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Epidemiologic studies have highlighted the relationship between hormones and carcinogenesis in the breast by identifying endocrine risk factors for breast cancer that are related to the timing of reproductive endocrine events, such as menarche and menopause. This relationship is illustrated by the observation that women who undergo an early first full-term pregnancy have a significantly reduced lifetime risk of breast cancer. We hypothesize that an early first full-term pregnancy results in a permanent change in the breast that confers a decreased risk for the subsequent development of breast cancer. We further hypothesize that parity-induced changes in the number and distribution of specific epithelial cell subtypes in the breast may contribute to parity-induced changes in breast cancer risk. To address these hypotheses, we are identifying and evaluating molecular biomarkers for parity-induced changes in the breast by isolating genes that are differentially expressed between the parous and nulliparous rodent breast using DNA chip technology. These differentially expressed genes are being used as biomarkers to determine the molecular and cellular changes that occur in the mammary gland as a result of parity. Ultimately, these studies will serve as a step towards determining the mechanisms by which breast cancer susceptibility is modulated by reproductive history.

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Lein L. Ellis MDPhD 7/10/00

Table of Contents

INTRODUCTION

Epidemiologic studies have highlighted the relationship between hormones and carcinogenesis in the breast by identifying endocrine risk factors for breast cancer that are related to the timing of reproductive endocrine events, such as menarche and menopause. This relationship is illustrated by the observation that women who undergo an early first full-term pregnancy have a significantly reduced lifetime risk of breast cancer. The recognition that specific reproductive endocrine events alter breast cancer risk in a predictable fashion raises the possibility that events associated with a decrease in breast cancer risk, such as early first full-term pregnancy, might be mimicked pharmacologically. As such, understanding the mechanisms by which these events influence breast cancer risk would facilitate the design of safe and effective hormonal chemoprevention regimens. In addition, the testing of such regimens would be facilitated by the identification of biomarkers that accurately reflect early biological changes in the breast associated with reproductive endocrine events that alter breast cancer susceptibility. Since the impact of reproductive endocrine history both on mammary gland development and on breast cancer risk most likely results from differential effects of hormones on particular subpopulations of cells, understanding these changes will almost certainly require a thorough understanding of the cell types present in the breast and the manner in which hormones affect their normal programs of differentiation and development. Unfortunately, pursuit of this goal has been hampered by the fact that few, if any, molecular biomarkers have been identified to date that are specific for changes in the breast that occur as a result of reproductive endocrine events known to influence breast cancer risk. Such biomarkers are essential both for understanding the impact of reproductive history on mammary epithelial biology in general, and the molecular and cellular basis of parity-induced protection against breast cancer in particular, as well as for the rational design and testing of hormonal chemoprevention regimens aimed at mimicking this naturally occurring protective event.

We hypothesize that an early first full-term pregnancy results in a permanent change in the breast that confers a decreased risk for the subsequent development of breast cancer. We further hypothesize that parity-induced changes in the number and distribution of specific epithelial cell subtypes in the breast may contribute to parity-induced changes in breast cancer risk. To address these hypotheses, the specific aims of this proposal are designed to identify and evaluate molecular biomarkers for parity-induced changes in the breast by isolating genes that are differentially expressed between the parous and nulliparous rodent breast using DNA chip technology. These differentially expressed genes will be used as biomarkers to determine the molecular and cellular changes that occur in the mammary gland as a result of parity. Ultimately, these studies are intended as a step towards determining the mechanisms by which breast cancer susceptibility is modulated by reproductive history.

BODY

We believe that generating a sufficiently robust panel of parous- and nulliparous-specific markers will require a more powerful parallel screening technique that permits the sensitive, reliable and reproducible detection of differentially expressed genes. To accomplish this aim, we have chosen to determine gene expression levels by hybridization of mRNA to high-density oligonucleotide arrays.

We initially compared the morphology of mammary glands from rodents that have undergone a single-round of pregnancy, 21 days of lactation and 4 weeks of regression to the morphology of mammary glands from nulliparous littermates (Fig. 1). It has previously been noted that mammary glands of parous woman have more differentiated lobules than seen in nulliparous woman[1]. Whole mount analysis of mammary glands from virgin and parous Sprague-Dawley rats demonstrate

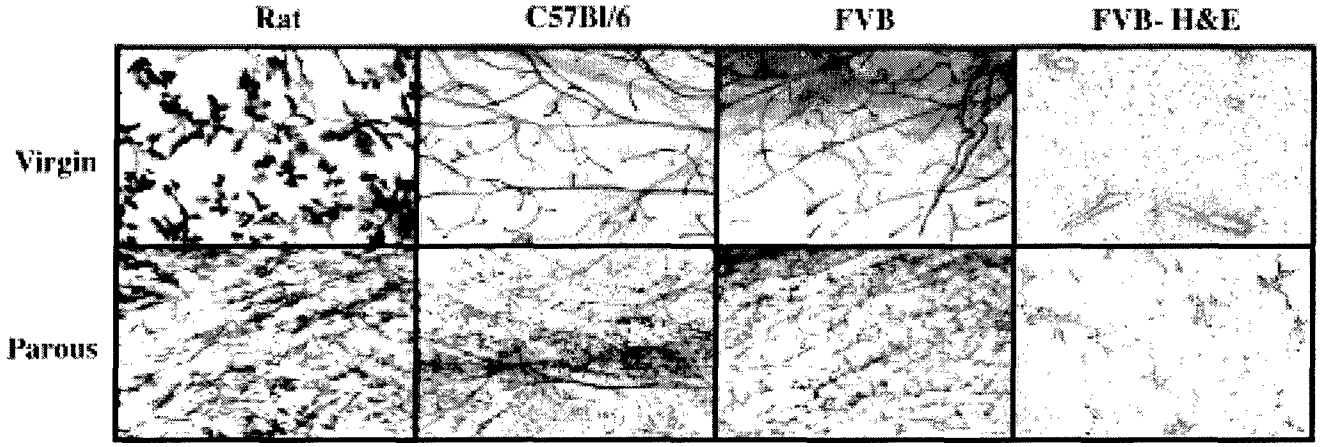

Figure 1: Parity-induced changes in mammary gland morphology

marked differences in the architecture of the virgin and parous glands. The parous gland is more highly branched, suggesting that it is permanently altered by parity-related changes. We have also examined two independent strains of mice, C57BL/6 and FVB to determine whether these morphological features appear in the murine system as well. Parous animals were mated at 4 wks of age, a time corresponding to puberty in order to approximate "early-parity". Although the virgin murine glands appear less developed as compared to the rat, similar overall differences are observed between the virgin and parous glands of mice and rats. Again the parous gland is more highly branched (Fig 1). Analysis of H&E stained glands demonstrate that there appears to be roughly the same amount of epithelium, an observation that has been confirmed by quantitation of epithelium by Cytokeratin 18 expression levels. The structural changes which accompany parity in the mammary gland appear to be permanent as the differences in the gland remain for essentially the life of the animal (data not shown).

Technical Objective I: Isolate genes expressed in the breast in a parity-specific manner.

Changes in the breast that are induced by reproductive events such as parity are likely to involve complex changes in the expression of multiple genes. Many techniques for comparing mRNA populations, such as differential display, subtractive hybridization, and serial analysis of gene expression (SAGE) have proven to be labor-intensive and relatively insensitive, particularly for genes expressed at very low levels. In addition, these techniques typically generate high percentages of false-positive clones. As such, we have chosen a technical approach that permits the direct assessment of mRNA expression profiles from large numbers of genes in parallel. This approach, referred to as Affymetrix GeneChip technology, involves the determination of the relative abundance of mRNAs based on hybridization of complex mRNA

populations to high-density arrays, or "chips" containing arrayed synthetic oligonucleotides[2-4]. Each chip is composed of 65,000 different oligonucleotides of defined sequence which are synthesized directly on derivatized glass slides. Each gene is represented on the array by 20 specific, unique 25-mer oligonucleotide probe pairs, such that each chip permits the detection of approximately 1,600 genes. A combination of arrays permits expression levels to be determined for more than 6,800 full-length genes in the UniGene, GenBank and TIGR databases. mRNA isolated from a sample of interest is used to generate an antisense poly(A)$^+$ probe with incorporated biotin-labeled nucleotides. This mRNA probe is then hybridized to the array using the GeneChip fluidics station which automates sample introduction, hybridization, incubation, and washing. Quantitative detection of mRNA bound to each oligonucleotide cell is achieved by measuring fluorescence of bound phycoerythrin-streptavidin using a Hewlett-Packard GeneArray scanner. Finally, the GeneChip operating system software automatically calculates intensity values for each probe cell. Expression for each gene is determined by analyzing the extent of binding to the 20 pairs of oligonucleotides specific for that gene. Fluorescence intensity from the hybridized arrays directly correlate with mRNA abundance levels. The GeneChip instrumentation system necessary for these experiments has recently been purchased from Affymetrix, Inc. by the Institute for Human Gene Therapy. This equipment will be located within the Chodosh laboratory.

GeneChip technology permits the quantitative, sensitive, specific and reproducible determination of gene expression profiles [2-4]. A high percentage of clones identified are true positives, thereby minimizing subsequent labor invested in pursuing false leads. Differences in expression levels of approximately 2 to 2.5-fold can be reliably distinguished. The detection limit is approximately 2 - 5 copies per cell, and is quantitative over three orders of magnitude [2-4]. Each array contains probes comlementary to multiple reference genes that permit data within and among experiments to be normalized and quantitated [2-4]. In addition, multiple internal reference points permit the normalization and quantitation of data from different experiments. Repeat hybridization with the same probe is reported to yield only one in 3,000 genes that differ by more than 3-fold [2-4]. Hybridization with probes independently prepared by different operators from the same sample source is reported to yield less than 1 in 1,000 genes that differ by more than a factor of three. Compared to array-based methods that involve the spotting of cDNAs onto nylon membranes, an additional advantage of the oligonucleotide-based approach is that highly specific binding and detection can be achieved even within conserved gene families. Finally, this parallel approach lends itself to increases in scale to permit large numbers of genes to be monitored and is thereby ideally suited for the extensive comparison of complex mRNA populations such as were expected in this proposal.

Task 1: *Months 1-12: Generation of mRNA samples representing reproductive endpoints.*

The primary endpoint of this task has been completed on schedule. Inbred FVB mice (which do not harbor active MMTV provirus) were bred and maintained in a barrier facility with a regular 12 hour light/12 hour dark cycle to facilitate regular estrous cycling. Animals were given food and water *ad libitum*. Mice were housed 4 per cage during virgin development and postlactational regression, and individually during pregnancy and lactation. Nulliparous controls were housed identically to parous animals. Daily vaginal smears were performed on animals to document regular cycling. Total RNA was isolated from the mammary glands of mice that had either undergone an early first full-term pregnancy followed by 3 weeks of lactation and 4 weeks of postlactational regression, or that were maintained as age-matched nulliparous controls. Animals were euthanized by carbon dioxide asphyxiation at specified developmental time points, and the number 4, 5 and 6 mammary gland pairs were harvested and snap frozen in liquid nitrogen, following removal of the lymph node embedded in gland 4. Mammary tissue from each animal was also fixed in 10% neutral buffered formalin and embedded in paraffin for *in situ* hybridization studies. Total RNA was isolated from tissues by homogenization in guanidinium isothiocyanate, followed by

ultracentrifugation through cesium chloride. In total, RNA was harvested from parous and nulliparous animals in three independent experiments with each experiment comprised of 20 parous and 20 nulliparous animals. Additional breeding of animals is ongoing in order to generate developmental time points representing other reproductive variables such as early vs. late first full-term pregnancy and duration of postlactational regression.

Task 2: *Months 6-18: GeneChip-based screening.*

Significant progress has been made in accomplishing this task during the initial budget period. Standard Affymetrix protocols were used to generate and hybridize probes prepared from nulliparous and parous RNA samples, and to quantitate and analyze gene expression levels. RNA isolated from nulliparous and parous animals was subject to reverse transcription to make first strand cDNA that incorporated a T7 RNA polymerase promoter at the 3' end. Following completion of the second strand of cDNA, this material was used in an in vitro transcription reaction to generate biotin-labeled cRNA representing each experimental pool of mammary gland RNA. This in vitro transcribed cRNA was then fragmented to facilitate hybridization. Hybridization reactions were prepared for each sample using fragmented cRNA in addition to standardized internal controls. Hybridization reactions representing each of the 3 nulliparous samples as well as each of the 3 parous samples were hybridized to murine 6500 A, B, C and D oligonuleotide arrays, representing approximately 5500 murine genes.

Using GeneChip software, expression levels were calculated for each of the 5500 genes represented on the Mu6500 chips for all six samples. This information was imported into a software database. Confidence intervals were generated for each gene in order to identify genes that were differentially expressed between the nulliparous and parous states at a confidence level > 90%. In addition, Affymetrix algorithms were applied to each individual nulliparous/parous pair to identify those genes that whose expression was considered to increase or decrease. This analysis was repeated for each of the three experiments. Genes that were considered to either increase in all three experiments or decrease in all three experiments were identified. As a supplementary analytical approach, genes that were considered to either increase in two of the three experiments or decrease in two of the three experiments were identified. To date, this approach identified 10 genes whose expression appears to be down regulated as a result of parity, as well as approximately 34 genes whose expression is upregulated as a result of parity. A comparable number of candidate genes in each category also exist based on other analytical algorithms.

The ascertainment of differentially expressed candidate genes based on GeneChip analysis is essentially complete for the Mu6500 chips. Additional experiments are currently underway to confirm and extend these results by analyzing these samples on oligonucleotide arrays representing approximately 11,000 murine genes.

Task 3: *Months 6-20: Testing of candidate genes by Northern hybridization and RNase protection.*

In order to test the differential expression of the candidate genes that we had identified, we generated 400-600 base pair fragments from the designated genes using RT-PCR and probed Northern blots containing one virgin and one parous sample identical to material that was used in the GeneChip analysis, as well as two independent virgin and parous samples to control for biological variability. This approach confirmed the differential expression of all of the genes in which expression either increased or decreased in all three parous/nulliparous sets, as well as many of the genes whose expression either increased or decreased in two of the three parous/nulliparous sample sets, that also had a confidence level > 90% (Fig. 2). Overall, the true-positive rate ascertained to date is approximately 70%. This true-positive rate is considerably higher than that from any of the procedures that we have previously used to screen for

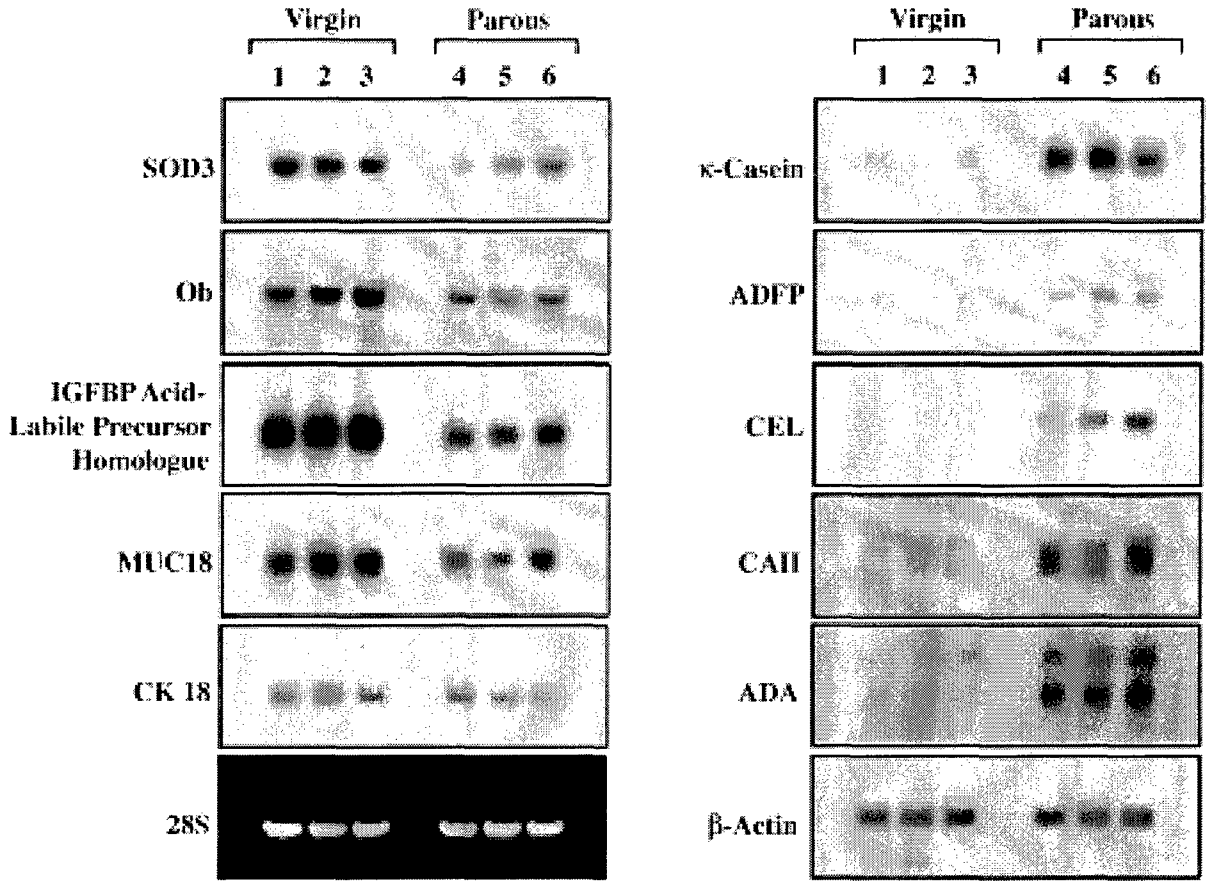

Figure 2: Differential expression of genes isolated by oligonucleotide microarrays

differentially expressed genes. Approximately 60 genes have been tested by Northern hybridization to date.

Since the most widely used and accepted rodent models for parity-induced protection against breast cancer employ the rat, we have begun testing selected genes that are differentially expressed in the mouse as a function of parity, in order to determine if they are also differentially expressed in the rat. To accomplish this, the corresponding rat genes were first cloned using RT-PCR. This analysis is currently ongoing, but initial experiments indicate that a significant fraction of genes that are expressed in a parity-dependent manner in the mouse are expressed in a similar manner in the rat (Fig. 3).

Technical Objective II: Analyze genes expressed in a developmental stage-specific manner.

Task 1: *Months 6-24: Determine the developmental expression pattern of cDNA markers.*

We propose to use the genes that we have identified as being differentially expressed to define parity-induced cellular and molecular changes that occur in the breast. Therefore, we have begun to examine the developmental expression pattern for those genes that we have confirmed as being differentially expressed as a function of parity. Gene expression patterns are being determined during mammary gland development using Northern hybridization. Developmental time points analyzed include virgin development from a point preceding puberty through the completion of ductal morphogenesis (2 , 5 , 10 , and 15 weeks); early, mid and late pregnancy (day 7, 14, and 21); mid lactation (day 9), and early, mid

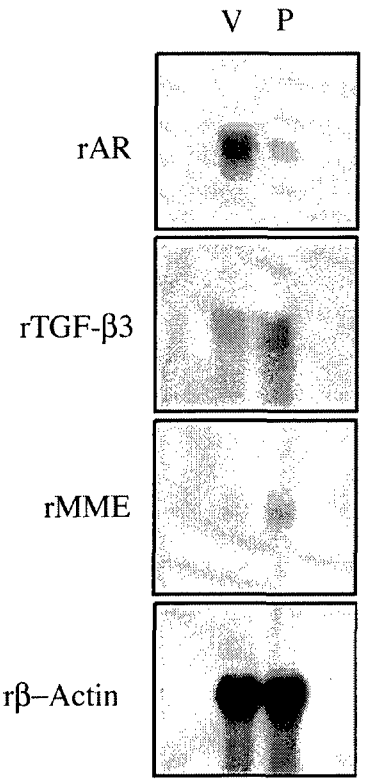

Figure 3: Differential expression of selected genes in Lewis rats

and late postlactational regression (day 2, 7, and 28). This analysis is ongoing. As an example, the developmental expression of the epidermal growth factor, amphiregulin, is shown in Figure 4. In adult animals, amphiregulin is expressed in a nulliparous-specific manner. Developmentally, amphiregulin expression appeared to be induced during puberty and to remain elevated thereafter in the virgin gland. Interestingly, amphiregulin expression subsequently decreases during mid-pregnancy and remains

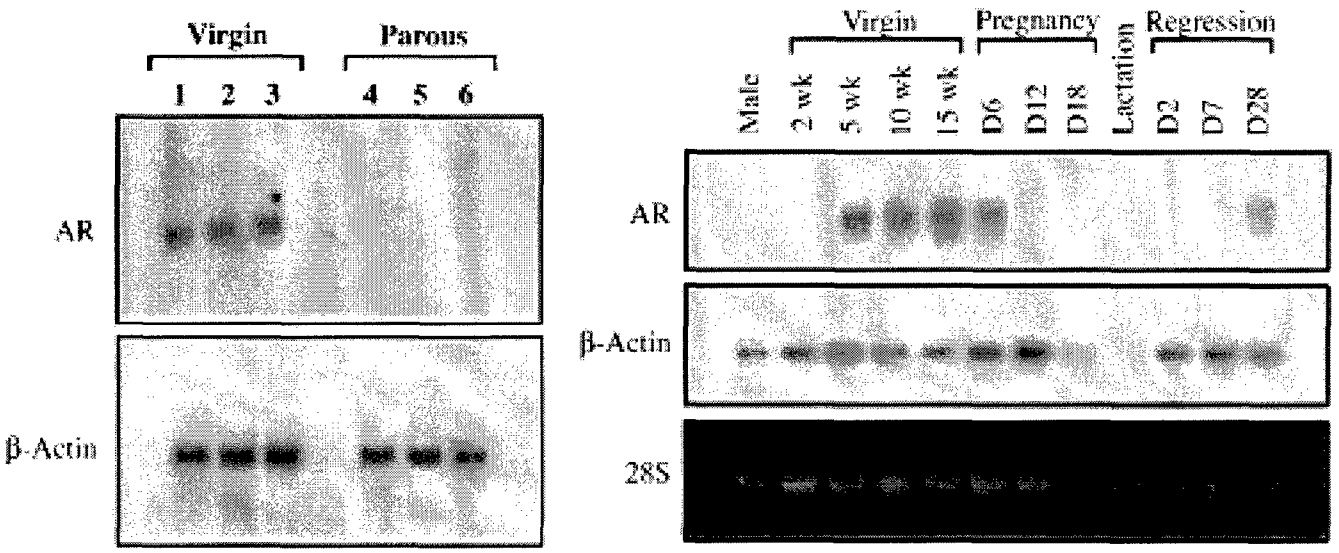

Fig. 4: Down-regulation of amphiregulin expression as a function of parity

suppressed during lactation and postlactational regression. The developmental expression patterns of the other genes identified in this screen are currently being examined.

Task 2: *Months 6-24: Determine the spatial pattern of expression of cDNA markers as a function of reproductive endocrine history.*

The parity-specific expression of a given gene could reflect a global increase in its expression in all expressing cells, an increase in the percentage of expressing cells in the breast, or both. In order to distinguish between these possibilities, we are performing in situ hybridization for those genes that have been identified as being differentially expressed as a function of parity. As an example, the spatial

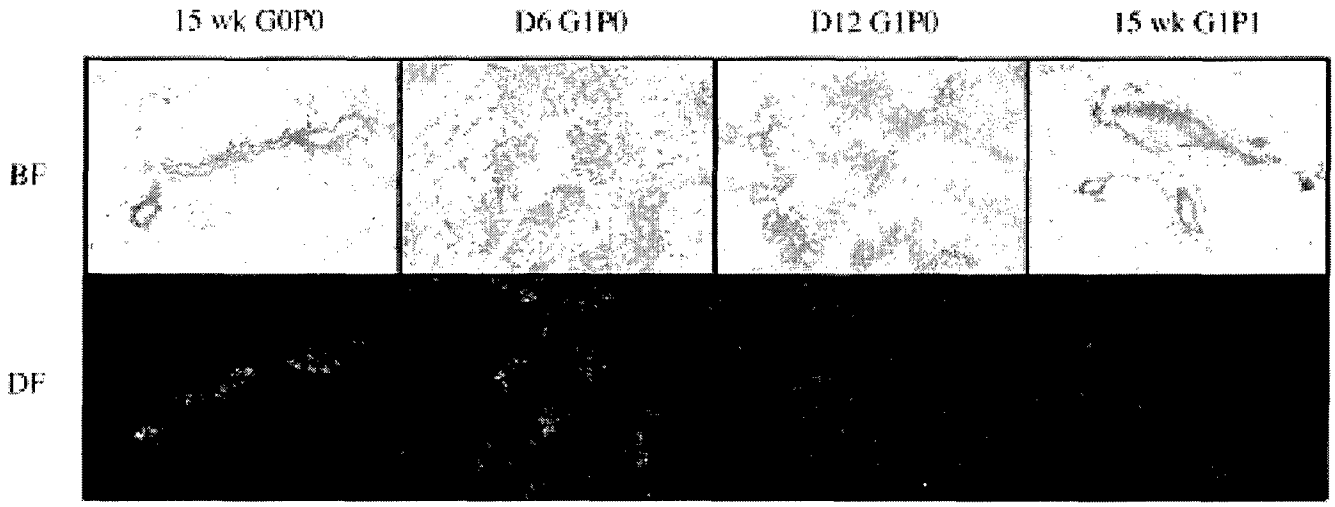

Fig. 5: Spatial expression of amphiregulin during development

expression of amphiregulin is shown during development (Fig. 5). This analysis demonstrates that the expression of amphiregulin decreases in each expressing cell, and also that the number of amphiregulin-expressing cells decreases as a result of parity. Similar approaches are now being applied to the other differentially expressed genes isolated in this panel.

Task 3: *Months 6-24: Determine the relationship between the expression of cDNA markers and reproductive endocrine variables known to affect breast cancer risk.*

We will determine how specific parity-related reproductive variables alter the expression of selected genes in the murine breast that we have identified as being differentially expressed as a function of parity. Biomarker expression levels will be quantitated in parous animals and relevant age-matched controls by Northern hybridization or RNase protection, as indicated based on prior studies with that biomarker. Animals are currently being generated that will permit the assessment of the impact of time of first full-term pregnancy, multiparity, duration of lactation, duration of postlactational regression and age, on the expression of these genes. Northern hybridization analyses using these mammary gland RNA samples will be performed during the next budget period.

KEY RESEARCH ACCOMPLISHMENTS

- Identification of genes that are down-regulated as a function of parity.
- Identification of genes that are up-regulated as a function of parity.
- Successful application of oligonucleotide microarray technology to gene expression in whole organs.
- Validation of the overall approach proposed in this application.

REPORTABLE OUTCOMES

- We are currently preparing a manuscript for submission detailing our results to date.

CONCLUSIONS

A number of important milestones have been accomplished during the first year of this project. Our principal advance has been the identification of molecular changes that occur in the mammary gland as a consequence of parity. Such genetic changes have not previously been identified. The identification and use of intermediate molecular endpoints that accurately identify changes in the breast associated with changes in breast cancer risk will ultimately facilitate the development of chemopreventative regimens that mimic parity-induced protection against breast cancer. 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 endpoint biomarkers. Such biomarkers are essential both for understanding the molecular and cellular basis of parity-induced protection against breast cancer, and for the rational design and testing of hormonal chemoprevention regimens aimed at mimicking this naturally occurring protective event. We hypothesize that understanding the biological mechanisms by which reproductive history influences breast cancer susceptibility will require a thorough understanding of the cell types present in the breast and the manner in which hormones affect their normal programs of differentiation and development. The goal of this proposal is to develop the molecular tools that are required to understand these complex changes in the breast.

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