Parity-Induced Decrease in Systemic Growth Hormone Alters Mammary Gland Signaling: A Potential Role in Pregnancy Protection from Breast Cancer

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Abstract
Early full-term pregnancy is an effective natural protection against breast cancer in both humans and experimental rodents. The protective effect of an early pregnancy is, in part, linked to changes in circulating hormones that are involved in both normal breast development and breast cancer. For example, a reduction in circulating growth hormone (GH) has been shown to protect rats from carcinogen-induced mammary tumors. We examined the ability of a full-term pregnancy to alter the endocrine GH/insulin-like growth factor-I (IGF-I) axis and how this change affected normal mammary gland function in two commonly used rat models (Sprague-Dawley and Wistar Furth). Circulating GH and IGF-I were measured in blood drawn every 30 minutes from parous and age-matched virgin female rats. Mean serum GH levels were significantly decreased (P < 0.01) in parous compared with age-matched virgin rats for both strains. Changes in GH levels were independent of estrous cycle, indicated by a significant (P < 0.05) reduction in circulating levels of GH during estrus and diestrus in both parous strains. Despite the decrease in circulating GH, pituitary GH mRNA levels were unaltered in parous rats. Circulating IGF-I and hepatic IGF-I mRNA were also unaltered by parity in either rat strain. Immunoblot analysis of mammary glands showed decreases in phosphorylation of signal transducer and activator of transcription 5A and Janus-activated kinase 2, suggesting reduced action of GH in the mammary gland. Therefore, although the parity reduction in circulating GH does not affect circulating IGF-I levels, it is possible that reduced GH acts directly at the mammary gland and may play a role in pregnancy protection from breast cancer.

Introduction
Numerous factors independently and/or synergistically influence the risk of developing breast cancer; after age and a woman’s genetic background, reproductive history is one of the strongest (1, 2). Elevated risk of breast cancer is linked to increased lifetime exposure to ovarian hormones, for example, early menarche and late menopause (2, 3). Supporting this, combined hormone therapy is associated with increased risk of breast cancer, and a dramatic reduction in risk is seen following ovariectomy (4, 5). An early full-term first pregnancy has been consistently found to be associated with a reduced risk of breast cancer and is one of the strongest and most effective natural protections against breast cancer in humans (1, 3, 6). For this reason, much research has focused on elucidating the mechanism of pregnancy protection from breast cancer, with a hope of mimicking this effect as a preventative intervention in the population.

One of the appealing aspects of pregnancy protection is that it is readily modeled in rodents. Many laboratories have shown that an early pregnancy, or treatment of mice with estrogen and/or progesterone, can protect rats and mice from carcinogen-induced mammary cancer (7–12). The majority of studies investigating the mechanism for this protection have focused on the mammary gland (in particular the mammary epithelium), and models suggest that the protective effect pertains to persistent changes in intracellular regulatory loops controlling cell proliferation and apoptosis (13–15).

Challenging the notion that pregnancy protection is solely due to changes in the mammary gland, two studies have shown that pregnancy alters the host environment to alter mammary carcinogenesis. First, mammary epithelial cells from rats treated with carcinogen showed increased progression to cancer when transplanted into carcinogen-naive age-matched virgins (AMV) compared with parous...
rats (16). Additionally, p53-null mammary epithelial cells also showed greater progression to neoplasia when transplanted into AMV compared with parous mice (17). Supporting a role for the host in regulation of pregnancy protection, Thordarson et al. (11) showed that parity persistently altered the levels of some hormones that are implicated in mammary gland development and cancer, including prolactin and GH.

The reduction in GH levels following pregnancy is compelling, given the increased evidence for a role of GH/insulin-like growth factor I (IGF-I) in both mammary gland development and breast cancer. Studies have established that normal pulsatile secretion of growth hormone (GH) and subsequent regulation of IGF-I is the cornerstone of mammary gland postnatal growth and differentiation (18–20). Furthermore, hyperactivation of the GH and/or IGF-I pathways leads to mammary hyperplasia and tumorigenesis (21, 22). Supporting this, inhibition of both pathways blocks tumor development (23–25). Most strikingly, the spontaneous dwarf rat, which has a mutation in the GH-releasing hormone (GHRH) gene, resulting in nondetectable circulating GH and subsequently low IGF-I, is completely resistant to chemically induced mammary tumorigenesis, which can be reversed by GH and/or IGF-I replacement (24, 26, 27).

Studies in human mammary epithelial cells and tissues also strongly support a role for the GH/IGF-I axis in breast cancer. Both GH and IGF-IR are oncogenes that can transform normal mammary epithelial cells (28, 29). IGF-IR and GHR are elevated and hyperactive in human breast cancers (30, 31), and IGF-IR is currently under intense investigation as a therapeutic target in cancer (32).

Most importantly, epidemiologic evidence indicates that individuals with circulating IGF-I levels at the higher end of the reference range predict increased risk, indicating that increased GH/IGF axis activity is associated with postmenopausal breast cancer (33). Furthermore, an analysis of IGF-I levels in women from the Nurses’ Health Study found that serum IGF-I levels are lower in parous compared with nulliparous women (34). Taken together, the animal and human data strongly support a role for downregulation of the GH/IGF-I axis as a mechanism for pregnancy-induced protection from breast cancer.

Data on the effect of parity on circulating GH are fragmentary, and one study found no effect on circulating IGF-I (35). To clarify if pregnancy protection from breast cancer is or can be associated with a persistent change in GH and/or IGF-I hormonal signaling, we assessed the ability of a single full-term pregnancy to persistently alter the GH/IGF-I axis (36). Most strikingly, inhibition of both pathways blocks tumor development (23–25). Most strikingly, the spontaneous dwarf rat, which has a mutation in the GH-releasing hormone (GHRH) gene, resulting in nondetectable circulating GH and subsequently low IGF-I, is completely resistant to chemically induced mammary tumorigenesis, which can be reversed by GH and/or IGF-I replacement (24, 26, 27).

Materials and Methods

Animals

This study used rats of the WF inbred line purchased from Harlan Laboratories, as well as rats of the SD outbred line purchased from Charles River. Adult female rats were housed two per cage on Sanichips (Sani-Pure Food Labs) until breeding, at which time individual dams were paired with a male (breed-specific) in wire-bottom cages. After detection of vaginal plugs, individual dams were again housed individually on Sanichips and remained so until their pups were weaned. At that time, dams were housed four per cage and allowed to naturally involute for 28 d. AMV controls for both strains were housed four per cage and remained so for the duration of the study. WF and SD rats were housed within the Laboratory Animal and Resources Facility at Texas A&M University. All animals were housed under controlled conditions of temperature (23°C), light (lights on: 6:00 a.m.; lights off: 6:00 p.m.), and ad libitum access to food (Harland Teklad Diet) and tap water.

After 28 d of involution (Fig. 1), parous SD and WF females and their respective AMV controls (all approximately 120–125 d of age, depending on when they became pregnant) were surgically inserted with silastic cannulae into the right external jugular vein according to a technique described previously by Harms and Ojeda (36). Following surgery, animals were allowed a full recovery and housed individually to minimize stress. The next day, cannula extensions were connected to each freely moving animal and flushed with heparinized saline before collecting each blood sample. To encompass GH pulsatile fluctuations and account for early-morning basal level increases in GH as previously reported (37), blood samples (250 μL) were collected at 8:00 a.m. every 30 min for 4.5 hours. All animals received blood cell replacement after 2 h of sampling to avoid anemia. After the last sample, blood was centrifuged, and sera were collected and stored at −80°C until assayed. All animals were assigned to their respective phase of estrous cycle by well-defined criteria established previously (38) and mammary glands and hepatic tissues were collected for further analysis.

Whole-gland morphologic and histologic analyses

Mammary gland whole mounts were processed similar to a procedure developed by Williams and Daniel (39) with the following modifications. Briefly, no. 4 inguinal mammary glands from the left side were removed from five parous and five AMV controls (in both strains) at 120 to 125 d of age and spread flatly on the inner surface of a 50-mL tube and fixed with 10% formalin in PBS. The next day, tissue was placed in a cassette and fat was
removed using acetone for 48 h. Samples were dehydrated in 100% ethanol for 1 h, 95% ethanol for 1 h, and stained with carmine alum. Mammary glands were destained as follows: H2O for 1 h; 70% ethanol for 1 h; 95% ethanol for 1 h; 100% ethanol 3× for 1 h; and cleared in xylene 3× for 1 h. Finally, tissues were permanently stored in glass vials filled with methylsalicylate until analyzed.

The no. 4 inguinal mammary gland from the right side (five versus five each strain) was harvested, placed in cassettes, and fixed in 4% paraformaldehyde in PBS overnight. The next day, parafomaldehyde was replaced with 70% ethanol and samples were embedded in paraffin. Serial sections (5 μm thick) cut from paraffin blocks were placed on Superfrost Plus slides (Fisher Scientific), deparaffinized, and gradually hydrated, and all sections were stained with H&E and then examined microscopically.

Immunoblot analysis

Frozen mammary glands and livers were first crushed under liquid nitrogen using a metal mortar and pestle. Crushed tissue was lysed in TNESV buffer and 100 μg of tissue protein lysate were immunooblotted as described previously (40). We used the phospho-specific antibodies phospho-Janus-activated kinase 2 (p-Jak2) 1:500 (Cell Signaling Technology), phospho-signal transducer and activator of transcription 5A/B (p-Stat5A/B) 1:500 (Upstate Group, Inc.), p-Akt 1:1,000 (Cell Signaling Technology), and phospho-extracellular signal–regulated kinase 1/2 (p-Erk1/2) 1:1,000 (Cell Signaling Technology), Jak2 1:1,000 (Cell Signaling Technology), Stat5A/B 1:1,000 (Santa Cruz Biotechnology), Akt 1:1,000 (Cell Signaling Technology), Erk1/2 1:4,000 (Upstate Group, Inc.), and β-catenin 1:4,000 (BD Biosciences). IR fluorescent-labeled antirabbit (IRDye 800) or antimouse (Alexa Flour 680) antibodies 1:5,000 (Rockland Immunochemicals) were used as a secondary antibody and images were acquired and densitometrically analyzed using the Odyssey IR imaging system (LI-COR Biosciences).

Quantitative reverse transcription-PCR

Total RNA was extracted from mammary gland, pituitary, and hepatic tissues using an RNeasy Mini kit (Qiagen) following the manufacturer’s protocol. Total RNA (3.125 μg) was treated with DNase (2 μL) in 10× PCR buffer (10.5 μL) with 50 mmol/L MgCl2 (Invitrogen) and RNase-free water (brining the total volume to 125 μL) for 30 min at 37°C followed by 10 min at 75°C before reverse transcription. DNase-treated RNA (50 ng/μL) was reverse transcribed using 5× first-strand buffer, 100 mmol/L DTT, 25 mmol/L deoxynucleotide triphosphates, 20 μmol/L reverse primer, distilled water, and 200 units/μL Moloney murine leukemia virus reverse transcriptase, following the manufacturer’s protocol. Next, the cDNA product was analyzed by TaqMan quantitative PCR under standard conditions using an Applied Biosystems 7700 Prism thermocycler. Data were analyzed using the comparative Ct method (ΔΔCt method) developed by Livak and Schmittgen (41), normalized with β-actin as an endogenous control. Sequences for primer/probe sets designed by Primer Express III (Applied Biosystems) and synthesized by Eurogentec are listed in Supplementary Table S1.

Hormone assays and statistical analysis

Growth hormone and prolactin levels were measured in sera using RIA by Dr. A.F. Parlow (Director, Pituitary Hormones and Antisera Center, Harbor-University of California at Los Angeles Medical Center, Torrance, CA). Circulating levels of total IGF-I were measured in sera by a single assay using a rat/mouse IGF-I immunoenzyometric assay purchased from Immunodiagnostics Systems and confirmed using a separate IGF-I RIA purchased from DSL. The assay sensitivity was 82 and 150 ng/mL, respectively.

The differences between parous and AMV controls were analyzed by unpaired Student’s t test assuming random sampling. Probability values <0.05 were considered to be statistically significant. Furthermore, differences between signaling protein expression in these groups were analyzed by both parametric and nonparametric Student’s t tests; results were the same and, thus, the parametric test was used to calculate all results. The IBM PC programs INSTAT and PRISM (GraphPad) were used to calculate and graph the results.
Results

Parity-induced changes in the GH/IGF-I axis

To clarify if a single full-term pregnancy reduces circulating GH levels, we conducted an extensive analysis of pregnancy-induced hormonal changes in parous compared with AMV in two different rat strains: SD (outbred) and WF (inbred). The overall design of the experiment is depicted in Fig. 1 and is similar to a protocol that many laboratories have shown can block chemical induced carcinogenesis. Figure 2 (A: WF; B: SD) illustrates the individual GH pulse profiles from 120- to 125-day-old parous and AMV rats from both rat strains. In Fig. 3A, we compared the average serum GH levels over the 4.5-hour time period between the AMV and parous animals depicted in Fig. 2. Specifically, in WF rats, the mean level of circulating GH was significantly \( P < 0.001 \) reduced in parous \( (8.80 \pm 0.45 \text{ ng/mL; mean } \pm \text{ SEM}) \) compared with AMV rats \( (11.91 \pm 0.59 \text{ ng/mL; mean } \pm \text{ SEM}) \). Similarly, parity in SD rats significantly decreased \( P < 0.001 \) mean serum GH levels, with parous rats having \( 7.37 \pm 0.81 \text{ ng/mL} \) (mean \( \pm \text{ SEM} \)) compared with 11.67 \( \pm 0.74 \text{ ng/mL} \) (mean \( \pm \text{ SEM} \)) in AMV rats (Fig. 3B; average). Additionally, in Fig. 3B, we compared the mean of the single highest concentration of GH in AMV and parous rats (the peak pulse release) shown in Fig. 2. As expected, parity significantly decreased peak GH release from 16.84 \( \pm 1.13 \text{ ng/mL} \) (AMV; mean \( \pm \text{ SEM} \)) to 12.13 \( \pm 0.79 \text{ ng/mL} \) (parous; mean \( \pm \text{ SEM} \)) in WF rats \( P < 0.01 \) and from 16.79 \( \pm 1.32 \text{ ng/mL} \) (AMV; mean \( \pm \text{ SEM} \)) to 11.57 \( \pm 1.69 \text{ ng/mL} \) (parous; mean \( \pm \text{ SEM} \)) in SD rats \( P < 0.05 \). Additionally, parous and AMV females were grouped based on their estrous cycle stage as depicted in Fig. 3C and D. Parity significantly reduced \( P < 0.05 \) GH levels in both estrus and diestrus compared with AMV in both rat strains, indicating no confounding effect of estrous cycle.

To determine if the pregnancy-induced reduction in serum GH was associated with changes in GH-producing somatotrophic cells in the pituitary, we analyzed the expression of GH-mRNA in the anterior pituitary. Figure 4A illustrates the average fold induction of GH-mRNA levels normalized to \( \beta \)-actin determined by quantitative reverse
transcription-PCR (RT-PCR) in the pituitary between parous and AMV rats. A single full-term pregnancy had no effect on GH-mRNA levels in the pituitary compared with controls (Fig. 4A), despite the decrease in serum levels of GH in the same animals (Fig. 4B).

One of the major effects of GH is to increase the hepatic output of IGF-I and thus increase its circulating levels. We therefore measured circulating IGF-I in the same rats. Because IGF-I does not exhibit episodic secretion like GH, levels were measured in three serum samples (8.30 a.m., 10 a.m., and 12 p.m., from the nine that were collected) and the mean level was calculated (Fig. 5A). Surprisingly, there was no significant change in circulating levels of IGF-I due to parity in either WF or SD females. Supporting this, IGF-I and acid-labile subunit (ALS; a GH-responsive gene) mRNA levels in the liver were unaltered (Fig. 5B). Similarly, immunoblot analysis showed that serum protein levels of IGF binding protein 3 (IGFBP-3), another
GH-regulated protein, were not altered by full-term pregnancy (data not shown). The lack of effect of the parity-induced downregulation of GH on IGF-I, ALS, or IGFBP-3 levels indicates that the reduction in circulating GH is not sufficient to alter hepatic regulation of these genes. Interestingly, there was a modest suppression in liver GH receptor (GH-R) mRNA levels associated with parity in WF rats.

Parity-induced changes in circulating GH are associated with decreased activation of downstream signaling intermediates in the rat mammary gland

To determine if the parity-induced reduction in circulating GH levels is associated with altered mammary gland function, we analyzed the changes in the signal transduction pathways. Whole mounts (top) and H&E-stained (bottom) mammary glands from AMV (right) and parous (left) WF rats showed morphologic changes in the mammary gland due to parity (Fig. 6A). An analysis of key signaling proteins involved in GH signal transduction in WF mammary gland extracts (Fig. 6B) revealed that a single full-term pregnancy decreased phosphorylation of several GH-R signaling modulators including p-Jak2 (P < 0.05) and p-Stat5A (P < 0.01; Fig. 6C). Furthermore, parity induced a reduction in p-Akt (P < 0.05) mammary gland protein expression compared with age-matched controls. Surprisingly, p-Erk1/2 expression in the mammary gland was not significantly different (P = 0.098) between parous and age-matched controls. Similar results were observed in SD females (data not shown). Interestingly, quantitative RT-PCR analysis of WF mammary glands revealed that a single full-term pregnancy had no effect on GH-R or IGF-I mRNA expression compared with controls (Fig. 6D). It is important to note that, for all experiments, mammary cell protein levels were uniform in all groups as confirmed by the loading control β-actin (Fig. 6B).

Discussion

Current dogma suggests that a full-term pregnancy results in permanent changes in key proliferative (IGF-I and transforming growth factor β) and tumor suppressor (p53) genes in the mammary gland, protecting it from carcinogenesis (13–15, 42). Although these alterations undoubtedly contribute to the protective effect, more recent evidence suggests that other systemic changes may independently modulate the sensitivity of the mammary glands to carcinogenesis. Specifically, rodent studies have shown that mammary tumorigenesis is decreased when either p53-null mammary epithelial cells (17) or AMV rat mammary epithelial cells exposed to the chemical carcinogen N-methyl-N-nitrosourea (16) are transplanted into estrogen/progesterone-pretreated or parous rodents. These studies suggest that pregnancy, at least in part, changes the systemic environment, altering endocrine hormones known to regulate mammary gland signaling, thus reducing the susceptibility of the mammary gland to tumors.

In the current study, we show that a single full-term pregnancy results in a decrease in basal circulating levels of GH in two different strains of rats. This confirms and extends a previous observation by Thordarson et al. (11) that showed that a single time point measurement of serum GH in 120-day-old parous SD females indicated lower levels compared with AMV. Given the episodic secretion of GH in the female rat (37), our study represents a more accurate depiction of circulating GH levels and shows that the parity-induced change is due to an overall reduction in GH secretion and is not caused by alterations in pulse frequency or pulse amplitude. Importantly, the alteration in circulating GH levels was associated with decreased p-Jak2 and p-Stat5A signaling within the mammary gland, suggesting that a global suppression of GH secretion may, in part, reduce the mammary gland sensitivity to GH. Based on other studies indicating a reduction in carcinogen-induced mammary tumorigenesis in rats with low levels of GH and increased tumorigenesis following GH replacement (24, 26, 27), our data would support the concept that pregnancy reduction of GH may, in part, be responsible for parity-induced protection from breast cancer.

Significant data implicate GH and IGF-I actions in both mammary gland development and tumorigenesis. The...
most compelling evidence linking GH with mammary carcinogenesis comes from studies using the SD spontaneous dwarf rat, which has a mutation in the GHRH gene resulting in nondetectable circulating GH and subsequently low IGF-I levels (24). Recently, Shen et al. (27) showed that female spontaneous dwarf rats treated with the chemical carcinogen N-methyl-N-nitrosourea do not develop mammary tumors; however, rat or bovine GH replacement in these animals initiated mammary tumorigenesis comparable to N-methyl-N-nitrosourea–treated controls. Similar results were shown in Lewis spontaneous dwarf rats, which have a less severe GH deficiency but are still resistant to chemically induced mammary tumorigenesis (43). Furthermore, transgenic overexpression of GH in mice results in spontaneous mammary tumors (21), whereas growth of human breast cancer cells is severely retarded in mice lacking GH secretion compared with wild-type controls (44). In addition, GH administration to aging primates results in mammary gland hyperplasia (45). However, relatively little data exist implicating GH-induced breast cancer in humans. A recent review of numerous clinical studies suggested that height positively correlates with an increase in breast cancer incidence (46). More importantly, GH-R expression is increased in neoplastic breast tumors (30) and GH is also expressed in breast cancers (47, 48).

In this study, we showed that pregnancy in the rat resulted in a moderate reduction (26% in WF and 37% in SD) in circulating GH levels. Although GH is known to be a major modulator of circulating IGF-I levels, the reduction in GH was not associated with decreased IGF-I. In fact, we actually observed a small increase in circulating IGF-I in parous rats compared with age-matched controls. The reduction in GH was not associated with decreased IGF-I. In fact, we actually observed a small increase in circulating IGF-I in parous rats compared with age-matched controls. The reduction in GH was not associated with decreased IGF-I. In fact, we actually observed a small increase in circulating IGF-I in parous rats compared with age-matched controls.

Fig. 6. Parity downregulates classic GH-R downstream signaling molecules. A, representative whole mounts (top) and H&E staining (bottom) of WF mammary glands. B, representative immunoblots of WF mammary glands (four AMV and four parous) stained for common mammary gland signaling proteins. β-Actin was used as a loading control. C, densitometric analysis revealed that a full-term pregnancy significantly downregulated phosphorylation of the signaling modulators Jak, Stat5A, and Akt compared with AMVs. Notably, there was no change in Erk1/2 signaling between groups. D, fold GH-R and IGF-I levels in parous mammary glands compared with age-matched controls; mRNAs from parous and AMV WF rats were isolated and analyzed by real-time quantitative RT-PCR. It is important to note that a single full-term pregnancy had no effect on GH-R or IGF-I mRNA mammary gland expression. *, $P < 0.05$; **, $P < 0.01$. Black columns, AMV females; white columns, parous rats. $n = 4$ animals in each group.
IGF-I similar to that previously reported by Thordarson et al. (35). Although unexpected, human obesity studies have shown that GH suppression can exist without subsequent reduction in circulating IGF-I levels (49–52). It is possible that the reduction does not reach a threshold necessary for altering IGF-I levels, or that IGF-I secretion is mainly modulated by alterations in the amplitude and frequency of GH release, both of which we found to be unchanged by IGF-I (data not shown). Our result could be explained, in part, by examining the paracrine feedback loops in the liver responsible for IGF-I production and secretion. We showed that the moderate reduction (26% in WF and 37% in SD) in GH due to parity was not sufficient enough to alter the liver mRNA expression levels of ALS, a protein that has been shown to be independently regulated by GH (53, 54). Furthermore, parity had no effect on IGFBP-3 protein concentrations in sera (data not shown), thus suggesting that paracrine feedback loops regulating IGF-I secretion in the liver remained intact. Supporting this are previous studies in mice showing that both ALS and IGFBP-3 produced in the liver are required for the stability of IGF-I (55, 56). Overexpression of IGFBP-3 results in increased plasma IGF-I (56), and knock out of ALS results in decreased levels of circulating IGF-I and IGFBP-3 (55). Therefore, a single full-term pregnancy may not alter IGF-I, or it could be that that assessing GH levels 28 days after weaning was premature, not allowing for the body to conform to new homeostatic conditions, and subsequent analyses of parous animals at a later date may reveal more dramatic changes in IGF-I signaling due to chronic suppression of GH. In addition, extending our current animal model to include multiple pregnancies may also positively reflect a previous report showing that women having four or more pregnancies have significantly reduced circulating levels of IGF-I (34), but a single full-term pregnancy only resulted in a 12 ng/mL nonsignificant difference versus nulliparous women, which, translated to our current study, is similar.

Although we found that decreased circulating GH did not affect circulating IGF-I levels, we still detected a decrease in liver GH-R mRNA in parous animals. Previous studies have shown that hepatic GH-R mRNA is decreased during pregnancy and this coincides with a significant decrease in circulating levels of IGF-I (57). Hepatic GH-R levels remain suppressed throughout lactation; however, plasma IGF-I levels return to normal when compared with early pregnancy levels (57). We did not measure pre-pregnancy levels of liver GH-R or serum IGF-I; however, it is possible that the parity-induced decrease in liver GH-R mRNA levels may reflect a persistent decrease that first occurred during pregnancy. Furthermore, the normalization of IGF-I levels that occurs during lactation is presumably carried through to involution, and parity and may in part explain the paradox of decreased liver GH-R mRNA but unaltered circulating IGF-I level.

The mechanism whereby a full-term pregnancy regulates the episodic release of GH from the anterior pituitary is largely unknown. In WF rats, pituitary GH mRNA expression and serum GH are increased during mid-pregnancy, but by day 8 of lactation, both mRNA expression and serum levels are similar to early pregnancy (57). At the end of gestation, the cellular composition of the anterior pituitary changes dramatically due to the increase in prolactin-producing lactotrophic cells (necessary for lactation), thus reducing the availability of GH-producing somatotrophic cells (57). Therefore, the cellular balance in the pituitary may never fully be restored. However, permanent pregnancy-induced alterations in the pituitary are unlikely because we observed no change in pituitary GH mRNA levels in parous WF rats compared with AMV. This would suggest that, after pregnancy, GH transcriptional activity returns to normal, as well as the ratio of somatotrophs to lactotrophs in the anterior pituitary. Therefore, the more likely scenario is that the parity-induced reduction in serum GH levels is due to changes in regulatory inputs responsible for GH secretion. GH production and secretion from the anterior pituitary are primarily regulated by hypothalamic hormones GHRH and somatostatin (inhibitory) as well as negatively regulated by circulating IGF-I (for review, see ref. 58). Given that we found no change in circulating levels in IGF-I, it is unlikely that IGF-I is inhibiting GH release. Furthermore, GHRH directly controls the production and release of GH during pregnancy (59). Therefore, pregnancy could directly alter the hypothalamic secretion of GHRH and/or somatostatin, resulting in the observed decreased circulating levels of GH without directly altering GH transcription. How this is occurring is difficult to speculate. GHRH and somatostatin are highly regulated by hypothalamic neurotransmitters and neuuropeptides, which integrate to determine the pulsatile secretion of GH (60, 61), thus presenting an arduous challenge in deciphering the mechanistic regulation of GHRH-induced GH release in the parous animal. Additionally, metabolic peptides such as ghrelin (62) and glucocorticoids (63) can directly affect GH secretion from the pituitary and it might be that permanently pregnancy alters metabolic rates that account for the change in GH release.

Global gene expression studies have shown alterations in key pathways critical for mammary gland development and homeostasis, including GHR, IGF-I, amphiregulin, and transforming growth factor β (15, 42). We found that amphiregulin mRNA was lower in the parous mammary gland (data not shown); however, parity did not alter the levels of GH-R or IGF-I mRNA. This is in contrast to recent microarray data showing that GH-R and IGF-I mRNA expression levels are lower in rat mammary glands due to parity compared with age-matched AmV (42). However, the conflicting results may simply be explained by the analytic methods used in the two studies, given that we measured mRNA expression by quantitative PCR. However, Thordarson et al. (11) also showed that pregnancy reduced serum GH levels but did not alter GH-R mRNA expression in the mammary glands of SD rats compared with AmV.

Thus far, the effect of reducing the mRNA levels of growth factor signaling components in the mammary gland has not been correlated with the altered activation...
status of proteins in the same pathways. To this end, we examined signaling in the parous mammary gland compared with AMV and found that pregnancy reduces mammary gland levels of p-Jak2, p-Stat5A, and p-Akt, all key regulators of mammary cell proliferation and differentiation (64–66). Jak2 and Stat5A are essential downstream elements in GH-R signaling transduction, suggesting that the parous mammary gland maybe insensitive to GH action. This is an important observation, given that several studies have identified that alterations in the Jak2/Stat5 signaling pathway in the mammary gland lead to cancer (67).

In conclusion, we showed that a single full-term pregnancy significantly decreased circulating basal levels of GH, a hormone known to regulate mammary gland development (18–20, 68) and shown to play a role in mammary tumorigenesis (21, 24, 27, 43, 44). This reduction in serum GH was not sufficient enough to alter circulating IGF-I levels. However, the parity-induced reduction in serum GH downregulated key canonical GH signaling proteins critical for mammary gland epithelial proliferation. Our results suggest that pregnancy reduces circulating levels of GH, possibly by altering hypothalamic regulatory mechanisms. This correlates with reduced sensitivity to GH in mammary stromal cells, resulting in stromal-epithelial paracrine communication pathways that may lead to altered mammary gland function, making it less susceptible to tumorigenesis. Therefore, GH may play a role in pregnancy-induced protection from breast cancer.

Disclosure of Potential Conflicts of Interest

A.V. Lee: commercial research grant, Pfizer; honoraria from speakers bureau, Pfizer and Roche; consultant/advisory board, Pfizer and Roche. The other authors disclosed no potential conflicts of interest.

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