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Award Number: DAMD17-00-1-0137

TITLE: Intermediate Molecular Biomarkers for the Protective Effect of Pregnancy Against Breast Cancer

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REPORT DATE: May 2003

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	May 2003	Annual Summary (15 Apr 2000 - 14 Apr 2003)

4. TITLE AND SUBTITLE
Intermediate Molecular Biomarkers for the Protective Effect of Pregnancy Against Breast Cancer

5. FUNDING NUMBERS
DAMD17-00-1-0137

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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

Epidemiological studies have consistently shown an early full-term pregnancy is protective against breast cancer. We hypothesize that the hormonal milieu that is present during pregnancy results in persistent changes in the pattern of gene expression in the mammary gland leading to permanent changes in cell fate that determine the subsequent proliferative response of the gland. To investigate this hypothesis, we have used suppression subtractive hybridization (SSH) to identify genes that are persistently up-regulated in the glands of estrogen and progesterone-treated Wistar-Furth rats 28 days subsequent to steroid hormone treatment compared to age-matched virgins. Using this approach, a number of genes displaying persistent altered expression in response to prior treatment with estrogen and progesterone were identified. Two markers have been characterized in greater detail - RbAp46, and a novel gene (currently designated GB7). Both genes were persistently up-regulated in the lobules of the regressed gland and required prior treatment with both estrogen and progesterone for maximal persistent expression. Sequence analysis and expression studies suggest that GB7 is a novel, non-coding RNA; recent studies have shown that non-coding RNAs are an important class of regulatory molecules that may contribute to gene regulation through their association with chromatin remodeling, histone acetylation/deacetylation and transcription factor complexes as well as by RNA interference and by otherwise acting as inhibitory RNAs. RbAp46 has been implicated in a number of complexes involving chromatin remodeling - thus suggesting a mechanism whereby epigenetic factors responsible for persistent changes in gene expression may be related to the determination of cell fate, leading to protection against breast cancer. These results provide the first support on the molecular level for the hypothesis that hormone-induced persistent changes in gene expression are present in the involuted mammary gland and can affect the response of mammary epithelial cells to carcinogenic insults.

14. SUBJECT TERMS
Pregnancy, parity, rat model, Nap 1, RbAp, SSH-PCR, differential expression, molecular biomarkers, development

15. NUMBER OF PAGES
41

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction

Despite advances in the technologies for the diagnosis and treatment of breast cancer, strategies for the prevention of this disease are poorly defined. However, one fundamental concept arising from recent studies of mammary gland biology is the understanding that the processes involved in normal mammary development and carcinogenesis are intrinsically related (1). One of the most frequently cited examples of this principle is the protective effect of an early full-term pregnancy against breast cancer. There is strong epidemiological evidence that women who experience a full-term pregnancy early in their lives have a significantly reduced risk of developing breast cancer (3), (5). This is substantiated by studies in a rat model in which prior treatment with estrogen and progesterone, (to simulate the effects of pregnancy) confers resistance to tumorigenesis in animals subsequently challenged with chemical carcinogen reviewed in (7) .The E+P-treated rat is thus a well-established model to study the effects of parity-induced protection against mammary cancer. In spite of this fact, little is known of the specific mechanisms governing pregnancy-specific developmental changes in the mammary gland. In this respect, the elucidation of novel targets for estrogen and progesterone action in the mammary gland, is crucial to our understanding of how an aspect of normal development might mediate the response of the organ to future proliferative signals.

To address this question, we have used the well-characterized Wistar-Furth rat model described previously, (8), in conjunction with subtractive suppressive hybridization (SSH) methodologies (10) to identify markers that are persistently up-regulated in response to prior exposure to E and P in the mammary gland. As a result of these initial studies, we selected several specific markers to study in greater detail to investigate their role in relation to parity-related protection in the rodent mammary gland. The details of these findings are presented below.

Report Body

a) Summary of research objectives

The specific objectives of this project, described in the original proposal are listed below:

Objective 1: To characterize the role of candidate biomarkers and to investigate their expression in the rat mammary gland:
a) To determine their temporal and spatial pattern of expression in the rat mammary gland.
b) To determine whether these candidate genes are markers of differentiation, *per se* or of protection.

Objective 2: To investigate the function of candidate genes *in vivo:*
a) To investigate the function of GB7 and to determine its role during normal cellular processes
b) To investigate the function of GB7, RbAp and Nap1 in reconstituted mammary glands
c) To determine the role of systemic factors on parity-induced changes in the mammary gland.

Many of the experiments described in objective 1 of the proposal have been completed, and the results published in a peer-reviewed article (2). *In situ* hybridization analysis was used to determine the spatial expression of G.B7 and RbAp46. The same approach was also used to determine the effect of perphenazine treatment on the expression of these two markers. A quantitative reverse-transcriptase PCR method has been developed to examine the temporal expression of GB7, RbAp46 and another marker (Stra13), in the rat mammary gland.

The experiments described in Objective 2 are in progress. We have used comparative genomic analysis to identify conserved functional elements in the rat, mouse and human orthologues of GB7. We have used *in situ* hybridization to examine the role of GB7 in developmental process. We have developed an RNA interference strategy to knock down the expression of GB7 (and other candidate biomarkers) in a mouse mammary epithelial cell line. We have used retroviral- and lentiviral- mediated gene delivery to exogenously express a gene of interest in a mouse mammary epithelial cell line and primary mammary epithelial cells.

b) Results

Objective 1

a) To determine their temporal and spatial pattern of expression in the rat mammary gland.
b) To determine whether these candidate genes are markers of differentiation, per se or of protection.

In the original statement of work, we proposed to use Northern analysis and *in situ* hybridization to investigate the expression of three markers (G.B7, RbAp46 and Nap1) at different stages of development in the rat mammary gland. In addition, we proposed to use perphenazine treatment as an experimental paradigm to distinguish the molecular processes involved in protection of the gland from those that are inherent to pregnancy-related differentiation of the gland. In previous annual reports, we described the use of i*n situ* hybridization analysis to determine the spatial expression of G.B7 and RbAp46. We also described experiments to recapitulate the effect of perphenazine treatment in Wistar-Furth rats and the use of *in situ* hybridization analysis to determine the effect of perphenazine treatment on the expression of G.B7 and RbAp46 (please see previous progress reports, Appendix I and II). The results of these analyses were also reported in our paper (2) .

As noted in our paper (and previous progress reports) one of the limitations of any expression analysis is the inherent difficulties of detecting low abundance mRNAs such as G.B7 and RbAp46. In our preliminary expression studies, it was necessary to use PolyA+ RNA Northerns for this analysis. Hence, the inherent difficulties in isolating sufficient amounts of poly A+ RNA from mammary glands at different stages of development to satisfy this requirement imposes certain constraints on these experiments. To overcome these difficulties, we proposed two alternative approaches - microarray analysis and quantitative RT-PCR - to enable us to examine the expression of candidate markers without the necessity of isolating poly A+ RNA. To do this, we developed a custom microarray (containing 96 non-redundant clones from the E+P subtracted SSH library as well as appropriate controls) in conjunction with the Microarray Facility at Baylor College of Medicine. This would have enabled us to fulfill several requisites for this Objective: the rapid assessment of developmental expression profiles for these genes, the assessment of the hormone-dependent nature of their

expression, and verification of the differential basis of their expression. More importantly, this analysis would assist us in defining a smaller subset of additional biomarkers for further characterization. To investigate the effectiveness of this approach, we performed pilot experiments using the custom array and Cy3- and Cy5-labeled cDNA prepared from the mammary glands of E+P-treated, involuted rats (and their controls) to examine the sensitivity of the detection method. We conducted these experiments using a variety of hybridization conditions to obtain optimal detection with cDNA prepared by these means. Unfortunately, the results of these preliminary experiments showed that less that 30 % of the cDNA targets (which included G.B7, Nap1 and RbAp46) could be detected by this method, even though the library from which they were isolated was constructed from the same E+P RNA pool used for the probe synthesis. We believe that this is once again due to the inherent difficulties of detecting low abundance RNAs from a relatively quiescent tissue such as the involuted mammary gland. Accordingly, we began to investigate alternative methods of probe preparation using indirect labeling with Cy3/Cy5 or cDNA amplification to increase the sensitivity of detection. However, in the intervening period, the Microarray Facility at Baylor College of Medicine underwent a change in directorship. As a consequence, the facility suspended its services to reevaluate the methodologies used in array production.

As a result of this development we decided to defer any further investment in this technology, and to pursue an alternative strategy using real-time (quantitative) RT-PCR instead. To avoid the high cost associated with this technology, we have established conditions that allow us to use the less costly SYBR Green detection method (as opposed to 'Taqman' or FRET-based methodologies) to study the expression of candidate markers in the rat mammary gland. So far this approach has been extremely successful and we have applied this analysis to GB7, RbAp46, and another potentially interesting marker, Stra13. Expression of Cytokeratin-8 was used as a normalizing control, to correct for the epithelial content of mammary glands at different stages of development. Further details of this approach are described in the previous progress report contained in appendix II. We are currently optimizing the conditions for a similar assay to enable us to characterize the expression of the mouse orthologue of GB7 and will use this assay to examine the knock down of GB7 in siRNA experiments described

below. Results from our comparative genomic analysis of GB7 orthologues (described below) has been extremely important for enabling us to identify suitable targets for real-time PCR, as has the recent availability of a publicly accessible mouse genomic database. The latter has provided an invaluable resource for predicting the exon-intron structure of the rat orthologues we have studied, and thus for designing suitable assay conditions for real-time PCR.

Objective 2

a) To investigate the function of GB7 and to determine its role during normal cellular processes.

In the first part of this objective, we proposed experiments to determine the function of GB7 and to predict its role in normal cellular processes. As stated in the original proposal and in previous progress reports, GB7 is a novel cDNA whose pattern of expression suggests it is an excellent marker for parity-induced protection. In spite of this fact, we have been unable assign a function to this marker. In the previous progress reports, we described the isolation and sequencing of full-length clones for this mRNA, as well as the comparative genomic analysis that enabled us to identify both mouse and human orthologues of rat GB7 (please refer to previous progress reports, Appendix I and II). As a result of this analysis, we have been able to show: 1) that the mouse and human orthologues of GB7 exhibit conserved chromosomal localization on syntenous regions of mouse chromosome 1 and human chromosome 2, respectively. 2) That the human orthologue of GB7 overlaps with one exon of the ORF of another gene (Accession # AK024261), and that it is also close to a predicted imprinted region (9). 3) Using the predicted sequence of the human orthologue of GB7, we have designed primers for RT-PCR and shown that human GB7 is also expressed in several human mammary epithelial cell lines (including MCF-10A and MCF-7). By Northern analysis and RT-PCR, we have also shown that mouse GB7 is expressed in the mouse mammary epithelial cell line, HC11. In addition, *in silico* analysis (by identification of related EST sequences) has shown that GB7 is also expressed in the mammary gland of the mouse (IMAGE clone #s 4024645, 3465244, and 3465245) and in the brain (medulla) of the rat (rat clone UI-R-CA0-baa-b-12-0-UI). We have obtained the

aforementioned mouse EST clones and sequenced them in entirety, leading to the identification of two alternate splice forms of mouse GB7. Analysis of this sequence data indicates that, like the rat gene, mouse GB7 is also an alternatively spliced non-coding RNA. Moreover, the fact that differentially-spliced transcripts were identified from independent mouse mammary gland libraries – one prepared from lactating mouse mammary tissue, and the other from a transgenic tumor model – suggests that differential splicing be important for mediating the function of this RNA. As a further step in elucidating the function of GB7, we have sequenced five additional rat cDNA clones isolated from the same library as the original full-length clone (accession # AY035343). Each of these sequences corresponds to a differently spliced transcript; however, alignment of all six rat sequences, as well as those corresponding to the two mouse transcripts, has revealed a conserved core region that may be important for determining the function of this gene. This core region has been subjected to RNA secondary structure analysis, using the analysis algorithms of Mfold (http://www.bioinfo.rpi.edu/applications/mfold)

To identify conserved structural regions in the GB7 sequence. The results of this analysis have lead to the identification a number of stable stem-loop structures that may be important for mediating the function of GB7 – either through their interactions with RNA-binding proteins, or as a substrate for further RNA processing. As an additional approach to elucidating the function of GB7, we have performed expression analysis using mouse embryos. Embryos were isolated from 10.5-, 14.5-, 16.5- and 18.5- day pregnant BALB/c mice and *in situ* hybridization analysis performed with an RNA probe corresponding to both transcripts of mouse GB7. This strain of mice was selected because previous studies have shown that the protective effect of pregnancy observed in rats is recapitulated in BALB/c animals (6). Preliminary experiments indicate that GB7 is expressed in a variety of tissues in response to developmental stimuli – most strikingly at the growing margins of the limb bud (at day 10.5), but also in the somites, intranasal cleft, madibular arch, hyoid arch, lens and heart at this same time point. At 16.5 days of gestation, GB7 is localized to the nuclei of developing chondrocytes, while in other tissue it exhibits a perinuclear pattern of expression. Although these findings are very preliminary, the fact that GB7 expression is associated with development

suggests that it may play a role in some form of epigenetic regulation (for the implications of this finding see below).

b) To investigate the function of GB7, RbAp46 and Nap1 in reconstituted mammary glands

The second part of this objective proposed the use of reconstituted mammary gland experiments to elucidate the function of GB7, RbAp and Nap1 in an *in vivo* context. To address this goal, we have developed methods for growing rat mammary epithelial cells in primary culture and infecting them with a recombinant retrovirus expressing a gene of interest (please refer to previous progress reports – Appendix I and II). As an alternative strategy, we have also developed an approach based on RNA interference to 'knock down' expression of a gene of interest. So far, we have performed these experiments in the mouse mammary epithelial cell line, HC11. However, these experiments are ongoing and in the future we hope to extend this technology to achieve stable knock down using a mouse stem cell virus engineered to expressed small hairpin interfering RNAs under the control of an RNA polymerase III promoter.

Key Research Accomplishments

- Experiments involving perphenazine treatment have been reproduced in Wistar-Furth rats. Level of morphological development examined by whole mount analysis, in comparison to mammary glands for E+P-treated, pregnant and unstimulated control animals. (Objective 1)

- *In situ* hybridization analysis used to characterize the expression of G.B7 and RbAp46 in response to pregnancy, hormonal stimulation and perpehnazine treatment. (Objective 1).

- Screening of rat E+P SSH library complete. Twenty-one markers from this library characterized by Northern blot analysis to verify their pattern of expression. (Objective 1). Reverse subtracted library constructed, screened and 96 clones partially sequenced.

- Manuscript revised and accepted for publication (July, 2001)

- A quantitative reverse-transcriptase PCR method has been developed to examine the expression of candidate markers in the rat mammary gland (Objective 1).

- Isolation and sequencing of full-length clones for G.B7 completed. Sequence submitted to Genbank (accession # AY035343) (Objective 2).

- Identification of putative human orthologue of rat G.B7 (Objective 2).

- Identification several additional rat and mouse clones with homology to GB7. Full-length sequence analysis of these mouse clones indicates that the mouse orthologue of GB7 is also an alternatively spliced non-coding RNA.

- Developed a siRNA strategy to knock down the expression of candidate biomarkers in mouse and human mammary epithelial cell lines.

- Established methods for culturing mammary epithelial cells (MEC) from rats (Objective 2)

- Established methods for transducing rat MECs using a lentivirus vector, and for using these transduced MECs to produce reconstituted mammary glands expressing a b-galactosidase reporter gene (Objective 2).

- Preparation and publication of a commentary article: 'Pregnancy-induced changes in cell-fate in the mammary gland' that discusses recent publications concerning parity-related changes in the mammary gland, and a model of epigenetic regulation leading to a change in cell fate in the parous mammary gland.

Reportable Outcomes

Manuscripts:

Melanie R. Ginger and Jeffrey M. Rosen, 2003. Pregnancy-induced changes in cell-fate in the mammary gland. Breast Cancer Res. 5:192-197

Melanie R. Ginger, Maria F Gonzalez-Rimbau, Jason P. Gay and Jeffrey M. Rosen. 2001. Persistent Changes in Gene Expression Induced by Estrogen and Progesterone in the Rat Mammary Gland. *Mol. Endocrinol.*, Nov. **15** (11): 1993-2009

Medina D, Sivaraman L, Hilsenbeck SG, Conneely O, Ginger M, Rosen J, O'Malley BW. 2000. Mechanisms of hormonal prevention of breast cancer. *Ann N Y Acad Sci* Dec; **952**:23-35

Abstracts:

- AACR Meeting, San Francisco, April, 2000
- International Congress of Endocrinology Meeting, Sydney, Australia, October, 2000
- Hormones and Cancer Meeting, Port Douglas, Australia, November 2000
- Era of Hope Meeting, Orlando, September 2002.

Presentations:

- AACR Meeting, San Francisco, April, 2000. Oral presentation
- International Congress of Endocrinology Meeting, Sydney, Australia, October, 2000. Oral presentation
- Hormones and Cancer Meeting, Port Douglas, Australia, November 2000. Poster.
- Breast Disease Research Group, Baylor College of Medicine, March 20, 2002. Seminar.
- Era of Hope Meeting, Orlando, September 2002.

Informatics:

- Full-length G.B7 sequence submitted to Genbank database, under the accession # AY035343

Funding applied for, based on work supported by this award:

We have used the work presented in this report to successfully apply for a renewal of NIH Breast Cancer Program Project grant CA 64255, which commenced in May 2002 under grant # CA64255-06A

Summary and Conclusions

The E+P-treated rat is a well-established model to study the effects of parity-induced protection against mammary cancer. In spite of this fact, little is known of the specific mechanisms governing pregnancy-specific developmental changes in the mammary gland. We have hypothesized that the normal hormonal milieu that is present during pregnancy results in persistent changes in the molecular pathways governing cell fate in

a defined population of cells in the mammary gland. These changes accordingly dictate the type of response that is elicited by subsequent exposure to hormones or chemical carcinogens. A critical aspect in understanding these processes is the elucidation of target genes for E and P in the mammary gland. Such information is necessary for determining how the molecular pathways involved in normal mammary development and tumorigenesis converge with systemic hormones to mediate parity-specific protection. In this regard, the identification of relevant molecular biomarkers for this developmentally-regulated change in the rodent mammary gland is a fundamental step in furthering our understanding of molecular processes governing parity-related protection against breast cancer.

In this study, we have investigated the role of a small number of biomarkers for the protective effect of parity. We have used *in situ* hybridization, Northern analysis and real-time PCR to characterize their expression in a temporal and spatially specific manner in order to understand how expression of these markers might be regulated by developmental processes. We have shown that these markers are expressed in the mammary epithelium, but not the stroma. As part of this investigation, we have developed alternative methods for characterizing the expression of markers of this nature and optimized techniques such as real-time PCR, so that they can now be routinely used for this purpose in our laboratory. Since one of the markers examined is a novel gene, we have had to develop an approach to examine the role of this gene in the parous mammary gland, without any prior knowledge of the function it encodes. GB7 is a differentially-spliced, developmentally-regulated non-coding RNA. Our studies have shown that it is conserved between mouse , rat and human and database analysis has shown that its loss (through chromosomal damage) results in cancer. The fact that it is a developmentally regulated non-coding RNA suggests that it may exert some kind epigenetic regulation (such as tissue-specific imprinting). Together GB7 and RbAp46 (a member of chromatin remodeling, histone acetylase/deacetylase, histone methylation and DNA methylation binding complexes) might contribute to an epigenetic mechanism that could be responsible for the enduring changes observed in the parous mammary gland. This hypothesis is explored in greater detail in our recent commentary article.

What are the implications of this work?

The observation that pregnancy confers protection against breast cancer has lead to the proposal that breast cancer may be prevented by pharmacologically mimicking the specific endocrine events at a defined window of development. However, the advancement of such technology is currently limited by our ignorance of the complex molecular and cellular events that are responsible for this developmentally specific protection. Another factor impeding the implementation of such strategies is the prolonged nature of any study that must rely on a defined clinical endpoint (such as tumorigenesis) as a measure of the efficacy of any chemopreventative treatment. In this respect, the identification of relevant intermediate biomarkers for a defined population of resistant and susceptible cells is of critical importance, and will greatly facilitate the short-term evaluation of any chemopreventative measures and could ultimately improve imaging techniques for assessing breast cancer risk in women.

The elucidation of markers that show persistent changes in gene expression in response to exposure to E and P is critical for understanding the molecular pathways that are altered in the parous gland and modulate the response of the gland to further proliferative stimuli. In this study, we have identified a number of such markers that warrant further study and that may be of relevance to parity-related protection to breast cancer. In particular, the identification of a non-coding RNA is an adventitious discovery. Non-coding RNAs represent a new and important class of regulatory molecules; non coding RNAs have been implicated as important mediators of a number of cellular processes including X-inactivartion (*xist*), chromatin remodeling (*Rox 1* and *2*), intracellular localization (BC1), steroid hormone signalling, and developmental regulation through the actions of microRNAs, to name but a few(4). The expression a non-coding RNA in the involuted mammary gland of parous rats implies that a higher tier of epigenetic regulation is present, and may be important for mediating parity-related protection against tumorigenesis. Such changes might elicit an enduring effect on the molecular pathways governing cell fate and may ultimately lead to the change in

proliferative response that is observed following subsequent exposure to carcinogenic assault.

In addition to the identification of suitable markers for following these parity-specific changes in the mammary gland, it is critical to develop methods for analyzing the function of these genes in an *in vivo* model – for testing the efficacy of any preventive treatment. To this end, we are developing a strategy for transducing mammmary epithelial cells from rats and producing reconstituted mammary glands expressing a gene of interest. In addition, we are developing methods for knocking down the expression of candidate genes to determine the effect of their loss. These approaches will enable us to examine the role of a single gene - in conferring protection against carcinogenesis -in the absence of the altered hormonal status that is present during pregnancy and without germline manipulation of the host animal. These techniques will greatly benefit any future research endeavours and – if widely applied in experimental settings – could dramatically expand the research tools available for examining the role of individual genes in the mammary gland.

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Appendix I

Annual Summary Report: April 15, 2000 - April 14, 2001

Introduction

Despite advances in the technologies for the diagnosis and treatment of breast cancer, strategies for the prevention of this disease are poorly defined. However, one fundamental concept arising from recent studies of mammary gland biology is the understanding that the processes involved in normal mammary development and carcinogenesis are intrinsically related (1). One of the most frequently cited examples of this principle is the protective effect of an early full-term pregnancy against breast cancer. There is strong epidemiological evidence that women who experience a full-term pregnancy early in their lives have a significantly reduced risk of developing breast cancer (2, 3). This is substantiated by studies in a rat model in which prior treatment with estrogen and progesterone, (to simulate the effects of pregnancy) confers resistance to tumorigenesis in animals subsequently challenged with chemical carcinogen (4-12). The E+P-treated rat is thus a well-established model to study the effects of parity-induced protection against mammary cancer. In spite of this fact, little is known of the specific mechanisms governing pregnancy-specific developmental changes in the mammary gland. In this respect, the elucidation of novel targets for estrogen and progesterone action in the mammary gland, is crucial to our understanding of how an aspect of normal development might mediate the response of the organ to future proliferative signals.

To address this question, we have used the well-characterized Wistar-Furth rat model (described previously, 4), in conjunction with subtractive suppressive hybridization (SSH) methodologies (13) to identify markers that are persistently up-regulated in response to prior exposure to E and P in the mammary gland. As a result of these initial studies, we selected several specific markers to study in greater detail to investigate their role in relation to parity-related protection. To achieve this goal, we proposed to use mRNA expression analysis to study the temporal and spatial expression of each candidate biomarker, to evaluate their role as a function of reproductive history in relation to both differentiation and parity-related protection of the mammay gland. Furthermore, since the role of many of these markers has not yet been described in the mammary gland, we described experiments to further elucidate their function in relation to parity-related changes within the gland. In part, this approach will involve the use of novel retroviral transduction methods to overexpress the gene of interest in a parity-independent manner in the reconstituted mammary glands of recipient animals. If this approach is succesful,

then we have proposed experiments to investigate the influence of overexpression of a gene of interest in relation to protection against tumorigenesis following carcinogen challenge.

Report Body

c) Summary of research objectives

The specific objectives of this project, described in the original proposal are listed below:

Objective 1: To characterize the role of candidate biomarkers and to investigate their expression in the rat mammary gland:
a) To determine their temporal and spatial pattern of expression in the rat mammary gland.
b) To determine whether these candidate genes are markers of differentiation, *per se* or of protection.

Objective 2: To investigate the function of candidate genes *in vivo:*
a) To investigate the function of GB7 and to determine its role during normal cellular processes
b) To investigate the function of GB7, RbAp and Nap1 in reconstituted mammary glands
c) To determine the role of systemic factors on parity-induced changes in the mammary gland.

The experiments described in objective 1 of the proposal are in progress and have already led to the preparation, and submission of a manuscript for publication. However, because of technical difficulties encountered in the first part of this objective, we have had to reorder the original statement of work to allow us to implement an alternative procedure to perform some of the analyses. This is described in greater detail in the Results section, below. In addition, because of the technical complexity of some of the experiments described in Objective 2, in particular those employing rat mammary epithelial cell culture and retroviral transduction methodology, we have already begun work on the experiments employing this technology. This has allowed us to take advantage of methods for the retroviral transduction of mouse mammary epithelial cells, established by two graduate students in Dr. Rosen's laboratory. Since one of these graduate students will depart shortly, to begin postdoctoral work in another laboratory, it is matter of expedience that we begin these experiments ahead of the original schedule to take advantage of his considerable technical expertise. This does not represent a significant departure from the original statement of work, however it has been necessary to reorder the time scale indicated in the proposal, such that objectives 1 and 2 are being investigated somewhat concurrently.

d) Results

Objective 1

a) To determine their temporal and spatial pattern of expression in the rat mammary gland.
b) To determine whether these candidate genes are markers of differentiation, per se or of protection.

In the original statement of work, we proposed to use Northern analysis and *in situ* hybridization to investigate the expression of three markers (G.B7, RbAp46 and Nap1) at different stages of development in the rat mammary gland. In addition, we proposed to use perphenazine treatment as an experimental paradigm to distinguish the molecular processes involved in protection of the gland from those that are inherent to pregnancy-related differentiation of the gland. Because of the low abundance of these mRNAs, it was necessary to use poly A+ RNA to perform this analysis. One of the obstacles that we encountered was the technical difficulty of isolating sufficient amounts of poly A+ RNA from mammary glands at different stages of development to perform this analysis.

Because of these technical difficulties, we propose two alternative approaches to enable us to quantitate the expression of these mRNAs without the necessity of isolating poly A+ RNA. Firstly, Baylor has recently acquired the facilities for performing quantitative real-time RT-PCR. This will allow us to assess the levels of each mRNA in a total RNA sample isolated at different stages of development. This approach has the advantage of being extremely rapid and sensitive to small changes in the concentration of a reverse-transcriptase amplified cDNA product. This will circumvent some of the shortcomings associated with using poly A+ RNA Northern blots (such as the large amount starting material required to perform a few experiments and the long exposure time that is often necessary to detect hybridization signals). In addition, this should eliminate the necessity of pooling RNA samples from many animals, such that we can obtain a better measure of the variation in expression of each marker, by performing multiple replicates with samples from individual animals.

Secondly, we wish apply the use of cDNA microarray technology to quantitate the expression each marker at different stages of development. We have already begun experiments to investigate the effectiveness of this approach by constructing a pilot array, containing 96 non-redundant clones from the E+P subtracted SSH library (including G.B7, Nap1 and RbAp46) in conjunction with the Microarray Facility at Baylor College of Medicine. The cDNA inserts of

these clones were amplified using the nested primers in the SSH library backbone, purified by gel filtration chromatography and arrayed using silanized glass technology by means of the 384 well format array printer in the Microarray Core Facility. This approach has the advantage that it allows us to examine the expression of 96 markers in a single experiment, and to investigate changes in their expression, as a group, at different stages of development. From this analysis, it may be possible to predict markers that are involved in related pathways and this will be of great assistance to the functional studies described in Objective 2. Currently, we are optimizing the hybridization conditions for this technique, using the pilot E+P array described above. To this effect, we have investigated a different method of probe labeling that does not rely on direct incorporation of the Cy3- or Cy5-modified nucleotide. However, because the sensitivity of this method is still being evaluated, we propose to add a number of additional controls to examine the limits of detection of the array. To address this issue, we will prepare cDNA probes from known amounts of RNA derived from the *in vitro* transcription of an individual E+P clone and use this to screen the array. This will enable us to determine the lowest level of detection of the array and to predict its sensitivity with respect to low abundance clones. Secondly, we will screen the array with RNA isolated from the mammary glands of individual animals to obtain a measure of variability for the assay. Thirdly, to measure the linearity of the response we will compare the Cy5/Cy3 ratio from a hybridization containing the same probe labeled with both fluorescent nucleotides.

In the second part of this objective, we proposed to use an experimental paradigm based on previously published methods (11) to distinguish between the molecular processes involved in parity-related protection and differentiation of the mammary gland. This treatment results in an increased serum level of prolactin and progesterone, but not estrogen, which permits differentiation, but does not confer protection against carcinogenesis (11). To compare the morphological differentiation resulting from this treatment, parallel experiments were performed in which animals were treated with either E+P, blank pellets, perphenazine, or the vehicle alone for a period of three weeks. At the end of the treatment period, mammary glands were removed and the degree of morphological development examined by whole mount analsyis. Mammary glands from 6-, 12- and 18-day pregnant animals were used as positive controls for pregnancy-specific differentiation of the gland. At the gross structural level these glands appear to be very similar, although a few minor differences were observed. These observations are reported in

greater detail in our manuscript which is appended to this report (Appendix ii, Fig 1 and text). At the end of the 21 day-treatment period, hormonal stimuli were withdrawn and remaining animals in each cohort allowed to undergo involution for 28 days. This resting phase mimics the period of involution that occurs after a normal pregnancy and lactation and represents a period of extensive tissue remodeling. At the end of involution, the gland generally reaches a quiescent state, resembling that of the mature virgin animal. As reported in previous studies (4), similar morphological characteristics were observed in all of the treatment groups, indicating that there were no persistent structural differences as a consequence of hormonal stimulation of the gland. Please refer to the manuscript appended (Appendix ii, Fig 1 and text) for a detailed description of these observations.

In situ hybridization analysis was then used to examine the expression of two markers, G.B7 and RbAp46, in response to hormonal stimulation of the gland. The results of these experiments demonstrate that both markers are highly expressed during pregnancy. However, prior exposure to E+P (which simulates the protective effects of pregnancy) is required for maximal persistent expression of both genes 28 days following hormone withdrawal. Perphenazine alone (ie exposure to progesterone and prolactin) is not sufficient for persistent maximal expression of these genes (Fig 4 and 5 of manuscript, Appendix ii). These observations, and our interpretation of them, are discussed in greater detail in the appended manuscript (Appendix ii).

In the final part of this objective, we proposed to continue to characterize candidate markers by sequencing and Northern blot analysis. To address this goal, we have sequenced a total of 203 clones from the E+P SSH library (including those already presented in the original proposal). In addition, we have characterized 21 of these clones by Northern blot analysis, 18 of which were confirmed as differentially expressed, based on quantitative Northern analysis of their expression in the E+P-treated gland vs. AMV. The results of this analysis are presented in greater detail in Tables I-III and Fig 3 of the manuscript (Appendix ii).

Objective 2

b) To investigate the function of GB7 and to determine its role during normal cellular processes.

In the first part of this objective, we proposed experiments to determine the function of GB7 and to predict its role in normal cellular processes. Clone GB7 is a novel cDNA whose pattern of

expression suggests it is an excellent marker for parity-induced protection. However, preliminary database searches using in the ~750 bp of sequence obtained from the SSH library clone revealed no homology to any known cDNA or protein. Therefore, to further elucidate the function of this cDNA, we constructed and screened an E+P-treated mammary gland cDNA library and isolated several full-length clones corresponding to the different sized transcripts observed by Northern analysis (refer to Fig. 3 of manuscript, Appendix ii). Three of these clones (6.3, 2.4 and 2.2 kb in size) were sequenced in their entirety. Analysis of the resultant sequences suggested that the different-sized transcripts observed by Northern blot analysis arose as a result of differential splicing of a single gene product. The full-length sequence of the largest of these clones (6.3 kb in length) was deposited in Genbank, with the accession # AY035343.

In an attempt to determine the function of this gene, we submitted the full-length, 6.3 kb sequence to database searching using BLAST and TBLASTN search algorithms (with six-frame translation and BEAUTY post-processing). The results of this analysis did not reveal significant homology to any known gene or protein motif. However, we found that a short section of the 5' and 3' region of this gene (n.t. 1125-1490 and 5920-6203 of the full-length sequence) exhibited homology with several rat and mouse EST clones. The best matches were to accession #s BG079981, BE119249 and BG079981 and revealed an identity of 85-97 % to these regions of ~300 bp in the full-length G.B7 sequence. In addition, we performed homology searching using the recently assembled human genome sequence database and found four regions of homology to human chromosome 2. These homologous regions corresponded to n.t. 2334-2419, 2435-2601, 2745-2789 and 5157-5437 of the full-length G.B7 sequence, with identities of 93, 83, 95 and 80 % respectively ($E < 1^{-9}$). This sequence maps to region 2q33 of the human genome and spans a known chromosomal break-point, which is associated with a number of human cancers, including breast adenocarcinoma (14). No open reading frames (ORF) or ESTs have been identified in the human sequence encompassing the region of homology with G.B7. However this may be a consequence of incomplete annotation of genome database or the failure of the search paradigms to detect a non-translated RNA. Indeed, we have been unable to detect any significant ORFs longer than ~200 n.t. in the full-length G.B7 cDNA. *In vitro* translation experiments, with appropriate positive controls run in parallel to validate the assay, also failed to detect any translation product for either the 6.3 kb, 2.4 kb or 2.2 kb forms of this RNA.

b) To investigate the function of GB7, RbAp and Nap1 in reconstituted mammary glands

The second part of this objective proposed the use of reconstituted mammary gland experiments to elucidate the function of GB7, RbAp and Nap1 in an *in vivo* context. To determine the role of a single gene in conferring parity-specific protection, it would be advantageous to develop a system that would permit the over-expression of the molecule of interest in the absence of the altered hormonal status that is present during pregnancy. To achieve this goal, we have recently developed methods for growing rat mammary epithelial cells (MEC) in primary culture based upon technology for isolating epithelial cells from mouse mammary glands (which is now well established in our laboratory). Our approach is to use an HIV-derived lentivirus vector (15) pseudotyped with the vesicular stomatitis virus (VSV) G protein to enhance the efficiency and host range of infection. Lentivirus has advantages over many conventional retroviral strategies, such as the ability to transduce slowly- or non-dividing cells – features which are of particular importance for conferring the ability to transduce a population of stem cells (16). However, although this retroviral vector has been used to transform intact tissues (16), it has not been applied to the infection of MECs from rats. As a preliminary measure to examine the efficacy of this approach for infecting MECs from rats, we will use a control retrovirus expressing the bacterial *lacZ* gene to determine the efficiency of infection and viability of the isolated rat MECs. Once we have established the success of this strategy, we will develop a retroviral construct expressing a gene of interest (ie RbAp46 or G.B7) and use this construct to infect rat MEC, to determine the effects of overexpression of that gene on normal mammary gland development and in repsonse to carcinogen challenge.

Appendix II

Annual Summary Report: April 15, 2001 - April 14, 2002

Introduction

Despite advances in the technologies for the diagnosis and treatment of breast cancer, strategies for the prevention of this disease are poorly defined. However, one fundamental concept arising from recent studies of mammary gland biology is the understanding that the processes involved in normal mammary development and carcinogenesis are intrinsically related (1). One of the most frequently cited examples of this principle is the protective effect of an early full-term pregnancy against breast cancer. There is strong epidemiological evidence that women who experience a full-term pregnancy early in their lives have a significantly reduced risk of developing breast cancer (2, 3). This is substantiated by studies in a rat model in which prior treatment with estrogen and progesterone, (to simulate the effects of pregnancy) confers resistance to tumorigenesis in animals subsequently challenged with chemical carcinogen (4-12). The E+P-treated rat is thus a well-established model to study the effects of parity-induced protection against mammary cancer. In spite of this fact, little is known of the specific mechanisms governing pregnancy-specific developmental changes in the mammary gland. In this respect, the elucidation of novel targets for estrogen and progesterone action in the mammary gland, is crucial to our understanding of how an aspect of normal development might mediate the response of the organ to future proliferative signals.

To address this question, we have used the well-characterized Wistar-Furth rat model (described previously, 4), in conjunction with subtractive suppressive hybridization (SSH) methodologies (13) to identify markers that are persistently up-regulated in response to prior exposure to E and P in the mammary gland. As a result of these initial studies, we selected several specific markers to study in greater detail to investigate their role in relation to parity-related protection. To achieve this goal, we proposed to use mRNA expression analysis to study the temporal and spatial expression of each candidate biomarker, to evaluate their role as a function of reproductive history in relation to both differentiation and parity-related protection of the mammay gland. Furthermore, since the role of many of these markers has not yet been described in the mammary gland, we described experiments to further elucidate their function in relation to parity-related changes within the gland. In part, this approach will involve the use of novel retroviral transduction methods to overexpress the gene of interest in a parity-independent manner in the reconstituted mammary glands of recipient animals. If this approach is succesful,

then we have proposed experiments to investigate the influence of overexpression of a gene of interest in relation to protection against tumorigenesis following carcinogen challenge.

Report Body

e) Summary of research objectives

The specific objectives of this project, described in the original proposal are listed below:

Objective 1: To characterize the role of candidate biomarkers and to investigate their expression in the rat mammary gland:
a) To determine their temporal and spatial pattern of expression in the rat mammary gland.
b) To determine whether these candidate genes are markers of differentiation, *per se* or of protection.

Objective 2: To investigate the function of candidate genes *in vivo:*
a) To investigate the function of GB7 and to determine its role during normal cellular processes
b) To investigate the function of GB7, RbAp and Nap1 in reconstituted mammary glands
c) To determine the role of systemic factors on parity-induced changes in the mammary gland.

Many of the experiments described in objective 1 of the proposal have been completed, and the results published in a peer-reviewed article in November, 2001 (14). *In situ* hybridization analysis was used to determine the spatial expression of G.B7 and RbAp46. The same approach was also used to determine the effect of perphenazine treatment on the expression of these two markers. A quantitative reverse-transcriptase PCR method has been developed to examine the temporal expression of these markers in the rat mammary gland.

The experiments described in Objective 2 are in progress. However as you may be aware, the Tropical Storm that struck Houston in June 2001 caused significant impairment to researchers at Baylor. This included not only closure of the research facilities in the immediate aftermath of the disaster, but also an extended period of downtime due to the losses incurred as a result of the disaster. It is estimated that members of our laboratory lost two months of research time due to the constraints imposed due to lack of access to the research facility, cataloguing, ordering and attempting to salvage damaged research materials, and waiting for the replacement materials to arrive before we could recommence work.

f) Results

Objective 1

a) To determine their temporal and spatial pattern of expression in the rat mammary gland.
b) To determine whether these candidate genes are markers of differentiation, per se or of protection.

In the original statement of work, we proposed to use Northern analysis and *in situ* hybridization to investigate the expression of three markers (G.B7, RbAp46 and Nap1) at different stages of development in the rat mammary gland. In addition, we proposed to use perphenazine treatment as an experimental paradigm to distinguish the molecular processes involved in protection of the gland from those that are inherent to pregnancy-related differentiation of the gland. In the previous Annual Report, we described the use of i*n situ* hybridization analysis to determine the spatial expression of G.B7 and RbAp46. We also described experiments to recapitulate the effect of perphenazine treatment in Wistar-Furth rats and the use of *in situ* hybridization analysis to determine the effect of perphenazine treatment on the expression of G.B7 and RbAp46. The results of this anlysis was also reported in a recent manuscript (14). We also described the difficulties of detecting low abundance mRNAs such as G.B7 and RbAp46, the necessity of using PolyA+ RNA for Northern analysis and technical difficulty of isolating sufficient amounts of poly A+ RNA from mammary glands at different stages of development to satisfy this requirement.

To overcome these difficulties, we proposed two alternative approaches to enable us to quantitate the expression of these mRNAs without the necessity of isolating poly A+ RNA (as described in the previous progress report). In the first case, we proposed to apply the use of cDNA microarray technology to quantitate the expression each marker at different stages of development. We begun experiments to investigate the effectiveness of this approach by constructing a pilot array, containing 96 non-redundant clones from the E+P subtracted SSH library (including G.B7, Nap1 and RbAp46), in conjunction with the Microarray Facility at Baylor College of Medicine. The cDNA inserts of these clones were amplified using the nested primers in the SSH library backbone, purified by gel filtration chromatography and arrayed using silanized glass technology by means of the 384 well format array printer in the Microarray Core Facility. To investigate the effectiveness of this approach, we screened the pilot array with

fluorescently-labeled first strand cDNA probes prepared by reverse transcription of total RNA isolated from mammary glands of E+P and age-matched virgin (AMV) rats. We conducted these experiments using a variety of hybridization conditions to determine the optimal conditions for sensitivity of the detection method. Unfortunately, the results of these preliminary experiments showed that less that 30 % of the cDNA targets (including those of G.B7, Nap1 and RbAp46) could be detected by this method, even though the library from which they were isolated was constructed from the same E+P RNA pool used for the probe synthesis. We believe that this is once again due to the inherent difficulties of detecting low abundance RNAs from a relatively quiescent tissue such as the involuted mammary gland. Accordingly, we began to investigate alternative methods of probe preparation using indirect labeling with Cy3/Cy5 or cDNA amplification to increase the sensitivity of detection. However, in the last year the Microarray facility at Baylor ceased its array screening services to reevaluate the method of array production.

As an alternative approach, we decided to apply the technique of real-time PCR analysis to investigate the expression of these markers at different stages of development, and as a consequence of protection or differentiation of the gland. This approach has the advantage of being extremely rapid and sensitive to small changes in the concentration of a reverse-transcriptase amplified cDNA product. The application of this technique was previously limited by lack of access to the facilities for performing this analysis and the cost of purchasing commercial reagents such as the double-labeled fluorescent probes and proprietary amplification kits required for these experiments. However, recently our department has acquired the facilities for performing quantitative real-time RT-PCR. Furthermore, a number of recent publications have described the use of SYBR Green (as opposed to 'Taqman' or FRET-based) detection methods, combined with self-assembled PCR reagents (15, 16). Thus we decided to adapt this approach to study the expression of candidate markers in the rat mammary gland. So far we have applied this analysis to the RbAp46, and another potentially interesting marker, Stra13 (17). Expression of Cytokeratin-8 was used as a normalizing control, to correct for the epithelial content of mammary glands at different stages of development. Using the human genomic database sequences (http://www.ncbi.nlm.nih.gov/genome) and a search algorithm to predict exons by aligning genomic and cDNA sequences (18), we predicted the exon-intron structure of the homologous rat genes, and then designed mRNA-specific primer pairs. These primers either

spanned an exon-intron boundary, or if this was not possible then the two primers were encoded by separate exons of the gene of interest. In the latter case, amplification times were shortened to prevent the amplification of a long genomic fragment. We designed the primers to produce short amplicons (150-200 bp) to allow rapid cycling conditions, which should also prevent the amplification of non-specific products. Primers were then rigorously tested by BLAST analysis to confirm the specificity of the primer sequence and in a standard RT-PCR reaction (using no-RT and genomic DNA as controls and the same amplification conditions as the quantitative assay) to ensure the absence of any contaminating genomic products. The resulting amplicons were analyzed by gel electrophoresis on 2 % agarose gels to confirm specificity of the reaction and the absence of primer-dimers. Synthetic RNA standards were also incorporated into the assay to allow absolute quantitative of the levels of the gene of interest (19). Synthetic standards were generated by in vitro transcription from a vector possessing a T7 promoter adjacent to the sense strand of the cDNA target. The transcript was quantitated by incorporating trace amounts of [α^{32}P] rUTP into the transcription reaction and the transcript analyzed by polyacrylamide gel electrophoresis to ensure that full-length transcripts are produced. The use of an RNA standard instead of a DNA standard circumvents any problems due to differences in amplification as a result of the reverse-transcription step.

The assay was then conducted as a two-step process: the first step involving reverse-transcription of RNA in a standard thermocycler, and in the second step the reverse-transcription products were simultaneously amplified and the amplification products detected using an ABI Prism 7700 Sequence Detection System. Duplicate samples were analyzed in same assay along with serial dilutions of reverse-transcribed synthetic standard (as described above). At the end of the reaction, amplification plots were inspected to make sure that none of the templates had been overloaded, that a single amplification product was produced, and that replicate samples produced overlapping amplification curves (figure 1). A standard curve was generated by plotting C_T values for each concentration of standard, versus the number of target molecules in that reaction. The linearity of the response was examined by calculating the regression coefficient for the plot (figure 2). As stated above, we have used this approach to examine the expression of RbAp46 and Stra13 at different stages of development and as a consequence of differentiation or protection of the gland. We hope to extend these studies to G.B7, using the recently assembled draft mouse genomic sequence database

(http://www.ensembl.org/Mus_musculus) to predict the exon-intron stucture of this gene in mouse and rat.

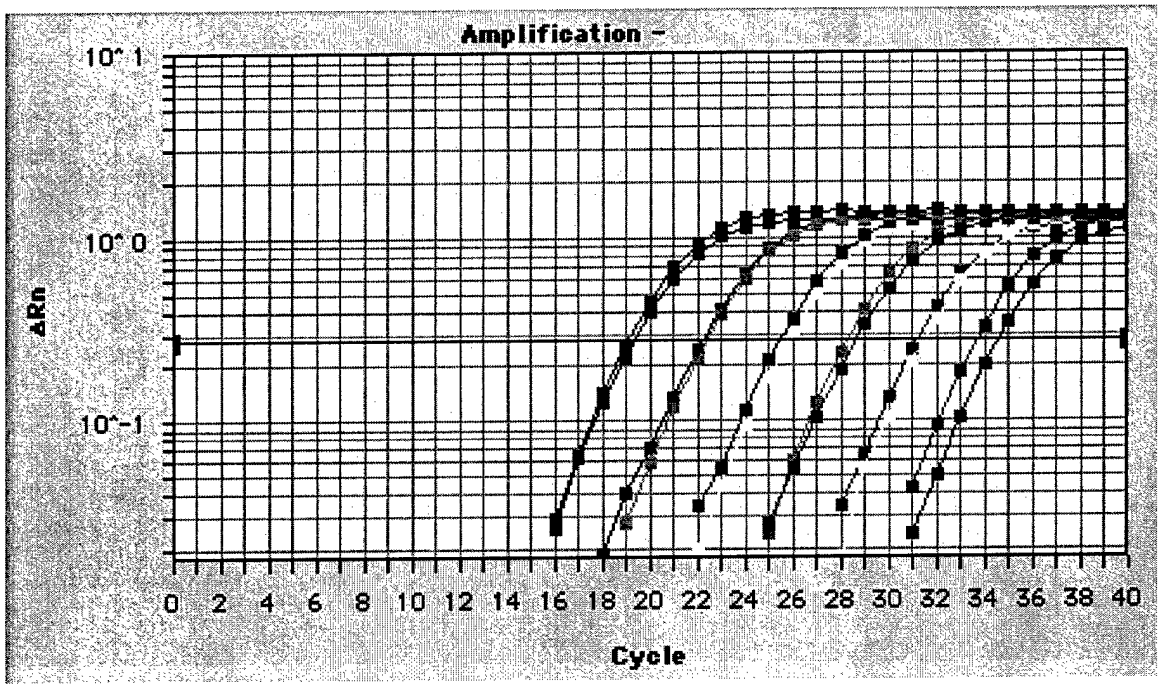

Figure 1. Amplification plot for varying concentrations of RbAp46 standard

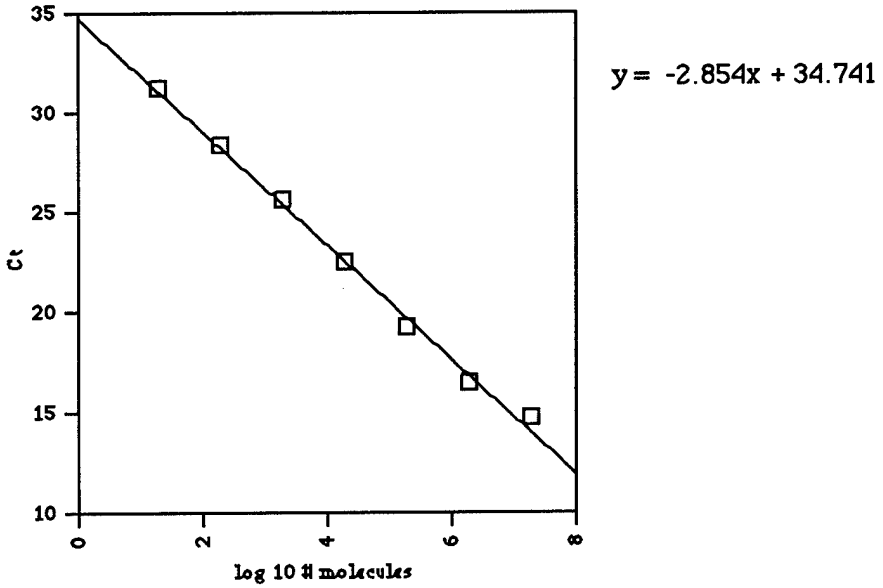

Figure 2. Standard curve generated from the data above. R^2=0.995

In the final part of this objective, we proposed to continue to characterize candidate markers by sequencing and Northern blot analysis. In addition to the data published in our manuscript in November, we have also sequenced 96 clones from a reverse subtracted (E+P driver, AMV tester) cDNA library which was constructed and screened using the same approach described for the E+P subtracted library.

Objective 2

c) To investigate the function of GB7 and to determine its role during normal cellular processes.

In the first part of this objective, we proposed experiments to determine the function of GB7 and to predict its role in normal cellular processes. As started in the original proposal and previous progress report, GB7 is a novel cDNA whose pattern of expression suggests it is an excellent marker for parity-induced protection. In spite of this fact, we have been unable assign a function to this marker. In the previous progress report, we described the isolation and sequencing of full-length clones for this mRNA. We then conducted extensive database analysis in an attempt to elucidate the function of G.B7; however, this search yielded little further information other than the identification of a potential human homologue for this gene, which mapped to a region spanning a known chromosomal break-point associated with a number of human cancers (20).

Recently, we decided to repeat this analysis with the hope if identifying newly characterized human ESTs with similarity to G.B7. The full-length G.B7 mRNA sequence (Genbank Accession # AY035343) was subjected to lower stringency alignment computation using the analysis algorithms of Pipmaker and Multipipmaker. In the first case, the full-length rat mRNA sequence was aligned with the sequence of the entire human BAC pertaining to the region of homology. In the second analysis, sequence from two overlapping rat BACs with homology to G.B7 were added to the alignment. These additional sequences were added to strengthen possibility of producing a good alignment, since the first alignment required

homology between orthologous mRNA and genomic sequences. This analysis was performed in collaboration with Dr. Monique Rijnkels, a senior postdoctoral fellow in Dr. Rosen's laboratory. The results of this analysis confirm the original prediction of a homologous human G.B7 sequence, located on human chromosome 2. Furthermore, it suggested that the putative human gene is encoded for the most part by a single large exon of 4503 bp and two smaller exons of 147 and 376 bp respectively. This information should enable to predict suitable regions to design primers for real-time PCR analysis (as described above). Finally, the results of this analysis showed that a) the G.B7 coding region overlaps with one exon of the ORF of a hypothetical human gene; and b) that it is close to a predicted imprinted region (22). As we have stated previously, we believe that this gene may function as a non-translated regulatory RNA (14). In the last few years, the importance of non-coding RNA genes has grown in recognition, with more and more examples of this class of molecules being discovered (23). Although this class of molecules may be of diverse function, there is evidence linking a number of non-coding RNAs with the regulation of imprinting of certain genes or loci. The fact that the human homologue of GB7 overlaps with a distinctly separate putative OFR suggests that G.B7 may also play a role in tissue- or developmentally-specific imprinting. The results of the human genomic database analysis may, therefore, enable us to design further experiment to investigate the function of G.B7.

b) To investigate the function of GB7, RbAp46 and Nap1 in reconstituted mammary glands

The second part of this objective proposed the use of reconstituted mammary gland experiments to elucidate the function of GB7, RbAp and Nap1 in an *in vivo* context. To achieve this goal, we have recently developed methods for growing rat mammary epithelial cells in primary culture and infecting them with a recombinant retrovirus expressing a gene of interest. In these preliminary experiments, we have tested several retroviral constructs and one lentiviral construct expressing a β-galactosidase reporter gene to determine the efficiency of the transduction with different vector systems. MEC were prepared from 96 day old virgin rats and maintained in culture as previously described (24). After 48 hours of culture, primary MEC were infected with freshly prepared virus stocks. These stocks were filtered before use to

remove residual virus particles and a constant volume of each applied to 48 h primary cultures. After five days of culture, and four rounds of infection, MEC were stained with the chromogenic substrate for, X-gal, and counterstained with nuclear fast red stain to estimate infection efficiency. Using the lentiviral vector, we have been able achieve infection of five to ten percent of the total cell population – measured by the nuclear localization of blue-staining following incubation of the cultured MECs with X-gal (figure 3). This is five to ten times higher than the efficiency of infection achieved by conventional retroviral strategies employing, as an example, the Moloney Murine Leukemia Virus (MMLV) derived vector pS2.

In subsequent experiments MECs were again cultured for five days, and during that time subjected to four rounds of infection with the same β-galactosidase-expressing lentivirus as described above. MECs were then removed from the culture plates, washed several times in serum-free media and injected into the cleared fat pads of 21 day old syngeneic host animals. As a control, non-transfected MECs were also injected into the contra lateral gland of two of the same rats. Eight weeks following transplantation, the recipient animals were sacrificed and the outgrowths removed and stained with X-gal to detect those resulting from transduced MECs. In this experiment, two out six outgrowths from the transduced MECs resulted in blue-staining outgrowths, indicating that we can produce transduced mammary outgrowths by this method.

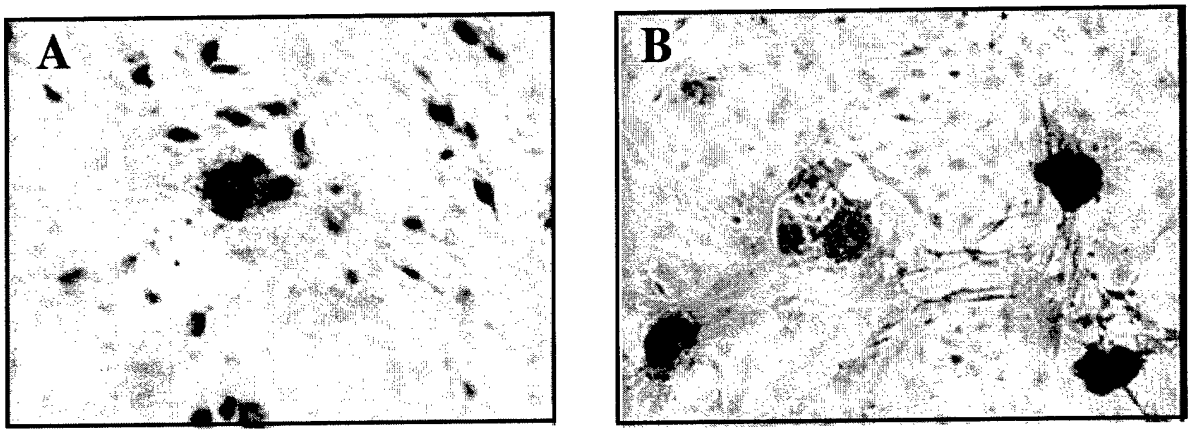

Figure 3: β-galactosidase staining of primary MEC cultures following two rounds of infection with recombinant lentivirus vector expressing *LacZ*

Commentary

Pregnancy-induced changes in cell-fate in the mammary gland

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Published: 25 April 2003

Breast Cancer Res 2003, **5**:192-197 (DOI 10.1186/bcr603)
 (Print ISSN 1465-5411; Online ISSN 1465-542X)

Abstract

The protective effect of an early full-term pregnancy is a well established phenomenon; in contrast, the molecular and cell-specific mechanisms that govern parity-specific changes in the mammary gland have not been well described. Recent studies signify a dramatic advance in our understanding of this phenomenon, and indicate a 'cell fate' model for parity-related changes that lead to protection against breast cancer.

Keywords: breast cancer, cell fate, epigenetic, mechanism, pregnancy, protection

Introduction

On the basis of epidemiologic studies and pathology, breast cancer appears to be an extremely complex, multifactorial disease. In spite of our growing understanding of the molecular aberrations that lead to cancer, and the role of endocrine signaling pathways in the progression of the disease, there are few common links pointing to an individual's susceptibility to breast cancer. However, one consistent finding is the correlation between the timing of normal endocrine-related developmental events and breast cancer risk [1]. In particular, there is strong epidemiologic evidence that women who experience a full-term pregnancy early in their lives have a significantly reduced risk for developing breast cancer [2–5]. This has been corroborated by numerous experimental studies in rodents that resoundingly demonstrated the protective effect of an early full-term pregnancy against mammary tumors (reviewed in [6]). In addition, hormonal mimicry, by treatment with estrogen and progesterone (E+P) or human chorionic gonadotropin for a period of at least 21 days, can be equally effective for inducing a refractory state that is similar to that afforded by an early full-term pregnancy [6–13].

The observation that the protective effect of an early full-term pregnancy can be accurately reproduced in rodents has lead to the development of defined animal models for studying this parity-related phenomenon. For many years, tumor-susceptible rat models were the mainstay of this experimental effort; however, there is a growing body of evidence suggesting that several strains of mice may also be appropriate models for these studies. Despite the wealth of literature supporting the role of endocrine-mediated processes in parity-related refractoriness, little is known of the molecular mechanisms that govern pregnancy-specific developmental changes in the mammary gland. In this commentary, we consider the contributions made by several recent publications to the advancement of our understanding of the molecular and cell-specific changes apparent in the parous (protected) gland. In this context, 'persistent changes' are defined as alterations that occur as a result of parity but that endure far beyond the initial stimulatory event. Taken together, these findings provide important insights into the strategies that might be employed for future studies of this parity-related phenomenon.

A global profile for molecular changes in the parous mammary gland

As a preliminary step in elucidating the molecular mechanisms that underlie parity-related protection, we and others have searched for molecular changes in the parous mammary gland. In the first study of this kind [14], we employed the Wistar–Furth rat model in conjunction with

AMV = age-matched virgin; E+P = estrogen and progesterone; MNU = methylnitroso urea; TDLU = terminal ductal lobular-like structure; TGF = transforming growth factor; WAP = whey acidic protein.

suppression subtractive hybridization to isolate molecular biomarkers for the protective effect of an early full-term pregnancy. Using this approach, we identified a set of 100 independent (nonredundant) markers that were persistently upregulated in the glands of 21-day E+P treated (20 μg estrogen/20 mg progesterone), 28-day involuted rats, as compared with age-matched virgin (AMV) control animals.

In an elaborate and rigorously executed study, D'Cruz and coworkers [15] applied global profiling metholodologies to examine persistent changes in the pattern of gene expression, as a function of parity, in the rodent mammary gland. Using oligonucleotide arrays, they performed a quantitative assessment of the expression levels of 5500 genes in the mammary glands of parous and nonparous FVB mice. In addition, they verified the expression of a subset of candidate markers by Northern analysis.

One concern raised by this type of analysis is that the findings may not be applicable across different species or even across different strains of mice [6]. Furthermore, although the protective effect of parity has been well characterized in many rat models of chemical carcinogenesis, these studies have only been performed in three strains of mouse [16,17]. To corroborate their findings, D'Cruz and coworkers examined the expression profile of a smaller subset of these markers as a function of parity in the mammary glands of 129SvEv and Balb/c mice, as well as Lewis and Sprague–Dawley rats. As a result of this analysis, they identified a subset of 38 genes that exhibited persistent alterations in their pattern of expression in parous versus nonparous rodents.

Global profiling: a predictive tool for parity-specific change in mammary gland

To extend their findings, D'Cruz and coworkers [15] tested whether the expression pattern of this subset of markers was sufficient to predict, in a blinded manner, the reproductive history of a sample. To examine the predictive merit of their approach, they performed microarray analysis with an independent group of mammary gland RNA samples and then used hierarchical clustering, based on the expression profiles of their 38 genes, to determine the reproductive history of the tissues of origin. These results elegantly demonstrate that this data set can be effectively and reproducibly used as a predictive indicator of parity-specific protection; such findings raise the prospect of developing a prognostic tool that is also translational to human studies.

Parity results in changes to growth-regulatory pathways in rodent mammary gland

D'Cruz and coworkers [15] showed that genes that encode growth factors such as amphiregulin (*A-reg*), pleiotrophin (*Ptn*), leptin (*Ob*) and insulin-like growth factor-1 (*Igf-1*), which may potentially be involved in epithelial proliferation, are downregulated in the parous involuted gland. In the case of *Ptn* and *A-reg*, at least, downregulation is observed as early as mid-pregnancy (day 12), and these genes remain persistently downregulated following 21 days of lactation and 28 days of postlactational involution. Conversely, genes that encode markers of the transforming growth factor (TGF)-β_3 signaling pathway are persistently upregulated following parity and remain elevated (as compared with AMV control animals) for up to 30 weeks following postlactational involution. Previous studies have shown that TGF-β_3 mRNA is markedly upregulated during involution, reaching peak expression during the second phase of this process [18]. Therefore, the persistent expression of TGF-β_3 observed by D'Cruz and coworkers could be a function of involution rather than parity. From our own studies (Ginger M, Rosen J, unpublished observations) we have also observed the downregulation of certain growth-promoting molecules; in contrast, markers involved in cell cycle control (*Cdc42*, *Rb*) or the modulation of the TGF-β signaling pathway (*Fstl-1*, *Nrln-1*) are upregulated in the parous mammary gland [14]. Interestingly, the observed changes in the level of insulin-like growth factor-1 mRNA may reflect an alteration in systemic growth hormone levels reported previously [10]. Altogether, these findings tend to support the notion that there are persistent changes in growth regulatory pathways in the parous mammary gland.

The downregulation of mRNA for certain growth-stimulatory molecules is a tantalizing observation, in particular *Ptn*, *Igf-1* and *A-reg*, because over-expression of these growth factors has been associated with human breast cancer. However, these findings alone do not provide sufficient explanation for the refractory nature of the parous gland because it has been adequately demonstrated that the gland can re-enter the proliferative phase during subsequent pregnancies. However, as suggested by D'Cruz and coworkers [15] and previously by Sivaraman and colleagues [17], the role of these molecules may only become evident once the gland is exposed to further proliferative stimuli. Moreover, it is possible that the downregulation of growth-promoting molecules and the upregulation of the TGF-β_3 signaling pathway may work in concert with one another, as well as other factors, to confer an altered response to subsequent proliferative stimuli.

p53 expression is correlated with a proliferative block

Previously, Sivaraman and coworkers [9] showed that one of the earliest consequences of carcinogen exposure is a proliferative burst in the epithelial compartment of the nonparous gland. In contrast, proliferation is abrogated in glands of parous or E+P-treated rats, although the pathways that govern this differential proliferative response have not yet been elucidated. In their recent publication, a potential mechanism for this proliferative block is explored.

Using the Wistar–Furth rat model described in their previous study [9], those investigators examined the expression pattern of p53 protein in response to hormonal stimulation and exposure to the chemical carcinogen methylnitroso urea (MNU) [17]. Rats aged 45 days were subjected to a priming dose of estradiol benzoate (to synchronize estrus) and then treated with E+P (20 μg estrogen/20 mg progesterone, subcutaneously) for a period of 21 days. This represents a modest dose of hormones, but it is sufficient to confer 82% protection against MNU-induced tumorigenesis. Treatment with E+P induced the upregulation and nuclear sequestration of p53 protein; p53 was up-regulated as early as 3 days after E+P treatment and remained elevated for the remainder of the 21-day treatment period. Strikingly, these levels were persistently elevated 28 days after hormone withdrawal, as compared with nonparous AMV, and remained elevated for at least 3 days following exposure to MNU. Pregnancy induced a similar pattern of expression; p53 was upregulated during pregnancy, remained persistently elevated after a 28-day involution period, and was localized to the nucleus of mammary epithelial cells. Nuclear accumulation of p53 preceded the proliferative block described previously and resulted in transcriptional activation of p53 target genes (MDM2 and $p21^{CIP1/WAF1}$) at 3 days post-MNU exposure.

To evaluate the mouse as a suitable model for these parity-related changes, Sivaraman and coworkers [17] repeated their experiments using BALB/c mice, and were able to recapitulate their findings from the E+P-treated Wistar–Furth rat. Once again, nuclear accumulation of p53 and induction of p21 preceded the proliferative block following carcinogen exposure, suggesting that they may contribute mechanistically to this event. These observations are strengthened by the finding that p53-mediated apoptosis, in response to γ-irradiation, in the pregnant and involuting gland differs significantly from that in the glands of virgin animals [19]. Given the fact that aberrations in p53 expression and/or function are often associated with breast cancer, parity-specific changes in p53 expression are likely to play an important role in protection against tumorigenesis.

Pregnancy results in changes in the cellular composition of the mammary gland

As a consequence of their profiling studies, D'Cruz and coworkers [15] provided evidence to suggest that there are persistent alterations in the cellular composition of the parous mammary gland: the persistent presence of hematopoietic cells, or cells with heomatopoietic-like properties, as well as changes in cytokine signaling pathways within the epithelium. At this point it is not clear whether the presence of cells with hematopoietic-like properties is due to the existence of lymphocytes and macrophages within the gland, or to the recruitment of certain epithelial cells to express markers of a hematopoietic-like population.

By independent means, Wagner and coworkers [20] also demonstrated alterations in the epithelial population of the parous mammary gland. Using double transgenic mice, expressing Cre-recombinase under the control of the whey acidic protein (WAP) gene promoter and carrying a floxed-Stop-*lacZ* knock-in cassette (Rosa-lox-Stop-lox-*lacZ*; hereafter referred to as Rosa-LacZ), those investigators were able to track the fate of WAP-Cre-expressing mammary epithelial cells during pregnancy and involution. The elegance of this approach derives from the ability of the Cre-recombinase to 'inscribe' its effect (i.e. recombination between loxP sequences), leading to permanent activation of the *lacZ* reporter gene in cells in which WAP-Cre expression has occurred. Thus, any cell that responds to the appropriate hormonal and developmental signal to express WAP-Cre retains a lasting signature (β-galactosidase expression) of this developmentally specific event.

Because expression of the WAP-Cre transgene closely mirrors that of the endogenous WAP gene, hormonal and developmental changes typical of mid to late pregnancy are required for its expression. During involution the intricate, differentiated alveolar structure of the epithelium diminishes through a combination of apoptotic and remodeling processes, and the gland regresses to a more quiescent state that is similar to that of the mature virgin gland. WAP gene, and therefore presumably WAP-Cre, expression ceases during the first few days of involution. However as Wagner and coworkers [20] demonstrated, a specific component of the epithelium retains β-galactosidase expression 3–4 weeks after postlactational involution.

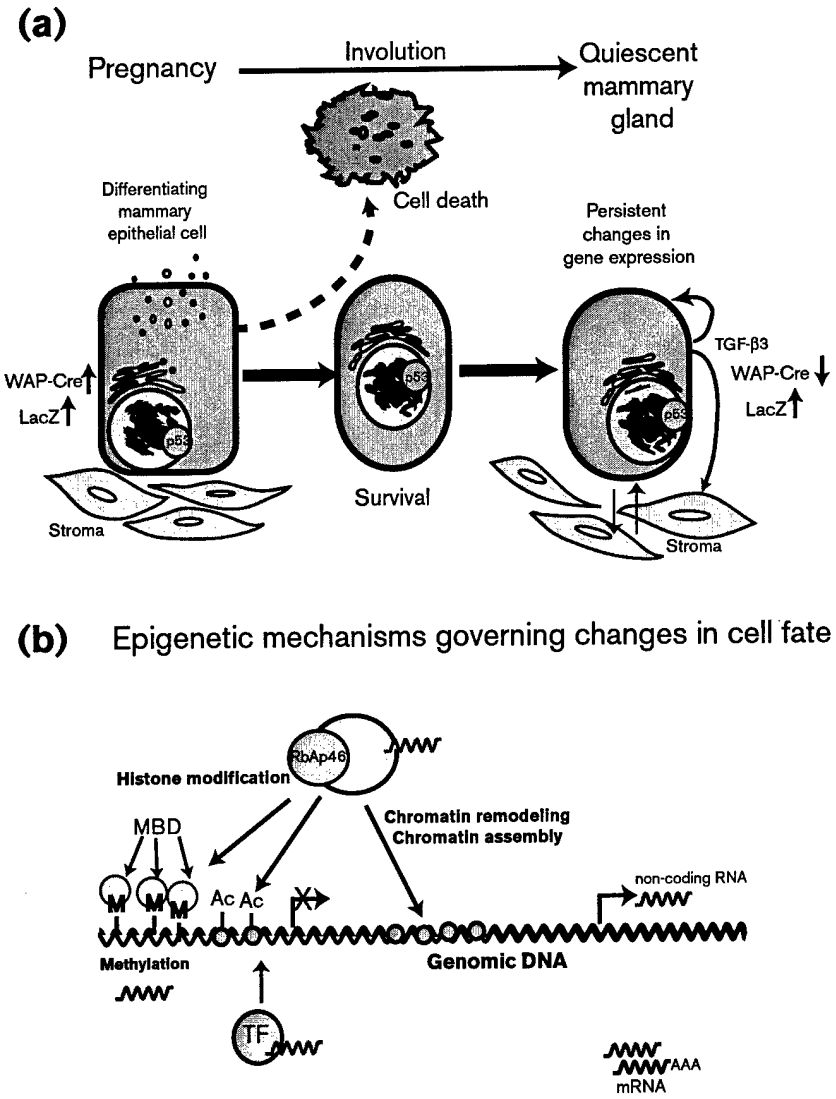
Thus, they identified a novel epithelial cell population originating from a parity-specific event, but which is distinct from the majority of the cells in the pregnant mammary gland. The ability of these cells not only to differentiate but also to survive involution distinguishes them as a unique subpopulation of the mammary epithelium. Furthermore, this observation implies that differentiation alone is not the basis of their unique properties, but that some other factor is responsible for their enduring presence in the parous mammary gland.

Strikingly, these β-galactosidase-expressing cells are confined primarily to the regressed terminal ductal lobular-like structures (TDLUs in humans) at the extremity of the ductal tree. As we have previously shown, two markers of parity-related protection, RbAp46 (a component of the Sin3A/HDAC, Mi-2/NuRD and histone methyltransferase complexes) and G.B7 (a noncoding RNA), are likewise expressed in a localized manner in the TDLU-like structures of the involuted mammary gland [14].

A unifying hypothesis

Taken together, these findings suggest that there are permanent alterations in the epithelial compartment of the

Figure 1



Unified model for parity-specific changes in the mammary gland. **(a)** Summary of reported parity-dependent changes in the mammary gland. Pregnancy induces multiple changes in the mammary epithelial cells, including nuclear accumulation of p53 and induction of whey acidic protein (WAP)-Cre expression (and subsequently β-galactosidase expression, indicated by blue cells) in WAP-Cre/Rosa-LacZ mice, and alteration in gene activity perhaps leading to changes in cell fate. During involution, a large component of the epithelium is eliminated through programmed cell death; however, a specific subpopulation is able to circumvent this process. The involuted mammary gland is characterized by persistent changes in gene expression, nuclear localization of p53, and an altered proliferative capacity in response to carcinogen. **(b)** Potential mechanism for the parity-specific changes in cell fate: Pregnancy invokes epigenetic changes affecting cell fate in the parous mammary gland. Epigenetic changes may be induced by a number of mechanisms, including chromatin remodeling, DNA methylation/demethylation, and histone modification, and as a result of *de novo* chromatin assembly. RbAp46 is a component of a number of complexes responsible for these processes, including the NuRD complex [23], which also contains methyl-CpG binding domain proteins (MBD). The common presence of RbAp46 in these complexes might thus provide a mechanism for sequential shuttling between these different functions, and in addition provides a link between DNA methylation and chromatin regulation. Noncoding RNAs have also been implicated in these changes in gene expression, through their association with chromatin remodeling, histone acetylation/deacetylation, and transcription factor complexes, as well as RNA interference [24,25]. Epigenetic changes can regulate cell fate in a number of ways, for instance by altering gene activity, by providing a signal for survival or proliferation, and by mediating responses to DNA damage. In addition, it is likely that there is continual cross-talk between the epithelium and the stroma, thus providing an additional level of epigenetic regulation in the parous mammary gland. Ac, acetylated histones; M, methylated cytosine; TF, transcription factor.

parous mammary gland (Fig. 1a). These changes include, but are not limited to, the following: an alteration in the signaling pathways governing cell-growth (and possibly also chemoattractant properties or the expression of hematopoietic markers); the ability to differentiate, survive apoptosis, and still retain the propensity for self-renewal; and the ability to circumvent proliferative stimuli, possibly through the transcriptional activation of p53 target genes. These changes endure beyond the primary phase of stimulation due to the hormonal manipulation or pregnancy, persisting 1 or even 6 months after this initial inductive event. In addition, changes in systemic hormone levels, as well as in the mammary stroma [21], may also participate in the protective effects of an early pregnancy.

How does pregnancy impose such persistent changes on the mammary gland? Wagner and coworkers [20] suggested that pregnancy confers a 'functional memory' on the mammary epithelium. Sivaraman and colleagues [17] hypothesized that pregnancy results in changes to the 'developmental fate' of a subpopulation of epithelial cells. Together, these studies suggest that pregnancy alters the fate of a specific population of epithelial cells, but how is cell fate altered by pregnancy? We propose that epigenetic factors might provide a unifying mechanism for this process (Fig. 1b) [14]. Epigenetic factors tell a cell who it is, where it resides, and what type of function it can perform. Changes in epigenetic regulation (through the processes of DNA methylation, histone modification, and chromatin remodeling) frequently accompany developmental processes, and thus provide a lasting signal that restricts the pattern of gene expression in those cells, long after the inductive event has been removed [22]. Such changes could thus provide an enduring 'memory' that determines cell fate and prevents cell-lineage aberrations that lead to cancer.

Conclusion

Taken in concert, these studies, as well as our own findings, provide several important conclusions. First, parity results in persistent changes in the pattern of gene expression in the rodent mammary gland. Second, the molecular changes induced by E+P treatment (hormonal mimicry) are mirrored by similar changes in the parous gland. Third, the molecular, proliferative, and cell-specific changes observed in the parous (or E+P-treated) mouse recapitulate the parity-related alterations seen in the rat. Fourth, parity results in an altered epithelial population in the rodent mammary gland. Such conclusions provide new insights into the strategies we might use to approach this question in the future and, in particular, they point to a new experimental model that recognizes the utility of transgenic and knockout mice as a complement to the traditional rat model. Finally, the generality of this model offers the hope that we can translate these findings to the prevention of human disease, possibly by mimicking these developmental events pharmacologically for the chemoprevention of breast cancer.

Competing interests

None declared.

Acknowledgments

We wish to thank Daniel Medina and Kathryn Schwertfeger for their critical reading of the manuscript. These studies were supported by U.S. Department of Defense BRCP grant DAMD17-00-1-0137 and by NIH grant CA 64255.

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