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***Proceedings***

# Basic and Clinical Aspects of Breast Cancer

**A Special Conference of the**
**American Association for Cancer Research**

**Supported by a Generous Grant from the**
**National Institute of Environmental Health Sciences**
**(N.I.E.H.S.)**

*The Keystone Resort*
*Keystone, Colorado*
*March 7-12, 1997*

*Conference Co-Chairpersons*

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Research Triangle Park, NC

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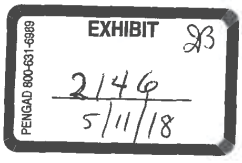




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# Contents

# Conference Sponsors

*This Conference is supported by a generous grant from the National Institute of Environmental Health Sciences. In addition, the American Association for Cancer Research wishes to express its gratitude to the following corporations for their generous support of this conference.*

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# BASIC AND CLINICAL ASPECTS OF BREAST CANCER
# KEYSTONE CONFERENCE CENTER
# KEYSTONE, COLORADO
# MARCH 7-12, 1997

## Co-Chairpersons


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## Scientific Program and Tentative Conference Schedule

**FRIDAY / MARCH 7**

4:00 – 9:30 pm **Registration**
7:00 – 9:00 pm **Keynote Talks**

**Chairperson: J. Carl Barrett, National Institute of Environmental Health Sciences, Research Triangle Park, NC**

**MARY-CLAIRE KING,** University of Washington, Seattle, WA *Topic to be Announced*
**KAREN S. H. ANTMAN,** Columbia University, New York, NY *"Breast Cancer, a Major Health Problem, Controversies and Public Policy."*

9:00 – 10:30 pm **Mixer**

**SATURDAY / MARCH 8**

8:00 – 11:00 am **Session 1: Basic Biology of the Breast**

**Chairperson: Jean M. Gudas, Amgen, Inc., Thousand Oaks, CA**

**JOSÉ RUSSO,** Fox Chase Cancer Center, Philadelphia, PA *"Cellular Basis of the Breast Susceptibility to Carcinogenesis."*
**MARC E. LIPPMAN,** Georgetown University Medical Center, Washington, DC *"EGF Superfamily in the Progression of Human Breast Cancer."*
**C. KENT OSBORNE,** UT Health Science Center, San Antonio, TX *"Molecular Markers as Prognostic and Predictive Factors in Breast Cancer."*
**CHARLES W. DANIEL,** University of California, Santa Cruz, CA *"Homeobox Genes in the Breast."*

4:00 – 6:00 pm **Poster Session A**

8:00 – 10:30 pm **Session 2: Genetic Predisposition to Breast Cancer**

**Chairperson: Donald M. Black, Beatson Institute for Cancer Research, Glasgow, Scotland**

**P. ANDREW FUTREAL,** Duke University Medical Center, Durham, NC *"The Genetics of Familial Breast Cancer: Role of BRCA1, BRCA2, and Evidence for Additional Susceptibility Loci."*
**SIMON A. GAYTHER,** CRC Human Cancer Genetics Research Group, Cambridge, England *"Variation of Risks of Breast and Ovarian Cancer Associated with Different Germline Mutations of the BRCA1 and BRCA2 Genes."*
**EVAN R. SIMPSON,** UT Southwestern Medical Center, Dallas, TX *"Mesenchymal-Epithelial Interactions and Breast Cancer: Role of Locally-produced Estrogens."*

**SUNDAY / MARCH 9**

8:00 – 11:00 am **Session 3: Molecular and Cellular Aspects of Breast Cancer**

**Chairperson: Stephen P. Ethier, University of Michigan Medical School, Ann Arbor, MI**

**JERRY W. SHAY,** UT Southwestern Medical Center, Dallas, TX *"The Role of p53 and Telomerase in Human Breast Cancer."*
**MINA J. BISSELL,** Lawrence Berkeley National Laboratory, Berkeley, CA *"Epithelial Biology and Breast Cancer: The Role of Integrins and Cyto-skeletal Signal Transduction in Genesis of Normalcy and Malignancy."*
**MARTHA R. STAMPFER,** Lawrence Berkeley National Laboratory, Berkeley, CA *"Gradual Phenotypic Conversion during Immortalization of Cultured Human Mammary Epithelial Cells."*
**JOYCE M. SLINGERLAND,** Sunnybrook Health Science Centre, Toronto, Ontario, Canada *"Altered Regulation of the Cell Cycle Inhibitor $p27^{Kip1}$ and $p15^{INK4B}$ in a TGF-β Resistant Human Mammary Epithelial Cell Line."*

4:00 – 6:00 pm **Poster Session B**

8:00 – 10:30 pm **Session 4: Mechanisms of Hormone Action**

**Chairperson to be Announced**

**V. CRAIG JORDAN,** Northwestern University Medical School, Chicago, IL *"The Regulation of Transforming Growth Factor (TGF) α."*
**MYLES A. BROWN,** Dana-Farber Cancer Institute, Boston, MA *"Role of Coactivators in Estrogen Receptor Function."*
**KENNETH S. KORACH,** National Institute of Environmental Health Sciences, Research Triangle Park, NC *"Influence of Estrogen Receptor Gene Knock-out on Mammary Gland Development and Tumorigenicity."*

**MONDAY / MARCH 10**

8:00 – 11:00 am **Session 5: BRCA1 and BRCA2 Function/Biochemistry**

**Chairperson: Cynthia Afshari, National Institute of Environmental Health Sciences, Research Triangle Park, NC**

**ROY A. JENSEN,** Vanderbilt University Medical Center, Nashville, TN *"Mechanisms of Tumor Suppression by BRCA1."*
**WEN-HWA LEE,** UT Health Science Center, San Antonio, TX *"Biological Function of BRCA Gene Products."*
**DAVID M. LIVINGSTON,** Dana-Farber Cancer Institute, Boston, MA *"Functional Analysis of BRCA1."*
**FRANK J. CALZONE,** Amgen Inc., Thousand Oaks, CA *"Differential Subcellular Localization, Expression, and Biological Activity of BRCA1 and the Splice Variant BRCA1-Δ11b."*

8:00 – 10:30 pm **Session 6: Experimental Models of Breast Cancer**

**Chairperson: Lewis A. Chodosh, University of Pennsylvania School of Medicine, Philadelphia, PA**

**TAK W. MAK,** Ontario Cancer Institute, Toronto, Ontario, Canada *"BRCA1 and BRCA2 Mutant Mice."*
**MICHAEL N. GOULD,** University of Wisconsin, Madison, WI *"Genetics of Mammary Cancer Susceptibility in the Rat: Potential Relevance to the Prevention and Genetics of Human Breast Cancer."*
**ROGER W. WISEMAN,** National Institute of Environmental Health Sciences, Research Triangle Park, NC *"Mouse Models for Breast Cancer Susceptibility."*

**TUESDAY / MARCH 11**

8:00 – 11:00 am **Session 7: Epidemiology of Breast Cancer**

**Chairperson: Kenneth Olden, National Institute of Environmental Health Sciences, Research Triangle Park, NC**

**WALTER C. WILLETT,** Harvard School of Public Health, Boston, MA *"Diet, Nutrition, and Breast Cancer."*
**MAUREEN HENDERSON,** Fred Hutchinson Cancer Research Center, Seattle, WA *"Prevention of Breast Cancer by Dietary Modification: The Women's Health Initiative (WHI) and Its Rationale."*
**MALCOLM C. PIKE,** University of Southern California, Los Angeles, CA *"Chemoprevention by Reducing Sex-Steroid Exposure, Dose-Response Relations and Control of Endometrial Hyperplasia."*
**MARY S. WOLFF,** Mount Sinai School of Medicine, New York, NY *"Environment and Breast Cancer."*

4:30 - 6:30 pm **Session 8: Clinical Aspects of Breast Cancer**

**Chairperson: Karen S. H. Antman, Columbia University, New York, NY**

**JUDY E. GARBER,** Dana Farber Cancer Institute, Boston, MA *"Issues in Testing for Hereditary Breast Cancer Susceptibility."*
**JEFFREY T. HOLT,** Vanderbilt University Cancer Center, Nashville, TN *"Retroviral Vector Therapies for Breast and Ovarian Cancer."*
**M. JOHN KENNEDY,** The Johns Hopkins Oncology Center, Baltimore, MD *"What Is the Future of Breast Cancer Chemotherapy?"*

7:00 - 11:00 p.m. **Reception and Banquet**

**WEDNESDAY / MARCH 12**

**DEPARTURE**

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# List of Travel Grant Awardees

## YOUNG INVESTIGATOR TRAVEL GRANTS

Nine presenters of meritorious abstracts have been selected by the Program Committee to receive travel awards to attend this conference. Predoctoral students, postdoctoral fellows, and physicians-in-training were eligible for consideration. The names of these persons, their affiliations, and the numbers and titles of their abstracts are given below.

**Mark S. Chapman**, The Salk Institute, San Diego, CA, Poster A-18:
"Transcriptional Activation by Wildtype but Not Mutant BRCA1 Sequences."

***Robert W. Fisher**, University of North Carolina, Chapel Hill, NC, Poster A-27:
"Modulation of Apoptosis in Human Mammary Epithelial Cells by Prostaglandin G/H Synthase 2."

**Dorota M. Gertig**, Harvard School of Public Health, Boston, MA, Poster A-30:
"A Prospective Study of Acetylation Genotype, Meat Intake and Risk of Breast Cancer."

**Bettina Hanstein**, Dana Farber Cancer Institute, Boston, MA, Poster A-34:
"Characterization and Cloning of an Estrogen Receptor-β-Splice Variant."

**Limin Li**, Stanford University School of Medicine, Stanford, CA, Poster A-48:
"Identification of a New Tumor Suppressor: The TSG101 Gene and Human Breast Cancer."

***Karen L. Mitro**, University of North Carolina, Chapel Hill, NC, Poster B-4:
"An Abrogated p53-Mediated Response to Ionizing Radiation in Human Mammary Epithelial Cells."

**Manuel L. Penichet**, University of California, Los Angeles, CA, Poster B-13:
"Establishment of Her2/neu Expressing Tumor Models in Immunocompetent Mice."

**Monja L. Proctor**, UT Southwestern Medical Center, Dallas, TX, Poster B-17:
"Analysis of a Gene Encoding a BRCA1 Associated Protein in Breast and Lung Cancer Cell Lines."

***Steinunn Thorlacius**, Icelandic Cancer Society, Reykjavik, Iceland, Poster B-39:
"Varied Penetrance and Expression of a Founder BRCA2 Mutation in a Well-Defined Population."

***Travel awards sponsored by a generous grant from AFLAC**

# Basic and Clinical Aspects of Breast Cancer

## *Abstracts of Oral Presentations*

## Breast Cancer, a Major Public Health Problem, Controversies and Public Policy

Karen H. Antman, M.D., Director, Herbert Irving Comprehensive Cancer Center at Columbia University

Breast cancer is common in industrialized nations, currently developing in one of eight American women. While less common in Asia, the incidence there also is increasing. In addition to the carriers of mutated Brca 1 and 2, ras, Li Fraumeni (P53) and possibly the Cowden's disease and ATM genes, risk factors associated with a 3 to 4 fold increased relative risk include early menarche or late menopause, alcohol consumption, radiation exposure especially in puberty, nuligravida or age >30 at first pregnancy, and prior breast cancer. Prognostic factors in some but not other studies include DES during pregnancy, the use of oral contraceptives by young women prior to first pregnancy, high consumption of calories or animal fat, weight gain after age 18 and low vitamin A levels.

**Diagnosis and Prognosis:** Current recommendations for screening asymptomatic women with no risk factors include q1-3 year mammography for women aged 50 to 70 years. Controversies include mammography for women 40-50, older than 70, optimal interval between mammograms, and role of mammography or MRI in younger high risk women. All suspicious masses should be biopsies. ~20% of patients with cancer will have a normal mammogram. The current percent of positive biopsies is 50% in Europe and 20% in the US, producing major differences in cost per year of life saved.

**Local therapy:** For resectable disease, the various local therapies produce differences in local control rates but do not affect survival. (1-3). Locally advanced cancers, inoperable at diagnosis, are treated with chemotherapy and radiation or mastectomy (4-7) In 2 randomized trials, survival and local recurrence were not statistically different for surgery or radiotherapy after chemotherapy.(8, 9) Radiation therapy appears most effective when administered at a relatively high dose (6000R) (10).Primary chemotherapy for 157 women with tumors of 3 cm or more allowed a breast serving procedure to be performed for 88% with an acceptable local failure rate. (11) Therefore, lumpectomy and radiotherapy, mastectomy, or for larger tumors a combination of chemotherapy and a local modality provide adequate local control. As local control rates do not affect survival women may chose the type of local therapy, in consultation with their physicians.

**Adjuvant Chemotherapy:** While most women can be rendered disease-free by mastectomy or with lumpectomy and radiation, relapse rates at 10 years increase proportionally to the number of axillary lymph nodes, from approximately 20% for patients with no positive lymph nodes, 60% for 1-3 involved lymph nodes and more than 85% for 4 or more axillary lymph nodes. Women with stage two disease with more than 10 positive lymph nodes, and locally advanced or inflammatory breast cancer have a very poor prognosis with conventional dose therapy. Relapse tends to occur earlier for patients with higher numbers of lymph nodes involved, and some risk of relapse remains for at least 20 years after mastectomy. Prognosis is worse for patients with poorly differentiated tumors, high nuclear grade, greater than a diploid number of chromosomes, high fraction of cells synthesizing DNA (S-phase fraction) and those with extra copies of Her 2/ neu oncogene.

**Metastatic breast cancer:** Women with metastatic breast cancer are essentially incurable with standard therapy with a median survival of about 2 years after documentation of metastases.(12, 13) The best prognosis using a MD Anderson data base reports a 17% rate of complete response with 18% of the Crs without progression at 5 years (3%) and 1.9% ultimately disease free (for a population selected by referral to a tertiary care center). (14) The median survival of women with metastatic disease has not changed in the 5 decades for which statistics are available. Women with estrogen receptor positive tumors (median 2.3 year survival), and those who achieve a complete response with standard dose therapy (median 2.5 years) or who have only small amounts of local disease (median > 4 years) have a

somewhat better survival.(13) Metastatic breast cancer therefore represents a major public health problem, as well as a frightening personal situation for women afflicted with the disease.

*Patient Care Issues:* What treatment goals should the physician caring for the patient with metastatic breast cancer strive for? A high clinical response rates? Prolongation of survival? Palliation of symptoms? Palliation is the accepted goal since affecting median survival has proven elusive, although a small percentage of patients who achieve CR live long-term.

*Clinical research issues:* Should research focus on therapeutic ratio (maximizing response; minimizing toxicity), view metastatic breast cancer as a model for regimens that might prove useful in an adjuvant (potentially curative) or cure of some subset of patients with metastatic disease? Each of these approaches results in a different, mutually exclusive, research strategy. Appropriate research focuses include new agents or new classes of agents, combinations incorporating new agents, higher doses of effective agents with strategies to ameliorate toxicity. From the experience gained so far, several high dose regimens designed for breast cancer yield both a high complete response rate and durable remissions in patients with metastatic disease responding to standard dose chemotherapy. Transplant early in the course of the illness, after a good response to standard dose therapy yields a complete response rate substantially higher than the 10 to 20% reported with standard dose therapy. With follow-up intervals of up to 8 years from the time of transplant, these unmaintained responses appear durable.

Current Status: Mortality from breast cancer peaked about 1990 and has fallen 3-6% between 1990 to 1995 chiefly for the premenopausal age cohort. This may result from changes in lifestyle and habits, better screening, or better treatment. However, incidence has remained stable for premenopausal breast cancer (while continuing to increase for older women), and the effect of screening on survival in this age group is controversial. In Canadian populations based studies, mortality for premenopausal breast cancer decreased in the interval after adjuvant chemotherapy was proven effective in large clinical trials and became the standard of care. Thus I believe the decreasing mortality derives from earlier diagnosis (because of higher physician and patient awareness and possibly mammographic screening) as well as the introduction of effective adjuvant therapy based on large clinical trials.

Collaborations among researchers in clinical, laboratory and populations has allowed identification of patients at substantial familial risk. Subsequent identification of moderate risk genes, and further study of their interaction, together and with environmental carcinogens and endogenous and exogenous hormonal exposures will allow us to more precisely define levels of risk, and thus appropriate screening and preventing interventions. Even relatively expensive but targeted screening and prevention strategies in this population could prove highly cost effective.

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## CELLULAR BASIS OF THE BREAST SUSCEPTIBILTY TO CARCINOGENESIS

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Breast cancer has become the most frequent malignancy diagnosed in American women, and the number one cause of cancer-related death in non-smokers. The cure of this disease remains in the realms of early detection and surgery, and its prevention depends upon our ability to understand its biology. This possibility, however, is hindered by deficiencies in our knowledge of the factors that influence the initiation and progression of breast cancer, namely: 1) lack of identification of a specific etiologic/causal agent(s); 2) lack of understanding of the mechanism(s) responsible for its initiation; 3) lack of identification of the precise time and site of initiation of the neoplastic process, and 4) lack of understanding of the mechanisms regulating tumor progression. Only genetic predisposition and exposure to ionizing radiations at a young age have been identified to be causally linked to the initiation of breast cancer. These factors, however, account for less than 5% of the overall cancer incidence, and they do not provide a satisfactory explanation for the continuous increase in breast cancer incidence in the industrialized societies. Thus the need to recur to experimental animal models in order to uncover the basic mechanisms involving the initiation, progression and cure of the neoplastic process in this highly specialized organ. The induction of mammary cancer in rodents is a model that has allowed us to determine that polycyclic aromatic hydrocarbons induce cancer by affecting undifferentiated terminal ductal structures, the terminal end buds (TEB), which are characteristics of the young and virgin animal. We have found the TEB to be equivalent to the undifferentiated lobule type 1 (Lob 1) of the female breast, which is the site of origin of ductal carcinomas. Both the Lob 1 and the TEB are characterized by having a high rate of cell proliferation. Parity in the rat inhibits the initiation of cancer, mainly through the induction of differentiation of the mammary gland, in which the TEB are progressively replaced by secretory lobules with a low proliferative activity. The breast of parous women contains a larger concentration of differentiated structures or lobules type 3 (Lob 3) in which cell proliferation is also markedly reduced. In addition to parity, the architecture of the breast is profoundly modified by menstrual status and age. At menopause the breast undergoes involution, resulting in the regression of the Lob 3 of the parous woman breast to Lob 1. Although the breast of postmenopausal nulliparous and parous women contains the same proportion of Lob 1, their proliferative activity is significantly higher in nulliparous than in parous women. These findings indicate that although the hormonal milieu at menopause is identical in women of different parity, the developmental changes undergone by the breast at younger ages are still exerting a greater influence on the lobular proliferative activity than circulating hormonal levels. Cell replication in Lob 1 of the human breast has its highest peak during early adulthood, at a time during which the breast is more susceptible to carcinogenesis, and decreases considerably with aging. The TEB of the rat mammary gland also has its highest proliferative activity and binding of carcinogen to the DNA, and lowest reparative capabilities when the animals are young and more susceptible to chemical carcinogens. The TEB affected by the carcinogen evolves to intraductal proliferation (IDP), ductal carcinoma *in situ* (CIS), and invasive ductal carcinoma. The undifferentiated epithelial cells composing the TEB represent the "stem cells" of the neoplastic process. Several factors regulate their

susceptibility to neoplastic transformation, such as their specific topographic location within the mammary gland tree, as well as the age and reproductive history of the animal at the time of carcinogen exposure. Epidemiological findings indicate that a parallelism exists between this experimental animal model and cancer incidence in the human population. Early menarche, nulliparity and late parity are associated with higher incidence of breast cancer, whereas women completing one or more full term pregnancies before age 24 have a reduced cancer incidence. The timing of these events indicates that a lengthening in the period between the beginning of ovarian function and full differentiation of the breast plays an important role in increasing breast cancer risk. The protection afforded by early pregnancy has been mimicked in rodents by treatment of virgin rats with the placental hormone human chorionic gonadotropin (hCG). This hormone induces differentiation of the mammary gland. The differentiated gland, however, does not lose its ability to proliferate under an adequate hormonal stimulus, such as a second pregnancy. Although these cells have been already primed by the first cycle of differentiation, if a cancer causing agent acts on them they will respond to the insult, since they are capable of metabolizing the carcinogen, thus becoming a second type of "stem cell". These cells, however, can repair the DNA damage induced more efficiently, originating tumors that are more differentiated. The fate of the mammary epithelial cell that has been affected by the carcinogen and progresses to IDP and CIS can be modulated by the same hormone that inhibits cancer initiation. Treatment with hCG of virgin rats previously exposed to the chemical carcinogen 7,12-dimethylbenz(a)anthracene (DMBA) reduces by 12 fold the number of CIS, as well as the overall tumorigenic response. These results indicate that hCG is capable of inducing differentiation of both the normal and the carcinogen-initiated mammary glands, resulting in permanent structural changes which overcome the initiation and the progression of the carcinogenic process. The relevance of our work lies in the validation of the experimental model system by vis-à-vis comparisons with the human disease, and the demonstration that both differentiation and cell proliferation, which are important factors in the susceptibility of the breast to the initiation of carcinogenesis, can be modulated through the utilization of physiological endocrinological mechanisms, leading to the development of rationale strategies for breast cancer prevention.

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EGF SUPERFAMILY IN THE PROGRESSION OF HUMAN BREAST CANCER. Marc E. Lippman, Careen Tang, David Goldstein, and Dajun Yang, Lombardi Cancer Center, Georgetown University Medical Center, Washington, DC 20007

Breast cancer is overwhelmingly the most common malignancy of women, afflicting approximately 185,000 American women in 1995 with nearly 46,000 deaths directly attributable to the disease. A clearer understanding of the pathogenetic significance of specific oncogenes, particularly those that can function directly as growth factors so that their receptors may have important diagnostic and prognostic and eventually therapeutic significance for the management of the disease. A wealth of recently derived information has strongly implicated the protooncogene erbB2 (also termed her2 or neu) and its protein product as critically involved in human breast cancer as well as other important epithelial malignancies. Because of its substantial homology with the EGF receptor, erbB2 has long been assumed to encode a growth factor receptor, although definitive identification of ligand(s) has remained elusive. ErbB2 is capable of inducing malignant transformation of many target cells including immortalized breast epithelium when overexpressed in a non-mutated form or in a mutated form. Overexpression of erbB2 is seen in a high proportion of intraductal human breast cancers particularly of the large cell comedo type. The precise significance of this finding is as yet unknown. On the other hand, overexpression in invasive breast cancer which occurs far less frequently (approximately 25%) is associated, independently of other prognostic variables, with a far worse prognosis. EGF receptor and erbB3 and erbB4 are overexpressed even more commonly in breast cancer. The EGF receptors can be activated by growth factors which bind to it (EGF, TGF$\alpha$, etc.) And erbB4 can be activated by the heregulins. Complex and confusing patterns of activation of erbB2 and erbB3 apparently occur by heterodimerization. We have recently exploited otherwise indifferent cells transfected singly and doubly with various members of the EGFR superfamily providing substantial clarification of pathways of receptor activation by various ligands. We have attempted to modulate signaling through this pathway using a series of approaches including receptor antibodies, antisense and ribozyme strategies, and signal transduction inhibitors. Several of these approaches are now in clinical trial and the translational potential of the strategies will be discussed.

Abstract—Keystone Meeting, March 7-12, 1997

MOLECULAR MARKERS AS PROGNOSTIC AND PREDICTIVE FACTORS IN BREAST CANCER. C. Kent Osborne, M.D., The University of Texas Health Science Center at San Antonio.

Invasive breast cancer is the result of a series of genetic changes that accumulate in an individual patient's tumor. These genetic alterations help to determine the aggressiveness of a patient's tumor and its likelihood of response to treatment. Identification of these specific genetic changes in a patient's tumor could provide a molecular fingerprint that would allow more accurate diagnostic and treatment approaches.

Standard prognostic factors now used in the clinic include histologic type and grade, lymph node status, tumor size, the presence or absence of estrogen and progesterone receptors, and a measure of cell proliferation such as S-phase fraction by flow cytometry. Using these features, patients can be classified into relatively good or relatively poor prognostic categories. Newer potentially useful prognostic factors include expression of polypeptide growth factors and their receptors, measures of angiogenesis, markers indicative of metastatic potential, expression of genes regulating apoptosis, expression of various heat shock proteins, and alterations in oncogenes or tumor suppressor genes. Many of these markers are able to segregate patients into relatively good and poor prognosis but none have been validated to the extent that they are now routinely used in the clinic.

The biomarker most likely to find its way to the clinic in the near future is the HER-2/neu oncogene. The protein is a growth factor receptor that appears to be involved in cell proliferation and differentiation. High expression has been shown in some studies to relate to poor breast cancer prognosis. However, HER-2 oncogene expression may relate more to response to chemotherapy or hormonal therapy than it does to the natural history of the disease. Several studies have shown that patients overexpressing the HER-2 oncogene (about 20 to 25% of all breast cancer) do not respond to adjuvant chemotherapy with cytoxan, methotrexate, 5-FU, but may respond to more aggressive therapy with adequate doses of Adriamycin and cytoxan. Some studies also suggest that patients overexpressing HER-2/neu may be less responsive to the antiestrogen tamoxifen although these data are less consistent and convincing.

New molecular analyses of breast tumors are providing clues to other genes involved in patient prognosis. Loss of heterozygosity on Chromosome 14q is found frequently in primary breast cancers suggesting the presence of a tumor suppressor gene at this locus. However, LOH at this locus is rarely found in metastatic tumors suggesting the presence of a nearby gene that is crucial for metastasis, and when down-regulated by LOH, results in a less metastatic phenotype.

In the future, molecular analysis of a patient's tumor will allow clinicians to accurately predict patient outcome and will facilitate individualization of treatment which should improve the management of this common disease.

## HOMEOBOX GENES IN THE BREAST

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Homeobox-containing genes are important developmental regulators in embryos of many species. We have recently begun to investigate the expression of homeobox genes in the mouse and human mammary gland. Using degenerate probes to highly conserved regions of the homeobox, we used PCR to amplify sequences from cDNA of mammary gland in various developmental stages and in malignancy. Representatives of each of the four Hox clusters were expressed, as well as others outside of these major groupings. Several characteristics of homeobox gene expression suggest that they may play a significant role. (1) Their expression is developmentally regulated. (2) Certain homeobox genes are influenced by ovarian hormones. (3) Gene expression is associated with regions of epithelium-stroma interactions, and transplant experiments in mice showed that stromal transcription was strictly dependent upon the presence of contiguous mammary epithelium. (4) Misexpression of certain genes was found in both preneoplastic and malignant mouse tissues. Hoxa-1 and Hoxa-2 are strongly expressed in mammary tumors, but not in normal tissues and not in the preneoplastic tissues from which the cancers were derived. This indicates a possible role for these two genes in later, rather than earlier, stages of tumor progression.

Recently we have expanded these studies to the human. Using surgical samples from breast cancers and from adjacent normal tissues, we have confirmed expression of several HOX genes in the breast. In connection with these studies, we have cloned and partially characterized a large new family of human homeobox-containing genes, which we have termed IRX (Iroquois Related). This family, which consists of at least five new genes, contains a DNA binding homeobox that is only distantly related to the HOX genes, but which has close homology with a recently discovered family in Drosophila, the IROC (Iroquois) complex. In the human, one of these genes, IRX-2, is abundantly expressed in a subpopulation of lumenal mammary epithelium, but not in myoepithelium or stromal cells. In breast cancers the gene is often misexpressed. The discovery of the IRX family opens the door to an unexpected area of investigation, with interesting possibilities for both normal and malignant aspects of breast development.

Speaker Abstract for "Basic and Clinical Aspects of Breast Cancer"
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The genetics of familial breast cancer: Role of BRCA1, BRCA2 and evidence for additional susceptibility loci

P. Andrew Futreal

The genetic basis of familial breast cancer is the subject of intense investigation. The last three years have seen the identification of two breast cancer susceptibility genes, BRCA1 and BRCA2. The contribution of these loci to familial breast cancer is of great interest on multiple fronts. The impact of the ability to screen for mutations in these two genes is now becoming a major focus at both the clinical, research and ethical/legal levels. More detailed understanding of the role these genes play in contributing to familial breast (and breast/ovarian) cancer syndromes will hopefully lead to better testing, screening, and hopefully therapeutic approaches to this disease. The focus of the laboratory is the elucidation of the molecular genetic contribution to familial breast cancer. Our approach has been to work with a large group of families representing the spectrum seen in what could be considered familial breast cancer. To date we have screened BRCA2 for mutations in 196 families. We have been using a SSCA/heteroduplex assay approach coupled with PTT for the two large exons in BRCA2. Our results to date are 23/196 (12%) families with detectable BRCA2 mutations. We have detected mutations in all families in the set which have positive linkage results for BRCA2, as well as detecting the reported polymorphisms, ruling out the possibility of a simple technical deficit being responsible for these results. This family set is composed a wide spectrum of phenotypes ranging from an early onset case (diagnosed under age 50) in the presence of later onset cancers (diagnosed over age 50) to families with multiple cases of breast cancer diagnosed under age 50. As such, whilst not being necessarily indicative of the contribution of BRCA2 to very high risk families, this data is more relative to the bulk of familial breast cancer. The data are suggestive of other genetic components of breast cancer susceptibility. To further investigate this possibility, we have examined BRCA2 and BRCA1 on a subset of our family set with the cutoff criteria for inclusion being 3 or more cases of breast cancer diagnosed under age 50, this criteria yielded a subset of 85 families. This subset is the most indicative of what may be true dominant susceptibility alleles segregating in breast cancer families. We have found that in 3 case families 24% had BRCA1/2 mutations, in 4 case families 32% with BRCA1/2 mutations, in 5 case families 67% with BRCA1/2 mutations, and in 6+ case families 63% had BRCA1/2 mutations. Taking into account the caveats of mutation detection sensitivity issues and of chance sporadic clustering of early onset cases, these data are still quite suggestive of additional breast cancer susceptibility genes contributing to familial breast cancer in the population. Efforts underway to more specifically investigate this possibility will be discussed.

**Variation of risks of breast and ovarian cancer associated with different germline mutations of the BRCA1 and BRCA2 genes.**

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Mutations in the *BRCA1* and *BRCA2* genes are associated with a strong predisposition to breast cancer in women; a gene carrier in a high-risk family has a roughly 50% chance of developing breast cancer by the age of 50, and 80-90% chance by the age of 75. It is likely that these risks are modified both by genetic background and environmental factors, but as yet data about this are lacking. In addition to female breast cancer, mutations of *BRCA1* also confer a substantially increased risk of epithelial ovarian cancer and probably a slightly increased risk of prostate and colon cancer, although current evidence suggests that the lifetime risk of these cancers in *BRCA1* carriers is probably less than 10%. Mutations in *BRCA2* confer, in addition to female breast cancer, an increased risk of male breast cancer and ovarian cancer in some families and probably increased risks of cancer of the prostate, pancreas and larynx, although the magnitude of these risks is not yet clear.

Data from the International Breast Cancer Consortium sugges that the risks of breast and ovarian cancer varies between different *BRCA1* linked families. In the majority of these families there appears to be a

high risk of breast cancer but a relatively low risk of ovarian cancer, while in a minority of families there appears to be an equally high risk of both breast and ovarian cancer. The isolation of the *BRCA1* gene has provided the opportunity to search for a correlation between specific *BRCA1* mutations and the incidence of breast and ovarian cancer in the family, as predicted by the previous data. To date, the results suggest that when mutations that result in a truncated *BRCA1* protein occur in the first two thirds of the *BRCA1* gene, the risk of ovarian cancer relative to breast cancer in the family is significantly higher than when truncating mutations occur in the last third of the gene. There is as yet no clear explanation for these findings although there is functional evidence to support the epidemiological data.

The risks of ovarian cancer in *BRCA2* linked families are less significant than for *BRCA1*. *BRCA2* is associated with a much greater risk of male breast cancer; the majority of multiple case breast cancer families containing one or more cases of male breast cancer appear to be due to mutations in the *BRCA2* gene. A genotype-phenotype correlation in respect of risks of breast and ovarian cancer similar to that observed for *BRCA1* is seen in *BRCA2*. However, in *BRCA2* , the data suggests that mutations that occur in families with a high risk of ovarian cancer are clustered in a central portion of the gene which has been termed the "ovarian cancer cluster region". There does not appear to be a correlation between the location of the mutation in *BRCA2* and the occurrence of male breast.

**MESENCHYMAL-EPITHELIAL INTERACTIONS AND BREAST CANCER: ROLE OF LOCALLY-PRODUCED ESTROGENS**; Evan R. Simpson, The University of Texas Southwestern Medical Center, Cecil H. and Ida Green Center for Reproductive Biology Sciences, 5323 Harry Hines Blvd., Dallas, Texas 75235-9051

In post-menopausal women, the ovaries cease to produce estrogens, and local synthesis is the main source. Thus, adipose tissue and various sites in the brain are major sites of estrogen biosynthesis in post-menopausal women In adipose tissue, estrogen biosynthesis increases with obesity, but also with advancing age, and correlates with the increased incidence of endometrial and breast cancer in elderly women. A considerable body of evidence suggests that local production within the breast is the main source of estrogen which stimulates the development and growth of breast carcinomas in such women. Estrogen biosynthesis is catalyzed by an enzyme known as aromatase cytochrome P450 (P450arom, the product of the *CYP19* gene). Aromatase is a member of the P450 superfamily of genes which currently contains over 300 members in some 36 gene families. In the human, aromatase expression occurs in a number of human tissues and cell types, including syncytiotrophoblasts of the placenta, ovarian granulosa cells and corpus luteum, testicular Leydig cells, various sites in the brain, as well as in adipose tissue. In women, aromatase activity and transcripts have been detected both in breast adipose and breast tumor tissues. Within adipose as well as tumor tissue, aromatase expression appears to occur primarily, if not exclusively, in mesenchymal cells rather than in the adipocytes or tumorous epithelium.

Blockage of estrogen representation is the most common and effective form of adjuvant therapy in the management of breast cancer. In this context, the estrogen antagonist tamoxifen has been used most commonly, but recently a number of pharmaceutical companies have developed aromatase inhibitors of high specificity and affinity which are currently in phase 3 clinical trials. A major disadvantage of these compounds is that they inhibit aromatase globally, that is, in all tissue sites of expression. In post-menopausal women, the ovary is no longer functioning to produce estrogens, however these continue to be produced in adipose tissue as well as in various sites in the brain, and estrogens produced within the brain have been implicated in a number of important roles including imprinting of sex-related behavior, regulation of gonadotropin release, but perhaps most significantly, they may be important in the prevention of Alzheimer's disease. For this reason, a drug which could specifically block the source of estrogen which is directly implicated in breast cancer development in post-menopausal women, namely estrogen synthesized in breast adipose, without inhibiting estrogen biosynthesis in other tissues, would be highly desirable.

A few years ago, we and others cloned and characterized the *CYP19* gene which encodes P450arom. The coding region spans nine exons beginning with exon II. Tissue-specific expression of the *CYP19* gene is determined, at least in part, by the use of tissue-specific promoters, which give rise to transcripts with unique 5'-noncoding termini. Placental transcripts contain at their 5'-end untranslated exon I.1 which is located at least 40 kb upstream from the start of translation in exon II. This is because placental expression is driven by a powerful distal placental promoter, I.1, upstream of exon I.1. On the other hand, transcripts in the ovary contain sequence at their 5'-end that is immediately upstream of the start of translation. This is because expression of the gene in the ovary utilizes a proximal promoter, promoter II. By contrast, transcripts in adipose tissue contain primarily another distal untranslated exon, I.4, located in the gene 20 kb downstream from exon I.1. A number of other untranslated exons have been characterized by ourselves and others, including one that is apparently specific for brain.

A number of studies have attempted to relate tumor site to either aromatase activity or else aromatase expression in adipose tissue of breast quadrants. In most cases, a direct relationship has been

found between tumor proximity and aromatase expression in the tumor-bearing quadrant. Moreover, we have recently demonstrated that aromatase expression in adipose of a tumor-containing breast is considerably higher than in tissue from cancer-free patients. These results suggest that tumors may secrete substances which influence aromatase expression in adjacent stroma to establish a gradient of aromatase expression. Recently a number of cytokines have been shown by us to stimulate aromatase expression in adipose fibroblasts or else tumor-derived fibroblasts in culture. These include members of the class I cytokine family, namely interleukin-6 (IL-6), interleukin-11 (IL-11), oncostatin M (OSM), and leukemia inhibitory factor (LIF), as well as TNFα. Members of this family have been shown to be elaborated either by cells derived from adipose tissue or else by tumor-derived fibroblasts, or breast tumor tissue. The latter may include in addition to transformed mammary epithelial cells, infiltrating macrophages and monocytes. When adipose fibroblasts are placed in culture, the proportions of promoter I.4-, II- and I.3-specific transcripts depend on the stimulatory factors present in the culture medium. Thus when aromatase expression is stimulated by members of the class I cytokine family, such as IL-6 or IL-11, or else TNFα, the transcripts which are present are those derived from promoter I.4. Recently we have defined the second messenger pathway whereby class I cytokines stimulate aromatase expression via promoter I.4. This stimulation is mediated by a Jak 1 kinase as well as a STAT 3 transcription factor which binds to a GAS (interferon-γ–activating sequence) element within promoter I.4 of the P450arom gene. On the other hand, stimulation by TNFα appears to involve the mediation of ceramide, and activation of a fos/jun complex which binds to an AP1 site upstream of the GAS element associated with promoter I.4.

Recently we found that prostaglandin $E_2$ is also a powerful stimulator of aromatase expression in adipose stromal cells. In contrast to the factors described above, $PGE_2$ acts via a cAMP-dependent pathway to stimulate expression via the proximal promoter II.

Since aromatase expression is confined to the adipose fibroblasts, we have considered it to be a marker of the undifferentiated preadipocyte phenotype. Consistent with this concept is the observation that factors which stimulate aromatase expression in adipose stromal cells, such as IL-6, IL-11, TNFα, are those which either inhibit or reverse the differentiation process. Furthermore, the desmoplastic reaction could be a consequence of release of factors by the tumor which not only stimulate fibroblast proliferation, but also inhibit or reverse the differentiation process. Thus, it is possible to envision the establishment of a local positive feedback loop whereby fibroblasts surrounding a developing tumor produce estrogens which stimulate the tumor to produce a variety of cytokines and other factors. Some of these act to stimulate the further growth and development of the tumor in a paracrine and autocrine fashion. Additionally, these or other factors act to stimulate proliferation of the surrounding fibroblasts (the desmoplastic reaction) and expression of aromatase in these cells. Thus a positive feedback loop is established via mesenchymal-epithelial interactions, which leads to the continuing growth and development of the tumor.

Since these pathways of regulation of aromatase expression are unique to adipose tissue, factors which block these pathways could be candidates for roles as novel tissue-specific aromatase inhibitors, which would leave expression in other sites such as brain and possibly bone, untouched. Such factors could conceivably find utility as new tools in adjuvant therapy for breast cancer.

## The Role of p53 and Telomerase in Human Breast Cancer

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The following model integrates the phenomenon of telomere-shortening and the two-stage hypotheses for *in vitro* cellular senescence. The repression or down regulation of telomerase activity during development initiates the process of telomere shortening in all human somatic cells. A signal that induces the M1 (Mortality Stage 1) mechanism is produced when telomeres still have several kilobase pairs of telomeric repeats remaining on most chromosomes. This signal may reflect the activation of a DNA-damage pathway produced from a rare telomere lacking protective repeats, or the activation of regulatory factors located in the subtelomeric DNA and previously silenced by trapping in telomere-induced heterochromatin. The key antiproliferative components of the M1 mechanism (p53 and a pRB-like protein) or one of their downstream effectors such as p21 or p16 prevents cell division in senescent cells. If the M1 mechanism is blocked (usually by mutation in p53 and/or RB) cells continue to divide (extended lifespan) and have progressive telomere shortening until a second proliferative blockade, the M2 mechanism (crisis) is activated. M2 probably represents the direct consequences of terminally shortened telomeres. Immortalization occurs when cells escape M2, usually following the reactivation or upregulation of telomerase activity but sometimes via a telomerase-negative pathway that may involve recombination of telomeres. In support of this model we have observed the following: 1) telomerase is downregulated/repressed in most human tissues between 16 weeks gestation and birth; 2) telomerase activity is detected in approximately 85% of more than 2000 primary human tumor biopsies; 3) telomerase is repressed in immortal/normal cell hybrids; and 4) manipulation of telomere length in the immortal parent prior to hybrid formation alters hybrid lifespan.

Our studies on the development of human breast cancer initially focused on a model of cellular immortalization using human mammary epithelial cells. The two independent mortality mechanisms controlling fibroblast senescence and immortalization (M1 and M2) were also found in human mammary epithelial cells. However, perhaps due to the conditions required for establishing normal human breast epithelial cells in culture, only p53 appears to be involved in the M1 mechanism of breast epithelial cells. Thus, expression of HPV16 E6 or dominant/negative p53 mutations is sufficient to overcome M1 in human breast epithelial cells but not breast stromal cells. In addition, we have been successful in obtaining a spontaneously immortalized cell line from "normal" breast tissue obtained from a patient with Li-Fraumeni Syndrome (germline mutation in p53). Primary cultures of breast epithelial cells initiated from this patient did not have detectable telomerase activity. During serial passage, the cells continued to lose telomeric repeats, lost the second p53 allele, and after a prolonged crisis period a rare cell in the population immortalized which had detectable telomerase activity and short but stable telomeres.

Our recent studies have sought to determine when telomerase activity is upregulated or reactivated in the progression to invasive breast cancer in an effort to decide if this knowledge may contribute to the early diagnosis and improved management of breast cancer patients. In these studies none of 54 breast tissue specimens from normal women or those with benign fibrocystic disease had detectable telomerase activity. However, 233/257 or approximately 91% of women with invasive breast carcinoma had telomerase activity. In areas adjacent to the excised cancers telomerase activity was detected in 2/84 or 2% of specimens suggesting that in supposedly cleared margins there may still be a few cancer cells remaining (occult micrometastases). In addition, we detected telomerase activity in fine needle aspirates prior to surgery and the results correlated well with microscopic cytopathology.

To improve the diagnostic value of telomerase determinations, new approaches to examine specimens include the application of *in situ* hybridization methods for the demonstration of the human telomerase RNA component (hTR) on archival paraffin embedded materials. In our initial studies, *in situ* hybridization for hTR expression easily distinguished cancer from normal cells and correlated well with telomerase activity. In summary, research disclosing how telomerase is regulated may not only lead to the development of methods for the accurate and early diagnosis of breast cancer but also to the development of novel anti-telomerase cancer therapeutics.

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***EPITHELIAL BIOLOGY AND BREAST CANCER: THE ROLE OF INTEGRINS AND CYTO-SKELETAL SIGNAL TRANSDUCTION IN GENESIS OF NORMALCY AND MALIGNANCY***

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Our studies in the last decade have established the importance of the extracellular matrix (ECM) and tissue structure as dominant regulators of mammary epithelial cell (MEC) gene expression. We have used "designer microenvironments" *in culture* and studied how the ECM regulates growth, differentiation and apoptosis in murine and human MEC's. From these studies we have determined that the ECM directs a hierarchy of transcriptional events which involve the intricate interplay between soluble and physical signaling pathways. Moreover, we have shown that these pathways control, and in turn are influenced by the tissue structure. Using transgenic mice we have shown that loss of tissue structure and alterations in ECM components lead to the formation and dissemination of breast tumors. Thus we have argued that cell-ECM pathways must contain tumor suppresser checkpoints. To identify these critical control points, we have established a unique human MEC model of tumorigenesis which recapitulates many of the genetic and morphological changes observed in breast cancer *in vivo*. Using a 3-dimensional (3-D) reconstituted basement membrane assay, we have shown that as long as cells continue to interact and respond to the ECM, and are able to form organotypic structures, they will not form tumors, despite extensive chromosomal aberrations and mutations. Specifically we have determined that as these cells progress towards malignancy, they sustain perturbations in their cell-ECM responsiveness as reflected in altered integrin levels and ratios. This is reflected by a progressive loss of growth control and increased apoptosis, but only subtle changes in structure. Loss of tissue structure in culture appears to be a prelude to a tumorigenic phenotype *in vivo*. By manipulating cell-ECM interactions through inhibitory function blocking integrin antibodies, we are now able to modify the morphology and behavior of these tumor cells, such that they can revert to their early passage, non-malignant counterparts. Whereas the untreated tumor cells which possess multiple genetic defects, form large disorganized colonies and do not growth arrest when cultured in 3-D, the modified tumor cells are able to reorganize their cytoarchitecture, resume cell-cell interactions and form polarized, growth arrested acinar structures. These treated tumor cells also have significantly reduced number and size of tumors in nude mice. Since these studies illustrate that growth as well as malignant behavior are fundamentally regulated at the level of the tissue (acini) organization, we have initiated studies to understand how structure can alter gene expression.

## Gradual Phenotypic Conversion during Immortalization of Cultured Human Mammary Epithelial Cells

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Examination of early passages of immortally transformed human mammary epithelial cell (HMEC) lines developed in our laboratory has uncovered a novel, apparently epigenetic step involved in the immortal transformation of HMEC in culture.

Primary cultures of HMEC from specimen 184 were exposed to the chemical carcinogen benzo(a)pyrene. Treated cells gave rise to around 6-10 different extended life (EL) cultures, which subsequently lost proliferative capacity. Only two cells, from different EL cultures, maintained proliferation, giving rise to the two lines, 184A1 and 184B5. Both of these lines showed a few specific karyotypic abnormalities, indicating their distinct clonal origins. With continued passage, 184A1 and 184B5 displayed a very low level of gross chromosomal instability. No defect has been detected in either line in the regulation of RB phosphorylation, or in the sequence of the p53 gene. Both 184A1 and its EL precursor have homozygous mutations of the p16 gene. Although no mutations in the p16 gene have been detected in 184B5 and its EL precursor, both have low or non-detectable expression of p16 protein. Neither line is tumorigenic in nude mice, or displays sustained anchorage independent growth.

When 184A1 and 184B5 were initially characterized in the 1980's, we observed two growth patterns with no obvious mechanistic explanations:
(1) Although both immortal lines maintained continuous growth in mass culture following their initial emergence, growth was slow and non-uniform for the first 20-30 passages. Visual observation indicated that many cells lost proliferative capacity.
(2) While absolutely no finite lifespan HMEC maintained growth in TGFβ, populations of 184A1 and 184B5 which maintained continuous growth in TGFβ could be isolated. However, the pattern of expression of resistance to growth inhibition by TGFβ was unusual. 184A1 mass cultures exposed to TGFβ at passages (p) 28-35 displayed severe growth inhibition, but a small subpopulation of cells maintained active growth. Assuming these resistant cells represented rare mutations, we attempted to obtain pure populations by clonal isolation. However, like the parental uncloned population, all four clones isolated displayed a small subpopulation of cells capable of unlimited growth in TGFβ. 184B5 exposed to TGFβ at p26-40 maintained good growth, but most clones isolated at p13-16 were growth inhibited. One particular severely growth inhibited clone, B5T1, repeatedly underwent an apparent "crisis" around p30 during which almost all the cells died. The populations derived from the few surviving cells maintained growth in TGFβ.

The lack of growth inhibition by TGFβ was not due to loss of the ability to respond to TGFβ. All 184A1 and 184B5 cultures showed morphologic alterations in the presence of TGFβ, and all cells tested displayed TGFβ receptors and induction by TGFβ of extracellular matrix associated proteins.

In an effort to understand (1) why so many early passage cells from immortal lines failed to maintain proliferation, and (2) how clonal isolates rapidly produced cell populations heterogeneous for growth in TGFβ, we particularly noted the association of TGFβ resistance with an indefinite lifespan in B5T1. Since telomerase activity had recently been shown to be associated with an indefinite lifespan, we considered the possibility that TGFβ resistance and

telomerase activity might be related. We therefore carefully characterized and correlated morphology, growth capacity in the absence and presence of TGFβ, telomerase activity, and telomere length in 184A1 and 184B5 at different passage levels to ascertain possible associations among these phenotypes.

These studies have provided the following results:
(1) Early passage cells of these lines are only "conditionally" immortal, i.e., although the mass culture maintains indefinite growth, most individual cells do not remain proliferative. These cells also do not express telomerase activity, show continued telomere shortening with passage, and display no sustained growth in TGFβ.
(2) With continued passage, both mass cultures and clonal isolates show a gradual increase in growth capacity, in the number of cells displaying progressively increased ability to maintain growth in TGFβ, and in telomerase activity. Telomere length becomes stabilized or increases slightly.
(3) This gradual change in phenotypic expression is first detected when the conditionally immortal cells have extremely short telomeres (~2.0 kb) and display slow non-uniform growth.
(4) There is a strong correlation between capacity to maintain growth in the presence of TGFβ and expression of telomerase activity.
(5) During the time conditionally immortal cells display poor non-uniform growth, there is an accumulation of the cyclin dependent kinase inhibitor p57. This inhibitor does not accumulate in fully immortal cells showing uniform good growth.

We have used the term "conversion" to describe this process of gradual acquisition of increased growth capacity in the absence or presence of TGFβ, and reactivation of telomerase. Conversion occurs in cells that have overcome replicative senescence, but have not obtained uniform indefinite proliferative potential. The consistent manifestation of conversion by clonal cell isolates, and the very gradual nature of the conversion process, suggest an epigenetic mechanism. We speculate that the development of extremely short telomeres may result in gradual changes in heterochromatin structure and/or gene expression, by mechanisms similar to those responsible for the derepression of silenced genes in yeast model systems.

Acquisition of a fully immortal phenotype in these HMEC requires overcoming the growth restrictions encountered by conditionally immortal cells and completing the conversion process. Preliminary experiments in which conditionally immortal cells have been infected with viral oncogenes has indicated that the ability of these oncogenes to simultaneously disable many cellular checkpoint controls may circumvent the growth restraints encountered by conditionally immortal cells.

Our data indicate that conditionally immortal cells can undergo a very large number of population doublings before becoming fully converted. If an extended period of conversion occurs in vivo, it would provide a continuous pool of slowly dividing cells able to accumulate errors unrelated to immortalization but which might promote angiogenesis, growth factor independence, and/or invasion. It will therefore be of interest to determine if the slow heterogeneous growth observed during development of many primary carcinomas in vivo is mechanistically related to the slow heterogeneous growth we observe in conditionally immortal HMEC in vitro. Understanding the underlying mechanisms of HMEC conversion in vitro may provide new insight into the process of carcinogenic progression in vivo, and offer novel modes for therapeutic intervention.

**Altered Regulation of the Cell Cycle Inhibitors $p27^{Kip1}$ and $p15^{INK4B}$ in a TGF-β Resistant Human Mammary Epithelial Cell Line**

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Cell cycle transitions are governed by a family of cyclin dependent kinases (cdks), whose activity is regulated by association with positive effectors, the cyclins, with negative regulators, the cdk inhibitors and by phosphorylation [1,2]. The cdks control a series of biochemical pathways, or checkpoints, which integrate extracellular mitogenic and growth inhibitory signals, monitor chromosome integrity and coordinate cell cycle transitions [3,4]. Passage through G1 into S phase is regulated by the activities of cyclin D-, cyclin E- and cyclin A-associated kinases. D-type cyclin dependent kinases play a major role in phosphorylation of pRb [5] which is required for G1 to S phase transition.

Two different families of cdk inhibitory proteins have been identified (for reviews see [6] and [2]). The KIP (kinase inhibitor protein) family is presently composed of three members, p21 (also identified as Cip1, Waf1, Sdi1), p27 (Kip1) and p57 (Kip2). The KIP family of cdk inhibitors play roles both in the assembly of cyclin D/cdk4 and cdk6 complexes [7] and to regulate responses to mitogenic and growth inhibitory signals by binding and inhibition of cyclin/cdks.

The INK4 family of cdk inhibitors bind specifically and inhibit cdk4 and cdk6 activities [2]. The first of the INK4 family to be cloned, the $p16^{INK4A}$ gene, or MTS1 (multiple tumor suppressor 1) is mutated in many human tumors and cell lines. The $p15^{INK4B}$ gene (also known as MTS2), located adjacent to the $p16^{INK4A}$ gene, is induced by TGF-β and may also be a target for inactivation in human tumors. Other INK family members, including p18, p19 and p20 are not known to be regulated by TGF-β.

TGF–β is a potent inhibitor of normal epithelial cells [8] and inhibits mammary duct development in vivo [9, 10]. TGF–β mediates G1 arrest through a number of mechanisms. Inhibition of retinoblastoma (pRb) protein phosphorylation [11] by TGF–β involves downregulation of cyclin D1 and cdk4 proteins in certain cell types [12-14]. Reduction of cyclin A and cyclin E mRNA levels and of cyclin A protein [15, 16] contribute to inhibition of cyclin E and A dependent kinases [16-18]. Furthermore, G1 cyclin-dependent kinases are inhibited by the heat stable cdk inhibitor, $p27^{Kip1}$ [16-18]. In epithelial cells, TGF–β causes up-regulation of $p15^{INK4B}$ mRNA, increased binding of a 15kD protein to cdks 4 and 6 [19, 20]. This is accompanied by a reduction in cdk4 associated $p27^{Kip1}$ and increased association of $p27^{Kip1}$ with cdk2 complexes [20]. The present study extends these observations in the HMEC model.

Inappropriate proliferation, due to loss of normal responsiveness to extracellular signals and deregulation of cell cycle checkpoints, is the hallmark of cancer. Loss of sensitivity to the growth inhibitory effects of TGF–β is common in human breast cancer derived cell lines, and this is thought to confer an advantage in breast carcinogenesis and progression [21]. In the present study, we have examined normal finite lifespan and immortal human mammary epithelial cells that differ in their sensitivity to TGF–β in order to better understand the mechanisms by which TGF–β mediates G1 arrest and to uncover how these regulatory mechanisms may be deranged in cells that are resistant to growth inhibition by TGF–β.

In the present study [22], the effects of TGF–β were studied in closely related human mammary epithelial cells (HMEC), both finite lifespan 184 cells and immortal derivatives,

184A1$^{S}$, and 184A1L5$^{R}$ which differ in their cell cycle response to TGF–β, but express type I and type II TGF–β receptors and retain TGF–β induction of extracellular matrix [23]. The arrest resistant phenotype was not due to loss of cdk inhibitors. TGF–β was shown to regulate p15$^{INK4B}$ expression at at least two levels: mRNA accumulation and protein stability. In TGF–β arrested HMEC, there was not only an increase in p15 mRNA, but also a major increase in p15$^{INK4B}$ protein stability. As cdk4 and cdk6-associated p15$^{INK4B}$ increased during TGF–β arrest of sensitive cells, there was a loss of cyclin D1, p21$^{Cip1}$, and p27$^{Kip1}$ from these kinase complexes. Furthermore, the equilibrium of p27$^{Kip1}$ binding shifted from cdk4 to cyclin E/cdk2 during TGF–β mediated arrest.

In HMEC, p15$^{INK4B}$ complexes did not contain detectable cyclin. p15$^{INK4B}$ from both sensitive and resistant cells could displace *in vitro* cyclin D1, p21$^{Cip1}$ and p27$^{Kip1}$ from cdk4 isolated from sensitive cells. Cyclin D1 and KIP proteins could not be displaced from cdk4 in the resistant 184A1L5$^{R}$ cell lysates. We propose a model in which a component of the cdk4 complexes in the resistant cells is altered and fails to allow complex dissociation. Thus, in TGF–β arrest, p15$^{INK4B}$ may displace already associated cyclin D1 from cdks and prevent new cyclin D1/cdk complexes from forming, but it can only do so where cdk complexes have been "prepared" for dissociation by another molecular event. The importance of post-translational regulation of p15$^{INK4B}$ and of its target cdk complexes by TGF–β is underlined by the observation that in TGF–β resistant 184A1L5$^{R}$, although the p15 transcript increased, p15$^{INK4B}$ protein was not stabilized, did not accumulate and cyclin D1/cdk association and kinase activation were not inhibited [22].


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# The Regulation of Transforming Growth Factor (TGF) α

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The estrogen receptor (ER) signal transduction pathway is pivotal in our understanding of estrogen regulated processes. Antiestrogens block the ER pathway and therefore are useful for the treatment of breast cancer (1) and new drugs are being developed to exploit differentially the target site specific actions of drugs like tamoxifen (2). At present there is no adequate explanation for the fact that tamoxifen has estrogen-like effects expressed in bone, liver and endometrial cancer, but antiestrogenic effects in breast (1).

The absence of the ER in a target cell usually indicates that the cell is refractory to estrogen action. There have, however, been numerous attempts to re-introduce the ER into hormone independent cells to determine whether control by estrogen can be reasserted (3). We have prepared stable transfectants with cDNAs from wildtype and mutant ERs using the hormone independent breast cancer cell line MDA-MB-231 (4,5). The natural mutant receptor was derived from a tamoxifen-stimulated breast tumor grown in athymic mice (6).

We have reported (7) that estrogen will increase TGFα mRNA in transfectants and since this is a central gene in the regulation of estrogen action we have focused upon this signal transduction pathway. We belive this is an important new model because the target is a gene *in situ* rather than an artificial construct of a reporter gene with an inappropriate estrogen response element (ERE). Of specific interest, we have noted a differential action by antiestrogens at the target gene: the pure antiestrogen ICI 182,780 blocks TGF α mRNA, raloxifene is an antiestrogen with wildtype but an estrogen with mutant receptors (8). In contrast, 4-hydroxytamoxifen is an estrogen with both wildtype and mutant receptors. The fact that an antiestrogen and an estrogen can act as an estrogen at the same promoter *in situ* in the same cell with the same receptor is a unique finding that could establish a mechanism for the target site specific actions of antiestrogens.

We have dissected the signal transduction pathway into ER and promoter regulation. Firstly, to establish direct ER action at the TGFα gene we have used site-directed mutagenesis at the DNA binding domain of the ER. Receptors that cannot interact with the ERE should not induce TGFα mRNA. Secondly, we have made a systematic study of the TGFα promoter (9) and the putative EREs. However the MDA-MB-231 cell line is particularly difficult to use routinely for both transient or stable transfection so we will report our progress using the novel cell line T47D:C42. Prolonged estrogen deprivation of T47D cells results in the eventual loss of ER (10) because of the requirement for estrogen to maintain ER levels in this cell line (11). The ER negative T47D:C42 cells are an ideal model for study. We will consider receptor concentrations and additional cell factors as reasons for the differential action of antiestrogens at various target sites.

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Mechanisms of Tumor Suppression by BRCA1
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BRCA1 encodes for an 1863 amino acid protein of unknown function. BRCA1 exhibits very little homology to any known gene, but has been noted to contain a zinc binding motif of the ring finger type. This has lead to speculation that BRCA1 might function as a transcription factor and in fact a C-terminal fragment of BRCA1 has been shown to be active in a transactivation assay. However, zinc binding domains of the ring finger type have not been shown to mediate DNA binding and appear to be responsible for protein-protein interactions only. In addition, full length BRCA1 was not active in the transactivation assay leading to speculation regarding the physiologic relevance of this observation. Thus, the role of BRCA1 as transcription factor remains unconfirmed.

In addition, it was presumed that once identified, a significant proportion of sporadic breast cancer cases would be explained by somatic mutations in the BRCA1 gene. Unfortunately, very few sporadic ovarian cancers contain somatic mutations in BRCA1 and such mutations have never been identified in truly sporadic breast cancer cases. The first evidence that BRCA1 might play a role in sporadic breast tumors was in the work of Thompson et al, who showed that the expression of BRCA1 mRNA was greatly down regulated in at least half of all sporadic breast cancers. This observation would suggest that despite the lack of somatic mutation in BRCA1 in these cases, inadequate levels of BRCA1 could contribute to the neoplastic phenotype in at least a subset of cases. Supporting this hypothesis is the finding by numerous groups that the BRCA1 locus undergoes loss of heterozygosity (LOH) in as many as 50% of sporadic invasive breast cancers. Furthermore, it was demonstrated that treatment of breast cancer cell lines with antisense oligonucleotides accelerated growth implying that BRCA1 may function as a growth inhibitor.

In light of this finding, it is interesting to note that an excess of medullary cancers and tumors with a high S-phase fraction, high mitotic rate, and aneuploidy have been identified in BRCA1-linked cases. These characteristics would be expected in tumors that had lost an essential mechanism of growth inhibition. In contrast it has been reported that BRCA2 tumors appear to show an excess of tubular and lobular cancers. An explanation for this difference in phenotype is not yet evident, but suggests that the molecular pathways for BRCA1 and BRCA2 tumorigenesis may not be coincident. An additional genotype/phenotype correlation is the observation that the site of a truncating mutation in the BRCA1 gene may strongly influence the level of ovarian cancer risk in patients that carry this mutation in the germline. This observation is also supported by in vitro data demonstrating that BRCA1 constructs lacking the C-terminus are still capable of inhibiting the growth of ovarian cancer cell lines, but not breast cancer cell lines.

Alternatively, Lee's group has presented data suggesting that aberrant subcellular localization of BRCA1 may contribute to the pathogenesis of sporadic breast cancer, although this work remains unconfirmed and is in conflict with other studies. BRCA1 has also been noted to contain a granin motif at the end of exon 11 and this in conjunction with certain biochemical properties of BRCA1, suggested that BRCA1 is a granin and may function as a secreted growth inhibitor. Granins are expressed in neuroendocrine cells and are proteolytically cleaved to yield bioactive peptides. An example of which is the cleavage of chromogranin A to pancreastatin, a peptide demonstrated to inhibit insulin secretion. Critical to the granin hypothesis however, is the demonstration that purified BRCA1 added

exogenously to cells inhibits their growth and thus far, these studies have not been performed. In addition, the C-terminus of BRCA1 shares a strongly immunogenic epitope with the epidermal growth factor receptor leading to antibody cross reactivity between BRCA1 and EGFR protein. Additional antibodies directed at N-terminal residues and functional epitope-tagged constructs do not suffer from this problem and support the original conclusions of Jensen et al. However, the precise subcellular localization and functional mechanism of BRCA1 remains controversial. It is known that women with germline mutations in BRCA1 have an 85-90% lifetime risk of developing breast cancer and a 50% lifetime risk of developing ovarian cancer. In addition, nearly all hereditary tumors show LOH at the BRCA1 locus and the LOH event nearly always involves the wild type allele. These findings strongly implicated that BRCA1 was a tumor suppressor gene, and in fact transfection of BRCA1 into breast and ovarian cancer cells markedly inhibits their growth and ability to form tumors confirming this hypothesis. This presentation will review recent data regarding BRCA1 and discuss attempts to translate these findings into novel therapeutic approaches.

## Biological Function of BRCA Gene Products

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Mutations in the *BRCA1* gene are known to predispose members of affected families to breast cancer. Unlike typical tumor suppressor genes, mutations in *BRCA1* are rarely observed in sporadic tumors. To address this issue, we identified BRCA1 as a 220 kDa nuclear phosphoprotein in normal cells, and one which is aberrantly located in the cytoplasm of most breast and some ovarian tumor cells. Immunostaining with new, highly specific mAbs to two different epitopes and expression of tagged BRCA1 has confirmed its mislocation in breast cancer cells. As an animal model of breast cancer, mice deficient for the *Brca1* gene were developed. Unexpectedly, *Brca1*$^{-/-}$ mice die early in embryonic development indicating that BRCA1 is critical for proper cell determination and/or differentiation during development. The finding that *Brca1*$^{-/-}$ blastocysts are growth-impaired *in vitro* is consistent with other data showing that the BRCA1 220 kDa phosphoprotein is expressed and phosphorylated in cell cycle-dependent manner. These combined data indicate that BRCA1 has a role in regulating cellular proliferation during development and differentiation of animal tissues, and that subcellular localization is an important aspect of BRCA1 regulation. Using recently developed cDNA and immunological reagents, the product of BRCA2 gene was identified as a nuclear 380 kDa protein. Data indicate that the levels of BRCA2 RNA, like that of BRCA1, coordinate with the cell cycle. rtPCR-Southern analysis demonstrated that cells express a variant RNA encoding a translatable BRCA2 that lacks exon three (BRCA2$^{-3}$). Activation of transcription is mediated by the N-terminus of BRCA2 that, curiously, is abolished in the BRCA2$^{-3}$ variant. The potential biological significance of these data on BRCA2 will be discussed.

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**Differential Subcellular Localization, Expression and Biological Activity of BRCA1 and the splice variant BRCA1-Δ11B.** Frank J. Calzone[1], Cindy A. Wilson[2], Cynthia Afshari[3], Dennis J. Slamon[2]. [1] Molecular Biology, Amgen, 1840 DeHavilland Dr. Thousand Oaks, CA 91320, [2] Department of Medicine, Division of Hematology-Oncology, UCLA School of Medicine, Los Angeles, CA 90050, [3] NIEHS, Research Triangle Park, NC 27709.

The mechanism of *BRCA1* tumor suppression in human breast and ovarian cells is the focus of intense investigation. We have attempted to more fully characterize BRCA1 mRNA and proteins and address some of the controversial issues in the literature regarding this important molecule. We describe the isolation of an alternatively spliced BRCA1 message in which the majority of exon 11 (905-4215) has been deleted (BRCA1-Δ11b). The Δ11b variant is present in both the human and the mouse. BRCA1 and BRCA1-Δ11b mRNA and protein appear to be expressed at similar levels in most cells, however, BRCA1-Δ11b is underrepresented, or absent, in breast and ovarian cancer cell lines relative to exon 11 containing transcripts. Protein subcellular localization was examined using CMV promoter vectors that express BRCA1 proteins with or without epitope tags (myc, HA, GFP). These analyses demonstrate that a functional nuclear localization signal (NLS) is encoded in exon 11b and that BRCA1-Δ11b which lacks this sequence is constitutively cytoplasmic. The subcellular localization of endogenous BRCA1 proteins was also examined with BRCA1 antibodies. Overexpression of BRCA1, but not BRCA1-Δ11b transgenes frequently appeared toxic to cells. The toxicity seen with full length BRCA1 expression, as well as N-terminal and C-terminal deletion constructs, was associated with accumulation of the protein in the cytoplasm, nuclear fragmentation and cell death. RNA probe excess titrations indicate that BRCA1 and BRCA1-Δ11b are encoded by rare mRNA, thus, the physiological significance of BRCA1 toxicity associated with overexpression remains an open question.

**Genetics of Mammary Cancer Susceptibility in the Rat: Potential Relevance to the Prevention and Genetics of Human Breast Cancer.** Michael Gould, Margaret Benton, Hong Lan, Jill Haag and Laurie Shepel, Department of Human Oncology, University of Wisconsin, Madison, WI 53792.

Mammary cancer in the rat is a good animal model for human breast cancer. Mammary cancers in rats and humans consist of a mixture of ER+ and ER- tumors while only ER- are found in mouse mammary cancer. In addition, the histopathology of rat mammary cancer more closely resembles the human than does the mouse mammary tumor. These observations suggest the use of the rat mammary model to help delineate and characterize the inherited genetic components of human breast cancer.

The ability to identify and characterize genes that influence susceptibility to human breast cancer is constrained by the limitation of epidemiology to identify genetically prone individuals and families. Thus far, identification of inherited genes that predispose individuals to breast cancer have been limited and accounts for only a small percentage of breast cancers. While it is possible that the remaining breast cancers do not have an inherited genetic component in their etiology, we feel that this is unlikely. Furthermore, it is also unlikely that many additional inherited genes that control breast cancer risk will be identified in population based studies. In light of the above, we have begun the study of the genetics of breast cancer susceptibility in the rat.

Rat strains vary considerably in their propensity to develop spontaneous and induced breast cancer. Strains such as the Wistar Furth (WF) rat have a very high risk of developing mammary cancer, while the Fisher 344 (F344) rat has an intermediate risk. Two rat strains, the Copenhagen (Cop) and Wistar Kyoto (WKy), are almost completely resistant to both induced and spontaneous cancer. Using a DMBA-induction model, we have shown that the WF's high susceptibility to the development of breast cancer is dominant while the resistance phenotypes of the Cop and WKy are both dominant and epistatic over the WF susceptibility phenotype.

We have begun to explore both the genetics and mechanisms underlying the resistance of the Cop and WKy strains to induced and spontaneous mammary cancer. We chose to investigate the resistant rats first because it is unlikely that this class of genes will be found in population-based linkage studies. In addition, such genes could provide insight into the development of breast cancer prevention drugs and strategies.

Using backcross and intercross mapping studies, we have identified and confirmed the existence of Mcs-1 (mammary cancer suppressor) near the centromere of rat chromosome 2. In addition, Mcs-2 and Mcs-3 have tentatively been identified on chromosomes 7 and 1, respectively. These three loci likely contribute to the observed mammary cancer resistance of the Cop rat. In addition, a suggestion of a breast cancer enhancing locus was found on chromosome 8.

In contrast, the other mammary resistant rat strain (WKy) appears to have a more complex inherited basis for its resistance to mammary cancer than the Cop strain. For example, in the (WFxCop) $F_1$xWF backcross the total yield of tumors in

the backcross population was, as expected, between that of the Cop and WF rat strains. In contrast, the (WFxWKy) $F_1$xWF backcross population had even more tumors than the highly susceptible WF rat population. Thus far, Mcs-1, -2, and -3 have not been found to control resistance in the WKy rat. Interestingly, the enhancer gene on rat chromosome 8 which was suggested to exist in the Cop was clearly identified in the WKy. Additional resistance and enhancer genes are being scanned for in the WKy.

The construction of chimeric rats has shown that the major Cop suppressor genes are expressed within the mammary parenchyma and do not function outside the cell in which they are synthesized. Subtraction hybridization studies suggested that the mammary cells in virgin rats of the resistant strain overexpress mammary differentiation markers such a β-casein. Recently, we have shown that cells from the mammary gland of resistant rats require fewer growth factors for differentiation in a matrigel culture. For example, in the absence of EGF WKy but not WF mammary cells differentiate. Interestingly, however, they still require these growth factors for proliferation. These data suggest the hypothesis that the Mcs genes may function as precocious lineage switching genes that convert transformation sensitive target ductal cells to more highly differentiated non-target cells.

Knowing the structure and function of the Mcs genes may provide insight into the design of breast cancer prevention drugs. The identification of human homologs of these genes may also contribute to our understanding of inherited human breast cancer susceptibility. Based on our on-going studies, it is hypothesized that rat strains inherit a series of both susceptibility (enhancer) and resistance (suppressor) genes. The specific mixture of these genes in inbred rats and their genetic segregation pattern in backcross and intercross generations determine the susceptibility of individual rats to breast cancer. A similar situation may also exist in humans. If so, individuals developing both late onset and early onset breast cancer may have a complex multigenic inherited predisposition to breast cancer. Understanding these genetics will both help identify such individuals and possibly lead to individualizing new prevention drugs for them.

# DIET, NUTRITION, AND BREAST CANCER

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Breast cancer rates vary at least five-fold among various countries around the world and migrants from low to high incidence areas tend to adopt rates of the new environment after several generations. This evidence, coupled with large changes in rates within countries, clearly indicates that nongenetic factors are the major determinant of breast cancer rates.

The observation that breast cancer rates are strongly correlated with national per capita fat consumption has raised the hypothesis that dietary fat may be a primary causal factor, but many alternative explanations for differences among countries exist. Recently, the relation of dietary fat to breast cancer risk has been examined in a number of large prospective studies in which confounding factors could be more carefully controlled. These studies have consistently shown little or no relation. In a pooled analysis of the large prospective studies, collectively comprising approximately 300,000 women among whom nearly 5,000 breast cancers were diagnosed, no association with breast cancer risk was seen between 15 and 50% of energy from fat; below 15% of energy from fat risk was actually significantly increased. These data do not exclude the possibility that fat intake earlier in life may influence breast cancer risk; however, the fact that breast cancer rates among counties in China with 25% of energy are still only about 1/5 of the rate among U.S. women with a similar percent of energy from fat provides additional strong evidence that fat per se is not a major cause of breast cancer. Recently, several studies have suggested that monounsaturated fat may actually reduce the risk of breast cancer.

Of the various dietary factors examined, the most consistent observation has been an increase in risk of breast cancer with regular alcohol consumption, even at moderate levels. Small increases appear to exist for even 1 drink/day and are about 30% higher than nondrinkers at 2 drinks/day. Alcohol consumption increases endogenous estrogens in blood, thus providing a plausible mechanism.

Animal studies conducted during the last 50 years have consistently shown that reduction of energy intake (calories) profoundly reduces occurrence of mammary tumors. Epidemiologic studies provide substantial evidence that energy intake in relation to expenditure, such as in physical activity, is also an important determinant of human breast cancer. Greater height, which is an indirect indicator of nutritional status during childhood, has been associated with risk in ecological, case-control and cohort studies. Also, more rapid weight gain during childhood is a strong determinant of early age at menarche, a known risk factor for breast cancer. Weight gain as an adult has been a risk factor for postmenopausal breast cancer in several recent studies, particularly among those who have not used estrogen

replacement therapy after menopause. This observation supports the concept that estrogen production by adipose tissue is the primary mechanism by which excess body fat measures risk.

Thus, available evidence strongly implicates nutritional factors in the etiology of breast cancer. However, energy balance, which influences age at menarche during childhood and raises endogenous estrogen levels after menopause, appears to be the most important aspect of diet. The effects of age at menarche and adult weight gain are sufficient to explain most of the differences in breast cancer incidence between Asia and Western Countries. Furthermore, avoidance of weight gain as an adult, which is best accomplished by regular physical activity and modest restraint of both carbohydrate and fat intake, is an immediately available method for reducing risk of breast cancer.


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**Environment and Breast Cancer**

AACR conference on breast cancer, Keystone, Colorado March 1997

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Wide variations are seen in international incidence of breast cancer, and nationally, differences also exist among ethnic groups. Thus, in the U.S., Hispanic women have lower rates of breast cancer than black and white women, lower than Japanese-Americans, but higher than other women of Asian ancestry. These disparities are not entirely explained by genetics, age and reproductive history. Therefore, it has long been supposed that environmental factors, broadly cast to encompass diet and lifestyle, are involved in one or more of the possible pathways leading to breast cancer.

We have known for centuries that breast cancer risk is strongly associated with reproductive hormones. Therefore, any role for environmental exposures to either increase or decrease risk for breast cancer is likely to act in concert with endogenous hormones. The exact hormonal mechanisms are not entirely worked out, but it is known that hormones determine onset of puberty, alter mammary cell development, promote bone growth, ameliorate menopausal symptoms, increase cell proliferation, and promote tumorigenesis.

In experimental models, dietary and environmental agents can exhibit either protective or adverse effects on tumor incidence and growth. Yet, exactly how diet influences risk for breast cancer in humans is far from settled. Ecologic studies have suggested that dietary fat and fiber intake are major risk factors, but analytic studies have not supported these correlations for breast cancer. At the same time, certain environmental exposures are established risk factors, including radiation and alcohol intake. But in general, environmental exposures have not been accepted as having significant contributions to breast cancer risk. A serious impediment to eliciting associations between environment and breast cancer is precise determination of exposures, which is especially difficult when they occur long before cancer diagnosis.

Like hormones, from the mechanistic point of view, environmental agents may influence carcinogenesis in at least four ways: as genotoxins, as tumor promoters, via carcinogen metabolizing enzymes, and through effects on reproductive development and function.

Estrogen and environmental exposures have the capacity to cause DNA damage. Through chemical or physical insults that cause mutations, such exposures can initiate tumors and alter oncogene function. Experimental and epidemiologic evidence suggests that tumor-initiating exposures are particularly potent early in life. Radiation is the best example of this. Polycyclic aromatic hydrocarbons (PAH) are another, although only animal data are now convincing. Very limited data on smoking history have been interpreted as showing a risk for breast cancer among women who began as teenagers.

Perhaps the most well-recognized effect of hormones is their ability to act as tumor promoters and growth modulators. Persistent organochlorines and other substances may also act as mammary tumor promoters via a hormonal mechanism, as seen in animal models. Reports from the U.S., Finland, Mexico and Canada since 1992 have suggested that organochlorine exposures

may be a risk factor for breast cancer. A slew of additional studies now underway on this topic will clarify the issue within the next few years. From a few of at least thirty on-going investigations, early data show confirmatory results in some, inconclusive results in others, and protective effects in yet others. Further investigations are examining associations between breast cancer risk and organochlorine exposures in occupational circumstances, which should be helpful in sorting out these findings.

Individual response to both environmental insults and hormones may be governed by one's metabolic capacity for activation or detoxification of chemicals in the body. A wide array of enzymes can metabolize xenobiotics, drugs and endogenous substances that are related to mammary tumors. Genotype as well as phenotypic expression of these enzymes differ among racial/ethnic groups, which may contribute to their variable risk. As an example, frequencies of the "minor" genetic variants for CYP1A1, NAT2, and GST range from 10%-60% among Asians, Hispanics, African-American, and Caucasians. The combination of adverse genotype with high exposure may constitute elevated risk, as reported recently among women with null NAT2 plus intense smoking history. Similarly, racial/ethnic groups may have risks related to genetic polymorphisms and pertinent exposures. In addition to their inherited variation, these enzymes may also be modulated by environmental exposures, an effect that could either reduce or increase risk.

Certain life events are linked to both reproductive hormones and subsequent risk for breast cancer, such as ages at menarche and menopause. Some epidemiologists have speculated that exposures as early as *in utero* may determine susceptibility for breast cancer. Recently more emphasis has been placed upon investigation of how hormones in early life may affect later risk, for example by altering the age at onset of puberty, by affecting the differentiation of mammary epithelium in adolescence, or even by changing gender. Experimental findings and sparse human data suggest that environmental contaminants and dietary constituents may modify these reproductive milestones, including cycle regularity, age at onset of puberty and menarche, gonadal development, and sexual behavior. For TCDD and DDT exposures, there are parallel findings in animals and humans of retardation or acceleration of puberty.

Breast cancer etiology is complex, because tumorigenesis can arise from a combination of many different mechanisms over a very long time. Crucial exposures as well as reproductive endpoints related to breast cancer may occur years before a tumor is found. Therefore, epidemiologic and ecologic investigations require a study design that is appropriate to combinations of exposures and to outcomes relevant to breast cancer. Our understanding of environmental contributions to breast cancer risk has not come very far. But the idea is intriguing, and ongoing research on environment and breast cancer will increase our knowledge of the causes of breast cancer and may lead to new approaches to primary prevention.

Issues in Testing for Hereditary Breast Cancer Susceptibility. **Judy E. Garber, M.D., Dana Farber Cancer Institute, Boston MA 02115.**

Multiple cancer susceptibility genes have been identified which, when mutated in the germline, confer an increased risk of breast among other cancers. These include those associated with the highest cancer risks, *BRCA1*, *BRCA2*, *p53* (Li-Fraumeni syndrome), likely additional *BRCAX* genes, as well as the HNPCC-associated genes, and a group of genes associated with lesser risk, including Cowden's gene and possibly *ATM*. Interest is increasing in low penetrance alleles of these genes, or of p450 or other metabolizing genes and their potential interaction with environmental exposures for their contribution to attributable breast cancer incidence.

Germline mutations in *TP53* are rare in early breast cancer patients in the absence of other rare tumors which are features of Li Fraumeni. Estimating the prior probability that small families will carry *BRCA1/2* genes has proved difficult: the genetic heterogeneity of breast cancer and the high frequency of phenocopies due to the population frequency of the disease make it likely that the majority of tested individuals will have a negative, but not entirely reassuring, test result.

The *BRCA1* and *BRCA2* genes are long; their function is not yet understood in a manner sufficient to permit functional analytic strategies. The size of these genes and the distribution of mutations throughout their lengths contribute to the technical challenges in their analysis. A false negative or positive result is obviously undesirable in clinical testing. A large number of polymorphisms are identified, and a group of results not yet interpretable, which are the most difficult for patients to comprehend. Data from Gayther et al. suggest that *BRCA1* mutations more 5' confer relatively greater ovarian cancer risk, while those more 3' confer relatively more breast cancer risk - a notable exception is the 5382insC mutation which, while extremely 3', is associated with high ovarian risk. These data have not been reproduced in all datasets. The same group suggests that *BRCA2* contains a region in exon 11 which confers greater ovarian risk. The data are not yet sufficiently established to permit allele-specific risk counseling for mutation carriers.

The data on cancer risk associated with *BRCA1* and *BRCA2* mutations come from studies of kindreds assembled for linkage, so reasonably dramatic, and may represent the upper bounds of risk estimates. Easton et al (1995) estimates breast cancer risks approach 60-85% by age 85 (50% by age 50); ovarian risk is 40-60% with *BRCA1*, 10-15% with *BRCA2*. Breast cancer risk in male carriers of *BRCA2* mutations is increased, but the frequency of BRCA2 mutations in male breast cancer patients is low. Penetrance figures for less selected populations will be forthcoming shortly. The spectrum of cancers associated with *BRCA1/2* mutations is incomplete: *BRCA1* includes small prostate and colon risks; *BRCA2* pancreas, stomach, larynx, ocular melanoma among others.

A group of mutations (*BRCA1* 185delAG, 5382insC; *BRCA2* 6174delT) has been found in about 2% of individuals of Ashkenazi Jewish descent. These represent founder effects; the estimated frequency of *BRCA1* mutations in the general population is about 0.0006. In a large collaborative study, we found a high prevalence of these mutations in families with appropriate history: data suggest the delT mutation is considerably less penetrant than the others. This would have significant implications for risk counseling. Data suggest that: not all at-risk individuals choose to be tested; motivations include information for children; to learn not a mutation carrier; to improve cancer risk management. Most undergoing testing and genetic counseling adjust to the information in the short term: some require additional psychological support. Data regarding surveillance and prophylactic surgical options will be briefly reviewed.

**Retroviral Vector Therapies for Breast and Ovarian Cancer**

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Although effective treatments for breast cancer predated the identification of causative molecular defects in humans, it is widely hoped that an understanding and/or manipulation of the key genetic events will lead to even more effective therapies. Powerful methods of positional cloning and gene identification have provided the breast cancer genes, BRCA1 and BRCA2, which are together responsible for the majority of cases of hereditary breast and ovarian cancer. Although the BRCA1 gene is rarely mutated in sporadic breast or ovarian cancer, levels of BRCA1 mRNA and protein are markedly decreased in the majority of sporadic cancers. This suggests that hereditary and sporadic breast cancer share common genetic themes and that treatments aimed at increasing levels of BRCA1 or BRCA2 may be useful for both hereditary and sporadic cancers.

A variety of gene-based approaches have been proposed as therapies for cancer. Retroviral vectors show specificity for tumor cells because the vector nucleic acids only integrate into dividing cells, providing a rationale for retroviral vector gene therapy for cancer. We have demonstrated that gene transfer of the retroviral vector LXSN-BRCA1 inhibits the growth of sporadic breast and ovarian cancer cells and suppresses growth of established breast and ovarian tumor models in nude mice. Mutant BRCA1 genes do not growth inhibit or tumor suppress providing additional evidence that BRCA1 is a tumor suppressor gene.

As an initial step towards gene therapy for ovarian cancer we conducted a Phase I/II trial to assess the pharmacokinetics and toxicity of intraperitoneal vector therapy. Clinical grade retroviral vector was produced under cGMP (current Good Manufacturing Practices) and tested for titer (5 x 107/ml), sterility, and in vitro efficacy. Following placement of an indwelling port-a-cath in patients, a dose escalation study was performed of four daily intraperitoneal infusions spanning doses from 3 mls to 300 mls at half-log intervals (23 cycles in 12 patients). Pharmacokinetics was assessed by PCR and southern blots detecting vector DNA and toxicity was evaluated by clinical exam and fluid analysis.Three of 12 patients developed an acute sterile peritonitis which spontaneously resolved within 48 hours. This presentation resembled that noted in immunocompetent mice given vector during oyster glycogen induced chronic peritonitis. Plasma antibodies to the retroviral envelope protein were detected in only 1 patient three months after initial treatment, but not in others despite repeat dosing for an interval of up to 4 months. PCR analysis of patient post-treatment peritoneal fluids revealed stable, transduction capable vector 24 hours after infusion. The presence of stable vector was inversely associated with peritoneal CH50 levels supporting the presumed link between complement activation and retroviral vector stability. Gene transfer was documented by PCR, southern blot and RT-PCR.

The vector-related complication of peritonitis was observed in three patients but resolved quickly as in preclinical mouse studies. Intraperitoneal infusion of retroviral vector produces stable vector, particularly in a subclass of patients with low peritoneal fluid CH50 levels. Ovarian cancer may provide an important model for retroviral gene therapy studies due to vector stability, lack of a significant antibody response, and access to tumor by intraperitoneal therapy.

## WHAT IS THE FUTURE OF BREAST CANCER CHEMOTHERAPY?

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Systemic combination chemotherapy administered to women with breast cancer in the adjuvant or metastatic settings has to date had only a modest impact on clinical outcome. For women with metastatic disease palliation from symptoms is possible, and there is likely a small improvement in survival, confined to patients with a partial or complete response. For young women who are at risk for recurrence after primary surgery, adjuvant therapy will reduce the annual odds of death by about a third. For women with involved lymph nodes this translates into an absolute improvement in survival of around 6% at 10 years after diagnosis. While these absolute numbers are small, the public health impact of such therapy can be substantial due to the frequency of this malignancy. It follows that any improvements which can be made in cytotoxic breast cancer therapy in future will have an impact on substantial numbers of women.

Strategies to improve these results will continue for the foreseeable future to focus on 3 broad areas of research: Optimizing currently available agents, identifying novel cytotoxics and developing non-cytotoxic therapies. For the past decade attempts to optimize available agents have concentrated on intensification of dose. Preclinical and retrospective clinical models have suggested that intensification of drug dose will increase log tumor cell kill and potentially have a beneficial impact on clinical outcome. Prospective evaluations of dose intensity have been reported. These suggest that inadequate doses will produce inadequate response rates and duration of remission. There are also intriguing data available to suggest that tumors which over express the transmembrane glycoprotein Her-2 are more likely to respond to dose intensification of doxorubicin-based therapy than tumors which are low expressors. Dose intensity finds its ultimate expression as high-dose therapy with autologous stem cell support. This strategy produces high response rates, and a couple of small randomized trials suggest that disease-freee survival may be prolonged. However, an impact on survival for women with metastatic disease has not yet been conclusively demonstrated. In the high-risk adjuvant setting single arm studies of high-dose therapy have reported good results for survival and disease-free survival after 5 years of follow-up. Whether these results are due to improved efficacy or to patient selection is not yet clear. Large Intergroup randomized trials comparing high-dose therapy with stem cell support to more conventional dose treatment are currently accruing patients. The future of role of high-dose therapy in the management of women with breast cancer will be determined by the results of these trials.

A number of novel cytotoxics, notably the taxanes and vinorelbine, have substantial activity in the treatment of both sensitive and resistant metastatic breast cancer. These agents may improve palliation for women with advanced disease. It is possible that they will also have a role to play in adjuvant therapy, as these agents, notably docetaxel, have activity in metastatic disease which is resistant to doxorubicin-based chemotherapy. Trials evaluating this hypothesis are just beginning. Combination chemotherapy with taxanes and doxorubicin is highly effective in inducing responses in women with advanced disease and the available data from small phase II studies report response rates of over 80%. While high-dose therapy and novel cytotoxics can clearly induce remissions in

the majority of patients with minimally pretreated metastatic disease, this has not reproducibly been translated to date into improvements in remission duration. The available data suggest that the residual burden of truly resistant cells is sufficiently large and capably of sufficiently rapid regrowth that the median duration of remission remains around a year at most. The challenge for cytotoxic therapy in the decade to come will be to improve on remission duration.

A variety of strategies offer the potential to augment cytotoxic drug activity and will be the focus of intensive study in the next several years. Current chemotherapeutic approaches can cause complete remission, providing a platform on which to investigate agents which might overcome drug resistance and prolong remission duration. Non-cross resistant therapies which are under investigation in this context include immune therapies following high-dose chemotherapy, antibodies against growth factor receptors, angiogenesis and matrix metalloproteinase inhibitors. Strategies which non-specifically reverse drug resistance (such as agents which reverse MDR activity or inhibit DNA repair) may be non-specifically toxic and not necessarily augment the therapeutic index of active cytotoxics. More specific strategies are, however, under investigation. Agents like UCN-01, which suppress the G2 checkpoint function in cells with abnormal p53, may specifically improve the therapeutic index of alkylating agents in tumors with this genotype. Similar specificity may be possible using antibodies to Her-2, which have been shown in *in-vitro* systems to augment the cytotoxicity of cisplatin against Her-2 overexpressing tumor target cells. Preliminary phase II studies of this strategy have been encouraging. The toxicity of cisplatin against normal non-Her 2 overexpressing cells would not be expected to be affected by this strategy and thus the therapeutic index may be improved. Encouraging preclinical data also support the hypothesis that agents which interfere with the function of the tumor matrix, such as angiogenesis inhibitors or matrix metalloproteinase inhibitors, may be capable of augmenting the activity of conventional cytotoxics. Such approaches should be investigated for their ability to prolong remission duration after or in conjunction with cytotoxic chemotherapy.

The challenge for the next several years will be to determine if the increases in reponse rate which have been observed with high-dose therapy and novel chemotherapeutic agents will correlate with improvements in disease-free and overall survival in the adjuvant setting. In addition, intense investigation of chemotherapy in conjunction with novel strategies based on an increased understanding of tumor cell biology can be anticipated in patients with metastatic disease.

# Basic and Clinical Aspects of Breast Cancer

## *Abstracts of Poster Presentations*

# Basic and Clinical Aspects of Breast Cancer

*Poster Session A*

*Saturday, March 8*
*4:00 p.m. - 6:00 p.m.*

Basic and Clinical Aspects of Breast Cancer

Down-regulation of an Alternately Spliced BRCA1 Protein in Various Breast and Ovarian Cancer Cell Lines. P. LouAnn Cable[1], Frank J. Calzone[2], and Cynthia A. Afshari[1]. [1]Laboratory of Molecular Carcinogenesis, National Institute of Environmental Health Sciences, Res. Tri. Pk., NC; [2]Molecular Biology, Amgen Inc., Amgen Center, Thousand Oaks, CA.

Individuals with inherited mutations in the BRCA1 gene have a predisposition to early onset breast and ovarian cancer. However, the function of the BRCA1 protein is not understood. Previous studies show that twenty-two exons encode for full length BRCA1 protein, but that alternately spliced forms exist. One splice variant, BRCA1-Δ11b, lacks most of exon 11, including a nuclear localization signal. The BRCA1-Δ11b transcript was shown to be expressed at the mRNA level in a variety of cell lines and tissue samples. In this study we demonstrate the expression of a 120 Kd BRCA1 protein product that we believe is translated from the BRCA1-Δ11b transcript. In addition, examination of a variety of human breast and ovarian carcinoma cell lines show down-regulation of the BRCA1-Δ11b protein. Cellular localization and putative function of the BRCA1-Δ11b protein will be discussed.

A-2

Basic and Clinical Aspects of Breast Cancer

Retinoic Acid Receptor-Alpha Expression Correlates with Retinoid-Induced Growth Inhibition of Human Breast Cancer Cells Regardless of Estrogen Receptor Status. Patrick Fitzgerald*, Min Teng¥, Roshantha A.S. Chandraratna¥, Richard A. Heyman* and Elizabeth A. Allegretto*. *Ligand Pharmaceuticals, Inc., Department of Retinoid Research, 10255 Science Center Drive, San Diego, CA 92121. ¥Allergan, Inc., Departments of Retinoid Chemistry and Biology, 2525 Dupont Drive, Irvine, CA 92715.

Retinoic acid receptor-alpha (RARα) has been shown to play a role in retinoid-induced growth inhibition of human breast cancer cell lines that express the estrogen receptor (ER). The dogma in the field has been that ER-positive breast cancer cell lines respond to retinoid treatment because they express RARα, whereas ER-negative breast cancer cell lines are refractory to retinoid treatment and have been thought to express little or no RARα. We set out to test several ER-negative breast cancer cell lines for expression of RARα protein and responsiveness to retinoids in growth inhibition assays. Of six ER-negative breast cancer cell lines that were tested, one (SK-BR-3), had high levels of RARα protein as measured by ligand-binding immunoprecipitation (~50 fmol/mg protein) and also displayed sensitivity to growth inhibition by retinoids (9-*cis*-retinoic acid $EC_{50}$ ≈3 nM). These cells were more sensitive than an ER-positive cell line, T-47D, which expressed ~35 fmol RARα/mg total protein (9-*cis* retinoic acid $EC_{50}$ ≈50-100 nM). Another ER-negative cell line, Hs578T, also expressed RARα (~22 fmol/mg) and was sensitive to retinoid-induced growth inhibition, albeit, to a lesser extent than SK-BR-3 or T-47D cells. In contrast, the other ER-negative cell lines tested expressed low (<10 fmol/mg) or no detectable levels of RARα protein and also did not respond to retinoids in growth inhibition assays. An RARα agonist displayed 100x greater potency than an RARβ,γ agonist in growth inhibition of both T-47D and SK-BR-3 cells, suggesting RARα involvement in the process. Furthermore, an RARα-specific antagonist completely abolished the growth inhibition induced by RAR agonists, implying that the activity of the agonists is exerted solely through RARα, not RARγ, which is also expressed in both cell lines. Additionally, while RXR compounds are weakly active in growth inhibition of the RARα-positive cell lines, they markedly increased the growth-inhibitory activity of RAR ligands. RXR compounds also potentiated the action of the anti-estrogen, 4-hydroxy-tamoxifen, to growth inhibit T-47D cells. These findings have clinical ramifications in that patients with ER-negative tumors which are RARα positive may be candidates for retinoid therapy. Additionally, combinations of RXR ligands with RAR ligands (especially RARα agonists) and/or anti-estrogens may have utility in the treatment of breast cancer.

LOSS OF BRCA1 EXPRESSION RESULTS IN NEOPLASTIC PROGRESSION OF A BREAST CANCER CELL LINE. Lois A. Annab, Greg Solomon, J. Carl Barrett, and Cynthia A. Afshari National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709

There has been limited evidence demonstrating BRCA1's involvement in the progression of tumor carcinogenesis.We have attempted to study the role of BRCA1 as a tumor suppressor gene by examining the effects of an antisense construct in the breast cancer cell line BT474, which is non-tumorigenic (0/14 sites positive, after 18 months) when injected subcutaneously into nude mice. BT474 cells were infected with a retroviral vector containing the inverted partial human cDNA sequence of BRCA1 from nucleotide 121 in exon 1 to nucleotide 1025 in exon 11. Three independent experiments injecting pooled populations of BT474 cells infected with the antisense construct resulted in the growth of tumors ranging from 17% (2/12) of sites injected positive to 60% (3/5) of sites injected positive, compared to no formation of tumors in sense or control vector construct injections. Tumors were found to have enhanced growth potential, be positive for alu sequences, be positive by PCR for vector and insert sequences, and have little to no detectable levels of BRCA1 protein by western blotting analysis. Our data suggests that the down regulation of BRCA1 in the breast cancer cell line BT474 induces the formation of tumors in nude mice.

**Association of CSK Homologous Kinase (CHK) (formerly MATK) with HER-2/ErbB-2 in Breast Cancer Cells.** Sheila Zrihan-Licht, Jinkyu Lim, Iafa Keydar*, Mark X. Sliwkowski**, Jerome E. Groopman and Hava Avraham. Divisions of Experimental Medicine and Hematology/Oncology, Beth Israel Deaconess Medical Center, Harvard Medical School, Boston, MA, *Department of Cell Research and Immunology, Tel Aviv University, Ramat Aviv, ISRAEL, and **Department of Protein Chemistry, Genentech, Inc., South San Francisco, CA.

Protein tyrosine kinases, such as HER-2/ErbB-2, have been specifically linked to breast cancer. The Csk Homologous Kinase (CHK), formerly MATK, is a tyrosine kinase which contains the SH2 and SH3 domains and demonstrates homology (~50%) to the Csk tyrosine kinase. Like Csk, CHK is able to phosphorylate and inactivate Src family kinases. In this report, we investigated whether CHK is expressed in breast cancer tissues and whether it participates in the ErbB-2 signaling pathway in T47D and MCF-7 breast cancer cell lines. Immunostaining of the CHK protein in breast tissues demonstrated that primary invasive ductal carcinomas stage II (29 out of 35 cases) and stage I (32 out of 41 cases) expressed the CHK protein, while this protein was not detected in the adjacent normal tissues from the same patients. To study the role of CHK in the ErbB-2 signaling pathway, glutathione S-transferase (GST) fusion proteins containing the SH2 and SH3 domains of CHK were generated. CHK-SH2 and CHK-SH3-SH2, but not CHK-SH3 or CHK-$NH_2$-SH3, precipitated the tyrosine-phosphorylated ErbB-2 upon stimulation with heregulin. EGF or IL-6 stimulation of T47D cells failed to induce CHK-SH2 association with either ErbB-2, the EGF-receptor or the IL-6 receptor. *In vivo* association of the tyrosine-phosphorylated ErbB-2 with CHK was observed in co-immunoprecipitation studies with anti-CHK antibodies. EGF-R, ErbB-3 and ErbB-4 were not detected in the CHK immunoprecipitates or in the precipitates of the GST-SH2 fusion proteins of CHK, suggesting that the association of CHK with ErbB-2 upon heregulin stimulation is receptor specific (ErbB-2) and ligand specific (heregulin). These results indicate that CHK might participate in signaling in breast cancer cells by associating, via its SH2 domain, with ErbB-2 following heregulin stimulation.

Web-based Repository of Breast Cancer Gene Information. David L. Steffen, Rudeina A. Baasiri and David A. Wheeler. Department of Cell Biology, Baylor College of Medicine, Houston, TX 77030.

We are developing a database of breast cancer gene information extracted primarily from the published biomedical research literature (1). The purpose is to assist students and researchers to gain rapid access to the latest findings in the field. In this regard, it constitutes an information resource akin to a continuously updated literature review on the subject of breast cancer genetics.

The data in this resource are accessible using Web Wide Web browsers. Data are entered into and retrieved from the database through a set of interactive forms. Thus, information can be deposited and retrieved from this repository in a platform-independent, universally accessible manner. The availability of Web-based data entry will facilitate a world wide collaborative authoring of the database.

Information is stored as a collection of findings (or "facts") in relational format. It is collected into topical categories organized by gene name. Each fact is displayed with pertinent comments and MEDLINE reference information. This enables one to search by gene name and rapidly obtain a listing of known molecular genetic facts characterizing the given gene. Alternatively the database can be searched by facts.

We take further advantage of the Web by linking information in the database to other on-line resources whenever possible. For example, all references in the database are presented to the user as links to MEDLINE abstracts and all gene names are linked to On-Line Mendelian Inheritance in Man. Links to other external sources of information (e.g., GenBank) will be created as appropriate.

The Breast Cancer Gene Database is an extension of previous, similar efforts (2). These previous efforts have been successful as judged by the frequency with which the repository is queried. We will discuss the utility of organization of this information in the context of the rapid accumulation of knowledge in the field of tumor gene biology and other related Internet-based information resources.

This work is supported by the National Action Plan on Breast Cancer, Grant Number CA70532, from the National Cancer Institute.

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**Neu Differentiation Factor (Heregulin) Activates a p53-Dependent Pathway in Cancer Cells.** Sarah S. Bacus, Yosef Yarden, Andrei Gudkov and Khandan Keyomarsi. Advanced Cellular Diagnostics, Inc., Elmhurst, IL 60126; Wadsworth Center for Laboratories and Research, New York State Department of Health, Albany, NY 12201; University of Illinois, Chicago, IL 60612, Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot 76100, Israel

Previously we reported that neu differentiation factor (NDF)/heregulin (HRG) elevates tyrosine phosphorylation of its receptors erbB-3, erbB-4, and erbB-2 (through heterodimer formation). We also showed that NDF/HRG as well as antibodies to erbB-2 can cause growth arrest and differentiation in breast cancer cells. In this study, we report on the possible mechanism of NDF/HRG induced cellular effects. We show that NDF/HRG and antibodies to erbB2 receptors up-regulate expression of p53, by stabilization of the protein. This is accompanied by up-regulation of the p53 inducible gene, p21 CIP1/WAF1/sdi1, in a variety of cell lines; MCF7 and their derivatives MCF7/HER2, (HER2 transfectants) in ZR75T, and LnCap cells. The induction of p21 is further enhanced when cells are treated with both NDF/HRG and DNA damaging chemotherapeutic agents (i.e. doxorubicin or cisplatin). The erbB mediated induction of p21 is dependent on wild type p53 as it fails to occur in cells expressing mutated or MCF7 cells containing dominant negative p53 (BT474 and MDD2 respectively). Furthermore, p21 induction is capable of inactivating cdk2 complexes as measured by Histone H1 phosphorylation assays. Lastly, we show that in primary cultures of breast cancers (and other cancers), p21 is significantly induced in response to NDF/HRG treatment. Collectively, these observations suggest that the mechanism of breast cancer cell growth inhibition and differentiation via erbB receptors activation is through a p53 mediated pathway.

## "Basic and Clinical Aspects of Breast Cancer."

"Investigating the role of the Ataxia Telangiectasia Gene in Breast Cancer." D Gwyn Bebb[1], Karen Gelmon[1], Brian H Weinerman[2], Richard Gatti[3], Barry W Glickman[4].
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**Background.**

The heritable nature of breast cancer has been known for more than two centuries and was recently confirmed with the uncovering of the role of the BRCA1 and 2 genes in familial cases. Nevertheless, the fact that even BRCA1 and 2 'negative' individuals diagnosed with breast cancer confer an increased risk of developing the disease on their first degree relatives suggests the existence of other, as yet undefined, hereditary factors in its aetiology.

**Hypothesis**

One such heritable factor could be the Ataxia Telangiectasia(AT) gene. An association between heterozygosity for the gene and predisposition to breast cancer has been proposed by Swift and reaffirmed by others but has never been confirmed cytologically or molecularly. If heterozygotes are, as suggested, at a six fold increased risk of developing breast cancer and make up about 1% of the general population, then they could account for more than of 10% of all breast cancer cases.

**Approach**

We have set out to investigate this association, initially with phenotypic based assays but also by molecular means. Using the recently acquired understanding of the AT gene we have identified AT heterozygotes among the breast cancer population by performing the protein truncation assay on peripheral blood lymphocyte(PBL) derived RNA. We are also in the process of sequencing the entire AT gene in all 120 patients. In addition, we are, by the use of novel markers, investigating loss of heterozygosity(LOH) at the AT locus by comparing paraffin embedded tumour DNA with normal lymph node DNA obtained from 80 node negative breast cancer patients

**Conclusions**

If our preliminary results are confirmed by sequencing analysis, then our work indicates that the AT gene does indeed have a role to play in the aetiology of breast cancer. It also suggests that LOH at the AT locus could be an underlying mechanism. Our experience also suggests that due to the complexity of the AT gene and its lack of mutational hotpots, screening tests for AT heterozygosity may yet have to be phenotypically based rather that genetically based. The implications of defining a role for the AT gene in breast cancer reach to the heart of current diagnostic and management protocols and require further, detailed study.

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**"Basic and Clinical Aspects of Breast Cancer"**

FUNCTIONAL AND STRUCTURAL INTERACTIONS BETWEEN C-SRC AND HER2: INVOLVEMENT IN HUMAN BREAST TUMOR FORMATION. Allison P.Belsches, J.S.Biscardi, S.J.Parsons. Dept. of Microbiology, University of Virginia Health Sciences Center, Charlottesville, VA.

Previous studies by several laboratories have shown that functional interaction between members of the human EGF receptor (HER) receptor tyrosine kinase family, and c-Src, a non-receptor tyrosine kinase, may be involved in breast tumor formation. Our laboratory has demonstrated that overexpression of two tyrosine kinases, HER1 and c-Src, in a murine fibroblast cell line, C3H10T1/2, results in an EGF-dependent synergistic increase in tumor formation in nude mice, as compared to overexpression of either protein alone. This increase in tumorigenicity correlates with the formation of an *in vivo* complex between HER1 and c-Src, the appearance of two novel phosphorylation sites on HER1, and enhanced phosphorylation of receptor substrates, suggesting that association of the HER1 receptor with c-Src causes a hyperactivation of receptor kinase activity. Hyperactivation of the HER1 receptor might then lead to enhanced intracellular signaling, resulting in augmented growth. The HER family of growth factor receptors, including HER1 and HER2, exhibit a high percentage of structural homology. HER2 is overexpressed in approximately 30% of human breast cancers, and this overexpression is correlated with poor patient prognoses. In addition, elevated levels and/or activity of c-Src have been demonstrated in human breast cancers. Because of their structural similarity and elevated expression in human breast cancers, we propose that HER1 and HER2 interact with c-Src through similar mechanisms to augment cellular growth and tumor progression. Existing data from other laboratories support this hypothesis. Wildtype HER2 has been shown to be weakly tumorigenic when overexpressed in NIH-3T3 cells (Science 237:178-182), and stable HER2/c-Src complexes have been detected in human and rodent mammary tumor cell lines and tissues. To examine the potential interactions between HER2 and c-Src, our approach has been two-fold. First, we have studied human breast tumor tissue, as well as a panel of human breast carcinoma cell lines, for expression of HER family members and c-Src by Western blotting. In almost all samples, either HER1 or HER2 is overexpressed, but not both simultaneously. These data are consistent with the hypothesis that different HER family members are expressed at different times during tumor progression, with HER2 being expressed primarily in earlier stage tumors, and HER1 being expressed in later, more invasive tumors. Lysates from tumors and cell lines have also been tested for heterocomplexes containing c-Src and HER2. Of thirteen tumor tissues examined, three exhibited heterocomplex formation, and in three of nine human breast tumor cell lines which overexpress both HER2 and c-Src, immunoprecipitation assays indicate an *in vivo* complex formed between c-Src and HER2, independent of EGF stimulation. The finding of c-Src/HER2 complexes in known tumor and carcinoma cell lines suggests structural and functional interactions between HER2 and c-Src. The second approach has been to test the hypothesis that HER2 and c-Src interact synergistically to potentiate tumor formation and growth, by generating model cell lines in a known genetic background (using C3H10T1/2 murine fibroblasts as a parental line), which overexpress wildtype HER2 and c-Src, either separately or in combination, and testing them for growth properties, heterocomplex formation and downstream signaling events. Preliminary results indicate an immune complex of HER2 and c-Src in a cell line which overexpresses both tyrosine kinases. No complex between HER2 and c-Src is seen in a cell line expressing c-Src alone. Cell lines generated will be tested for tumorigenicity in assays of [$^3$H]-thymidine incorporation, growth in soft agar, and tumor formation in nude mice. Performed both in model systems and in breast cancer cell lines, these investigations may identify potential mechanisms of action between HER family members and c-Src, as well as target molecules active in breast cancer formation and progression.

Basic and Clinical Aspects of Breast Cancer

Development of *Brca2*-Deficient Mice. L. Michelle Bennett, Kimberly A. McAllister, Gina Goulding, Donna O. Bunch, E. Mitch Eddy, and Roger W. Wiseman. National Institute of Environmental Health Sciences, National Institute of Health.

Hereditary breast cancer results largely from mutations in the BRCA1 and BRCA2 susceptibility genes. Women who inherit a germline BRCA2 mutation are at an 80-90% risk for breast cancer development during their lifetime. Alterations in the BRCA2 gene have been associated with cancers at other sites including the pancreas, prostate, larynx, colon and ovary in addition to an increased risk for male breast cancer. We have characterized the mouse and rat homologues of the BRCA2 gene and found that they are 58% identical to the putative human protein at the amino acid level, similar to our previous observations with BRCA1. Approximately 720 amino acids at the carboxy-terminal region are more highly conserved between rodents and humans (75% identity) indicating this may represent an important functional domain. In order to understand better the consequences of carrying a BRCA2 germline mutation we are developing mice that harbor a defective copy of the *Brca2* gene by homologous recombination. We designed a targeting vector that, when properly integrated into the murine genome, would replace the 3' region of exon 10 through sequences 5' in exon 11 with a pgkNEO cassette. The targeting vector also contained the TK gene for negative selection. Embryonic stem (ES) cells were electroporated with the linearized targeting vector and subjected to positive/negative selection. Of the forty colonies that survived selection, three clones were identified by PCR and Southern analyses as harboring a properly targeted *Brca2* allele. Injection of these cell lines into blastocysts has yielded 18 chimeric mice which are being bred to wild-type animals to establish lines of *Brca2* deficient mice. Brother/sister matings will be used to determine if the *Brca2* gene is required for normal growth and development. We will observe the $Brca2^{(+/-)}$ offspring for developmental and neoplastic phenotypes that arise spontaneously, after carcinogen treatment, or that have been generated in crosses with p53 transgenic mice. In addition to trying to generate *Brca2* knockout mice through genetic crosses, we have taken two approaches to establish a $Brca2^{(-/-)}$ cell line *in vitro*. A $Brca2^{(+/-)}$ ES cell line will be subjected to high G418 concentrations with or without a second electroporation of the *Brca2* targeting construct to select subclones in which both alleles of *Brca2* contain the targeted mutation. Future studies with $Brca2^{(-/-)}$ cells will include their injection into blastocysts as well as forced differentiation *in vitro* with subsequent analysis of the effect of functional *Brca2* loss. Using these two approaches we hope to gain a better understanding of the function of and the developmental requirements of the *Brca2* gene. Creation of a mouse model for BRCA2 defects should allow us to examine interactions between the loss of *Brca2* function and genetic co-factors.

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Basic and Clinical Aspects of Breast Cancer

CHEMOPREVENTION OF MAMMARY CARCINOMA BY TARGRETIN™ (LGD1069), a RXR- SELECTIVE LIGAND.

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Retinoids (derivatives of vitamin A) act through binding to and activating the transcriptional properties of the retinoic acid receptors (RAR). In addition to the RARs there is a second family of RAR-related receptors the retinoid X receptors (RXR). Synthetic RXR-selective ligands have been characterized for their receptor and biological activity. LGD1069, an RXR-selective ligand, is the first molecule of its class to be evaluated in human clinical trials. Our recent work (Cancer Res. 56:5566-5570, 1996) demonstrated the efficacy of LGD1069 in inhibiting the incidence and multiplicity of tumors in the NMU-induced hormone dependent rat mammary carcinoma model. Targretin was well tolerated at all doses tested. By comparison, mean animal weights in both TAM-treated groups were significantly less that mean weights of vehicle-treated animals. No apparent differences were noted in sex steroid serum levels and reproductive cycles for Targretin-treated animals compared to vehicle-treated animals. In addition to inhibiting tumor induction, we also observed that there was an inhibitory effect on uterine wet weight of LGD1069-treated animals. To more carefully evaluate the effects of LGD1069 in the rat uterus, we used the immature rat uterine wet weight model. For these experiments, 21 day old female rats were given estradiol (2 μg/animal) for three consecutive days with and without concurrent oral administration of LGD1069 (10, 30 or 100 mg/kg). As expected, administration of estrogen to immature rats increased their uterine wet weights when compared to controls. In contrast, administration of LGD1069 inhibited, in a dose dependent mannner, estrogen-induced increases in uterine wet weight. The blunting of the estrogen-induced increase in uterine wet weight is an RXR-specific effect. Retinoids that interact solely with the RARs (TTNPB) do not exhibit this same effect. To evaluate the mechanism of LGD1069 inhibition, we are examining changes in uterine morphology, proliferation, apoptosis and interference with estrogen-induced signal transduction. In addition, we are studying the effect of LGD1069 on established NMU-tumors, to determine its therapeutic, rather than chemopreventive , efficacy in this model.

## Chromosome 7q31 breast cancer tumour suppressor gene

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Alterations in oncogenes and tumour suppressor genes are critical events in the multistep process leading to the development of cancer. Many putative locations of tumour suppressor genes have been identified by tumour loss of heterozygosity (LOH) studies. In breast cancer, significant losses are seen on chromosomal arms 1p, 1q, 3p, 6q, 7q, 8p, 11p, 11q, 13q, 16q, 17p, 17q, 18q, 22q and Xq. The highest frequencies of loss (over 80% LOH) have been observed at 7q31, near the microsatellite marker D7S522. Markers from this region are also lost at high frequencies in colon, head and neck, ovarian, pancreatic and prostate cancer, suggesting that a multitissue tumour suppressor gene maps to 7q31.

Human cellular senescence genes have been mapped into four complementation groups (A-D) by cell fusion studies. Monochromosome transfer has show that the group B, C and D genes map to chromosomes 4, 1 and 7 respectively, each to a region showing LOH in human tumours. We have carried out a functional and positional cloning approach to try to identify the chromosome 7, complementation group D gene, as we believe it is likely to be the target of losses at 7q31 is many tumour types.

A tagged copy of human chromosome 7 was transferred into an immortal human fibroblast cell line, and into human tumour cell lines, by monochromosome transfer. Introduction of the chromosome caused the hybrids to senesce, as determined by senescence specific endogenous B-galactosidae activity. However, during the fusion, immortal segregants occasionally arose, which had presumably inactivated the senescence gene on chromosome 7. We have generated over 50 such immortal hybrid cell lines. These have been screened with over 100 polymorphic microsatellite markers from chromosome 7 to identify any losses in the transferred chromosome. We have found losses in about half the lines. The losses centre on 7q31, indicating that the chromosome 7 immortalisation gene and multitissue tumour suppressor gene are the same. We have defined a minimal region for this locus, and are currently identifying and characterising candidate genes.

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Abstract for "Basic and Clinical Aspects of Breast Cancer"

**Recurrent Breast Cancer Detected By Repeated Quantitative Antimalignin Antibody in Serum (AMAS) Determinations.**

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The American Cancer Society estimates that deaths due to cancer could be decreased by 35% with early detection. In the case of breast cancer, indication of recurrence is obtained through the appearance of a mass, on mammography, or through the signs or symptoms of metastases. It would be useful to have a quantitative molecular determination which could give notice in advance of the appearance of symptoms or signs. We have examined whether the concentration of antimalignin antibody in serum (AMAS) might be helpful in this regard.

AMAS is a highly specific IgM autoantibody against a 10,000 Dalton cancer cell peptide. This antibody is universally present in low concentrations in normal human serum, somewhat higher concentrations in as yet unaffected members of high-risk families, and is cytotoxic/cytostatic *in vitro* at concentrations of picograms (femtomoles) per cancer cell. Because AMAS concentration is markedly elevated early in malignancy, it is determined as an aid to differential diagnosis and in screening high risk patients[1,2].

We now report a blind longitudinal study of 82 patients, with two or more AMAS tests per patient, 1 to 30 years post-surgery for breast cancer, 67 of whom over time remained in remission and 15 of whom had a recurrence. Of those remaining in remission, 67/67 retained normal AMAS concentrations; and of those with recurrence of malignancy, 15/15 had elevated AMAS concentration.

This blind longitudinal study was done against the background of, and with repeat specimens interspersed with single blind AMAS determinations in 1,175 patients with benign and malignant breast disorders as follows: 172 patients with non-tumor inflammatory breast disease, 238 with histopathologically benign breast tumors, 379 with malignant tumors of breast, 386 in remission post-surgery, and 3,078 normal controls.

Other evidence indicates that AMAS elevation occurs within days of transformation of cells to the malignant state[1]. Repeated AMAS tests therefore can be useful as an early warning system, before symptoms and signs appear, of the recurrence of breast cancer.

References:

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Structure and regulation of the *BRCA1* gene

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In the human the breast-ovarian cancer susceptibility gene *BRCA1* is housed within a complex region of 17q21. A tandem duplication exists which includes the 5' end of the *BRCA1* gene along with the nearby *NBR1* gene (previously known as *1A1-3B*). This duplication results in the presence of a partial pseudocopy of *BRCA1* (referred to as pseudo-*BRCA1*) and *NBR1* (referred to as *NBR2*). In the mouse the structure of this region is very different. The region is not duplicated and thus *BRCA1* lies directly adjacent to *NBR1*. This poster will describe the analysis of this region in both the human and mouse, comparing and contrasting the structure and regulation of these genes. We also show that in the human the *BRCA1* gene undergoes considerable alternate splicing, resulting in the generation of multiple mRNA species. Alternate splicing at the 5'end of the human gene suggests the presence of two distinct *BRCA1* promoters, functional analysis of which will also be presented. Characterization of the human *NBR2* gene has also been performed, including its structure, RNA and protein expression and its mutation analysis in breast tumours. The potential significance of the genomic and transcript complexity to *BRCA1* mutation analysis, regulation of *BRCA1* expression, interpretation of *BRCA1* mouse models and to the etiology of sporadic breast cancer will also be discussed.

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"Basic and Clinical Aspects of Breast Cancer"

**Biosynthesis of Estrogen by Aromatase in Human Breast Cells Produce Estrogen-induced Responses *In Vitro*.** Robert W. Brueggemeier , Anne L. Quinn, and William E. Burak, Jr., The Ohio State University, Columbus, OH 43210.

Paracrine interactions between the stromal and epithelial components of the breast are critical to the sustained growth and progression of breast tumors. Local estradiol production is maintained by aromatase, the cytochrome $P450_{arom}$ complex, and this enzymatic activity has been found primarily in the stromal component of breast tissue. Current research is focusing on developing *in vitro* cell culture systems that more closely mimic *in vivo* interactions in order to dissect actual paracrine signaling between these two cell types. Human fibroblasts were isolated from normal breast tissue and were maintained in a cell culture system utilizing a collagen I matrix. This model supports cell maintenance and subsequent differentiation on collagen rather than maximal proliferation, therefore allowing for a more accurate environment for the study of hormonal control and cellular communication. The objectives of this study are to determine (1) if estrogen biosynthesis by breast stromal cells is influenced by culturing conditions and (2) if the estrogen produced is sufficient to result in estrogen-mediated responses. First, initial experiments compared aromatase activity of patient fibroblasts grown on plastic versus collagen I using the tritiated water release method. Constitutive aromatase activity was found to be lower when cells were grown on a collagen gel for 4-7 days (7.7 fold lower; n=4 patients) using DMEM/F12 containing 10% DCC stripped serum. Fibroblasts grown on collagen I also appeared to be significantly more responsive to stimulation by 100nM dexamethasone (plastic: 6.5 fold induction; collagen: 27.8 fold induction; n=8-10 patients) when pretreated for 12 hours prior to measurement of aromatase activity. Furthermore, co-culturing fibroblasts and epithelial cells (ratio 5:1) on the collagen I gel for 4 days appears to increase constitutive aromatase activity significantly. In the second objective, determination of induced pS2 mRNA levels by Northern analysis was utilized as a sensitive assays for the measurement of hormone-induced responses in breast cancer cells. In these assays, human MCF-7 cells were grown in 6-well plates and treated with conditioned media obtained from cultures of patient fibroblasts. Total RNA samples were isolated using phase lock gel tubes, the RNA precipitated, and dissolved in RNAse-free water. RNA samples (10 μg each) were separated on 1.2% agarose gels, transferred to Nytran Plus membranes, and hybridized with $^{32}$P-labeled pS2 probe (0.434 kb cDNA) and with $^{32}$P-labeled 36B4 (0.562 kb cDNA). The amount of radioactivity was quantified using a PhosphoImager. MCF-7 cells grown in conditioned media obtained from cultures of patient fibroblasts treated with testosterone (100 nM) demonstrated elevated levels of pS2 mRNA at approximately 70% of the response induced by estradiol. This induction of pS2 could be blocked by the antiestrogen tamoxifen. Furthermore, MCF-7 cells grown in conditioned media from fibroblasts treated with both testosterone and the aromatase inhibitor 7α-APTADD did not produce pS2 gene expression, suggesting that aromatase conversion of testosterone to estradiol is needed to stimulate pS2 gene expression. Thus, these *in vitro* methods using cultured primary breast stromal fibroblasts allow for a more meaningful examination of the role of locally produced estrogen in the paracrine interactions between stromal and epithelial breast cells. This research is supported by NIH Grants R21 CA66193 and T32 CA09498.

ROLE OF GLYCOSPHINGOLIPIDS IN REVERSING ADRIAMYCIN RESISTANCE IN BREAST CANCER – NEW TARGETS FOR DRUG DESIGN. Myles C. Cabot, Ralph C. Jones, Won I. Cho, Anthony Lucci, Armando E. Giuliano, and Tie-Yan Han. John Wayne Cancer Institute at Saint John's Hospital and Health Center, 2200 Santa Monica Blvd., Santa Monica, CA 90404

Drug resistance is the leading cause of cancer treatment failure, and 30-50% of breast cancers eventually become resistant to chemotherapy. Our recent work with multidrug resistant (MDR) cancer cells (J. Biol. Chem. 271, 19530-36, 1996) revealed that these cells accumulate glucosylceramide (glc-cer). Glc-cer is the first modified product of ceramide in the glycosphingolipid anabolic pathway. Because glycosphingolipids impact cell differentiation, proliferation, and tumor progression, it was of interest to evaluate the role of glc-cer in MDR disease. Cultured MCF-7-AdrR (Adriamycin-resistant) breast cancer cells, radiolabeled with glycosphingolipid precursors, were employed to assess the influence of MDR reversing agents on lipid metabolism and cellular response to Adriamycin. Tamoxifen (5 μM) potently blocked cellular glc-cer formation (70%), was not toxic alone, but increased by 2-fold cell sensitivity to Adriamycin. Chemical analogs of tamoxifen that did not retard glc-cer synthesis were ineffective in enhancing Adriamycin toxicity. The antifungal triazole, ketoconazole, chemically unrelated to tamoxifen, also blocked cell glc-cer formation (60% inhibition at 10 μM). Alone ketoconazole rendered cells 75% viable, as did Adriamycin at 2.5 μM, but when drugs were mixed, reversal of MDR ensued with a marked decrease to 17% cell viability. Reasoning that agents like tamoxifen and ketoconazole could potentially elevate cellular ceramide levels by retarding ceramide glycosylation, we evaluated cell response to ceramide, as ceramide is known to induce apoptosis. MCF-7-AdrR cells were essentially resistant to ceramide toxicity (tested at 1-10 μM); however, when tamoxifen was given in combination with ceramide, cell viability decreased to 40% of control. Because MDR cells accumulate glc-cer, cell resistance to ceramide toxicity may be related to the enhanced capacity of the cells to eliminate ceramide via glc-cer synthase [ceramide + UDP-glc --> glc-cer]. These results reveal an intriguing association between MDR, MDR reversal, and glycosphingolipids, and identify a new target for, and a unique approach to, the treatment of chemotherapy-resistant tumors. (Supported by the Breast Cancer Research Program of the University of California, grant 0211, and the Ben B. and Joyce E. Eisenberg Foundation).

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**Decreased levels of the cell cycle inhibitor $p27^{Kip1}$ protein: prognostic implications in primary breast cancer.**

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Breast cancer is the second leading cause of cancer death in North American women. There is considerable need for reliable prognostic markers to assist clinicians in making important management decisions. Although a variety of factors have been tested, only tumor stage, grade, size, hormone receptor status and S-phase fraction are used on a routine basis. The cell cycle is governed by a family of cyclin dependent kinases (Cdks) whose activity is positively regulated by associated cyclins and by phosphorylation. $p27^{Kip1}$ is a member of a new class of cyclin dependent kinase inhibitors that regulate cell cycle progression from G1 into S phase by binding to and inhibiting the Cdk/cyclin complexes. $p27^{Kip1}$ activity and/or protein levels are upregulated by a variety of growth inhibitory cytokines including transforming growth factor-beta (TGF-b) and thus, provide an important link between extracellular regulators of proliferation and the cell cycle. As a negative regulator of cell cycle progression, loss of $p27^{Kip1}$ may contribute to oncogenesis and progression. However, mutations of $p27^{Kip1}$ have not been found in human tumors studied thusfar. We have performed immunohistochemical analysis of primary breast carcinomas from 168 patients and shown a consistent and progressive decrease in the levels of the cell cycle inhibitor $p27^{Kip1}$ with increasing grade in both in *in-situ* and invasive disease. The decrease in $p27^{Kip1}$ levels was confirmed by Western analysis and shown to be regulated post-transcriptionaly by *in-situ* hybridization. In addition, cyclin E and cyclin A-associated kinase activities were increased in tumors with low $p27^{Kip1}$ underscoring the functional importance of this change. On multivariate analysis, reduced $p27^{Kip1}$ was a strong, independent predictor of disease relapse, and thus may be a useful adjunct to the current assessment of breast cancer patients, providing an aid to important treatment decisions.

**Stimulation of anchorage-independent growth of MCF-7 human breast cancer cells by preadipocyte co-culture**

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## Abstract

Breast stromal adipocyte differentiation proceeds in women between the age of 35 and 50. It has been estimated that most breast cancers develop over a 10 to 15 years period, suggesting that adipocytes differentiation may occuring at a time when breast cancers are also developing prior to clinical detection. The present study investigated the hypothesis that recruitment of preadipocytes to adipocytes stimulates breast cancer cell growth via cell to cell interaction. We studied the effect of co-cultured preconfluent, confluent 3T3-L1 preadipocytes and differentiated adipocytes on anchorage-independent growth of MCF-7 cells in soft agar. Our results indicated that co-cultured preconfluent 3T3-L1 cells increased colony growth of MCF-7 by two fold. The confluent 3T3-L1 cells did not have any significant effect, however differentiated 3T3-L1 cells inhibited colony growth by about 59%. The stimulatory effect was specific for 3T3-L1 cells since the parent fibroblasts (3T3 cells) from which 3T3-L1 cells were derived, had no effect on MCF-7 colony growth. Co-culturing of preconfluent 3T3-L1 cells also stimulated MDA-MB-231 and MDA-MB-436, two other breast cancer cell lines colony growth by 43% and 60% respectively. In contrast no significant change was detected in human pancreas (Panc1) or prostate (PC3) cancer cells. Our finding of stimulation of breast cancer cell growth by proliferating 3T3-L1 preadipocytes suggested cell to cell interaction between preadipocytes and breast cancer cells and raised the possibility of a positive correlation between obesity and breast cancer.

## Transcriptional Activation By Wildtype But Not Mutant BRCA1 Sequences

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Mutations in the breast cancer gene BRCA1 may account for as much as 90% of inherited breast and ovarian cancers in families predisposed to both maladies. BRCA1 encodes an 1863 amino acid protein containing a putative zinc ring finger domain suggesting that BRCA1 may bind DNA. Over 100 distinct mutations in BRCA1 in affected individuals have been described. To date, no function has been ascribed to BRCA1. Here we report that, in a human cell line, the carboxy-terminal portion of BRCA1 acts as a strong transcriptional transactivator when fused to the GAL4 DNA binding domain. This activity is completely absent in sequences corresponding to four different mutations found in BRCA1 families, including three point mutations and one small deletion. These findings suggest a possible role for BRCA1 as a transcriptional transactivator, and that loss of this function may contribute to breast and ovarian cancers.

## The PEA3 Group of Ets-Related Transcription Factors in Mammary Cancer

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The PEA3 group of transcription factors belongs to the Ets family and is composed of three known members, PEA3, ERM and ER81, which are more than 95% identical within the DNA-binding domain, the ETS domain, and demonstrate 50% aa identity overall. These transcription factors possess functional domains responsible for DNA-binding, DNA-binding inhibition and transactivation (Monté *et al.*, Oncogene, 9: 1397, 1994). These factors are targets of signaling cascades such as the Ras-dependent one and may thus contribute to the nuclear response upon stimulation of cells and also to cellular transformation due to Ras (Janknecht *et al.*, Oncogene, 13: 1745. 1996). Their expression in certain breast cancer cells let suggest the involvement of these genes in the development, progression and invasion of this disease. In fact, transgenic animals bearing the *neu* oncogene have been shown to overexpress PEA3 mRNA in mammary adenocarcinomas. By characterizing the level of mRNA and protein expression of the three PEA3 group members in human breast cancer cell lines, we showed a differential expression of the PEA3 group members in these cells and more particularly ER81 expression is inversely correlated to estrogen and progesterone receptor expression (Baert *et al.*, Int. J. Cancer, 70, 1997). These data let us to hypothesize potential roles in specific breast cancer regulation pathways. Several targets of the PEA3 proteins have been determined to be the matrix metalloproteinase enzymes. It has thus been shown by others that overexpressed human PEA3/E1AF, confers invasive phenotype on MCF-7 cells (Kaya *et al.*, Oncogene, 12: 221, 1996) probably by turning on the type IV collagenase expression. Here we present a tri-dimensional model where we culture NMuMg normal and MMT cancerous murine mammary cells in a collagen matrix. These epithelial cells are cultured either alone or under fibroblast conditioned medium. In basal conditions, normal cells do not undergo tubular morphogenesis nor significantly express *pea3* genes. Fibroblast conditioned medium induces both tubular branching and *pea3* gene expression specially in invasive terminal end buds as shown by *in situ* hybridization. MMT cancerous cells wich scatter in the gels constitutively express PEA3 group members and fibroblast conditioned medium increases both scattering and *pea3* gene expressions.

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Cripto-1 modulates growth and differentiation on mammary epithelial cells.
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Growth, morphogenesis and lactation in the mammary gland is regulated by an interplay of systemic hormones and locally derived growth factors. In this context, Cripto-1 (CR-1) a protein of the epidermal growth factor family was found to be a weak mitogen for the HC11 mouse mammary epithelial cell line and to block prolactin-induced expression of β-casein and other milk proteins in these cells and in primary epithelial explant cultures. Also, CR-1 can induce branching morphogenesis in NMuMG mouse mammary epithelial cells when embedded in collagen type I gels, suggesting a role in the development of the mammary gland. The signal transduction cascade for this ligand include tyrosine phosphorylation of the SH2-containing adaptor protein Shc and increase an mitogen -activated protein kinase (MAPK) activity in HC11 cells. In order to establish the signal transduction pathways used by CR-1 to modulate the differentation of this mammary epithelial cell line we studied the inhibitory response of CR-1 on β-casein expression after treatment with prolactin. We found that the inhibitory effect by CR-1 on prolactin induced β-casein expression can be significantly impaired when using a farnesyl transferase inhibitor that blocks p21ras farnesylation, also by a MEK inhibitor (PD 98059) and by a PI3-kinase inhibitor (LY 294002). As well, CR-1 induced the tyrosine-phosphorylation of the p85 subunit of PI3-kinase on HC11 cells. These results suggest that CR-1 regulates differentiantion of HC11 cells by activating the ras/raf/MEK/MAPK pathway and PI3-kinase.

The Steroid Receptor Coactivator SRC-1 Mediates Estrogen Responsiveness Via an Unusual Estrogen Response Element. James DiRenzo, Bettina Hanstein, Molly Yancisin and Myles Brown. Dana Farber Cancer Institute, 44 Binney Street Boston MA 02115.

Breast cancer is the most common cancer afflicting women in the United States and the second leading cause of cancer death. Critical predictions as to the biological behavior, and thus the appropriate therapeutic strategy, of breast cancers can be made based upon the status of the estrogen receptor (ER), a member of the nuclear receptor superfamily of hormone activated transcription factors. ER has been known to regulate target genes by binding to sequence-specific hormone response elements in the regulatory regions of such genes. However, other target genes including the gene encoding the transforming growth factor-beta (TGF-β3), have been shown to be responsive to estrogen but have no recognizable estrogen response element, suggesting that other factors may function to mediate the activities of ER in genes lacking classical EREs. The binding of estrogen to the estrogen receptor triggers a complex series of biochemical events which transmit the hormone binding signal to the general transcription machinery, resulting in the modulation of RNA polymerase II activity. A critical element of this coactivation is the recruitment of the recently identified steroid receptor coactivator, SRC-1. SRC-1 has been shown to interact with several members of the nuclear receptor superfamily in a hormone dependent manner. The identification of a domain of SRC-1 which contains significant homology to members of the basic helix loop helix family of transcription factors lead us to investigate the possibility that SRC-1 may itself contain intrinsic DNA binding activity. In this study we examine the role of SRC-1 in the regulation of an unusual estrogen response element contained in the 5' untranslated region of the gene encoding transforming growth factor β3. We present evidence that SRC-1 forms a complex on this element and is capable of activating transcription in a manner that is dependent upon this element. Furthermore, we present data which suggests that SRC-1 may mediate responsivness to estrogen via this element. These studies suggest that the presence of SRC-1 binding elements in the regulatory regions of genes may identify a novel class of estrogen responsive genes.

## SSCP, LOH ANALYSIS AND TISSUE MICRODISSECTION TO STUDY SPORADIC P53 MUTATIONS IN MULTISTEP BREAST TUMORIGENESIS

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The p53 tumor supressor gene is one of the most frequently mutated in human breast cancer.
To determine the stage of tumor development at which mutation occurs we are studying microdissected premalignant and preinvasive lesions from 17 cases whose tumors have been identified as having p53 mutations. These mutations were identified by SSCP analysis of exons 4 through 8 and sequence analysis of DNA from fresh frozen tumor samples. Two cases have been found to have mutations in exon 4, six in exon 5, four in exon 6, three in exon 7 and two in exon 8. All cases are from women who had node negative primary breast cancer. Sixteen of the cases are invasive ductal carcinoma and one invasive lobular carcinoma. In sixteen of the cases the invasive tumor is associated with DCIS. A range of premalignant changes from mild hyperplasia to atypical ductal hyperplasia is also present in several of these cases.
DNA is extracted following microdissection of lesions of interest. These samples are then analysed by SSCP and LOH is evaluated at two sites - TP53 and D17S513.
In cases we have analysed we have been able to demonstrate the same shift in the formalin fixed paraffin embedded (FFPE) tumor as in the unfixed frozen sample. Shifts in DCIS are accompanied by LOH at TP53 in preliminary cases studied.
SSCP and LOH analysis can be performed on microdissected FFPE tissue samples. The results of SSCP on FFPE tissue agree with SSCP analysis of unfixed frozen tumor samples. p53 mutations can be detected in DCIS suggesting that it is an early event the development of some invasive ductal carcinomas.

Disrupting insulin-like growth factor-1 (IGF-1) receptor inhibits breast cancer invasion. Dunn, Sandra E.[1], Doerr, Monica E.[2], Palmantier, Remi.[1], Baserga, Renato[3], and J. Carl Barrett[1].

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Metastasis is the major cause of treatment failure in breast cancer. The initial steps in metastasis involve cellular adhesion, and invasion through the basement membrane. Growth factors may play an important role in metastasis. Inhibition of the insulin-like growth factor-1 (IGF-1) receptor results in a loss of the metastatic phenotype in murine carcinomas cells. Further, IGF-1 was recently reported to stimulate invasion of the breast cancer cell lines MCF-7 and MDA-MB-231, although the mechanism in which IGF-1 transmits its signal to invade remains unknown. In this study, we show that the human breast cancer cell lines MDA-MB-231 and MDA-MB-435 are stimulated 2-3 fold to invade through type IV collagen in the presence of IGF-1 (10 ng/ml). Furthermore, IGF-1 increased cellular adhesion of both cell lines to collagen IV. Invasion was blocked by the dominant negative soluble receptor 486/STOP and by inhibitors of signal transduction. MDA-MB-435 cells stably transfected with the 486/STOP construct adhered and invaded through collagen IV less than the vector controls. Wortmannin (1.0 uM), a phosphotidylinositol 3 kinase (PI3K) inhibitor blocked tumor invasion by 93%. In contrast, rapamycin, a pp70 S6K inhibitor had no effect on tumor invasion. In conclusion, disruption of the IGF-1 pathway through PI3K inhibits breast cancer invasion.

## DELETIONS AT CHROMOSOME 13q12-q13 ASSOCIATE WITH BREAST TUMOR PROGRESSION AND PATIENT SURVIVAL

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Human breast tumors were analyzed with polymorphic microsatellite markers from chromosome 13q12-q13 carrying the BRCA2 and RB1 genes, one of the markers is located within the RB1 gene. Loss of heterozygosity (LOH) at 13q was observed in 55 (39%) of the 142 tumors analyzed and 16% of tumors when analyzed with the RB1 marker alone. LOH at 13q associated with a high fraction of cells in the S-phase ($p < 0.001$) and low progesterone receptor content ($p = 0.04$). No association was found between LOH at 13q and axillary lymph-node involvement, tumor size, tumor type, estrogen receptor content, aneuploidy, or patient age. Patients with tumors exhibiting LOH at 13q had early recurrence of the disease and lower survival rate than other patients. There was about 25% difference in disease-free survival between the groups at 5-years follow-up. Multivariate analysis adjusting for tumor size, lymph-node involvement, S-phase fraction, and estrogen receptor content showed that LOH at 13q is an independent prognostic variable. Patients with LOH at 13q have a threefold risk of recurrence and death. A significant association was found between LOH at 13q and LOH at chromosomal arms 3p, 6q, 9p, 11q, and 17p. Our results suggest that the BRCA2 gene or another gene in the vicinity of BRCA2 participates in the suppression of tumor growth in breast tissue. Inactivation of the gene results in a high rate of cell proliferation and poor prognosis.

Growth regulation in human breast cancer cells: Common themes and novel observations with new human breast cancer cell lines. Kathleen W. Ignatoski, Cheryl A. Dilts, Michele L. Dziubinski, Carolyn I. Sartor, and Stephen P. Ethier. Department of Radiation Oncology, Univ. of Michigan Medical School, 1331 E. Ann St., Ann Arbor, MI 48109-0582.

The broad aim of our work is to understand how genetic alterations that occur in human breast cancer (HBC) alter important growth regulatory mechanisms that are operative in normal mammary epithelial cells. To perform these studies, we have developed methods for the isolation and serum-free growth of human breast cancer cells from primary and metastatic sites. We have also developed culture methods for the serum-free growth of normal luminal mammary epithelial cells. To date, we have isolated 12 new human breast cancer cell lines; four lines were derived from primary tumors, three from recurrent chest wall lesions, four from pleural effusion specimens, and one from a skin metastasis. These cell lines have been characterized for overexpression of oncogenes and loss of tumor suppresser genes known to be important in HBC. In addition, since these cells are cultured under well defined conditions, specific altered growth phenotypes, relative to normal luminal cells, can be studied. Thus, experiments with erbB-2 overexpressing HBC cells have indicated a role for this oncoprotein in the progressive acquisition of independence of both EGF-like growth factors and insulin-like growth factors. These experiments also demonstrated a role for activated erbB-3 in the growth factor independence phenotype. Some of the HBC cell lines we have developed express erbB-4. Studies with these cells suggest that expression of erbB-4, in combination with other members of the erbB family, influences whether HBC cells are growth stimulated or growth inhibited by heregulins or antibodies against the ectodomain of erbB-2. Another important subset of HBC cell lines in our panel overexpress the epidermal growth factor receptor (EGFR), and these cells exhibit distinctive growth phenotypes. Most notably, EGFR overexpressing breast cancer cells express constitutively activated STAT-3, independent of erbB-2 expression or activation status. In addition, our data indicate the presence of novel STAT-3 isoforms that are constitutively activated in these HBC cells. Most of our EGFR overexpressing HBC cell lines require activation of the receptor for continuous growth. In some cells (SUM-1315, SUM-229), EGFR activation by exogenous ligand is required. In cell lines where the EGFR gene is amplified and receptor levels are dramatically overexpressed (SUM-16), constitutive activation occurs in a ligand-independent manner. In still other of our cell lines, most notably SUM-102, EGFR activation occurs as a result of juxtacrine growth factor signaling. These cells synthesize heparin-binding EGF in the presence of progesterone, which yields constitutive activation of the EGFR. Finally, all of our HBC cell lines express constitutively tyrosine phosphorylated p125-focal adhesion kinase (p125-FAK), whereas normal mammary epithelial cells cultured under identical conditions do not. The presence of tyrosine phosphorylated p125-FAK correlates with survival and growth of HBC cells in soft agar. Thus, these studies indicate that the use of new human breast cancer cell lines that are studied at early passage and under defined conditions, can yield novel insights into how HBC cells regulate growth. Furthermore, by studying a panel of well characterized cell lines, one can begin to understand the cellular consequences of important genetic alterations that occur in HBC.

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uPA Inhibition and Suppression of Pulmonary Metastases of Rat Mammary Cancer: Comparison of Inhibitory Agents. Douglas M. Evans, M.D. and Kimberly D. Sloan-Stakleff,M.S. Calhoun Research Laboratory, Akron General Medical Center, Akron,Ohio

The role of plasminogen activators in the initiation of the proteolytic cascade required for cancer cell invasion and metastases has been clearly established. Inhibition of urokinase plasminogen activitor [uPA] is a proven method of suppression of experimentally induced pulmonary metastases of rat mammary cancer cells. Dose dependency of uPA inhibition has also been previously demonstrated with both PAI2 and amiloride [Evans and Lin, The AM Surg'95;61:692-697. Evans and Sloan-Stakleff, Abstract AACR Conf. March 1996]. The extent to which more potent uPA inhibition will result in more effective suppression of metastases is examined in this study. To this end a synthetic uPA inhibitor, 4-iodo benzo{b}thiophene-2-carboxamidine [B428], is compared to the known uPA inhibitory effects of amiloride. B428 has been shown to be a tenfold more potent inhibitor of uPA than amiloride in vitro. The level of amiloride used was doubled over the highest level in the prior study in order to approximate the 10:1 ratio of inhibitory potency. **Methods:** Two groups of Fisher 344 female rats were inoculated with $10^5$ MATB rat mammary cancer cells via the jugular venous system. Each group consisted of saline treated controls, B428 and amiloride treated animals. Group 1 received their respective drugs commencing at the time of inoculation at dosages of 20mg/kg/day of amiloride and 2.0mg/kg/day of B428 via continuous intraperitoneal infusion for a period of 10 days utilizing Alzet osmotic pumps. Group 2 received the same dose of each drug administered in an identical fashion commencing 3 days post inoculation. No external indications of toxicity were observed in animals receiving either drug. The study was terminated following ten days of delivery of inhibitors. The middle lobe of the right lung was harvested and sagittal sections were examined histololgically for the numbers of metastases by two blinded observers. The numbers for each group were averaged and compared to controls using the Student's T-test. **Results:**

| | n | #Mets | Std Error | |
|---|---|---|---|---|
| Group 1 Controls | 8 | 36.125 | +/- 5.47 | |
| Amiloride | 10 | 19.150 | +/- 5.21 | p=0.02 |
| B428 | 10 | 18.3 | +/- 3.67 | p=0.0065 |
| Group 2 Controls | 6 | 16.75 | +/- 1.56 | |
| Amiloride | 10 | 11.9 | +/- 4.26 | p=0.154 |
| B428 | 10 | 11.2 | +/- 2.10 | p=0.04 |

**Conclusions:** Suppression of pulmonary metastases by uPA inhibition is again demonstrated. B428 provides a greater level of suppression of pulmonary metastases than amiloride, with significant suppression when administered post inoculation of cancer cells, and at one tenth the dosage.

Modulation of Apoptosis in Human Mammary Epithelial Cells by Prostaglandin G/H Synthase 2. Robert W. Fisher and Steven A. Leadon, Curriculum in Toxicology and the Department of Radiation Oncology, University of North Carolina, Chapel Hill, NC, USA

An important component of the ability of cells to resist carcinogenic insult is the maintenence of genomic integrity in response to DNA damage. Metabolic activation of the chemical carcinogen benzo[a]pyrene generates reactive oxygen species that damage DNA, and in normal human mammary epithelial cells (HMEC184) the production of reactive oxygen species is transient and returns to basal levels when benzo[a]pyrene treatment is ended. These reactive oxygen species appear to be produced by the arachidonic acid cascade since treatment of cells with benzo[a]pyrene in the presence of inhibitors of prostaglandin G/H synthase (PGHS), indomethacin or ibuprofen, reduces the levels of one type of oxidative base modification, thymine glycols. In contrast, a human mammary epithelial cell line (HMEC184B5) immortalized by multiple treatments with benzo[a]pyrene produces high levels of reactive oxygen species that produce thymine glycols even in the absence of benzo[a]pyrene. These reactive oxygen species also appear to be produced by the arachidonic acid cascade since treatment with either indomethacin or ibuprofen significantly reduces the amount of thymine glycols. The high background levels of oxidative DNA damage in the immortalized HMEC184B5 cells was not due to slower removal of this base damage relative to the normal HMEC184 cells. When thymine glycols are induced by treatment with hydrogen peroxide, both HMECs removed thymine glycols with a similar efficiency. We investigated whether the high levels of thymine glycols in the immortalized breast cells could be due to the induction of the pathway which produces this oxidative DNA damage when the cells are treated with B[a]P, namely the arachadonic acid cascade. We examined the expression of the inducible form of PGHS, PGHS2, in the immortalized HMEC184B5 cells. We found that there are high levels of both PGHS2 mRNA and protein in the HMEC184B5 cells relative to the normal HMEC184 cells. Overexpression of PGHS2 has been shown to decrease the apoptotic response of rat intestinal epithelial cells. Apoptosis or programmed cell death is one mechanism whereby cells with high levels of DNA damage are removed from the general cell population. Loss or attenuation of this protective pathway may result in an increased number of cells harboring DNA damage, thereby increasing the probability of engendering genomic instability. We hypothesize that the overexpression of PGHS2 will attenuate the ability of the HMEC 184B5 cells to undergo apoptosis. We exposed HMEC184B5 cells to ionizing radiation to induce apoptosis. Apoptosis was measured by visualizing the production of a laddering of DNA fragments 180 base pairs or multiple in size, which is characteristic of apoptosis. Following treatment with either 8 or 16 Gy of ionizing radiation, no induction of DNA ladders was detectable by either 24 or 46 hours post-treatment. However, when HMEC184B5 are exposed to increasing doses of staurosporine, a protein kinase inhibitor shown to induce p53-independent apoptosis, DNA fragmentation is detected. Our results with ionizing radiation suggest that that HMEC184B5 cells cannot undergo apoptosis via a p53-dependent pathway. In order to further characterize these observations, experiments are in progress to quantitate apoptosis following staurosporine treatment in HMEC184 and HMEC184B5 cells using flow cytometry. The majority of research on apoptosis has been carried out in hematopoetic or fibroblast cell lines, however, it is becoming increasingly evident that there may be important distinctions in how breast epithelial cells modulate their response to DNA damage or other insults (see Mirto and Leadon abstract). Since the majority of human cancers are derived from epithelial cells, the characterization of pathways influencing apoptosis in this specific cell type should provide important information on the etiology of human breast cancer.

DNA Topoisomerase IIα and DNA Topoisomerase IIβ as Markers for Human Breast Carcinomas. Foglesong, Paul D. (1), Connolly, RitaT. (2), D'Andrea, Michael R. (2), and Reyes, Alonzo. (1) University of the Incarnate Word, San Antonio, TX (2) Rutgers University, Camden, NJ

The levels of DNA topoisomerase IIα (Topo IIα) and DNA topoisomerase IIβ (Topo IIβ) have been measured by immunohistochemistry with specific monoclonal antibodies in formalin-fixed, paraffin-embedded specimens of 49 human breast carcinomas. Topo IIα was elevated in 41 of these tissues (84%). However, high levels of Topo IIα were observed in only 2 of 10 normal tissues. The levels of Topo IIα correlated with those of Ki-67, a marker of cellular proliferation. Topo IIβ was elevated in 20 of 49 breast carcinomas tested (41%). However, high levels of Topo IIβ were observed in only 3 of 10 normal tissues. The levels of the two isozymes of Topo II were not correlated. Elevated levels of both isozymes were observed in 39% of the tumors.

The levels of mRNAs for Topo IIα and Topo IIβ were determined by *in situ* hybridization using specific oligonucleotides as probes in formalin-fixed, paraffin-embedded specimens of 44 human tumors. The mRNAs for Topo IIα and Topo IIβ were elevated in 33 tumors (75%) and 38 tumors (86%), respectively. Both mRNAs were elevated in 7 of 8 breast carcinomas tested. In contrast, only 1 of 6 normal tissues examined (normal thyroid) contained high levels of mRNA for either isozyme of Topo II. The levels of mRNA for both isozymes were highly correlated in all 50 tissues examined except 2 breast carcinomas. However, the levels of protein for the two isozymes of Topo II correlated for only 50 % of the specimens. These results suggest that the expression of the isozymes of Topo II may be regulated translationally.

The levels of mRNA and protein for the two isozymes of Topo II were measured simultaneously by double labeling using *in situ* hybridization followed by immunohistochemistry. These procedures were performed for 44 human tumors and 6 normal tissues. The cells of these tissues were classified into four groups: those with high levels of both mRNA and protein, those with low levels of both, those with high mRNA levels but low levels of protein, and those with low mRNA levels but high levels of protein. Of the 8 breast carcinomas tested for Topo IIα 7 exhibited cells most of which had high levels of protein but low levels of mRNA; the other specimen revealed cells that had low levels of protein but high levels of mRNA. However, for Topo IIβ 6 of the specimens exhibited cells most of which had low levels of protein but high levels of mRNA, 1 contained cells most of which were elevated in both protein and mRNA, and 1 was negative for both. These results are consistent with those previously obtained by *in situ* hybridization alone which indicated that the levels of mRNA for Topo IIβ are higher in some breast carcinomas (3 of 8) than the corresponding levels of mRNA for Topo IIα.

Thrombospondin-1 and -2 mRNA levels in normal, benign and neoplastic human breast tissues. Correlation with prognostic factors, tumor angiogenesis, and fibroblastic desmoplasia. Lucien Frappart, Nicolas Bertin, Philippe Clezardin and Robert Kubiak, Department of Pathology and CNRS UMR 5641 (N. B., L.F.) and INSERM Research Unit 403, Pavillon F (P. C.), Edouard Herriot Hospital, Lyon, France and Department of Oncology, Medical Academy of Lodz, Poland (R. K.).

Thrombospondin-1 (TSP1) is a 450-kDa extracellular matrix glycoprotein which modulates tumor growth, angiogenesis, and metastasis. Of the five structurally different TSPs described to date, only TSP2 is similar to TSP1 in terms of its molecular architecture, and TSP2 also modulates angiogenesis. Angiogenesis plays a relevant role in the biologic aggressiveness of breast cancer, and TSP1 is present in the tumor stroma (termed desmoplasia) of invasive human breast ductal carcinoma NOS. The present study was designed to identify and quantify TSP1 and TSP2 mRNAs in normal, benign and neoplastic human breast tissues using the reverse transcriptase PCR technique. We found that TSP2, like TSP1, was expressed in human breast tissues, and that TSP1 and TSP2 mRNA levels in invasive breast carcinoma NOS were significantly increased compared to those observed in normal and benign tissues.The expression of TSP1 and TSP2 in invasive breast carcinoma NOS does not significantly correlate with any of the prognostic factors studied (tumor size, lymph node status, morphology, hormone receptor status). However, when our study population is divided according to the quantity of tumor stroma, TSP1 (and possibly TSP2) mRNA levels and microvessel counts in desmoplastic-rich stroma of breast carcinoma NOS are significantly increased compared to those observed in desmoplastic-poor stromata.

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A PROSPECTIVE STUDY OF ACETYLATION GENOTYPE, MEAT INTAKE AND RISK OF BREAST CANCER. D.Gertig, S.Hankinson, H.Hough, K.Kelsey, W.Willett, D. Hunter & the NHS Research Group. Harvard University, Boston MA 02115

The metabolism of certain carcinogens, in particular heterocyclic amines, is influenced by polymorphisms in the N-Acetyltransferase-2 gene. Among Caucasians rapid acetylators are defined by presence of one or two wild type alleles; slow acetylators are homozygous for any combination of 3 of the mutant alleles. Heterocyclic amines (HCA's) are produced by high temperature cooking of meat and protein and have been found to be mammary carcinogens in rodents. Although data in humans are scarce, some epidemiologic studies have found positive associations with meat intake and breast cancer risk. The associations between meat intake (including cooking method), acetylator genotype and risk of breast cancer were examined in a case control study, nested within the Nurses' Health Study, a large cohort study of women in the U.S. 240 women who developed incident breast cancer after blood draw in 1989 and prior to June 1 1992, were matched to 240 controls. The multivariate OR for rapid acetylators compared with slow acetylators was 0.9 [0.6-1.4]. Rapid acetylators in the highest category of red meat intake did not have an increased risk compared with slow acetylators with the lowest red meat intake OR 0.8 [0.4-1.6]. The multivariate OR's for rapid acetylators in the highest category of chicken and fish intake were 0.7 [0.2-1.9] and 0.7 [0.4-1.3], respectively. Frequent intake of charred meat among rapid acetylators was not associated with an increased risk, multivariate OR 1.1 [0.4-2.7], although there was a non-significant elevation in risk for rapid acetylators in the highest category of roasted meat intake, multivariate OR 2.4 [0.7-8.0]. There were no significant interactions between either red meat intake or cooking method and acetylation genotype. Although in this population there is no association with acetylation, meat intake and breast cancer, other polymorphisms involved in metabolism of HCA's, in particular N-Acetyltransferase-1, may also be important and should be considered in future studies.

**RALOXIFENE HCL IN ER + METASTATIC BREAST CANCER.** Charles L.Vogel[1], Joan F. Glusman[2], George W. Sledge[3], Janine L. Mansi[4], Kenneth R. Kunz[2], William J. Gradishar[5]. [1]Comprehensive Cancer Research Group, Miami, FLA, [2]Eli Lilly, Indianapolis, IN., [3]Indiana University Hospital, Indianapolis, IN, [4]St. Georges Hospital, London, UK, [5]Northwestern University Hospital, Chicago, Ill.

Pharmacologic agents whose profile can mimic the favorable effects of estrogen on the skeleton and cardiovascular systems, without increasing the risk of cancer in the breast and uterus are needed. Raloxifene, a novel, nonsteroidal compound acts as a selective estrogen receptor modulator (SERM) producing estrogen agonist activity in selected target tissues such as bone and liver, together with estrogen antagonist activity in reproductive tissues such as the breast and uterus. Raloxifene has been clearly shown to be an estrogen antagonist, both *in vitro* and *in vivo*, in experimental breast cancer models. In an ongoing Phase II study of raloxifene in patients with recurrent estrogen receptor positive (ER +) breast cancer, patients receive 150 mg of raloxifene hydrochloride orally twice a day. Tumor response rates for the first 14 evaluable patients are reported. Eligibility criteria include: postmenopausal status, no prior chemotherapy for recurrent locoregional or metastatic disease, and bidimensionally measurable lesions. Prior adjuvant tamoxifen or chemotherapy must have been completed at least 1 year prior to study entry. Four patients received prior tamoxifen. For efficacy analysis, patients must have received at least 4 weeks of raloxifene treatment. Sixteen postmenopausal women have been enrolled (median age of 56 years). Of 14 evaluable patients, 2 (14%) attained partial responses, and 5 (36%) had stable disease for >6 months. The 2 partial responses have been durable, lasting 23+ and 17+ months, respectively. Both of these patients, who had lytic bone lesions were able to discontinue narcotic analgesics. Raloxifene was well tolerated, less than 1/3 reported mild vasomotor symptoms. Stable disease of >6 months has been recognized as a clinically meaningful parameter in hormonal treatment of metastatic breast cancer. Therefore, in addition to beneficial effects on bone mineral density and lipid profiles, raloxifene demonstrates antitumor activity in selected postmenopausal women with advanced breast cancer.

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CONTRIBUTION OF MOUSE MAMMARY TUMOR VIRUS (MMTV) SEQUENCES IN ACTIVATING CELLULAR ONCOGENES. Sandra L. Grimm and Steven K. Nordeen, Program in Molecular Biology and Department of Pathology, University of Colorado Health Sciences Center, Denver, CO 80262.

A consequence of mouse mammary tumor virus (MMTV) infection is the eventual formation of mammary adenocarcinomas, even though this retrovirus does not encode an oncogene. Although integration of the provirus is random, analysis of integration sites in MMTV-induced tumors demonstrated preferential integration near a limited set of genes. These genes have been termed *int*, for integration, and expression of these *int* genes plays a key role in the disruption of cellular homeostasis leading to mammary tumorigenesis. It is quite striking that the provirus almost always integrates upstream of these *int* genes in the opposite transcriptional orientation or downstream of the genes in the same orientation. This positions the 5' end of the MMTV long terminal repeat (LTR) closest to the *int* promoter, with MMTV-driven transcription directed away from the *int* gene. Integration is almost always outside of the *int* coding region, allowing expression of an unaltered gene product. In these cases, expression occurs from the authentic promoter and NOT the MMTV promoter. Thus, increased oncogene expression occurs via an enhancer insertion mechanism rather than a promoter insertion event.

We constructed a series of vectors that recapitulate MMTV integration at the *int-2* genomic locus and allow monitoring of expression from both the *int-2* and MMTV promoters. These vectors contain the mouse *int-2* promoter driving expression of a luciferase reporter gene. Upstream of the *int-2* promoter, in the opposite transcriptional orientation, the MMTV LTR, or mutants thereof, controls expression of the CAT reporter gene. The arrangement of these vectors mimics the arrangement of transcriptional control sequences found in MMTV-induced tumors. We have employed these vectors as a model to show that MMTV sequences strongly enhance *int-2* promoter activity and that the elements critical for mammary specificity in the 5' region of the MMTV LTR are also critical to this enhancement.

In particular, we have identified a strong enhancer element in the 5' end of the MMTV promoter, from -948 to -872 relative to the start of transcription. Through transient transfection assays comparing mammary and non-mammary cell lines, we have shown that this enhancer element is absolutely critical for basal transcription of MMTV in mammary cells, but is not required for expression in a non-mammary cell line. DNaseI footprinting experiments have identified three functional domains within this 76 base pair enhancer. Point mutations in any one of these functional domains, leaving the other two domains intact, abrogates mammary-specific expression. To better understand the mechanisms governing the mammotropism of MMTV, we are currently characterizing an unknown factor that binds to one of these functional domains.

## *BASIC AND CLINICAL ASPECTS OF BREAST CANCER*

### QUALITY ASSURANCE IN SCREENING MAMMOGRAMS : first results of the Brussels Project for Breast Cancer Screening.

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2. Public Health School of UCL; Brussels

**Context and objectives** : the Brussels Project for Breast Cancer Screening aims to implement a quality assurance system for screening mammograms, through the organization of double reading and the follow up of quality indicators.

**Material and methods** : at 13/3/96, 15 months after the project started, 10 radiology centers actively participated; in this first period, the quality indicators were studied only for centers who uniquely practised screening. 8946 double reading have been realized within this period. Follow up results for women with a positive screening was searched by contacting the radiologist or the practitioner.

**Results** : recall rates was 7.8 %, biopsy rate was 0.5 %, crude cancer detection rate was 3 for thousand, positive predictive value of biopsy indication was 57 %, malignant to benign biopsy ratio is 1.3.

**Conclusion** : in the studied centers, recall rate is slightly too high, but this does not result in an excess of invasive exams : cancer detection rate corresponds to the expected rate in a screening programme. In the future, notification of the type of mammogram should allow to calculate quality indicators for all participating centers.

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CHARACTERIZATION AND CLONING OF AN ESTROGEN RECEPTOR-ß-SPLICE VARIANT, Bettina Hanstein, Hong Liu, Molly Yancisin and Myles Brown. Dana-Farber Cancer Institute, Boston, Ma 02115.

Recently a new member of the nuclear receptor family with highest homology to the estrogen receptor (ERα) has been cloned and was therefore termed ERß. In situ hybridization showed a high concentration of ERß in prostate, ovary and thymus. By screening of a rat prostate c-DNA library we identified a novel ERß transcript. Being otherwise 100% homologous to the previously published amino-acid sequence, it contains an insert of 18 amino-acids in the hormone binding domain. We have initiated functional studies to compare signaling by these two ERß forms to ERα. Hormone binding assays show a decreasing affinity for ERα > ERß (-insert) > ERß (+insert) to 17ß-estradiol. We expressed the hormone binding domain of both forms of ERß as a GST-fusion protein and performed affinity interaction assays for proteins associating with the hormone binding domain of these receptor forms compared to ERα. We were able to show that coactivators such as ERAP160 bound ERß as they bind ERα, but did not bind the alternative splice variant containing the insert. Data from transient transfections will be presented aimed to clarify the function of this unique example of regulation in the nuclear receptor family.

**Cytochrome P450 in the Rat and Human Breast**

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In order to resolve the issue of whether P450 in the breast plays a role in the sensitivity and/or resistance of the breast to hormones and hormone antagonists, studies of the P450 profiles in the rat and normal human breast were initiated. Human breast samples were obtained from reduction mammoplasties and P450 isoforms were detected by RT-PCR and Western blotting. The P450 content of the human breast was between 0.5 and 7.8 pmol/g wet weight of tissue. In the rat it varied between 5 pmol/g in the 6-week-old to 30 pmol/g in the lactating animals. This is approximately 0.1% of the liver level. Based on the ease with which their mRNAs could be detected in the breast of both humans and rats, the P450s could be classified into two groups. Group 1; P450 1B1, 2E1, 2C and 4A were ubiquitous and relatively abundant i.e. were easily detected when 10% of the PCR reaction was used for Southern blotting. Group 2; members of the subfamilies P450 1A, 2A, 2B, 2D, 3A and aromatase were of lower abundance and could only be detected on Southern blots when 100% of the PCR product was loaded onto gels. Group 2 could be subdivided into 2a; (2A6, 2D6 and aromatase) which in humans were present in over 50% human samples and in the rat (2A3, 2D4 and aromatase) which were present only at specific ages. In the peripubertal period; P450 2A3, in 1-3 week old rats; P450 2D4 and in rats over 9 weeks of age; aromatase. Group 2b; in humans P450 1A1, 2B6 and 3A, which were expressed in less than 35% of the samples. In rats group 2b contained P450 1A1 and 2B1, which appeared to be hormonally regulated. P450 1A1 was present only during pregnancy and lactation and P450 2B1 in post lactation. Western blots revealed that the relative abundance of the mRNAs was reflected in that of the proteins. Samples of human breast epithelium revealed a P450 profile similar to that in the reduction samples which are mostly fat. The data shows that certain forms of P450, which can activate procarcinogens and convert estradiol and tamoxifen to inactive as well as toxic metabolites, are present in the breasts of some but not all women. These differences could be related to the sensitivity of the breast to both the beneficial and toxic effects of estrogen and antiestrogens. We conclude that future studies on the rat breast are likely to elucidate the hormonal mechanisms of regulation of these enzymes. Furthermore, we conclude that a survey of the P450 profile in breast fat biopsies of women undergoing antihormone therapy is warranted.

Inhibition of Grb2 function and EGF induced mitogenesis by micro-injection of non-phosphorylated small peptides. Mai M. Hijazi[1], Feng-Di T. Lung[2], Peter P. Roller[2], Tin Cao[1], Lakshmi Sastry[1] and C. Richter King[1]. [1]Lombardi Cancer Center, Georgetown University Medical Center, Research Building, E508, 3970 Reservoir Rd., N.W., Washington, DC 20007; [2]Laboratory of Medicinal Chemistry, Division of Basic Sciences, NCI, Bldg. 37, 5C/02, NIH, Bethesda, MD 20892.

Binding of Grb2 to phosphotyrosine (pTyr) motifs on growth factor receptors such as EGFR and erbB2 leads to downstream activation of the ras signaling pathway. This provides a promising therapeutic target to develop anti-cancer agents for tumors over-expressing growth factor receptors. Novel structural motifs capable of binding to Grb2 and inhibiting its function were identified by screening a phage peptide display library. The library was generated with a variable nine amino acid region flanked by disulfide-bonded cysteine residues forming cyclized peptides with limited conformational flexibility. A high-affinity binding phage, G1, was identified, sequenced and shown to have a nonphosphorylated tyrosine residue. Experiments using Biacore analysis and ELISA assays demonstrate G1 association with the SH2 domain of Grb2 and its inhibition of the Grb2/SHC phosphopeptide interaction, but did not show G1 binding to src. Reduction of the disulfide link in the G1 peptide diminished its ability to inhibit Grb2 association with the SHC phosphopeptide suggesting a required conformational constraint. In order to test G1 in cell systems and prevent reduction of the disulfide bond, a G1-TE molecule was generated in which the disulfide structure was replaced with a thioether bond. G1-TE added to cell lysates was able to reduce the amount of pTyr proteins co-immunoprecipitating with Grb-2. Initial experiments using microinjection have investigated potential biological effects of the G1-TE peptide on cell proliferation. NIH/3T3 fibroblasts expressing the hEGFR receptor (NIH/3T3.EGFR) were selected for testing since mitogenesis can be specifically induced with EGF stimulation. Synthesis of DNA in the microinjected cells was measured by immunostaining of bromodioxyuridine (BrDU) incorporated into newly synthesized DNA. G1-TE or G1.cys-ser, a non-active linearized control peptide, were micro-injected with rabbit IgG (R IgG) as a tracking agent into 300-400 serum-starved cells followed by addition of BrdU (10 mM) and EGF (50 ng/ml) for 24 h. Cells were then fixed in 70% ethanol and stained with an anti-BrdU monoclonal Ab, anti-mouse Ig-fluorescein and anti-R IgG Texas Red-conjugated secondary antibody. Nuclear BrdU labeling in microinjected cells was analyzed by fluorescence microscopy. G1-TE inhibited BrdU uptake in 70% of the microinjected NIH/3T3.EGFR fibroblasts treated with EGF, while microinjection of the G1.cys-ser control peptide or R IgG in absence of peptide showed only 18% and 16% inhibition of BrdU uptake respectively. These results demonstrate that the G1-TE peptide has in vivo functionality in blocking Grb2 binding to the activated EGFR tyrosine kinase and that it significantly inhibits ($p<0.001$) downstream signaling leading to cell proliferation.

"Basic and Clinical Aspects of Breast Cancer"

**A Novel Approach to Isolate "Pure" Populations of Apoptotic Breast Cancer Cells.** Christine Y. Rauh and Shuk-mei Ho, Biology Department, Tufts University, Medford, MA 02155

Studies have shown that a derangement in the regulation of apoptosis is associated with breast cancer and it has been hypothesized that the ability to promote or control the cell death pathway may lead to more successful breast cancer therapies. However, investigations of the genetic regulation of apoptosis in breast cancer cells have been hampered, in part, by the difficulty in identifying and isolating *pure* populations of apoptotic cells. This difficulty is due primarily to the asynchronous nature of apoptosis. In fact, most gene expression studies to date have used mixed populations of apoptotic and non-apoptotic cells. This limits and often obscures comparisons of gene expression in apoptotic cells to that occurs in non-apoptotic cells.

Utilizing light scatter parameters and a combination of fluorochromes that serve as markers for apoptosis/necrosis, we have developed a novel flow cytometric cell sorting procedure to isolate *pure* populations of apoptotic cells and non-apoptotic cells for use in gene expression studies. Cells undergoing early apoptosis are separated from non-apoptotic (viable) cells with the use of the following selection criteria: 1) when analyzed by flow cytometry, apoptotic cells exhibit increased side scatter as a result of chromatin condensation and increased granularity, and reduced forward light scatter due to cytoplasmic condensation and alteration in plasma membrane asymmetry, 2) due to chromatin condensation, apoptotic cells show enhanced nuclear staining with the supravital, non-intercalative DNA binding dye Hoechst 33342, and 3) as a result of membrane perturbation apoptotic cells externalize phosphatidylserine which is readily detectable with FITC-labeled Annexin V, a high affinity binding protein for this aminophospholipid. Late apoptotic cells and necrotic cells are identified and removed from the former two cell populations by virtue of propidium iodide staining in their nuclei, due principally to the loss of selective permeability of their plasma membrane. Using a computer-assisted multiple-parameter, parallel-analyses sorting procedure, we are able to reliably and reproducibly obtain *pure* populations of apoptotic and non-apoptotic cells for our studies.

Cultured MDA MB 231 (estrogen receptor negative) breast cancer cells were treated with the calcium ionophore A23187 to induce apoptosis. Ninety minutes after treatment with the ionophore, cells were harvested and incubated at room temperature with Hoechst dye 33342, FITC-labeled Annexin V, and propidium iodide. The cells were then analyzed and sorted by the flow cytometry procedure described above. After obtaining *pure* populations of apoptotic and non-apoptotic MDA MB 231 cells, RNA was extracted from each population and RT-PCR performed to semiquantitatively measure transcript levels of several known apoptosis-associated genes. Transcript levels of C-*myc*, Bax, and TGFβ1 type II receptor in apoptotic cells were found to be comparable to those observed in non-apoptotic cells. In contrast, expression levels of Bcl-2, TGFβ1, and TRPM-2 were found to be downregulated in apoptotic cells when compared to those present in non-apoptotic cells. The fact that the two populations of cells exhibit differential expression levels of selected apoptosis-associated genes strengthens our believe that our newly developed protocol is suitable for use in future studies of gene regulation in breast cancer cells. Supported in part by NCI grants# CA60923, CA15776 and CA62269.

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## The effect of truncated BRCA1 proteins: mouse model and overexpression studies

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Since the identification of *BRCA1* in 1994 [1], over 140 different germline mutations in this gene have been described. Most of these mutations are predicted to result in a truncated protein, either through frame shift or nonsense mutations. Only a minority of the mutations found so far result in missense amino acid substitutions, often found in domains which are believed to be of functional importance to the protein.

To examine the effects of of truncated BRCA1 proteins and study genotype - phenotype correlations at this locus, we have generated a mouse model, Brca1-1700HAT, which carries a targeted nonsense mutation in exon 20 of the endogenous *Brca1* gene. The mutation is predicted to result in a 178 kDa truncated protein encompassing both the RING finger and the putative NLS domain. At present, several chimeras have been generated and are being tested for germline transmission.

Three different Brca1 mouse models have already been described [2-4]. Interestingly, although these mutations have been shown to be embryonic lethal, none of these three seems to confer predisposition to mammary tumorigenesis to heterozygous animals. The Brca1 proteins, if any, predicted to result from these targeted mutations lack the putative nuclear localization signals found in exon 11 of the gene. The latter might result in the in the cytoplasmic localization of the mutated protein. Recently it has been shown that the C-terminus of the BRCA1 protein (exon 20-24) can function as a transcriptional activation doman [5,6]. In our mouse model, both putative NLS's and the N-terminally located RING finger are intact, whereas the transactivation domain is absent.

To further investigate the effect of truncated BRCA1 proteins, NIH3T3 cells are being transfected with different constructs expressing the *Brca1* RING finger, known to bind the BARD1 protein [7], with or without a SV40-derived NLS. Stable transfectants will be tested for phenotypic transformation.

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THERAPEUTIC ADVANTAGE OF CHEMOTHERAPY DRUGS IN COMBINATION WITH RECOMBINANT, HUMANIZED, ANTI-HER-2/*NEU* MONOCLONAL ANTIBODY (rhuMAb HER-2) AGAINST HUMAN BREAST CANCER CELLS AND XENOGRAFTS WITH HER-2/*NEU* OVEREXPRESSION

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We have previously demonstrated a synergistic interaction between rhuMAb HER-2 and the cytotoxic drug cisplatin in human breast and ovarian cancer cells (Pietras, *et al*, *Oncogene*, **9**: 1829-38, 1994). To characterize the nature of the interaction between rhuMAb HER-2 and other classes of cytotoxic drugs, we used multiple drug effect analysis (Chou, *et al*, *Adv Enz Reg*, **22**: 27-55, 1984) to determine combination index values for drug/antibody combinations in an *in vitro* cytotoxicity assay. SKBR-3 cells, human breast cancer cells with HER-2/*neu* amplification/overexpression, served as the target cell line in these experiments. In the cytotoxicity assay, rhuMAb HER-2 exhibited synergistic effects in combination with cisplatin, thiotepa, and etoposide. When used in combination with doxorubicin, taxol, or vinblastine, rhuMAb HER-2 had additive cytotoxic effects. One drug, 5-fluorouracil, was found to be antagonistic with rhuMAb HER-2 *in vitro*. *In vivo* studies were conducted in an athymic mouse model with HER-2/*neu*-transfected MCF-7 human breast cancer xenografts which, in contrast to SKBR-3 cells, are tumorigenic in athymic mice. Combinations of rhuMAb HER-2 plus cyclophosphamide, doxorubicin, methotrexate, etoposide, and vinblastine resulted in a significant reduction in xenograft volume compared to drug alone or rhuMAb HER-2 alone controls ($p<0.05$). Xenografts treated with rhuMAb HER-2 plus taxol or 5-fluorouracil were not significantly different from drug alone controls with the doses and dose schedules tested in this model. A phase III, randomized clinical trial is in progress, testing chemotherapy alone vs. chemotherapy plus rhuMAb HER-2 in patients with advanced, HER-2/*neu*-overexpressing breast cancer. (Supported by K12 CA01714, RO1 CA36827, R29 CA60835, the U.S. Army Breast Cancer Research Program DAMD 17-94-J-4370, and the Revlon/UCLA Women's Cancer Research Program)

YEAST-BASED FUNCTIONAL ASSAYS OF HUMAN *BRCA1* DETECT CANCER-PREDISPOSING MUTATIONS OF *BRCA1* AND DISTINGUISH THEM FROM BENIGN POLYMORPHISMS.

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Inherited inactivating mutations of the tumor suppressor gene BRCA1 result in Hereditary Breast-Ovarian Cancer syndrome (HBOC) which may account for 2.5-5% of the total incidence of breast cancer. Genetic testing currently relies on DNA analysis by full sequencing, SSCP, PTT, DNA chip analysis and related techniques to detect sequence alterations associated with cancer. During the course of two-hybrid studies, we discovered an activity of *BRCA1* in yeast. Yeast transformed with plasmid expressing a fusion protein of the *GAL4* activation domain to *BRCA1*, GAD-BRCA1, grew slowly and formed colonies considerably smaller than vector-transformed controls. This activity was localized by deletion analysis to the BRCT domains contained within the C-terminal 305 amino acids of BRCA1.

A nonsense mutation of BRCA1 at codon 1853, Y1853x (5677 insA), truncates BRCA1 by only 11 amino acids and has been identified as a mutation associated with HBOC. The Y1853stop mutation and a 5382 insC (Q1756C+) frameshift mutation abrogated the small-colony activity of GAD-BRCA1, demonstrating that the small-colony phenotype is specific to fusions of GAD to wild-type BRCA1 sequences. These data suggest the small-colony assay may be used to detect truncating mutations of *BRCA1*.

Missense mutations detected during evaluation for HBOC are difficult to interpret and may represent either functionally silent polymorphisms or inactivating, cancer-predisposing mutations. Population data necessary to make the distinction is often unavailable. We tested three disease-associated mutations that presumably inactivate *BRCA1* function. These mutations (A1708E, P1749R, and M1775R) completely or partially inactivated the small colony function of a GAD-BRCA1 Δ2, a fusion of GAD to the c-terminal 305 a.a. of pBRCA1, in the small colony assay. We then tested three common polymorphisms not associated with an increased cancer risk. These polymorphisms (S1613G, P1637L, and M1652I) did not alter the small colony function of GAD-BRCA1 Δ2 in the assay. Thus, the assay detected inactivating mutations by the appearance of colonies of normal or near-normal size after transformation with plasmid encoding the mutated GAD-BRCA1. In contrast, transformation with GAD-BRCA1 Δ2 altered by common polymorphisms resulted in small colonies indistinguishable from GAD-BRCA1 Δ2 transformed controls. These results were confirmed in part by a second assay of BRCA1 transcriptional activity reported in a second abstract (Salim, *et al.*).

The small colony assay may be a viable alternative to or useful addition to labor intensive, DNA based evaluations of *BRCA1* currently in use, and may provide helpful information regarding the functional effects of missense mutations.

## BASIC AND CLINICAL ASPECTS OF BREAST CANCER

**A role for thrombospondin during growth and regression of the mammary gland: Regulation of vessel growth in mammary tumors.** M.Luisa Iruela-Arispe, Maria Asuncion Ortega, Sarah Oikemus, Jack Lawler, and Sunjay Patil.
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The ability of tumors to recruit new vascular growth is a critical stage in cancer progression, and tumor vascularity has been correlated with a poor prognosis for breast cancers in women. Thrombospondin-1 (TSP-1) is an extracellular matrix glycoprotein implicated as a tumor-suppressor gene by virtue of its deletion in tumors that lost p53. Data from several laboratories indicate that TSP-1 inhibits endothelial cell cycle progression, focal adhesion formation, and suppresses angiogenesis directed by basic FGF. Nevertheless, the role of TSP-1 in physiological settings, or in tumor-mediated angiogenesis, has been difficult to address. To study the role of TSP-1 in vascular growth, we have examined the consequences of lack of TSP-1 expression on the development of the vasculature of the murine mammary gland and have tested the consequences of TSP-1 overexpressing on tumor growth.

Initially, we examined TSP-1 expression in the mammary glands of normal mice at several stages of growth and involution. We found that TSP-1 mRNA was present at relatively moderate levels throughout mammary development and pregnancy, but increased 7- to 12-fold during involution of the gland following weaning. By in situ hybridization, we found that TSP-1 transcripts were present in both the mammary epithelium and stroma. Interestingly, TSP-1 protein was associated primarily with the basement membranes of blood vessels and mammary ducts. These results suggested that blood vessels were a potential target of TSP-1 production during mammary growth and regression.

To study the function of TSP-1 in mammary development and vascularization, we compared the vascular density of glands from control and TSP-1 null mice produced by gene targeting. We found no significant differences in vascular density in virgin and lactating animals; however, there was a higher vascular density in involuting glands of TSP-1 null animals. Because a delay in vascular regression could have an impact on mammary epithelial viability, we examined the animals for changes in the onset of apoptosis following weaning. Mammary apoptosis was markedly delayed in TSP-1 deficient animals as compared with wild-type controls. These results indicate that a normal function of TSP-1 is to stimulate vascular regression at the onset of mammary involution and that delays in this process affect epithelial cell survival during the early stages of involution.

Since TSP-1 null mice show impaired vascular regression, we hypothesized that overexpression of TSP-1 would cause precocious regression of vascular cells and/or impairments in the ability to recruit new vascular growth. We therfore tested this hypothesis by over-expression of TSP-1 in a mammary tumor model. In this model, mammary cells overexpressing an activating form of *neu* are introduced into mice and tumor progression is monitored. When TSP-1 was stably transfected into these cells, we observed a marked decrease in tumor incidence and size. In addition, tumors displayed low vascular density, and higher levels of apoptotic cells, when compared with tumors generated from cells expressing vector alone.

These observations establish that TSP-1 plays a role in vascular regression during physiological remodeling of the mammary gland. In addition, over-expression of TSP-1 impaired vascular recruitment in tumors and delayed tumor growth. Taken together, the findings indicate that TSP-1 is a potential tool for regulation of mammary tumor vascularity and growth.

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Detection of heterozygous truncating mutations in the *BRCA1* and *APC* genes using Stop Codon (SC) Assay in yeast. Chikashi Ishioka*[†], Takao Suzuki*, Michael FitzGerald[‡], Michael Krainer[‡], Hideki Shimodaira*, Akira Shimada*, Tadashi Nomizu[§], Kurt J. Isselbacher[‡], Daniel Haber[‡] and Ryunosuke Kanamaru*. *Department of Clinical Oncology, Institute of Development, Aging and Cancer, Tohoku University, 4-1 Seiryo-machi, Aoba-ku, Sendai 980, Japan; [‡]Massachusetts General Hospital Cancer Center, Building 149, 13th Street, Charlestown, MA 02129; and [§]Department of Surgery, Hoshi General Hospital, 2-1-16 Ohmachi, Koriyama 963, Japan.

The detection of mutations in tumor suppressor genes is critical to their characterization, as well as to the development of clinical diagnostics. Most approaches for mutational screening of germline specimens are complicated by the fact that mutations are heterozygous and that missense mutations are difficult to interpret in the absence of information about protein function. Here, we describe a novel method for detecting protein truncating mutations in *BRCA1* and *APC* genes using *Saccharomyces cerevisiae*. The PCR-amplified coding sequence is inserted by homologous recombination into a yeast *URA3* fusion protein and transformants are assayed for growth in the absence of uracil. The high efficiency of homologous recombination in yeast ensures that both alleles are represented among transformants and achieves separation of alleles, which facilitates subsequent nucleotide sequencing of the mutated transcript. The specificity of translational initiation of the *URA3* gene leads to minimal enzymatic activity in transformants harboring an inserted stop codon, and hence to reliable distinction between specimens with wild-type alleles and those with a heterozygous truncating mutation. This method, yeast-based Stop Codon (SC) Assay, accurately detects heterozygous truncating mutations in the *BRCA1* gene in patients with early onset of breast cancer and in the *APC* gene in patients with familial adenomatous polyposis. This approach offers a rapid, reliable and less-expensive method for genetic diagnosis in individuals at high risk for germline mutations in cancer susceptibility genes.

Expression of Epidermal Growth Factor Receptor Family Members and Their Ligands in the Developing Mouse Mammary Gland.

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The *ErbB2* protooncogene is amplified and/or overexpressed in 15-40% of human breast carcinomas. Furthermore, ErbB2 amplification is associated with poor disease prognosis, especially in lymph node positive patients. ErbB2 is a member of the epidermal growth factor receptor (EGFR) family of receptor tyrosine kinases, which also includes ErbB3 and ErbB4. Although a ligand for ErbB2 has not been identified several EGF family members can activate ErbB2 through their target receptors. ErbB2 activation by these growth factors is thought to occur via growth factor driven receptor heterodimerization and cross-phosporylation also referred to as transmodulation. Based upon this model ErbB2 signaling in breast tissue would be dependent upon the tissue expression of 1) ErbB2 regulating growth factors and 2) ErbB2 heterodimer partners. To identify the receptors and ligands which may regulate ErbB2 signaling in normal and malignant breast tissue we have determined the expression profiles of EGFR family members and their ligands in developing breast tissue.

Using RNase protection assay we found that each EGFR family member was expressed at relatively constant levels throughout normal mammary gland development. In contrast, the expression of ErbB2 agonists examined to date appears to be developmentally regulated in the mammary gland. For example the EGFR ligand amphiregulin is only expressed in mammary glands from virgin mice whereas mammary gland expression of the erbB4 and erbB3 ligand heregulin (HRG) is only observed during pregnancy. These results suggest that differential regulation of ErbB2 agonists rather than ErbB2 heterodimer partners regulate ErbB2 signaling during normal mammary gland development and these agonists may drive mammary gland development. To test this hypothesis we implanted slow-release pellets containing HRG within the mammary glands of virgin female mice and assayed for epithelial differentiation by immunohistochemical detection of the milk protein β-casein. As predicted HRG implants induced the differentiation of mammary epithelium into milk protein secreting lobuloalveoli. In contrast, implants containing the EGF family member TGFα do not induce epithelial differentiation. In conclusion, evidence presented here indicates that expression of ErbB2 agonists are developmentally regulated and may play an important role in coordinating development of the mouse mammary gland.

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# The Cancer Predisposing C61G Mutation Disrupts the C-terminal Region of the BRCA1 RING-Finger Domain.

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The isolation and cloning of the tumor suppressor protein BRCA1 represents a significant milestone in breast cancer research. Mutations in the *BRCA1* gene are correlated with increased susceptibility to familial breast and ovarian cancer. Several predisposing mutations have been found in the N-terminal cysteine-rich region of BRCA1. This region is homologous to the recently described RING-finger motif, a $Zn^{2+}$-binding motif that has been identified in over 80 proteins of diverse size, function, and origin. At present, very little is known about the biological function or structure of the BRCA1 RING-finger domain. The goal of the present study is to define the BRCA1 RING-finger domain accurately and to determine the structural consequences of cancer-predisposing mutations.

Using limited proteolysis in conjunction with MALDI-TOF mass spectrometry we have identified a minimal folding domain that encompasses the RING-finger motif of BRCA1. The results show that two $Zn^{2+}$ atoms bind to the domain in a stepwise manner and that metal binding has a profound effect on the stability of the domain. Secondly, the observed proteolytic cleavage in the presence of $Zn^{2+}$ suggests that the BRCA1 RING-finger domain folds in a manner consistent with previously determined structures of other RING-finger domains. Mutation of cysteine 61 to glycine (C61G), a known cancer predisposing mutation of a putative $Zn^{2+}$-binding ligand, unmasks a previously protected proteolytic cleavage position adjacent to the lower affinity $Zn^{2+}$-binding site. Using $Co^{2+}$ as a spectroscopic probe and $Zn^{2+}$ substitute, we find that the C61G mutant binds only a single metal ion located at the intact higher affinity site. These results show that the C61G mutation eliminates the lower affinity metal-binding site and that the structural consequences are localized to the C-terminal portion of the domain.

Basic and Clinical Aspects of Breast Cancer

Expression of nm23 in breast cancer tissues. How does it relate to other clinical markers and what is it biological mechanism of action.

Rosaland L Russell, Timothy E. Kute, Nora Zbieranski, Brent Shelton, Rena Long, Kim Geisinger, Andres Peterson, Nils Brunner, Jason Kantor Wake Forest University Cancer Center, Winston-Salem, NC and Finsen Inst. Copenhagen, Denmark and Children's Hospital Boston, MA

Levels of nm23 have shown an inverse correlation to metastatic potential and poor prognosis in breast cancer patients. The expression of the two isoforms of nm23 (H1 and H2) in 150 patients using a quantitative Western blot analysis were measured and compared to expression of steroid receptors, ploidy, cell kinetics, cathepsin D, urokinase plasminogen activator (uPA), the uPA inhibitor, and the uPA receptor. nm23-H1 was directly correlated with nm23-H2 levels ($R= 0.84$, $p = 0.0001$) and uPAR ( $R= 0.25$, $p=0.008$). Patients whose tumors were diploid had significantly higher mean nm23-H1 values than those patients whose tumors were aneuploid ($p = 0.06$). The expression of nm23-H2 was directly correlated with uPA, and uPAR ( $p = 0.02$, 0.01, respectively). Neither isoform of nm23 was related to steroid receptor, cell cycle kinetic values or cathepsin D..

The antimetastatic gene, nm23 was transfected into the human breast cancer cell line MDA-MB-231 resulting in 4 and 8 fold induction of nm23-H1 protein. Analyses of the phenotypic changes associated with the overexpression of nm23-H1 protein were performed in both *in vitro* and *in vivo* experimental systems. The overexpression of nm23-H1 was not associated with alterations in proliferation *in vitro* or in the growth rate of solid tumors in nude mice. The nm23-H1 overexpressing clones were injected into nude mice and analyzed for metastatic potential as compared to the parental cell line. Whereas the parental cell line MDA-MB-231 produced metastatic disease in 71% of the animals, the transfected clones produced metastatic lesions in 38% (MDA-MB-231-40) and 40% (MDA-MB-231-47) of the animals. *In vitro* motility assays were performed on the MDA-MB-231 parent cell line and the two nm23-H1 overexpressing clones, using a modified Boyden Chamber assay. These experiments demonstrated that the clones had a statistically significant reduction response to motility-stimulating factors (PDGF, 0.5% serum, and LPA) as compared to the parental cell line.

In summary, We propose that the anti-metastatic potential of nm23 is not related to down regulation of proteolytic activity or of proliferative activity in solid tumors as might have been predicted. Secondly, the increase of nm23 expression in MDA-MB-231 breast cancer cell by tranfection, resulted in reduced metastatic disease in nude mice and a reduced response to motility-stimulating factors *in vitro*. The mechanism of action of nm23 could be related to decreased motility.

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Altered Growth and Cell Cycle Pattern in Human Breast Epithelial Cells Induced by a *BRCA1* Mutant

Jeffrey S. Larson, John L. Tonkinson and Mei T. Lai, Cancer Research Division, Lilly Research Laboratories, Lilly Corporate Center, Indianapolis, IN 46285

Mutations in the coding region of *BRCA1* confer susceptibility to breast and ovarian cancer. To study the effects of *BRCA1* mutants on the growth of human mammary epithelial cells, we transfected a *BRCA1* mutant into the immortalized but non-transforming AB184-A1 human breast epithelial cells. High-level expression of the mutant BRCA1 was demonstrated in the established individual clonal lines. These cells displayed accelerated growth, reduced dependence on growth factors and altered cell cycle patterns.

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**Basic and Clinical Aspects of Breast Cancer.**

***Wnt*-10b regulates mammary development and functions as a transforming gene in mammary glands of male and female transgenic mice.** Timothy F. Lane and Philip Leder. Howard Hughes Medical Institute, Department of Genetics, Harvard Medical School, Boston, MA 02115.

*Wnt*-10b mRNA is one of a family of closely related growth factors related to Wnt-1, a proto-oncogene implicated in breast cancer. Unlike *Wnt*-1, *Wnt*-10b is expressed during mammary development and has been identified in the mammary rudiment of mouse embryos. To study the function of *Wnt*-10b in breast development and transformation, we created lines of mice that express elevated levels of *Wnt*-10b cDNA under the control of the MMTV promoter/enhancer. Overexpression resulted in expanded glandular development and in the precocious appearance of alveoli in virgin females. Transgenic males also developed highly branched mammary ducts, bypassing the normal role of ovarian hormonal control in stimulating ductal growth. Transgenic mice from both sexes were highly susceptible to the development of mammary adenocarcinomas. To test whether *Wnt*-10b plays a critical role in mammary development, we used gene targeting to develop a line of mice which lack *Wnt*-10b. *Wnt*-10b null mice were viable and appear to lactate normally. However, inspection of developing glands show a decrease in ductal branching. The ability of *Wnt*-10b mice to develop normal mammary function is likely due to redundancy of function between *Wnt*-10b and other *Wnt* genes expressed during ductal development. Our results show that overproduction of *Wnt*-10b induces increased branching of mammary ducts, the precocious appearance of alveoli, and transformation of the mammary epithelium. Ablation of the gene has a reciprocal effect, displaying less branching in early stages of mammary development. These results provide direct evidence that *Wnt*-10b, along with other *Wnt* genes expressed early in mammary development, play a role in regulating sexual dimorphism and patterning of the mammary gland. In addition, elevated expression of this gene results in the rapid onset of adenocarcinomas of the breast.

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**Identification of a New Tumor Suppressor: the TSG101 Gene and Human Breast Cancer.** L. Li*, X. Li*, U. Francke+, and S.N. Cohen*. Deparment of Genetics* and Howard Hughes Medical Institute+, Stanford University School of Medicine, Stanford CA 94305-51220

Using a novel approach that identifies previously unknown genes whose homozygous inactivation yields a selectable phenotype in cells growing in culture, we recently discovered a gene, *tsg101,* whose functional knock out leads to malignant transformation and the ability to form metastatic tumors in nude mice (L. Li and S.N. Cohen, Cell, 85:319). The cellular transformation and tumorigenesis that result from inactivation of *tsg101* are reversible by restoration of *tsg101* function, suggesting that the gene suppresses abnormal cell proliferation. Sequence analysis showed that *tsg101* encodes a 43 kDa protein containing a proline-rich domain and DNA-binding motifs characteristic of transcription factors and a coiled-coil domain that interacts with stathmin,a cytosolic phosphoprotein elevated in a variety of malignancies and implicated in the regulation and relay of diverse signals associated with cell growth and differentiation. We isolated human *TSG101* genomic DNA and cDNA and mapped the gene*TSG101* to bands 15.1-15.2 of the short arm of chromosome 11. Seven of 15 uncultured primary human breast cancers obtained from patients not selected from breast cancer families showed*TSG 101* deletions that in each instance physically ablated or functionally inactivated the stathmin-interacting coiled-coil domain of TSG101; matched normal breast tissue from the same individuals showed no detectable *TSG101* defects. Our results strongly implicate abnormalities of the *TSG101* gene in human breast cancer.

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Selective Antitumor Therapy in Patients with Breast Cancer, Loefflmann, Adolf, Institute of Cancer Research and Treatment, Munich, Germany.

Malignancy can be reversed in different carcinomas by an oxygen evolving enzyme M¬ 67 000 (MDA) used in combination with biological proteinase inhibitors M¬ 62000, 43000 and 13800 (PI). The theoretical base of my studies is described in the paper "A Comprehensive Theory of Carcinogenesis and it's Reversal", Med. Organica, 1991, Heft 4, p. 122 -125.
In this paper the working hypothesis is that the origin of carcinogenesis is located in the cytochromoxidase in complex IV of the mitochondria. If the Fe 3+ centre in the cytochromoxydase is reduced in more than 80 % of the mitochondria of one cell to Fe 2+ the cell apparently has to go back to embryonal coding to cope with it's energy requirements. The idea is due to the similarities in structure and function of chloroplasts and mitochondria to purify a protein-bound metalloporphyrin from the photosystem of plants that could remove the excess electron in the respiratory chain of the malignant cell and to oxidize the ferrous ion centre to Fe 3+ by using the infrared irradiation of the body to make reaction proceed. But MDA has only very limited effect on malignant cells because as a protein it is cleaved by the membrane-bound proteinases of the malignant cells. Fetal proteinase inhibitors - M¬ 62000, 43000 and 13800 - are able to neutralize and faciliate the uptake of the enzyme into malignant cells. In animal tests with Wistar rats on 20 - Methylcholantren induced advanced fibrosarcomas this combined drugs caused hemorrhagic necrosis and the degradation of malignant cells.
In a preclinical trial 3 patients with different histories of mammary carcinoma were treated with this biochemical combination therapy.These combined drugs were applied as infusions twice a week. Patient I was treated since November 1991 with several courses of selective antitumor therapy after she has had a breast conserving operation. We detected and dissolved micrometastases in the liver. Patient II, who has got removed both breasts because of mammary carcinomas on both sides with multiple axillary metastases showed also dissolution of liver metastases. Patient III with systemic metastases of the skeleton was a responder of selective antitumor therapy. There was a dose dependent clinical response in releasing glutamatdehydrogenase, as indicator of selective degradation of mitochondria and an increase of Ca 15-3 , TPA and CEA as parameter for selective destruction of tumor cell membranes. An up-regulation of hematological parameters like reticulocytes, platelets and erythrocytes were observed. Primary side effects were not observed, secondary side effects were due to destruction of malignant cells inducing liberation of endogenous vasoactive mediators like histamin, prostaglandines and thromboplastin initiating the production of thrombocytes. Biochemical parameters indicated the complete destruction of all malignant cells. Patient III developed a leakage syndrom with a weight gain of 10 kg after one year of treatment. Standard of art in selective antitumor therapy is now that patients with various carcinomas of small amount are accessible to a complete degradation of malignant cells. The leakage syndrome caused by selective antitumor therapy in final stage patients is investigated.

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## Targeted Mutations of Breast Cancer Susceptibility Gene Homologues *Brca1* and *Brca2* in Mice

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Departments of [1]Anatomy and Cell Biology and [2]Genetics and Development
Columbia University, New York, NY 10032

Mutations of the human *BRCA1* and *BRCA2* genes encoding tumor suppressors have been implicated in inherited predisposition to breast and other cancers. We have inactivated the homologous mouse genes *Brca1* and *Brca2* by homologous recombination in embryonic stem cells to investigate their functions, and showed that both genes play indispensable roles during embryogenesis, since nullizygous embryos become developmentally retarded and disorganized, and die early in development. A detailed analysis of embryos at different developmental stages revealed that the onset of abnormalities is earlier by one day in *Brca1* mutants, while their phenotypic features and time of death are highly variable. In contrast, the phenotype of *Brca2* null embryos is much more uniform, and they all survive for at least 8.5 embryonic days. Consistent with these *in vivo* observations, blastocysts homozygous for the *Brca1* or *Brca2* mutations are severely compromised when cultured *in vitro*.

*Brca1/Brca2* double nullizygotes fall phenotypically within the range of *Brca1* single mutants. This apparently epistatic behavior of the *Brca1* mutation; the temporal overlap of *Brca1* and *Brca2* gene actions; and the predominant feature of growth retardation affecting more severely the embryonic than the extraembryonic portion of the conceptus, which the phenotypes of the mutations share, point to a functional relationship, potentially involving overlapping developmental pathways. Interestingly, both classes of single mutants can be partially rescued in a *p53*-null background. Although overtly retarded and abnormal in comparison with wild-type controls and *p53* nullizygotes, *Brca1/p53* and *Brca2/p53* double nullizygous mutants are further advanced than embryos lacking only *Brca1* or *Brca2*. Thus, in addition to their functions that are antagonistic to *p53* in early embryos, as revealed from this genetic evidence, the *Brca1* and *Brca2* genes should play roles that are not *p53*-related, since complete rescue does not occur in the double mutants.

Regardless of the embryonic lethality of *Brca1* and *Brca2* nullizygotes, it is expected that, if the human and mouse proteins encoded by these genes are functionally equivalent, females heterozygous for either mutation will develop mammary and/or other tumors stochastically and with long latency from somatic mutation of the intact allele. Until now, females heterozygous for *Brca1* or *Brca2* are still tumor-free at the ages of 14 and six months, respectively.

# Basic and Clinical Aspects of Breast Cancer

*Poster Session B*

*Sunday, March 9*
*4:00 p.m. - 6:00 p.m.*

Basic and Clinical Aspects of Breast Cancer

**Evidence of Decreased Expression of Transforming Growth Factor Beta Type II Receptor and Its Isoform in Human Breast Tumors.** M. A. Lynch, W. Burak*, G. D. Stoner, and C. M. Weghorst. Division of Environmental Health Sciences, School of Public Health and *Department of Surgery, The Ohio State University College of Medicine, Columbus, Ohio 43210.

Growth factors that regulate normal breast epithelial cell growth and proliferation are also important mediators of malignant transformation and metastatic progression in human breast cancer.Transforming growth factor beta (TGF-β) is a potent inhibitor of breast epithelial cell growth and proliferation. Loss of sensitivity to TGF-β inhibition correlates with malignant progression in studies of breast cancer cell lines and in clinical breast cancer specimens. The biological effects of TGF-β are mediated by the TGF-β receptor complex, which is composed of two essential independent and interacting subunits, TGF-β receptor type I (TβR-I) and TGF-β receptor type II (TβR-II). Recently, a TβR-II isoform transcript, TβR-$II_2$, containing a specific 75 base pair insert in the 5' coding region has been described. Its functional significance is unknown. Evidence from breast cell culture studies suggests that loss of expression of the TβR-II component of the TGF-β receptor complex is implicated in the loss of sensitivity to TGF-β mediated growth inhibition.

We analyzed six primary infiltrating ductal carcinomas, one primary in situ ductal carcinoma, one axillary lymph node metastasis, and one recurrent skin metastasis, each with matched normal adjacent tissue, for mRNA expression of TβR-II and TβR-$II_2$. Using semi-quantitative multiplex RT-PCR, the relative expression of TβR-II and TβR-$II_2$ mRNA, normalized to HPRT mRNA levels, was determined for tumor and matched normal adjacent tissue. Two independent studies were conducted with consistent results. Decreased expression of TβR-II in tumor compared to matched adjacent normal tissue was observed in 7 of 7 primary tumors [28% to 64% decreased expression], in the 1 axillary lymph node metastasis[41% decreased expression], and in the 1 recurrent skin metastasis [41% decreased expression]. Similarly, decreased expression of TβR-$II_2$ in tumor compared to matched adjacent normal tissue was observed in 7 of 7 primary tumors [51% to 78% decreased expression], in the 1 axillay lymph node metastasis [50% decreased expression], and in the 1 recurrent skin metastasis [46% decreased expression]. The decreased expression of TβR-II and TβR-$II_2$ observed in this sample of breast tumors compared to matched normal tissue was statistically significant by the paired Student's T Test [$p<0.001$]. The consistent decrease in expression seen in all tumor samples compared to matched normal adjacent tissue provides compelling evidence that decreased TβR-II expression is associated with malignant progression in human breast carcinogenesis. Further studies are in progress to confirm this finding in larger number of clinical specimens, to examine correlations of decreased mRNA expression with TβR-II protein expression, and to determine possible causes of the decreased mRNA expression observed in this sample of human breast carcinomas.

**FGF-8 Expression In Breast Oncogenesis.** Dorit Daphna-Iken, Avril Lawshé, and Craig A. MacArthur. Departments of Pediatrics and Pathology, Washington University School of Medicine, St. Louis, MO 63110.

Prior studies have identified murine *Fgf8* as a likely proto-oncogene in mouse mammary tumorigenesis. *Fgf8* encodes eight potential protein isoforms by alternative splicing of the of RNA. RNA for seven of the eight isoforms have been identified during the examination of murine development, and similar binding of FGF receptors by the different recombinant FGF-8 isoforms has been demonstrated. We and others have identified the FGF-8b protein isoform as the most potent transforming isoform in NIH 3T3 cells. We now report on the generation of MMTV-FGF-8b transgenic mice that express the FGF-8b isoform from a minigene driven by the mouse mammary tumor virus long terminal repeat. Male and female MMTV-FGF-8b mice are viable and fertile. RNA for FGF-8b is detected in RNA from mammary gland and salivary gland tissues of transgenic mice by northern blot analysis. Although the analysis of these animals is preliminary, transgenic female mice have developed mammary carcinomas, while non-transgenic littermates have not. These results demonstrate that FGF-8b production in the mammary gland contributes to oncogenesis.

We have recently cloned the human *FGF8* gene, and have analyzed *FGF8* expression in human breast cancer cell lines. We prepared total RNA from 24 human breast cancer cell lines, available through the American Type Culture Collection, and analyzed these RNAs for *FGF8* expression by reverse transcription-polymerase chain reaction (RT-PCR) techniques. PCR primers were designed so that only human *FGF8*, and not murine *Fgf8*, would amplify under the conditions employed. *FGF8* is not detected in RNA from normal human mammary tissue by the RT-PCR conditions employed. RNA from day 10 mouse embryo also does not give a positive signal, as expected by the design of the human-specific primers. Twelve of 24 human breast cancer cell lines tested express *FGF8*. These results indicate that *FGF8* expression is common in human breast cancer cell lines, and suggests that *FGF8* may be an important growth factor involved in human mammary oncogenesis.

Work supported by NIH R01 CA70601 and Edward Mallinckrodt, Jr., Foundation.

**Semi-quantitative analysis of *Bcl-2* Protein in Breast Cancer**

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We have developed a novel semi-automated image cytometric method for estimation of cellular interfaces in tissue sections stained immunohistochemically. This method allows consistent measurement of cytoplasmic antigens in tissue sections on a cell-by-cell basis and therefore provides information supplementary to typical qualitative analysis.

Initial studies have now been performed on several human breast cancer sections immunohistochemically stained for the *bcl-2* proto-oncogene product. Intra-observer and inter-observer variability in the application of these techniques were <2.6% and <4.4% respectively. The mean staining densities of slides correlated well with qualitative scoring performed by two independent pathologists. Cell-by-cell analysis however, identified significant variation of staining intensities between regions within individual slides, and between individual cells in regions of both DCIS and invasive carcinoma. These results indicate that this method may provide unique information regarding the heterogeneity of *bcl-2* expression in breast cancer.

An Abrogated p53-Mediated Response to Ionizing Radiation in Human Mammary Epithelial Cells. Karen L. Mitro, Suzanne M. Hess, and Steven A. Leadon, Department of Radiation Oncology, Curriculum in Toxicology, University of North Carolina at Chapel Hill, Chapel Hill, NC 27599

Cells have evolved a complex array of signaling mechanisms that actively respond to DNA damage to maintain their genomic integrity. An increasing body of evidence suggests that these signaling networks that detect DNA damage, promote DNA repair, induce cell cycle checkpoints, and trigger apoptosis are inter-related. The tumor suppressor protein p53 plays a central role in the cellular response to DNA damage by mediating G1-S cell cycle arrest, apoptosis, and emerging evidence suggests that it also participates in DNA repair. Several laboratories have shown that the G1 arrest in cells treated with ionizing radiation (IR) exhibit a p53-dependent response, whereby p53 accumulates and subsequently transactivates downstream target genes $p21^{WAF1}$ and GADD45. However, most of the studies have examined cells of a mesenchymal origin. Cells from patients with the recessive, cancer-prone and radiosensitive disorder, ataxia-telangiectasia (AT) are deficient in the G1 checkpoint following treatment with IR; furthermore, the induction of p53, $p21^{WAF1}$, and GADD45 proteins, key participants in the G1-S checkpoint, are suboptimal upon exposure to IR. These studies lead to the proposal that the A-T gene product plays a crucial role in the DNA damage response and functions upstream of p53. Recent studies in our lab indicate that although normal human mammary epithelial cells (HMEC) express wild-type p53, they fail to arrest at the G1 checkpoint after treatment with 4 Gy of IR, as evaluated using flow cytometry. Therefore, we investigated further the p53-mediated response of normal HMEC following treatment with IR, and compared these results to those obtained from matched sets of normal mammary fibroblasts. Two HMEC lines (HMEC 184 and 161) and their fibroblast (Fb) counterparts were irradiated with 4 Gy of IR or sham-irradiated, and incubated for various times from 0-16 hrs post-irradiation. After isolation of whole cell lysates, Western analyses were performed to examine the levels of p53 and one of its downstream targets, $p21^{WAF1}$. Following exposure to IR, both 184 Fb and 161 Fb show a rapid induction of p53 protein, with maximal increases occurring within 3 hrs. The $p21^{WAF1}$ protein levels also increase in these cells, with nearly a 10-fold induction in the 161 Fb occurring by 8 hrs post-treatment, and a 3-fold induction in the 184 Fb occurring between 6-10 hrs post-treatment. In marked contrast to the results with the fibroblasts, the levels of p53 and $p21^{WAF1}$ in HMEC 184 and 161 are not significantly increased over the time course examined. Thus, the lack of induction of p53 and $p21^{WAF1}$ proteins in the HMEC following IR supports our previous findings showing the absence of a G1 checkpoint in response to IR. Our results show that normal breast epithelial cells exhibit a different p53-mediated response to IR than that found in cells of a mesenchymal origin.

These findings suggest that other signaling pathways both upstream and downstream of p53 may be altered in HMEC. Currently, experiments are underway in our lab to examine the p53-mediated apoptotic response to IR in HMEC lines (see R. W. Fisher and S. A. Leadon abstract). In addition, experiments are being performed to examine the response of GADD45, another downstream target gene of p53 important in G1 arrest and implicated in DNA repair. Previous studies show that this gene is induced within 4 hrs after exposure to IR. Therefore, Northern analyses will be done to determine whether the expression of this gene is upregulated in normal HMEC post-treatment with IR. Furthermore, there are ongoing studies investigating the pathway upstream of p53, where the ATM (mutated in AT) protein is implicated, to determine its expression level in epithelial cells.

## EXPRESSION OF ONCOGENES AND TUMOR SUPPRESSOR GENES IN USUAL DUCTAL HYPERPLASIA OF THE BREAST

Ellen CM Mommers, Angelique M Leonhart, Paul J van Diest, Chris JLM. Meijer, Jan PA Baak
Department of Pathology, Free University Hospital, Amsterdam

Invasive breast cancer is thought to develop through a spectrum of increasingly aberrant lesions from normal tissue to hyperplastic lesions to carcinoma *in situ*. Little is known about cell - and molecular biological events that play a role in the progression of ductal hyperplastic lesions.

The aim of this study is to determine the expression of oncogenes and tumor suppressor genes, like HER-2/*neu*, Ki-67, cyclin D1, p53 and p21, in ductal hyperplastic breast lesions without atypia to identify early events in breast cancer oncogenesis that may indicate high risk lesions.

Immunohistochemistry has been performed on 67 cases of usual ductal hyperplasia. No cases showed membrane expression of HER-2/*neu*. Approximately 61% of cases showed expression of Ki-67, a proliferation associated antigene, of which 12 % had more than 10% positive nuclei. Expression of cyclin D1, p53 or p21 was also seen in ductal hyperplastic lesions, although the percentages of positive cases were much lower. Cyclin D1 was overexpressed in 10% of cases with low percentages of positive nuclei (less than 5%). Accumulation and expression of p53 and p21 was detected in 13% and 24% of cases respectively. In only 1 case, expression was found of 4 of 5 genes tested in this study.

In conclusion, overexpression of some oncogenes and altered expression of tumor suppressor genes is already present in some 'early stage' preinvasive breast lesions. These genes may therefore play a role in progression of ductal hyperplastic lesions and may indicate high risk lesions.

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Alcohol Consumption and Serum Estradiol in Premenopausal Women

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The authors analyzed the relationship between alcohol intake and estradiol (E2) in 60 premenopausal women from a cohort study on hormones and breast cancer (ORDET Study). Measurements were made twice in each subject, one year apart. Blood was drawn fasting from 8 to 9.30 am and between the 20th and the 24th day of the cycle. Both blood samples were obtained on the same day of that interval during the luteal phase of the menstrual cycle, in the same month and in the same hour and minute of the day. Sample from the first examination were frozen at -80°C for 12 months and analyzed simultaneously in blind fashion with serum from the second examination. The intra-assay and inter-assay CVs of E2 were less than 14%. Alcohol intake, estimated by questionnaire, showed a good agreement between the first and the second interview (Pearson's r=.88). The means of serum E2 in all women showed no significant difference from the two time points (360±166.6 pmol/L and 368.7±166.9 pmol/L), indicating the absence of storage effects. Since no correlation was found in E2 levels between these two measurements (intraclass correlation coefficient=0.05), to control for over-time intra-individual variability of E2, participant were then divided into tertiles of hormones distribution for each of the two sets of measurements and classified based on their consistency in E2 across the two visits.. Women showing consistent high E2 at both visits (n=22) were characterized by a significant higher alcohol intake (92.8 g/wk) in comparison with those showing consistently low E2 (n=19) (31.6 g/wk). No significant difference between the two groups in age, BMI and body fat distribution was observed. These results indicate that when the variability of E2 in premenopause is considered, it is possible to identify a relation between alcohol intake and E2. If alcohol consumption causes breast cancer, its action could be, at least in part, mediated through an effect on estrogen metabolism.

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**Differential expression of the orphan nuclear receptor ARP-1 in breast cancer.** HARIKRISHNA NAKSHATRI[1,2], POORNIMA BHAT-NAKSHATRI[3], ROBERT J. GOULET, JR.[1], MARC S. MENDONCA[4], KENNETH CORNETTA[3], and GEORGE SLEDGE JR.[3]. Departments of Surgery[1], Biochemistry and Molecular Biology[2], Medicine[3], Radiation Oncology[4], Indiana University School of Medicine. Indianapolis, IN 46202.

The nuclear hormone receptors which include receptors for steroid hormones, thyroid hormones, retinoids, vitamin D3 and orphan receptors, function as transcription factors that exert profound effects on growth, development and differentiation. Impaired activity of estrogen receptors (ERs), progesterone receptors (PRs) and retinoic acid receptors (RARs) is believed to be responsible for anti-estrogen resistant and metastatic growth of breast cancers. The degree of transcriptional response by ERs and RARs is determined by a combinatorial function of other nuclear hormone receptors which includes two closely related orphan receptors chicken ovalbumin upstream promoter transcription factor 1 (COUP-TF1) and apolipoprotein regulatory protein -1 (ARP-1). Expression pattern of COUP-TF1 and ARP-1 were examined in ER-positive and ER-negative breast cancer cell lines. Constitutive expression of COUP-TF1 was observed in all cell lines examined. ARP-1 expression in ER-positive cell lines was either constitutive (MCF-7) or retinoic acid inducible (T47-D, ZR-75-1). In contrast, four out of nine ER-negative cell lines (MDA-MB-435, MDA-MB-468, SK-BR-3, MCF-10A) expressed very little ARP-1 while the expression in three other cell lines (MDA-MB-157, MDA-MB-436 and Hs 578T) was lower than that of MCF-7. Furthermore, four out of ten primary breast cancers displayed reduced ARP-1. Interestingly, unlike PRs and RARs whose loss of expression is a consequence of loss of ER activity, lack of ARP-1 expression is an independent event as MCF-7 and ER-negative cell lines MDA-MB-231 and HBL-100 expressed similar level of ARP-1 and estrogen did not induce ARP-1 in MCF-7 and T47-D cell lines. Reexpression of ARP-1 but not COUP-TF1 by retrovirus mediated gene transfer resulted in diminished growth of MDA-MB-435 cell line due to prolonged cell cycle length. These findings suggest that reduced ARP-1 expression may lead to imbalance in expression of cell cycle regulatory genes and rapid progression of breast cancer.

Tamoxifen induces a dramatic reduction of nucleotide pools and thymidine kinase levels in MCF-7 breast cancer cells, Kim O'Neill, Fan Zhang, Xiaowen Shao, Suzann Adams, Bryan Poe, Department of Microbiology, Brigham Young University, Provo, UT 84602

Tamoxifen (TAM) is the drug of choice for adjuvant therapy following breast cancer surgery due to its anti-estrogenic characteristics. However, some tumors are resistant to TAM and at present, there are no definitive markers to ascertain therapeutic efficacy of the drug.

Thymidine kinase (TK) is associated with DNA synthesis. TK levels have been shown to be correlated with the development and progression of various cancers. We now report that *in-vitro* exposure of MCF-7 breast cancer cells to increasing TAM concentrations results in a dramatic decrease in cellular TK levels in a dose-dependent manner within 24 hours. Growth of TAM treated cells showed significant inhibition compared to the control cells within 5 days. The overall nucleotide pool levels were shown to drop significantly within 24 hours. Genotoxic studies were also carried out using the comet assay and confirm that TAM (10 $\mu$M) is tumorstatic rather than tumorcidal. In concordance with cell viability, these studies indicate that the cellular depression of TK is not a result of cellular death pathways.

We hypothesize that down-regulation of TK by TAM is due to competitive inhibition of the estrogen receptor and subsequent alteration of transcription at the nuclear level. In conclusion, these data suggest that TK may be a valuable marker in monitoring adjuvant TAM therapy in breast cancer patients.

## INCREASED CARRIER RATE OF BRCA2 6174delT GERMLINE MUTATIONS IN PATIENTS WITH PANCREATIC ADENOCARCINOMA

Hilmi Özcelik[1,5], Beverly Schmocker[2,5], Nando Di Nicola[1,5], Xiu-Hong Shi[1,5], Bernard. Langer[2,6], Malcolm Moore[3,7], Bryce.R. Taylor[2,6], Gerrarda Darlington[9], Irene L. Andrulis[1,4,5], Steve Gallinger[2,5,6], and Mark Redston[1,6]

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Mutations of the hereditary breast cancer gene, BRCA2, was initally shown to be present in cancer families with very high risk of female breast cancer sometimes associated with male breast cancer. However, recently other cancers were suggested to be increased in BRCA2 families, including cancers of prostate, colon, esophagus and particularly pancreatic cancer. In one study pancreatic cancer has been shown to be a strong predictor for germline BRCA2 mutations. Because there is evidence of low penetrance of BRCA2 mutations, such as absence of family history in some male breast cancer patients with germline BRCA2 mutations, in this study we analyzed the presence of germline mutations in a cohort of pancreatic cancer patients which were collected independent of family history. We have analyzed the entire coding region of BRCA2 gene in 41 pancreatic cancers by Protein Truncation Test (PTT) using DNA and RNA extracted from peripheral blood samples. Mutations in exon 11 of BRCA2 gene have been identified in 2 patients (4.9%), one of which was the 6174delT Ashkenazi Jewish mutation. We further characterize the prevelence of this mutation in formalin fixed paraffin-embedded normal tissue DNA samples from 26 Jewish pancreatic cancer patients. Heteroduplex analysis revealed the presence of 6174delT mutation in 3 patients. In total, we identified 4 germline *BRCA2* 6174delT mutations in 39 Jewish individuals (paraffin and blood) with pancreatic cancer (10% carrier rate; 95% confidence interval 3 to 24%). This is a significant increase in the 6174delT heterozygote rate compared to 1.36% in the Ashkenazi Jewish population (Fisher Exact $p=0.002$). While additional studies are required to confirm our findings, we suggest that germline *BRCA2* 6174delT mutations may be associated with an increased risk for the development of pancreatic cancer, and that an important subgroup of pancreatic cancer could be associated with germline *BRCA2* mutations.

Clinical and Genetic Evaluation of Ovarian Cancer Families. Ronald P. Zweemer, Rene H. M. Verheijen, Johan J. P. Gille, Paul J. van Diest, Fred H. Menko, Maria J. Worsham, Patricia L. Christopherson, S. David Nathanson, Sandra R. Wolman, Gerard Pals. Departments of Clinical Genetics, Gynecology and Pathology, University Hospital Vrije Universiteit Amsterdam, and Departments of Pathology and Surgery, Henry Ford Health Care System, Detroit, MI

We examined the prevalence of BRCA1 and BRCA2 germline mutations in 53 patients from 32 ovarian cancer families in relationship to age at diagnosis, histology and FIGO stage. The 32 ovarian cancer families were subdivided into four groups based on the tumor types and disease pattern in each family. Disease was classified as 1) hereditary breast and ovarian cancer (HBOC) and 2) hereditary ovarian cancer (HOC) when: a) in the case of maternal transmission, at least three family members from two successive generations had a confirmed diagnosis of breast or ovarian cancer (or ovarian cancer only in HOC), one case being a first-degree relative of the other two cases, or b) in case of paternal transmission, at least two first-degree relatives and one (paternal) second degree family member from two successive generations with confirmed breast or ovarian cancer (or ovarian cancer only in HOC). When there was familial clustering and the pedigree data did not fulfill the criteria for either HBOC or HOC, the families were described as 3) familial breast and ovarian cancer (FBOC) and 4) familial ovarian cancer (FOC). We compared relevant clinical characteristics in each group with those of the presumably sporadic cases in a cancer registry group. BRCA1 (exons 2-24) and BRCA2 (exon 11) germline mutation detection was performed by protein truncation testing and sequencing of BRCA1 exon 2 (185delAG mutation). Fifteen (51.7%) of 29 families tested revealed a germline BRCA1 or BRCA2 mutation (11 BRCA1 mutations, 3 BRCA2 mutations). Mutations were detected in 7 of 13 (53.8%) HBOC families, in 6 of 11 (54.5%) of FBOC, and 1 of 5 (20%) FOC families. Patients with ovarian cancer from the study group had significantly lower age at diagnosis (mean age of 54.3 years (range 31-77)), more advanced disease and a relatively higher frequency of serous adenocarcinoma. The finding of 3 BRCA2 mutations suggests that perhaps the frequency of ovarian cancer in BRCA2 families is higher than has been appreciated.

**Protein tyrosine phosphatase activity and apoptosis in a hormone-dependent/hormone-independent cancer progression model.** Carl Y. Sasaki and Antonino Passaniti, Laboratory of Biological Chemistry, National Institute on Aging, N.I.H., Baltimore, Maryland

Acquisition of resistance to apoptosis maybe associated with tumor progression. We have previously isolated a spontaneous rat mammary tumor cell line from aged mice (RM22 E-type) which is hormone-dependent and retains many characteristics of a well-differentiated epithelial cell. From these cells a hormone-independent cell line was derived (RM22 F-type) exhibiting increased vimentin expression and tumorigenicity (a fibroblastic phenotype). Here we show that treatment of the hormone-dependent E-type cell with protein tyrosine phosphatase inhibitors results in apoptosis whereas the hormone-independent F-type cell is resistant to this treatment. Sodium orthovanadate, a protein tyrosine phosphatase inhibitor, induced cell death in a dose dependent manner either in the presence or absence of serum. Treated cells exhibited nuclear morphological alterations and DNA fragmentation consistent with apoptosis. Bromotetramisole, also a protein tyrosine phosphatase inhibitor, induced cell death of the E-type cells whereas okadaic acid, a protein serine/threonine phosphatase inhibitor, had no effect. Protein tyrosine phosphatase inhibitor-induced cell death occurred within 24 hours and was not dependent on *de novo* protein synthesis. These findings suggest that selection for more invasive and tumorigenic cells during tumor progression results in acquired resistance to apoptosis.

## Development and analysis of human breast and ovarian cancer cell lines constitutively expressing antisense-BRCA1

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BRCA1 has been shown to be a tumour suppressor gene, important in development, and is implicated in early onset breast ovarian cancer syndrome. The mechanism for its role in breast cancer, however, has not been precisely defined. For this purpose, model systems have to be developed in which BRCA1 expression can be manipulated experimentally. Mouse models are often used as a paradigm for human systems but, in the case of BRCA1, these systems may not reflect what is observed in humans. For example, knockout of murine Brca1 results in death *in utero*, which has not been observed in humans (even doubly mutant). Here we present the initial stages of development of an alternative model system to create human cells which are deficient in BRCA1 protein.

In order to create this system, a mammalian expression vector was constructed which consists of the 5' fifth of BRCA1 transcribed in an antisense orientation by a CMV promoter. This expression construct was then transfected into MCF7 and SKOV3 cells, stably integrated by selection with G418, and resistant cells clonally expanded. The MCF7 cell line is an immortal human breast adenocarcinoma of epithelial-like morphology, which has maintained several characteristics of differentiated mammary epithelium, including the capability to form domes and expression of functional oestrogen receptor. SKOV3 is a human ovarian adenocarcinoma of epithelial like morphology. Transcription of the BRCA1 element of the construct has been confirmed by RT-PCR, and northern analysis. Phenotypic characterisation of the transfected cell lines has begun and extensive studies are proposed. Comparision of paried transfected and parental cell lines should help to validate the specificity of putative anti-BRCA1 antibodies..

Basic and Clinical Aspects of Breast Cancer.

## Establishment of Her2/neu expressing tumor models in immunocompetent mice.

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The aberrantly expressed her2/neu oncogene has been found to be amplified in as many as 30% of human breast and 10-30% of ovarian cancers. Overexpression of her2/neu in breast and ovarian cancers has also been shown to be associated with poor prognosis. To provide an immunocompetent animals model for breast cancer we have developed antigen-specific tumor models by transducing murine cell lines with human her2/neu.

The mouse tumor adenocarcinoma MC38 and T-lymphoma EL4 cell lines (derived from the mouse strain C57Bl/6) were transfected with a retroviral vector containing the her2/neu cDNA. MC38 and EL4 cells sorted for high her2/neu expression were injected in the flank of C57Bl/6 mice. The kinetics of tumor growth of both human-her2/neu expressing and parental cells was similar. When the tumor was dissected from the mouse and the cells were adopted to culture, more than 90% of the cells expressed her2/neu over several passages.

To determine the tumorogenic potential, different doses of MC38 and EL4 cells expressing her2/neu were injected intraperitoneal, subcutaneous or intravenuos. MC38 and EL4 cells expressing her2/neu and the parent cell lines show similar tumorogenic potential.The induced tumor displayed a histology of malignant tumor and the *in vivo* expression of her2/neu was also detected.

Our results suggest that our tumor models might be a useful tool for the study of breast cancer and/or breast cancer therapy.

## BASIC AND CLINICAL ASPECTS OF BREAST CANCER

Title: **Family history and the risk of breast cancer: A systematic review and meta-analysis**

Authors: **Paul D.P. PHAROAH[1,2,*], Nicholas E. DAY[1], Stephen DUFFY[1], Douglas F. EASTON[1], Bruce A.J. PONDER[2]** (1 Institute of Public Health, Robinson Way, Cambridge, CB2 2SR, U.K. 2 CRC Human Cancer Genetics Group, Box 238, Addenbrooke's Hospital, Hills Road, Cambridge, CB2 2QQ, U.K.)

**Introduction**

An increased risk of breast cancer in women with a family history of breast cancer has been demonstrated by many studies using a variety of study designs. The magnitude of this risk varies according to the nature of the family history: type of relative affected and number of relatives affected may all alter risk. In addition, the risk may be modified by the age of the individual and age at diagnosis of the relative. The aim of this study was to identify all the published studies which have quantified the risk of breast cancer associated with a family history of the disease, and to summarise the evidence from these studies, with particular emphasis on the age specific risks according to subject and relative age. All studies which published data on familial risk of breast cancer were eligible for inclusion.

**Methods**

Published studies were identified using the *Medline* (National Library of Medicine, USA) databases for 1966-1996 using the search terms "breast neoplasms" and "family". In order to identify studies in which family history had been recorded but was not a focus of the write-up, a search on the terms "breast neoplasms" and "cohort" or "case-control" was also performed. Studies identified in this way were then read to abstract any reported family history data. The computer aided searches were complemented by following up the citations from all studies located. Pooled estimates of risk were calculated as a weighted average of the log relative risk estimates. Studies were weighted according to the inverse of the variance of the log relative risk, which was estimated from the confidence interval for that relative risk.

**Results**

74 eligible studies were identified -52 case-control studies and 22 cohort studies. The pooled estimate of relative risk associated with various family history categories was as follows: any relative, RR = 1.9 (95% CI 1.7-2.0); a first degree relative, RR = 2.1 (CI = 2.0,2.2); mother, RR = 2.0 (CI = 1.8,2.1); sister, RR = 2.3 (CI = 2.1,2.4); daughter, RR = 1.8 (CI = 1.6,2.0); mother and sister RR = 3.6 (2.5,5.0); a second degree relative RR = 1.5 (CI = 1.4,1.6). Risks were increased in subjects under the age of 50 and when the relative had been diagnosed before the age of 50.

The risk estimates varied according to the study type and design. For example the pooled risk estimates for a history of breast cancer in any first degree relative was 1.6 (CI = 1.4,1.9) for the prospective cohort studies was compared to 2.2 (CI = 2.1,2.3) for the retrospective study designs. The pooled risk estimate for the case-control studies with population controls was 2.2 (CI = 2.1,2.3) compared to 2.5 (CI = 2.2,2.7) for the case-control studies using hospital controls.

**Discussion:**

In the discussion, potential causes of bias (eg publication bias) that may affect the validity of these results are considered as are The implications of the age specific risk differences and the differences in risks estimated by different study designs.

Alternative Splicing of the p53-Binding Domain of MDM2 during Normal Mammary Gland Development and Mammary Tumorigenesis, Jan Pinkas[1], Changli He[2], Charlotte Kuperwasser[2], Steven P. Naber[3] and D. Joseph Jerry[1,2]. [1]Program in Molecular and Cellular Biology, [2]Department of Veterinary and Animal Science University of Massachusetts, Amherst MA and [3] Department of Pathology, Baystate Medical Center, Springfield, MA.

Activation of MDM2, a cellular antagonist of p53, has been observed in human tumors. Analysis of human breast carcinomas by immunohistochemistry using amino terminal antibodies demonstrated that MDM2 is overexpressed in approximately 25% of tumors tested, while being undetectable in normal breast tissue. However, multiple forms of MDM2 have been observed, some of which lack the amino terminus and are unable to bind p53. Therefore, experiments were conducted to determine if the pattern of mdm2 expression is altered during breast tumor progression. Mammary tumors would be expected to express mdm2 isoforms containing the p53-binding domain, while this epitope would be absent from mdm2 expressed in quiescent mammary tissue. In murine mammary tissues at least three distinct mRNA transcripts (3.3, 1.6 and 1.5 kb) were detected by Northern blot analysis. The 3.3 kb transcript was expressed constitutively in normal mammary tissues, as well as in hyperplasias (HAN) and tumors, whereas, the 1.6 and 1.5 kb transcripts appeared to be differentially expressed. The 1.5 kb transcript was the major form detected in nulliparous and hyperplastic mammary tissue, while expression of the 1.6 kb transcript was induced during tumor progression. The 1.6 kb transcript was also found to be induced during lactation resulting in a pattern of transcripts similar to that found in tumors. The structures of the *mdm2* mRNA transcripts was addressed through RT-PCR analysis and cDNA library screening. The RT-PCR analysis indicated that the transcripts expressed in nulliparous mammary tissue and 4/5 HAN lacked exon 3 which codes for the p53-binding domain, but they contain 3' sequences coding for the carboxy terminus. Fragments containing complete coding sequences were identified by RT-PCR in mRNA from 5/5 mammary tumors examined and from lactating mammary tissue. Similar analyses were performed on a panel of normal and malignant human breast tissue. The RT-PCR results paralleled those seen with murine mammary tumors, with 12/12 human breast carcinomas and 0/6 normal breast tissue samples expressing transcripts containing exon 3. A fragment corresponding to 3' coding sequences was amplified from RNA from all of the normal and malignant human breast tissue. Multiple alternatively spliced cDNA clones were isolated from virgin and lactating 1 day murine mammary cDNA libraries. Apparent full-length 3.3 kb cDNA clones were obtained, but were found to be heterogeneous due to alternative splicing. The splice variants included deletion of the 5' exons encoding the p53-binding domain, deletion of internal exons, and an alternate exon in the 3'UTR. Smaller clones bearing internal deletions appear to represent the 1.5 and 1.6 kb transcripts observed by Northern blot analysis. The data demonstrate that multiple alternatively spliced *mdm2* mRNA transcripts are expressed and differentially regulated during normal and neoplastic breast development. This analysis of *mdm2* transcripts directly implicates inclusion of 5' exons encoding the p53-binding domain as a consistent etiological factor in breast tumorigenesis in both mice and humans.

"Basic and Clinical Aspects of Breast Cancer"

Estrogen-Dependent Regulation of the Cell Cycle in Breast Cancer Cells

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Whitehead Institute for Biomedical Research, Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02142

Estrogen is a key factor controlling growth and development of normal mammary gland and breast tumors. However, the molecular mechanisms by which estrogen affects proliferation are not well understood. In order to determine mechanisms by which estrogens and antiestrogens modulate the growth of breast cancer cells, we have characterized the changes induced by estradiol that occur during the G1 phase of the cell cycle of MCF-7 human mammary carcinoma cells. Addition of estradiol relieves the cell cycle block created by tamoxifen treatment, leading to marked activation of cyclin E-cdk2 complexes within 6 hr. The kinetics of cyclin E-cdk2 activation parallel the phosphorylation of the retinoblastoma protein. Cyclin D1 levels increase significantly while the levels of cyclin E, cdk2, and the p21 and p27 cdk inhibitors are relatively constant. However, the p21 cdk inhibitor shifts from its association with cyclin E-cdk2 to cyclin D1-cdk4, providing an explanation of the observed activation of the cyclin E-cdk2 complexes. These results support the notion that cyclin D1 has an important role in steroid-dependent cell proliferation and that estrogen, by regulating the activities of G1 cyclin-dependent kinases, can control the proliferation of breast cancer cells.

ANALYSIS OF A GENE ENCODING A BRCA1 ASSOCIATED PROTEIN IN BREAST AND LUNG CANCER CELL LINES. Monia L. Proctor[1], Yoshitaka Sekido[1], Gail Tomlinson[1], David Jensen[2], Frank Rauscher III[2] and John D. Minna[1]. [1] Hamon Center for Therapeutic Oncology Research, Univ. of Texas Southwestern Med. Ctr., Dallas and [2] Wistar Institute, Philadelphia

Half of familial breast cancers are associated with *BRCA-1* gene mutations and the BRCA-1 protein appears mislocalized in many sporadic breast cancers which have no detectable *BRCA-1* mutations. The *BAP-1* gene is located on chromosome 3p21, a region deleted in breast cancer. It encodes a protein which binds to the zinc-finger region of the BRCA-1 protein and may participate in the *BRCA-1* tumor suppressor pathway. We studied breast cancer (BC) cell lines for mutations in the *BAP-1* gene. Small cell lung cancer (SCLC) and non small cell lung cancer (NSCLC) cell lines were also evaluated for mutations given the frequent genetic abnormalities in the 3p21 region seen in these cancers.

RT-PCR SSCP analysis was used to screen the 1.7 kb open reading frame of *BAP-1* for mutations with fourteen overlapping primer pairs. Sixteen BC, 39 SCLC and 19 NSCLC cell lines were studied and abnormal SSCP bands were reamplified by PCR and sequenced by dye terminator chemistry. Southern and Northern blot analyses were performed to detect gross structural and expression abnormalities respectively in BC and LC lines.

One SSCP shift was found in the 16 BC cell lines which consisted of a silent mutation without amino acid alteration; no other significant *BAP-1* mutations were found. Five *BAP-1* alterations were found in 39 SCLC cell lines: one expressed a shortened transcript resulting in an 18 amino acid deletion; another contained a single base pair change that altered the amino acid; and three cell lines had silent polymorphisms. In 19 NSCLC cell lines, one cell line contained an eight base pair deletion which resulted in a truncated protein. An additional NSCLC cell line was found to have a large homozygous deletion in the *BAP-1* region by Southern blot analysis. *BAP-1* was ubiquitously expressed in BC, SCLC, and NSCLC cell lines; expression was absent only in the NSCLC cell line with the *BAP-1* truncation mutation. These studies suggest that genetic alterations in *BAP-1* may occur in some human cancers. Further analysis of *BAP-1* mutations may help characterize BAP-1 function as well as the BAP-1/BRCA-1 interaction.

Tumor-cell based vaccine decreases metastatic potential of 4T1, a highly spontaneously metastatic mammary carcinoma. Beth A. Pulaski and Suzanne Ostrand-Rosenberg. University of Maryland, Baltimore County. Baltimore, MD 21250.

While primary breast tumors can be surgically removed, typically disseminated metastatic tumor is responsible for a patient's demise. Therefore, we developed a gene transfection strategy in which tumor cells themselves can serve as a vaccine to activate both $CD4^+$ and $CD8^+$ T-cells against metastatic breast cancer. Since optimal activation of $CD8^+$ T-cells requires "help" from $CD4^+$ T-cells, we hypothesized that co-activation of both types of cells should provide a potent anti-tumor immune response. Previously we found that class II MHC gene transfection increases the ability of sarcoma cells to present antigen and activate $CD4^+$ T-cells. Thus, we developed a strategy which includes the genes for class II MHC and B7-1, which has also been shown to significantly facilitate optimal activation of $CD4^+$ T-cells. To model metastatic cancer in mice, we are using the 4T1 mammary carcinoma, a poorly immunogenic tumor that is highly spontaneously metastatic in syngeneic BALB/c mice. This tumor line expresses suitable levels of class I MHC for presentation of tumor antigen to $CD8^+$ T-cells and no detectable class II MHC and is readily transfectable with foreign genes. Our data reveals that the effects of B7-1 or class II MHC expression on spontaneous metastases are different from the effects on progressive solid (primary) tumor formation. While a dose of $5x10^3$ wild type 4T1 tumor cells injected subcutaneously can form primary tumors in 100% BALB/c mice, only 20-60% of mice develop primary tumors from $B7\text{-}1^+$ tumor cells depending on the level of B7-1 expression. However, regardless of B7-1 expression levels, the metastatic potential analyzed at day 55 showed that 19/20 of these same mice contained <10% of the levels of metastatic cells as compared to wild type controls. The primary tumor growth of class $II^+$ tumor cells was varied. Progressive tumor formation was either delayed, completely eliminated, or partially reduced to 40% in BALB/c mice as compared to 100% growth in wild type controls. However, the early onset of metastatic disease (day 32) by these transfectants was completely eliminated in 14/15 mice and partially reduced in the remaining mouse. Groups of *nu/nu* mice injected with four different (2 class $II^+$ and 2 $B7\text{-}1^+$) transfectants developed progressive tumors indicating the requirement of T-cells for control of primary tumor growth. In contrast, the metastatic potential of 1/2 class $II^+$ transfectants was completely eliminated and significantly reduced (>90%) for 1/2 $B7\text{-}1^+$ transfectants, suggesting the involvement of T-cells and/or other effector cells. More importantly, vaccination of BALB/c mice with just one dose of class $II^+$ tumor cells alone or mixed together with the $B7\text{-}1^+$ tumor cells offered complete or partial (>80% reduction) protective immunization against metastatic disease from a wild type tumor challenge in 9/12 or 3/12 mice, respectively. Interestingly, this vaccination did not protect against the primary growth of wild type tumor. Therefore, these data demonstrate that class $II^+$ and $B7\text{-}1^+$ transfectants are effective immunotherapeutic agents and that different immune cells control metastatic disease vs. primary tumor growth.

"Basic and Clinical Aspects of Breast Cancer"

THE ROLE OF ERBB2 EXTRACELLULAR DOMAIN IN PREDICTING RESPONSE TO HIGH-DOSE CHEMOTHERAPY IN BREAST CANCER PATIENTS. Michael J. Ramirez, Donald Berry, James J. Vredenburgh, Lyndsay N. Harris, Duke University Medical Center, Durham, NC.

ErbB2 is overexpressed in nearly 30% of human breast cancer specimens and is associated with poor outcome in most studies. This relationship is also demonstrated in patients with extracellular domain (ECD) of erbB2, thought to be a surrogate marker for receptor expression. Clinical studies support an association between erbB2 receptor overexpression and resistance to alkylating agents. Other data indicate a dose-response relationship of doxorubicin in erbB2 overexpressing patients, such that tumors which overexpress erbB2 are more effectively treated with higher doses of doxorubicin containing FAC than non-overexpressing tumors.

**Study**: From February 1987 to January 1991, a population of 85 (study #8782) patients at Duke University Medical Center with high risk breast cancer underwent high-dose cyclophosphamide, cisplatin, carmustine with autologous bone marrow support after four cycles of cyclophosphamide, doxorubicin and fluorouracil. Serum samples drawn after standard adjuvant therapy but before high-dose chemotherapy were available for study and analyzed for ECD using an ELISA assay with a cut-off value of 30 U/ml. (Serum ECD's generously performed by Chiron Diagnostics).

**Results**: 82 patients were evaluable for study. 0/82 samples were positive for erbB2 ECD after induction therapy with standard FAC. The minimum value was 1.57, and the highest value was 16.8 U/ml. The median was 5.94 U/ml and the mean 6.42 U/ml. The standard deviation was 2.88. Using a Cox proportional hazards model, there was no predictive value for erbB2 ECD for OS or DFS. Comparing Kaplan-Meier curves for patients above and below the median also showed no statistical difference in DFS or OS. Finally, there was no statistical correlation with erbB2 and tumor size, ER/PR hormone receptor status, age, S phase or p53.

**Conclusions**: ErbB2 extracellular domain was not a useful predictor of response to high dose chemotherapy after standard-dose adjuvant therapy with FAC for high risk primary breast cancer. No positive serum samples for erbB2 ECD were obtained in this cohort of 82 patients pretreated with standard FAC. Whether or not induction chemotherapy including doxorubicin altered the expression of the erbB2 ECD is an interesting question. We intend to analyze serum samples for erbB2 ECD from this cohort before induction therapy for comparison and to compare these results with the original tissue erbB2 by immunohistochemistry. Additionally, we plan to investigate the role of erbB2 ECD in predicting response to high-dose chemotherapy in the metastatic setting.

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"Basic and Clinical Aspects Of Breast Cancer"

**Analysis of *BRCA*1 and *BRCA*2 mutations in Scottish breast cancer families**

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We have been screening the lymphocyte DNA of index cases from breast and ovarian cancer families in Scotland for germline *BRCA*1 and *BRCA*2 mutations. These families were identified as "at risk" by the Dept. Of Medical Genetics. In an attempt to increase the speed of mutation detection, Protein Truncation Testing (PTT) was carried out on 146 cases to identify which truncating mutations were present in exon 11 of *BRCA*1 and *BRCA*2 in the Scottish population. Single Stranded Conformation Polymorphism (SSCP) analysis of the remaining coding exons was carried out on a further 40 families. Any mutations found were also screened for in a total of 276 patients.

To date we have identified 8 different mutations in 13 families. Six of these families show the same mutation, an AA deletion in exon 11 of *BRCA*1 at position 2800 (2800 del AA). This results in an in frame stop codon at nucleotide 2820. One individual was shown to be homozygous for this mutation, thereby giving rise to a naturally occurring human "knockout" for this gene. This (2800 del AA) mutation appears to be common in the Scottish population and all six families share a common haplotype around the *BRCA*1 gene. The other mutations were found in individual families; a 185 AG deletion, a C insertion at position 5382. Those mutations found in *BRCA*2 were 5445 del 7, 5574 del AA, and an 8525 del C all resulting in truncated protein products.

The results demonstrate which germline mutations are present in familial breast cancer cases in Scotland. This has been of help to the patient, geneticist and surgeon allowing them to make more informed decisions with regards to the risk of developing breast cancer not only in a single patients but in subsequent generations.

We have now cloned portions of wild-type and mutant *BRCA*1 genes into bacterial expression plasmids, and isolated recombinant proteins. Using these antigens, we have developed antibodies to the carboxy terminal of the 2800 del AA *BRCA*1 and the 5445 del 7 *BRCA*2 mutation gene products, and to 3 portions of the wild-type protein. These are currently being tested to see if they are of use in identifying mutation carriers and in identifying interacting proteins.

Basic and Clinical Aspects of Breast Cancer

Allelic losses of *BRCA1*, *BRCA2* and *ATM* genes in sporadic invasive ductal breast carcinoma are associated with different clinicopathological parameters.

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To study genetic changes and the respective involvement of the *BRCA1*, *BRCA2* and *ATM* genes in sporadic breast cancer, we assayed for loss of heterozygosity (LOH) in 59 sets of invasive ductal carcinoma, compared to matched normal control DNA. Microsatellite markers which are intragenic to *BRCA1*, *BRCA2* and *ATM* were simultaneously used. In addition, four more markers centromeric and telomeric to *ATM* were analysed.

Thirty two per cent of the informative cases showed LOH for the *BRCA1* gene, 40% for *ATM* and 18% for *BRCA2*. LOH involving the *ATM* region were associated with large size tumors (p=0.01) and showed a good trend, although statistically not significant, in association with high lymph node metastasis number. LOH of *BRCA1* and/or *BRCA2* significantly correlated with high grade tumors plus negative hormone receptors. *BRCA2* negatively correlated with low number of metastatic lymph nodes (p=0.01).

These results may indicate that *ATM* alterations and *BRCA1/BRCA2* alterations do not occur in the same pathways in the pathogenesis of breast carcinoma.

Breastfeeding and risk of breast cancer in the offspring. Matti A. Rookus, Thea M. Mooij, Flora E. van Leeuwen (Netherlands Cancer Institute, 1066CX Amsterdam, Netherlands)

Breastfeeding as opposed to bottle-feeding may affect the risk of breast cancer in the offspring. Differences in milk content, feeding practices or a retrovirus may play a role. Two recent US studies found a protective effect for being breastfed, but a Swedish study did not find an association (Lancet 1992;340:1015-8, Epidemiol 1994;5:324-31, 1995;6:198-200).

The relationship between being breastfed and the risk of breast cancer was investigated in a Dutch population-based case-control study of breast cancer and oral contraceptives. The study included 918 breast cancer patients (20-54 yrs, diagnosed 1986-1989). Population controls were pair-matched on age. During home interviews the women provided information on possible risk factors of breast cancer. In case the participant did not know whether or not she had been breastfed, she was asked to question her mother if she was still alive.

Information about being breastfed was provided by 88.5% of the cases and 92.2% of the controls. The duration of lactation was available for 75.1% of the cases and 76.6% of the controls. The proportions of breastfed women among cases and controls were 74.7% and 76.9%, respectively. The median period of breastfeeding had been 12 weeks in cases and 13 weeks in controls. There was no association between breastfeeding and risk of breast cancer in the offspring; with a Relative Risk (RR) for being breastfed as compared to being bottle-fed of 1.0 (95% Confidence Interval (CI): 0.8-1.4), adjusting for level of education and family history of breast cancer. No duration-response relationship was found: a period of breastfeeding of more than 30 weeks as compared to not being breastfed was not associated with a substantially decreased risk (RR=0.9; 95%CI: 0.6-1.3).

Our findings do not support a protective effect of breastfeeding on risk of breast cancer in the offspring, as was found in two other recent studies.

"BASIC AND CLINICAL ASPECTS OF BREAST CANCER"

**GROWTH FACTOR DEPRIVATION THERAPY OF HORMONE INSENSITIVE PROSTATE AND BREAST CANCERS UTILIZING ANTISENSE OLIGONUCLEOTIDES,** Marvin Rubenstein, Ph.D., Yelena Mirochnik and Patrick Guinan, M.D., Division of Cellular Biology, Hektoen Institute for Medical Research, Chicago, Illinois, 60612.

Antisense oligonucleotides (oligos) are artificial sequences of nucleotide bases which may be synthesized complementary to known regions within specific mRNAs. When these constructed oligos interact with protein encoding mRNA they may regulate expression of various growth factors and/or their receptors. Oligos directed against transforming growth factor-α (TGF-α) and its binding site, the epidermal growth factor receptor (EGFR), were employed: A) *in vitro* to affect the growth of hormone insensitive human derived PC-3 prostate cancer cells as well as the human derived UACC-893 breast cancer cell line; and B) *in vivo* to treat tumors established by these cell lines in athymic nude mice.

The *in vitro* results for each oligo, and their combination, produced significant inhibition of both prostate and breast cell lines. In addition, the combination of oligos most efficiently diminished the immunohistochemical expression of both TGF-α and EGFR in PC-3 cells. Direct *in vivo* inoculation of oligos into established PC-3 or UACC-893 tumors in nude mice produced hemorrhagic necrosis within 2-3 days.

Such therapy could represent a new tier of therapy for recurrent, hormone insensitive, tumors based upon the concept of growth factor deprivation.

**BRCA1 is a cell cycle regulated nuclear phosphoprotein**

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We have characterized the BRCA1 gene product using polyclonal antibodies raised against peptides from four different regions of the protein. The antibodies specifically recognize a ~220 kD BRCA1 protein. The specifity of the antibodies was confirmed by comparing two-dimensional tryptic peptide maps of *in vitro* synthesized BRCA1 protein and immunoprecipitated endogenous and overexpressed BRCA1 protein. BRCA1 is predominantely expressed in the nucleus of both normal and neoplastic breast cancer cells. It is a serine phosphoprotein which undergoes hyperphosphorylation during late G1 and S phases and is transiently dephosphorylated early after M phase. Moreover, BRCA1 mRNA and protein levels are cell cycle regulated.

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## THE POLYPROLINE REGION OF P53 IS REQUIRED TO ACTIVATE APOPTOSIS BUT NOT CELL GROWTH ARREST

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The mechanism by which p53 induces apoptosis is poorly understood but crucial to an understanding of its function. We report that a p53's polyproline region (PP), which has putative SH3 binding sites (PxxP), is necessary to induce cell death but not growth arrest.

PP is located between p53's transactivation and DNA binding domains. PP deletion did not affect the ability of p53 to bind DNA, suppress E1A+RAS transformation of rat embryo fibroblasts and primary baby rat kidney epithelial cells (BRKs), or inhibit the growth of SAOS-2 osteosarcoma cells. Moreover, a temperature sensitive (ts) dominant inhibitory p53 mutant lacking PP ($p53^{ts}\Delta PP$) retained its ability to cooperate with adenovirus E1A in immortalization of primary BRK cells. However, while E1A+$p53^{ts}$-immortalized BRKs died following activation of wild-type p53, E1A+$p53^{ts}\Delta PP$-immortalized cells arrested in the G1 phase when shifted to the permissive temperature for wild-type p53 activity but remained viable. Similarly, PP deletion abolished apoptosis in LoVo, a colon carcinoma cell line which is killed by introduction of wild-type p53. Consistent with its lack of effect on cell cycle arrest, PP deletion reduced but did not abolish transactivation from an artificial promoter or the WAF1 promoter. When fused to the GAL4 DNA binding domain, PP transactivated only a subset of GAL4-responsive promoters, suggesting that it may have a selective role in transactivation.

Taken together, we concluded that 1) the PP region plays a crucial role in p53-mediated apoptosis, possibly through effects on transactivation of certain p53 target genes or direct participation in signaling pathways activating cell suicide, or both, and 2) p53's ability to induce apoptosis is dispensable for inhibiting cell growth and transformation. A model in which PP region acts as a binding site for nuclear SH3 proteins will be presented.

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## A *HIS3* TRANSCRIPTIONAL ACTIVATION ASSAY FOR THE CHARACTERIZATION OF BRCA1 MUTATIONS

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The hereditary breast and ovarian cancer gene, *BRCA1*, contains two tandem copies of a highly acidic domain near the C-terminus. These newly recognized BRCA1 C-terminal domains (dubbed 'BRCT' domains) have recently been shown to activate transcription when fused to a heterologous DNA binding domain (GAL4). Transcriptional activation was shown to be specific to fusions of wild-type BRCA1 C-terminal sequence containing the tandem BRCT domains. Several missense and a truncating mutation found in patients with inherited breast or ovarian cancer had severely impaired transcriptional activity. Here we confirm these findings and use this approach to investigate two additional classes of sequence alterations: a common polymorphism and putative splice-site mutant of BRCA1. We show that missense mutations of BRCA1 which are not cancer-predisposing (i.e., wild-type polymorphisms) are transcriptionally active when fused to the GAL4 binding domain (GBD).

Utilizing a *HIS3* reporter in *Saccharomyces cerevisiae* strain HF7c, we confirmed that a fusion of the GBD to wild-type BRCA1 is transcriptionally active. In contrast, expression of GBD fused to BRCA1 containing an 11-amino acid truncation (Y1853stop) was not. Likewise, expression of GBD alone, GBD fused to another tumor suppressor (VHL), or BRCA1 fused to the GAL4 activation domain did not activate transcription.

The impairment of transcriptional activation has previously been correlated to missense mutations found in patients (e.g., M1775R). However, whether single amino acid substitutions not correlated with cancer would also fail to activate transcription has not been investigated. We thus fused GBD to BRCA1 containing the common S1613G polymorphism. This construct activated transcription of the *HIS3* reporter, while a cancer-predisposing missense mutation (P1749R) did not.

We then examined a putative splice-site mutation of BRCA1 which cosegregates with breast cancer. As the nucleotide change (G5193A) is thought to abrogate correct splicing at the exon 17-18 boundary, it was not known whether its representation in a correctly spliced message as a single amino acid substitution (D1692N) would inactivate the protein. We found that expression of GBD fused to BRCA1 containing the D1692N substitution activated transcription in this assay, suggesting that disruption of BRCA1 function occurs by splice site disruption and not by an amino acid substitution.

It is interesting to note that the ability of functional but not cancer-predisposing forms of BRCA1 to activate transcription as GBD fusions correlates strongly with a finding which we present elsewhere (Humphrey et al.), namely, that yeast expressing a GAL4 activation domain-BRCA1 fusion exhibit a small colony phenotype specific to wild-type and non-cancer-predisposing polymorphisms forms of the protein. Both of these approaches may thus be useful for the functional characterization of BRCA1 mutations.

**Mechanisms of Cdk Inhibition in Senescent Human Mammary Epithelial Cells.** Charanjit S. Sandhu [1], Martha R. Stampfer [2], Joyce M. Slingerland [1] Sunnybrook Health Science Centre, University of Toronto, Toronto, Ontario, Canada, M4N 3M5 [1]; Lawrence Berkeley National Laboratory, Berkeley, California, USA 94720 [2]

The progression of finite lifespan human mammary epithelial cells (HMEC) from early passage to G1 arrest in senescence is accompanied by gradual alterations in the activity and steady state levels of key cell cycle regulators. These alterations may bring about the increased proportion of HMEC with 2N DNA content and the gradual decrease in %S phase cells seen as HMEC approach senescence. We observe a progressive reduction in G1/S phase cyclin associated kinase activities as the HMEC population ages. The levels of cdk4 and cdk6 proteins and their associated cyclin, cyclin D1 remained constant. However, a marked reduction in cyclin A underlies the progressive loss of cyclin A/cdk2 activity as these cells approach senescence. In contrast to observations in aging fibroblasts, there is no increase in the cdk inhibitor, $p21^{Cip1}$, in HMEC at senescence. The $p27^{Kip1}$ levels are unchanged during aging of the HMEC population and the association of $p21^{Cip1}$ and $p27^{Kip1}$ with target cdks is not increased. However, there is a notable increase in the INK family inhibitor, $p15^{INK4B}$ due to a progressive increase in its rate of protein synthesis. The association of $p15^{INK4B}$ with both cdk4 and cdk6 is markedly increased in senescent cells. However, the increased binding of $p15^{INK4B}$ did not result in displacement of cyclin D1, $p21^{Cip1}$ and $p27^{Kip1}$ from cdk4 and cdk6 complexes. In senescent cells, p15/cdk complexes do not contain cyclin or Kip proteins. Consequently, there remains in senescent cells a substantial quantity of inactive cyclin D1 bound cdk4 and cdk6 in which the stoichiometry of Kip protein binding (p21 and p27) is not increased compared to that in early passage cells. Similarly, cyclin E/cdk2 inactivation in senescent cells is not due to loss of associated cyclin or increased p21 or p27 binding. At present we are investigating whether the inhibition of cyclin E and cyclin D1 associated kinases in senescent cells occurs through increased inhibitory phosphorylation of cdks by Wee1 or results from loss of cdc25 phosphatase activity

**Invasion of a hormone-independent breast cancer cell line is regulated by protein tyrosine phosphatases.** Carl Y. Sasaki and Antonino Passaniti, Laboratory of Biological Chemistry, National Institute on Aging, N.I.H., Baltimore, Maryland

Vimentin expression is associated with increased tumor cell invasion and metastasis. A highly invasive hormone-independent breast cancer cell line (RM22 F-type) was found to express higher levels of vimentin than its hormone-dependent parental cell line (RM22 E-type). Colonies of RM22 F-type exhibited increase migration and invasion through Matrigel extracellular matrix. RM22 F-type invasion measured in Matrigel-coated Boyden chambers was also two-fold higher than invasion of RM22 E-type. Sodium orthovanadate, a protein tyrosine phosphatase inhibitor, inhibited RM22 F-type invasion in a dose dependent manner. Bromotetramisole, a noncompetitive inhibitor of protein tyrosine phosphatase, also inhibited RM22 F-type invasion. Furthermore, zymographic evidence indicated that RM22 F-type cells activate gelatinase B. Gelatinase B activation was inhibited by both vanadate and minocycline, a gelatinase inhibitor. Taken together, protein tyrosine phosphatases and gelatinases are required for the activation of gelatinase B during RM22 F-type invasion.

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Tissue Remodeling of the Rat Mammary Gland, Implications for Chemoprevention

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We are investigating periods of tissue remodeling and programmed cell death in the mammary gland of the female Sprague Dawley rat. We have identified elevated levels of tissue remodeling and cell death during post-lactational involution, the estrous cycle, and peri-menarche. The tissue remodeling appears to be characterized by increases in epithelial cell death which, in order to maintain tissue size homeostasis, balance a preceding period where epithelial cell number is increased. Changes in tissue remodeling are measured by mammary gland histology, proliferative and apototic indices of epithelial cells, and regulation of matrix metalloproteinases. To determine whether tissue remodeling can be altered by dietary components we evaluated the impact of vitamin A deficiency on peri-menarche mammary gland development. Vitamin A deficiency during peri-menarche was associated with a delay in maturation of terminal end buds into ducts and alveolar buds suggesting an inhibition of tissue remodeling. Alterations in mammary gland remodeling as a result of dietary deficiency of vitamin A could contribute to mammary carcinogenesis by changing the size of the epithelial component of the gland and subsequent number of targets available for transformation

# ALL-*TRANS*-RETINOIC ACID MEDIATES G1 ARREST BUT NOT APOPTOSIS OF NORMAL HUMAN MAMMARY EPITHELIAL CELLS

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Retinoids mediate the normal growth of a variety of epithelial cells and may play an important role in the chemoprevention of certain malignancies. Loss of retinoic acid receptor-beta (RARβ) function may be an important event in mammary carcinogenesis since the majority of breast cancers, in contrast with normal mammary epithelial cells, fail to express this receptor. We previously reported that all-*trans*-retinoic acid (RA) mediates $G_1$ arrest as well as apoptosis in certain RARβ-transduced breast cancer cell lines. We now report the effect of RA on normal human mammary epithelial cells (HMECs), which express functionally active retinoid receptors. We observe that RA induces growth suppression and $G_1$ arrest of these HMECs but find no evidence that RA mediates apoptosis in these normal cell strains. This RA-induced $G_1$ arrest is temporally associated with decreased levels of hyperphosphorylated retinoblastoma protein (pRB) without any significant changes in c-myc, p53, p21, or p27 expression. Expression of cyclin D1, cdk4, and cyclin E protein, however, decreased in association with RA-mediated $G_1$ arrest. Cdk2 levels appeared to slightly decrease. Our studies suggest that growth inhibition, rather than apoptosis, may be a mechanism by which RA and RA receptors act to prevent the malignant transformation of normal mammary epithelial cells. The molecular targets(s) of the activated RA receptors that mediate this $G_1$ arrest in HMECs appear to be associated with an Rb-dependent pathway.

SEQUENCING, MAPPING, EXPRESSION PATTERN, AND FUNCTIONAL ANALYSIS OF MOUSE *Brca2* GENE

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Breast cancer is one of the most common malignancy in women, affecting one in nine during their lifetime. While a single major cause of breast cancer has not been identified, there are certain factors that contribute to the risk, the most important being a family history of the disease. This factor is particularly striking for early onset breast cancer. Recently cloned *BRCA1* and *BRCA2* genes are estimated to contribute equally to about 90% of families with significantly high incidence of breast cancer. *BRCA1* is predicted to be involved in more that 80% percent of families with high frequency of early onset breast as well as ovarian cancer. In contrast to *BRCA1*, *BRCA2* is not associated with ovarian cancer. However, *BRCA2* is linked to male breast cancer. Based on autosomal dominant pattern of inheritance, both genes have proposed to be tumor suppressor genes. Recent cloning and mutational analyses of these genes in humans have confirmed these predictions. As a first step toward the functional analysis of the *BRCA2* gene we have cloned the mouse homologue of this gene. The mouse cDNA was cloned by screening an embryonic cDNA library with human cDNA sequence as probe under low stringency condition. A cDNA walk was undertaken to clone full length cDNA and completed in seven steps. The mouse Brca2 protein sequence consists of 3328 amino acids and is 90 amino acids shorter than the human protein. The mouse protein shows an overall identity of 59% and 72% similarity with the human BRCA2 protein. At nucleotide level the identity between the human and mouse cDNAs is 74%. In the context of this relatively low identity, there are several regions that exhibit a much higher degree of conservation and therefore, may be functionally important. None of these conserved domains show significant homology to any known protein. Using the *Mus musculus* and *M. spretus* backcross DNA panel at the Jackson Laboratory we have mapped the mouse *Brca2* gene to chromosome 5. By Northern analysis, *Brca2* has been found to be expressed in adult thymus, testis and ovary. A single 11-12 kb transcipt was detected in these tissues. In addition, *Brca2* transcript was detected in embryos between 9.5 and 14.5 days of gestation. To conduct functional analysis of *Brca2* in mice we have mutated the gene by targeted mutagenesis in ES cells. We have obtained heterozygous mice and found them to be viable and fertile. Currently, we are in the process of the characterizing the phenotype associated with complete loss of *Brca2* in homozygous mice. We are also monitoring the heterozygous mice for tumor formation.

STRUCTURE AND EXPRESSION OF MOUSE AND RAT HOMOLOGUES OF THE BREAST CANCER SUSCEPTIBILITY GENE BRCA2. Jacques Simard[1], Martine Dumont[1], Martine Tranchant[1], Carolle Samson[1], Genevieve Legris[1], Marc Desrochers[1], Jean-François Leblanc[1], Marianne Schroeder[2], Michelle Baumgard[2], Mark Skolnick[2], Sean Tavtigian[2], and Fernand Labrie[1]. 1: MRC Group in Molecular Endocrinology, CHUL Research Center & Laval University, Québec, G1V4G2, Canada. 2: Myriad Genetics, 390 Wakara Way, Salt Lake City. Utah 84108.

It is estimated that about 5% to 10% of breast cancer cases may be due to inherited autosomal dominant susceptibility genes with high penetrance. Two genes, BRCA1 on 17q21 and BRCA2 on 13q12-13, account for the large majority of breast cancer families. Recently, the partial (Wooster et al. Nature 378:789-792) and complete (Tavtigian et al. Nature Genetics, 12: 333-337, 1996) structure of BRCA2 was elucidated. The human BRCA2 coding sequence is huge, composed of 26 exons that span 10443 bp and encodes a protein of 3418 amino acids. Development of breast/ovarian tumors in linked families is associated with the loss of the wild-type BRCA2 allele, which supports its predicted role as a tumor suppressor gene. The biological function of BRCA2 and factors modulating tissue- and cell-specific expression, gene-hormonal milieu, and gene-gene interactions, may be more effectively investigated in appropriate animal model. The aim of the present study was to elucidate the structure of murine and rat BRCA2 homologues, and to determine the tissue distribution of the BRCA2 gene expression. We have initially used 9 labelled fragments of the human BRCA2 cDNA as probes to isolate 4 cDNA clones from a mouse testis λgt11 cDNA library. Thereafter, 4 non-overlapping mouse cDNA clones were used as probes to isolate 16 cDNA clones from a rat testis λgt11 cDNA library and 2 of them were then used to isolate 18 additional mouse cDNA clones. The missing regions were completed in both species using a RT-PCR-based strategy. The mouse BRCA2 cDNA encodes a protein of 3328 amino acids, whereas the rat BRCA2 cDNA predicts a 3344-amino acid protein. The mouse BRCA2 protein shares 72.5% and 89.1% similarity and 59.2% and 82.9% identity with the human and the rat homologues, respectively, while the rat BRCA2 protein shares 71.8 % similarity and 58.1% identity with the human protein. The elucidation of these rodent full-length cDNAs led to the characterization of several highly conserved motif sequences, especially in exon 11. Hybridization of labelled mouse cDNA fragment to mouse multiple tissue Northern filters revealed an approximately 11 kb transcript detectable predominantly in testis, spleen, thymus, epididymis and seminal vesicles. Moreover, as revealed by RT-PCR using a rodent BRCA2-specific primer pair that yields a 1.4 kb amplicon overlapping exons 13 to 19, based on alignment of the cDNAs to the human BRCA2 gene, the transcript was found in all 12 mouse and rat tissues tested. It is also of interest to note that several smaller transcripts were detected in all mouse tissues tested using a probe of 823 nucleotides overlapping the sequence from exon 3 to 10, but not with probes in exon 11, as we previously reported in human tissues (Couch et al. Genomics 36: 86-99, 1996). The elucidation of the murine and rat BRCA2 cDNA thus provides the necessary molecular tools to study its biological function in these species and to gain essential information concerning the evolution and the structural biology of this protein and might suggests the existence of alternatively spliced transcripts.

**Differential Staining for Urokinase Plasminogen Activator Receptor in Patients with Ductal Carcinoma *In Situ*.** Kimberly D. Sloan-Stakleff and Daniel P. Guyton. Akron General Medical Center, Calhoun Research Laboratory and Department of Surgery, Akron, Ohio 44307.

Urokinase plasminogen activator (uPA) and receptor (uPAR) are involved in tumor cell invasion through interactions with the extracellular matrix and basement membrane. UPAR is essential for binding uPA and activates the conversion of plasminogen to plasmin at the surface of the tumor cell. Studies have correlated increased levels of soluble uPA with prognosis and relapse and have demonstrated increased presence of uPA and uPAR in invasive breast carcinoma tissue. Comparisons have been made between uPA and uPAR levels of benign lesions and invasive carcinomas, but characterization and grading of those levels have not been assessed among the progressive stages of breast carcinoma. Benign breast lesions display a spectrum of histologic changes which may precede invasive breast disease. Progressive changes may correlate alterations in biochemical events as indicated by uPA/uPAR levels. Since invasive breast carcinoma is thought to be preceded by ductal carcinoma in situ (DCIS), we evaluated DCIS patients for the presence of uPA and uPAR to investigate quantitative differences and/or evidence of possible transitional stages.

Step sections of formalin-fixed, paraffin-embedded tissue were cut at 5 um, mounted and heat dried for proper fixation. Following rehydration, tissue sections were microwaved for appropriate antigen retrieval. Slides for uPAR were pretreated with an acid wash to remove bound uPA from the receptor. Mouse-anti-human monoclonal antibodies for uPA (#3689) and uPAR (#3937) (American Diagnostica Inc.) and control mouse IgG1 were applied at 10 ug/ml and incubated for 18 hours at 4 °C. Biotinylated rabbit-anti-mouse secondary antibody was used for detection with streptavidin (DAKO, LSAB 2 kit) and the peroxidase reaction was visualized by diaminobenzaldehyde. All sections were counterstained with methyl green, observed and scored for intensity (+ to ++ = low; and, +++ or above = high).

Normal and ductal hyperplasia patients (10 per group) were stained as controls for comparison to DCIS (30 patients). UPA was observed with high intensity in the ductal structures of all patients with no notable distinction in levels between the three groups. However, differences in the intensity scores were observed for uPAR. Nine of ten (9/10) normal and ten of ten (10/10) hyperplasia patients demonstrated low levels of uPAR; whereas staining for DCIS patients could be distinguished as either low (21 patients = 70%) or high (9 patients = 30%).

We have identified a subset of DCIS patients that demonstrate high uPAR levels similar to that found in the invasive breast carcinoma tissues. Differences in staining intensities for uPAR within the DCIS group suggest that *in situ* lesions may have a greater invasive potential. Evaluation of DCIS for uPAR may provide a method to ascertain progressive changes between *in situ* and invasive lesions. Identification of patients with *in situ* lesions which may have a greater potential for invasion would aid in designing individualized therapeutic strategies for patients.

## Isolation and Characterization of Single Independent YAC Clones Containing Full-Length ***BRCA2*** Gene Using the Transformation-Associated-Recombination (TAR) technique

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In the study of genetically related diseases, such as early onset breast cancer, one of the most difficult steps in understanding and characterizing the disease is to easily isolate disease associated genes or large specific DNA sequences containing genes of interest. The cloning of large chromosomal fragments is first necessary to properly identify possible genes within genomic DNA. Identification of the disease genes will lead to a better understanding of molecular mechanisms that cause cells to function abnormally. A common method used to isolate large fragments of genomic DNA is yeast artificial chromosome vectors commonly referred to as YACs. There are some problems associated with using YACs, such as they can have rearrangements or random deletions and they may be mitotically unstable. Also it may take a large number individually isolated YACs to form a contig which would cover the area of interest.

Recently an alternative method for manipulating genomic DNA and its transformation into yeast cells has been developed which should limit the common problems associated with YACs, making them even more powerful tools to isolate a specific human genes. This method is known as transformation-associated-recombination or TAR cloning . Using this approach YAC's can be created *in vivo* by homologous recombination between human genomic DNA and a linear plasmid TAR vector containing a specific human sequence for a gene of interest. We generated a linear plasmid TAR vector which contained on one end of the vector of 670 bp piece of human genomic sequence that corresponds to a location approximately 7000 base pairs upstream of the *BRCA2* gene on human chromosome 13q. The other end of the vector contained a 310 bp fragment corresponding to the 3′ end of the genomic sequence for *BRCA2*. Using this *BRCA2* specific targeting vector, normal YAC transformation protocols were followed, yielding 34 pools of clones that contained approximately 30 clones in each. Of these 34 pools, 3 tested positive by PCR for the presence of *BRCA2* exon 11. Isolation of individual clones from these pools resulted in 3 independent, but identical sized clones all corresponding to the known full length size of the *BRCA2* gene. PCR of these clones for 26 different exons in *BRCA2* yielded a positive result in all cases. Regions corresponding to the 5′ and 3′ ends were also isolated by PCR and sequenced resulting in an exact match for each region. It was further demonstrated that these clones are mitotically stable as 5 subclones from each resulted in the same genetic profile.

## Regulation of Bcl-2 Phosphorylation and Apoptosis in Breast Cancer Cells

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The Bcl-2 family of proteins plays a significant role in regulation of apoptosis. In the present study, chemotherapeutic drugs taxol or vincristine induced Bcl-2 phosphorylation and apoptosis in MCF-7 and MDA-MB-231 cells. Phosphorylation of Bcl-2 inhibits its binding with Bax since less Bax was immunoprecipitated with Bcl-2 in taxol- or vincristine-treated cells. Overexpression of Bcl-2 in these cell lines counteracts the effects of low doses of taxol (0.01 - 1μM) or vincristine (0.01 - 1 μM). The phosphatase inhibitor okadaic acid, but not vanadate, induced Bcl-2 phosphorylation suggesting its phosphorylation on serine/threonine residues. In addition, forskolin induced Bcl-2 phosphorylation suggesting the involvement of cAMP-dependent protein kinase A. These findings support the use of the anticancer drugs taxol and vincristine for the treatment of breast cancers expressing Bcl-2, which may be resistant to other anticancer agents.

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Expression of Transforming Growth Factor β Type III Receptor Inhibits the Growth of Human Breast Cancer MCF-7 Cells. LuZhe Sun and Changguo Chen. University of Kentucky College of Medicine, Lexington, KY 40536.

Human breast cancer MCF-7 cells express a high level of transforming growth factor $\beta_1$ (TGF$\beta_1$) and very low levels of TGFβ type II receptor (RII) and type III receptor (RIII). They do not show any detectable autocrine TGF$\beta_1$ activity because of the low level of RII whose activation is necessary for signal transduction. Since RIII, a non-signaling receptor, has been shown to enhance TGF$\beta_1$ binding to RII, we investigated the role of RIII in regulating autocrine TGF$\beta_1$ activity and consequently the growth of MCF-7 cells. A tetracycline-repressible RIII expression vector was stably transfected into a limiting dilution clone of this cell line. Expression of RIII increased TGF$\beta_1$ binding to both TGFβ type I receptor (RI) and RII. Treatment with tetracycline suppressed RIII expression and abolished TGF$\beta_1$ binding to RI and RII. Growth of RIII-transfected cells was reduced by 40% when plated at low density on plastic. This reduction was reversed by tetracycline treatment and was partially reversed by treatment with a TGF$\beta_1$ neutralizing antibody. The activity of a TGFβ-responsive promoter construct when transiently transfected was more than three-fold higher in the RIII-transfected cells than in the control cells. Treatment with tetracycline or the TGF$\beta_1$ neutralizing antibody also significantly attenuated the increased promoter activity. These results suggest that expression of RIII restored autocrine TGF$\beta_1$ activity in MCF-7 cells by enhancing endogenous TGF$\beta_1$ binding to signaling receptor RI/RII complex. The RIII-transfected cells were also much less clonogenic in soft agarose than the control cells indicating a reversion of progression. Thus, RIII may be essential for an optimal level of the autocrine TGFβ activity in some cells, especially in the transformed cells with reduced RII expression.

**A Transcriptional Enhancer Required for the differential Expression of the Human Estrogen Receptor in Breast Cancers**, Zuoqin Tang, Isabelle Treilleux, and Myles Brown, Division of Neoplastic Disease Mechanisms, Dana-Farber Cancer Institute, Boston, MA 02115

Estrogen receptor status is used clinically both as a prognostic factor and as a target in the therapy of breast cancer. However, only half of all breast cancers express high levels of ER. We have been analyzing the ER gene in order to identify the important elements that may be responsible for the differential transcription of ER in human breast cancers.

Using transfection assays we identified a transcriptional enhancer element located between -4.1 and -2.7 kb upstream of the major ER mRNA start site. A 35 bp element located from -3778 to -3744 demonsted strong differential enhancer activity in ER+ versus ER- human breast cancer cell lines. We termed it ER-EH0 (for estrogen receptor enhancer). Sequence analysis revealed an AP-1 site. Gel retardation assays identified multiple DNA-protein complexes which could be specifically formed on this enhancer with ER+ and ER- nuclear extracts. One of those three bands could be supershifted when anti-Fos or anti-Jun antibodies were added in the gel shift assay. Methylation interference assays and footprinting suggest binding of factors to both the AP-1 site and adjacent sites. Mutations in either the AP-1 site or the adjacent site abolish enhancer activity. Mutations introduced into ER-ER0 and the recently described proximal promoter element ERF-1 in the context of the full length promoter confirm ER-EH0 as the dominant cis-acting element involved in differential ER expression. Current studies are focused on identifing which member(s) of the Fos or Jun family are involved in binding to the AP-1 site and which other factors bind to the sequences adjacent to the AP-1 site in ER-EH0.

## Induction of Phosphorylation on BRCA1 Following Exposure to DNA Damaging Agents.

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Recent evidence suggests that the breast cancer susceptibility gene, *BRCA1*, may play a role in modulating cell proliferation and the cell cycle. We found that BRCA1 immunoprecipitated from MCF7 cells blocked in G1/S or progressing through S phase of the cell cycle was hyperphosphorylated and migrated more slowly through SDS gels than BRCA1 immunoprecipitated from cells maintained in serum supplemented media, serum-free media for 24 hr or delayed in G2/M by treatment with colchicine. Hyperphosphorylation of BRCA1 also occurs when MCF7 cells or MDA-MB-468 cells are exposed to 2-5 mJ UV light, 4.4 Grays of X-rays or are grown in the presence of 5 mM hydrogen peroxide. Induction of phosphorylation on BRCA1 following exposure of MCF7 cells to 4.4 Grays X-rays or UV irradiation was over a similar time course as the increased expression of p53 in these cells. Interestingly, the punctate nuclear staining pattern normally observed for BRCA1 was eliminated following UV-irradiation, however, biochemical fractionation indicated that both the hypo- and hyperphosphorylated BRCA1 proteins were localized predominantly to the nucleus. These data suggest that phosphorylation on BRCA1 may be a regulatory response of the cell to changes in the cell cycle or following DNA damage.

**Varied penetrance and expression of a founder BRCA2 mutation in a well defined population.**

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Germline changes in the cancer predisposition gene BRCA2 are found in a small proportion of breast cancers. The importance of germline BRCA2 mutations in individuals without a family history of breast cancer is unknown. The majority of inherited breast cancer in Iceland seems to be due to a founder mutation in the BRCA2 gene (999del5) which can be traced back to a common ancestor at least as early as the beginning of the 16th century.
Screening for the 999del5 mutation was performed on DNA samples from 1182 Icelanders, 520 randomly selected samples from a near population based collection, 632 unselected samples from women with breast cancer (22.6% of female breast cancer patients in Iceland in the last 40 years and 61.4% of patients diagnosed in the study period) and 30 samples from males with breast cancer (all male breast cancer patients diagnosed in the last 40 years). The 999del5 mutation was found in 3 of 520 controls or 0.6% (95% CI; 0.1-1.7) indicating that one in 170 individuals in this population is a 999del5 mutation carrier. 7.7% of female breast cancer patients and 40% of males with breast cancer tested positive for 999del5. The median age at onset for the breast cancer in the female BRCA2 positive group was 45 years but 60 years for the patients without 999del5, whereas no age difference was found in male breast cancer patients. Germline BRCA2 mutation can be present without a strong family history of breast cancer (all 1st, 2nd and 3rd degree relatives checked). A number of cancers, other than breast cancer, were found to be increased in relatives of mutation carriers, including prostate and pancreatic cancer. We found 12 female mutation carriers who had a mother with breast cancer. Age at onset for breast cancer and disease stage at diagnosis was obtained for the 12 mother/daughter pairs There was a suggestive trend for an earlier age at diagnosis among the daughters indicating that age at onset is going down in the younger generation. Male breast cancer incidence has icreased considerably during the latter half of the study period and the proportion of BRCA2 mutation positive male breast cancer cases has also been increasing (20% in 1975-1984, 50% in 1985-1994). The same BRCA2 mutation clearly has varied penetrance and expression in the Icelandic population. Increased breast cancer incidence and lower age at onset in the younger generations suggest that there could be some enviromental factors affecting the phenotypic expression of this mutation.

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"Basic and Clinical Aspects of Breast Cancer"

SYNERGY BETWEEN CELLULAR SRC AND THE EPIDERMAL GROWTH FACTOR RECEPTOR IN TUMORIGENESIS. David A. Tice, Ming-Chei Maa and S. J. Parsons. Department of Microbiology, University of Virginia, Charlottesville.

The evidence linking tyrosine kinase proto-oncogenes to the induction or the progression of human malignancies is steadily increasing. Two of these proto-oncogenes, the epidermal growth factor receptor (EGFR), and cellular Src (c-Src), have been implicated in breast cancer and often linked to a worse prognosis or increased metastatic potential. A synergistic interaction between c-Src and the EGFR was discovered in both mitogenic and tumorigenic studies in murine model fibroblast systems. C-Src was shown to be required for EGF-induced mitogenesis and to potentiate EGFR-mediated tumorigenesis. This potentiation was exemplified in tumor formation studies where a cell line that overexpressed both c-Src and the EGFR formed 1+ cm tumors in 100% of the injections, while cells overexpressing either molecule alone formed only 1 mm tumors in 50% of the cases. The characterization of this interaction uncovered an EGF-dependent in vivo association between the two kinases, two novel phosphorylations on the receptor upon association, and a resulting hyperactivation of the receptor suggesting that physical association is linked to enhanced tumorigenesis. To gain further insights into the mechanism of c-Src synergy with the EGFR, stable cell lines containing various c-Src mutants and overexpressed *wt* EGFR are being examined for decreased mitogenic and tumorigenic responses with the ultimate goal of determining which regions of c-Src are required for the interaction with the receptor. C-Src is composed of 6 domains: 1) The myristylation domain responsible for tethering c-Src to the membrane; 2) The unique domain has no known function; 3) The Src Homology 3 (SH3) domain is responsible for binding proline rich regions on target molecules; 4) The Src Homology 2 (SH2) domain is responsible for binding phosphotyrosine residues on target molecules; 5) The kinase domain is responsible for phosphorylating tyrosine residues on target molecules; 6) And the negative regulatory domain which diminishes the kinase activity upon tyrosine phosphorylation. Stable mouse embryo fibroblasts overexpressing both the *wt* EGFR and either myristylation deficient, SH2 mutant or kinase deficient c-Src, have been created and were injected subcutaneously into nude mice and monitored for tumor growth. Preliminary evidence suggests that both the SH2 and kinase mutant containing cell lines exhibit decreased tumor formation compared with *wt* double overexpressors, while the myristylation mutant has increased tumor formation. By determining if the biochemical characteristics, (that is, association between the kinases, phosphorylation of the receptor, and hyperactivation of the receptor), correlate with the biological phenomenon, an indication will be given as to whether c-Src and the EGFR are acting together or through independent mechanisms. Once the mechanism of c-Src synergy with the EGFR is uncovered, a strategy of interdiction using c-Src peptides can be pinpointed to a specific region and tested for the ability to inhibit this interaction in breast cancer cell lines.

INCREASED EXPRESSION OF PROLACTIN RECEPTOR GENE IN HUMAN BREAST TUMORS *VERSUS* CONTIGUOUS NORMAL BREAST TISSUES. Philippe Touraine[1*], Jean-François Martini[1], Brigitte Zafrani[2], Jean-Claude Durand[3], Françoise Labaille[1], Catherine Malet[4], André Nicolas[2], Marie-Catherine Postel-Vinay[1], Frédérique Kuttenn[2] and Paul A. Kelly[1]. [1]INSERM Unité 344, Faculté Medecine Necker, 156 rue de Vaugirard, 75730 Paris Cedex 15, [2] Institut Curie, Paris, France, [3]Clinique St Jean de Dieu, Paris, France, [4]Département d'Endocrinologie, Hôpital Necker, Paris, France,

Prolactin (PRL) is involved in breast differentiation and proliferation and its role in breast carcinogenesishas been widely studied, especially in rodents. However, the role of PRL in human breast carcinogenesis is less well understood. One of the limits is the difficulty to accurately measure prolactin receptor (PRLR) in human tissues, due to the low level of expression. We established a Quantitative PCR method (Q-PCR) in T-47D human breast cancer cells and applied it to 30 patients, 25 of which presented either cancer or fibroadenoma. Five patients underwent a mammoplasty and normal epithelial cells were cultured before Q-PCR. For Q-PCR, an internal control was constructed by inserting a 50-base pair fragment of the human GH receptor cDNA into a portion of the human PRLR cDNA. The internal control and the target tissue were amplified together with the same set of primers. PCR of 25 cycles was used to obtain an exponential phase of amplification. In T-47D cells, 31 x $10^6$ molecules were detected per µg of total RNA. In all patients, expression of PRLR gene was detected, varying from 1500 to 1 x $10^6$ molecules/µg of RNA in normal tissues and from 4500 to 4.7 x $10^6$ molecules/µg of RNA in tumors. Expression was always greater in tumor versus normal contiguous tissue and was comparable in mammary epithelial cells to *in vivo* normal breast tissues. Estradiol receptor and progesterone receptor negative patients expressed low levels of PRLR transcripts as did menopausal women. Immunocytochemical analysis of PRLR confirmed stronger staining in almost all tumor samples compared to normal tissues. Finally, in all samples tested, a mRNA encoding hPRL was also identified by RT-PCR. Our results confirm PRLR gene expression in all tissues and moreover this expression is increased in tumors *versus* normal tissues, suggesting a potential role of PRLR in the process of tumor development.

TANDEM HIGH DOSE CHEMOTHERAPY (HDC) WITH ESCALATING PACLITAXEL (P), MELPHALAN (M) AND CYCLOPHOSPHAMIDE, THIOTEPA, AND CARBOPLATIN (CTCB)WITH PERIPHERAL BLOOD PROGENITOR (PBP)SUPPORT IN RESPONDING METASTATIC BREAST CANCER (MBC). LT Vahdat, C Balmaceda, KP Papadopoulos, TJ Garrett, D Savage, A Tiersten, T McGovern, L Kaufman, KH Antman and CS Hesdorffer. College of Physicians and Surgeons, Columbia Presbyterian Medical Center. NY, NY.

Despite complete remission (CR) rates of @ 50% to HDC, the majority of patients(pts) with metastatic breast cancer relapse and ultimately die. Analysis of prognostic factors associated with durable remissions include a CR at the end of high dose therapy. In an attempt to increase the proportion of pts achieving a CR at the end of therapy, we designed a tandem HDC program which incorporates a dose-escalation of P as the first high dose cycle of chemotherapy. All pts with responding MBC have a minimum of 3 x $10^6$ CD34+ cells/kg leukapheresed after mobilization with chemotherapy and filgrastim(G). **Study**: P (400- 825 mg/ $m^2$) administered over 24 hrs. Second intensification is M at 180 mg/$m^2$ and third intensification CTCb (cyclophosphamide 6000 mg/$m^2$, thiotepa 500 mg/$m^2$, carboplatin 800 mg/$m^2$). All cycles supported with PBP and G. **Results**: pts registered: 34, 4 removed (inadequate PBP collection-2, progression-1, social reasons-1), Twenty-eight pts completed all 3 cycles. No toxic deaths. P dose levels, toxicity and final response as follows:

| P dose | | maximal neurotoxicity(NCI) | | | | Final Response | | | |
|---|---|---|---|---|---|---|---|---|---|
| (mg/$m^2$) | #pts | 1 | 2 | 3 | 4 | NE | CR | PR | SD |
| 400 | 5 | 3 | 2 | 0 | | 4 | 1 | 0 | 0 |
| 480 | 3 | 0 | 1 | 2 | | 1 | 1 | 0 | 1 |
| 525 | 3 | 0 | 1 | 2 | | 2 | 0 | 1 | 0 |
| 690 | 5 | 0 | 3 | 1 | 1 | 1 | 0 | 3 | 1 |
| 725 | 9 | 0 | 2 | 7 | | 5 | 2 | 2 | 0 |
| 775 | 6 | 0 | 3 | 2 | | 4 | Too early | | |
| 825 | 1 | | 1 | | | Too early | | | |

Two pts developed transient atrial fibrillation at the 400 and 725 mg/$m^2$ dose levels. Neurotoxicity appears to be unrelated to extent of pretreatment with P or site of metastases and is reversible. Ten of 12 evaluable pts responded (4CR, 6PR). 70% of pts are progression-free at median f/u of 9mos. Dose escalation continues.

Smoking and Breast Cancer Risk. Karin van der Kooy, Matti A. Rookus, Frederike de Vries, Flora E. van Leeuwen. Netherlands Cancer Instituut, Amsterdam, The Netherlands.

Women who smoke may have lower urinary estrogen levels and an earlier menopause compared to women who do not smoke. This suggests that smoking may lower the risk of breast cancer through an anti-estrogenic effect. Conversely, carcinogens from cigarette smoke found in breast fluid may affect breast cancer risk in the opposite way. The association between smoking and breast cancer has been investigated in more than 20 studies, but the findings are inconsistent. Only 3 studies found a protective effect of smoking.

We studied the association between smoking and breast cancer in a population-based case-control study of breast cancer and oral contraceptives. The study included 918 breast cancer cases (aged 20-54 years; diagnosed 1986-1989) and 918 age-matched population controls. Information on risk factors (e.g. history of benign breast disease and familial breast cancer, reproductive and menstrual history, and hormone use) and lifetime smoking history was obtained during in-person interviews.

69% of cases and 65% of controls had ever smoked. The ex-smokers had, in contrast to the current smokers, an slightly increased breast cancer risk compared to never smokers (relative risk (RR):1.3, 95% confidence interval (BI):1.0-1.6 and RR:1.1 BI:0.8-1.3, respectively). The relative risk of breast cancer was not associated with the average amount of cigarettes smoked per day (adjusted for duration of smoking), and the risk hardly increased with longer duration of smoking (> 25 years versus never-smoking RR:1.2 BI:0.8-1.9 adjusted for cigarettes per day). Women who always smoked filter cigarettes had a slightly lower breast cancer risk than women who always smoked cigarettes without filter (RR:1.1 BI: 0.8-1.4 and RR:1.4 BI:1.0-1.9 versus never-smoking, respectively). The difference increased with longer duration of smoking (p for interaction:$p = 0.017$). Finally, we divided the cases in those having breast tumors with and without overexpression of p53 protein and we found no differences in associations with smoking status.

In conclusion, if smoking is associated with breast cancer risk, it appears to increase the risk slightly rather than to lower the risk of breast cancer.

An automated image processing based method for microvessel counting in invasive breast cancer

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Microvessel counting was shown to be of prognostic value in invasive breast cancer in different retrospective studies. Up to now the number of microvessels is assessed mainly manually according to a standardized but strict protocol. However, the assessment of the number of microvessels and the selection of the most vascularized area (hot spot) is subjective when done manually and therefore not perfectly reproducible. In this study we have developed an automated method for microvessel counting and selection of the hot spot based on image processing.
In 10 cases of invasive breast cancer, microvessels were stained with CD31 immunohistochemistry in 4 $\mu$m paraffin-embedded sections. In the whole tumour area, microvessels was automatically counted by image processing, and a microvessel map was composed revealing the hot spots. In addition, microvessels were counted manually..
Automated and manual microvessel counts within the whole tumour area as well as in the hot spots were very well correlated ($r=0.92$) The reproducibility of the automated method was excellent ($r=0.95$). The percentage false negatives varied between 5-15% depending on the quality of the specimen.
In conclusion, automated counting of microvessels is possible and preferable above the manual method because of the reduction in measurement time, increased accuracy and objectivity of hot spot selection and counting, and possibility of visual inspection and relocation of each measurement field afterwards.

Microsatellite Instability in DCIS of the breast.

Tom Walsh, Steve A Chappell, Rosemary A Walker, Jacqui A Shaw. Breast Cancer Research Unit, Department of Pathology, University of Leicester, Glenfield Hospital, Leicester, UK.

Microsatellite instability (MI), first described in hereditary non-polyposis colorectal cancer (HNPCC), may lead to an increase in strand-slippage induced mutations and consequently altered gene expression. Although this phenomenon has been described in a number of different tumours, including breast cancers, the significance of MI in breast cancer is poorly understood, particularly in the early stages of the disease.

We have investigated the frequency and type of MI in individual ducts microdissected from 23 cases of ductal carcinoma in situ (DCIS) of the breast and correlated our findings with patholobiological data. Mono-, di-, and trinucleotide repeats from seven chromosomal regions were analysed. Five cases (22%) displayed MI at two or more loci for all ducts examined. These tumours are candidates for mutation analysis in candidate mismatch repair (MMR) genes. No changes in hPMS2 expression have been found using immunohistochemistry, and hMSH2 is currently being evaluated.

In addition, 7 other cases (30%) showed MI at a single locus, the DM-1 trinucleotide repeat, which is located in the 3' untranslated region of the DMPK gene and these might indicate a different mechanism for instability and different pathway for tumour development. Moreover, alterations in the number of (CTG)n repeats may alter the mRNA stability or translational controls that normally function to regulate DMPK. DMPK expression is under investigation, initially in breast cancer cell lines that have different length DM-1 alleles.

The DCIS cases that showed MI were predominantly high grade, suggesting that aberrations to DNA repair functions may lead to the acquisition of a more aggressive phenotype. No significant associations were found with c-*erb*B-2 and p53 protein. Adjacent morphologically normal ducts within these tumours are currently being analysed to determine whether MI alterations represent a 'field change', as has been described recently for allelic loss. MI appears to be an important mechanism in the development of certain types of ductal carcinoma in situ.

The Role of Estrogen Metabolism in Aging-Related Diseases: Breast Cancer, Osteoporosis, and Coronary Heart Disease. Kim C. Westerlind. AMC Cancer Research Center, Denver, CO 80214.

Estrogen is strongly implicated in the etiology of the three major aging-related causes of death and disability in women: breast cancer, osteoporosis, and coronary heart disease. To date, establishing a direct link between a woman's estrogen levels and her risk for developing these diseases has eluded investigators and has resulted in conflicting reports. In the breast cancer literature, it has been suggested that how a woman metabolizes her estrogen may be predictive of her risk for developing breast cancer. The two primary metabolites, 2-hydroxyestrone and 16α-hydroxyestrone, are characterized by different degrees of estrogenicity and mutagenicity and it has been speculated that the ratio of 2 to 16α-hydroxyestrone may be a marker of disease susceptibility, presence, or prognosis. Until recently, it has been difficult to assay the two metabolites. Further, the question of whether estrogen metabolism is related to risk for developing osteoporosis or coronary heart disease, has not been evaluated. This laboratory has performed several studies using humans and animal models to begin to address these questions. Initial human studies have been focused on establishing the reliability of a urine-based enzyme immunoassay (Estramet, ImmunaCare) and assessing the potential effects of confounders including the type of sample (24 hour vs. single time point) and diurnal and menstrual cycle variation. Serum assays for the two metabolites are currently underway. Establishing the utility of these assays has been of critical initial importance prior to initiating risk group comparison studies and/or lifestyle intervention studies designed to impact estrogen metabolism. Two animal studies have been performed to date, in which ovariectomized and ovary-intact rats were treated with 2-hydroxyestrone, 16α-hydroxyestrone, 17β-estradiol, or vehicle for three weeks. Estrogen-responsive tissues (mammary glands, uteri, livers, and tibiae/femora) were excised and histologically examined for estrogen action. In the ovariectomized animals, 2-hydroxyestrone had no effect on any of the tissues, whereas 16α-hydroxyestrone was a full estrogen on the skeleton and liver and a partial estrogen agonist on the mammary gland and uterus. Antagonistic effects of the two metabolites are currently being evaluated in tissues from the ovary-intact animals. Human and animal data will be presented and implications associated with breast cancer, osteoporosis, and coronary heart disease will be discussed.

Biochemical and *in situ* analysis of BRCA1 protein expression.

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Inherited mutations in the *BRCA1* gene predispose individuals to early onset breast and ovarian cancer. The genetic and molecular evidence indicates that *BRCA1* functions as a tumor suppressor gene. However, despite intense investigation, little is known about the cellular pathways or regulatory mechanisms involved and there is still considerable debate as to the subcellular localization of the BRCA1 protein(s) in normal and malignant cells. Alternatively spliced BRCA1 transcripts have been described including one product missing the exon 11 encoded nuclear localization signal. This mRNA, BRCA1-Δ11b, was shown to be underrepresented in some breast and ovarian cancer derived cell lines. In this study we have extended previous observations by surveying the expression levels and subcellular localization of the endogenous 220 kDa full-length BRCA1 protein as well as a 120 kDa product that appears to be the BRCA1-Δ11b protein. Roughly equal amounts of the BRCA1 proteins (220 kDa and 120 kDa) were detected in HBL100 cell lysates using a panel of monoclonal and polyclonal antibodies specific for the N and C-terminus of BRCA1. The expression levels of both BRCA1 proteins were markedly reduced in several breast cancer cell lines compared to HBL100, CV-1 and DU145 cells. Several antibodies, including one specific for exon 11, were used in immunohistochemical and cellular fractionation experiments. These results demonstrate that full-length BRCA1 is a nuclear protein in HBL100 cells as well as in all breast and ovarian cancer cell lines examined.

ANALYSIS OF EXPRESSION PATTERN OF THE BRCA1 PROTEIN IN SPORADIC BREAST CANCER CELLS.

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The BRCA1 gene, which was cloned as a tumor suppressor gene responsible for familial breast and ovarian cancer syndrome, showed mutation only in hereditary cases and sporadic ovarian cancer patients. In sporadic breast cancer, decreased BRCA1-mRNA expression in addition to allelic loss in the BRCA1 locus has been reported. However, intracellular localization and function of the BRCA1 protein are not well elucidated. To analyze expression patterns of the BRCA1 protein, frozen normal and tumor tissues from over 100 cases of sporadic breast cancer were used for immunohistochemistry and Western blotting. Four polyclonal antibodies from Santa Cruz Biotechnology Inc. and four monoclonal antibodies (kindly provided by Dr. Ralph Scully, Dana Farber Institute) were used. Most breast cancer tissues showed heterogeneous nuclear staining pattern while normal duct epithelium and stromal fibroblasts showed mostly homogeneous and diffuse nuclear staining. The results were almost the same among the antibodies used. All antibodies recognized 220, 200, 180, and 160 kDa bands of the BRCA1 protein by Western blotting. Relationship between the BRCA1 expression and the cell cylcle is currently under investigation. Our data indicate changes of BRCA1 expression levels might have an important role in the development of spordic breast cancer.

**A Screening for BRCA1 Mutations in Breast and Breast Ovarian Cancer Families from the Stockholm Region.** Moraima Zelada-Hedman[1], Brita Wasteson Arver[1], Antonio Claro[1], Jindong Chen[1], Barbro Werelius[1], Helen Kok1, Kerstin Sandelin[2], Sara Håkansson[3], Tone Ikdahl Andersen[4], Anne-Lise Børresen Dale[4], Åke Borg[3], and Annika Lindblom[1]. [1]Dept. of Clinical Genetics and [2]Dept. of Surgery, Karolinska Hospital, S-171 76 Stockholm, Sweden; [3]Institute of Oncology, University Hospital, S-221 85 Lund, Sweden; [4]Dept. of Genetics, Insitute for Cancer Research, The Norwegian Radium Hospital, N-0310 Oslo, Norway.

In order to identify BRCA1 mutations in the breast and breast ovarian cancer families in the Stockholm region a total of 127 families were screened. Blood samples from 174 patients from these families were studied using mutation screening techniques followed by direct DNA sequencing to search for germline mutations in the BRCA1 gene. Mutations were identified in seven of 20 families with breast and ovarian cancer and in one family with ovarian cancer only, whereas only one family out of 106 with breast cancer showed a mutation. Thus, germline mutations in BRCA1 were found in one third of the families with both breast and ovarian cancer, but in only 1% of the breast cancer families. The low frequency of germline mutations in the site specific breast cancer families means that other genes are likely to segregate in these families.

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# CLINICAL AND PATHOLOGICAL OBSERVITION OF BREAST CANCER

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36 patients of breast cancer with 40 tumors diagnosed by needle biopsy in 5different pathologic types were treated with a microwave hyperthermotherapy apparatus of 2450MHz. The radiator was circular with adiameter of 10cm. The treatment lasted 30min. in every 2 days. A temperature measuring device was used to measure tumor's temperature and was connected to a temperature contral device to maintain the temperature at 44℃. They were treated 10-12 times. After that, the size of every tumor was measured, then biopsies were taken for examination with optic and electronic microscopy.

The sizes of the tumors changed after treatment: 6 tumors had complete regression, 28 tumors had partial regression, 6 tumors were found no changes in size.

The changes in pathology after treatment: In 6 cases, cancer cells can not be found by pathologic examination, the changes of cnacer cell were granular and vacular degeneration, cytolysis, cytopyknosis, karyolysis and karyopyknosis. Interstitial changes were fibroplasia and inflitration of inflammatory cells. The ultrastructure changes after treatment: the cell membrane is not clear, the interstitial spaces are widened and with oil droplets, the mitochondria showed agglutinated type of degrneration.

Conclusion: It was found that breast cancer can be reduced even eliminated by hyperthermotherapy and the damaging and curative effect of both hypermia and radiotherapy were similar. Superficial tumor was more sensitive than profound tumor to hyperthermia.

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