

AD \_\_\_\_\_

Award Number: DAMD17-00-1-0138

TITLE: Gene Targets of C/EBPB Involved in Mammary Gland  
Development and Breast Cancer

PRINCIPAL INVESTIGATOR: Sandra L. Grimm, Ph.D.  
Jeffrey Rosen

CONTRACTING ORGANIZATION: Baylor College of Medicine  
Houston, Texas 77030

REPORT DATE: August 2003

TYPE OF REPORT: Annual Summary

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

DISTRIBUTION STATEMENT: Approved for Public Release;  
Distribution Unlimited

The views, opinions and/or findings contained in this report are those of the author(s) and should not be construed as an official Department of the Army position, policy or decision unless so designated by other documentation.

20040311 024

# REPORT DOCUMENTATION PAGE

Form Approved  
OMB No. 074-0188

Public reporting burden for this collection of information is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and maintaining the data needed, and completing and reviewing this collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Davis Highway, Suite 1204, Arlington, VA 22202-4302, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503

<b>1. AGENCY USE ONLY</b> (Leave blank)	<b>2. REPORT DATE</b> August 2003	<b>3. REPORT TYPE AND DATES COVERED</b> Annual Summary (1 Jul 2000 - 31 Jul 2003)	
<b>4. TITLE AND SUBTITLE</b> Gene Targets of C/EBPβ Involved in Mammary Gland Development and Breast Cancer		<b>5. FUNDING NUMBERS</b> DAMD17-00-1-0138	
<b>6. AUTHOR(S)</b> Sandra L. Grimm, Ph.D Jeffrey M. Rosen		<b>8. PERFORMING ORGANIZATION REPORT NUMBER</b>	
<b>7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)</b> Baylor College of Medicine Houston, TX 77030  E-Mail: sgrimm@bcm.tmc.edu			
<b>9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)</b> U.S. Army Medical Research and Materiel Command Fort Detrick, Maryland 21702-5012		<b>10. SPONSORING / MONITORING AGENCY REPORT NUMBER</b>	
<b>11. SUPPLEMENTARY NOTES</b>  Original contains color plates: ALL DTIC reproductions will be in black and white			
<b>12a. DISTRIBUTION / AVAILABILITY STATEMENT</b> Approved for Public Release; Distribution Unlimited			<b>12b. DISTRIBUTION CODE</b>
<b>13. ABSTRACT (Maximum 200 Words)</b>  Targeted deletion of the bZIP transcription factor, C/EBPβ, was shown previously to result in aberrant ductal morphogenesis and decreased lobuloalveolar development, accompanied by an altered pattern of progesterone receptor (PR) expression. Similar changes in the level and pattern of prolactin receptor (PrIR) and estrogen receptor alpha (ERα) expression were also observed in C/EBPβ KO mice. PR patterning was also altered in PrIR KO mice, and in mammary tissue transplants from both PrIR KO and Stat5a/b-deficient mice, with concomitant defects in hormone-induced proliferation. Along with decreased BrdU-incorporation and decreased expression of Ki67 were increases in the cyclin-dependent kinase inhibitors p21 and p27. IGFBP-5, IGF-II, and IRS-1 all displayed altered patterns and levels of expression in C/EBPβ KO mice, suggestive of a change in the IGF signaling axis. Increased expression of markers of ductal epithelium, such as NKCC1, aquaporin 5 and keratin 8, were observed in C/EBPβ KO glands. In addition, SPRR2A, a marker of epidermal differentiation, and keratin 6 were misexpressed in the mammary epithelium of C/EBPβ KO mice. Together, these data suggest that C/EBPβ is a master regulator of mammary epithelial cell fate and that the correct spatial pattern of PR and PrIR expression is a critical determinant of hormone-regulated cell proliferation.			
<b>14. SUBJECT TERMS</b> C/EBP beta, progesterone receptor, mammary gland development, knockout mouse, progenitor cell markers			<b>15. NUMBER OF PAGES</b> 30
<b>17. SECURITY CLASSIFICATION OF REPORT</b> Unclassified			<b>16. PRICE CODE</b>
			<b>20. LIMITATION OF ABSTRACT</b> Unlimited
<b>18. SECURITY CLASSIFICATION OF THIS PAGE</b> Unclassified	<b>19. SECURITY CLASSIFICATION OF ABSTRACT</b> Unclassified		

## Table of Contents

Cover.....	1
SF 298 Report Documentation Page .....	2
Table of Contents .....	3
Introduction .....	4
Body.....	4
Key Research Accomplishments.....	8
Reportable Outcomes.....	10
Conclusions .....	11
References .....	11
Appendices:	
Appendix I: Current Contact information.....	13
Appendix II: Grimm et al (2002) Mol Endo 16: 2675-91.....	14

## • Introduction

The study of mouse knockout (KO) models has helped elucidate the *in vivo* function of many different genes involved in the various stages of mammary gland development. Previous analyses of C/EBP $\beta$  KO mice have demonstrated an important role for this transcription factor in ductal morphogenesis and lobuloalveolar development of the mammary gland, and suggested that detailed molecular studies of this mouse model might provide new insights into the mechanisms responsible for these processes. The correlation of increased PR expression coupled with an inhibition of proliferation provides a novel way of viewing PR signaling events. The identification of genes and signaling pathways involved in the normal mammary gland development may provide novel targets for the treatment of breast cancer.

## • Body

***Objective 1:*** Investigate the potential role of known genes that may mediate the paracrine action of PR required for alveolar proliferation using the C/EBP $\beta$ <sup>-/-</sup> mouse model.

- Task 1A**
- Isolate total RNA from virgin and E+P treated mammary glands from C/EBP $\beta$  wildtype and knockout mice
  - Construct riboprobe vectors
  - Perform RPA and Northern analyses

Riboprobe constructs were obtained or made to a number of candidate genes, including progesterone receptor (PR), prolactin receptor (PrIR), estrogen receptor alpha (ER $\alpha$ ), p27<sup>Kip1</sup>, and insulin receptor substrates (IRS) 1 and 2. RPA analyses using these probes were performed to determine the levels of mRNA expression in wildtype (WT) versus C/EBP $\beta$  KO mammary glands from virgin animals or those treated for 2 days with estrogen and progesterone (E+P). These experiments demonstrated increased expression of PR, PrIR, ER $\alpha$ , and p27 in the mammary glands of C/EBP $\beta$  KO mice, while the levels of IRS-1 and IRS-2 did not change. Northern blot analyses were also performed to validate the differential expression of PR and PrIR. As we learned more about the molecular changes in the mammary glands from C/EBP $\beta$  KO mice, the focus of our studies switched from mRNA expression to protein expression, especially the co-localization of these candidate molecules in the intact gland (Task 1C).

- Task 1B**
- Perform *in situ* hybridization on paraffin-embedded mammary gland sections, comparing WT and KO, using riboprobes from above

In collaboration with Teresa Wood at Penn State and Russell Hovey in Barbara Vonderhaar's lab at the NIH, we performed *in situ* hybridization experiments to analyze the expression pattern of IGFBP-5, IGF-II, prolactin receptor and progesterone receptor in sections of mammary glands from WT and C/EBP $\beta$  KO mice. The experiments from Task 1A showed an increase in total

PR and PrIR mRNA by RPA and Northern blot analyses, but could not tell us whether it was an increase in expression per cell or an increase in the number of cells expressing the gene. *In situ* hybridization experiments revealed the normal pattern of PR, PrIR, and IGF-II expression during development. In a 6-week, pre-pubertal gland, expression of these genes is uniform along the ducts in both WT and C/EBP $\beta$  KO. However, as the mice reach puberty and the levels of circulating ovarian hormones increase, expression of PR, PrIR, and IGF-II are downregulated and the expression pattern becomes punctate and non-uniform along the ducts in WT mice. In contrast, the expression of these genes remains uniform in glands from mature C/EBP $\beta$  KO mice. Therefore, it appears that more cells are expressing these genes, but there may also be more expression per cell. Finally, IGFBP-5 was identified by suppressive subtractive hybridization [SSH; (1)] to be downregulated in the C/EBP $\beta$  KO and *in situ* hybridization validated this finding. These results have now been published since the last annual report (2).

**Task 1C** • Obtain antibodies to candidate proteins and perform immunohistochemistry.

Our immunostaining experiments have been the most informative, allowing us to visualize the pattern of expression of candidate proteins along the ducts and establish co-localization or dissociation of certain proteins by double immunofluorescence. These studies have continued in the third year, resulting in new findings. In addition to the nearly three-fold increase in PR expression in C/EBP $\beta$  KO glands we have previously observed, staining for ER $\alpha$  showed a similar pattern and increase in expression. Expression of ER $\alpha$  did not co-localize with BrdU expression in either the WT or C/EBP $\beta$  KO, also similar to PR. Expression of the epithelial-specific keratin, K8, was also increased in C/EBP $\beta$  KO glands and co-localized with the steroid-receptor expressing cells. The sodium-potassium-chloride cotransporter NKCC1 has been identified as a marker of ductal epithelium (3) and was also found to have increased expression in the C/EBP $\beta$  KO. Aquaporin 5 (aqp5) is a small integral membrane protein expressed on the luminal surface of secretory epithelial cells which functions to facilitate water transport [reviewed in (4)]. Aqp5 is also a marker of ductal epithelial cells, whose expression is downregulated during pregnancy (5). Expression is punctate along the apical surface of wildtype ducts and uniformly expressed in the ducts from C/EBP $\beta$  KO mice. Previous experiments have shown a 10-fold decrease in BrdU incorporation in the C/EBP $\beta$  KO, and more recent staining for the proliferation marker Ki67 confirms the defect in proliferation. Along with the decrease in proliferation, increases in expression of the cyclin-dependent kinase inhibitors (CKIs) p21 and p27<sup>Kip1</sup> have been observed in the ducts from C/EBP $\beta$  KO glands, which may be one mechanism to account for the block in proliferation.

Three distinct patterns of protein expression have emerged during our comparison of wildtype and C/EBP $\beta$  KO mammary glands. The first, where there is increased and more uniform expression in C/EBP $\beta$  KO glands, has been seen not only for PR and ER $\alpha$ , but also for PrIR and IGF-II (by *in situ* hybridization), the ion transporters NKCC1 and aquaporin 5, p21, p27, and keratin 8. It appears it is the failure to downregulate normal protein levels that leads to this apparent increase in expression. The second pattern observed is the aberrant

expression of a protein in C/EBP $\beta$  KO glands that is not normally expressed during that stage of mammary gland development. Two examples of this pattern of staining are keratin 6, normally expressed earlier in the terminal end bud (TEB) structures, and small proline-rich protein 2A (SPRR2A), a marker of skin differentiation. The third pattern of expression demonstrates decreased levels of proteins in the C/EBP $\beta$  KO, such as IGFBP-5, IRS-1, IRS-2, Ki67, and BrdU incorporation.

It appears likely that the expression of these markers and the alterations in the normal patterns of protein expression may represent a block in the normal cell fate determination and development pathway as a consequence of the germline deletion of C/EBP $\beta$ . To further investigate this possibility, I analyzed both wildtype and C/EBP $\beta$  KO embryos at E15.5-E16.5 to determine the expression of various proteins in the developing mammary placodes. Some of the proteins that were analyzed by immunostaining include keratin 6, keratin 8, keratin 14, p63, C/EBP $\beta$ , androgen receptor, and tenascin C. Mammary buds from both genotypes developed normally and there was no difference in staining of these markers, which argues that the deletion of C/EBP $\beta$  affects a later lineage of mammary progenitor cells, namely the putative lobuloalveolar progenitors (6).

**Task 1D** • *Implant hormone-containing pellets in mammary glands of C/EBP $\beta$  -/- mice.*

**Task 1E** • *Mix C/EBP $\beta$  -/- and ROSA26 mammary epithelium, transplant into RAG1 -/- females. Impregnate engrafted mice and harvest mammary glands at timepoints during pregnancy.*

These aims were proposed to verify the hypothesis of a paracrine or juxtacrine mechanism of action where PR-positive cells influence the neighboring cells to induce proliferation. Similar experiments have been performed using PRKO, PrIR KO and Wnt-4 KO tissues (7-9). Considering the technical difficulties of this technique, the number of mice required, and the discoveries we have made in identifying molecular changes in the C/EBP $\beta$  KO mammary gland, we did not pursue these tasks. In light of our data supporting the hypothesis of a block in the progenitor cell lineage, we instead focused on earlier developmental events during embryonic mammary bud formation and ductal elongation during the first three weeks of mammary gland development, as well as comparing the pattern of protein expression in the ductal mammary epithelial cells between WT and C/EBP $\beta$  KO glands.

**Objective 2:** *Identify and characterize novel downstream gene targets differentially expressed in the C/EBP $\beta$ -/- mammary gland.*

**Task 2A** • *Screen KO tester and WT tester libraries for additional differentially expressed partial cDNA clones*  
• *Confirm change in gene expression of SSH-PCR clones by Northern*  
• *Construct normalized midpregnant mouse mammary gland cDNA library [Revised to virgin mammary gland cDNA library]*

Suppressive subtractive hybridization (SSH) was performed to identify genes that are preferentially expressed in either the WT or C/EBP $\beta$  KO mammary glands (1). We performed SSH using either virgin mammary glands or glands treated with E+P for 21 days. Genes more highly expressed in the WT are found in the KOsub library and genes with higher expression in the C/EBP $\beta$  KO are in the WTsub library. Our more recent findings that PR expression is changing early in mammary gland development have led us to focus more heavily on the virgin SSH libraries. Approximately 60 genes have been sequenced from the virgin WTsub and KOsub libraries. About half the genes were known and the other half were ESTs (expressed sequence tags) or unique genes. Half of the clones/ESTs were expressed in mammary tissues, which is a good indication that they will truly be differentially expressed in the C/EBP $\beta$  KO mammary gland. Two of the clones, WTsub F28 and WTsub F80, are homologous to ESTs and were confirmed by Northern blot analysis to be upregulated in the KO. Screening of a cDNA library for the full-length F28 gene has been performed (discussed below). Other examples of genes identified by this screen that have been investigated further are SPRR2A, IGFBP-5, NKCC1, and aquaporin 5, as described above.

Because of the focus on events in early mammary gland development, a virgin cDNA library was generated instead of a mid-pregnant mammary gland cDNA library. PolyA+ RNA was isolated from the mammary glands of mature virgin C57Bl/6 mice and 5  $\mu$ g was used to reverse transcribe into cDNA. Linkers were added to the ends of the cDNAs and after digestion with EcoRI and XhoI, the cDNAs were directionally cloned into the Zap Express vector from Stratagene. The cDNA library was then packaged into phage and plated for screening. Aliquots of the library were frozen at  $-80^{\circ}\text{C}$ .

- Task 2B**
- Screen cDNA library for full length cDNA clones
  - Sequence analysis of full length cDNAs

Based on the EST distribution and the confirmation of overexpression in the C/EBP $\beta$  KO gland by Northern blot analysis, WTsub clone F28 was selected to screen the virgin mammary gland cDNA library to isolate a full-length cDNA. Approximately  $9 \times 10^5$  cDNA clones were plated for the initial screen and after performing three rounds of screening 8 clones were isolated. There were three different size inserts ranging from 1.2 to 4.3 kilobases, and after sequencing it was found that all three inserts had a common 3' end. However, Northern blot analysis shows that clone F28 corresponds to a cDNA of approximately 8 Kilobases in size, so only a partial F28 cDNA was isolated.

The F28 clone was most closely related to a gene found in many species, including CRP-ductin in mouse (10), hensin in rabbit (11), DMBT1 in human (12), and ebnerin in rat (13). With the sequence of the mouse and human genomes available, a BLAST search using the 4.3 Kb of F28 sequence identified the genomic mouse sequence that matched the partial cDNA. The F28 gene is located on mouse chromosome 7 (band F4) and the human F28 gene is found on the syntenic region of human chromosome 10 (q25-26). Analysis of the genomic sequence using Genescan (<http://genes.mit.edu/GENSCAN.html>) predicts approximately 55 exons spanning 135 Kb, placing F28 50 Kb

downstream of the CRP-ductin gene. The partial cDNA was aligned to the genomic sequence using the SIM4 program (<http://biom3.univ-lyon1.fr/sim4.html>) to test the validity of the cDNA prediction with Genescan. The predicted cDNA of 8610 bp encodes an open reading frame (ORF) of 2699 amino acids (aa). Predominant characteristics of the predicted protein include a good Kozak consensus sequence surrounding the translation start site (14), a signal peptide at amino acid 24, 4.7% cysteine residues, and 17.6% serine/threonine residues that have the potential to be glycosylated. The Ser/Thr-rich region is concentrated to approximately 400 aa at the C-terminus. Domain mapping using SMART (Simple Modular Architecture Research Tool; <http://smart.embl-heidelberg.de>) identified twelve SRCR (scavenger receptor cysteine rich) domains (15), four CUB domains (16), one ZP (Zona pellucida) domain (17), but no transmembrane domain at the C-terminus. The conservation of the domain content and order between F28 and CRP-ductin/DMBT1/hensin implies that F28 will have a similar function to these known proteins. There was also 65% identity between first 500 bp of the 5' UTR of the mouse and human sequences, suggesting the promoter regions are conserved.

**Task 2C** • *Preliminary gene characterization*

Northern blot analysis was performed using RNA from various stages of mammary gland development (virgin, d10 pregnancy, d18 pregnancy, d3 lactation and d4 involution) and comparing WT and C/EBP $\beta$  KO RNA from untreated or 21d E+P-treated mammary glands. F28 message was only detected in the sample from virgin C/EBP $\beta$  KO mammary gland and was downregulated in response to chronic hormone treatment. A multiple tissue blot revealed F28 transcripts in heart, liver, skeletal muscle and brain from WT mice.

• **Key Research Accomplishments**

**First year:**

- Identification of a number of genes whose expression levels and patterns change in the C/EBP $\beta$  KO mammary gland using a combination of RPA, Northern blot, *in situ* hybridization, Western blot and immunostaining analyses.
- Progesterone receptor expression is increased and proliferation is decreased in two other mouse knockout models: Prolactin receptor KO and Stat5A/B double KO.
- Construction of virgin mammary gland cDNA library from C57Bl/6 mouse strain completed.
- Isolation and sequencing of partial cDNA for a novel gene (designated F28) has been completed. Domain mapping and exon boundary mapping are complete for the 3' end of the gene. 5' RACE underway to isolate the 5' end of gene.

### **Second year:**

- Altered expression and localization of IGF axis molecules (IGF-II, IRS-1, IGFBP-5) in mammary glands of C/EBP $\beta$  KO mice.
- Hormonally-regulated signaling pathways in the mammary glands of C/EBP $\beta$  KO mice are functional as shown by hormone-induced downregulation of PR and phosphorylation of Stat5.
- Increased expression of NKCC1, a marker of ductal epithelium, in C/EBP $\beta$  KO mammary glands.
- Increased expression of p27, a cell-cycle inhibitor, in C/EBP $\beta$  KO mammary glands correlates with a 10-fold decrease in proliferation.
- SPRR2A, a marker of epidermal differentiation, and keratin 6 are inappropriately expressed in the mammary glands of C/EBP $\beta$  KO mice, as determined by immunohistochemistry.
- Identification of a novel gene (designated F28) that is related to CRP-ductin/DMBT/hensin. Domain mapping and exon boundary mapping are complete for genomic sequence, predicted cDNA and putative ORF.

### **Third year:**

- Increased expression of keratin 8 in the ductal epithelium of C/EBP $\beta$  KO mice co-localizes with PR-positive, but not BrdU-positive, cells.
- Aquaporin 5 and ER $\alpha$  expression are increased in the C/EBP $\beta$  KO.
- Increased expression of cyclin-dependent kinase inhibitor proteins (p21 and p27) may account for defect in cell proliferation in C/EBP $\beta$  KO glands.
- Ki67 expression, like BrdU incorporation, is similarly decreased in C/EBP $\beta$  KO glands.
- No difference between embryonic mammary buds from WT or C/EBP $\beta$  KO embryos. Both develop normally and express the same levels of markers, such as keratin 6, keratin 14, androgen receptor, p63, keratin 8, and C/EBP $\beta$ .
- Preliminary gene characterization of clone F28 shows it is preferentially expressed in the C/EBP $\beta$  KO mammary gland and is down-regulated in response to estrogen and progesterone.

## •Reportable Outcomes

### Presentations/Abstracts:

- Gordon Conference on Mammary Gland Biology, Barga, Italy (May 2000)  
"C/EBP $\beta$  Controls Cell Fate Determination During Mammary Gland Development"  
**ORAL presentation**
- International Congress of Endocrinology Meeting, Sydney, Australia (Oct. 2000)  
"Mammary gland development and oncogenesis"
- Hormones and Cancer Meeting, Port Douglas, Australia (November 2000)  
"Mammary Gland Development and Breast Cancer: Insights from Transgenic and Knockout Mouse Models"
- Molecular Mechanisms of Apoptosis, Keystone, Colorado (January 2001)  
"Unique and redundant roles of Stats 5a and 5b in the proliferation and differentiation of mammary epithelial cells"
- Endocrine Society Meeting, Denver, Colorado (June 2001).  
"Using mouse knockout models to understand signal transduction pathways required for early mammary gland development"  
**Poster presentation**
- Gordon Conference on Mammary Gland Biology, Barga, Italy (April 2002)  
" Disruption of steroid receptor patterning correlates with a block in lobuloalveolar development in multiple mouse knockout models"  
**Poster presentation**
- Mouse Models of Cancer (AACR), Lake Buena Vista, Florida (February 2003)  
"Functional characterization of mammary stem cells in development and breast cancer"

### Manuscripts:

- **Signal Transducer and Activator Transcription 5 (Stat5) controls the specification of mammary alveolar epithelium.**

Keiko Miyoshi, Jonathan Shillingford, Gilbert H. Smith, Sandra L. Grimm, Kay-Uwe Wagner, Takami Oka, Jeffrey M. Rosen, Gertraud W. Robinson and Lothar Hennighausen.  
Journal of Cell Biology **155**: 531-542 (2001)

- **Jak2 is an essential tyrosine kinase involved in pregnancy-mediated development of mammary secretory epithelium.**

Jonathan Shillingford, Keiko Miyoshi, Gertraud W. Robinson, Sandra L. Grimm, Jeffrey M. Rosen, Hans Neubauer, Klaus Pfeffer, Lothar Hennighausen. *Molecular Endocrinology* **16**: 563-570 (2002)

- **Disruption of steroid receptor patterning correlates with a block in lobuloalveolar development in multiple mouse knockout models.**

Sandra L. Grimm, Tiffany N. Seagroves, Elena B. Kabotyanski, Russell C. Hovey, Barbara K. Vonderhaar, John P. Lydon, Keiko Miyoshi, Lothar Hennighausen, Christopher J. Ormandy, Adrian V. Lee, Malinda A. Stull, Teresa L. Wood, Jeffrey M. Rosen.

*Molecular Endocrinology* **16**:2675-2691 (2002)

- **Developmental and hormonal signals dramatically alter the localization and abundance of insulin receptor substrate proteins in the mammary gland.**

Lee AV, Zhang P, Ivanova M, Bonnette S, Oesterreich S, Rosen JM, Grimm SL, Hovey RC, Vonderhaar BK, Kahn CR, Torres D, George J, Mohsin S, Allred DC, and Hadsell DL.  
*Endocrinology* **144**(6): 2683-2694 (2003)

•**The role of C/EBP beta in mammary gland development and breast cancer.**

Sandra L. Grimm and Jeffrey M. Rosen

Journal of Mammary Gland Biology and Neoplasia, 2003, In Press.

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

We have used the work presented in this report to receive a competitive renewal of NIH grant CA16303. This funding was used to supplement costs associated with this project.

•**Conclusions**

By analyzing the early events in mammary gland development, we hope to pinpoint changes in gene expression that are responsible for, or markers of, the lack of lobuloalveolar development in the C/EBP $\beta$  KO mice. So far, we have observed changes in the expression of a number of genes that are already known to influence mammary gland development. However, the genes identified from SSH analysis may provide the greatest insight into this process. Preliminary results indicate that it may not be a block in lobuloalveolar development, per se, but an alteration in cell fate at an earlier stage that may be responsible for the phenotype observed. Support for this hypothesis is the observation of overexpression of SPRR2A, a marker of skin differentiation, and the inappropriate expression of keratin 6 in C/EBP $\beta$  KO mammary glands. The approach we are taking should be able to identify genes and signaling pathways involved in the normal development of the mammary gland, which may provide novel targets for the treatment of breast cancer. Some of the genes identified may also be markers of early mammary epithelial progenitors, such as keratin 6.

• **References**

1. **Diatchenko L, Lau YF, Campbell AP, et al.** 1996 Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. Proc Natl Acad Sci U S A 93:6025-30
2. **Grimm SL, Seagroves TN, Kabotyanski EB, et al.** 2002 Disruption of steroid and prolactin receptor patterning in the mammary gland correlates with a block in lobuloalveolar development. Mol Endocrinol 16:2675-91
3. **Shillingford JM, Miyoshi K, Flagella M, Shull GE, Hennighausen L** 2002 Mouse mammary epithelial cells express the Na-K-Cl cotransporter, NKCC1: characterization, localization, and involvement in ductal development and morphogenesis. Mol Endocrinol 16:1309-21
4. **Verkman AS, Mitra AK** 2000 Structure and function of aquaporin water channels. Am J Physiol Renal Physiol 278:F13-28
5. **Shillingford JM, Miyoshi K, Robinson GW, et al.** 2003 Proteotyping of mammary tissue from transgenic and gene knockout mice with immunohistochemical markers: a tool to define developmental lesions. J Histochem Cytochem 51:555-65

6. **Wagner KU, Boulanger CA, Henry MD, Sgagias M, Hennighausen L, Smith GH** 2002 An adjunct mammary epithelial cell population in parous females: its role in functional adaptation and tissue renewal. *Development* 129:1377-86
7. **Briskin C, Park S, Vass T, Lydon JP, O'Malley BW, Weinberg RA** 1998 A paracrine role for the epithelial progesterone receptor in mammary gland development. *Proc Natl Acad Sci U S A* 95:5076-81
8. **Briskin C, Kaur S, Chavarria TE, et al.** 1999 Prolactin controls mammary gland development via direct and indirect mechanisms. *Dev Biol* 210:96-106
9. **Briskin C, Heineman A, Chavarria T, et al.** 2000 Essential function of Wnt-4 in mammary gland development downstream of progesterone signaling. *Genes Dev* 14:650-4
10. **Matsushita F, Miyawaki A, Mikoshiba K** 2000 Vomeronasal/CRP-Ductin is strongly expressed in the glands associated with the mouse vomeronasal organ: identification and characterization of mouse vomeronasal ductin. *Biochem Biophys Res Commun* 268:275-81
11. **Takito J, Yan L, Ma J, et al.** 1999 Hensin, the polarity reversal protein, is encoded by DMBT1, a gene frequently deleted in malignant gliomas. *Am J Physiol* 277:F277-89
12. **Mollenhauer J, Wiemann S, Scheurlen W, et al.** 1997 DMBT1, a new member of the SRCR superfamily, on chromosome 10q25.3-26.1 is deleted in malignant brain tumours. *Nat Genet* 17:32-9
13. **Li XJ, Snyder SH** 1995 Molecular cloning of Ebnerin, a von Ebner's gland protein associated with taste buds. *J Biol Chem* 270:17674-9
14. **Kozak M** 1987 At least six nucleotides preceding the AUG initiator codon enhance translation in mammalian cells. *J Mol Biol* 196:947-50
15. **Resnick D, Pearson A, Krieger M** 1994 The SRCR superfamily: a family reminiscent of the Ig superfamily. *Trends Biochem Sci* 19:5-8
16. **Bork P, Beckmann G** 1993 The CUB domain. A widespread module in developmentally regulated proteins. *J Mol Biol* 231:539-45
17. **Bork P, Sander C** 1992 A large domain common to sperm receptors (Zp2 and Zp3) and TGF-beta type III receptor. *FEBS Lett* 300:237-40

## **Appendix I: Current contact information**

Sandra L. Grimm, Ph.D.

Department of Molecular and Cellular Biology  
Room M637  
Baylor College of Medicine  
One Baylor Plaza  
Houston, TX 77030-3498

Phone: 713-798-6211  
Fax: 713-798-8012  
Email: [sgrimm@bcm.tmc.edu](mailto:sgrimm@bcm.tmc.edu)

## Disruption of Steroid and Prolactin Receptor Patterning in the Mammary Gland Correlates with a Block in Lobuloalveolar Development

SANDRA L. GRIMM, TIFFANY N. SEAGROVES\*, ELENA B. KABOTYANSKI, RUSSELL C. HOVEY†, BARBARA K. VONDERHAAR, JOHN P. LYDON, KEIKO MIYOSHI‡, LOTHAR HENNIGHAUSEN, CHRISTOPHER J. ORMANDY, ADRIAN V. LEE, MALINDA A. STULL, TERESA L. WOOD, AND JEFFREY M. ROSEN

*Department of Molecular and Cellular Biology (S.L.G., T.N.S., E.B.K., J.P.L., J.M.R.) and Breast Center (A.V.L.), Department of Medicine, Baylor College of Medicine, Houston, Texas 77030; Molecular and Cellular Endocrinology Section (R.C.H., B.K.V.), Center for Cancer Research, National Cancer Institute, National Institutes of Health, and Laboratory of Genetics and Physiology (K.M., L.H.), National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892; Cancer Research Program (C.J.O.), Garvan Institute of Medical Research, Darlinghurst, Australia; and Department of Neuroscience and Anatomy (M.A.S., T.L.W.), Pennsylvania State University College of Medicine, Hershey, Pennsylvania 17033*

Targeted deletion of the bZIP transcription factor, CCAAT/enhancer binding protein- $\beta$  (C/EBP $\beta$ ), was shown previously to result in aberrant ductal morphogenesis and decreased lobuloalveolar development, accompanied by an altered pattern of progesterone receptor (PR) expression. Here, similar changes in the level and pattern of prolactin receptor (PrIR) expression were observed while screening for differentially expressed genes in C/EBP $\beta$ <sup>null</sup> mice. PR patterning was also altered in PrIR<sup>null</sup> mice, as well as in mammary tissue transplants from both PrIR<sup>null</sup> and signal transducer and activator of transcription (Stat) 5a/b-deficient mice, with concomitant defects in hormone-induced proliferation. Down-regulation of PR and activation of Stat5 phosphorylation were seen after estrogen and progesterone treatment in both C/EBP $\beta$ <sup>null</sup>

and wild-type mice, indicating that these signaling pathways were functional, despite the failure of steroid hormones to induce proliferation. IGF binding protein-5, IGF-II, and insulin receptor substrate-1 all displayed altered patterns and levels of expression in C/EBP $\beta$ <sup>null</sup> mice, suggestive of a change in the IGF signaling axis. In addition, small proline-rich protein (SPRR2A), a marker of epidermal differentiation, and keratin 6 were misexpressed in the mammary epithelium of C/EBP $\beta$ <sup>null</sup> mice. Together, these data suggest that C/EBP $\beta$  is a master regulator of mammary epithelial cell fate and that the correct spatial pattern of PR and PrIR expression is a critical determinant of hormone-regulated cell proliferation. (*Molecular Endocrinology* 16: 2675-2691, 2002)

**M**OUSE MAMMARY GLAND development occurs postnatally under the control of systemic hormones and local growth factors. Mice are born with a rudimentary ductal structure, and between 3 and 8 wk of age levels of systemic ovarian hormones increase during puberty resulting in the penetration of the ducts into the surrounding fat pad (1). During pregnancy, exposure to estrogen (E), progesterone (P), and the lactogenic hormones, prolactin (PrI) and placental lactogen, induces lobuloalveolar development. By the

end of pregnancy, the fat pad is completely filled with secretory epithelium.

Previous studies have determined that the CCAAT/enhancer binding protein- $\beta$  (C/EBP $\beta$ ) transcription factor is required for normal ductal morphogenesis and lobuloalveolar development during pregnancy (2, 3). The mammary glands of C/EBP $\beta$ <sup>null</sup> mice exhibit enlarged, cystic ducts with decreased side-branching and an inhibition of alveologenes in response to E + P. In addition, increased levels of progesterone receptor (PR) mRNA and protein were detected in the mammary epithelial cells (MECs) of C/EBP $\beta$ <sup>null</sup> mice, with an altered distribution of PR-expressing cells along the ducts. This alteration in PR expression correlated with a 10-fold decrease in proliferation induced by an acute, 2-d treatment with E + P (4). This was unexpected because progesterone acts a mitogen to stimulate proliferation of MECs (5), and PR in MECs has been demonstrated to be essential for lobuloalveolar development (6). Furthermore, transplantation experi-

Abbreviations: BrdU, Bromo-deoxyuridine; C/EBP $\beta$ , CCAAT/enhancer binding protein- $\beta$ ; DAPI, 4',6 diamidino-2-phenylindole; E, estrogen; ER, E receptor; IGFBP, IGF binding protein; IRS, insulin receptor substrate-1; ISH, *in situ* hybridization; Jak, Janus kinase; K6, K10, K14, or K18, keratin 6, 10, 14, or 18; MECs, mammary epithelial cells; NKCC1, sodium potassium chloride; P, progesterone; PR, progesterone receptor; PrI, prolactin; PrIR, PrI receptor; RPA, ribonuclease protection assay; SSH, suppression subtractive hybridization; SPRR2A, small proline-rich protein; Stat5, signal transducer and activator of transcription 5; TBS, Tris-buffered saline.

ments with chimeras containing PR<sup>nu11</sup> MECs tagged with lacZ along with wild-type MECs indicated that alveolar development in PR<sup>nu11</sup> cells occurred when the two cell types were in close proximity, thus demonstrating that PR acts in a paracrine fashion to stimulate proliferation of neighboring cells (7).

The study of gene-targeted mouse models has helped identify other systemic hormone and local growth factors and their cognate receptors required for mammary gland development (reviewed in Ref. 8). Deletion of many of these genes results in mammary gland phenotypes that exhibit defective lobuloalveolar development. For example, the PrI receptor (PrIR) is required for pregnancy-induced lobuloalveolar development. PrIR<sup>nu11</sup> mice exhibit a phenotype similar to that observed in the PR<sup>nu11</sup> mice (9). This is supported by evidence that PR can regulate PrIR expression (10, 11). The side-branching defect observed in PrIR<sup>nu11</sup> mammary glands can be rescued with P treatment, but lobuloalveolar development still did not occur (12).

Signal transducer and activator of transcription 5 (Stat5) is an essential component of the PrIR signal transduction pathway (13). Stat5a-deficient mice exhibit impaired lobuloalveolar development, which could be partially compensated by Stat5b after multiple pregnancies (14, 15). Stat5b, however, was not essential for lactation, and no significant mammary gland phenotype was observed in Stat5b-deficient mice (16). Because of this potential redundancy, a deletion of both Stat5 genes was generated (16). The mammary gland phenotype of these mice was more severe than in Stat5a-deficient mice, with a complete block of lobuloalveolar development and the absence of functional differentiation (17).

Although PR is required for lobuloalveolar development, E receptor  $\alpha$  (ER $\alpha$ ) is required at an earlier stage to induce ductal elongation (18). Mammary glands from ER $\alpha$ <sup>nu11</sup> mice have a rudimentary ductal tree that does not fill the fat pad, although it can undergo lobuloalveolar development in response to a pituitary isograft or E + P treatment (19). In separate studies of the normal mammary gland, it has been shown that PR and ER $\alpha$  are coexpressed in MECs approximately 96% of the time (20). These steroid receptor-positive cells are often located adjacent to proliferating cells but rarely colocalize (4, 20, 21). It is thought that proliferating MECs eventually give rise to more differentiated, steroid receptor-expressing MECs (20, 22).

In the current study, additional changes in gene expression were identified in the mammary glands of C/EBP $\beta$ <sup>nu11</sup> mice that provide further insight into the mechanisms by which systemic hormones and local growth factors regulate lobuloalveolar development. Alterations in the level and pattern of PrIR concomitant with the changes in PR were observed in C/EBP $\beta$ <sup>nu11</sup> mice. These studies suggest that the correct distribution of both PR and PrIR is required to facilitate hormone-induced proliferation, and that defects in PR expression and proliferation are common features among several mouse models in which impaired lobu-

loalveolar development is observed. Changes in IGF binding protein (IGFBP)-5, IGF-II, and insulin receptor substrate-1 (IRS-1) expression and alterations in their distribution also were observed in C/EBP $\beta$ <sup>nu11</sup> mice, suggesting a role of the IGF axis in the paracrine regulation of lobuloalveolar development. Finally, the inappropriate expression of an epidermal differentiation marker, small proline-rich protein (SPRR2A), and K6 suggests that the germline deletion of C/EBP $\beta$  not only disrupts the necessary hormone receptor patterning but also results in an alteration of MEC cell fate.

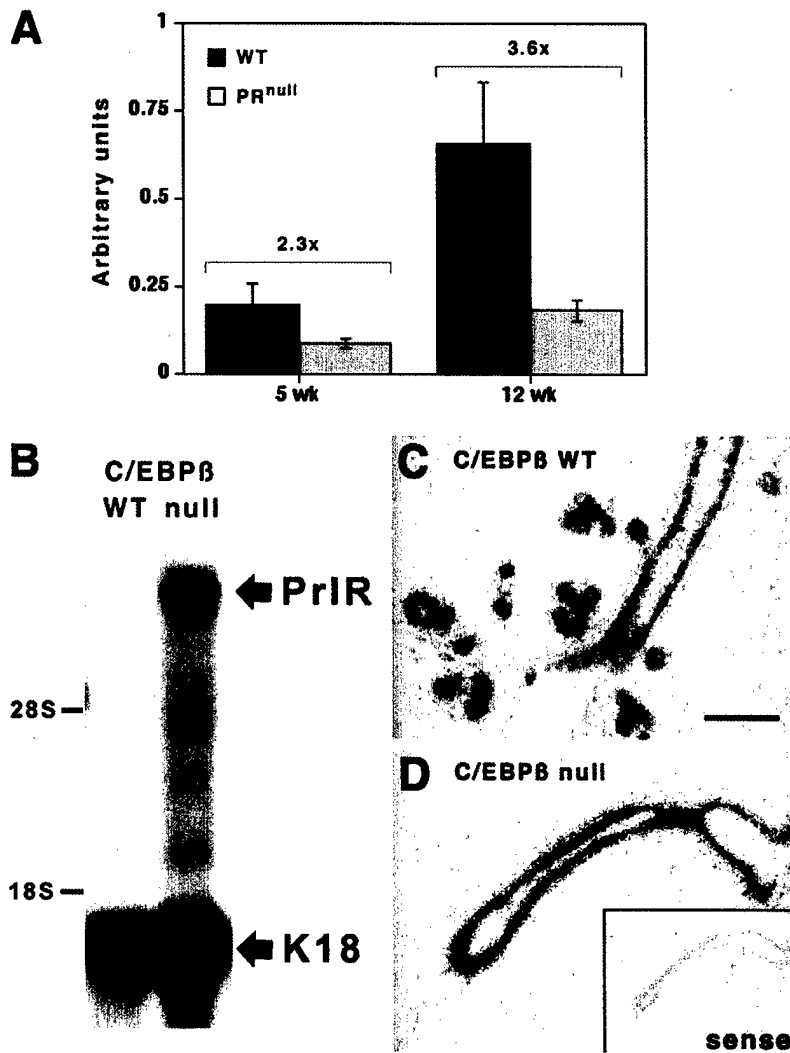
## RESULTS

### Coordinate Regulation of PrIR and PR Expression in C/EBP $\beta$ <sup>nu11</sup> Mice

To identify additional molecular changes in the mammary glands of C/EBP $\beta$ <sup>nu11</sup> mice that might indicate mechanisms by which systemic hormones and local growth factors regulate lobuloalveolar development, a candidate gene approach in which genes known to be important for lobuloalveolar development were examined. PrIR is a likely candidate gene because its expression has been shown to be regulated by progesterone (10, 11), and deletion of PrIR also results in a lack of lobuloalveolar development (9, 23).

Additional evidence that PrIR expression is mediated through activation of PR is shown in Fig. 1A. Semiquantitative RT-PCR analysis of the long form of PrIR was performed using mammary gland RNA from either wild-type or PR<sup>nu11</sup> mice at 5 or 12 wk of age. In the mammary glands of wild-type mice there was an approximate 3-fold increase in the amount of PrIR mRNA between 5 and 12 wk of age, coinciding with the increase in circulating ovarian hormones that occurs during this time (1). PR<sup>nu11</sup> mice expressed a much lower level of PrIR at 5 and 12 wk of age, suggesting that PrIR expression depends, at least in part, on PR. These data and previous *in situ* hybridization (ISH) studies suggest that PR and PrIR may be coregulated during early mammary gland development (24).

Accordingly, the levels of PrIR mRNA were examined in the mammary glands of C/EBP $\beta$ <sup>nu11</sup> and wild-type mice. Northern blot analysis was performed using poly(A) RNA isolated from mice treated acutely with E + P for 2 d to measure the level of PrIR mRNA (Fig. 1B). E, acting through ER $\alpha$ , is known to elevate serum PrI levels (25), and P, acting through PR, increases PrIR mRNA expression (10, 11). A marked increase in the amount of the long form of PrIR mRNA was detected in the mammary glands from C/EBP $\beta$ <sup>nu11</sup> mice relative to wild-type mice. Hybridization to a keratin 18 (K18) probe demonstrated both similar mRNA loading and levels of mammary epithelium, which was expected after only 2 d of E + P treatment. ISH was also performed to determine the cellular distribution of PrIR mRNA in C/EBP $\beta$ <sup>nu11</sup> mammary glands (Fig. 1, C and D). Signal was detected in a nonuniform, punctate



**Fig. 1.** Altered Expression of PrIR mRNA in Mammary Glands from PR<sup>null</sup> and C/EBP $\beta$ <sup>null</sup> Mice

Semiquantitative RT-PCR was performed to assess the levels of PrIR in wild-type and PR<sup>null</sup> mice (A). Mammary tissue from four to six mice for each age and genotype was pooled to isolate total RNA, which was then reverse transcribed and PCR amplified using primers for the long form of PrIR. PrIR levels were normalized to the amount of GAPDH mRNA expressed. Values are representative of three reverse transcription reactions with the *error bars* indicating the SEM. There was a significant difference between the wild-type and PR<sup>null</sup> samples at 12 wk ( $P < 0.05$ ). Northern blot analysis (B) using 2  $\mu$ g of poly(A) RNA per lane demonstrated an increase in the long form of PrIR mRNA in the C/EBP $\beta$ <sup>null</sup> mice after treatment with E + P for 2 d. Hybridization to a K18 cDNA probe was used as a control for loading and epithelial cell content. The positions of 18S and 28S rRNAs are indicated. Differences in the levels and cellular distribution of PrIR mRNA were shown by ISH using paraffin-embedded sections from mammary glands treated 2 d with E + P. There was a nonuniform, punctate pattern of expression in wild-type sections (C), but the levels increased and became more uniform in C/EBP $\beta$ <sup>null</sup> mice (D). The *inset* in panel D shows the nonspecific background observed with the control sense riboprobe (*bar*, 200  $\mu$ m).

pattern in MECs along the ducts in mammary glands from wild-type mice, similar to the pattern observed previously for PR (4, 24). However, PrIR mRNA levels were not only increased in C/EBP $\beta$ <sup>null</sup> mice, but the pattern of expression along the ducts was also more uniform, similar again to alterations observed previously for PR expression (4). Based on the results of the semiquantitative ISH, there was an increased level of

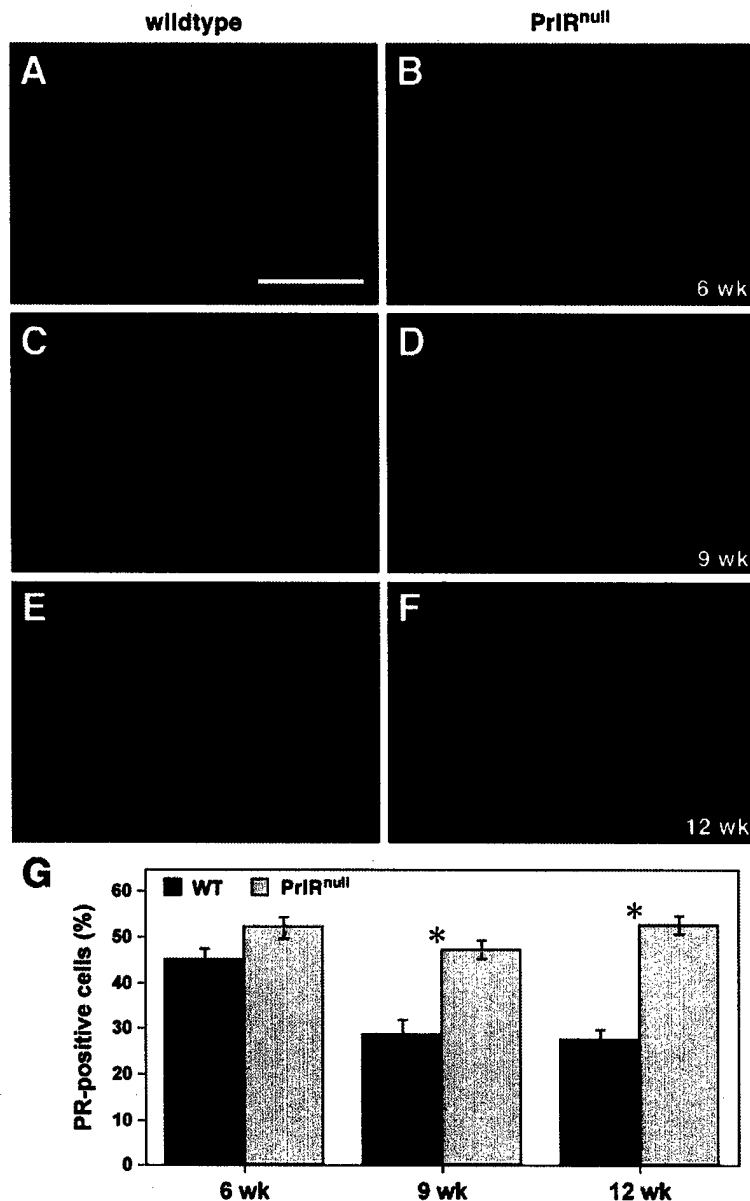
PrIR mRNA per cell in the C/EBP $\beta$ <sup>null</sup> gland, in addition to a higher percentage of cells expressing PrIR.

#### Increased PR and Decreased Proliferation in the Mammary Glands of PrIR<sup>null</sup> Mice

Because the levels and pattern of PrIR expression were altered in C/EBP $\beta$ <sup>null</sup> mice, and because PR and

PrIR appear to be coordinately regulated, the levels of PR were examined in the mammary glands from intact, untreated PrIR<sup>null</sup> animals. Between 6 and 9 wk of age, PR expression went from a uniform pattern to a heterogeneous, punctate pattern in the MECs of wild-type mice (Fig. 2, A and C), consistent with previous obser-

variations (4). By 12 wk, the percentage of PR-positive ductal cells had decreased to approximately 25% (Fig. 2, E and G). In contrast, PR expression was not down-regulated between 6 and 12 wk in PrIR<sup>null</sup> mice, retaining a level of approximately 50% positive MECs (Fig. 2, B, D, and F). The difference in the percent of



**Fig. 2.** Disrupted Pattern of PR Expression during Development of Mammary Glands in Nulliparous PrIR<sup>null</sup> Mice

Immunofluorescence staining for PR was performed on sections of untreated wild-type or PrIR<sup>null</sup> glands taken at 6 (A and B), 9 (C and D), and 12 (E and F) wk of age. PR expression was down-regulated in the wild-type between 6 and 9 wk, and appeared nonuniform by 12 wk (A, C, E). PR expression remained elevated in the PrIR<sup>null</sup> MECs during this time frame (B, D, F). The images were taken at  $\times 60$  magnification (*bar*, 50  $\mu$ m). Quantitation of PR-positive MECs is plotted in the *bar graph* (G), with the *error bars* showing the SEM. Statistically significant differences were observed between wild-type and PrIR<sup>null</sup> mice at 9 and 12 wk of age (indicated by the *asterisks*). Four animals from each age group and genotype were analyzed, and an average of 1400 nuclei were counted for each data set.

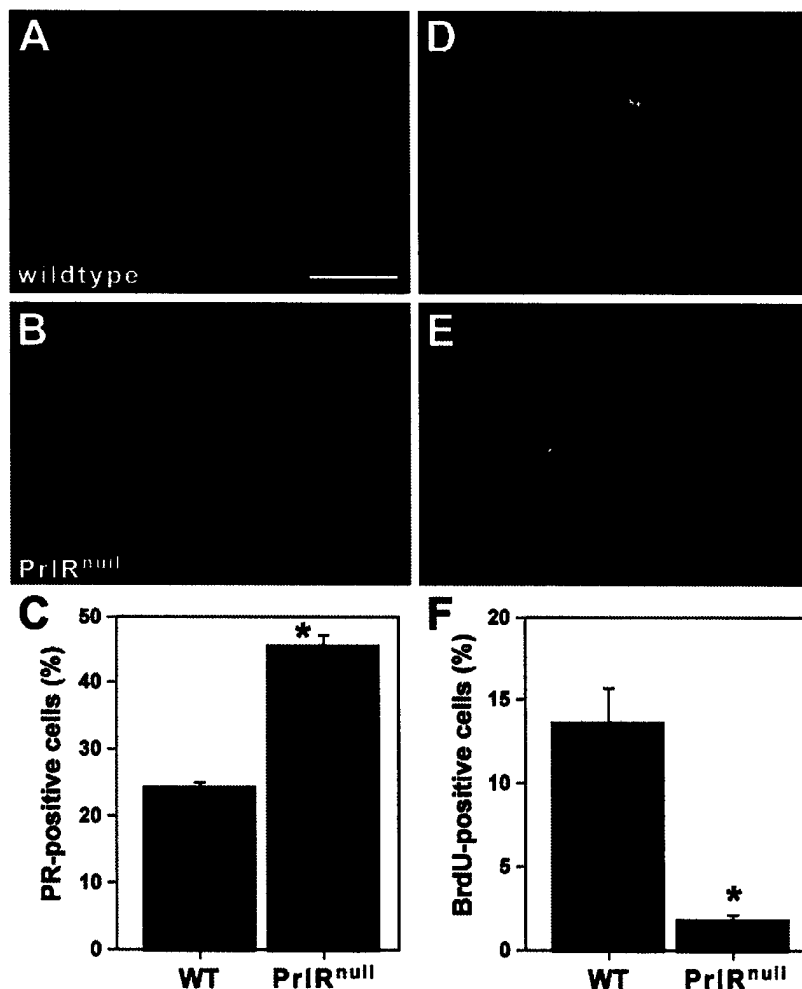
PR-positive MECs between the two genotypes was statistically significant at 9 and 12 wk of age (Fig. 2G).

Because an increase in PR expression was associated with a decrease in the number of proliferating MECs in mature C/EBP $\beta$ <sup>null</sup> animals after an acute E + P treatment (4), the level of proliferation was also examined in PrIR<sup>null</sup> mice. PR expression and bromodeoxyuridine (BrdU) incorporation were analyzed in mammary glands from 12-wk PrIR<sup>null</sup> animals that were treated for 2 d with E + P. As above, there was an increased percentage of PR-positive MECs in PrIR<sup>null</sup> mice (Fig. 3B) as compared with wild-type animals (Fig. 3, A and C). When the incorporation of

BrdU was measured, there was a significant decrease in the number of proliferating MECs in the PrIR<sup>null</sup> mice (Fig. 3, E and F). The finding that 13% of MECs were BrdU-positive in wild-type mice (Fig. 3, D and F) was consistent with previous results (4).

#### Transplanted PrIR<sup>null</sup> and Stat5ab-Deficient Mammary Tissue Also Exhibit Increased PR and Decreased Proliferation

Stat5 is a signaling molecule downstream of PrIR, and is important for lobuloalveolar development. Because Stat5ab-deficient and PrIR<sup>null</sup> mice are infertile and



**Fig. 3.** Increased PR Expression and Decreased Proliferation in the Ductal Epithelium of Mammary Glands from PrIR<sup>null</sup> Mice after 2 d of E + P

Immunofluorescence staining for PR (red) and BrdU (green) was performed on mammary gland sections from 12-wk-old wild-type or PrIR<sup>null</sup> mice after treatment for 2 d with E + P. PR expression was increased and proliferation was decreased in the PrIR<sup>null</sup> (B, E) compared with wild-type (A, D). Note the PR- and BrdU-positive cells rarely colocalized. The images were taken at  $\times 60$  magnification (bar, 50  $\mu$ m). Quantitation of PR- and BrdU-positive MECs is plotted in the bar graphs (C and F), with the error bars showing the SEM. Statistically significant differences were observed between wild-type and PrIR<sup>null</sup> for both PR and BrdU ( $P < 0.0001$ ; indicated by the asterisks). Four animals from each group were used and an average of 1400 nuclei were counted for each data set.

have deficiencies in circulating ovarian hormones (9, 16), mammary tissue from both genotypes was transplanted into the cleared fat pads of recipient nude mice. This approach permitted a direct determination of the effects of these gene deletions on MECs in the absence of any systemic effects, as well as a direct comparison of the PrIR<sup>null</sup> and Stat5ab-deficient MECs exposed to the same hormonal milieu in the same host animal. Nine to 10 wk after transplantation, host animals were treated for 2 d with E + P and the outgrowths collected. Analysis of PR expression revealed that the percent of PR-positive MECs in the endogenous no. 3 control gland was significantly higher than the value of 25% usually observed (Fig. 4A). When mammary gland tissue from wild-type mice was transplanted into nude mice and analyzed for PR expression, the percent PR-positive MECs closely resembled the endogenous gland shown in Fig. 4A (data not shown), suggesting that the lowered levels of circulating E + P in nude mice (26) accounted for the higher level of PR in the controls. Despite the higher basal level of PR expression in nude mice, both Stat5ab-deficient and PrIR<sup>null</sup> epithelium had increased levels of PR-positive MECs relative to the control (Fig. 4, B and C). There were statistically significant differences between the two gene-deleted outgrowths and the control, as well as between Stat5ab-deficient and PrIR<sup>null</sup> outgrowths (Fig. 4D). Proliferation in Stat5ab-deficient outgrowths was also lower relative to the control (Fig. 4, F vs. E), but not to the extent in PrIR<sup>null</sup> outgrowths (Fig. 4G). This difference is most likely because PrIR acts through multiple signaling pathways, including Janus kinase (Jak) 2, phosphatidylinositol-3 kinase, and MAPK, as discussed previously (17). In all cases, the amount of PR expressed was inversely proportional to the rate of proliferation.

#### Altered Expression and Localization of IGF Axis Molecules in Mammary Glands of C/EBP $\beta$ <sup>null</sup> Mice

Suppression subtractive hybridization (SSH) PCR was performed to identify differentially regulated genes from the mammary glands of mature, nulliparous C/EBP $\beta$ <sup>null</sup> mice (3–6 months old) as compared with wild-type mice (27). At this stage of development, differences in gene expression should reflect changes in ductal morphology in these mice, rather than alterations in the epithelial/stromal cell ratio. Several hundred clones were randomly chosen for high throughput reverse Southern blotting (WT-subtracted, 200 clones; C/EBP $\beta$ <sup>null</sup>-subtracted, 350 clones). To identify potentially differentially expressed genes, these clones were probed with enriched total cDNAs prepared from mammary glands of either wild-type or C/EBP $\beta$ <sup>null</sup> mice. The 60 clones identified by this method were then sequenced. A summary list of selected genes identified in this screen is published as

supplemental data on The Endocrine Society's Journals Online web site at <http://mend.endojournals.org>.

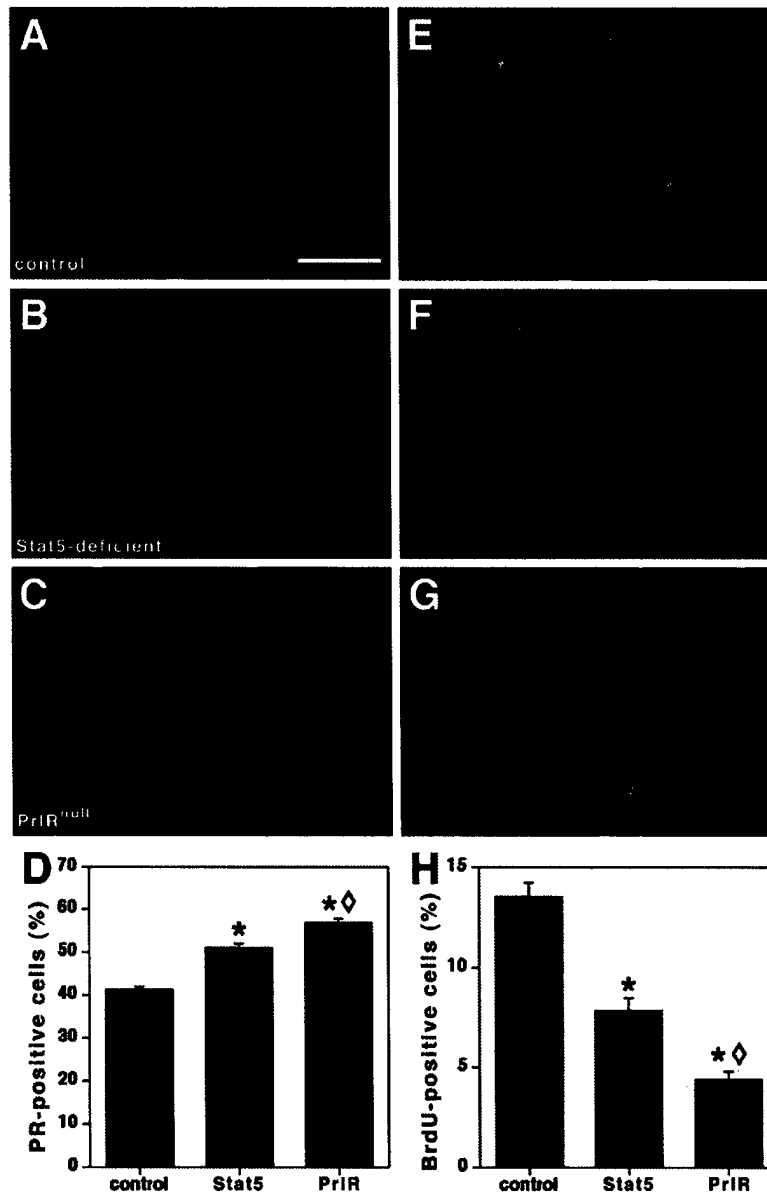
One of the genes identified was IGFBP-5. IGFBP-5 was also identified during a screen using the CLONTECH Laboratories, Inc. (Palo Alto, CA) Atlas array as a gene down-regulated in the mammary glands of C/EBP $\beta$ <sup>null</sup> mice (data not shown), and this observation was confirmed by Northern blot analysis of mammary gland mRNA from untreated C/EBP $\beta$ <sup>null</sup> mice (Fig. 5A). ISH was performed on frozen sections of mammary glands from untreated, nulliparous wild-type or C/EBP $\beta$ <sup>null</sup> mice to determine the cellular distribution of the IGFBP-5 mRNA (Fig. 5, B–E). Opposite to the pattern of PR and PrIR expression, IGFBP-5 mRNA changed from a relatively uniform pattern of expression in wild-type mammary glands to a nonuniform, punctate pattern in C/EBP $\beta$ <sup>null</sup> mice. This effect was more pronounced in the C/EBP $\beta$ <sup>null</sup> after treatment for 2 d with E + P (Fig. 5, C and E).

Because of the alteration in IGFBP-5 expression, we examined the expression of two other molecules in the IGF signaling axis: IGF-II and IRS-1. Frozen sections of mammary glands from untreated 6-wk or 12-wk animals or 2-d E + P-treated mature mice were analyzed for IGF-II expression by ISH (Fig. 5, F–K). At 6 wk, expression of IGF-II was uniform in the ductal epithelium from both wild-type and C/EBP $\beta$ <sup>null</sup> mice (Fig. 5, F and I). By 12 wk, the pattern of expression became punctate in the wild-type (Fig. 5G) but remained uniform in the ducts of C/EBP $\beta$ <sup>null</sup> mice (Fig. 5J), reminiscent of the change in patterning of PR and PrIR expression. The difference in IGF-II distribution became more pronounced in wild-type mice after 2 d of E + P treatment (Fig. 5H).

IRS-1 is a cytoplasmic signaling molecule downstream from the insulin and IGF-I receptors. No significant differences in IRS-1 mRNA levels were observed between untreated or 2-d E + P-treated wild-type and C/EBP $\beta$ <sup>null</sup> mice (Fig. 5L) as determined by ribonuclease protection assay (RPA). However, when the expression of IRS-1 protein was analyzed by Western blot, a 2- to 3-fold decrease between wild-type and C/EBP $\beta$ <sup>null</sup> mice was observed in both the untreated and 2-d E + P-treated animals (Fig. 5M). A change in the expression pattern of IRS-1 from uniform in the wild-type (Fig. 5N) to nonuniform in the ducts of C/EBP $\beta$ <sup>null</sup> mice (Fig. 5O) was observed by immunostaining and correlated with the results obtained by Western blotting.

#### Hormonally Regulated Signaling Pathways in the Mammary Glands of C/EBP $\beta$ <sup>null</sup> Mice Are Functional

Whereas the levels of PR and PrIR increased in the mammary glands of C/EBP $\beta$ <sup>null</sup> mice, it is not clear if the signaling pathways in these cells are functional. Turnover and down-regulation of PR protein results from prolonged exposure to P (28, 29). Therefore, wild-type and C/EBP $\beta$ <sup>null</sup> mice were implanted with

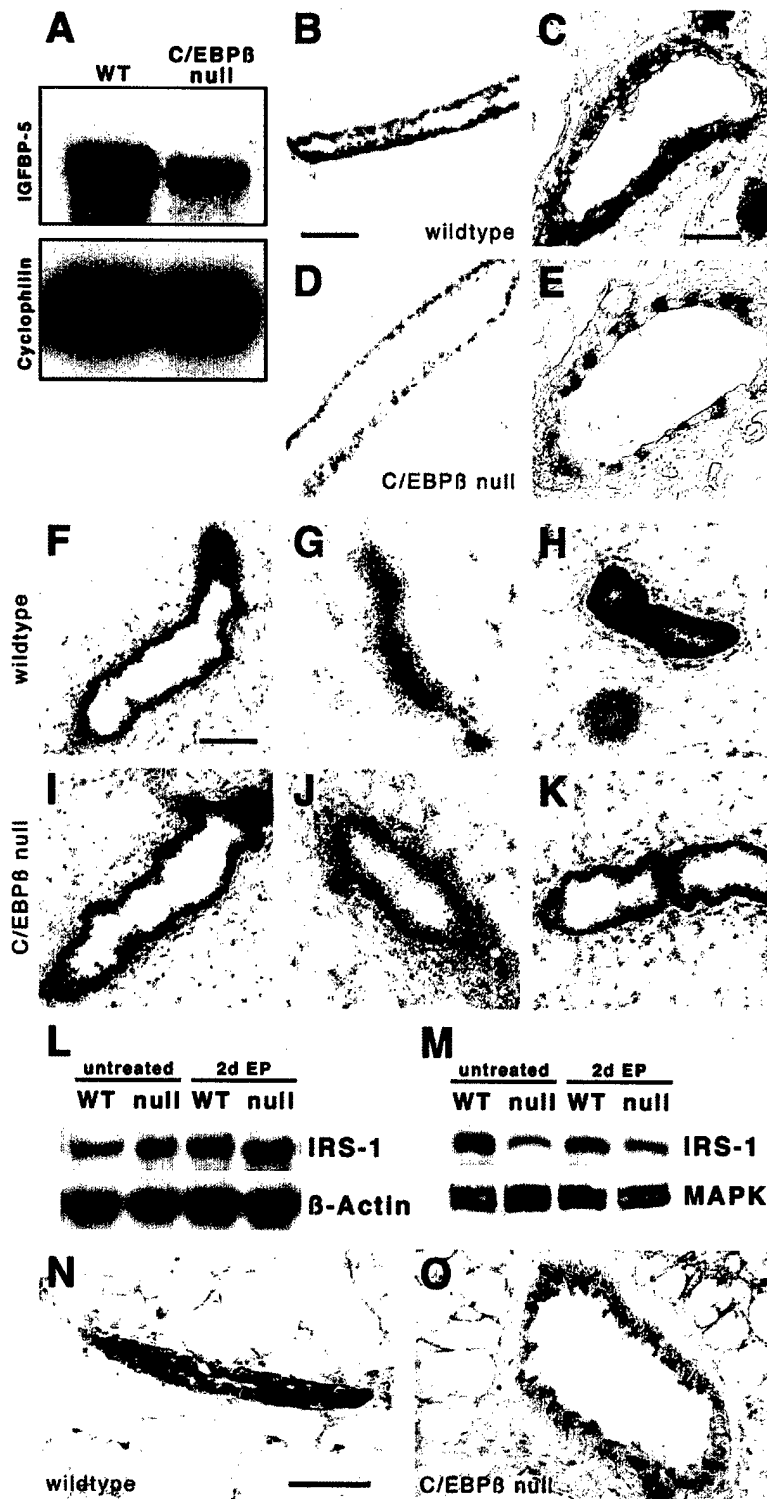


**Fig. 4.** Increased PR and Decreased Proliferation in Mammary Outgrowths from Stat5ab-Deficient and PrIR<sup>null</sup> Mammary Tissue Transplants

Nine to 10 wk after transplantation, host mice were treated for 2 d with E + P. Stat5ab-deficient and PrIR<sup>null</sup> transplants and the endogenous no. 3 mammary glands (control) were analyzed for PR staining (red) and BrdU incorporation (green). The nuclei were stained with DAPI (blue). PR expression was increased and proliferation was decreased in both the Stat5ab-deficient (B and F) and PrIR<sup>null</sup> (C and G) outgrowths, as compared with the control (A and E). The images were captured at  $\times 60$  magnification (bar, 50  $\mu$ m). The percentage of PR- and BrdU-positive MECs is plotted in the bar graphs (D and H), with the error bars showing the SEM. Statistically significant differences were observed between control and Stat5ab-deficient or PrIR<sup>null</sup> samples for both PR and BrdU (indicated by the asterisks). There were also statistically significant differences between Stat5ab-deficient and PrIR<sup>null</sup> samples (diamond symbols). A Student's *t* test was used to analyze the data ( $P < 0.0001$ ). Between 5 and 7 tissue samples were analyzed, and at least 6000 nuclei were counted for each genotype.

E + P pellets for 21 d to determine if PR levels could be down-regulated after chronic exposure to steroid hormones. Comparing the percentage of PR-positive MECs from untreated animals with those treated for

21 d with E + P showed a 2-fold decrease in the glands from wild-type mice (Fig. 6, A vs. B). Although the level of PR was almost three times greater in C/EBP $\beta$ <sup>null</sup> mice before treatment, the fold down-



**Fig. 5.** Disrupted Expression of IGF Axis Molecules in the Mammary Glands of *C/EBPβ*<sup>null</sup> Mice

Northern blot analysis (A) demonstrated a decrease in the amount of IGFBP-5 mRNA from the mammary glands of mature, untreated *C/EBPβ*<sup>null</sup> mice, as compared with wild type. Cyclophilin mRNA was used as a loading control. Nonradioactive ISH on frozen sections showed that IGFBP-5 mRNA localizes mainly in the epithelial cells. In untreated mice (B and D), the pattern



from glands from either untreated or hormone-treated wild-type mice. Interestingly, no Stat5 tyrosine phosphorylation was detected in mammary gland extracts from untreated C/EBP $\beta$ <sup>null</sup> mice, but phosphorylation was observed after acute hormone treatment, which may be due to estrogen-induced Prl expression (25).

#### **SPRR2A, a Marker of Epidermal Differentiation, and K6 Are Expressed in the Mammary Glands of C/EBP $\beta$ <sup>null</sup> Mice**

Surprisingly, another gene identified by the SSH-PCR screen was SPRR2A, a marker of epidermal differentiation that is normally expressed in the cornified layer of the skin (30). Northern blot analysis confirmed the differential expression of SPRR2A and demonstrated that the level of SPRR2A mRNA was markedly increased in the mammary glands from nulliparous C/EBP $\beta$ <sup>null</sup> mice (Fig. 7A). Immunohistochemistry was performed to determine the levels and pattern of SPRR2 protein expression in the mammary gland (Fig. 7B). There was no staining in wild-type glands but a punctate, nonuniform pattern of expression was observed in the MECs from C/EBP $\beta$ <sup>null</sup> glands, similar to the pattern seen for PR and PrIR. Although this staining was performed on tissue treated for 2 d with E + P, similar results were seen in tissues from untreated animals (data not shown).

This finding led us to investigate whether other epidermal markers, such as keratins, were expressed in the mammary glands of C/EBP $\beta$ <sup>null</sup> mice. K10 staining was negative in both wild-type and C/EBP $\beta$ <sup>null</sup> glands, but was positive on a section of newborn skin (data not shown). K6 is involved in wound healing and is associated with hyperproliferative diseases (31). Whereas K6 is expressed in the body cells of the terminal end buds in the pubertal mammary gland, it is rarely detected in ducts of the mature gland (32, 33). Consistent with these findings, we saw no K6 immunoreactivity in wild-type MECs (Fig. 7C). However, K6-positive MECs were readily apparent in ducts from mature C/EBP $\beta$ <sup>null</sup> mice. Whereas the immunoreactivity was more uniform than that observed for SPRR2A, not all MECs were K6-positive. In contrast, no K6-positive cells were identified in ducts from transplanted PrIR<sup>null</sup> or Stat5ab-deficient tissue (data not shown). K14 expression is normally detected in the myoepithelial cells surrounding the ducts, and no differences were detected in the staining patterns in the myoepithelium between wild-type and null mice (Fig. 7D).

## **DISCUSSION**

### **Correct PR and PrIR Patterning Is Required for Normal Lobuloalveolar Development**

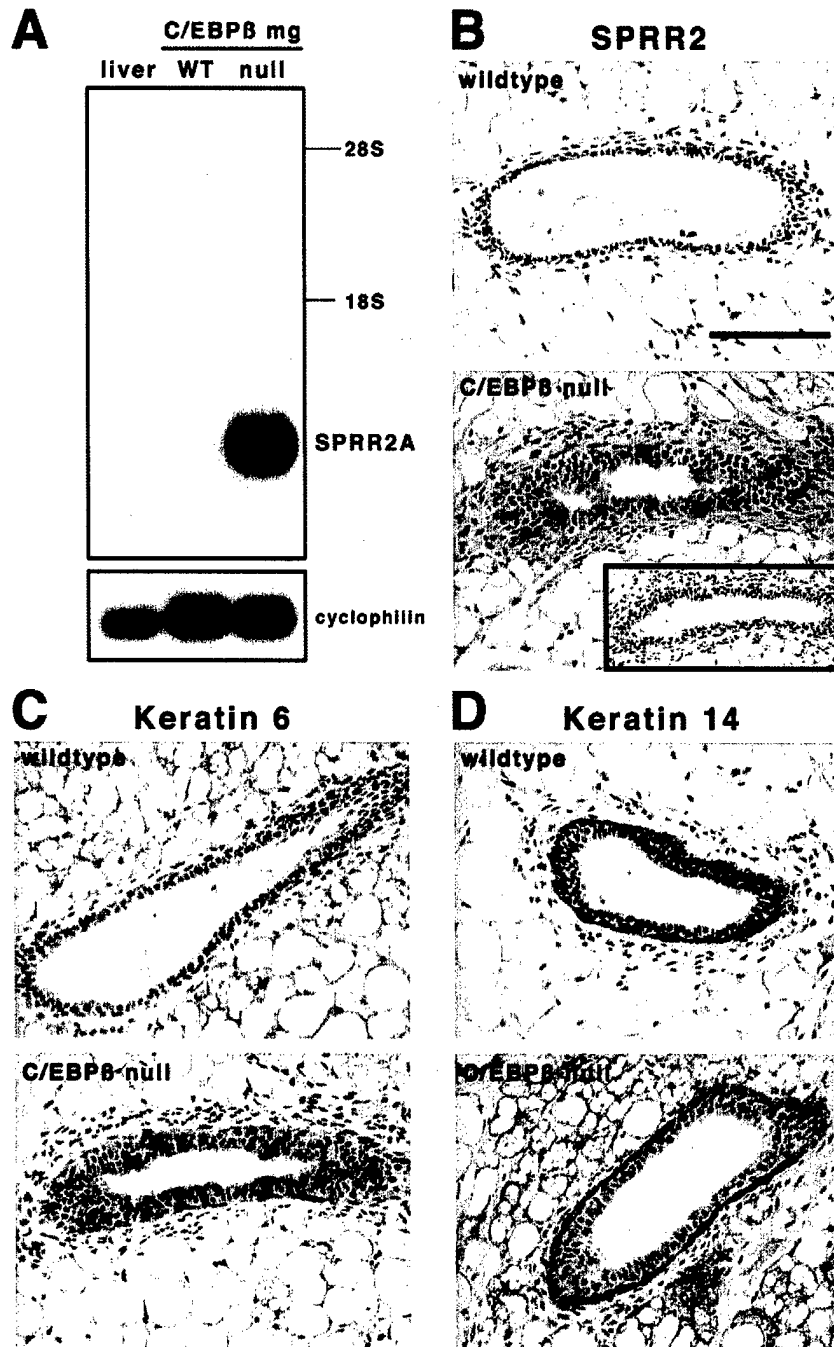
These studies illustrate the importance of establishing the correct pattern of PR and PrIR expression during ductal morphogenesis to facilitate the proliferative re-

sponse to steroid and lactogenic hormones during pregnancy. Disruption of PR and PrIR patterning and a concomitant decrease in proliferation was observed in mammary glands from several different gene targeted mouse models, all of which display defects in lobuloalveolar development. Although it has not yet been definitively established that PR and PrIR are expressed in the same cells, ISH experiments using serial sections have demonstrated a very similar pattern of expression for both mRNAs (24). In the current experiments, the level of PrIR mRNA was substantially reduced in the PR<sup>null</sup> mice, particularly at 12 wk of age when PrIR expression is normally increased in response to rising circulating ovarian hormone levels (1). PrIR mRNA is uniformly expressed in the MECs before puberty, and its expression becomes heterogeneous in the mature gland, similar to the pattern reported for PR during the same time period (24). However, in C/EBP $\beta$ <sup>null</sup> mice, PrIR mRNA remains uniformly expressed in mature mammary glands.

More recently, it has been possible to detect the pattern of PR expression in mice using  $\beta$ -galactosidase staining wherein lacZ has been inserted in place of the PR gene (34). The pattern of lacZ staining observed in mature nulliparous mice was also heterogeneous and was regulated by ovarian steroids during normal development. Furthermore, PR expression has even been observed in the mammary anlage as early as embryonic d 14 (34).

When proliferation rates were quantitated after acute E + P treatment, the Stat5ab-deficient and PrIR<sup>null</sup> outgrowths contained fewer BrdU-positive MECs than wild-type mice. However, even with fewer BrdU-positive MECs, the dissociation between PR expression and proliferation was maintained in these gene-targeted mouse models. It has been proposed that p27<sup>Kip1</sup> expression in steroid-receptor positive cells may block cell division (35). Our preliminary results show an increase in the number of p27 positive cells along the ducts of the C/EBP $\beta$ <sup>null</sup> glands (Grimm, S. L., and J. M. Rosen, unpublished observation), which correlates both with the increased PR expression and decreased proliferation.

Although the MECs from C/EBP $\beta$ <sup>null</sup>, PrIR<sup>null</sup> and Stat5ab-deficient mouse models all display a decreased proliferative response to steroid hormones, the inability of these cells to proliferate is probably not due to defective signaling pathways. The down-regulation of PR protein after prolonged exposure to P is mediated through serine phosphorylation by MAPK, resulting in the ubiquitin-mediated degradation of PR (29). Even though C/EBP $\beta$ <sup>null</sup> mice contain approximately three times the number of PR-positive MECs, chronic exposure to E + P resulted in a 2-fold down-regulation of PR in both wild-type and C/EBP $\beta$ <sup>null</sup> mice. These data rule out the possibility that defects in this pathway account for the increased level of PR in the C/EBP $\beta$ <sup>null</sup> mammary gland. The PrIR/Stat5 pathway also appears to be functional in C/EBP $\beta$ <sup>null</sup> mice. There was no detectable tyrosine phosphorylation of



**Fig. 7.** Expression of SPRR2A, an Epidermal Differentiation Marker, and K6 in the Mammary Glands from C/EBP $\beta$ <sup>null</sup> Mice

Northern blot analysis (A) demonstrated a substantial increase in the amount of SPRR2A mRNA in mammary glands from untreated C/EBP $\beta$ <sup>null</sup> compared with wild-type mice. Cyclophilin mRNA was used as a loading control. Staining for SPRR2 (B) and K6 (C) by immunohistochemistry was observed in C/EBP $\beta$ <sup>null</sup> sections treated for 2 d with E + P, but not in wild-type sections. The *inset* in panel B shows a control where no primary antibody was added. K14 immunostaining (D) of the myoepithelium surrounding the ducts was as expected for both wild-type and null tissues. Images were taken at  $\times 40$  magnification (bar, 100  $\mu$ m).

Stat5 observed in the mammary glands of untreated C/EBP $\beta$ <sup>null</sup> mice, most likely due to defective ovarian function in the C/EBP $\beta$ <sup>null</sup> mice (36). However, Stat5

protein was tyrosine phosphorylated after acute hormone treatment, most likely as a consequence of E-induced Prl production (25). However, chronic hor-

more treatment, which can activate Stat5, was not sufficient to rescue lobuloalveolar development, suggesting that other downstream responses to the Jak/Stat pathway might be altered in *C/EBP $\beta$ <sup>null</sup>* mice.

These observations have led to the development of a testable autoregulatory model depicted in Fig. 8. During embryonic development, epithelial/mesenchymal interactions required for the development of the mammary anlage may lead to the expression of PR. PR expression then results in the induction of PrIR; whether this occurs via a direct transcriptional mechanism or via an indirect mechanism remains to be determined. PrIR may then act through the Jak/Stat pathway to help regulate the level of ER $\alpha$  expression. This is consistent with the observation that Prl can activate ER $\alpha$  transcription in corpus luteum via Stat5a or 5b (37). Whether Prl directly activates ER $\alpha$  transcription in MECs is not known. Additionally, PrIR may feed back to control PR expression. Finally, ER $\alpha$  may then regulate the level of PR gene transcription both via indirect interactions with other transcription factors, such as SP1, as well as binding to ER $\alpha$  response element half-sites (38, 39). Although this model helps explain the coregulation of ER $\alpha$ , PR, and PrIR observed in MECs in mature, nulliparous mice, the mechanism by which the nonuniform pattern of receptor expression is established in response to E and P remains to be determined. *C/EBP $\beta$*  does not appear to

play a direct role in this autoregulatory loop; the level of *C/EBP $\beta$*  mRNA does not change in the *PrIR<sup>null</sup>* mammary gland (4), nor does it differ in transplanted *PrIR<sup>null</sup>* or *Stat5ab*-deficient tissue at parturition (17). Instead, *C/EBP $\beta$*  appears to act at a much earlier stage of development and may be required for the specification of mammary epithelial progenitors.

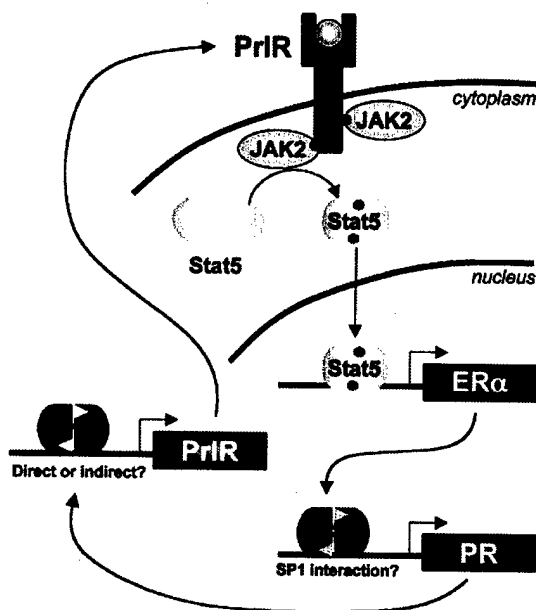
#### Altered Gene Expression in *C/EBP $\beta$ <sup>null</sup>* Mice

PR and PrIR were not the only genes whose levels and patterns of expression were altered in the ductal epithelium of *C/EBP $\beta$ <sup>null</sup>* mice. A decreased level of IGFBP-5 mRNA coupled with a shift to a nonuniform, punctate pattern of expression was observed in the mammary ducts of *C/EBP $\beta$ <sup>null</sup>* mice. These changes were exactly opposite from those observed for PR and PrIR. Little is known concerning the function of IGFBP-5 during early mammary gland development, although this protein is expressed during this period (40). The down-regulation of IGFBP-5 in the mammary glands of *C/EBP $\beta$ <sup>null</sup>* mice was coincident with the decrease in proliferation observed, suggesting that IGFBP-5 might facilitate the proliferative response. This initially seemed counterintuitive because IGFBP-5 expression in the mammary gland has been mainly associated with increased apoptosis during involution. IGFBP-5 levels increase markedly at the onset of involution in response to the activation of Stat3 (41), and IGFBP-5 is thought to function to sequester IGF-I, which acts as a cell survival factor during lactation (42).

However, IGFBP-5 has been shown previously to augment the effects of IGF-I on the migration and proliferation of smooth muscle cells (43). Recent studies have also demonstrated a positive role for IGFBP-5 in *Xenopus* neural tube induction (44). In this system, it has been suggested that IGFBP-5 potentiates the activity of endogenous IGFs to facilitate signaling through the IGF receptor.

During lactation, Prl is thought to suppress IGFBP-5 expression possibly through the activation of Stat5 (42). The increase in PrIR levels observed in *C/EBP $\beta$ <sup>null</sup>* mice might, therefore, result in the decreased levels of IGFBP-5 mRNA expression. Changes in the level of IGFBP-5 expression may also reflect the deletion of *C/EBP $\beta$* , which may act as a direct regulator of IGFBP-5 transcription in the mammary gland, as it does in osteoblasts (45). Again, the mechanisms responsible for the observed changes in the spatial pattern of IGFBP-5 expression remain to be established.

Along with changes in IGFBP-5, the expression patterns of other molecules in the IGF axis were altered in *C/EBP $\beta$ <sup>null</sup>* mice. IGF-II patterning in wild-type mice was reminiscent of PR and PrIR, with a uniform pattern of expression detected initially at 6 wk that became punctate by 12 wk. However, the pattern of IGF-II mRNA expression remained uniform in *C/EBP $\beta$ <sup>null</sup>* mice throughout this period of development. IGF-II is known to be a mitogen in the mammary gland



**Fig. 8.** Testable Model of Autoregulation between PR, PrIR, and ER $\alpha$  Expression

Potential autoregulatory loop between PR, PrIR, and ER $\alpha$ . PR can up-regulate PrIR expression, and there may be feedback from PrIR back to PR. PRL-mediated activation of the Jak/Stat pathway, through phosphorylation of Stat5, may then activate ER $\alpha$  in the mammary gland, which in turn leads to increased PR expression.

(reviewed in Ref. 40) and may be a direct target of the PrI-induced Jak/Stat pathway (Ormandy, C., unpublished). Whereas the level of IGF-II mRNA was increased at 12 wk post partum in C/EBP $\beta$ <sup>null</sup> mice as compared with wild-type, the decreased expression of IGFBP-5 and IRS-1 may inhibit the paracrine effects of IGF-II in MECs, as well as IGF-I in the mammary stroma.

IRS-1 expression was also decreased in the mammary glands of C/EBP $\beta$ <sup>null</sup> mice. Again, the change in the pattern of IRS-1 expression resembled that observed for IGFBP-5 expression. The decrease in IRS-1 protein expression appears to be regulated at the post-transcriptional level, as reported previously during normal mammary gland development (46). However, this is probably not the result of ligand-mediated activation of IRS-1 (47), based on the marked inhibition of E + P-induced proliferation in the C/EBP $\beta$ <sup>null</sup> mice. With the availability of epitope-specific antibodies directed against the myriad of phosphotyrosines and phosphoserines in IRS-1, it may be possible to determine directly the mechanisms responsible for the different patterns of IRS-1 expression observed in the mammary gland.

Additional evidence for involvement of the IGF axis with PrIR signaling comes from comparing RNA isolated from transplanted mammary tissue from wild-type and PrIR<sup>null</sup> mice using Affymetrix microarrays. IGF-II expression was decreased at d 6 of pregnancy in the PrIR<sup>null</sup> transplants, with no change observed at d 2 or 4 of pregnancy (Ormandy, C., unpublished). Thus, it is conceivable that IGF-II may act as one of several local growth factors, including Wnt-4 (48, 49), receptor activator of nuclear factor  $\kappa$ B ligand (50), TGF $\alpha$ , and/or amphiregulin (51), all of which may be important mediators of the paracrine/juxtacrine action of steroid hormones and PrI on proliferation during lobuloalveolar development.

#### Altered Cell Fate in the Mammary Glands of C/EBP $\beta$ <sup>null</sup> Mice

These results suggest that the germline deletion of C/EBP $\beta$  may result in a change in cell fate preventing ductal epithelial progenitors from responding appropriately to hormone-regulated signal transduction pathways. In this regard, one of the genes identified in the SSH screen that is up-regulated in the mammary gland of C/EBP $\beta$ <sup>null</sup> mice is the sodium potassium chloride (NKCC1) cotransporter, which has been demonstrated previously to represent a marker of the ductal epithelium (17, 52, 53). Ductal morphogenesis is delayed in the mammary glands of NKCC1<sup>null</sup> mice, and this effect is MEC autonomous (53). Both PrIR<sup>null</sup> and Stat5ab-deficient mice fail to undergo lobuloalveolar development, perhaps because of the absence of lobuloalveolar progenitors (17, 54). The expression of NKCC1 is also retained in the ductal epithelium of these mice during pregnancy (53). Thus, it is conceivable that despite the presence of PR and PrIR, their

ability to activate the appropriate cellular response is due to a deficiency in the lobuloalveolar progenitors in C/EBP $\beta$ <sup>null</sup> mice.

Additional evidence for a change in cell fate in the mammary glands of C/EBP $\beta$ <sup>null</sup> mice is the misexpression of SPRR2A, a marker of epidermal differentiation. SPRR2A is a protein normally expressed in the cornified layer of the epidermis and is involved in skin barrier function (30). No SPRR2A expression was detected by Northern blot analysis of mRNA from wild-type mice, or by immunohistochemical staining in the ductal epithelium, but high levels of expression were observed in the MECs of C/EBP $\beta$ <sup>null</sup> mice in a nonuniform pattern. K6 expression was also observed in the MECs of C/EBP $\beta$ <sup>null</sup> glands but not in the wild-type MECs or in transplanted tissue from PrIR<sup>null</sup> or Stat5ab-deficient mice. The expression of K6 was more uniform than the expression of SPRR2A but was not detected in every cell. It has been proposed that K6/K14-positive MECs exist in mature mammary glands and may represent stem cells (32). Because K6 expression is normally observed in the body cells of terminal end buds (32, 33), this result suggests that deletion of C/EBP $\beta$  may prevent further differentiation of these ductal progenitors. It appears likely that the expression of these markers may represent a block in the normal cell fate determination and development pathway as a consequence of the germline deletion of C/EBP $\beta$ , which was not observed in the PrIR<sup>null</sup> or Stat5ab-deficient mice.

Overall, these studies have illustrated the importance of appropriate receptor patterning in normal mammary gland development and have helped provide support for the model by which steroid hormones and PrI regulate lobuloalveolar development via a paracrine/juxtacrine mechanism. Disruptions in the pattern of these receptors and diminished proliferative responses were observed after the targeted deletion of several receptors, downstream signaling molecules and transcription factors, suggesting that this is a required mechanism for alveolar development. Although there are common defects between these knockout models, in particular increased PR and decreased proliferation, there are additional alterations in C/EBP $\beta$ <sup>null</sup> mice that suggest an earlier role for this transcription factor in controlling MEC cell fate. The study of C/EBP $\beta$ <sup>null</sup> mice continues to provide useful insights into the steps governing lobuloalveolar development. However, additional experiments are required to understand the precise molecular mechanisms mediating this complex developmental process.

## MATERIALS AND METHODS

### Animals and Tissue Collection

C/EBP $\beta$ <sup>null</sup>, PR<sup>null</sup>, PrIR<sup>null</sup>, and Stat5ab-deficient mice have been described (2, 6, 9, 16). The genetic backgrounds of all the mice were as follows: C/EBP $\beta$  (C57BL/6  $\times$  129Sv x

MF-1), PR (C57BL/6 × 129Sv), PrIR (129OlaHsd × 129SvPas), and Stat5ab (C57BL/6). All animal experimentation was conducted in accord with accepted standards of humane animal care.

The C/EBP $\beta$  mice were genotyped by PCR, rather than by Southern blot analysis as described previously (55). The primers for genotyping span the insertion site of the Neo cassette in the 3' end of the C/EBP $\beta$  gene (55). This results in two different size products, either 367 bp for the wild-type allele or 1.3 kb for the mutant allele. Heterozygous animals will have both size bands. The forward primer was 5'-AGAA-GACGGTGGACAAGCTG-3' and the reverse primer was 5'-CTCGGTGCAGGTGCAGGT-3'. The 30- $\mu$ l PCR contained 1.25  $\mu$ M MgCl<sub>2</sub>, 0.2  $\mu$ M of each primer, and 1/20th vol of dimethylsulfoxide. Thirty cycles were performed using a denaturing step at 94 C for 30 sec, an annealing step at 55 C for 45 sec, and an extension time of 90 sec at 72 C.

Ovary-intact, nulliparous mice were treated for 48 h with a single intrascapular sc injection of 17 $\beta$ -estradiol benzoate (1  $\mu$ g) and P (1 mg) in 100  $\mu$ l of sesame oil (all from Sigma, St. Louis, MO). For chronic E + P treatment, mice between 18 and 22 wk of age were treated for 21 d with E + P pellets as previously described (2). The transplantation of Stat5ab-deficient and PrIR<sup>nu/nu</sup> mammary tissue has been described elsewhere (17). Nine to 10 wk after transplantation into athymic NCr-nu/nu mice, an acute treatment of E + P was given. Two days later, both of the transplanted no. 4 inguinal mammary glands (Stat5ab-deficient and PrIR<sup>nu/nu</sup>) and an endogenous no. 3 thoracic gland (control) were removed. Two hours before they were euthanized, all E + P-treated animals were injected ip with 0.3 mg BrdU per 10 g body weight (Amersham Pharmacia Biotech, Arlington Heights, IL). Tissues were fixed in 4% paraformaldehyde for 2 h at 4 C. Paraffin-embedded tissues were sectioned (5–7  $\mu$ m) onto Probe-On Plus charged slides (Fisher Scientific, Pittsburgh, PA). Alternatively, mammary gland tissues were flash frozen in liquid nitrogen and stored at –80 C before cryostat sectioning. Ten-micron frozen sections were collected from all glands, mounted onto Superfrost Plus microscope slides (Fisher Scientific) and stored at –80 C.

#### RNA Analyses

Details for PrIR semiquantitative RT-PCR and ISH, including RNA preparation, primers and probes, have been described previously (24).

For Northern blot analyses, total RNA was first isolated from frozen mammary gland tissue using RNazolB reagent (Tel-Test, Inc., Friendswood, TX). Poly(A) RNA isolation from total RNA was performed using the PolyATract I kit (Promega Corp., Madison, WI) according to manufacturer's instructions. Probes for PrIR-L, K18, SPRR2A, and IGFBP-5 were prepared by digesting the appropriate expression vectors, and the cyclophilin probe was purchased from Ambion, Inc. (Austin, TX). Inserts were labeled with  $\alpha$ <sup>32</sup>P-deoxy-ATP using the Prime-A-Gene kit (Promega Corp.). The blotting protocol was performed as previously described (4).

Radioactive ISH for IGF-II was performed on frozen sections as described previously (56). Nonradioactive ISHs were performed as for the radioactive ISH with the following modifications: the cRNA probe to IGFBP-5 was transcribed according to standard protocols (Roche Molecular Biochemicals, Indianapolis, IN) using a linearized mouse IGFBP-5 cDNA (57). Hybridizations were done overnight at 60 C with 400 ng/ml of digoxigenin-labeled IGFBP-5 cRNA probe. Sections were rinsed in Tris-buffered saline (TBS), blocked in 1 $\times$  blocking reagent (Roche) in TBS and incubated in anti-DIG AP (1:500; Roche) in blocking reagent for 1 h at 37 C. Sections were then rinsed in TBS and incubated in detection buffer (100 mM Tris; 100 mM NaCl; 50 mM MgCl<sub>2</sub>, pH 9.5) with levamisole (1.2 mg/ml) for 10 min. Sections were then incubated in nitro-blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate toluidine salt substrate system (Sigma) in

the dark for 30 min. Slides were rinsed with water for 10 min, dehydrated through ethanols and xylenes, and coverslipped in Permount mounting media (Fisher Scientific).

RPAs were performed as described in the manufacturer's protocol (Ambion, Inc.). Twenty micrograms of total RNA were incubated with 3 fmol of <sup>32</sup>P-uridine triphosphate-labeled antisense IRS-1 (46) or  $\beta$ -Actin (Ambion, Inc.) riboprobes overnight at 55 C. Samples were run in triplicate and signal intensity was quantitated by PhosphorImager analysis using ImageQuant software (both from Amersham Biosciences, San Diego, CA).

#### Immunostaining

Sections were deparaffinized in xylenes, then rehydrated through a graded ethanol series. Indirect immunofluorescence for PR and BrdU and IRS-1 immunohistochemistry were performed as previously described (4, 46). Immunohistochemical staining was performed without antigen retrieval and used 5% goat serum (Sigma) in PBS as blocking buffer. Sections were incubated with the following primary antibodies overnight at room temperature:  $\alpha$ -SPRR2 rabbit polyclonal antibody at 1:3000 (30),  $\alpha$ -K6 (no. 66) rabbit polyclonal antibody at 1:5000 (kindly provided by Dennis Roop, Baylor College of Medicine, Houston, TX), and  $\alpha$ -K14 rabbit polyclonal antibody at 1:10,000 (Covance, Richmond, CA). Immunoperoxidase staining was detected using the Vectastain Elite ABC kit and the diaminobenzidine substrate kit according to manufacturer's instructions (Vector Laboratories, Inc., Burlingame, CA).

#### Cell Counting and Statistical Analysis

Fluorescent images were digitally captured using an Olympus Corp. BX50 microscope connected to a Hamamatsu C5810 charge-coupled device. At least 6–16 individual 60 $\times$  microscopic fields per sample were captured using the appropriate fluorescein isothiocyanate, Texas Red, and 4',6-diamidino-2-phenylindole (DAPI) filters. The number of PR- and BrdU-positive MECs in a given field was expressed as a percentage of total number of DAPI-stained MECs. Statistical significance was determined by the Student's paired *t* test with all *P* values below 0.0001.

#### Immunoprecipitation and Western Blot Analyses

Frozen mammary gland tissues from three to four animals (ages 21–25 wk) for each genotype and treatment were pooled to prepare whole cell extracts as previously described (58). Immunoprecipitation assays were performed as previously described (58) using 1.5 mg of whole cell extract incubated with 800 ng of  $\alpha$ -Stat5 rabbit polyclonal antibody (N-20; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). Antiphospho-tyrosine antibody PY20 (BD Biosciences, San Diego, CA) was used to evaluate the phosphorylation status of Stat5. The blot was then stripped and reprobed with the  $\alpha$ -Stat5 rabbit polyclonal antibody used for the immunoprecipitation. IRS-1 protein was detected by Western blot analysis using 80  $\mu$ g of whole cell extract per lane and the same antibody as used for immunohistochemistry, diluted 1:1000. MAPK p42/44 protein was used a loading control (1:1000; Cell Signaling Technology, Beverly, MA). Chemiluminescence was performed using SuperSignal reagents from Pierce Chemical Co. (Rockford, IL).

#### SSH PCR

The PCR-select cDNA Subtraction kit (CLONTECH Laboratories, Inc.) was used according to the manufacturer's instructions to screen for genes differentially regulated in the

mammary glands of mature, untreated C/EBP $\beta$ <sup>null</sup> mice. Briefly, 2  $\mu$ g of poly(A) RNA from either wild-type or C/EBP $\beta$ <sup>null</sup> mammary glands (pooled tissue; 3–6 months old) were used to synthesize double-stranded cDNA. After subtraction and PCR amplification using nested primers, the cDNA inserts were cloned into pGEM-T Easy vectors (Promega Corp.), transformed into XL2-Blue ultracompetent cells (Stratagene, La Jolla, CA), and plated for blue/white color selection. Individual colonies ( $n = 672$ ) were picked randomly and the inserts were PCR-amplified and screened by reverse Southern blot analysis using probes made from either enriched wild-type or C/EBP $\beta$ <sup>null</sup> total cDNA. Approximately 60 clones showing detectable differences in expression levels by reverse Southern were then sequenced (59).

### Acknowledgments

We would like to thank Liz Hopkins and Maria Gonzalez-Rimbau for histology support and Shirley Small for animal handling support. Thanks to Dr. Darrell Hadsell for providing the IRS-1 riboprobe vector and to Dr. Dennis Roop, Maranke Koster, and Dr. Zhijian Zhou for helpful advice and the SPRR2 and keratin 6 antibodies.

Received July 9, 2002. Accepted September 16, 2002.

Address all correspondence and requests for reprints to: Jeffrey M. Rosen, Department of Molecular and Cellular Biology, Baylor College of Medicine, Houston, Texas 77030. E-mail: jrosen@bcm.tmc.edu.

S.L.G. and R.C.H. are supported by the U.S. Army Medical Research and Materiel Command (DAMD17-00-1-0138 and DAMD 17-99-19311). J.M.R. and A.V.L. are supported by National Cancer Institute Grants CA-16303 and CA-4118. C.J.O. is supported by the New South Wales State Cancer Council, National Health and Medical Research Council Australia, and Department of Defense Breast Cancer Research Program. J.P.L. received support from the NIH and the U.S. Army Medical Research and Materiel Command (CA-77530-01 and DAMD 17-01-1-0138).

\* Present address: Department of Biology, Molecular Biology Section, University of California San Diego, La Jolla, California 92093.

† Present address: Department of Animal Science, University of Vermont, Burlington, Vermont 05405.

‡ Present address: Department of Biochemistry, School of Dentistry, the University of Tokushima, Tokushima, Tokushima 770-8504, Japan.

### REFERENCES

- Daniel CW, Silberstein GB 1987 Postnatal development of the rodent mammary gland. In: Neville MC, Daniel CW, eds. The mammary gland: development, regulation, and function. New York: Plenum Press; 3–36
- Seagroves TN, Krnacik S, Raught B, Gay J, Burgess-Beusse B, Darlington GJ, Rosen JM 1998 C/EBP $\beta$ , but not C/EBP $\alpha$ , is essential for ductal morphogenesis, lobuloalveolar proliferation, and functional differentiation in the mouse mammary gland. *Genes Dev* 12:1917–1928
- Robinson GW, Johnson PF, Hennighausen L, Sterneck E 1998 The C/EBP $\beta$  transcription factor regulates epithelial cell proliferation and differentiation in the mammary gland. *Genes Dev* 12:1907–1916
- Seagroves TN, Lydon JP, Hovey RC, Vonderhaar BK, Rosen JM 2000 C/EBP $\beta$  (CCAAT/enhancer binding protein) controls cell fate determination during mammary gland development. *Mol Endocrinol* 14:359–368
- Haslam SZ 1988 Progesterone effects on deoxyribonucleic acid synthesis in normal mouse mammary glands. *Endocrinology* 122:464–470
- Lydon JP, DeMayo FJ, Funk CR, Mani SK, Hughes AR, Montgomery Jr CA, Shyamala G, Conneely OM, O'Malley BW 1995 Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. *Genes Dev* 9:2266–2278
- Brisken C, Park S, Vass T, Lydon JP, O'Malley BW, Weinberg RA 1998 A paracrine role for the epithelial progesterone receptor in mammary gland development. *Proc Natl Acad Sci USA* 95:5076–5081
- Hennighausen L, Robinson GW 2001 Signaling pathways in mammary gland development. *Dev Cell* 1:467–745
- Ormandy CJ, Camus A, Barra J, Damotte D, Lucas B, Buteau H, Ederly M, Brousse N, Babinet C, Binart N, Kelly PA 1997 Null mutation of the prolactin receptor gene produces multiple reproductive defects in the mouse. *Genes Dev* 11:167–178
- Ormandy CJ, Graham J, Kelly PA, Clarke CL, Sutherland RL 1992 The effect of progestins on prolactin receptor gene transcription in human breast cancer cells. *DNA Cell Biol* 11:721–726
- Ormandy CJ, Hall RE, Manning DL, Robertson JF, Blamey RW, Kelly PA, Nicholson RI, Sutherland RL 1997 Coexpression and cross-regulation of the prolactin receptor and sex steroid hormone receptors in breast cancer. *J Clin Endocrinol Metab* 82:3692–3699
- Binart N, Helloc C, Ormandy CJ, Barra J, Clement-Lacroix P, Baran N, Kelly PA 2000 Rescue of preimplantatory egg development and embryo implantation in prolactin receptor-deficient mice after progesterone administration. *Endocrinology* 141:2691–2697
- Watson CJ, Burdon TG 1996 Prolactin signal transduction mechanisms in the mammary gland: the role of the Jak/Stat pathway. *Rev Reprod* 1:1–5
- Liu X, Robinson GW, Wagner KU, Garrett L, Wynshaw-Boris A, Hennighausen L 1997 Stat5a is mandatory for adult mammary gland development and lactogenesis. *Genes Dev* 11:179–186
- Liu X, Gallego MI, Smith GH, Robinson GW, Hennighausen L 1998 Functional rescue of Stat5a-null mammary tissue through the activation of compensating signals including Stat5b. *Cell Growth Differ* 9:795–803
- Teglund S, McKay C, Schuetz E, van Deursen JM, Stravopodis D, Wang D, Brown M, Bodner S, Grosfeld G, Ihle JN 1998 Stat5a and Stat5b proteins have essential and nonessential, or redundant, roles in cytokine responses. *Cell* 93:841–850
- Miyoshi K, Shillingford JM, Smith GH, Grimm SL, Wagner KU, Oka T, Rosen JM, Robinson GW, Hennighausen L 2001 Signal transducer and activator of transcription (Stat) 5 controls the proliferation and differentiation of mammary alveolar epithelium. *J Cell Biol* 155:531–542
- Korach KS, Couse JF, Curtis SW, Washburn TF, Lindzey J, Kimbro KS, Eddy EM, Migliaccio S, Snedeker SM, Lubahn DB, Schomberg DW, Smith EP 1996 Estrogen receptor gene disruption: molecular characterization and experimental and clinical phenotypes. *Recent Prog Horm Res* 51:159–186; discussion 186–188
- Bocchinfuso WP, Lindzey JK, Hewitt SC, Clark JA, Myers PH, Cooper R, Korach KS 2000 Induction of mammary gland development in estrogen receptor- $\alpha$  knockout mice. *Endocrinology* 141:2982–2994
- Clarke RB, Howell A, Potten CS, Anderson E 1997 Dissociation between steroid receptor expression and cell proliferation in the human breast. *Cancer Res* 57:4987–4991
- Russo J, Ao X, Grill C, Russo IH 1999 Pattern of distribution of cells positive for estrogen receptor  $\alpha$  and progesterone receptor in relation to proliferating cells in the mammary gland. *Breast Cancer Res Treat* 53:217–227

22. Zeps N, Bentel JM, Papadimitriou JM, D'Antuono MF, Dawkins HJ 1998 Estrogen receptor-negative epithelial cells in mouse mammary gland development and growth. *Differentiation* 62:221-226
23. Brisken C, Kaur S, Chavarria TE, Binart N, Sutherland RL, Weinberg RA, Kelly PA, Ormandy CJ 1999 Prolactin controls mammary gland development via direct and indirect mechanisms. *Dev Biol* 210:96-106
24. Hovey RC, Trott JF, Ginsburg E, Goldhar A, Sasaki MM, Fountain SJ, Sundararajan K, Vonderhaar BK 2001 Transcriptional and spatiotemporal regulation of prolactin receptor mRNA and cooperativity with progesterone receptor function during ductal branch growth in the mammary gland. *Dev Dyn* 222:192-205
25. Maurer RA 1982 Estradiol regulates the transcription of the prolactin gene. *J Biol Chem* 257:2133-2136
26. Kopf-Maier P, Mboneko VF 1990 Anomalies in the hormonal status of athymic nude mice. *J Cancer Res Clin Oncol* 116:229-231
27. Diatchenko L, Lau YF, Campbell AP, Chenchik A, Moqadam F, Huang B, Lukyanov S, Lukyanov K, Gurskaya N, Sverdlov ED, Siebert PD 1996 Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes and libraries. *Proc Natl Acad Sci USA* 93:6025-6030
28. Nardulli AM, Katzenellenbogen BS 1988 Progesterone receptor regulation in T47D human breast cancer cells: analysis by density labeling of progesterone receptor synthesis and degradation and their modulation by progesterin. *Endocrinology* 122:1532-1540
29. Lange CA, Shen T, Horwitz KB 2000 Phosphorylation of human progesterone receptors at serine-294 by mitogen-activated protein kinase signals their degradation by the 26S proteasome. *Proc Natl Acad Sci USA* 97:1032-1037
30. Hohl D, de Viragh PA, Amiguet-Barras F, Gibbs S, Backendorf C, Huber M 1995 The small proline-rich proteins constitute a multigene family of differentially regulated cornified cell envelope precursor proteins. *J Invest Dermatol* 104:902-909
31. Wojcik SM, Bundman DS, Roop DR 2000 Delayed wound healing in keratin 6a knockout mice. *Mol Cell Biol* 20:5248-5255
32. Smith GH, Mehrel T, Roop DR 1990 Differential keratin gene expression in developing, differentiating, preneoplastic, and neoplastic mouse mammary epithelium. *Cell Growth Differ* 1:161-170
33. Sapino A, Macri L, Gugliotta P, Pacchioni D, Liu YJ, Medina D, Bussolati G 1993 Immunophenotypic properties and estrogen dependency of budding cell structures in the developing mouse mammary gland. *Differentiation* 55:13-18
34. Ismail PM, Li J, DeMayo FJ, O'Malley BW, Lydon JP 2002 A novel LacZ reporter mouse reveals complex regulation of the progesterone receptor promoter during mammary gland development. *Mol Endocrinol* 16:2475-2489
35. Clarke RB, Howell A, Potten CS, Anderson E 2000 p27(KIP1) expression indicates that steroid receptor-positive cells are a non-proliferating, differentiated subpopulation of the normal human breast epithelium. *Eur J Cancer* 36(Suppl 4):S28-S29
36. Sterneck E, Tessarollo L, Johnson PF 1997 An essential role for C/EBP $\beta$  in female reproduction. *Genes Dev* 11:2153-2162
37. Frasor J, Barkai U, Zhong L, Fazleabas AT, Gibori G 2001 PRL-induced ER $\alpha$  gene expression is mediated by Janus kinase 2 (Jak2) while signal transducer and activator of transcription 5b (Stat5b) phosphorylation involves Jak2 and a second tyrosine kinase. *Mol Endocrinol* 15:1941-1952
38. Nardulli AM, Greene GL, O'Malley BW, Katzenellenbogen BS 1988 Regulation of progesterone receptor messenger ribonucleic acid and protein levels in MCF-7 cells by estradiol: analysis of estrogen's effect on progesterone receptor synthesis and degradation. *Endocrinology* 122:935-944
39. Petz LN, Nardulli AM 2000 Sp1 binding sites and an estrogen response element half-site are involved in regulation of the human progesterone receptor A promoter. *Mol Endocrinol* 14:972-985
40. Wood TL, Richert MM, Stull MA, Allar MA 2000 The insulin-like growth factors (IGFs) and IGF binding proteins in postnatal development of murine mammary glands. *J Mammary Gland Biol Neoplasia* 5:31-42
41. Chapman RS, Lourenco PC, Tonner E, Flint DJ, Selbert S, Takeda K, Akira S, Clarke AR, Watson CJ 1999 Suppression of epithelial apoptosis and delayed mammary gland involution in mice with a conditional knockout of Stat3. *Genes Dev* 13:2604-2616
42. Tonner E, Barber MC, Travers MT, Logan A, Flint DJ 1997 Hormonal control of insulin-like growth factor-binding protein-5 production in the involuting mammary gland of the rat. *Endocrinology* 138:5101-5107
43. Nam T, Morales A, Clemmons D 2002 Vitronectin binding to IGF binding protein-5 (IGFBP-5) alters IGFBP-5 modulation of IGF-I actions. *Endocrinology* 143:30-36
44. Pera EM, Wessely O, Li SY, De Robertis EM 2001 Neural and head induction by insulin-like growth factor signals. *Dev Cell* 1:655-665
45. Ji C, Chen Y, Centrella M, McCarthy TL 1999 Activation of the insulin-like growth factor-binding protein-5 promoter in osteoblasts by cooperative E box, CCAAT enhancer-binding protein, and nuclear factor-1 deoxyribonucleic acid-binding sequences. *Endocrinology* 140:4564-4572
46. Hadsell DL, Alexeenko T, Klemmendis Y, Torres D, Lee AV 2001 Inability of overexpressed des(1-3)human insulin-like growth factor I (IGF-I) to inhibit forced mammary gland involution is associated with decreased expression of IGF signaling molecules. *Endocrinology* 142:1479-1488
47. Lee AV, Gooch JL, Oesterreich S, Guler RL, Yee D 2000 Insulin-like growth factor I-induced degradation of insulin receptor substrate 1 is mediated by the 26S proteasome and blocked by phosphatidylinositol 3'-kinase inhibition. *Mol Cell Biol* 20:1489-1496
48. Bradbury JM, Edwards PA, Niemeyer CC, Dale TC 1995 Wnt-4 expression induces a pregnancy-like growth pattern in reconstituted mammary glands in virgin mice. *Dev Biol* 170:553-563
49. Brisken C, Heineman A, Chavarria T, Elenbaas B, Tan J, Dey SK, McMahon JA, McMahon AP, Weinberg RA 2000 Essential function of Wnt-4 in mammary gland development downstream of progesterone signaling. *Genes Dev* 14:650-654
50. Fata JE, Kong YY, Li J, Sasaki T, Irie-Sasaki J, Moorehead RA, Elliott R, Scully S, Voura EB, Lacey DL, Boyle WJ, Khokha R, Penninger JM 2000 The osteoclast differentiation factor osteoprotegerin-ligand is essential for mammary gland development. *Cell* 103:41-50
51. Luetke NC, Qiu TH, Fenton SE, Troyer KL, Riedel RF, Chang A, Lee DC 1999 Targeted inactivation of the EGF and amphiregulin genes reveals distinct roles for EGF receptor ligands in mouse mammary gland development. *Development* 126:2739-2750
52. Shillingford JM, Miyoshi K, Robinson GW, Grimm SL, Rosen JM, Neubauer H, Pfeffer K, Hennighausen L 2002 Jak2 is an essential tyrosine kinase involved in pregnancy-mediated development of mammary secretory epithelium. *Mol Endocrinol* 16:563-570
53. Shillingford JM, Miyoshi K, Flagella M, Shull GE, Hennighausen L 2002 Mouse mammary epithelial cells express the Na-K-Cl cotransporter, NKCC1: characterization, localization, and involvement in ductal development and morphogenesis. *Mol Endocrinol* 16:1309-1321

54. Wagner KU, Boulanger CA, Henry MD, Sgagias M, Hennighausen L, Smith GH 2002 An adjunct mammary epithelial cell population in parous females: its role in functional adaptation and tissue renewal. *Development* 129:1377–1386
55. Screpanti I, Romani L, Musiani P, Modesti A, Fattori E, Lazzaro D, Sellitto C, Scarpa S, Bellavia D, Lattanzio G, Bistoni F, Frati L, Cortese R, Gulino A, Ciliberto G, Costantini F, Poli V 1995 Lymphoproliferative disorder and imbalanced T-helper response in C/EBP  $\beta$ -deficient mice. *EMBO J* 14:1932–1941
56. Richert MM, Wood TL 1999 The insulin-like growth factors (IGF) and IGF type I receptor during postnatal growth of the murine mammary gland: sites of messenger ribonucleic acid expression and potential functions. *Endocrinology* 140:454–461
57. Green BN, Jones SB, Streck RD, Wood TL, Rotwein P, Pintar JE 1994 Distinct expression patterns of insulin-like growth factor binding proteins 2 and 5 during fetal and postnatal development. *Endocrinology* 134:954–962
58. Kazansky AV, Kabotyanski EB, Wyszomierski SL, Mancini MA, Rosen JM 1999 Differential effects of prolactin and src/abl kinases on the nuclear translocation of STAT5B and STAT5A. *J Biol Chem* 274:22484–22492
59. Ginger MR, Gonzalez-Rimbau MF, Gay JP, Rosen JM 2001 Persistent changes in gene expression induced by estrogen and progesterone in the rat mammary gland. *Mol Endocrinol* 15:1993–2009

---

### NEUROPEPTIDES 2003

Joint Meeting of the 13th Annual Meetings of the American Summer  
Neuropeptide Conference & the European Neuropeptide Club (ENC)

June 8–12, 2003

Montauk, NY, USA

#### Main Topics

1. Alzheimer's Disease
2. Storage and Secretion of Neuropeptides
3. Neuropeptides and Obesity
4. Drug Development in the Peptide Field
5. Neuropeptides and Anxiety
6. CGRP
7. Neuropeptides in the Pathogenesis and Control of Pain
8. Functional Genomics of Neuropeptides
9. Neuroendocrinology and Neuropeptides
10. Neuropeptides in the Gastrointestinal System
11. Biotechnology
12. Neuropeptides in Chronic Disease
13. Neuropeptides in Cognitive Functions
14. Mitogenic and Trophic Functions of Neuropeptides
15. Neuropeptides in Neuro-Immune Communication
16. Other

#### Meeting Chairs

Illana Gozes, Ph.D. (Israel)

Douglas E. Brenneman, Ph.D. (USA)

#### NEUROPEPTIDES 2003 Secretariat

c/o Unitours Ltd.

P.O. Box 3190, Tel Aviv 61031, Israel

Tel: 972-3-5209999

Fax: 972-3-5239299, 5239099

E-mail: meetings@unitours.co.il

#### IMPORTANT DATES

February 15, 2003

Deadline for Submission of Abstracts

March 2003

Notification of Acceptance of Abstracts

March 15, 2003

Deadline for Early Registration

June 8–12, 2003

NEUROPEPTIDES 2003