

Hormone Receptor Patterning Plays a Critical Role in Normal Lobuloalveolar Development and Breast Cancer Progression

Jeffrey M. Rosen

Department of Molecular & Cellular Biology, Baylor College of Medicine, 1 Baylor Plaza, Houston, TX 77030-4498, USA

Tel.: +1 713 798 6210; Fax: +1 713 798 8012; E-mail: jrosen@bcm.tmc.edu

URL: <http://public.bcm.tmc.edu/rosenlab/>

Abstract. Studies initially focused on understanding the hormonal regulation of milk protein gene expression have evolved using transgenic and knockout mouse models to help provide new insights into the mechanisms by which hormones regulate proliferation during normal mammary gland development, and how these regulatory mechanisms have deviated in breast cancer progression. During normal mammary development, a non-uniform pattern of expression of the estrogen receptor alpha, and the progesterone and the prolactin receptors in the ductal epithelium is established. All of these receptors apparently are co-expressed in non-proliferating ductal epithelial cells. Local growth factors, which are members of the Wnt, EGF and IGF families and Rank ligand, act as mediators of systemic steroid and lactogenic hormones to stimulate proliferation of adjacent ductal epithelial cells via a paracrine mechanism. During early breast cancer progression a more uniform pattern of steroid receptor expression is observed accompanied by an apparent switch to an autocrine mechanism regulating cell proliferation.

A BRIEF HISTORY

One of the key questions, which has driven the research in our laboratory over the past three decades, is “Why do the same hormones which promote growth and differentiation in the normal mammary gland result in aberrant growth in breast cancer?”. In order to understand the alterations which have occurred in breast cancer, it was apparent that we needed first to elucidate the mechanisms by which hormones regulated the growth and differentiation of the normal mammary gland. Twenty five years later a similar conclusion was echoed by the National Cancer Institute Program Review Group Summary Report entitled, “Charting the Course: Priorities for Breast Cancer Research” by Drs. Harold Moses and Nancy Davidson, which stated that “Our understanding of the biology and developmental genetics of the normal mammary gland is a barrier to progress. . . a more complete understanding of the normal mammary gland at each stage of development. . .

will be a critical underpinning of continued advances in detecting, preventing and treating breast cancer”.

At the time we initiated our studies on mammary gland development, one of the few *in vivo* systems in which to study the hormone-dependence of breast cancer was the dimethylbenz(a)anthracene (DMBA)-induced mammary tumor model in Sprague-Dawley rats [1]. Therefore, even before cDNA cloning was in vogue, we decided to synthesize cDNAs encoding the caseins and other major whey proteins from messenger RNA isolated from the mammary gland of lactating rats, as markers of hormone-induced differentiation [2]. With the application of recombinant DNA technology to the mammary gland it was possible to isolate both the individual cDNAs and subsequently the genes encoding the major milk proteins [3,4]. A comparison of the phylogenetically conserved regions in the putative casein gene promoters led to the identification of the binding sites for a number of transcription factors, although at the time the identity of these factors was un-

known [5]. Comparative genomic analysis is now the approach of choice for the identification of conserved regulatory elements, which may be critical for developmental or tissue-specific gene expression [6]. We have recently used this approach to analyze the entire casein gene locus in the human, mouse, rat and bovine genomes [7]. These studies have identified not only conserved transcription factor binding sites in the proximal promoter, but also a conserved β -casein enhancer, which is located at distances ranging from -1.6 to -6.4 kb from the start site of transcription in the bovine and mouse genomes, respectively. The casein locus also contains a number of other novel secretory genes as well as evolutionarily conserved noncoding regions.

In addition to the application of recombinant DNA technology to the mammary gland, a second major advance was the development of transgenic and knockout mouse technologies. Only a limited number of breast cancer cell lines were available which retained hormonal responsiveness and it was not yet feasible to efficiently introduce gene constructs directly into primary mammary epithelial cell cultures to study their properties in three dimensional cell culture models. Therefore, in order to study the developmental and hormonal regulation of milk protein gene expression, lines of transgenic mice were developed using the regulatory elements from the β -casein and whey acidic protein (WAP) genes [8–10]. These studies established that the promoters and enhancers in these genes were able to confer appropriate tissue-specific and hormonal regulation on a heterologous reporter gene.

More detailed deletion mapping and DNA footprinting studies on the β -casein gene were facilitated by studies using the HC11 mammary epithelial cell line [11]. These and other studies [12] helped define the concept of Composite Response Elements (CoRE), which consist of a series of clustered, often nonconsensus DNA binding sites, which mediate the appropriate hormonal and developmental regulation of the milk protein genes through protein-protein as well as protein-DNA interactions. CoRE are present in both the proximal promoters and upstream enhancers of the casein and whey protein genes. Unexpectedly, the transcription factors that recognize and bind to these sites are not exclusively expressed in the mammary gland. These include the CCAAT-enhancer binding protein (C/EBP) β , nuclear factor (NF) 1, signal transducers and activators of transcription (Stat) 5, and the glucocorticoid receptor (GR). Thus, to date there does not appear to be a “mammary-specific” transcription factor which regulates the identity of the mammary epithelial cells (MECs) and milk protein gene expression.

INSIGHTS FROM TRANSGENIC AND KNOCKOUT MOUSE MODELS

The early application of transgenic mouse models to mammary gland biology was fortuitous because it forced several laboratories, including our own, to move into the field of developmental biology. This was essential due to the complex cell-cell interactions required for hormonal signaling and normal mammary gland development. Surprisingly, germline deletion of many of these transcription factors identified in the milk protein CoRE (e.g. C/EBP β and Stat5a & b) exerted profound effects not only on milk protein gene expression and lactation as expected, but also on earlier stages of mammary gland development [13–15]. Careful analysis of these germline knockouts, specifically the direct visualization of signaling pathways *in situ* in wildtype and null mice, has provided some new insights into factors regulating normal mammary gland development. Our laboratory has focused on studying the role of C/EBP β in mammary gland development.

C/EBP β is a member of the basic leucine zipper family of transcription factors. Encoded by an intronless gene, *cebpb* is expressed as several distinct protein isoforms whose expression is regulated by the differential use of a number of in-frame translation start sites (reviewed in [16]). Increased expression of C/EBP β has been detected in breast cancer, ovarian tumors and colorectal tumors. In contrast, C/EBP β null mice are refractory to Ras-mediated skin tumorigenesis [17]. Thus, disruption of signaling through C/EBP β appears to contribute to malignant transformation. Support for this hypothesis has been obtained by recent studies by Lamb et al. [18] using a combination of molecular genetics, DNA microarray and data mining of human tumor expression databases. These investigators have identified a cyclin dependent kinase (cdk)-independent mechanism by which the interaction of cyclin D1 with C/EBP β may mediate tumorigenesis.

Targeted deletion of all the C/EBP β isoforms results in a severe inhibition of lobuloalveolar development and a block to functional differentiation, as well as more subtle changes in ductal morphogenesis [13,14]. The altered expression of a number of molecular markers, including the progesterone, estrogen, and prolactin receptors, the transporter proteins (NKCC1 and aquaporin 5), and several markers of skin differentiation (Sprr2A and keratin 6), suggests that germline deletion of C/EBP β results in an altered cell fate [19]. Thus, C/EBP β appears to play a role in the specification of progenitor cell fate in the mammary gland, as well as in a number of other tissues (reviewed in [20]).

THE IMPORTANCE OF HORMONE RECEPTOR PATTERNING

C/EBP β null mice display an unusual pattern of steroid and prolactin receptor expression, as well as a defect in lobuloalveolar development [19,21]. Because deletion of the progesterone receptor [22] was previously shown to block lobuloalveolar development, we suspected that deletion of C/EBP β might result in a loss of PR expression. In fact, the opposite phenomenon was observed, and there was an increased level of PR expression and an increased percentage of MECs expressing PR observed in the C/EBP β null mice. Furthermore, there was greater than a ten-fold reduction in the number of cells entering S phase as determined by BrdU incorporation. The key observation was obtained by double immunofluorescent confocal microscopic analysis of Texas Red-labeled PR and FITC-labeled BrdU positive cells, which revealed that the PR-positive cells were not proliferating, but were adjacent or nearby to the proliferative cells [21]. These studies indicated that the spatial distribution of steroid receptors is critical to normal mammary gland development.

Similar observations in the normal ductal epithelium have been reported in the human, bovine and rodent mammary glands [21,23–25]. Thus, while the PR and ER α co-localize in over 96% of normal breast epithelial cells, proliferating cells are ER α and PR-negative. Approximately, 25% of the ductal cells in the mature virgin are steroid receptor positive, and this distribution appears to be established during development as a consequence of the increase in progesterone (P) levels (see below). This non-uniform pattern of steroid receptors is also observed for PrIR, and it has been suggested, therefore, that ER α , PR and PrIR are all co-localized in the same cells [26]. The non-uniform pattern of PR expression along the mammary duct has been observed not only by immunostaining using specific anti-PR antibodies, but also by *in situ* hybridization to detect PR mRNA [21], and finally by using a unique lacZ reporter mouse to directly visualize PR promoter transcriptional activity [27].

The distribution of lacZ positive cells reflecting PR promoter activity can even be visualized nicely in mammary gland wholemounts. Studies using ovariectomized mice have suggested that chronic administration of estrogen (E) alone results in a uniform pattern of lacZ expression in the ductal epithelium, but that simultaneous administration of E + P resulted in a non-uniform pattern of expression. Thus, the increase

in serum progesterone levels, which begins at around 6 weeks of age in most mouse strains, may account for the switch observed in the pattern of steroid and prolactin receptor expression during normal mammary gland development, and this presumably reflects PR transcription in a subset of ductal MECs [21]. Confocal microscopy studies have revealed that the PR-positive cells are not always directly adjacent to a proliferating cell, but are usually no more than 2–3 cells away from a PR-positive cell, suggesting a paracrine mechanism controls MEC proliferation.

Recent analysis of PRKO and PrIR KO mice have suggested that there may be an autoregulatory pathway involved in the co-expression of ER, PR, and PrIR in ductal MEC [19]. Receptors for both E and P, which play an essential role for postnatal mammary gland development, are expressed in the embryonic mammary gland [27,28]. Both isoforms of the ER have been detected in the mammary mesenchyme of mouse embryos beginning at E 12.5, while PR is expressed in the epithelium in the mammary bud. This has led us to postulate that PR, perhaps even in its unliganded form, may induce the PrIR in the mammary epithelium, and that the PrIR through the Jak/Stat pathway may then regulate the level of the ER α in MECs. Finally, ER regulates the level of PR leading to an autoregulatory loop. Interestingly, no embryonic mammary phenotype has been reported for mice with ablation of either of the ER or PR isoforms [22,29], although they both are critical for postnatal development.

Progesterone and prolactin (Prl) are the principal mediators of lobuloalveolar development in the mammary gland [30]. Deletion of Prl, PrIR or PR completely inhibits alveolar development and ductal lateral branching, but does not significantly compromise the process of ductal outgrowth and bifurcation [31,32]. Exogenous administration of P and Prl partially rescue lobuloalveolar development in ERKO mice [33]. Two distinct progesterone receptor isoforms, PR-A and PR-B, which arise from a single gene are expressed in the mammary gland (reviewed in [34]). Analysis of mice null for either the PR-A or PR-B isoform suggests that PR-B alone is sufficient to elicit normal proliferation and differentiation [35]. Ductal side branching and lobuloalveolar development are markedly reduced in the PRBKO mice. Defects in both PrIR- and PR-mediated development have been localized to the mammary epithelium. Elegant PR $+/+$ and PRKO-lacZ-tagged MEC chimera reconstitution experiments have demonstrated that PR acts via a paracrine mechanism to induce alveolar proliferation [31]. Alveolar

development can be rescued if PRKO MECs mixed with an excess of PR^{+/+} MECs are reconstituted in close proximity within the cleared fat pads of syngeneic hosts. Recombination of PR wild-type epithelium and PRKO stroma indicates that the stroma does not play a critical role in alveolar morphogenesis, further emphasizing the importance of epithelial-epithelial paracrine interactions, rather than epithelial-stromal interactions, in PR action. Interestingly, deletion of PR results in decreased cyclin D1 expression, providing one mechanism of impaired development in the PRKO mouse. Mice lacking cyclin D1 also display an epithelial cell-autonomous defect in lobuloalveolar development [36, 37]. However, the effects of PR on cyclin D1 appear to occur in cells adjacent to those expressing the steroid receptor, again suggesting a paracrine mechanism [31].

LOCAL PARACRINE GROWTH FACTORS

Establishment of the correct patterning of steroid and prolactin receptors in the mammary ducts is, therefore, required for local growth factors to stimulate the proliferation of nearby or adjacent steroid receptor negative MECs for normal lobuloalveolar development. The phenotypic consequences of deletion of either PR or PrlR are quite similar suggesting that their downstream signaling pathways likely converge as some point. In support of this hypothesis, gene arrays performed using these knockout models have identified amphiregulin, IGF-II, Wnt-4 and Receptor Activator of NF κ B-ligand (RANKL) as potential downstream targets of both pathways. Thus, members of the IGF, EGF, Wnt gene families and RANKL all may be the local growth factors, which mediate the paracrine effects of P and Prl. Support for this model has been obtained by the analysis of specific knockout mice.

The importance of the Wnt family of secreted glycoproteins was shown by ectopic expression of Wnt-1, not normally expressed in the mammary gland, which rescued the lobuloalveolar defect in PRKO mice [38]. Additional studies comparing mammary epithelium transplants from Wnt-4 null and wild-type donor mice into the cleared fat pad suggested that Wnt-4 acts as a local mediator of progesterone action in early pregnancy. However, the defects in lobuloalveolar development observed in the Wnt-4 null transplants were not seen in late pregnancy most likely due to compensation by other members of the Wnt gene family, such as Wnt-5b.

The importance of EGF family members in regulating lobuloalveolar development has been obtained by

the analysis of the triple knockout of TGF α , EGF and amphiregulin [39]. In triple null glands, alveoli were poorly organized and undifferentiated, and milk protein gene expression was decreased. Again because of compensation by other family members, there are minimal effects of single deletions of these EGF family members. Estrogens have been suggested to regulate the transcription of TGF α [40] while progestins have been shown to regulate amphiregulin expression [41].

Mice lacking RANKL or its receptor also fail to form lobuloalveolar structures during pregnancy [42]. RANKL also appears to be an essential mediator of PR-dependent alveolar proliferation and survival, and recently is co-expressed in PR positive MECs, but not in the cyclin D1 proliferative cells [35]. Studies using a mutant *Ikka* (the *Ikka* subunit of I κ B kinase involved in the NF κ B signaling pathway) knockin allele replacing serine residues with alanines in the activation loop have suggested that *Ikka* is a critical intermediate in a pathway that controls mammary epithelial proliferation in response to RANK signaling via cyclin D1 [43]. IGF-2 also has been suggested to be a mediator of prolactin-induced lobuloalveolar development [44,45]. Ectopic IGF-2 expression restores alveologenesis in PrlR null epithelium, and lobuloalveolar development is retarded, but not prevented, in IGF-2-deficient MECs [44]. IGF-1 and -2 are also expressed in the mammary stroma [46], and may partially compensate for the loss of IGF-2 in the MECs. Alterations in the IGF signaling axis have been observed in the C/EBP β null mice [19].

Thus, it appears that the precise patterning of steroid and prolactin receptors in the normal mammary gland is required to elicit the appropriate paracrine response to local growth factors in order to regulate alveologenesis. A transition from a paracrine to an autocrine mechanism has been suggested to be an early step in preneoplastic progression [21] as illustrated in Fig. 1. Supporting this hypothesis, ER-positive proliferating cells are rare in premenopausal lobules, but increase with age in the normal breast. However, the percentage of dual-expressing cells was significantly increased in all of the *in situ* proliferations examined, such as ductal carcinoma *in situ* (DCIS), which correlated positively with the level of risk of developing breast cancer [47, 48]. Interestingly, in rodent models a similar increase in dual-expressing cells has been observed in aged rats, which could be prevented by an early exposure to estrogen and progesterone, which exerts protective effects similar to an early pregnancy [49]. Finally, a mouse model which displays hormone-dependent preneoplas-

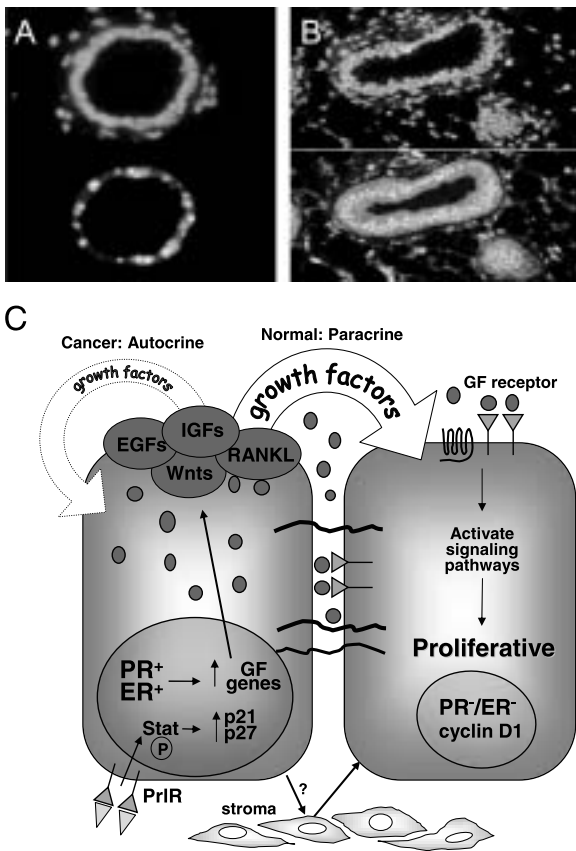


Fig. 1. Mechanisms regulating hormone-induced proliferation during breast cancer progression. A. Non-uniform distribution of PR expression in a normal duct. Dapi staining of nuclei is shown above in blue and the non-uniform pattern of PR staining is shown below in red. Note the single layer of epithelial cells lining the duct. B. Uniform staining for PR in p53 null mammary epithelial outgrowths. Staining is as shown in A. Note the multilayered cells beginning to form a DCIS-like lesion. C. A model depicting the putative switch from a paracrine to an autocrine mechanism during early breast cancer progression. The nonproliferative cell expressing ER, PR and the PrIR depicted in red may secrete local growth factors, which are members of the Wnt, EGF, IGF families and Rankl, but may not be able to divide because of the expression of cdk inhibitors such as p27 and p21. The adjacent cells depicted in green respond to these local growth factors and activate receptor tyrosine kinases and downstream MAPK pathway, as well as the frizzled/ β -catenin pathway resulting in increased proliferation.

tic progression, involving transplantation of p53 null MECs from Balb/c mice into the cleared fat pad of syngeneic recipients, has been developed by Medina and his colleagues [50]. In this system, an increase in the percentage of ERalpha and PR positive cells also is observed in the transition from normal ductal epithelium to early DCIS lesions. Estrogen and progesterone, both singly and in combination enhance tumorigenesis in the p53 null MEC transplants, and a markedly re-

duced tumor incidence was observed in the absence of the progesterone receptor, observed by crossing with PRKO mice. Thus, this model may be useful in elucidating the molecular mechanisms responsible for these early alterations in hormonal patterning and proliferation observed during breast cancer progression.

FUTURE QUESTIONS

These studies of normal mammary gland development performed primarily in genetically engineered mouse models have provided new insights into the mechanisms by which hormones regulate growth in the normal mammary gland and how these mechanisms have been altered in breast cancer. However, at the same time these studies raise a number of critical questions: First, how is the pattern of steroid- and prolactin- receptor expressing cells established along the normal mammary ducts? Are there different progenitors, which give rise to the approximately 25% of these receptor-positive ductal epithelial cells. Third, why don't the receptor positive cells proliferate in response to the secreted local growth factors? Finally what are the early changes that disrupt these patterns in breast cancer progression?

One testable hypothesis is that receptor-positive cells are prevented from entering the cell cycle and S phase because of the expression of cdk inhibitors such as p27 and p21. Decreased expression of p27 in breast cancer is associated with a poor prognosis. Careful analysis of p27 null MECs has suggested that p27 regulates both proliferation and survival in MECs, but there were no obvious morphological defects in the p27 null mice or MEC transplants derived from these mice [51]. Partial compensation by p21 in p27 null MECs suggests that it may be important to examine double null MECs for effects on receptor patterning and proliferation. As mentioned, deletion of the p53 tumor suppressor gene in MECs, a known regulator of p21, can disrupt correct receptor patterning.

Recent progress in the isolation of functional mammary stem cells [52], suggests it may be possible to isolate specific progenitors by FACS and follow their fate following transplantation back into the cleared mammary fat pad. Insertion of EGFP, instead of lacZ, into the PR gene may also facilitate the isolation of PR-positive cells at different stages of development and tumorigenesis (J. Lydon, personal communication). Use of microarrays to identify genes expressed in specific mammary epithelial cell types may help provide impor-

tant lineage markers required for these studies. Finally, the increasing availability of phospho-specific antibodies should permit a more detailed analysis in situ of the signaling pathways activated by local growth factors to determine if they are activated in both the receptor-positive as well as proliferative cells, and what happens during early breast cancer progression.

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