Mammary Gland Development, Reproductive History, and Breast Cancer Risk

Lewis A. Chodosh, Celina M. D’Cruz, Heather Perry Gardner, Seung I. Ha, Sandra T. Marquis, Jayant V. Rajan, Douglas B. Stairs, James Y. Wang, and Man Wang

Departments of Molecular and Cellular Engineering [L. A. C., C. M. D., H. P. G., S. I. H., S. T. M., J. V. R., D. B. S., J. Y. W., M. W.], and Department of Medicine, Division of Endocrinology, Diabetes and Metabolism [L. A. C.], Stellar-Chance Laboratories, University of Pennsylvania School of Medicine, Philadelphia, Pennsylvania 19104-6100

Abstract

The observation that normal pathways of differentiation and development are invariably altered during the process of carcinogenesis implies an intrinsic relationship between these processes. This relationship is particularly evident in the breast, as exemplified by the existence of endocrine risk factors for breast cancer that are related to the timing of normal developmental events. Understanding the mechanisms by which normal developmental events alter breast cancer risk is a central focus of our laboratory. Herein, we describe three approaches being taken in our laboratory toward defining the molecular basis of this relationship. These include: determining the roles played by the tumor suppressor genes, BRCA1 and BRCA2, in the normal differentiation and development of the breast; studying the function of three novel protein kinases identified in our laboratory in mammary epithelial development; and defining the molecular and cellular changes that occur in the breast as a result of reproductive events known to influence breast cancer risk.

Introduction

A basic tenet emerging from studies in cancer biology is that normal pathways of differentiation and development are inevitably disrupted during the process of carcinogenesis. This implies an intrinsic relationship between these processes. The existence of endocrine risk factors for breast cancer that are related to the timing of normal developmental events such as menarche, menopause, and age at first full-term pregnancy epitomizes this relationship. The recognition that breast cancer risk is determined in part by the same reproductive endocrine events that drive mammary gland development argues that mammary gland development and mammary carcinogenesis are fundamentally related.

One of the most intriguing examples of this principle is the observation that women who undergo their first full-term pregnancy early in life (i.e., early parity) have a significantly reduced lifetime risk of breast cancer (1). The magnitude of this parity-induced protection against breast cancer is similar in many countries and ethnic groups, regardless of endemic incidence. This suggests that protection results from an intrinsic effect of parity on the biology of the breast rather than from extrinsic factors specific to a particular environmental, genetic, or socioeconomic setting. This conclusion is bolstered by the observation that rats that have previously undergone a full-term pregnancy are resistant to the induction of breast cancer by administration of the carcinogen DMBA, as compared to age-matched nulliparous controls (2, 3). Therefore, both human epidemiology and animal model systems support the conclusion that an early first full-term pregnancy results in a permanent change in the breast, either directly or indirectly, that confers a decreased risk for the subsequent development of breast cancer. Although this effect has been hypothesized to result from the impact of terminal differentiation on the susceptibility of the mammary epithelium to carcinogenesis, the molecular and cellular basis for this phenomenon is unknown.

A second illustration of this principle comes from the observation that breast cancer risk attributable to exposure to ionizing radiation is a function of age at the time of exposure. Specifically, studies of women who received mantle irradiation for Hodgkin’s disease or who underwent repeated fluoroscopy in the course of treatment for tuberculosis have demonstrated that breast cancer risk is significantly greater in women who were exposed to ionizing radiation during adolescence as compared to women exposed at later ages (4, 5). Analogously, nulliparous rats fed DMBA are more likely to develop breast cancer if they are exposed during puberty rather than as mature adults (6). Interestingly, epidemiological studies suggest that the increased susceptibility of the immature human breast to early events in carcinogenesis may occur prior to as well as during puberty. Studies of survivors from Hiroshima and Nagasaki indicate that the greatest increase in breast cancer risk occurred in women who were less than 10 years old at the time of exposure (7). The observed increase in breast cancer incidence in women irradiated during the first year of life for presumed thymic enlargement is perhaps an even more impressive illustration of this principle, given the rudimentary state of the mammary gland at this age (8). Together, these studies suggest that the susceptibility of the mammary gland to carcinogenesis is related to the gland’s developmental state at the time of exposure to mutagenic agents and that the immature breast is particularly susceptible to early events in carcinogenesis.

Understanding the molecular and cellular mechanisms by which normal developmental events alter breast cancer risk is a central goal of our laboratory. We believe that achieving this goal requires a more complete understanding of the manner in which hormones and reproductive history alter subpopulations of epithelial cell types present in the breast and of the roles played by key regulatory molecules in these processes. Toward this end, we are currently focusing on: (a) determining the roles played by the tumor suppressor genes, BRCA1 and BRCA2, in the normal differentiation and development of the breast; (b) studying the function of three novel protein kinases identified in our laboratory in mammary epithelial development and carcinogenesis; and (c) defining the molecular and cellular changes that occur in the breast as a result of reproductive events known to influence breast cancer risk.

Tumor Suppressor Genes: BRCA1 and BRCA2

The epidemiological relationship between development and carcinogenesis is illustrated on a molecular and mechanistic level by the existence and function of tumor suppressor genes such as p53, the Wilms’ tumor gene (WT1), and the retinoblastoma susceptibility gene (RB). Germ-line mutations in these genes are associated with inherited cancer predisposition syndromes (9). The cloning and analysis of several tumor suppressor genes has revealed that they frequently

Received 11/11/98; accepted 2/4/99.

1 Presented at the "General Motors Cancer Research Foundation Twentieth Annual Scientific Conference: Developmental Biology and Cancer," June 9–10, 1998, Bethesda, MD. This work was supported by: NIH Grants CA71513 (to L. A. C.) and CA78410 (to L. A. C.) from the National Cancer Institute and the National Institute of Diabetes and Digestive and Kidney Diseases; American Cancer Society Grant RPG-97-097-01-CCG (to L. A. C.); United States Army Breast Cancer Research Program Grants DAMD17-96-1-6113 (to L. A. C.), DAMD17-96-1-6112 (to H. P. G.), DAMD17-98-1-8227 (to C. M. D.), DAMD17-98-1-8235 (to D. B. S.), and DAMD17-96-1-8226 (to L. A. C.); and the Charles E. Culpeper Foundation (to L. A. C.). L. A. C. is a Charles E. Culpeper Medical Scholar.

2 To whom requests for reprints should be addressed, at Department of Molecular and Cellular Engineering, Stellar-Chance Laboratories, Room 309A, University of Pennsylvania School of Medicine, 422 Curie Boulevard, Philadelphia, PA 19104-6100. Phone: (215) 898-1321; Fax: (215) 573-8590; E-mail: chodosh@mail.med.upenn.edu.

3 The abbreviations used are: DMBA, 7,12-dimethylbenz[a]anthracene; EGFR, epidermal growth factor receptor.
encode proteins that act as negative regulators of cell proliferation, exert cell cycle checkpoint control function, or maintain genome integrity (10, 11). In addition, the targeted deletion of these genes in mice frequently results not only in increased susceptibility to cancer but also in abnormalities in proliferation, apoptosis, differentiation, and development (10, 12). As such, one approach to elucidating the relationship between mammary gland development and carcinogenesis is to determine the function of tumor suppressor genes known to be involved in the pathogenesis of breast cancer.

Genetic analysis of families in which multiple individuals have developed breast cancer suggests that 5–10% of breast cancer cases result from the inheritance of germ-line mutations in autosomal dominant susceptibility genes (13, 14). Over the past 4 years, several of these breast cancer susceptibility genes have been isolated by positional cloning, including BRCA1 and BRCA2 (15–19). Tumors arising in patients with germ-line mutations in either BRCA1 or BRCA2 typically display loss of the corresponding wild-type allele, suggesting that BRCA1 and BRCA2 are tumor suppressor genes (20–22). Interestingly, BRCA1 and BRCA2 mutations have not been identified in sporadic breast cancers, despite the fact that 25–30% of sporadic breast cancers show loss of heterozygosity at these loci (16, 23–26). This raises the intriguing possibility that the normal functions of these genes are temporally and/or developmentally restricted.

Recently, important clues to BRCA1 and BRCA2 function have come from biochemical studies demonstrating that treatment of cells with a variety of DNA-damaging agents leads to the rapid phosphorylation of BRCA1 (27, 28). Moreover, both BRCA1 and BRCA2 have been shown to directly or indirectly bind to RAD51, a homologue of RecA that has been implicated in DNA repair and recombination (29–32). These and other observations have led to the hypothesis that BRCA1 and BRCA2 are involved in the cellular response to DNA damage. Consistent with this hypothesis, embryonic cells from mice homozygous for mutations in the Brca2 locus have an increased sensitivity to DNA-damaging agents (30, 33, 34). It is interesting to speculate that the developmental regulation of BRCA1 and BRCA2 expression or function may contribute to the age-dependent susceptibility of the breast to ionizing radiation-induced carcinogenesis described above.

The markedly elevated risk of breast cancer observed in women carrying germ-line mutations in BRCA1 and BRCA2 strongly suggests that these genes are critical for the properly regulated growth of mammary epithelial cells. As a first step toward understanding the developmental role of BRCA1 and BRCA2, we have analyzed the spatial and temporal expression of the murine homologues of these genes during embryogenesis, in the mammary gland during postnatal development, and in adult tissues (35, 36). These studies reveal that expression of both Brca1 and Brca2 are tightly regulated during mammary gland development. For example, Brca1 and Brca2 expression levels in the mammary glands of adolescent female mice undergoing ductal morphogenesis are significantly higher than those found in the mammary glands of mature females in whom ductal morphogenesis has been completed (35, 36). This temporal pattern of expression is explained in part by the observation that Brca1 and Brca2 are expressed at high levels in terminal end buds, which are puberty-specific structures that contain rapidly proliferating cells undergoing differentiation (35–37). Brca1 and Brca2 mRNA levels are also markedly up-regulated in the mammary gland early in pregnancy, a period during which alveolar buds begin the process of rapid proliferation and differentiation to form mature, milk-producing alveoli (35–38). This up-regulation of Brca1 and Brca2 expression occurs preferentially in developing alveoli as compared to adjacent epithelial ducts, consistent with patterns of proliferation (35, 36). Indeed, at virtually all stages of development, Brca1 and Brca2 expression are restricted to cellular compartments actively involved in proliferation and differentiation. These patterns of expression suggest that these tumor suppressor genes may play a role in the normal development of the breast and other tissues.

The spatial and temporal patterns of Brca1 and Brca2 expression during development likely reflect the fact that expression of these genes is tightly regulated as a function of proliferation. We have shown that Brca1 and Brca2 mRNA levels are high in exponentially growing cells and low in quiescent cells (39). During progression through the cell cycle, Brca1 and Brca2 mRNA levels increase during G1 and attain maximal levels at the G1–S transition (39). Similar observations have been made for human BRCA1 and BRCA2 at both the mRNA and protein levels (39–46). These findings clearly demonstrate that proliferative stimuli modulate the expression of these genes. Despite the strong correlation between Brca1 and Brca2 expression and proliferative status, the expression of these genes also appears to be influenced by factors other than proliferation. For example, we have shown that Brca1 and Brca2 mRNA levels are coordinately up-regulated in postconfluent HC11 mammary epithelial cells during differentiation as well as following treatment with insulin and glucocorticoids (39). Brca1 and Brca2 expression increase in this setting to levels as high as those found in actively proliferating cells, despite the fact that cellular proliferation rates remain low under these experimental conditions. Together, these observations imply that Brca1 and Brca2 may be involved in the processes of proliferation and differentiation in the breast.

A particularly intriguing finding of our studies has been the striking degree to which Brca1 and Brca2 are temporally and spatially coexpressed at the mRNA level (36). We have found that Brca1 and Brca2 are expressed at similar levels in a similar set of tissues and in similar cellular compartments within those tissues. In fact, the developmental expression patterns of these two putative tumor suppressor genes are essentially identical during embryogenesis and in multiple tissues of the adult. This similarity is particularly evident during postnatal mammary gland development as Brca1 and Brca2 expression are each up-regulated during puberty and pregnancy. The coordinate induction of these genes in proliferating and differentiating mammary epithelial cells in vitro may provide a cellular basis for this similarity (39). These findings suggest that similar pathways and stimuli regulate the expression of Brca1 and Brca2 in multiple cell types. Taken together with the fact that inherited mutations in either BRCA1 or BRCA2 predispose mammary epithelial cells to transformation, the striking similarities in Brca1 and Brca2 expression patterns formed the initial basis for speculation that these genes may function in overlapping pathways and may even directly interact.

As alluded to above, no somatic mutations have been identified in BRCA1 or BRCA2 in sporadic breast cancers. This puzzling observation could be explained if the function of these cancer susceptibility genes in the mammary gland were restricted to specific developmental stages, as might be suggested by the tightly regulated expression that these molecules exhibit during mammary gland development. Similarly, in light of the proposed relationship between normal mammary gland development and reproductive risk factors for breast cancer, it is interesting to note that Brca1 and Brca2 may each up-regulate in the breast during puberty and pregnancy because these stages of development are each associated with increases in cellular proliferation as well as increases in breast cancer risk. Potentially, the induction of Brca1 and Brca2 expression during these developmental stages may be a protective response to proliferation or to DNA damage that accompanies proliferation, as suggested by the observation that Rad51 is also up-regulated in proliferating cells (35, 47).

Our laboratory has chosen to focus on understanding BRCA1 and BRCA2 function in mammary epithelial cells because considerably
less is known about their function in this context and because breast cancer is the most important clinical phenotype associated with germ-line mutations in these genes. Specifically, we are interested in those aspects of mammary gland biology responsible for the observation that women carrying germ-line mutations in BRCA1 and BRCA2 preferentially develop cancer of the breast. Because this may ultimately relate to mammary-specific functions of these molecules, a complete understanding of the role played by these genes in breast cancer susceptibility will almost certainly require that their functions be studied directly in the mammary epithelium. As such, we are analyzing the impact of altering BRCA1 and BRCA2 expression levels on proliferation, differentiation, and DNA repair in the mammary epithelium using in vivo and in vitro model systems. These studies may provide insight into mechanisms of growth control and DNA damage response in normal mammary epithelial cells as well as serve as a foundation for understanding how the absence or mutation of these molecules promotes carcinogenesis.

**Novel Protein Kinases**

A second approach to investigating the relationship between development and carcinogenesis in the breast is to study members of a family of regulatory proteins that are typically involved in differentiation, development, and carcinogenesis. Analysis of these processes in a variety of model systems has underscored the key role frequently played by protein kinases. Many protein kinases function as intermediates in mitogenic signal transduction pathways or encode growth factor receptors whose overexpression, aberrant expression, or mutation to ligand-independent activated forms results in transformation. Several members of the protein kinase family have been shown to be involved in the development of breast cancer both in humans and in rodent model systems including the epidermal growth factor receptor, the insulin-like growth factor-I receptor, the fibroblast growth factor receptor family, HER2/Neu, Met, and Src. For instance, amplification and overexpression of HER2/Neu and EGFR have each been correlated with aggressive tumor phenotype and poor clinical prognosis. Similarly, overexpression of certain protein kinases or of their ligands in transgenic animals results in malignant transformation of the mammary epithelium. To date, however, evidence for a causal role of protein kinases in the initiation and progression of breast cancer exists for only a few members of this family of proteins. For this reason, we embarked on a screen designed to identify tyrosine kinases and serine-threonine kinases expressed in the murine breast during normal development and in breast cancer.

First-strand cDNA was prepared from mRNA isolated either from mammary glands of mice at specific developmental stages or from a series of mammary epithelial cell lines derived from breast tumors that arose in transgenic mice expressing either the activated neu, c-myc, H-ras, or int2 oncogenes (48–50). Degenerate PCR was used to amplify kinase catalytic subdomains VI–IX, and the resulting cDNA clones were screened to identify those harboring catalytic domain fragments of protein kinases (51–53). This screen identified 41 kinases: 33 tyrosine kinases and 8 serine-threonine kinases, 3 of which are novel.4 We have characterized the temporal and spatial expression of these kinases during mammary gland development as well as in a panel of mammary epithelial cell lines derived from breast tumors arising in transgenic mice expressing either the activated neu, c-myc, H-ras, or int2 oncogenes.4 This analysis has revealed that many of these kinases are preferentially expressed in the breast during specific stages of puberty, pregnancy, lactation, and postlactational regression.

Our laboratory has subsequently focused on the function of three novel serine-threonine kinases identified in our screen: Hunk, Punc, and Krcp. The novel protein kinase, Hunk, was initially isolated from a mammary epithelial cell line derived from a breast tumor that arose in a transgenic mouse expressing the neu oncogene (54). Analysis of sequence homology within a portion of the catalytic domain of Hunk suggests that it is a serine/threonine kinase with highest homology to the SNF1 kinase family. The novel protein kinase, Punc, was initially isolated from the mammary glands of mice undergoing early postlactational regression.4,5 The catalytic domain of Punc is 60% identical at the amino acid level to calcium/calmodulin-dependent protein kinase I and shares a lower homology with other members of the calcium/calmodulin-dependent kinase family (55).6 Krcp appears to represent a new family of mammalian protein kinases and is most closely related to a protein kinase recently identified by the yeast genome project that does not fall into any of the families of protein kinases previously identified in yeast (54).

**Hunk and Punc** appear to be particularly relevant to studies of the relationship between mammary gland development and carcinogenesis by virtue of their patterns of expression.7,8 Specifically, Hunk is expressed at low levels in the mammary glands of immature and mature virgin animals and undergoes a dramatic up-regulation of expression during early pregnancy. Hunk expression rapidly drops to basal levels by midpregnancy and decreases further during lactation and early postlactational regression. Like Hunk, Punc expression is also up-regulated in the mammary epithelium during pregnancy. However, unlike Hunk, maximum levels of Punc expression occur late in pregnancy just prior to parturition.

To determine whether the developmental changes in Hunk and Punc expression observed during pregnancy represent global changes in expression occurring throughout the mammary gland or changes in the abundance of an expressing subpopulation of cells, we have defined the spatial pattern of expression of these kinases.7,8 This was of particular interest because the expression of several protein kinases has been shown to be cell lineage restricted, thereby permitting their use as markers for biologically interesting subpopulations of cells. Examination of the spatial pattern of Hunk and Punc expression revealed that throughout the course of mammary development both kinases are expressed predominantly in the mammary epithelium. Interestingly, the expression of each of these kinases in the mammary epithelium is strikingly heterogeneous, with the greatest number of Hunk-expressing cells being observed at day 7 of pregnancy and the greatest number of Punc-expressing cells being observed at day 20 of pregnancy. This pattern of expression does not appear to be due to the heterogeneous distribution of cells through the cell cycle. Analogously, studies of the expression of these kinases in a variety of other tissues suggest that Hunk and Punc expression may also identify subsets of cells in other organs besides the breast. These observations suggest that Hunk and Punc are differentially expressed in distinct

---


epithelial cell subtypes in the breast that are differentially regulated during pregnancy.

To further investigate this hypothesis, we have examined Hunk and Punc expression in a panel of mammary epithelial cell lines derived from independent mammary adenocarcinomas arising in transgenic mice expressing the neu, c-myc, H-ras, or int2 oncogenes.7,8 Surprisingly, all eight cell lines derived from breast tumors that arose in transgenic mice expressing the neu or H-ras oncogenes were found to express high levels of Hunk mRNA, whereas none of the seven cell lines derived from breast tumors that arose in transgenic mice expressing the c-myc or int2 oncogenes expressed detectable levels of Hunk. Conversely, all seven cell lines derived from breast tumors that arose in transgenic mice expressing the c-myc or int2 oncogenes expressed Punc mRNA, whereas none of the eight cell lines derived from breast tumors that arose in transgenic mice expressing the neu or H-ras oncogenes expressed detectable levels of Punc. In each case, kinase expression levels observed in tumor cells were significantly higher than those observed in nontransformed mammary epithelial cells.

The heterogeneous spatial patterns of Hunk and Punc expression in the breast, along with the mutually exclusive patterns of expression of these two kinases in transgenic mammary epithelial cell lines, suggest that these novel serine/threonine kinases may be differentially expressed in distinct mammary epithelial cell subtypes that are themselves differentially regulated during pregnancy. The observation that Hunk and Punc are overexpressed in cell lines derived from breast cancers induced by the neu or c-myc oncogenes, respectively, suggests either that Hunk and Punc are downstream targets of the neu and c-myc oncogenes or that these kinases identify epithelial cell subtypes that are preferentially transformed either by neu or c-myc.

Each of these hypotheses is based on our observations suggesting that the normal mammary epithelium appears to be composed of distinct Hunk- and Punc-expressing cell types. The first hypothesis postulates that Hunk mRNA expression is activated by the neu and/or H-ras pathways, whereas Punc mRNA expression is activated by the c-myc and/or int2 pathways. In this model, neu (or c-myc) transgene expression in the mammary epithelium induces Hunk (or Punc) expression in all mammary epithelial cell types that express the transgene. As a consequence, tumors that arise from the epithelium display the same differential pattern of expression exhibited by the parental normal transgenic mammary epithelium. The second hypothesis postulates that neu and c-myc preferentially transform two different mammary epithelial cell types, one of which (in the case of neu) is marked by Hunk expression and the other of which (in the case of c-myc) is marked by Punc expression. In this model, overexpression of Hunk in neu-induced tumors reflects the selection and outgrowth of an Hunk-expressing epithelial cell subtype that otherwise represents a minor fraction of cells in the normal mammary epithelium. That is, Hunk and Punc expression may be restricted to distinct epithelial cell subtypes that are preferentially transformed by these oncogenes.

Our data suggest that the novel serine/threonine kinases identified in our laboratory may serve as markers for biologically interesting subpopulations of epithelial cells in the breast that are relevant both to development and carcinogenesis. Current work in our laboratory on Hunk, Punc, and Kret focuses on placing these kinases in known or novel signal transduction pathways and on determining their role in mammary development and carcinogenesis using transgenic and knockout animal models as well as tissue culture model systems. In addition, we have cloned the human homologues for each of these genes and are currently determining whether Hunk, Punc, and Kret are mutated, amplified, or overexpressed in human tumors or tumor cell lines.

Parity-induced Changes in the Breast

A third approach that our laboratory is taking to explore the relationship between development and carcinogenesis in the breast is to focus on the molecular and cellular changes that occur in the breast as a result of reproductive events known to influence breast cancer risk. Epidemiological studies have consistently shown that women who undergo an early first full-term pregnancy have a significantly reduced lifetime risk of breast cancer (1, 56–64). This association is independent of parity (i.e., number of live births). In contrast, women who undergo their first full-term pregnancy after the age of 30–35 years appear to have a risk of breast cancer that is actually higher than that of nulliparous women. This suggests that parity-induced protection against breast cancer is principally dependent upon the timing of a first full-term pregnancy rather than on its occurrence per se. These observations imply that an early first full-term pregnancy results in a change in the breast, either directly or indirectly, that confers a decreased risk for the subsequent development of breast cancer. Because aborted pregnancies are not associated with a decreased risk for breast cancer, it has been hypothesized that the protective effect of parity requires attaining the terminally differentiated state of lactation (2, 3, 6, 59, 65–71). Unfortunately, the biological basis of parity-induced protection against breast cancer is unknown. In principle, the protective effect of early first childbirth could result from the pregnancy-driven terminal differentiation of a population of target cells at increased risk for carcinogenesis, from the preferential loss of a population of target cells during postlactational regression or from a permanent systemic endocrine change affecting the breast in such a way as to reduce the risk of carcinogenesis. Clearly, a more thorough elucidation of the molecular and cellular changes that take place in the breast as a result of parity will be required to fully understand this phenomenon.

The realization that specific reproductive endocrine events alter breast cancer risk in a predictable fashion raises the possibility that events known to decrease breast cancer risk might be mimicked pharmacologically. The desire to pursue this objective is heightened by the fact that, although it is now possible by genetic means to identify women who are at elevated risk for developing breast cancer, interventions between the extremes of more frequent mammographic screening and prophylactic bilateral mastectomy are only now beginning to be considered. As such, reducing breast cancer risk via hormonal manipulations designed to mimic naturally occurring endocrine events could represent a feasible alternative. It is to this end that both early first full-term pregnancy and early menopause have been proposed as logical paradigms on which to model the hormonal chemoprevention of breast cancer. The achievement of this goal, however, has been hampered by current ignorance regarding the mechanism by which reproductive history alters breast cancer risk. As such, the rational design of hormonal chemoprevention regimens would benefit from a better understanding of the influence of development on breast cancer risk. An additional stumbling block in the development of chemoprevention regimens aimed at reducing breast cancer risk has been the prolonged and costly clinical trials required to determine the efficacy of these regimens due to reliance on the development of breast cancer as a clinical end point (72–75). As such, the identification and use of intermediate molecular end points that accurately identify changes in the breast associated with changes in breast cancer risk would facilitate the development of such chemopreventive regimens. To this end, we have chosen to exploit the relationship between development and carcinogenesis in the breast to generate rational and biologically plausible candidate surrogate end point biomarkers.

The mechanism of parity-induced protection against breast cancer
is likely to involve complex genetic and epigenetic processes that may be influenced by reproductive endocrine variables as well as by inherited genotypes. In this context, it is useful to analyze complex processes such as this in model systems that recapitulate relevant epidemiological findings, permit critical aspects of reproductive history to be rigorously controlled, reduce genetic variation, and permit the examination of molecular and cellular events at defined developmental stages of interest in normal tissue. The use of animal models to study the impact of mammary gland development on breast cancer risk is facilitated by the fact that the structure, function, and developmental stages through which the mammary gland passes are similar in humans and in rodents (76, 77). Administration of the carcinogen DMBA to nulliparous Sprague Dawley rats induces mammary adenocarcinomas that are hormone dependent and histologically similar to human breast tumors. In contrast, rats that have previously undergone a full-term pregnancy are highly resistant to the induction of breast cancer by carcinogen administration, as compared with age-matched nulliparous controls (2, 6, 78–83).

Paralleling these functional differences, there are also marked morphological differences between the adult nulliparous mammary gland and the mammary glands of age-matched parous littersmates that have undergone a single cycle of pregnancy, lactation, and regression. These parity-induced morphological changes are permanent because nulliparous and parous glands may be distinguished easily even after 1 year of postlactational regression (3). Similar morphological changes are also seen in mice and in rats and are analogous to those reported in the parous human breast (70, 77). These observations support the hypothesis that parity results not only in a permanent change in the functional state of the breast (i.e., susceptibility to carcinogenesis) but also in permanent structural changes in the breast. Finally, the fact that the Sprague Dawley DMBA model system mirrors complex epidemiological phenomena observed in humans, and that numerous molecules believed to play important roles in the pathogenesis of human breast cancer have similar effects in rodents, suggests that rodent model systems such as this can be a valuable tool for understanding fundamental aspects of mammary gland biology and breast cancer etiology.

We hypothesize that understanding the impact of parity on breast cancer risk will require a thorough understanding of the manner in which reproductive history affects subpopulations of cell types present in the breast. To address this hypothesis, we are using rodent model systems to identify and evaluate genes that are differentially expressed in the breast as a function of parity. Candidate genes that are specifically expressed in either the parous or the nulliparous rodent breast are being isolated and identified using a variety of approaches. These differentially expressed genes are being used as biomarkers for the cellular and molecular changes that occur in the breast as a result of an early first full-term pregnancy to define the impact of early parity on the development and differentiation of specific cell types in the breast. Finally, biomarkers that are found to be biologically informative in the rodent model system are being tested for their ability to detect parity-associated changes in histologically normal breast tissue obtained from nulliparous and parous women with known reproductive history and hormone exposures. The level and spatial pattern of expression of each of these candidate biomarkers is being analyzed in human tissue and evaluated with respect to parity as well as other parameters of reproductive endocrine history, such as age, age at first full-term pregnancy, menopausal status, and exogenous hormone use. These studies will determine whether candidate biomarkers characterized in rodent model systems can specifically detect parity-induced changes in the human breast.

To date, this approach has yielded a variety of genes that are expressed at higher levels in the mammary glands of parous animals as compared with age-matched virgin controls, confirming the utility of this approach for isolating genes that are specifically expressed in the breast as a function of reproductive history. Several of the parity-specific genes that we have isolated and identified are markers of mammary epithelial cell differentiation, such as milk proteins. This finding suggests that the parous breast is more “differentiated” than the nulliparous breast and, as such, is consistent with the proposal made by Russo and Russo (2, 84) that parity protects against breast cancer by virtue of the differentiation that it induces. The developmental patterns of expression of milk protein genes are notably heterogeneous because each is up-regulated at a specific point in the alveolar differentiation pathway (85). Interestingly, we have found that the expression patterns of several of these genes reflect subtle aspects of reproductive history. As such, studying the regulated expression of this class of genes as a function of reproductive history may provide insights into parity-related events in the breast. In addition, we have isolated a number of genes that are as yet unidentified. Given their interesting developmental patterns of regulation and parity-specific pattern of expression, these genes appear to represent an informative pool of candidate biomarkers for detecting changes in the breast associated with reproductive events.

In theory, the parity-specific pattern of expression for a given biomarker could reflect a global increase in expression of the gene in all mammary epithelial cells, an increase in the percentage of expressing cells in the breast, or both. We are analyzing the developmental pattern of expression of candidate genes by in situ hybridization to distinguish between these mechanisms. Our results indicate that parity-specific patterns of expression for different genes result from distinct developmental pathways. For example, these studies reveal examples of parity-dependent global changes in expression as well as parity-dependent changes in the abundance of expressing cells. This latter example is suggestive of a permanent pregnancy-induced expansion in the number of cells expressing a given biomarker in the breast. These findings are consistent with the hypothesis that reproductive events may permanently alter the biology of the breast by differentially affecting subpopulations of cells.

We have also determined the impact of several reproductive parameters on the differential pattern of expression of these genes. These experiments reveal that the parity-specific pattern of expression for some genes is independent of age, duration of postlactational regression, and age at first full-term pregnancy. In contrast, other genes we have identified are expressed in a parity-specific manner in the mammary glands of animals that have been mated as adolescents but not in the mammary glands of animals that have been mated as adults. These results suggest that the regulation of expression of such genes reflects developmental events in the mammary gland that are specific for age at first full-term pregnancy. These findings suggest that candidate cDNA biomarkers generated by these approaches may provide insight into subtle aspects of the molecular and cellular changes that occur in the breast as a result of parity. Ultimately, these studies are intended to gain sufficient understanding of the molecular pathways responsible for parity-induced protection against breast cancer in order to permit this naturally occurring protective event to be mimicked pharmacologically.

Summary

The current aims of this laboratory are designed to develop the molecular tools required to understand the relationship between nor-
mammary gland development and mammary carcinogenesis, as reflected in the epidemiology of reproductive endocrine risk factors for breast cancer. We have taken three approaches toward understanding this relationship, including: determining the role normally played by breast cancer susceptibility genes in mammary epithelial development; studying the function of three novel protein kinases in the breast; and identifying and analyzing genes that are specifically expressed in the breast during developmental stages associated with changes in breast cancer risk. We anticipate that these approaches will ultimately lead to a clearer understanding of the mechanisms by which breast cancer susceptibility is modulated by reproductive history.

Acknowledgments

We thank members of the Chodosh laboratory for helpful discussions and Barbara Handelin for critical reading of the manuscript.

References

Discussion

Dr. Andrew Feinberg: I have a really simple-minded question. These are very elegant studies, but I worry a bit about transgene-induced tumors, because in a sense you’re starting with loaded dice. Aren’t there any models of spontaneous mammary tumorigenesis? I thought there were some dogs or other species that developed cancer in a similar epidemiological manner as you mentioned for humans at the beginning. But, I don’t know this field, so I may be totally wrong.

Dr. Chodosh: It is true that there are certain breeds of dogs that do develop breast cancer spontaneously, though I am not aware of any that show parity-induced protection against breast cancer. Obviously, how you choose a model to study a particular question is a central issue. Regarding transgenic rodent models of breast cancer there are a couple of points worth making.

The first is that our main experimental thrust is to look at the normal developmental biology of the breast. There is no question that the developmental stages through which the breast passes for both the mouse and the rat are exceedingly similar to what one finds in the normal developmental biology of the breast. There is no question that the molecular pathways involved are highly conserved. So, while tumor development in the way that we think of for human breast cancers, I would argue that the molecular pathways involved are highly conserved.

The second is that it’s quite clear from transgenesis experiments that many of the pathways that are altered during the process of carcinogenesis in the human breast cause similar problems in the rodent breast when altered by transgenic approaches. That is, the molecular pathways involved are highly conserved.

The third is that it’s not “spontaneous” in the same way that we think of for human breast cancers, I would argue that the molecular pathways involved are highly conserved.
history of cancer biology suggests that they are still quite useful models to examine pathways involved in development and carcinogenesis. So at the moment, as far as animals that we can work with, particularly those that we can genetically manipulate, we have mice. Similarly, in the rat, one is somewhat restricted to carcinogen-induced models, which may or may not faithfully mimic the processes involved in human carcinogenesis.

We think about the suitability of our model systems a great deal, and it’s not clear to me that there’s another in vivo system available at the present time that’s more appropriate.

Speaker: Do you have any evidence these kinases play similar roles in the human breast? Because human breast cancer is quite different. Pathological studies are quite different from real breast cancer, because it’s quite complicated by different pathways. So, my interest at the moment is that even if we are able to link these kinases to the set of human reactants, it is different with different types of breast cancer and different kinases being expressed. How do you plan to address these potential differences?

Dr. Chodosh: A very important question, which explains why we are moving into human tissue and human breast cancer cell lines to address some of these issues. This is information that we’re currently gathering. The data that I showed you in human breast cancers and cancer cell lines are quite recent, so it’s too preliminary for us to know whether there is some correlation between the expression of our kinases and Erb2 status or ER status, or a particular histological cell type. Regarding tumors that are marked by Hunk or Punc expression, clearly we would want to know whether they behave differently in terms of patient prognosis or response to therapy. We don’t know that yet, though that’s certainly something that we’re very interested in.

Dr. Robert Ryan: I would like to ask, have you considered perhaps doing something like the chip-based assay where now you use the MMTV-neu and MMTV-c-myc breast cancer cell lines and test those samples for changes by looking at the various genes that are up-regulated or down-regulated. It might give you a handle on that, do you think?

Dr. Chodosh: Yes, that’s certainly a possibility. In the context of DNA chip technology, I think we’d probably want to make the fewest possible changes that we could, starting with the most normal cells we can, then induce expression of a Hunk or Punc transgene and ask what genes are downstream, as opposed to using as a starting point tumor cell lines that obviously have undergone many unrelated changes over the long period of time they have been in culture. Certainly, I agree it’s an important new technology.
Mammary Gland Development, Reproductive History, and Breast Cancer Risk

Lewis A. Chodosh, Celina M. D'Cruz, Heather Perry Gardner, et al.

_Cancer Res_ 1999;59:1765s-1772s.

**Updated version**

Access the most recent version of this article at:

http://cancerres.aacrjournals.org/content/59/7_Supplement/1765s

**E-mail alerts**

Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**

To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**

To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.