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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/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 aged-matched virgin (AMV) female rats. Mean serum GH levels were significantly decreased ($p<0.01$) in parous compared to AMV in both rat 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 Stat5A and Jak2, suggesting reduced action of GH in the mammary gland. Therefore, while the parity reduction in circulating GH doesn't impact upon circulating IGF-I levels, it is possible that reduced GH action directly at the mammary gland and may play a role in pregnancy protection from breast cancer.

Keywords

pregnancy; parity; breast cancer; growth hormone; IGF-I

INTRODUCTION

Numerous factors independently and/or synergistically influence the risk of developing breast cancer, and after age and a women'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 e.g. early menarche and late menopause (2–3). Supporting this, combined hormone

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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 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 to parous rats (16). Additionally, p53-null mammary epithelial cells also showed greater progression to neoplasia when transplanted into AMV compared to parous mice (17). Supporting a role for the host in regulation of pregnancy protection, Thordarson *et al.* (11) showed that parity persistently altered 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/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 (SDR), which has a mutation in the GH releasing hormone gene, resulting in non-detectable 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 tissue 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, epidemiological evidence indicates that individuals with circulating IGF-I levels at the higher end of the normal range predict increased risk, indicating increased GH/IGF axis activity is associated with postmenopausal breast cancer (33). Furthermore, an analyses of IGF-I levels in women from the Nurses Health study found that serum IGF-I levels are lower in parous compared to 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 is fragmentary, and one study found no effect upon 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-1 hormonal signaling, we assessed the ability of a single full term pregnancy to persistently alter the GH/IGF endocrine axis. We

compiled extensive hormone pulse profiles from two commonly used pregnancy protection rat models: Sprague Dawley (outbred) and Wistar Furth (inbred). In the current study, we provide evidence that a single full term pregnancy is sufficient to lower basal levels of circulating GH in both rat strains but that this change is not associated with a change in pulse amplitude or frequency. Furthermore, we show parity-associated downregulation of key GH signaling proteins in the mammary gland. A greater understanding of how the GH/IGF-I axis is normally altered by pregnancy and how this is maintained following involution may highlight novel mechanisms for the prevention of human breast cancer.

MATERIAL AND METHODS

Animals

This study used rats of the Wistar Furth (WF) inbred line purchased from Harlan Laboratories, Houston, TX, as well as rats of the Sprague Dawley (SD) outbred line purchased from Charles River, Boston, MA. Adult female rats were housed two per cage on Sanichips (Sanipure food labs, Saddlebrook, NJ) 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 4 per cage and allowed to naturally involute for 28 days. AMV controls for both strains were housed 4 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), lights (lights on: 0600h; lights off: 1800h) and ad libitum access to food (Harland Teklad Diet, Madison, WI) and tap water.

After 28 days of involution (Figure 1), parous SD and WF females and their respective AMV controls (all approximately 120–125 days 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 prior to collecting each blood sample. To encompass GH pulsatile fluctuations and account for early A.M. basal level increases in GH as previously reported (37), blood samples (250μl) were collected at 8:00 am every 30min for four and a half hours. All animals received blood cell replacement after 2hrs of sampling in order to avoid anemia. After the last sample, blood was centrifuged and serum collected and stored at −80°C until assayed. All animals were assigned to their respective phase of estrous by well-defined criteria established previously (38) and mammary glands and hepatic tissue were collected for further analysis.

Whole gland morphological and histological analysis

Mammary gland whole mounts were processed similar to a procedure developed by Williams and Daniel (39) with the following modifications. Briefly, #4 inguinal mammary glands from the left side were removed from 5 parous and 5 AMV controls (in both strains) at 120–125 days of age and spread flatly on the inner surface of a 50ml tube and fixed with with 10% Formalin in PBS. The next day, tissue was placed in a cassette and fat was removed using acetone for 48 hrs. Samples were dehydrated in 100% ethanol (EtOH) for 1 hr, 95% EtOH for 1hr, and stained with Carmine Alum. Mammary glands were destained as follows: H_20 for 1hr.; 70% EtOH for 1hr.; 95% EtOH for 1hr.; 100% EtOH 3x for 1hr.; and cleared in xylene 3x for 1hr. Finally tissues were permanently stored in glass vials filled with methylsalicylate until analyzed.

The #4 inguinal mammary gland from the right side (5 vs. 5 each strain) was harvested placed in cassettes and fixed in 4% paraformaldehyde in PBS overnight. The next day, paraformaldehyde was replaced by 70% EtOH and samples were embedded in paraffin. Serial sections (5 μm thick) cut from paraffin blocks were placed on Superfost Plus slides (Fisher Scientific, Fair Lawn, NJ), deparaffinized, gradually hydrated and all sections stained with Hematoxylin-Eosin (H&E) and then examined microscopically.

Immunoblot analysis

Frozen mammary glands and livers were first crushed under liquid nitrogen using a metal pestle and mortar. Crushed tissue was lysed in TNESV buffer and 100μg of tissue protein lysate was immuonblotted as described previously (40). We used phospho-specific antibodies: p-Jak2 1:500 (Cell Signaling Technology, Beverly, MA, USA), p-Stat 5 A/B 1:500 (Upstate Group, Inc., Lake Placid, NY, USA), p-AKT 1:1000 (Cell Signaling Technology, Beverly, MA, USA), p-ERK1/2 1:1000 (Cell Signaling Technology, Beverly, MA, USA), Jak2 1:1000 (Cell Signaling Technology, Beverly, MA, USA), Stat 5 A/B 1:1000 (Santa Cruz Biotechnology, Santa Cruz, California, USA), AKT 1:1000 (Cell Signaling Technology, Beverly, MA, USA), ERK1/2 1:4000 (Upstate Group, Inc., Lake Placid, NY, USA), and β-catenin 1:4000 (BD Biosciences, San Jose, CA, USA). Infrared (IR) fluorescent-labeled anti-rabbit (IRDye 800) or anti-mouse (Alexa Flour 680) antibodies 1:5000 (Rockland Immunochemicals, Gilbertsville, PA, USA) were used as a secondary antibody and images were acquired and densitometrically analyzed using the Odyssey infrared imaging system (Li-Cor biosciences).

Quantitative RT-PCR

Total RNA was extracted from mammary gland, pituitary, and hepatic tissue using a RNeasy Mini kit (Qiagen) following the manufacturer's protocol. Total RNA (3.125 μg) was treated with DNAse (2μl) in 10x PCR buffer (10.5 μl) with 50nM of MgCl (Invitrogen) and RNase free water (bring the total volume to 125μl) for 30 minutes at 37°C followed by 10 minutes at 75°C prior to reverse-transcription. DNase treated RNA (50ng/μl) was reverse transcribed using 5x First Strand Buffer, 100mM DTT, 25mM dNTPs, 20μM reverse primer, dH_20, and 200 μ/μl MMLV Reverse Transcriptase following the manufacturer's protocol. Next, the cDNA product was analyzed by TaqMan quantitative PCR (Q-PCR) in standard conditions using an Applied Biosystems 7700 Prism thermocycler. Data was 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, Foster City, CA, USA) and synthesized by Eurogentec (San Diego, CA, USA) are listed in Supplemental Table 1.

Hormone Assays and Statistical Analysis

Growth Hormone and prolactin levels were measured in serum using radioimmunoassay (RIA) by Dr. A.F. Parlow, Director, Pituitary Hormones & Antisera Center, Harbor-UCLA Medical Center, Torracne, CA, USA. Circulating levels of total IGF-I were measured in serum by a single assay using a rat/mouse IGF-I immunoenzymometric assay (IEMA) purchased from Immunodiagnostic Systems (Boldon, Tyne & Wear, UK) and confirmed using a separate IGF-I RIA purchased from DSL (Webster, TX). The assay sensitivity was 82 ng/ml and 150ng/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 non-parametric Student's *t* test; results were the same and thus the parametric test was used to calculate all results. The IBM PC programs INSTAT

and PRISM software (GraphPad, San Diego, CA, USA) 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 to AMV in two different rat strains: Sprague Dawley (SD; outbred) and Wistar Furth (WF; inbred). The overall design of the experiment is depicted in Figure 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 120–125 day old parous and AMV rats from both rat strains. In Figure 3A, we compared the average serum GH levels over the 4½ hour time period between the AMV and parous animals depicted in figure 2. Specificallly, in WF rats, the mean level of circulating GH was significantly ($p<0.001$) reduced in parous (8.80 ± 0.45 ng/ml; mean ± SEM) compared to AMV rats (11.91 ± 0.59 ng/ml: mean ± SEM). Similarly, parity in SD rats significantly decreased ($p< 0.001$) mean serum GH levels with parous rats having 7.37 ± 0.81 ng/ml (mean ± SEM) compared to 11.67 ± 0.74 ng/ml (mean ± SEM) in AMV rats (Figure 3B; average). Additionally, in Figure 3B we compared the mean of the single highest concentration of GH in AMV and parous rats (the peak pulse release) shown in figure 2. As expected, parity significantly decreased peak GH release from 16.84 ± 1.13 ng/ml (AMV; mean ± SEM) to 12.13 ± 0.79 ng/ml (parous; mean ± SEM) in WF ($p< 0.01$) and 16.79 ± 1.32 ng/ml (AMV; mean ± SEM) to 11.57 ± 1.69 ng/ml (parous; mean ± SEM) in SD ($p< 0.05$) rats. Additionally, parous and AMV females were grouped based on their estrous cycle stage as depicted in Figure 3(C and D). Parity significantly reduced ($p<0.05$) GH levels in both estrus and diestrus compared to 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 β-actin determined by Q-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 to controls (Figure 4A), despite the decreased in serum levels of GH in the same animals (Figure 4B).

One of the major effects of GH is to increase hepatic output of IGF-I and thus raise circulating levels. We therefore measured circulating IGF-I in the same rats. As IGF-I does not exhibit episodic secretion like GH, levels were measured in 3 serum samples (8.30 am, 10am and 12pm from the 9 that were collected) and the mean level was calculated (Figure 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 (Figure 5B). Similarly, immunoblot analysis showed that serum protein levels of 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 upon 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 growth hormone 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 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 morphological changes in the mammary gland due to parity (Figure 6A). An analysis of key signaling proteins involved in GHR signal transduction in WF mammary gland extracts (Figure 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 ($p< 0.01$) (Figure 6C). Furthermore, parity induced a reduction in p-AKT ($p< 0.05$) mammary gland protein expression compared to age-matched controls. Surprisingly, p-ERK 1/2 expression in the mammary gland was not significantly different ($p= .098$) between parous and age-matched control. Similar results were observed in SD females (data not shown). Interestingly, Q-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 to controls (Figure 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 (Figure 6B).

Discussion

Current dogma suggests that a full term pregnancy results in permanent changes in key proliferative (IGF-I & TGF β) and tumors 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 (MNU) (16) are transplanted into estrogen/progesterone pre-treated 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.* which showed that a single time point measurement of serum GH in 120 day old parous SD females was lower compared to AMV (11). 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 are due to an overall reduction in GH secretion and not to 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 implicates GH and IGF-I action 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 (SDR), which has a mutation in the GHRH gene resulting in non-detectable circulating GH and subsequently low IGF-I levels (24). Recently, Shen *et al.* showed that SDR females treated with the chemical carcinogen MNU do not develop mammary tumors, however rat or bovine GH replacement in these

animals initiated mammary tumorigenesis comparable to MNU-treated controls (27). Similar results were shown in Lewis SDRs, 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 to 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. While 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 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 didn't reach a threshold necessary for altering IGF-I levels, or that IGF-I secretion is mainly modulated by alterations in 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 liver ALS mRNA expression levels, a protein which has been shown to be independently regulated by GH (53–54). Furthermore, parity had no effect on IGFBP-3 protein concentrations in serum (data not shown); thus, suggesting that paracrine feedback loops regulating IGF-I secretion in the liver remained enacted. 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 knockout 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 4 or more pregnancies have significantly reduced circulating levels of IGF-I (34), but a single full term pregnancy only resulted in a 12ng/ml non-significant difference vs. nulliparous women, which translated to our current study, is similar.

While we found that decreased circulating GH didn't affect circulating IGF-I levels, we did still detect 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 to early pregnancy levels (57). We didn't 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 are 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 is 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 since we observed no change in pituitary GH mRNA levels in parous WF rats compared to 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 is primarily regulated by hypophysiotropic hormones GH-releasing hormone (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 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 neuropepites which integrate to determine the pulsatile secretion of GH (60–61), thus presenting an arduous challenge in deciphering the mechanic 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 alteration in key pathways critical for mammary gland development and homeostasis including GH-R, IGF-I, amphiregulin, and TGF-β (15, 42). We found that amphiregulin mRNA was lower in parous mammary gland (data not shown), however, parity didn't alter 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 to age matched AMVs (42). However, the conflicting result may simply be explained by the analytical methods used in the two studies given we measured mRNA expression by Q-PCR. However, Thordarson *et. al* also showed that pregnancy reduced serum GH levels but did not alter GH-R mRNA expression in the mammary glands of SD rats compared to AMVs (11).

Thus far, the effect of reducing mRNA levels of growth factor signaling components in the mammary gland hasn't been correlated with altered activation status of proteins in the same pathways. To this end, we examined signaling in the parous mammary gland compared to 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 to the Jak2/Stat5 signaling pathway in the mammary gland leads to cancer (67).

In conclusion, we demonstrated 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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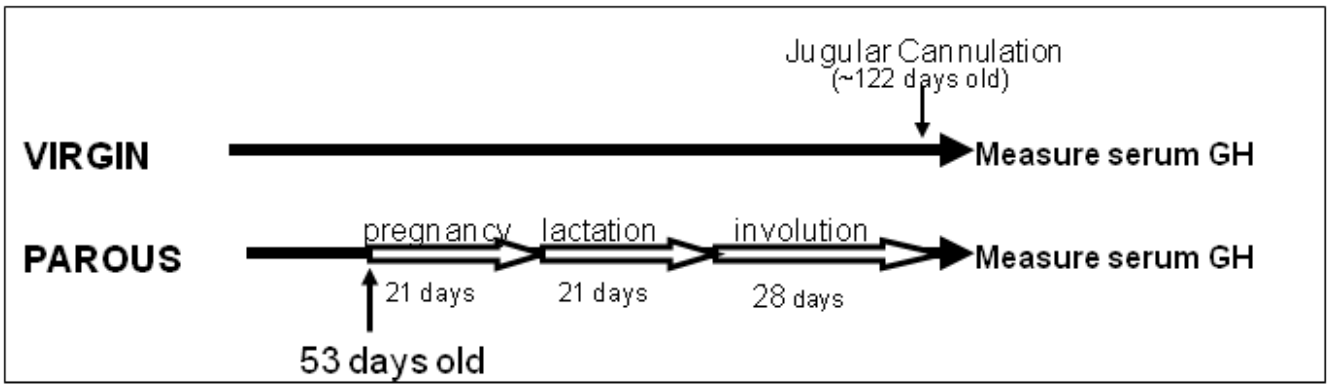

Figure 1. Schematic illustration of the experimental protocol

In brief, parous animals were bred on 53 days of age and underwent a single full term pregnancy, lactation, and were allowed 28 days for the mammary gland to involute. The next day jugular cannulas were inserted, 24hrs later GH was measured for 4 ½ hours. All females were between 120–125 days old (range due to conception) when serum and tissue was collected.

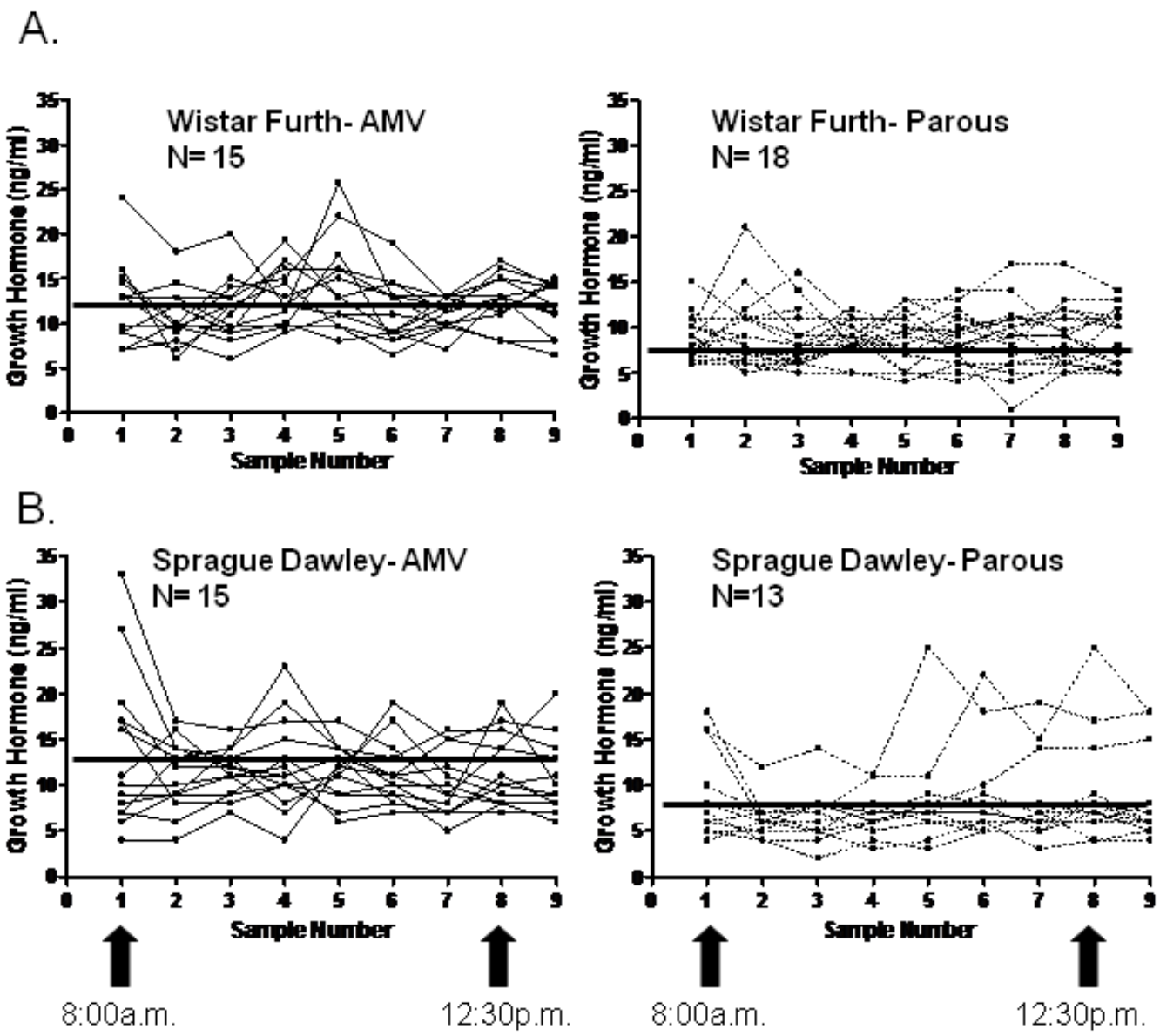

Figure 2. Individual serum growth hormone pulse profiles

Serum GH levels plotted at every time point for individual Wistar Furth (A) and Sprague Dawley (B) AMV (left panel) and parous (right panel) females after involution. Note that parity in both rat strains results in an overall basal decrease in GH levels throughout the 4 ½ hour pulse period (8:00 am – 12:30 am). Thick black horizontal line represents the mean GH levels for each group.

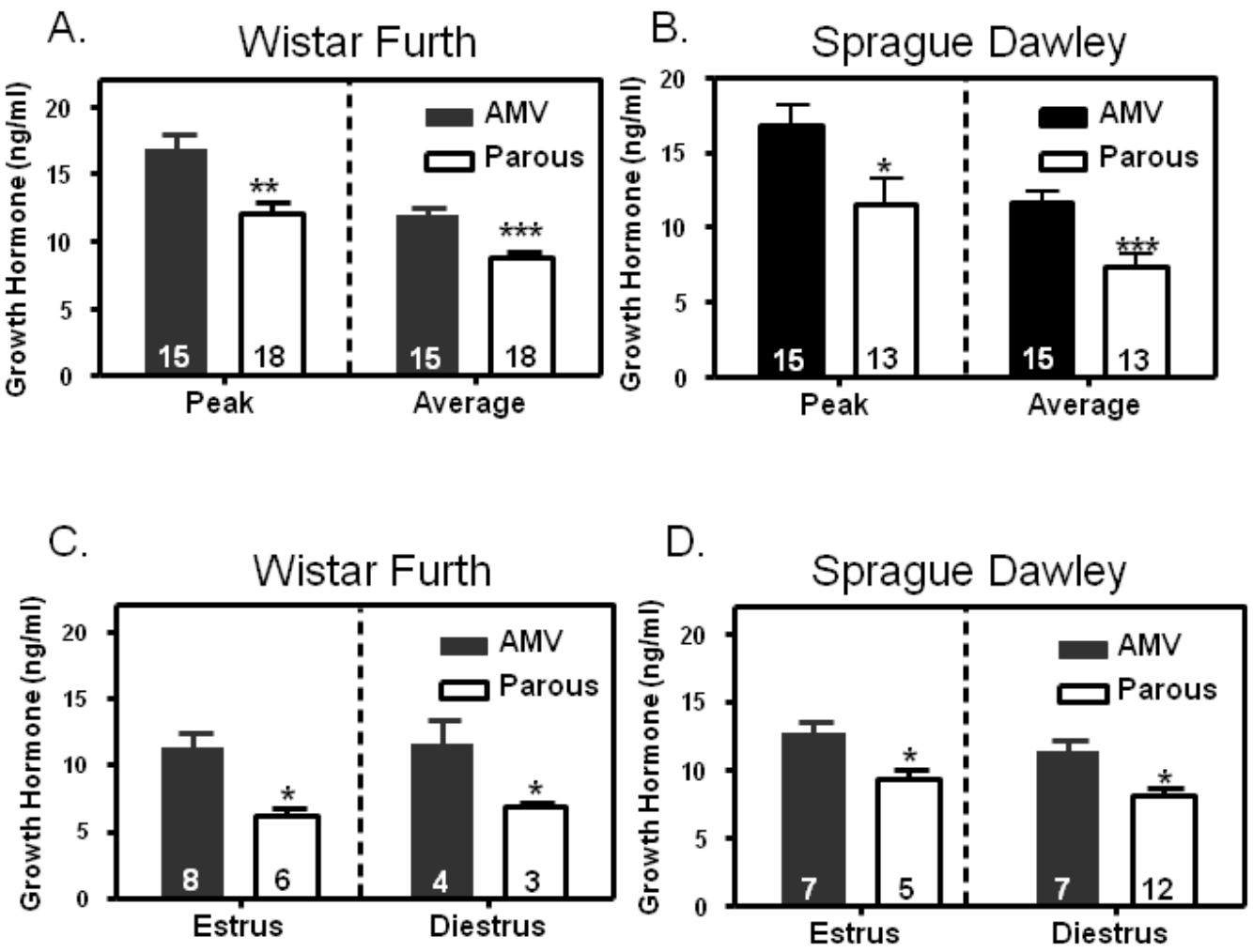

Figure 3. Parity lowers growth hormone in Wistar Furth and Sprague Dawley rats

A and B) Parity results in a decrease in average (±SEM) circulating levels of GH in both Wistar Furth (A.) and Sprague Dawley (B) rat strains compared to AMVs. In addition, mean (±SEM) peak pulse release of GH levels were significantly decreased in parous vs. controls over the 4 ½ hour pulse period in both rat strains. C and D) Estrous cycle had no effect on parity induced decrease in circulating growth hormone levels of Wistar Furth (C) and Sprague Dawley (D) AVM and parous females were separated based on stage of estrous. As expected, stage of cycle did not alter the pregnancy effect on mean (±SEM) serum GH concentrations, in that parity significantly reduced ($p<0.05$) GH levels in both estrus and diestrus compared to age matched AMVs in both rat strains. * $p< 0.05$; ** $p< 0.01$; *** $p<0.001$. Black bar represents AMV (age-matched virgin) females and white bar indicates parous rats. Number of animals per group indicated within bars.

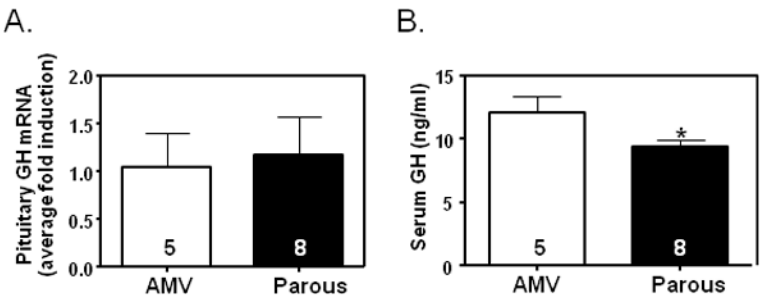

Figure 4. Pregnancy-induced reduction of serum GH is not associated GH-mRNA expression in the pituitary

A.) Compares the mean (± SEM) GH-mRNA fold induction normalized to β-actin between parous and AVM Wistar Furth females. Note there was no change in pituitary GH mRNA expression due to pregnancy in the pituitary from individual AMV and parous Wistar Furth animals after 28 days of involution as determined by Q-RT-PCR, B.) The average (±SEM) circulating levels of GH over the 4 ½ hour pulse period from animals depicted in figure 4A. * $p < 0.05$. Black bar represents AMV (age-matched virgin) females and white bars indicate parous rats. Number of animals per group indicated within bars.

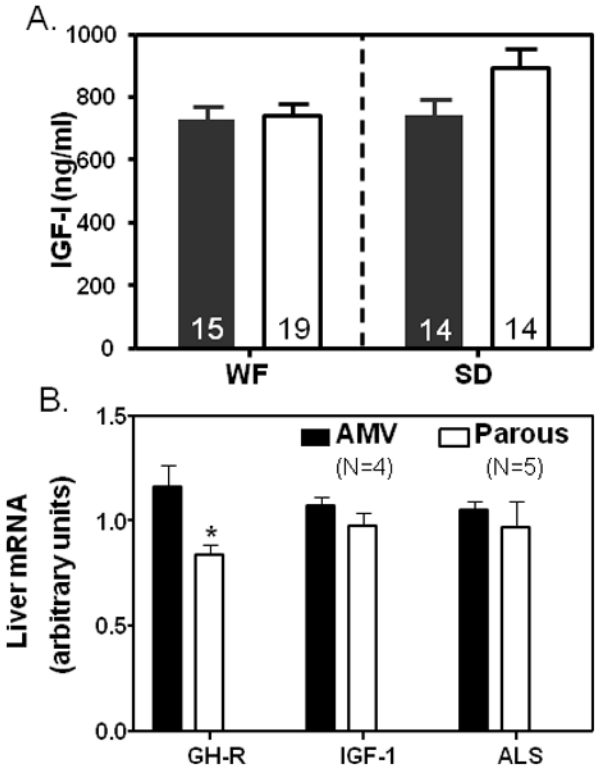

Figure 5. Single full term pregnancy does not alter circulating levels of IGF-I but decreases GH-R expression in the liver

A.) Serum IGF-I levels were not altered due to parity in either Wistar Furth (left panel) or Sprague Dawley (right panel) females compared to AMVs B.) Hepatic GH-R, IGF-I and ALS mRNA levels from parous and AMV Wistar Firth rats were isolated and analyzed by real time Q-RT-PCR. * $p < 0.05$; Black bar represents AMV (age matched virgin) females and white bar indicates parous females A.) Number of animals per group indicated within bars. B.) N= 4 animals in AMV (age-matched virgin) group and N=5 in parous group.

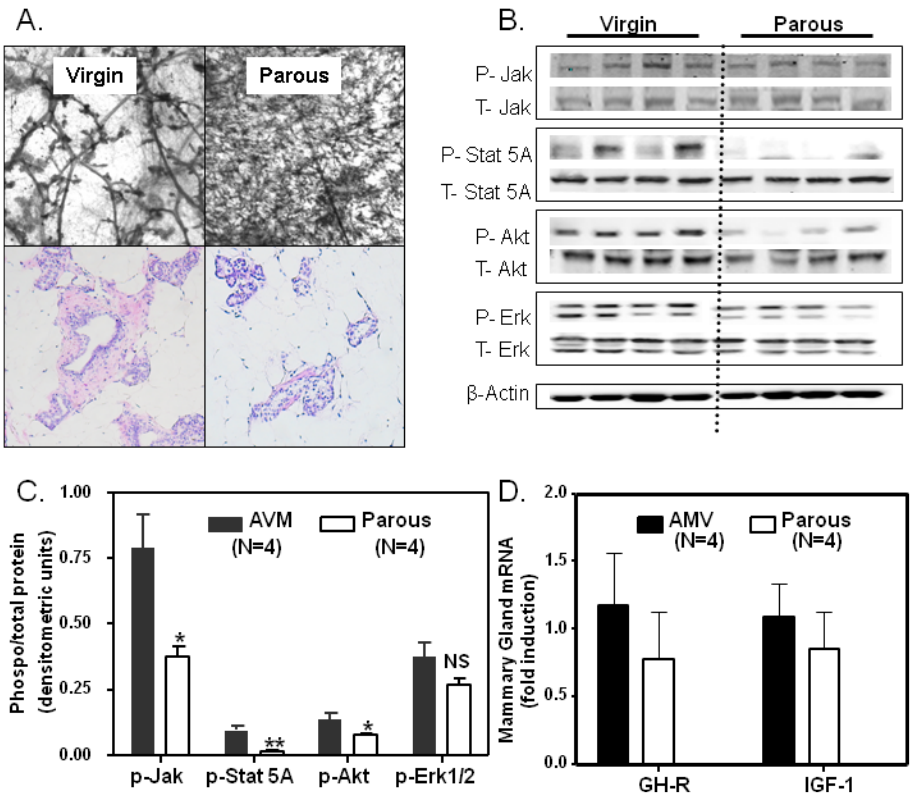

Figure 6. Parity downregulates classic GH-R downstream signaling molecules

A.) Representative whole mounts (top) and H&E staining (bottom) of Wistar Furth mammary glands. B.) Representative immunoblots of Wistar Furth mammary glands (4 AMV and 4 parous) stained for common mammary gland signaling proteins. β-Actin was used as a loading control, respectively. C.) Densitometric analysis revealed that a full term pregnancy significantly downregulated the phosphorylation of signaling modulators Jak, Stat 5A, and Akt compared to AMVs. Notably there was no change in Erk 1/2 signaling between groups. Densitometric units represent phosphorylated protein/total protein for each signaling molecule. D.) Fold GH-R and IGF-I levels in parous mammary glands compared to age-matched controls; mRNA from parous and AMV Wistar Firth rats were isolated and analyzed by real time Q-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 bar represents AMV (age-matched virgin) females and white bar indicates parous rats. N= 4 animals in each group.