**Endocrine**, *vol. 5, no. 1, 103–110, August 1996* 1046–3976/5/1:103–110/$7.00 

# Rat Placental Lactogens Initiate and Maintain Lactation Yet Inhibit Suckling-Induced Prolactin Release

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**Mammalian reproduction is dependent on both a successful pregnancy and on the subsequent period of lactation. In the rat, ovulation occurs shortly after parturition making it possible for a dam to be simultaneously pregnant and lactating. The present studies investigate the effect of placental hormones on suckling-induced prolactin (PRL) release and the contribution of placental hormones to milk synthesis and secretion. A rat choriocarcinoma cell line, Rcho-1, which secretes placental lactogens (PLs) following transplantation in vivo, attenuated suckling-induced PRL release on both d 9 and d 14 of lactation by 43 and 58%, respectively. When PRL secretion was completely inhibited by bromocriptine, a dopamine agonist, Rcho-1-bearing dams still maintained a normal litters weight gain, demonstrating that placental lactogens can continue an established lactation. The Rcho-1 tumors also initiated milk synthesis and secretion in nulliparous rats continuously exposed to pups. Whereas none of the 11 control virgins began lactating and had an average pup weight loss of 2.07 g, the Rcho-1-bearing rats began lactating, as evidenced by a significant reduction in pup weight loss. Thirty percent of these rats became fully lactationally competent. Northern blot analysis showed that the Rcho-1 tumors expressed both PL-I and PL-II mRNA in all experimental groups. These tumors also secreted PL-I into the circulation, as shown by radioimmunoassay.**

**Key Words:** Lactation; placental lactogens; mammary gland; prolactin.

## Introduction

The known role of prolactin (PRL) in mammals is primarily reproductive and includes maintenance of pregnancy and lactation. In rats, stimulation of the cervix during mating initiates a pulsatile release of PRL, with nocturnal surges (0300 h) and diurnal surges (1700 h) occurring during the first 8–10 d of pregnancy (Butcher et al., 1972; Smith and Neill, 1976). These PRL surges rescue and maintain the corpora lutea and their progesterone (P) secretion (Smith et al., 1976), without which pregnancy in the rat would be terminated (Csapo and Wiest, 1969). It follows, therefore, that premature termination of the PRL surges will also terminate pregnancy (Clemens et al., 1969a; Dang and Voogt, 1977). Under normal conditions, however, cessation of the mating-induced PRL surges is correlated with increased circulating levels of placental lactogen-I (PL-I), secreted by the developing placenta (Voogt et al., 1982; Tonkowicz and Voogt, 1984; Voogt and deGreef, 1989). Since PL-I can bind and activate the PRL receptor (Robertson et al., 1982), this peptide can assume the functions of PRL during pregnancy, including maintenance of the corpora lutea (Morshige and Rothchild, 1974; Tabarelli et al., 1982).

In contrast to pregnancy, PRL release during lactation involves a more typical neuroendocrine reflex with a direct one-to-one correspondence between suckling (stimulus) and PRL secretion (response) (Mena and Grosvenor, 1968; Grosvenor et al., 1979). In general, placental lactogens (PLs) are not present during lactation since the placenta is expelled, along with the pups, during parturition. Yet, because female rats ovulate shortly after parturition (Connor and Davis, 1980a; Connor and Davis, 1980b), it is possible for a dam to be simultaneously pregnant and lactating. Although embryo implantation is generally delayed by 4–6 d in a pregnant lactating rat (Mantalenakis and Ketchel, 1966; Yogev and Terkel, 1982), under such conditions PLs are still present and potentially influence suckling-induced PRL release and lactation. High levels of PLs can also be artificially induced in nonpregnant rats by transplantation of rat choriocarcinoma (Rcho-1) cells, that express PL-I in vivo (Faria and Soares, 1991).

Ovarian and pituitary hormones may also affect placental expression during a concurrent pregnancy and lactation. Previous studies of primiparous rats show that hypophysectomy increases placental hormonal release (Voogt et al., 1985). Whereas pituitary transplants given to hypophysectomized pregnant rats could not block an initial increase in PL levels, PLs were later reduced in treated

Received October 27, 1995; Revised December 20, 1995 and April 29, 1996; Accepted May 17, 1996.

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animals as compared to the hypophysectomized controls (Voogt et al., 1985). Steroids also modify placental hormonal release. Both estrogen (E) and P treatment can separately increase PL secretion, although P+E is more potent than either steroid alone (Tonkowicz and Voogt, 1984). Interestingly, removal of the steroids by ovariectomy will also temporarily raise PL levels before the fetuses and placenta are reabsorbed (Robertson et al., 1984). Individual hormones secreted by the placenta may also be differentially affected by various hormone treatments. For example, the Rcho-1 cells that secrete PL-I in vivo, are also capable of secreting PL-II in vitro (Faria and Soares, 1991). The environmental factor(s) that selectively inhibit or promote PL-II expression in the Rcho-1 cells have not yet been elucidated.

The aims of this study are fourfold: determine the ability of PLs to inhibit suckling-induced PRL release; investigate the lactogenic properties of PLs in the absence of PRL; determine whether PLs can initiate and maintain lactation in virgin rats; and identify the PRL-related mRNAs expressed in Rcho-1 transplants under different hormonal milieus.

## Results

### *PRL Release on Days 9 and 14 of Lactation*

Lactating dams bearing HRP-1 tumors placed under the right kidney capsule exhibited a strong PRL response to suckling on both d 9 and d 14 (Fig. 1). Blood samples were taken at 0, 5, 15, 30 and 60 min of suckling after 6 h of separation. On d 9, plasma PRL reached 267 ng/mL at 60 min of suckling and 172 ng/mL on d 14. In contrast, suckling-induced PRL release was significantly reduced in Rcho-1-bearing dams on both d. On d 9, plasma PRL levels were significantly lower in the Rcho-1-bearing dams at 5, 15, and 30 min of suckling ($p < 0.05$). On d 14, plasma PRL levels were significantly lower in the Rcho-1-bearing dams at 15, 30, and 60 min of suckling ($p < 0.05$). Two rats that were injected with Rcho-1 cells but did not develop tumors exhibited a PRL response similar to those found in HRP-1-bearing rats. Daily litters weight gains for Rcho-1-bearing and HRP-1-bearing dams were not significantly different.

### *Litter Weight Gains Following Bromocriptine Treatment*

In order to determine the lactogenic properties of the Rcho-1 tumor in lactating dams, bromocriptine, a dopamine agonist, was employed to reduce PRL levels. Bromocriptine (3 mg/kg sc) was given to Rcho-1-bearing and HRP-1-bearing dams at 12-h intervals; the first injection was at 1800 h on d 7 and the last at 0600 h, d 10. Another group of Rcho-1-bearing dams received vehicle injections. Bromocriptine treatment in both Rcho-1-bearing and HRP-1-bearing dams effectively inhibited suckling-induced PRL release (Fig. 2). In spite of the presence of very low PRL levels, the litters of Rcho-1-bearing, bromocriptine-treated rats maintained a constant daily weight gain (Fig. 3). The

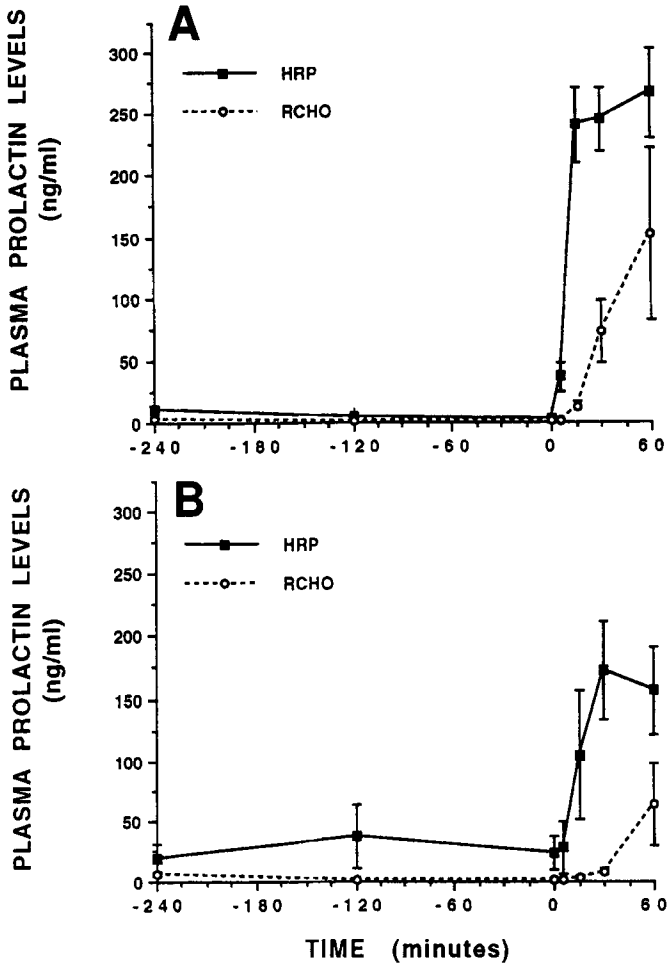


**Fig. 1.** Plasma PRL levels measured in lactating dams bearing an HRP-1 or Rcho-1 transplant during a 6-h period of pup separation followed by 1 h of suckling. **(A)** On d 9 of lactation the Rcho-1 tumor significantly ($p < 0.05$) reduced plasma PRL levels at 5, 15, and 30 min of suckling (upper panel). Each value represents a mean ± SE of determinations from 4–7 rats. **(B)** On d 14 of lactation the Rcho-1 tumor significantly ($p < 0.05$) reduced plasma PRL levels at 15, 30, and 60 min of suckling (lower panel). Each value represents a mean ± SE of determinations from 5–10 rats.

daily weight gain of litters suckled by Rcho-1-bearing, bromocriptine-treated dams was not significantly different from those of the Rcho-1-bearing, vehicle-treated dams. In contrast, the daily litters weight gains of the HRP-1-bearing, bromocriptine-treated dams was significantly reduced by d 9 and 10 of lactation ($p < 0.05$).

### *Lactational Competency in Nulliparous Rats*

The final experiment determined the ability of secretions from Rcho-1 tumors to initiate mammary development, maternal behavior, and lactation. Maternal behavior, defined as pup retrieval and crouching, was observed in all foster animals, including control virgin rats. Lactational competency, defined as a positive pup weight change over a 24-h time period, was only observed in Rcho-1-bearing rats and control dams. Six of the 20 (30%) Rcho-1-bearing virgin rats were able to induce a positive litters weight gain,

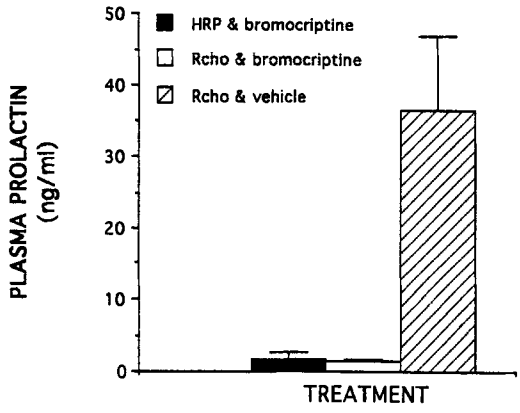


Fig. 2. Plasma PRL levels measured in lactating dams bearing an HRP-1 or Rcho-1 transplant following 4 d of bromocriptine or vehicle treatment beginning on d 7 of lactation. Blood samples were taken after 6 h of pup separation and 1 h of suckling. Bromocriptine treatment significantly (*$p < 0.01$) reduced plasma PRL levels in both HRP-1 and Rcho-1 transplanted animals. Each value represents a mean ± SE of determinations from 8–10 rats.

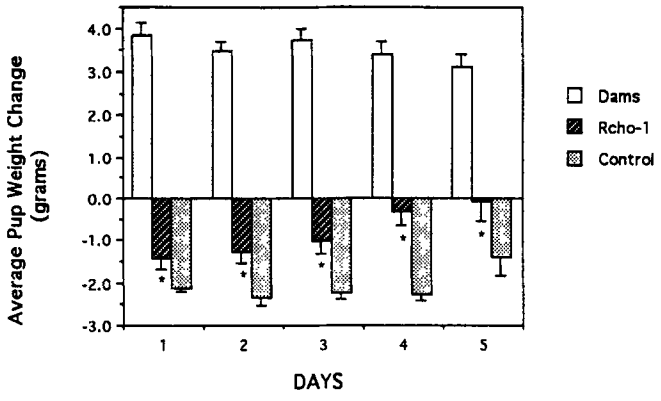


Fig. 4. Average pup weight change over the last 5 d of pup exposure in litters of lactating dams and foster virgin rats. The mean weight loss of pups exposed to Rcho-1 transplanted virgins was significantly less ($p < 0.05$) than the weight loss of pups exposed to control virgins on all 5 d shown. Each value represents the mean ± SE of the average pup weight change in 8–10 litters.

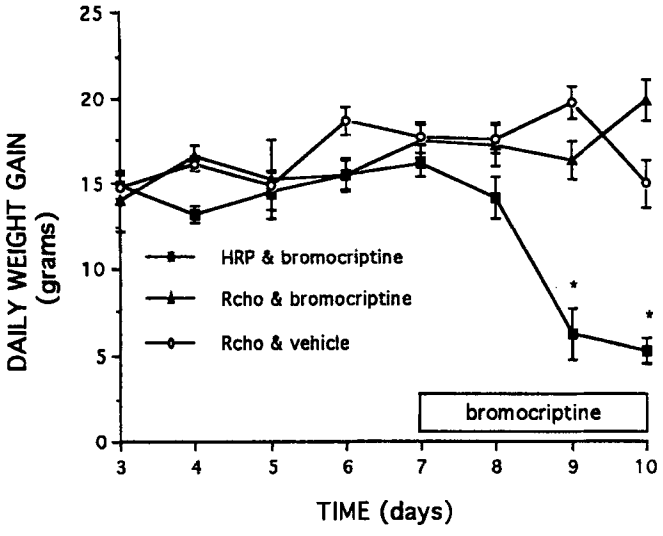


Fig. 3. Daily litter weight gain over 8 d of lactation in tumor-bearing dams treated with bromocriptine or vehicle for 4 d. Bromocriptine treatment significantly reduced the litter weight gain (*$p < 0.05$) of the HRP-1 transplanted dams, but did not affect litters weight gain of the Rcho-1 transplanted rats. Each rat suckled 8 pups; each value represents the mean ± SE of 8–10 litters.

while litters from all of the 11 control rats lost weight. As Fig. 4 shows, the average pup weight gain over the last 5 d each lactating dam was exposed to pups was fairly constant (+3.51 ± 0.12 g). Similarly, the average pup weight loss for the control virgin rats was consistent over time (–2.07 ± 0.12 g). During this same time period, the daily average pup weight loss in the Rcho-1-bearing group was significantly ($p < 0.05$) reduced each day as compared to the control virgins.

The ability of the Rcho-1-bearing animals to sustain pup growth is further indicated by examination of mammary tissue taken from the adult females. Mammary glands taken from control nulliparous animals displayed extensive connective tissue and small numbers of undifferentiated alveoli (Fig. 5). In contrast, the lactating dams exhibited numerous alveoli that were enlarged and contained milk. Similarly, mammary tissue taken from Rcho-1-bearing rats had large numbers of enlarged alveoli that contained milk. Even those animals that had not demonstrated sufficient lactation to cause a positive pup weight gain had extensive alveolar development and the presence of milk.

### *Protein and mRNA Levels of Lactogenic Hormones*

Messenger RNA expression of two PRL-GH family members, PL-I and PL-II, by the Rcho-1 cell line was evaluated by Northern blot analysis (Fig. 6). All of the Rcho-1 tumors expressed both PL-I and PL-II mRNA regardless of the hormonal condition (pubertal, ovx, pregnant, lactating) of the host animal. The expression of PL-II mRNA appears to be less than PL-I mRNA expression, although the exact levels of mRNA for both hormones was not measured.

Plasma levels of PL-I were measured by radioimmunoassay. The circulating levels of PL-I in HRP-1-bearing dams were below detection (5 ng/mL). Rcho-1-bearing dams had circulating PL-I levels of 133 ± 60 ng/mL, and virgin animals bearing Rcho-1 tumors, 144 ± 31 ng/mL.

## Discussion

In this study, we have shown for the first time that secretions from a rat choriocarcinoma (Rcho-1) are capable of both maintaining full lactational competency in primiparous dams and initiating lactation in nulliparous rats.

The Rcho-1 cell line phenotypically and genotypically resembles the trophoblast giant cells (TGC) of the rat placenta. The TGC express placental lactogen-I (PL-I) (Faria et al., 1990a) on d 7–12 of pregnancy. On d 10, PL-II mRNA expression is initiated by the TGC and this expression is

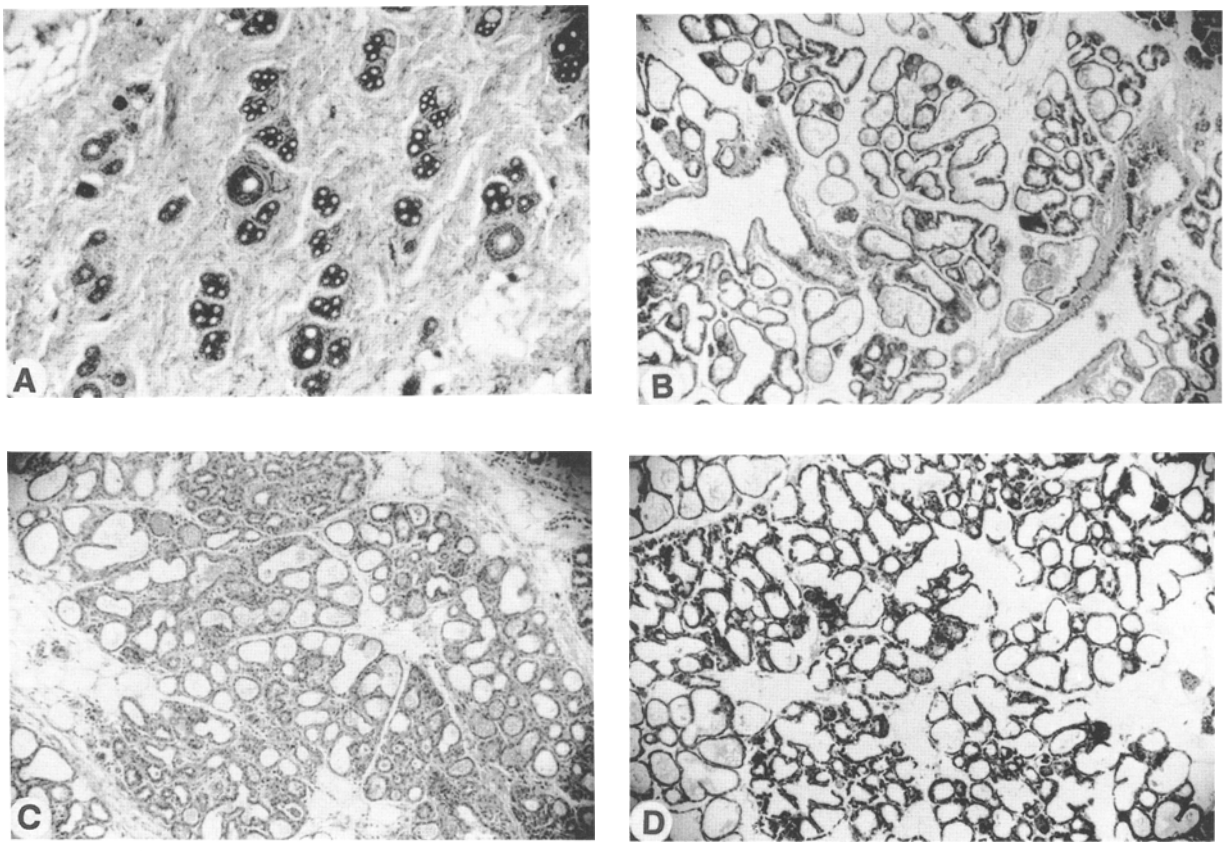


**Fig. 5**. Histological comparison of mammary tissue taken from a nonlactating virgin rat, lactating dam and lactating nulliparous rats. Tissues were fixed in Bouin's solution, embedded in paraffin, sectioned at 10 μm, and stained with hematoxylin and eosin. **(A),** mammary tissue from a control virgin rat. **(B),** mammary tissue from a lactating dam. **(C),** mammary tissue from a fully lactationally competent, Rcho-1 transplanted nulliparous rat. **(D),** mammary tissue from a lactating Rcho-1 transplanted virgin rat that was not fully lactationally competent. Note the extensive lobuloalveolar development and the presence of milk in (B), (C), and (D). Magnification, ×40.

maintained until parturition (Faria et al., 1990a). In vitro cultures of Rcho-1 cells maintain TGC sequential gene expression of PL-I and PL-II (Faria and Soares, 1991; HamLin et al., 1994). However, unlike the coexpression of PL-I and PL-II by the TGC in vivo, that is limited to 24 h (Faria et al., 1990b), coexpression of the PLs is maintained since PL-I expression is not terminated.

In contrast to their in vitro findings, Faria and Soares (1991) showed that Rcho-1 tumors maintained in pubertal female rats expressed only PL-I, and none of the other members of the PRL-GH family. In the present study, Northern blot analysis demonstrated that Rcho-1 tumors expressed not only PL-I mRNA, but also PL-II mRNA, in all hormonal conditions studied: lactation, ovariectomy, and puberty. Although the mRNA levels of PL-I and PL-II were not quantitated, it appears that PL-I expression is greater than PL-II expression in these Rcho-1 tumors. Possibly the PL-II mRNA levels in Rcho-1 tumors analyzed by Faria and Soares (1991) were too low to be detected by Northern blot analysis. Alternatively, the ability of Rcho-1 cells to secrete PL-II in vivo may have been acquired by further differentiation of the Rcho-1 cells over time and passaging of the cell line.

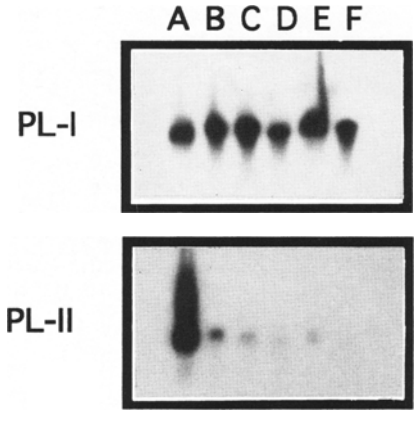


**Fig. 6**. Northern blot analysis of PL-I and PL-II expression by Rcho-1 tumors grown under the kidney capsules of female rats. Total RNA (10 μg/lane) was fractionated on a 1% agarose gel by electrophoresis and transferred to a Nytran membrane. Hybridizations were carried out using $^{32}$P-labeled PL-I or PL-II probes. **(A),** postconfluent cell culture; **(B),** cells transplanted in a lactating dam; **(C),** cells transplanted in a lactating dam treated with bromocriptine; **(D),** cells transplanted in a lactating virgin rat; **(E),** cells transplanted in an ovx rat; **(F),** cells transplanted in a pubertal animal. Autoradiograph for PL-I blot was exposed for 86 h; autoradiograph for PL-II blot was exposed for 115 h.

During pregnancy, the rat placental hormones act to maintain the pregnancy while terminating the PRL surges of early pregnancy. Several studies have correlated the rise in circulating levels of PL-I with cessation of the mating-induced PRL surges (Voogt et al., 1982; Tonkowicz and Voogt, 1983). PL-II is believed to continue the suppression of the PRL surges (Voogt, 1980). The ability of the PLs to inhibit the PRL surges of pregnancy is associated with their ability to bind and activate the PRL receptor (Robertson et al., 1982). In contrast to PRL release during early pregnancy, PRL levels in the lactating dam are continuously elevated. Although it is possible to inhibit both the PRL surges of pregnancy and suckling-induced PRL release with hypothalamic implants of PRL (Clemens et al., 1969a,b), there is evidence that PRL has a reduced ability to feedback on its own secretion at this time (Demarest et al., 1983; Arbogast and Voogt, 1996). Our study demonstrated that placental secretions also are capable of inhibiting suckling-induced PRL release. The inability of the Rcho-1 tumors to completely abolish suckling-induced PRL release may be because of the decreased sensitivity of the PRL feedback system during lactation. Additionally, the levels of circulating PLs produced by the Rcho-1 tumors in this study are somewhat lower than the peak levels present during a normal rat pregnancy. Circulating levels of PL-I peak at approx 400 ng/mL on d 12 of pregnancy (Robertson and Friesen, 1981). In comparison, the Rcho-1 tumors in lactating dams and lactating virgin rats produced circulating levels of PL-I of 133 ng/mL and 144 ng/mL, respectively.

Yogev and Terkel (1982) demonstrated that suckling-induced PRL release was reduced in pregnant lactating rats. At the same time PL-I and PL-II can inhibit PRL release, they can act at the mammary gland to stimulate milk synthesis and secretion (Lyons, 1944; Ray et al., 1955). Our study demonstrated that Rcho-1 tumor secretions can simultaneously reduce suckling-induced PRL release while assuming lactogenic properties. Even when PRL levels were maximally inhibited by bromocriptine, milk synthesis and secretion, as measured by the daily litters weight gain, was not reduced in Rcho-1-bearing dams. This maintenance of lactation occurs even though the circulating PL-I levels in experimental animals were lower than plasma PL-I levels present during a normal rat pregnancy. This suggests that an excess of PL-I may be produced by the pregnant dam in order to insure a successful pregnancy and lactogenesis. Whereas cytokines and growth factors secreted by the Rcho-1 tumors also contribute to lactation, certainly PLs from the Rcho-1 tumors play an important role in the maintenance of milk synthesis and secretion.

Finally, these studies demonstrate the ability of Rcho-1 tumor secretions to induce lactation in nulliparous rats. Mammary development in Rcho-1-bearing virgin rats was comparable to that of primiparous lactating dams. Milk was also visible within the alveoli of all Rcho-1-bearing and lactating rats, as well as in the stomachs of pups exposed to both groups. Pups exposed to Rcho-1-bearing animals that were not fully lactationally competent exhibited a reduction in weight loss that became increasingly less each d. The teats of the Rcho-1-bearing rats were elongated, enabling the pups to firmly attach. At the same time, none of the 11 animals in the control virgin group exhibited mammary development or reductions in the pup weight loss. The inability of some lactating foster animals to attain a lactational competency comparable to that of lactating dams is most likely a result of the time period of lactogenic hormone exposure and levels present in each animal. Primiparous animals are exposed to high levels of lactogenic hormones for the 22 d of rat pregnancy. The corresponding development of the mammary gland enables the dam to initiate and sustain high levels of milk synthesis and secretion during lactation. In contrast, PL secretion by the Rcho-1 tumors in virgin rats begins slowly, thereby delaying growth and differentiation of the mammary gland. Circulating levels of the PLs are also limited to the size and development of the Rcho-1 tumor, which occurs at variable rates in different rats. Rcho-1 tumors become necrotic as they enlarge, which may limit tumor size and PL secretion levels. Additionally, other components of lactation, such as the milk-ejection reflex or milk composition, may not be identical in both groups of rats.

Previously, Evans and Simpson (1928–29) reported that they could induce lactation in nulliparous rats by repeated injections of bovine anterior pituitary extracts. Suckling alone is not a sufficient stimulus for the induction of mammary development and lactogenesis (Bruce, 1961). This agrees with our findings of extensive mammary development, milk synthesis and milk secretion in nulliparous animals bearing Rcho-1 tumors.

In conclusion, placental hormone secretions from the Rcho-1 tumors partially inhibit suckling-induced PRL release. At the same time, these secretions can both maintain milk synthesis and secretion in PRL-deficient primiparous dams, and initiate lactation in nulliparous animals. The Rcho-1 tumors express both PL-I and PL-II mRNA. These experiments provide further proof that rat PLs can substitute for PRL at the mammary gland to stimulate milk synthesis and secretion.

## Materials and Methods

### *Animals*

Female Sprague-Dawley rats (Sasco, Omaha, NE), weighing 200–225 g on arrival, were housed at a controlled temperature (22°C) and an alternating 12-h light:12-h dark room (lights on at 0600 h), with food and water available ad libitum. Six prepubertal female rats, age 22 d, were also housed under the same conditions. One set of adult rats was ovariectomized and received a Rcho-1 transplant within a week of their arrival. Eight groups of rats were mated and delivered their litters 22 d later. Two other groups of rats

were not mated and were not exposed to pups prior to experimentation. Parturition was designated d 1 of lactation. Litters in the first three experiments were adjusted to eight pups on d 3 of lactation. All litters were weighed daily as a measure of lactational competency. Lactating rats were individually housed throughout the experiments. The animal procedures used in this study were approved by the Institutional Animal Care and Use Committee of the Kansas University Medical Center.

### *Surgery*

Rats received a transplant of $1 \times 10^6$ Rcho-1 or HRP-1 cells in a 50 μL vol by injection under the right kidney capsule. Animals used in the first three experiments received the tumor transplant 6–24 h following parturition. One group of nulliparous females used in the fourth experiment received a cell transplant approximately two weeks prior to pup exposure. For the Rcho-1 tumor expression study, prepubertal rats received a transplant at 22 d of age and adult rats were ovariectomized at the same time they received the transplant.

For experiments requiring multiple blood samples, carotid cannulation was performed under Ketamine (120 mg/kg im)/acepromazine maleate (1 mg/kg im) anesthesia. PE50 tubing filled with heparinized saline (20 IU/mL) was advanced 1 in. into the left carotid artery, exteriorized at the back of the neck, and protected by a stainless steel spring extended outside the cage.

### *Blood Sampling and Radioimmunoassay (RIA)*

Serial blood samples and trunk blood samples were collected in heparinized centrifuge tubes. For the serial samples, 0.3 mL vol of blood were taken and replaced with an equal volume of heparinized saline. Plasma PRL levels were determined by radioimmunoassay using RP-3 as the standard reference and $^{125}$I-PRL as the labeled antigen. Plasma was separated from whole blood by centrifugation and then stored at –20°C. Samples were assayed at 5 and 25 μL. The lower limit of sensitivity was 50 pg; the interassay coefficient of variation was 10.80%.

PL-I RIA was performed as described previously (Robertson and Friesen, 1981). Each tube contained 100 μL standard or unknown sample, 300 μL buffer (PBS containing 25 μM EDTA and 1.0% BSA), 100 μL rabbit anti-rPL-I serum at a dilution of 1:3000, and 100 μL [$^{125}$I]rPL-I (30,000–40,000 cpm). Standards ranged from 1–200 ng/mL, corresponding to 100–20,000 pg/tube. After incubation for 1 d at 23°C or 2 d at 4°C, 1% normal rabbit serum and sheep antirabbit γ-globulin were added, and 1 d later, bound and free [$^{125}$I]rPL-I were separated by centrifugation.

### *Cell Cultures*

Rcho-1 cells, which secrete PL-I in vivo, were maintained as previously described (Faria and Soares, 1991). Briefly, the Rcho-1 cells were cultured in RPMI-1640 culture media supplemented with 20% heat-inactivated fetal bovine serum at 37°C under humidified atmosphere of 95% air-5% $CO_2$. Cells were passaged by brief exposure (60 s) to 0.25% trypsin-0.02% EDTA, followed by mechanical scraping of cells from the culture dish. Cells were subsequently plated at a density of $1 \times 10^6$ cells/75 cm$^2$ tissue culture flask. Cells were briefly trypsinized, scraped, centrifuged, and resuspended in culture medium before they were injected under the kidney capsules of the rats.

HRP-1 cells served as a control for several of the experiments. This cell line was established from the midgestational chorioallantoic placenta of a Holtzman rat, and does not secrete any members of the PRL-GH family (Soares et al, 1987). The HRP-1 cells were maintained, passaged and transplanted as described for the Rcho-1 cells.

### *Drugs*

Bromocriptine was dissolved in 0.3% tartaric acid, 30% ethanol to a concentration of 6 mg/mL. This dopamine agonist was administered subcutaneously at a dose of 3 mg/kg body wt.

### *Histology*

Mammary gland tissue was fixed in Bouin's solution, dehydrated, cleared, and embedded in paraffin. Tissue samples were sectioned at 10 μm and stained with hematoxylin and eosin.

### *mRNA Analysis*

Rcho-1 and HRP-1 tumors were immediately frozen in liquid nitrogen and stored at –70°C. Tumor RNA was extracted by the modified method of Chomczynski and Sacchi (1987) as described by Xie and Rothblum (1991). In brief, approx 0.10 g tumor was lysed using a single-step acid guanidinium thiocyanate-phenol-chloroform extraction. RNAs were precipitated in isopropanol and stored as precipitates at –70°C until ready for use, then washed with 70% EtOH, dried under reduced pressure, and diluted in TE. Total RNA (10 μg/well) was fractionated on 1% agarose-formaldehyde gel and transferred to a Nytran membrane by capillary transfer (Davis et al., 1986). Denatured DNA probes were hybridized with prehybridized Nytran membranes containing RNA samples for 24 h at 42°C. Blots were then washed with high stringency (0.1X SSC and 0.5% SDS) at 65°C, air-dried, and autoradiographed.

cDNAs for members of the rat placental PRL family were generously provided by D. Linzer (PL-I; Northwestern University, Evanston, IL) (Colosi et al., 1987), and M. Duckworth and H. Friesen (PL-II; University of Manitoba, Winnipeg, Canada) (Duckworth et al., 1986). The cDNA inserts were used as templates for the synthesis of $^{32}$P-labeled cDNA probes using the Prime-It II Random Primer Labeling Kit (Stratagene).

### *Statistical Analysis*

Data are expressed as mean ± SE. A statistics program, StatView 512+ (Brainpower, Agoura Hills, CA), was used to analyze data with a two-way analysis of variance for repeated measures. Differences were considered significant if $p < 0.05$.

*Experiments*

The first two experiments determined the ability of Rcho-tumor secretions to inhibit suckling-induced PRL release during mid and late lactation. In order to insure sufficient tumor growth and development, these initial experiments were not carried out until 9 or 14 d after the transplantation of tumor cells.

In the first experiment, dams received an Rcho-1 or HRP-1 tumor on d 1 of lactation and were cannulated on d 8. On d 9 of lactation, litters were removed in the morning for 6 h and blood samples taken during the period of separation. Pups were then returned and blood was sampled at 5, 15, 30, and 60 min of suckling. The dams and litters were observed and pups weighed daily until d 14 to determine the dam's lactational competency. The second experiment was identical to the first, except that blood samples were taken on d 14 of lactation.

The third experiment examined the ability of the Rcho-1 tumor secretions to maintain lactation in the almost complete absence of PRL. Two groups of lactating dams received an Rcho-1 tumor on d 1 of lactation; a third group of dams received an HRP-1 tumor, also on d 1. All HRP-1-bearing dams and one group of Rcho-1-bearing dams were injected with bromocriptine (3 mg/kg body wt) at 1800 h on d 7 of lactation. The second group of Rcho-1-bearing animals received a vehicle injection instead of the bromocriptine. Additional injections were made every 12 h thereafter until the morning of d 10. Following the 0600 h injection on d 10, litters were removed for 6 h. Pups were returned and allowed to suckle for 1 h, at which times the dams were rapidly decapitated and trunk blood collected. Litters were weighed daily to determine lactational competency.

In the fourth experiment, the ability of the Rcho-1 tumor to initiate and maintain lactation in nulliparous foster animals was determined. Sixteen rats were mated and delivered their litters 22 d later. These animals served as a source of pups for the other two groups and as a positive control for lactational competency. A second group was made up of 20 nulliparous rats that had not been previously exposed to pups. They received an Rcho-1 tumor approx 2 wk prior to being exposed to the pups. A final group of 11 nulliparous, nonpup-exposed rats did not receive any tumor transplant. Each foster animal was exposed daily to three pups, aged 3–21 d, for 24 h. Both foster and lactating dams were observed for 15 min following introduction of the pups each morning, and behavioral responses recorded. They were then checked every 15 min thereafter for 2 h. In order to reduce novelty effects, foster animals generally received pups from only one or two different lactating dams. Once maternal behavior (defined as pup retrieval and crouching) was initiated in foster animals, pup exposure was increased to four to six pups daily. Pups that exhibited positive weight gain when placed with foster mothers remained with the foster animal for 4 d. Following 4 d of positive pup weight gain, the foster mother and the corresponding dam were rapidly decapitated and trunk blood, mammary gland tissue, and Rcho-1 tumors collected. Other dams and foster animals were sacrificed after approx three weeks of pup exposure.

A final experiment examined the gene expression of Rcho-1 tumor transplants under a variety of hormonal conditions. Adult virgin female rats were ovariectomized and simultaneously received a transplant of Rcho-1 cells under the kidney capsule. Rats were sacrificed by rapid decapitation 12 d later. Prepubertal female rats were transplanted with Rcho-1 cells at 22 d of age and were sacrificed by rapid decapitation at 34 d of age. Rcho-1 tumors were collected at the time of sacrifice from both groups. Messenger RNA expression from the Rcho-1 tumors collected from both groups was compared with the mRNA expressed by Rcho-1 tumors maintained in the lactating animals of the previous experiments. Plasma PL-I levels from Rcho-1-bearing and HRP-1-bearing lactating dams, and from Rcho-1-bearing lactating virgin rats were measured using a radioimmunoassay.

## Acknowledgments

The authors would like to thank G. Shah for his assistance with Northern blot analysis, and M. C. Robertson for performing the PL-I radioimmunoassay. We also thank the NIDDK, NICHHD, and the National Hormone and Pituitary Program (University of Maryland School of Medicine) for the gift of PRL RIA materials. This material is based on work supported under a National Science Foundation Graduate Fellowship (R. J. F.) and by NIH grant HD 24190 (J. L. V.).

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