRCA2 antibodies in differentiated normal epithelia of skin and thymus, as well as in squamous cell carcinoma of head and neck origin, but not in normal or malignant breast epithelium.

Materials and Methods

Antibodies: The anti-BRCA2 monoclonal antibody 2C9 was developed by immunization with a bacterially-produced gel purified 46 kD protein fragment derived from BRCA2 exon 11, termed BAC2 (bp 3748 to 4817, Genbank U43746), and hybridoma formation as described previously (Scearce and Eisenbarth, 1983). Anti-BRCA2 polyclonal rabbit antiserum #5814 was generated by immunization with a bacterially produced peptide derived from a non-overlapping portion termed BAC1 (bp 2716-3714) of BRCA2 exon 11. Additional anti-BRCA2 mAbs generated against sequences in exon 14 (mAbs 5F6 and 9D3) or against a peptide fragment encoded by bp 792-1917, corresponding to mid exon 7 through most of exon 10 (mAb 3E6) were kind gifts of Wen Hwa Lee. Anti-cytokeratin (CK) mAbs AE1 and AE3 (reactive with CK 1-2, 5, 6, 14) were obtained from Boehringer Mannheim. Anti-CK mAb AE2 (reactive with CK 1, 2, and 10 found in terminally differentiated cells) was a kind gift from Barton Haynes. Mib-1 mAb specific for the Ki-67 nuclear antigen expressed by all cells that are not in G_0 (Key *et al.*, 1993) was obtained from Immunotech Inc. Anti-phosphotyrosine mAb 2G8.D6 hybridoma cell line was obtained from the ATCC and was grown in IMDM+10% Fetal Clone I (Hyclone, Logan UT).

Tissue Culture. MCF-7, BT483, and T47D breast carcinoma cell lines and the SKOV3 ovarian carcinoma cell line, obtained from the ATCC, were grown in RPMI 1640+10% fetal calf serum (FCS). Cos-7 cells were obtained from the Duke University Tissue Culture Facility and were grown in IMDM + 5% FCS. Human foreskin keratinocytes (HFKs) were derived from discarded neonatal foreskins by trypsinization (0.25%, 4°C, overnight) and mechanical dissociation, and were grown in Keratinocyte Serum-free Medium (Life Technologies, Grand Island NY). All cultured cells were grown at 37°C in 5% CO_2.

BRCA2 cDNA and Transfectants. Full length BRCA2 cDNA was prepared as follows: a cDNA containing BRCA2 bp 23-10502, encoding full length BRCA2 protein (translated from bp 229 to bp 10485) was created by splicing partial cDNAs derived from an MCF-7 breast carcinoma cDNA library and PCR cloning (bp 9784 to 10502). This DNA was cloned into the pBSX/CMV/pA expression vector, a modified Bluescript under the control of the CMV immediate-

early promoter/enhancer and stabilized by SV40 polyA sequences, a kind gift of A. Amalfitano. 10 ug of this construct pBSX/BRCA2 was used to transfect Cos-7 cells, using the Fugene (Boehringer Mannheim, Indianapolis IN) transfection agent according to the manufacturer's protocol. Transfected cells were analyzed after 48 hours in culture by protein extraction and Western Blot (described below).

Immunohistochemistry. 5 μm frozen tissue sections were acetone-fixed and blocked with goat serum for 20 minutes at 37°C. The sections were reacted with primary antibody for 30 minutes at 37°C, followed by a 10 minute wash (10 mM $NaPO_4$, pH 7.5, 0.9% PBS). The bound antibodies were then reacted with biotinylated horse anti-mouse or goat anti-rabbit IgG (Vector Laboratories, Burlingame CA) for 30 minutes at 37°C, followed by a wash as described above. Finally, the sections were incubated with avidin and biotinylated horseradish peroxidase macromolecular complex (Vectastain Elite ABC Kit, Vector Laboratories, Burlingame CA) for 30 min at 37°C, washed, and then incubated with 3,3'-diaminobenzidine(DAB)/H_2O_2 substrate solution for 5 minutes. Sections were washed, counterstained with hematoxylin, and permanently mounted. To enhance sensitivity in some experiments, signals were amplified using the Tyramide Signal Amplification (TSA) system (NEN™ Life Sciences Products, Boston MA) according to the manufacturers instructions. Where indicated, formalin-fixed paraffin-embedded tissue sections were deparaffinized, then heated in Glyca antigen retrieval solution (BioGenex, San Ramon CA) for 2C9 or 0.01M citrate solution for cytokeratins, 2 x 5 minutes at 600 Watts using a microwave oven, prior to the blocking step and further immunostaining as described above.

Western Blot Analysis. Protein extracts were prepared using 10 mM Tris (pH 8.0), 150 mM NaCl, and 1% Triton X-100 in the presence of proteinase inhibitors (20 mM iodoacetamide, 0.02 mM E64, 0.03 mg/ml aprotinin, 0.07 mM pepstatin A, 1 mg/ml antipain, 0.5 mM phenylmethylsulfonyl fluoride (PMSF), and 0.1 mM Nα-p-tosyl-L-lysine chloromethyl ketone (TLCK) at a concentration of 50 μl per $10x10^6$ cells. Following a 20 minute incubation on ice, samples were centrifuged for 30 minutes at 14,000 rpm, 4°C to remove insoluble material. Detergent soluble proteins from $5x10^6$ cells were boiled in sample buffer for 5-10 minutes, separated on a 4% or 5% sodium dodecyl sulfate (SDS) polyacrylamide gel and transferred to nitrocellulose. Blots were then incubated with anti-BRCA2 or control antibodies, followed by HRP-conjugated goat anti-mouse or rabbit IgG, with intervening washes. Antibody-bound protein was visualized by exposure to a chemiluminescent substrate (ECL Western blotting protocol; Amersham Life Science, Arlington Heights, IL) and detection on film.

Immunoprecipitation. Lysates prepared as described above were precleared twice with 20 μl of control P3x63 supernatant, followed by 20 μl of goat anti-mouse IgG-agarose (Sigma, St. Louis MO). BRCA2 protein was then immunoprecipitated with 20 μl of 2C9 supernatant, followed by 20 μl of goat anti-mouse IgG-agarose. For all reactions with primary and secondary antibodies (except 2C9), lysates were incubated for one hour at 4°C with end-over-end mixing; lysates were incubated with 2C9 antibody overnight at 4°C. Following immunoprecipitation, the agarose pellets were washed sequentially with 500 μl of the following solutions: 1) TSA (0.01 M Tris, pH 8, 0.14 M NaCl, 0.025% NaN_3) + 0.1% Triton X-100, 2) TSA + 0.1% Triton X-100, 3) TSA, and 4) 0.05 M Tris-Cl, pH 6.8. The pellets were then boiled in sample buffer for 5-10 minutes, the agarose was pelleted, and the supernatant was analyzed by Western Blot as described above.

Results

mAb 2C9 Specifically Recognizes BRCA2 Protein. To determine the specificity of mAb 2C9, we first performed Western Blot analysis. 2C9 was strongly reactive against 49 kDa purified BAC2 peptide, but was not reactive against the non-overlapping purified BAC1 (Figure 1). In addition, when bacterial lysates containing BAC2 peptide fused to glutathione-S-transferase (GST) were blotted, 2C9 detected a shift in molecular weight to 79 kDa, further confirming the BAC2-specificity of 2C9. 2C9 was not reactive against protein from similar bacterial lysates containing the BAC1-GST fusion protein, or from those producing GST alone (not shown). To further define the BRCA2 epitope recognized by mAb 2C9, we prepared lysates from bacterial or yeast cells transfected with various BRCA2 expression constructs (Figure 2). These studies localized the reactivity of mAb 2C9 to aa 1264-1329, corresponding to bp 4021-4215 of BRCA2 exon 11. In addition, mAb 2C9 reacted with a band consistent with the predicted 384 kDa MW of BRCA2 in Cos-7 cells transfected with full length BRCA2 cDNA (pBSX/BRCA2) by Western blot. The band is identical to that detected by polyclonal rabbit antiserum #5815, raised against non-overlapping sequences of BRCA2. Negative control mAb P3x63 and normal rabbit serum do not detect bands at this molecular weight (Figure 3).

BRCA2 is a High Molecular Weight Protein Expressed at Very Low Levels in Breast and Ovarian Carcinoma Cells. No reactivity above background was demonstrable using mAb 2C9 on frozen sections of breast carcinoma or normal breast tissue by immunohistochemistry, even when using the extremely sensitive immunoenhancing technique of catalyzed signal reporter deposition. Similarly, BRCA2 protein was not detected using 2C9 in standard Western blot assays or flow cytometry of MCF-7 breast carcinoma cells, previously shown to express BRCA2 mRNA under conditions of serum-starvation and release (Vaughn *et al.*, 1996). These studies suggested that BRCA2 protein may be present at very low levels within cells, requiring the use of antigen concentration techniques for detection. Therefore we used 2C9 to immunoprecipitate (and thus concentrate) BRCA2 protein from lysates of breast (MCF-7, T47D, BT483) and ovarian (SKOV3) carcinoma cells, followed by Western blot detection with anti-BRCA2 immunological reagents (polyclonal anti-BAC2 mouse serum or mAb 2C9). These studies revealed identical high molecular weight bands specifically immunoprecipitated from each breast or ovarian carcinoma cell line, consistent with the predicted size of 384 kDa of the BRCA2 protein (Figure 4).

BRCA2 mAb 2C9 is Reactive with Terminally Differentiated Normal Epithelial Tissues and with Squamous Cell Carcinomas. Since mAb 2C9 was non-reactive with normal breast and breast carcinoma tissues, we next screened a panel of acetone-fixed frozen tissues, emphasizing tissues such as thymus, which have high expression of BRCA2 mRNA. The tissues analyzed included skin, thymus, small and large intestine, appendix, liver, kidney, heart, skeletal muscle, tonsil, lymph node, and pancreas. The majority of these tissues are non-reactive with BRCA2 mAb 2C9, as seen for normal breast and breast cancer tissues. However, 2C9 mAb reacted strongly with terminally differentiated thymic epithelial cells present in the Hassall's bodies of normal pediatric thymus (Figure 5B), as well as with the suprabasal layers of adult epidermis (Figure 6B). Similarities in expression of a variety of antigens have previously been observed for thymic epithelial cells and epidermal keratinocytes, which is not surprising as both consist of epithelial cells continually undergoing terminal differentiation in the adult (Laster *et al.*, 1986; Patel *et al.*, 1995). Incubation of the positively staining tissue sections with the BAC2 immunogen prior to the addition of mAb 2C9 blocked 2C9's reactivity, suggesting that the reactivity of 2C9 with BRCA2 sequences is specific (data not shown). To further determine the specificity of this pattern of reactivity, we then tested monoclonal antibodies directed against different portions of the BRCA2 molecule. MAb 5F6 (raised against a portion of exon 14) also reacts with Hassall's bodies in thymus and with epidermis, although 2 other monoclonal

antibodies (3E6 and 9D3) as well as the rabbit polyclonal serum (#5814) were non-reactive (not shown). These differences in reactivity may be due to the availability of the particular epitopes against which each antibody is directed in these tissues, or to differences in Ab reactivity with native protein conformations.

The pattern of reactivity of 2C9 with differentiating epithelium suggested that BRCA2 protein may be stabilized or upregulated under conditions favoring differentiation of epithelium. Therefore, we next examined the reactivity of this antibody with a panel of moderate to well differentiated squamous cell carcinomas (SCCs) of head or neck origin. MAb 2C9 demonstrated strong reactivity with 9 of 10 formalin-fixed paraffin-embedded well differentiated SCCs (Figure 7B). 2C9 was also reactive with 1 of 4 frozen, moderately differentiated SCCs. The frozen tumor which was reactive with mAb 2C9 also reacted with mAb 5F6, directed against a different portion of the BRCA2 molecule (data not shown), suggesting that these antibodies are specifically reacting with BRCA2 in SCCs.

BRCA2 mAb 2C9 Immunoreactivity Increases in Differentiating Keratinocytes. We further confirmed mAb 2C9 reactivity with differentiating epithelium using immunoperoxidase assays on epidermal keratinocytes cultured *in situ* onto glass slides. Keratinocytes cultured in media containing either increased calcium concentrations (1.2 mM) or fetal calf serum (FCS) undergo characteristic changes associated with differentiation, including changes to squamous morphology and upregulation of molecules that are considered classical markers of differentiation (high molecular weight cytokeratins 1, 2, and 10, filaggrin, loricrin, and transglutaminase). Little 2C9 immunoreactivity is seen in epidermal keratinocytes at baseline (t=0), which is limited to a few scattered cells which are larger, more spread out (squamous), and more differentiated-appearing than cells which are non-reactive with 2C9. Increased 2C9 immunoreactivity is noted after several hours of incubation in differentiation medium, and 2C9 immunostaining remains strongest in the large, squamoid, more differentiated cells (which are much more numerous after several hours of differentiation). The staining pattern in these cells is cytoplasmic with perinuclear accentuation and nuclear dots (Figure 8).

Because the immunohistochemical reactivity of 2C9 correlates with terminal differentiation of epithelial cells, we examined BRCA2 mRNA levels in terminally differentiating keratinocytes by Northern blot analysis. BRCA2 mRNA levels do not appear to change during differentiation of keratinocytes, suggesting that the upregulation of protein in these cells is occurring at a post-transcriptional level (data not shown).

Discussion

This study demonstrates that BRCA2 mAbs react with epithelial cells undergoing terminal differentiation, including skin, cultured epidermal keratinocytes, Hassall's bodies in the thymus, and squamous carcinomas. We did not see reactivity with either normal or malignant breast epithelium, suggesting that BRCA2 protein is expressed in these tissues at levels which are undetectable in immunohistochemical analyses. The role of BRCA2 in nuclear activities, primarily DNA damage repair, has been under intensive investigation; however, the involvement of BRCA2 in differentiation is poorly understood. A significant amount of evidence supports the concept that BRCA2 is important in the process of differentiation, in addition to its role in mitogenesis. Initial *in vitro* studies demonstrated that BRCA2 mRNA expression is cell cycle regulated and decreases to negligible levels during serum starvation or at confluency, when cells are no longer proliferating (Rajan *et al.*, 1996; Vaughn *et al.*, 1996). Rajan et. al. further demonstrated that BRCA2 is upregulated in response to lactogenic hormones in post-confluent, *non*-proliferative cells during mammary epithelial cell differentiation (Rajan *et al.*, 1996). *In situ* hybridization studies in the mouse have demonstrated that BRCA2 is expressed in many tissues in which cellular compartments are undergoing rapid proliferation, including ovary, testis, lymph node, spleen,

thymus, pancreas, endometrium, stomach mucosa, intestinal crypts, epithelium in the outer root sheath of hair follicles, and mammary gland, where expression is upregulated during pregnancy, again suggesting that this protein is important in normal control of the cell cycle (Rajan *et al.*, 1997; Connor *et al.*, 1997a; Blackshear *et al.*, 1998). However, Blackshear et. al. also demonstrated BRCA2 mRNA expression in some non-proliferating cell types, including terminally differentiated neurons and more differentiated cells undergoing spermatogenesis (Blackshear *et al.*, 1998). These studies suggest some function for BRCA2 in differentiation processes, unrelated to its involvement in the cell cycle. Further evidence for the role of BRCA2 in the process of differentiation is provided by the phenotype of the mice homozygous for truncated BRCA2 which survive embryogenesis (Connor *et al.*, 1997b), in which some tissues show evidence of improper differentiation, including skin, testes, ovaries, and thymocytes.

Clearly, BRCA2 has important roles in both cellular proliferation and differentiation; however, the mechanisms involved are still unclear. It is difficult to understand how BRCA2's "proliferative" role, as seen in the non-viable or growth retarded BRCA2-deficient mice, might be related to tumorigenesis in humans. Based on the finding that levels of the cyclin-dependent kinase inhibitor $p21^{Cip1/WAF1}$ are increased in BRCA2 mutant embryos, Suzuki et. al. suggested that lack of this protein in BRCA2-deficient mice results in DNA repair defects during the highly proliferative stages of embryogenesis that then activate pathways involved in checkpoint control, culminating in cell cycle arrest (Suzuki *et al.*, 1997). Connor et. al. also found that p21, as well as p53, are overexpressed in mouse embryonic fibroblasts derived from embryos homozygous for truncated BRCA2 (Connor *et al.*, 1997b). Furthermore, BRCA2/p53 nullizygotes have a less severe phenotype than mice with BRCA2-deficiency alone (with average survival time *in utero* increased from 8.5 days to 10.5 days), suggesting that the upregulation of p53 noted in the BRCA2-null mice, or some downstream effect of this upregulation, is partly responsible for the earlier embryonic lethality in these animals (Ludwig *et al.*, 1997). A model for human tumorigenesis due to loss of BRCA2 has been proposed in which both the loss of the wild-type allele in BRCA2 mutation carriers, as well as loss of the p53 pathway, are required for tumorigenesis (Connor *et al.*, 1997b; Bertwistle and Ashworth, 1998). This model requires that a mammary epithelial cell suffer three "hits" prior to malignant transformation (two hits to knock out the p53 pathway, and one hit to knock out the wild-type BRCA2 allele.) Furthermore, it is likely that the p53 pathway would have to be eliminated first, followed by loss of the wild-type BRCA2 allele, as mutations occurring in the opposite order would probably have the same effect as is seen in BRCA2-null mice, namely growth arrest rather than uncontrolled proliferation (Bertwistle and Ashworth, 1998).

Another possibility to consider in trying to understand BRCA2's contribution to tumor suppression is its putative role in the process of differentiation. Most normal cells in an adult are non-proliferative, unless tissue damage or response to particular hormones or mitogens stimulates these cells to re-enter the cell cycle. Furthermore, most cells that are actively proliferating, aside from the pure population of stem cells, are somehow programmed to exit the cell cycle at some point and enter the differentiation pathway. Mammary epithelial cells would be included in the latter category, in that a subpopulation of these cells is hormonally stimulated to develop into differentiated structures in the breast with each menstrual cycle. Thus, unchecked proliferation in these cells may be consequent to either unresponsiveness to signals that control cell cycle activity, or alterations in a signal that would normally instruct these cells to leave the cell cycle and enter the differentiation pathway. A recent report demonstrated that p21, which has traditionally been considered an inducer of differentiation, is initially upregulated in cultured mouse keratinoctyes following a differentiation signal, but that the protein levels must quickly be downregulated in order for differentiation to proceed (Di Cunto *et al.*, 1998). These data are consistent with a potential role for BRCA2 in a negative feedback mechanism required in differentiating epithelium to eliminate upregulated p21 protein and allow differentiation to proceed normally. This may provide an explanation for the abnormal differentiation of skin and other tissues noted in the mice

expressing truncated BRCA2, in which p21 levels are elevated (Connor *et al.*, 1997b). According to this hypothesis, loss of BRCA2 resulting in p21-mediated resistance to normal differentiation processes may be a mechanism for tumorigenesis. Whether p21 is functionally involved in the BRCA2 -/- mutant phenotype (and by correlation in tumorigenesis in humans); and if so, whether the effect of its upregulated expression is due to an anti-proliferative function or to an inhibition of normal differentiation processes has yet to be elucidated.

Bertwistle et al recently identified BRCA2 protein by immunoprecipitation using multiple polyclonal antisera directed against different portions of the BRCA2 molecule, and detection with a rat monoclonal antibody raised against the C-terminus (aa#3386-3400) of BRCA2. A single high molecular weight species was detected in each case by the rat monoclonal antibody (Bertwistle *et al.*, 1997). Similarly, Chen et al described a high molecular weight species immunoprecipitated from ^{35}S-labeled T24 bladder carcinoma cells that was consistent with BRCA2, using each of two BRCA2 polyclonal antisera (Chen *et al.*, 1998). In cell lines transfected with the full length BRCA2 construct, we detect a single high molecular weight band consistent with the predicted 384 kDa using our monoclonal and polyclonal antibodies. However, we also see lower molecular weight species in addition to the high molecular weight band in immunoprecipitation-Western blot experiments from breast cancer cell lines. Smaller molecular weight species may derive from alternative splicing events, differential RNA processing, BRCA2 protein processing, protein degradation, or cross-reactivity. Alternatively spliced mRNA species have not been reported for BRCA2, and we see single bands on Northern blot analysis, even in cases in which smaller protein sizes are detected, making alternative RNA splicing a less likely explanation. However, it is possible that our antibodies detect post-translationally processed BRCA2 species which, if these species did not include the C-terminal portion of BRCA2, would not be detected by the rat monoclonal antibody used in the Bertwistle report.

In both the Bertwistle and Chen reports, these investigators localized BRCA2 protein to the nucleus of carcinoma cells by differential purification of subcellular fractions (Bertwistle *et al.*, 1997; Chen *et al.*, 1998) This protein's interaction with RAD51 and its putative role in double stranded DNA repair are consistent with its localization to the nucleus. However, our experiments have consistently demonstrated BRCA2 mAb 2C9 reactivity in the cytoplasm of differentiating cells, in a perinuclear pattern with nuclear dots. Our results may not be incompatible with studies demonstrating nuclear localization of BRCA2 in carcinoma cells, as this molecule may have different functions, and thus different localization in different cell types or stages of development. Its function as a molecule involved in the process of differentiation may require its presence in the cytoplasm, whereas its function as a DNA repair molecule, or molecule otherwise involved in cell cycle processes, may require its presence in the nucleus. As BRCA1 and BRCA2 appear to be coordinately regulated, and mRNA for both genes appears to have a very similar distribution, it is likely that the protein expression pattern is also similar. A similar ongoing debate over the localization of the BRCA1 protein remains unresolved, with reports of nuclear staining as well as differential trafficking with localization to the cytoplasm in breast cancer cells (reviewed in (Bertwistle and Ashworth, 1998). A more recent report suggested that the "nuclear dot" staining pattern of BRCA1 mAbs was actually due to reactivity located in tube-like channels extending into the nucleus, likely originating from the perinuclear endoplasmic reticulum-Golgi complex (Coene *et al.*, 1997). Of note, the authors report that the nuclear tubes are seen in a fraction of the cell population, that they form a much more extensive branching network in cancer cells compared with non-malignant cells, and that the nuclear invaginations are often seen in close proximity to nucleoli, suggesting a possible involvement in the cell cycle. Coene et. al.'s description of a perinuclear staining pattern with nuclear dots for BRCA1, with distinct visualization of either nuclear staining or cytoplasmic staining being dependent on the fixation method, is consistent with the immunohistochemical staining pattern we observe with mAbs against BRCA2. The subcellular localization of BRCA2 in different cell types deserves further investigation.

In our studies, 2C9 clearly reacts immunohistochemically with a cytoplasmic protein in epidermis, as well as in cultured keratinocytes. The possibility that the observed 2C9 reactivity in these cells is entirely non-specific, due to cross reactivity with another protein present in skin, thymus, and differentiating keratinocytes, is very unlikely, as mAb 5F6, which is directed against an entirely separate part of the BRCA2 molecule, reacts with these same cells by immunohistochemistry. In addition, 2C9, 5F6, and 3E6 react with identical high molecular weight bands on Western blot from skin extracts, and 2C9 and polyclonal anti-BRCA2 rabbit serum #5815 react with identical bands from cultured keratinocytes undergoing differentiation (data not shown). Although BLAST searching revealed no significant homology to other differentiation-associated proteins at the nucleic acid level, the 65 aa portion of BRCA2 to which the 2C9 epitope has been narrowed demonstrated limited amino acid homology with the cytokeratin 2 (CK2) molecule (45% identity, with 76% similarity; longest stretch of identical aa = 3) over a 22 amino acid stretch (aa #1305-1327). However, our data clearly demonstrates lack of 2C9 cross reactivity with cytokeratins in Western blots, as well as a different immunohistochemical staining pattern compared with that of mAbs recognizing CK2 (Figure 6). Furthermore, CK2 is not found in foreskin epithelium, squamous carcinomas, or their derivative cell lines (Collin *et al.*, 1992), and cytokeratin staining patterns are exclusively cytoplasmic, with no nuclear reactivity. Thus, while it is impossible to totally exclude immunohistochemical cross-reactivity of 2C9 with CK2 in tissue sections, amassed evidence suggests that 2C9 is specifically detecting BRCA2 present in the cytoplasm of differentiating epithelial cells.

In conclusion, direct study of BRCA2 distribution and function within cells has been limited by the lack of appropriate reagents, as well as the fact that the BRCA2 protein is very large and appears to be expressed at very low levels in most tissues. Utilizing monoclonal antibody 2C9 and polyclonal antibody #5815, developed in our lab, as well as other monoclonal antibodies raised against BRCA2 sequences to confirm specificity, we have demonstrated a potential role for BRCA2 in cellular differentiation of keratinoctyes and thymic epithelium. Our data supports research showing that BRCA2 mRNA is upregulated in differentiating cells (Rajan *et al.*, 1996) and extends these findings to the level of BRCA2 protein expression. Furthermore, our findings are consistent with data which demonstrates that lack of BRCA2 results in abnormalities in differentiating tissues (Connor *et al.*, 1997b). Whether BRCA2 actually plays a role in the process of differentiation in these tissues, or whether this protein's presence in differentiating tissues is simply a result of accumulation due to extensive crosslinking, enhanced stability, or some other factor, has yet to be elucidated. However, BRCA2 likely plays multiple roles in the diverse processes of cellular proliferation, differentiation, and monitoring of the integrity of DNA.

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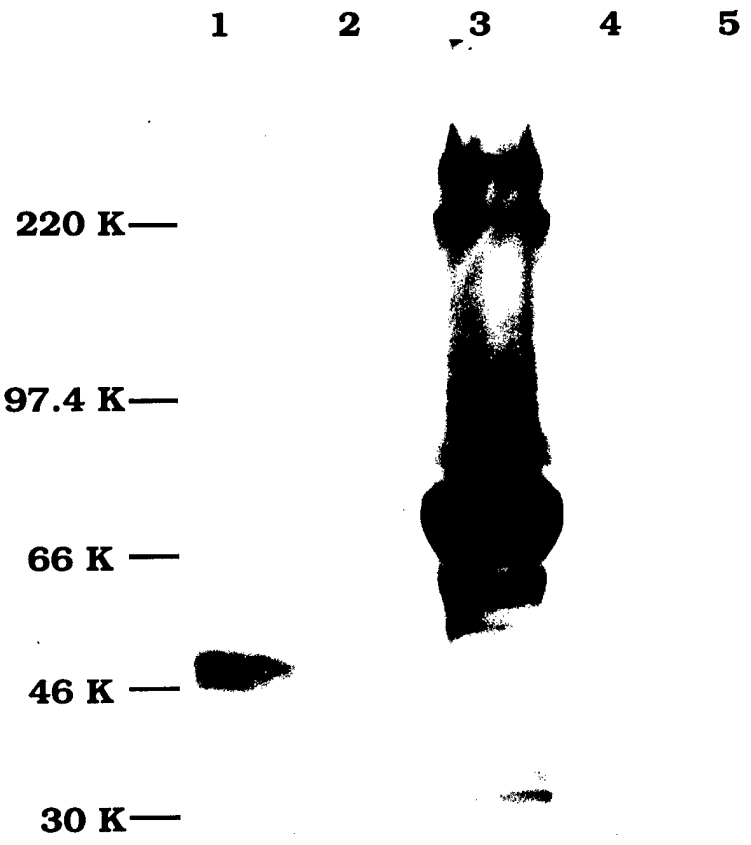

Figure 1: Mab 2C9 reacts with immunizing peptide BAC2 and its fusion protein BAC2-GST. Lysates were prepared from uninduced (lane 2) and induced (lane 3) bacteria producing the BAC2-GST fusion protein (BRCA2 bp 3748-4816) as well as from uninduced (lane 4) and induced (lane 5) bacteria producing the BAC1-GST fusion protein (BRCA2 bp 2716-3714). The lysates and GST-cleaved and purified BAC2 protein were separated on a 7.5% polyacrylamide gel. 2C9 was reactive with the purified BAC2 protein (lane 1) at the expected MW of 49 kDa as well as with the 79 kDa BAC2-GST fusion protein (lanes 2, 3). 2C9 was non-reactive with all components of the lysates from BAC1-GST producing bacteria (lanes 4, 5).

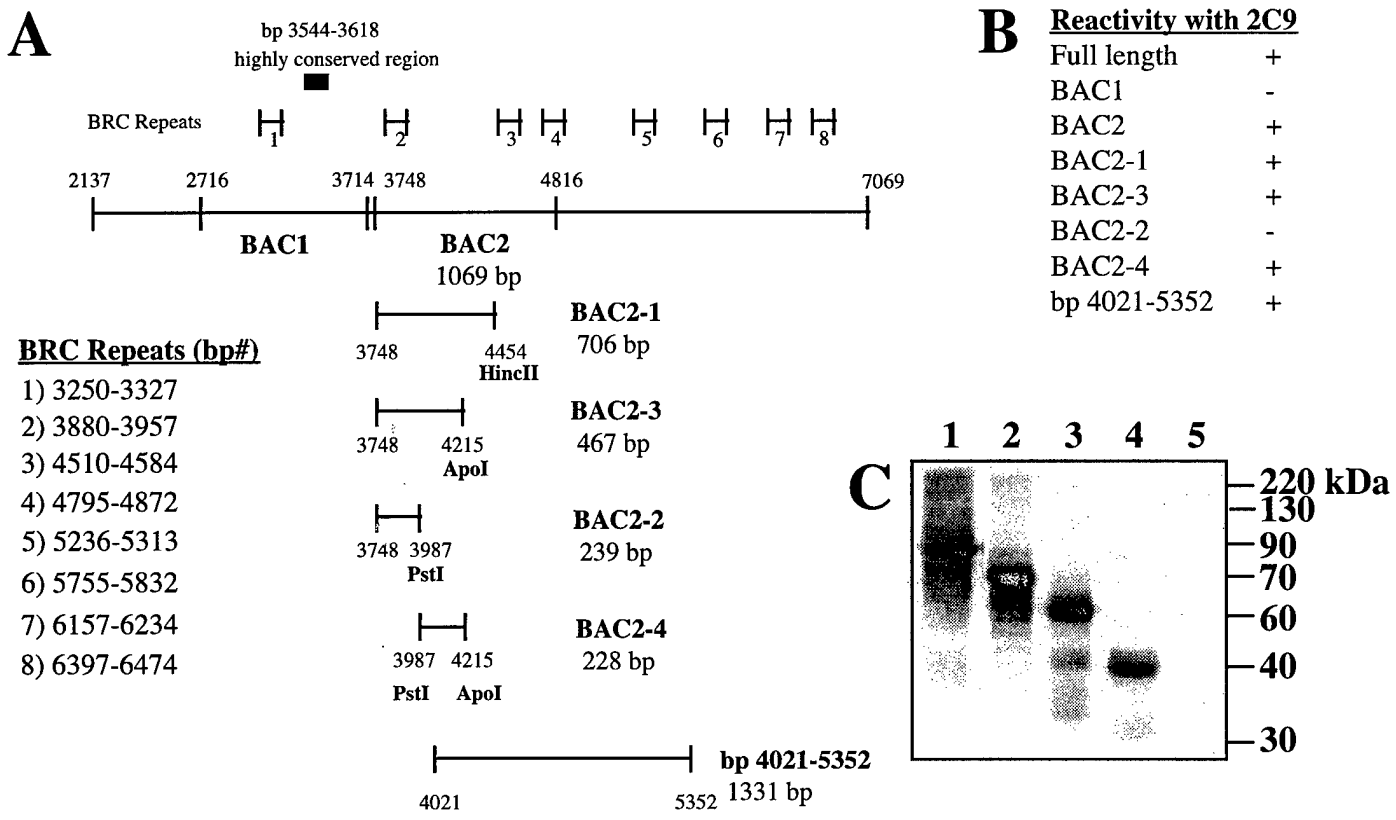

Figure 2: Reactivity of mAb 2C9 with BRCA2 Exon 11 Constructs.
A) The BRCA2 peptides studied are shown on this diagram of exon 11, along with structural features of this region of the BRCA2 gene, including BRC-repeats #1-8 (Bork *et al.*, 1996) and a region which is highly conserved among different species (Bignell *et al.*, 1997). C) Induced lysates from bacteria or yeast transformed with the indicated BRCA2 sequences were analyzed on 10% SDS-PAGE gels, transferred to nitrocellulose, and reacted with 2C9 in a Western blot assay. 2C9 was reactive with bacterial proteins containing the immunizing peptide BAC2, bp 3748-4816 (lane 1); deletion constructs BAC2-1, bp 3748-4454 (lane 2); BAC2-3, bp 3748-4215 (lane 3); BAC2-4, bp 3987-4215 (lane 4); and yeast-derived BRCA2 sequences, bp 4021-5352 (not shown); but not with deletion construct BAC2-2, bp 3748-3987 (lane 5) or BAC1, bp 2716-3714 (Figure 1), derived from a different portion of BRCA2 exon 11. These studies define the 2C9 epitope to be contained within BRCA2 aa 1264-1329, encoded by bp 4021-4215. Reactivity of BRCA2 deletion constructs with 2C9 is summarized in panel B.

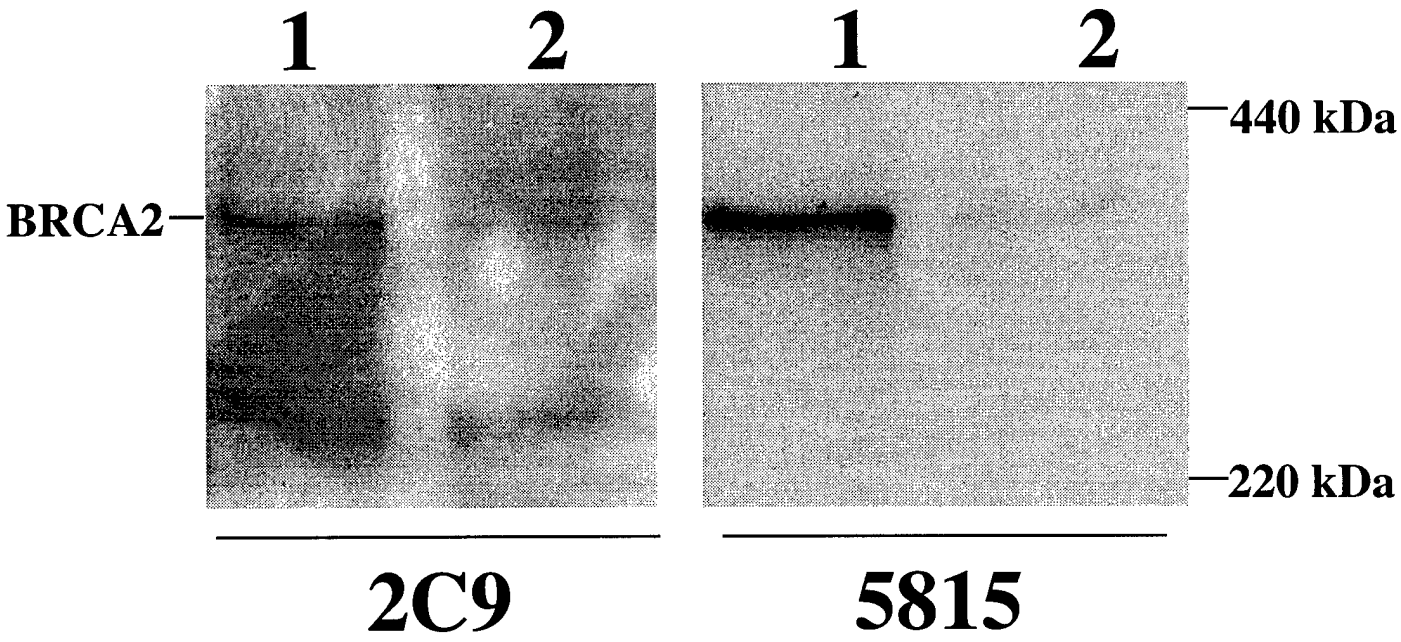

Figure 3: BRCA2 antibodies recognize full length BRCA2 in Cos-7 cells. Lysates from Cos-7 cells transfected with pBSX/BRCA2 (lane 1) or mock transfected (lane 2) were reacted with mAb 2C9 (left panel) or polyclonal rabbit serum #5815 (right panel) in Western blot assays. Identical bands consistent with the 384 kDa predicted molecular weight of BRCA2 were detected by both 2C9 and #5815. Extremely faint bands at the same molecular weight are also seen in lysates from mock transfected Cos-7 cells. As African green monkey BRCA2 is 94% identical (61 of 65 aa) with human BRCA2 in the 2C9 epitope region (aa 1264 to 1329) and 90% identical (304 of 338 aa) with human BRCA2 in the BAC1 region (aa 829 to 1162), this most likely represents detection of endogenous Cos BRCA2. No bands at this molecular weight were detected using control mAb P3x63 or normal rabbit serum.

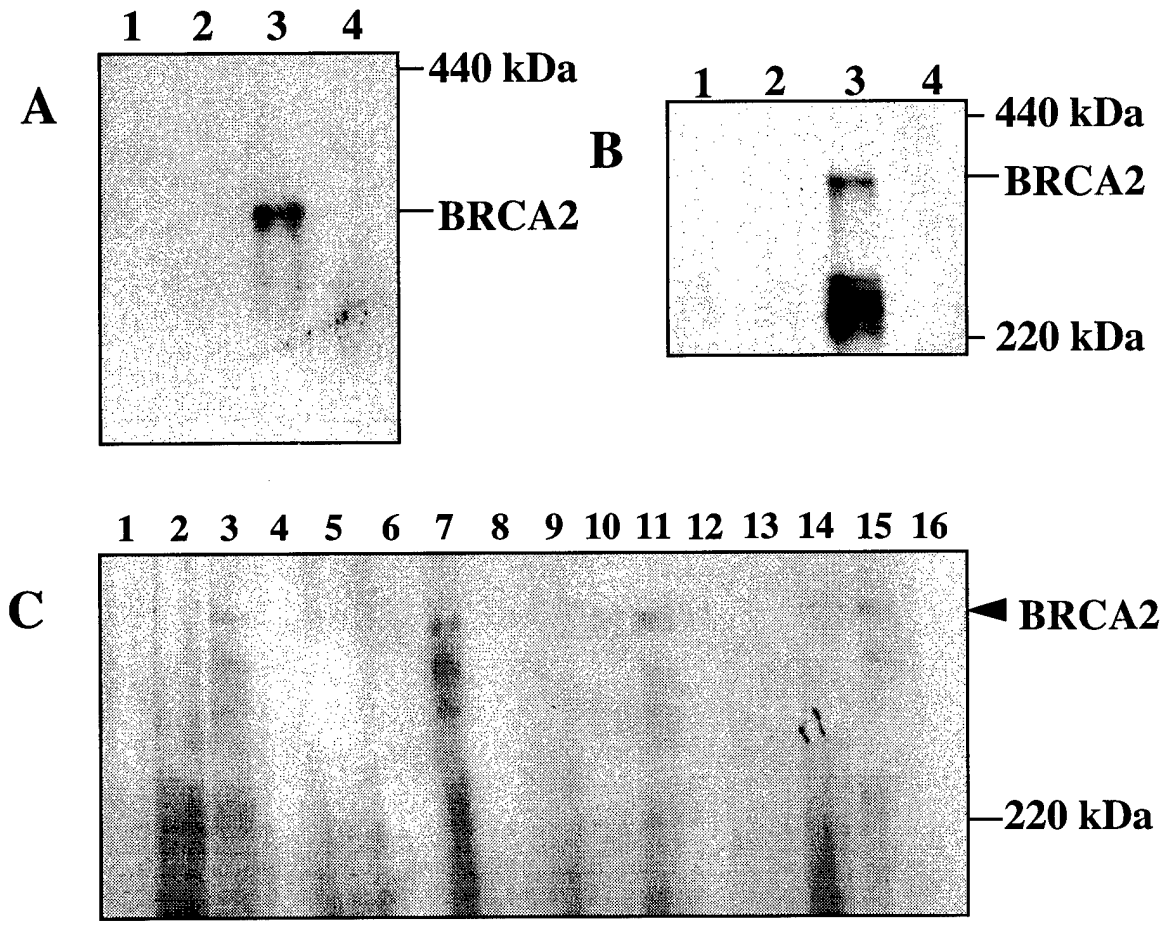

Figure 4: MAb 2C9 immunoprecipitates a high molecular weight protein consistent with BRCA2 from breast and ovarian carcinoma cells. MCF-7 cells (panels A and B) were serum starved for 3 days, then released from starvation for 1 day, followed by lysis and protein extraction. The lysate from 17 x 10^6 cells (panel A) or 56 x 10^6 cells (panel B) was immunoprecipitated with mAb 2C9 as described. Immunoprecipitates were separated on a 4% SDS polyacrylamide gel, and Western blot analysis was performed. Bands were detected with either polyclonal anti-BAC2 serum (panel A) or with 2C9 (panel B). In panel C, protein lysates from 61 x 10^6 T47D cells (lanes 1-4), 54 x 10^6 BT483 cells (lanes 9-12), 52 x 10^6 MCF-7 cells (lanes 13-16), and 60 x 10^6 SKOV3 cells (lanes 5-8) were immunoprecipitated with mAb 2C9, followed by separation on a 5% non-denaturing polyacrylamide gel, Western blot analysis, and detection with 2C9. A band consistent with the 384 kDa predicted MW of BRCA2 protein is specifically immunoprecipitated by 2C9 in all cases (lanes 3, 7, 11, 15). No specific reactivity is seen with proteins immunoprecipitated by negative control antibody (preclear #1-lanes 1, 5, 9, 13; preclear #2-lanes 2, 6, 10, 14), or with proteins remaining in the lysate following 2C9 immunoprecipitation (lanes 4, 8, 12, 16). In panels B and C, lower molecular weight bands likely correspond to products of mRNA splice variants, differentially processed protein, or protein degradation products. In panel A, the 220 kDa molecular weight marker was run off the gel; lower molecular weight bands may have been run off as well.

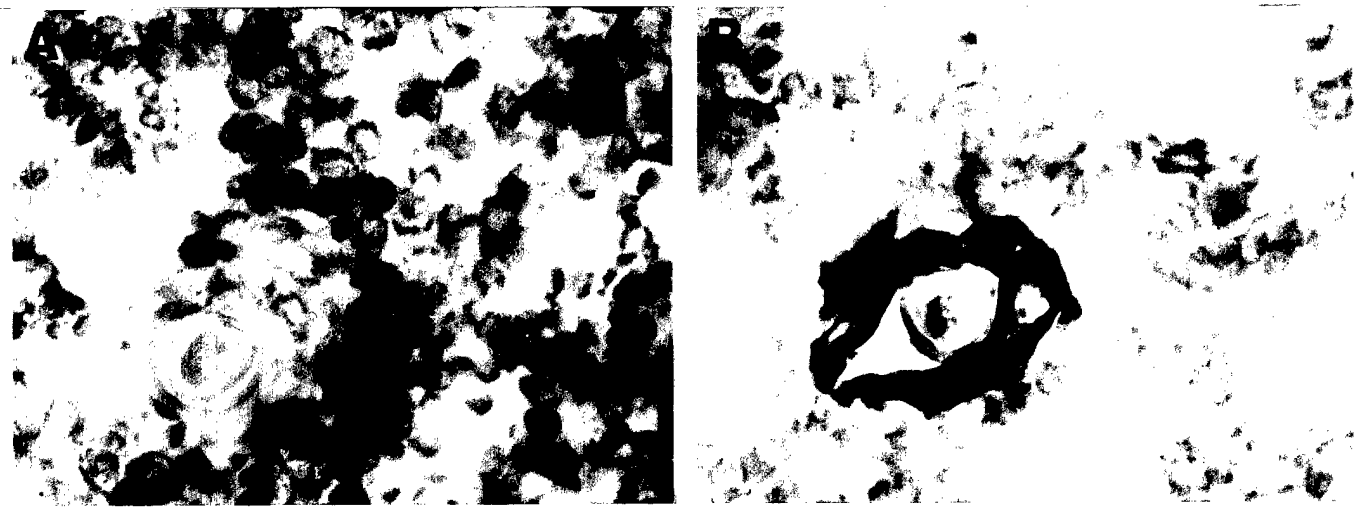

Figure 5: BRCA2 mAb 2C9 is reactive with terminally differentiated epithelial cells in thymus. In a comprehensive immunohistochemical screen of frozen acetone-fixed tissue sections to identify tissues with potentially higher levels of BRCA2 protein, we found strong cytoplasmic reactivity with 2C9 mAb in the terminally differentiating thymic epithelial cells present in the Hassall's bodies of normal pediatric thymus (panel B). Panel A shows reactivity with isotype-matched control mAb, P3X63. Immunoperoxidase method, DAB substrate, hematoxylin counterstain.

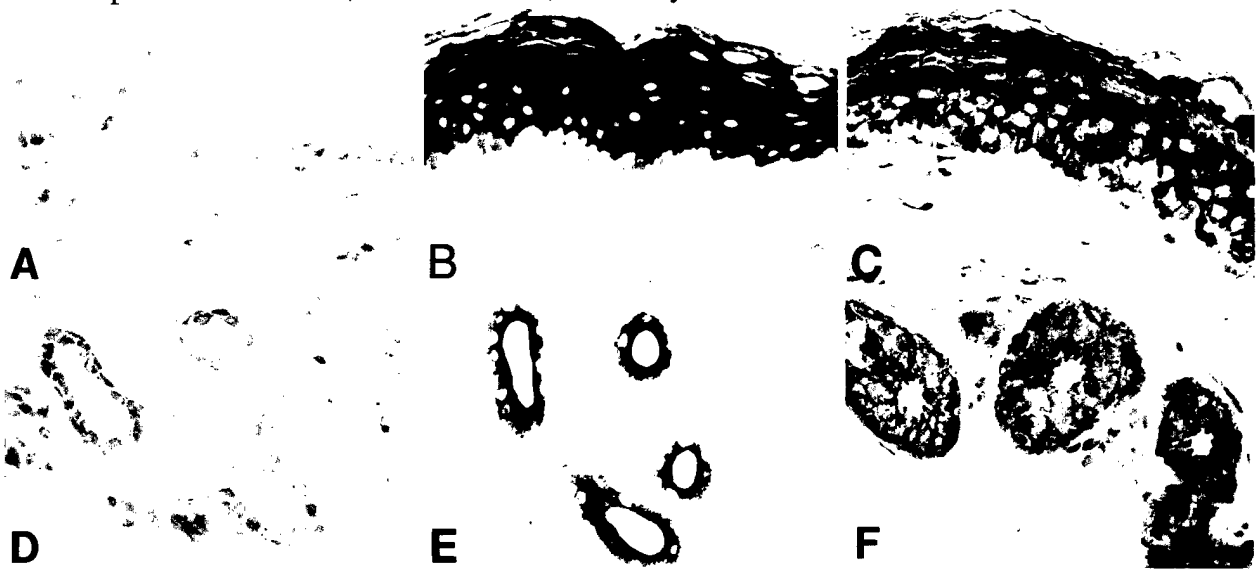

Figure 6: BRCA2 mAb 2C9 is reactive with terminally differentiated cells in adult human skin. MAb 2C9 reacts with the suprabasal layers of adult epidermis (panel B) and with eccrine ducts in the dermis (panel E). Panels A and D show corresponding negative reactivity with isotype-matched control mAb P3X63. For comparison, the pattern of reactivity with mAb AE-2 (panels C, F), specific for the 65-67 kDa and 56.5 kDa cytokeratins (CK 1, 2, and 10) characteristic of terminally differentiated epithelial cells (Moll 1982, Woodcock-Mitchell 1982), differs from that seen with mAb 2C9. MAb 2C9 reacts with eccrine ducts but not with the secretory portion of the eccrine coil (panel E), while AE-2 demonstrates significant reactivity with all portions of the eccrine gland (panel F). In addition, while 2C9 shows uniform reactivity with suprabasal layers of the epidermis (panel B), AE-2 shows granular staining, with increasing reactivity with distance above the basal epidermal layer (panel C). Immunoperoxidase method, DAB substrate, hematoxylin counterstain.

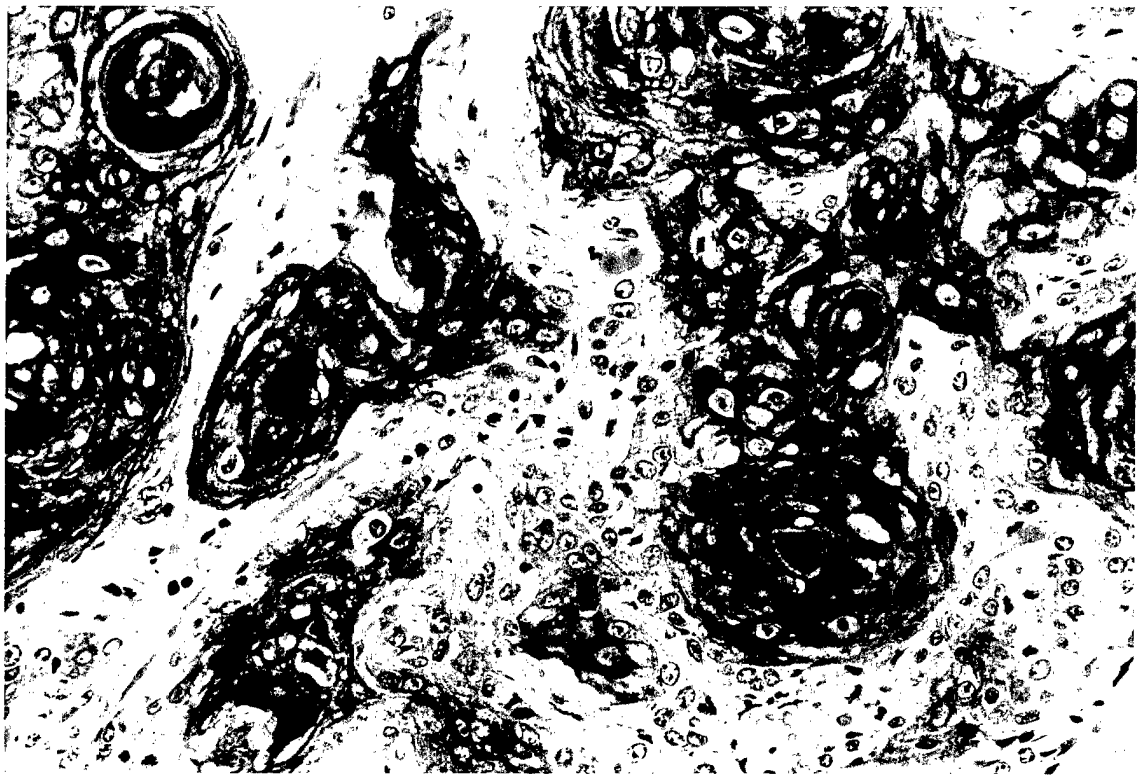

Figure 7: BRCA2 mAb 2C9 reacts with squamous cell carcinomas. MAb 2C9 demonstrated strong reactivity with 9/10 of well differentiated squamous cell carcinomas (SCCs) of head or neck origin. No reactivity was seen with isotype-matched negative control mAb P3X63 (not shown). Immunoperoxidase method, DAB substrate, hematoxylin counterstain.

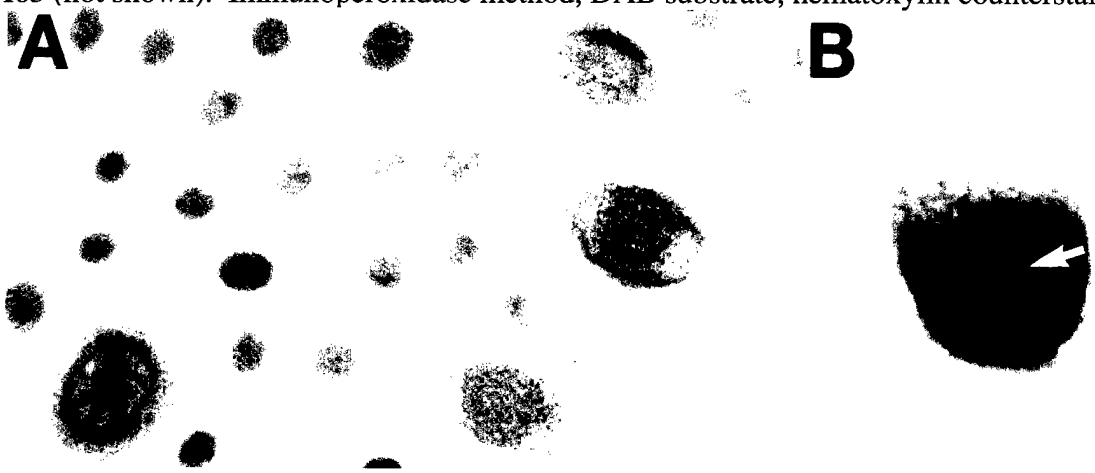

Figure 8: BRCA2 mAb 2C9 reactivity in cultured epidermal keratinocytes. 2C9 reactivity was localized using immunoperoxidase assays on epidermal keratinocytes derived from neonatal foreskins and cultured *in situ* onto glass slides. 2C9 immunoreactivity was cytoplasmic and in many cells, appeared to be perinuclear, with a thin rim of unstained cytoplasm present between the nucleus and the greatest intensity of staining. Nuclear dots were notable at high magnification (panel B, arrow). Reactivity was strongest on larger, more squamous cells. Similar results were obtained using immunofluorescence staining. The overall staining intensity of cultured keratinocytes is much lower than that seen in tissue sections of epidermis, suggesting that factors which up-regulate expression of BRCA2 protein *in vivo* are not present in our keratinocyte cultures. Immunoperoxidase method, DAB substrate, hematoxylin counterstain.