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Prolactin Regulates Mammary Epithelial Cell Proliferation Via Autocrine/Paracrine Mechanism

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Prolactin (PRL) is essential for a number of developmental events in the mammary gland. Work with PRL and PRL receptor knockout mice has shown that PRL indirectly regulates ductal side branching during puberty and directly controls lobuloalveolar development and lactogenesis during pregnancy. Anterior pituitary or placental PRL is thought to be responsible for these functions via an endocrine mechanism; however, PRL is also produced in a number of extrapituitary sites including the mammary gland. The physiologic relevance of mammary PRL remains unknown. In this study we utilized mammary recombination in Rag1$^{-/-}$ hosts, to determine whether mammary PRL plays a role in the regulation of mammary gland development. Mammary glands formed with the PRL gene deleted from either the epithelium, stroma, or both displayed normal development, on the basis of whole mount and hematoxylin and eosin histology, during puberty and lactation. At the end of pregnancy, a 2.8-fold decrease in bromodeoxyuridine incorporation was observed in the epithelial cells of mammary glands formed using PRL knockout epithelium compared with those formed using wild-type epithelium. No balancing alteration in the rates of apoptosis was detected. Thus, mammary-derived PRL influences mammary epithelial cell proliferation via an autocrine/paracrine mechanism, establishing a physiologic function for mammary PRL during mammopoiesis.

Key Words: Prolactin; mammary gland; autocrine; paracrine; development.

Introduction

Prolactin (PRL) is a polypeptide hormone that has an established role in the regulation of mammary gland development *(1)*. The generation of mice with null mutation of genes involved in the PRL signaling pathway has established the essential function of this pathway in indirect regulation of ductal side branching, and direct regulation of lobuloalveolar development and lactogenesis in the mammary gland *(2–5)*. This pathway is subject to positive and negative regulation, and the correct balance of these signals is essential for normal mammary gland development *(6)*. PRL is synthesized primarily in the anterior pituitary, and studies utilizing bromocriptine, which inhibits pituitary PRL synthesis, or pituitary isographs, which secrete large amounts of PRL, have established that endocrine PRL is largely responsible for PRL's reported physiologic functions *(7)*. However, PRL is also synthesized in several extrapituitary sites including mammary epithelial cells *(8–10)*, raising the possibility that in addition to PRL's demonstrated direct and indirect endocrine roles, it may also regulate mammopoiesis via an autocrine/paracrine mechanism.

PRL's mitogenic role during normal mammary development parallels a role for it in mammary carcinogenesis. Transgenic mice overexpressing rat PRL develop mammary carcinomas *(11)*. PRL and PRL receptor (PRLR) are expressed in human breast carcinoma *(12–14)* and increased serum PRL levels are associated with an increased risk of breast cancer in postmenopausal woman *(15)*. PRL influences the proliferation of normal and malignant breast cells in vitro *(16,17)*. Several in vitro studies have demonstrated that in breast cancer cell lines, PRL may act via an autocrine/paracrine mechanism, since the use of antibodies against PRL can inhibit breast cancer cell proliferation *(18)*.

A physiologic function of mammary-produced PRL is yet to be demonstrated in vivo. Using mammary gland recombination and transplantation in Rag1$^{-/-}$ hosts with normal endocrinology, we demonstrate that at the end of pregnancy epithelial cell proliferation is reduced 2.8-fold in epithelium carrying a null mutation of the PRL gene with no detectable change in the rate of apoptosis. The present study indicates that PRL acts via an autocrine/paracrine mechanism within the epithelium to regulate mammary epithelial cell proliferation from the onset of lactation.

Results

Recombined mammary gland transplantation using mammary glands from PRL knockout (PRL$^{-/-}$) and PRL wild-type (PRL$^{+/+}$) mice enabled the investigation of the role of

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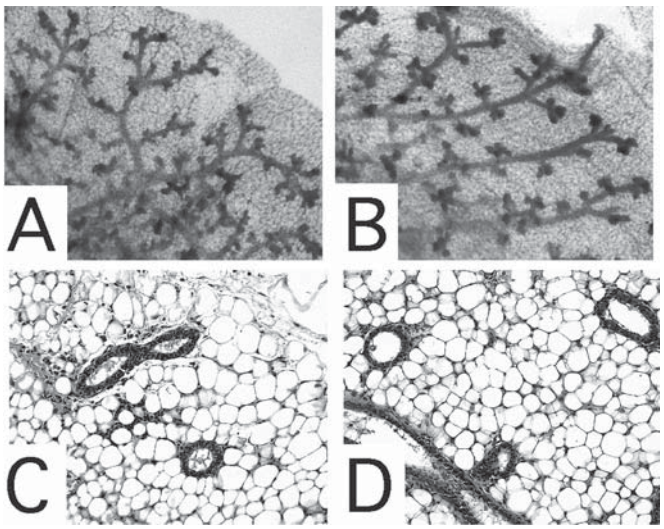

Fig. 1. Mammary PRL is not required for development in virgin animals. Analysis of the development of $PRL^{-/-}$ or $PRL^{+/+}$ mammary epithelium following puberty: (**A**) $PRL^{+/+}$ epithelium whole mount; (**B**) $PRL^{-/-}$ epithelium whole mount; (**C**) $PRL^{+/+}$ epithelium H&E histology; (**D**) $PRL^{-/-}$ epithelium H&E histology.

mammary PRL during mammopoiesis in a normal endocrine environment.

Deletion of the PRL gene from the epithelium did not alter ductal architecture (Fig. 1A,B) or histology (Fig. 1C,D) in mature virgin animals. The amount of cell proliferation, as assessed by bromodeoxyuridine (BrdU) staining in these glands, was not significantly different (percentage of epithelial cells positive for BrdU: 1.17 ± 0.09 for $PRL^{+/+}$ epithelium and 1.47 ± 0.47 for $PRL^{-/-}$ epithelium; $p = 0.56$). Deletion of the PRL gene from the stroma or from both the stroma and epithelium did not result in any differences in ductal architecture or histology (data not shown). These data demonstrate that in mice, mammary PRL does not have an essential role in regulation of mammary gland development in virgin animals and plays no detectable role in epithelial cell proliferation at this stage.

At the onset of lactation, normal lobuloalveolar development was observed in whole mounts of mammary glands carrying a null mutation of the PRL gene in the mammary epithelium (Fig. 2A,B). Hematoxylin and eosin (H&E)–stained sections of mammary glands formed from $PRL^{-/-}$ and $PRL^{+/+}$ epithelium at d 1 postpartum showed the presence of colostrum and oil droplets, indicating normal epithelial secretory function at this time (Fig. 2C,D). Cell proliferation was assessed by measuring BrdU incorporation in a subset of transplanted mammary glands on d 1 postpartum (Fig. 2E,F). Both epithelial and stromal cells were scored for BrdU staining; however, the number of proliferating stromal cells was too few to analyze. In mammary glands formed using $PRL^{+/+}$ epithelium, the percentage of proliferating epithelial cells was 7.94 ± 0.20 compared with 2.82 ± 0.08 in $PRL^{-/-}$ epithelium–derived glands (Fig. 2F). This

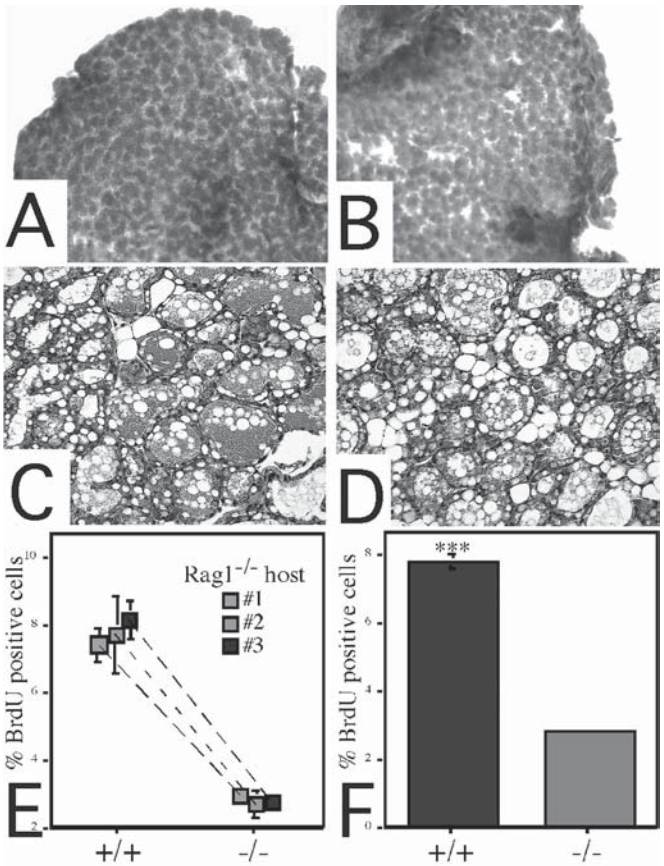

Fig. 2. Mammary PRL regulates epithelial cell proliferation at lactation. Analysis of $PRL^{-/-}$ or $PRL^{+/+}$ mammary epithelial transplants d 1 postpartum: (**A**) $PRL^{+/+}$ epithelium whole mount; (**B**) $PRL^{-/-}$ epithelium whole mount; (**C**) $PRL^{+/+}$ epithelium H&E histology; (**D**) $PRL^{-/-}$ epithelium H&E histology; (**E**) BrdU incorporation in epithelial cells of mammary transplants, same color, with dashed lines denoting transplants in the same $Rag1^{-/-}$ host; (**F**) BrdU incorporation, collective data, $***p < 0.0001$. Absence of error bars indicates that the standard error was less than the symbol size.

represents a 2.8-fold ($p < 0.0001$) decrease in epithelial cell proliferation in mammary glands unable to produce PRL, identifying an autocrine/paracrine mechanism for PRL during mammopoiesis.

The levels of apoptosis were also assessed using TUNEL assay in the mammary glands formed with $PRL^{-/-}$ and $PRL^{+/+}$ epithelium at d 1 postpartum, to determine whether the decreased rate of BrdU incorporation was also associated with a decreased rate of apoptosis. As expected for this stage of development, rates of apoptosis were very low (<1%) in the epithelium of both genotypes. No alteration in apoptosis rate was detected in the transplants of $PRL^{-/-}$ epithelium that showed reduced rates of proliferation.

Discussion

Previous work with PRL and $PRLR^{-/-}$ mice has demonstrated that the PRLR indirectly regulates ductal side branching during puberty *(5)*. This indirect effect is probably via

regulation of progesterone levels since progesterone treatment rescues ductal side branching in $PRL^{-/-}$ mice *(19)* and $PRLR^{-/-}$ mice (unpublished data). During pregnancy direct activation of epithelial PRLR is essential for lobuloalveolar development and lactogenesis *(5)*. In our study, we have shown that mammary gland–produced PRL is not essential for ductal side branching, alveolar bud formation, and lobuloalveolar development on the basis of morphologic and histologic examination. These data demonstrate that these developmental events are the result of a direct endocrine action of PRL on the mammary gland.

Mammary epithelial cell–produced PRL was shown to be essential for normal proliferation rates of the cells within the lobuloalveoli, identifying an autocrine/paracrine role for PRL during mammary development. This autocrine/paracrine effect of PRL is seen despite a normal endocrine environment during pregnancy, demonstrating the importance of this aspect of PRL-regulated mammopoiesis. Perplexingly, however, no difference in morphology was seen despite the large difference in proliferation rate. TUNEL assays detected no change in apoptosis rates in these glands, indicating that the decrease in cell proliferation rate was not balanced by decreased rates of cell death. Although this may simply reflect the difficulty of detecting a decrease in apoptosis from a low baseline, another explanation is possible. At this late stage of development, the mammary gland may undergo a shift from reliance on endocrine PRL to local PRL influence as part of the changes in regulatory control accompanying the shift from proliferation to milk production. Thus, the effect of an altered proliferation rate may not yet have had time to exert an effect on morphologic end points. This is consistent with the expression pattern of PRL in the mouse mammary gland during development, where mammary PRL is only detected at late pregnancy and lactation *(8–10)*. This is also consistent with our knowledge of the role of pituitary PRL during lactation. Although hypophysectomy or treatment with a dopamine agonist will stop lactation, the level of PRL secretion by the pituitary falls as lactation proceeds, without diminution in milk supply *(20)*. Synthesis of local PRL may play a role in the maintenance of lactation during falling pituitary PRL secretion.

Several studies for the treatment of breast cancer using inhibitors of pituitary PRL secretion either have been unsuccessful or have produced inconsistent findings *(21)*. It has been hypothesized that the reason these studies have failed is that these compounds do not inhibit extrapituitary synthesis or secretion *(21)*. Our data provide evidence in support of a role for mammary-produced PRL during the onset of lactation. Unlike rodents, human mammary epithelial cells secrete PRL outside of pregnancy *(22,23)*, so it is possible that PRL may also act in an autocrine or paracrine manner throughout the lifetime of women.

In conclusion, we have demonstrated a physiologic function for mammary PRL, regulating epithelial cell proliferation via an autocrine/paracrine mechanism.

Materials and Methods

Mice

The generation of $PRL^{-/-}$ mice has been previously described *(4)*. $Rag1^{-/-}$ mice *(24)* of the 129/C57BL/6J strain were purchased from Jackson. All animals were housed with food and water ad libitumin with a 12-h day/night cycle at 22ºC and 80% relative humidity.

Recombined Mammary Gland Transplantation

Recombined mammary gland transplantation is a variation of the mammary epithelium transplant technique *(25)*. Briefly, an approx 1-mm^3 section of mammary gland excised from 12-wk-old $PRL^{-/-}$ or $PRL^{+/+}$ donors from between the nipple and lymph node of the fourth mammary gland was inserted into the excised cleared fat pad from 3-wk old $PRL^{-/-}$ or $PRL^{+/+}$ mice. The recombined mammary epithelium–stroma complex was then grafted between the abdominal cavity and skin, placing the transplant between the third and fourth mammary glands of 3-wk-old $Rag1^{-/-}$ mice *(26)*. The following recombinations of mammary epithelium (Ep) and stroma (St) were performed: $PRL^{-/-}$ Ep and $PRL^{+/+}$ St; $PRL^{-/-}$ Ep and $PRL^{-/-}$ St; $PRL^{+/+}$ Ep and $PRL^{-/-}$ St; and $PRL^{+/+}$ Ep and $PRL^{+/+}$ St.

Transplants were examined by whole-mount histology at 6 wk postsurgery or mated and examined on d 1 postpartum. Examination showed that in all cases the new glands arose from the transplanted epithelium *(27)*. Mammary development during puberty was assessed in all of the possible epithelial-stromal combinations (n = 3–9 per combination), and development of $PRL^{-/-}$ Ep and $PRL^{+/+}$ St vs $PRL^{+/+}$ Ep and $PRL^{+/+}$ St was also assessed at d 1 postpartum (n = 6 for each combination).

Histology

Mammary whole mounts were made by spreading the gland on a glass slide and fixing in 10% formalin solution. Glands were defatted in acetone before carmine alum (0.2% carmine, 0.5% aluminum sulfate) staining overnight. The whole mount was dehydrated using a graded ethanol series followed by xylene treatment for 60 min and storage in methylsalicylate.

Cell Proliferation Analysis

Two hours before the harvesting of mammary glands, mice were injected with BrdU (30 mg/kg of body wt). BrdU staining was performed using BrdU antibody (Neomarkers) and Dako Animal Research Kit peroxidase. Approximately 2000 epithelial cells per transplanted gland (n = 3) were scored for the presence of BrdU staining. Each gland was counted blind, and in triplicate, by at least two researchers. Statistical significance between groups was determined using student's unpaired t-tests.

Cell Apoptosis Analysis

Cell apoptosis was measured by assessing nuclear DNA fragmentation using the DeadEnd Fluorometric TUNEL

System (Promega, Madison, WI) according to the manufacturer's instructions. Propidium iodide was used to stain all cells. Apoptotic cells were scored and counted using the same method as for assessing BrdU incorporation.

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