Leptin modulates fertility under the influence of elevated growth hormone as modeled in oMt1a-oGH transgenic mice

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Abstract

Elevated growth hormone (GH) concentrations suppress reproductive function in a variety of species, although it is unclear whether GH directly suppresses reproductive performance, or whether GH activates other pathways to achieve these effects. The ovine metallothionein 1a-ovine GH (oMt1a-oGH) transgenic mouse has been used to model the effects of GH on both body composition and reproductive function. A recent report has documented increased leptin levels in obese oMt1a-oGH mice. Given the importance of leptin in modulation of the reproductive endocrine axis, as well as the reports documenting reduced leptin signal transduction in animals with elevated leptin levels, we hypothesized that high leptin concentrations in response to elevated GH would reduce fertility. To determine the effects of high circulating leptin levels on the reproductive endocrine axis, we assessed hypothalamic neuropeptide Y (NPY) and GnRH expression. At weaning, oMt1a-oGH transgenic (TG) and wild-type (WT) female mice were allocated to one of four treatment groups: oMt1a-oGH females chronically expressing the transgene (TG ON); oMt1a-oGH females expressing the

transgene from 3 to 8 weeks of age (TG ON/OFF); WT females receiving the transgene stimulus from 3 to 8 weeks of age (WT ON/OFF); and WT females never receiving the transgene stimulus (WT OFF). Eight-week-old females were housed with males for a 2-week period, after which females were isolated from males and allowed to carry pregnancies to term. Body and gonadal fat pad (GFP) weights, along with plasma leptin concentrations, estrous cyclicity, pregnancy rate and litter characteristics, were recorded for each female. Chronic expression of the oMt1a-oGH transgene resulted in larger leaner mice, and inactivation of the transgene produced obese females. Pregnancy rate was reduced in TG ON females when compared with all other groups, and infertility was associated with elevated leptin levels. In addition, high leptin levels were associated with increased NPY expression, suggesting reduced leptin-signaling capacity, which may contribute to suppression of the reproductive axis in oGH animals.

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Introduction

High circulating growth hormone (GH) concentrations in females result in decreased reproductive performance in mice (Murray & Pomp 1995, Pomp *et al.* 1995, Thomas *et al.* 2001), humans (Jadresic *et al.* 1982) and livestock (McGuffey *et al.* 1991, Downer *et al.* 1993, Oldenbroek *et al.* 1993, Esteban *et al.* 1994, Buskirk *et al.* 1996, Chapula *et al.* 1996). It is still unclear whether elevated GH suppresses reproductive function directly or through an intermediary pathway.

The ovine metallothionein 1a-ovine GH (oMt1a-oGH) transgenic mouse model has been a useful tool for the study of GH effects on reproduction. While many of the GH transgenic constructs currently in use utilize promoters that constitutively drive expression of the GH transgene (Palmiter *et al.* 1983, Orian *et al.* 1989), the oMt1a

promoter is regulated by provision or exclusion of 25 mM ZnSO₄ in the drinking water (Shanahan *et al.* 1989). Oberbauer *et al.* (1997) reported average plasma oGH concentrations of 2279 ng/ml in animals overexpressing the transgene, but inactivation of the transgene results in a drop in oGH concentrations to unstimulated levels within 24 h (Shanahan *et al.* 1989). The oGH females overexpressing the transgene are subfertile and unable to support implantation, despite the fact that normal blastocysts are observed at day 3.5 of pregnancy (Pomp *et al.* 1995).

Expression of the oGH transgene results in a lean phenotype, while transgene inactivation promotes accrual of adipose tissue and eventual obesity (Pomp *et al.* 1996, Oberbauer *et al.* 1997, Oberbauer & Murray 1998). Oberbauer *et al.* (2001) reported significantly higher circulating leptin concentrations in oGH females with an inactivated oGH transgene than in mice chronically

overexpressing oGH. This was attributed to larger adipose deposits in the former group. Many of the clinical problems documented in women with polycystic ovarian syndrome (PCOS) are exacerbated by obesity (Jacobs & Conway 1999), and PCOS is often associated with increased leptin concentrations in subpopulations of affected women (Brzechffa *et al.* 1996). It was not until recently, however, that the link between body composition and reproduction was identified. The leptin (*ob*) gene cloned from white adipose tissue by Zhang *et al.* (1994) offered insight. Circulating leptin levels are proportional to the amount of energy stores contained within the body (Rosenbaum *et al.* 1996), and very high circulating leptin concentrations are associated with impaired reproductive function (Brzechffa *et al.* 1996). Collectively, these data suggest that leptin may play a role in suppressing reproductive function in obese individuals.

Under normal physiological conditions, leptin modulates neuropeptide Y (NPY) expression, which when chronically elevated suppresses fertility (Catzeffis *et al.* 1993, Pierroz *et al.* 1995). However, when circulating leptin levels are elevated, as seen in obese individuals, leptin signaling may be reduced (Campfield *et al.* 1995, 1996, Frederich *et al.* 1995, Halaas *et al.* 1997, Sinha & Caro 1998). Arcuate NPY neurons contain glucocorticoid receptors (Cintra *et al.* 1991), and the presence of a glucocorticoid response element (GRE) in the promoter region of several mammalian NPY genes (Moore *et al.* 1985, Misaki *et al.* 1992) indicates the potential for glucocorticoids to stimulate NPY expression. oMt1a-oGH transgenic mice actively expressing the transgene have elevated plasma corticosterone concentrations (Thomas *et al.* 2001), suggesting that glucocorticoids may elevate NPY expression in these mice. Elevated NPY expression, then, could contribute to the reduced fertility observed in oMt1a-oGH mice. In infertile oMt1a-oGH females, reduced leptin signal transduction may attenuate the ability of leptin to downregulate NPY, resulting in suppression of gonadotropin-releasing hormone (GnRH) release, gonadotropin release and reproductive function.

Previous reproductive studies utilizing oMt1a-oGH mice have evaluated females mated at 8–12 weeks of age (Murray & Pomp 1995, Pomp *et al.* 1995, Thomas *et al.* 2001) when differences in body composition and leptin concentrations are just becoming pronounced between groups experiencing various elevated GH-exposure regimens (Oberbauer *et al.* 2001). However, studies correlating reproductive function with body composition data, GH effects and leptin have not been done. The objective of this study was to elucidate the endocrine mechanism(s) by which the somatotrophic axis affects adipose metabolism and reproductive function. The first specific aim was to characterize fertility as a function of transgene status, body composition and circulating leptin concentrations. The second aim was to correlate leptin levels with hypothalamic NPY and GnRH expression in order to assess the

impact of varying levels of leptin on the reproductive endocrine axis.

Materials and Methods

Animals

The transgenic mice used in this study were produced by crossing mice homozygous for the oMt1a-oGH construct (Shanahan *et al.* 1989) in a CCX (C57/Bl6 x CBA) background to CCX wild-type (WT) mice. The resulting offspring were hemizygous for the transgene. WT cohorts were derived from CCX by CCX crosses. At 9–11 days of age, all female mice were toe-notched for identification purposes. At 3 weeks of age, females were weaned and allocated into one of four treatment groups: transgenic mice continuously overexpressing the GH transgene from 3 weeks of age (TG ON, $n=182$); transgenic mice expressing high levels of GH from 3–8 weeks of age (TG ON/OFF, $n=193$); WT mice receiving the transgene stimulus from 3–8 weeks of age but not expressing high GH levels (WT ON/OFF, $n=197$); and WT mice not receiving the transgene stimulus (WT OFF, $n=202$); the last two groups express normal levels of murine GH. Mice had free access to food (Purina Mouse Chow 5001, St Louis, MO, USA) and drinking water. Transgene expression was induced by inclusion of 25 mM ZnSO₄ (Sigma Chemical Inc., St Louis, MO, USA) in the drinking water; GH levels in the transgenics decrease to peak WT levels within 24 h after removal of the zinc stimulus (Shanahan *et al.* 1989). Animals were housed under controlled conditions of photoperiod (14 h light:10 h dark), humidity (55%) and temperature (65–70 °F) in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-approved animal facility in accordance with NIH guidelines for animal care.

Experiment 1

Body weights were recorded weekly from weaning to completion of the experiment. At 15, 20, 25, 30, 35 or 40 weeks of age, a subset of females ($n=30$) from each treatment group was placed with males of proven reproductive performance for 14 days. During this time, females were assessed daily for the presence of a vaginal copulatory plug. Females were isolated from males after the mating period. Pregnant females were allowed to maintain pregnancies to term, after which time pups were immediately removed from the dam. Reproductive data collected included total number of copulatory plugs observed, pregnancy rate and pregnancy characteristics, including gestation length and number of live and dead pups produced. Mice failing to produce litters were determined to be cycling if they demonstrated two periods of proestrus, punctuated by a period of diestrus. Proestrus was confirmed by the presence of epithelial cells, and diestrus by the presence of leukocytes in the vaginal smear.

Table 1 Sequence of PCR primers and real-time TaqMan probes specific for murine GAPDH, NPY and GnRH

	Primer name	Sequence (5'→3')	TaqMan probe name	Sequence (5'→3')
Target				
GAPDH	mGAPDH-167f mGAPDH-263r	GCACAGTCAAGGCCGAGAAT GCCTACCCCATTTGATGTT	mGAPDH-217p	ACCATCTTCCAGGAGCGAGACCCCA
NPY	mNPY2-165f mNPY2-247r	CATCAATCTCATCACCAGACAGAGAT TGCTTTCCTTCATTAAGAGGTCTGA	mNPY2-192p	TGGCAAGAGATCCAGCCCTGAGACA
GnRH	mGnRH-13f mGnRH-89r	CTGATGGCCGGCATTCTACT GGGCGCAACCCATAGGA	mGnRH-34p	GGAGCAGCCTTCCAAACACACAGTCAG

Females were weighed and killed by CO₂ asphyxiation 14 days after parturition. Mice failing to produce litters were killed at a comparable age. Blood was collected by cardiac puncture, and the right gonadal fat pad (GFP) dissected from the carcass and weighed for later correlation to plasma leptin levels and final body weight. The GFP is highly correlated with overall body fat content and can be used as an indicator of total body lipid (Rogers & Webb 1980, Eisen & Leatherwood 1981). Blood was transferred to EDTA tubes (Fisher Scientific, Santa Clara, CA, USA) and centrifuged at 1800 g for 20 min, and plasma was collected and stored at -70 °C. Plasma leptin concentrations were measured by ¹²⁵I radioimmunoassay kit according to instructions provided by the manufacturer (Linco Research, St Charles, MO, USA).

Experiment 2

A decline in reproductive performance was observed in females mated at 40 weeks when compared with those mated at earlier ages. Therefore, further reproductive studies were undertaken to elucidate the causes for reduced fertility. Elevated leptin concentrations were associated with infertility in the previous experiment, and as leptin is known to modulate NPY expression, which in turn modulates the reproductive endocrine axis, we also assessed hypothalamic NPY and GnRH expression as a function of leptin concentrations and transgene activation status.

At either 15 or 40 weeks of age, females from each treatment group were placed with males of proven reproductive performance for 14 days, and treated as in the previous experiment. At 14 days after parturition, or at a comparable time point in infertile animals, females were killed by CO₂ asphyxiation, and brain tissue was dissected and processed for either the real-time PCR or *in situ* hybridization procedures. The stage of the estrous cycle at time of death was assessed in order to ensure basal measurements of hypothalamic NPY and GnRH expression, and to avoid the transient increases in NPY and GnRH expression that occur during proestrus and estrus (Suzuki *et al.* 1995). Therefore, only animals demonstrating diestrus or metestrus on the day of death were assessed

for hypothalamic NPY and GnRH mRNA expression. Blood was collected by cardiac puncture, and plasma leptin levels were determined. Plasma leptin concentrations were measured by ¹²⁵I radioimmunoassay kit (Linco). The intra-assay and interassay coefficients of variation were 2.57% and 6.01% respectively. The detection limit of the assay was 0.86 ng/ml.

Real-time PCR

Real-time PCR was used to quantitate hypothalamic NPY and GnRH expression. Briefly, following blood collection, mice were decapitated and the brain was removed. The hypothalamus was excised within 2–3 min of death according to anatomical landmarks; it is bounded by the optic chiasm anteriorly, the mamillary bodies posteriorly and the floor of the third ventricle dorsally (Zeman & Innes 1963). Hypothalamic tissue was then snap frozen in liquid nitrogen, and stored at -70 °C until RNA extraction.

One 20–40 mg piece of hypothalamic tissue per animal was placed in a tube with liquid nitrogen and pulverized with a mortar and pestle. Total RNA was extracted from the samples, according to the manufacturer's directions for the RNeasy Mini Kit (Qiagen). RNA was eluted with nuclease-free water into tubes previously treated with RNase-free DNase I (Fisher, Santa Clara, CA, USA) to eliminate contaminating genomic DNA. Samples were heated for 5 min at 95 °C and then cooled on ice.

Synthesis of cDNA was achieved by adding RNA to a reverse transcriptase reaction consisting of 5 × first-strand buffer (Invitrogen, Carlsbad, CA, USA), 10 mM dNTPs, 0.1 M DTT, 40 U/μl RNaseOut (Invitrogen), 200 U/μl SuperScript II (Invitrogen), and 300 ng/μl random hexamers (Promega). The reaction was incubated at 42 °C for 50 min, heat inactivated for 5 min at 95 °C, cooled on ice, and stored at -20 °C until use.

For each target gene, two primers and an internal, fluorescent-labeled TaqMan probe (5' end, reporter dye FAM (6-carboxyfluorescein), 3' end, quencher dye TAMRA (6-carboxytetramethylrhodamine)) were designed, using Primer Express software (Applied Biosystems, Foster City, CA, USA) (Table 1). As an

endogenous control, a real-time PCR system was designed to target murine glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The length of the PCR products was restricted to very short fragments (73–170 bp) to enable high amplification efficiencies.

Each 12 µl PCR reaction contained 400 nM of each primer, 80 nM of the real-time probe, a commercially available PCR mastermix (TaqMan Universal PCR Mastermix, Applied Biosystems) containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 5 mM MgCl₂, 2.5 mM deoxynucleotide triphosphates, 0.625 U AmpliTaq Gold DNA polymerase per reaction, 0.25 U AmpErase UNG per reaction, and 5 µl diluted cDNA sample. The samples were placed in 96-well plates and amplified in an automated fluorometer (ABI PRISM 7700 Sequence Detection System, Applied Biosystems). Amplification conditions were 2 min at 50 °C, 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 60 s at 60 °C. Final quantitation was done by the comparative C_T method (User Bulletin no. 2, Applied Biosystems) and is reported as relative transcription relative to a calibrator cDNA. In brief, the signal of the endogenous control GAPDH was used to normalize the target gene signals of each sample. The difference in the C_T for the target and the C_T for the internal control, termed ΔC_T , was calibrated against a control group. The relative linear amount of target molecules relative to the calibrator, was calculated by $2^{-\Delta\Delta C_T}$. Therefore, all target gene transcription is expressed as an n-fold difference relative to expression in the calibrator group.

In situ hybridization

The *in situ* procedure was undertaken to confirm the NPY and GnRH expression data from the real-time PCR. Animals in either diestrus or metestrus on the day of death were assessed for hypothalamic NPY or GnRH expression. Within moments of death, brains were dissected free of the skull, and two transverse cuts at the level of the optic chiasm and mamillary bodies were employed in order to generate a coronal brain section encompassing the hypothalamus. These sections were placed, cerebellum-side down, in cryomolds, which were then filled with embedding media and frozen with a combination of isopentane and liquid nitrogen. A beaker of isopentane was suspended in a container of liquid nitrogen to facilitate cooling of the isopentane. Specimens were then immersed in the cold isopentane until frozen. Frozen sections were stored at –80 °C until sectioning. Coronal brain sections were cut at 14 µm through both the arcuate nucleus and the medial preoptic area with a cryostat and thaw-mounted onto poly-L-lysine-coated microscope slides (Electron Microscopy Services, Fort Washington, PA, USA). Sections were fixed in 4% paraformaldehyde and stored in 95% ethanol at 4 °C until use.

The *in situ* hybridization procedure was performed using oligonucleotides (Operon, Alameda, CA, USA)

synthesized to recognize unique coding sequences in the genes of interest. The specific probes used were anti-sense NPY (5' GAGTAGTATCTGGCC ATGTCCTCTGC TGGCGCGTCCTCGCCCGG 3') (Guan *et al.* 1998) and anti-sense GnRH (5' TTCAGTGTTCCTCTTTC CCCCAGGGCGCAACCCATAGGACCAGTGCTG 3') (Fueshko *et al.* 1998). Sense probes were also generated as controls. Probes were end-labeled with [³³P] dATP (New England Nuclear, Boston, MA, USA), using terminal transferase (Boehringer-Mannheim, Indianapolis, IN, USA). Hybridization and washing conditions were as described by Wisden and Morris (1994). Briefly, a diluted radiolabeled probe was applied to each slide and incubated overnight at 42 °C. Sections were then subjected to a series of 1 SSC and ethanol washes and allowed to air dry for 30 min. Tissue sections and the appropriate controls were then exposed to the same film (Amersham Hyperfilm; one film for NPY and one film for GnRH) for 3 weeks and then developed. For NPY, the arcuate hybridization area was calculated with SigmaScan Pro (SPSS Inc., Chicago, IL, USA). Anatomical localization was verified with a mouse brain atlas (Montemurro & Dukelow 1972).

Statistical analysis

Body weight, adipose, reproductive traits, and hypothalamic NPY and GnRH expression were analyzed by least-squares analysis of variance procedures for unequal subclass numbers, using PROC GLM of SAS (version 8.0 2001). In addition, Pearson coefficients were calculated to determine the correlation between plasma leptin and body weight, leptin and GFP weight, and final body weight and GFP weight in experiment 1.

Results

Experiment 1

Body composition A time 2 weeks after parturition (5–7 weeks after mating) was chosen to collect body weight, adipose weight, and plasma leptin concentrations, as this allowed the mice to return to pre-pregnancy body weight and circulating leptin values. In addition, assessing leptin concentrations at this time also eliminated the confounding factors of pregnancy and lactation when comparing fertile and infertile females. Infertile animals, that is, those that failed to maintain a pregnancy, were assessed at a comparable age.

The WT ON/OFF and WT OFF groups were statistically equivalent in both the body composition ($P>0.12$) and reproductive ($P>0.31$) analyses; therefore, these groups were pooled for all analyses unless otherwise noted. Body weight, as expected, differed between the genotype classes and was a function of age as well

Table 2 Pearson coefficient (r^2) for correlation between leptin and body weight, leptin and right gonadal fat pad weight, and body weight and right gonadal fat pad weight for transgenic females chronically expressing the transgene (TG ON) or from 3–8 weeks of age (TG ON/OFF), and pooled wild-type (WT) females

Treatment group	Leptin vs body weight (r^2)	Leptin vs fat pad weight (r^2)	Body weight vs fat pad (r^2)
TG ON	0.1356	0.4867	0.2311
TG ON/OFF	0.6213	0.5614	0.6699
Pooled WT	0.4462	0.6231	0.6992

as treatment. TG ON females were heavier than TG ON/OFF animals, and both transgenic groups were heavier than WT animals (44.63 ± 0.29 , 34.32 ± 0.28 and 29.75 ± 0.20 g respectively; $P < 0.0003$). While weight increased with age in both the TG ON/OFF and pooled WT groups, maximum weights were achieved by TG ON females mated at 20 weeks and did not appreciably increase thereafter ($P < 0.02$; data not shown).

There was a significant interaction between treatment and age at mating for GFP weights ($P < 0.02$). Fat pad weight at the last mating age (40 weeks) was greater for the TG ON/OFF and pooled WT animals than for TG ON females (0.762 ± 0.047 , 0.689 ± 0.034 and 0.382 ± 0.059 g respectively; $P < 0.0003$). For the TG ON females, GFP weight was unchanged over the course of the experiment (age of mating 15–40 weeks). In contrast, GFP weight increased with advancing age for both TG ON/OFF and WT females. When GFP weight was examined as a percentage of body weight, TG ON females

were leaner than TG ON/OFF animals regardless of age ($P < 0.001$) (data not shown). Furthermore, although WT females were initially leaner than TG ON/OFF animals, this difference was absent in females mated at 30 weeks of age and older. Finally, body weight and GFP weight were highly correlated with each other in all but the TG ON treatment groups (Table 2).

Plasma leptin concentrations were dependent upon treatment, age at mating ($P < 0.02$) and interaction between the two factors ($P < 0.04$). TG ON/OFF females had higher plasma leptin levels than pooled WT animals, and the levels of the latter were in turn greater than concentrations observed in TG ON females ($P < 0.02$). Furthermore, leptin levels continued to increase with age in both the TG ON/OFF and pooled WT females, while they remained unchanged in TG ON mice (Fig. 1). In general, leptin levels showed energy stores to be most closely correlated with fat pad weight (overall $r^2 = 0.6281$) rather than body weight ($r^2 = 0.0641$) across all treatments.

Figure 1 Plasma leptin concentrations for oMt1a-oGH transgenic females expressing the transgene chronically (TG ON) or from 3–8 weeks of age (TG ON/OFF) and pooled wild-type (WT) females mated at either 15, 20, 25, 30, 35 or 40 weeks of age. Values are means $\pm$ S.E.M. Bars within treatments with different superscripts are significantly different ($P < 0.04$).

Table 3 Estrous cycle characteristics observed in infertile transgenic females expressing the transgene from 3 weeks of age (TG ON) or from 3–8 weeks of age (TG ON/OFF) and infertile wild-type (WT) animals. Females were assessed for estrous cyclicity by vaginal saline lavage for 14 days

Treatment	No. females	No. infertile females	Estrus-metestrus recycling	Constant diestrus	Metestrus-diestrus recycling	Normal estrous cycle	Constant metestrus
TG ON	170	97	30	6	5	54	0
TG ON/OFF	172	28	6	1	1	18	0
Pooled WT	367	43	12	1	2	22	3

Numbers refer to the number of females exhibiting this estrous cycle characteristic.

When treatment groups were examined individually, GFP weight was the best predictor of plasma leptin concentrations except in the TG ON/OFF females, where body weight was more correlated with leptin ($r^2=0.6213$) than fat pad weight ($r^2=0.5614$) (Table 2). Thus, animals with a greater proportion of body fat had higher circulating leptin levels.

Reproduction The percentage of females mating did not differ between treatment groups ($P>0.22$), although the number of copulatory plugs recorded did. During the 14-day period of housing females with males, TG ON females exhibited more copulatory plugs than WT females ($P<0.01$), although plug number was not different between the two TG groups (1.25, 1.14 and 1.07 plugs for TG ON, TG ON/OFF and pooled WT females respectively). Females failing to establish pregnancy continued to cycle and rebreed, as more copulatory plugs ($P<0.003$) were observed in animals classified as infertile (data not shown). Those females that failed to mate or maintain a pregnancy to term displayed a variety of estrous cycle abnormalities (Table 3). The most common estrous

cycle irregularity was cycling between estrus and metestrus.

The percentage of pregnant females was significantly reduced in TG ON females when compared with other treatment groups ($P<0.0003$), although the pregnancy rate improved for TG ON females as they aged. That was not the case for TG ON/OFF or WT females ($P<0.03$) (Fig. 2). Of note was that 11 TG and seven WT females were found to be pregnant upon dissection, despite the fact that many of them had produced pups during the week prior to death or had exhibited no prior signs of parturition. In nearly all of these cases, a reabsorbing fetus was found to be blocking the birth canal. These 18 females were included in the pregnancy analysis, but excluded from the other reproductive statistics. In addition to transgene activation status, fertility was also correlated with plasma leptin concentrations. Lower plasma leptin levels were detected for fertile than infertile females in the TG ON and TG ON/OFF transgenic groups ($P<0.0001$) and in WT ON/OFF ($P<0.05$) females (Table 4). This correlated with the infertile females having larger fat masses in these three treatment groups.

Figure 2 Pregnancy rate of oMt1a-oGH transgenic and WT female mice mated at 15, 20, 25, 30, 35 or 40 weeks of age. Values are means $\pm$ S.E.M. Different superscripts within treatment groups designate statistical differences ($P<0.03$).

Table 4 Plasma leptin concentrations (ng/ml) presented as means $\pm$ S.E.M. in fertile and not pregnant oMt1a-oGH transgenic and wild-type female mice

	TG ON	TG ON/OFF	WT ON/OFF	WT OFF
Plasma leptin (ng/ml)				
Fertile	1.57 $\pm$ 0.17 ^a	3.67 $\pm$ 0.25 ^a	2.64 $\pm$ 0.19 ^c	2.74 $\pm$ 0.19
Infertile	2.60 $\pm$ 0.14 ^b	6.38 $\pm$ 0.56 ^b	3.82 $\pm$ 0.55 ^d	3.31 $\pm$ 0.51

^{a,b} $P < 0.0001$, fertile vs infertile within treatment groups.^{c,d} $P < 0.05$, fertile vs infertile within treatment groups.

Gestation length was dependent upon both treatment group and age at mating, but there was no significant interaction between the terms ($P > 0.39$). TG ON females had a longer gestation length than WT animals ($P < 0.05$) (20.01 ± 0.10 , 19.84 ± 0.07 and 19.75 ± 0.05 days for TG ON, TG ON/OFF and pooled WT females respectively), and gestation length increased as the age of the dam advanced ($P < 0.05$) (Table 5). In addition, females produced fewer live ($P < 0.05$) and more dead ($P < 0.01$) pups with increasing age (Table 5). Transgenic groups produced more dead offspring than the pooled WT animals ($P < 0.003$; 1.22 ± 0.13 , 0.91 ± 0.09 and 0.33 ± 0.06 dead produced by TG ON, TG ON/OFF and pooled WT females respectively).

Experiment 2

Real-time TaqMan PCR Real-time PCR results showed an increase in hypothalamic NPY transcription in infertile relative to fertile animals ($P < 0.04$). Upon further examination, this relative elevation of transcription was due to increased message in nonfertile transgenic females when compared with fertile WT animals ($P < 0.05$) (Fig. 3). NPY transcription did not differ significantly between fertile and nonfertile animals within treatment groups ($P > 0.23$), nor was it significantly different when examined as a function of age within groups ($P > 0.40$; data not shown).

Table 5 Gestation data for oGH transgenic and wild-type mice presented as means $\pm$ S.E.M. Statistical comparisons are made within columns and between age groups

	Gestation length (days)	No. live offspring	No. dead offspring
Age at mating (weeks)			
15	19.27 $\pm$ 0.13 ^a	8.91 $\pm$ 0.38 ^a	0.427 $\pm$ 0.157 ^a
20	19.46 $\pm$ 0.11 ^a	8.16 $\pm$ 0.35 ^a	0.589 $\pm$ 0.143 ^a
25	19.66 $\pm$ 0.10 ^a	7.54 $\pm$ 0.32 ^{ab}	0.499 $\pm$ 0.132 ^a
30	20.09 $\pm$ 0.10 ^b	6.36 $\pm$ 0.32 ^b	0.765 $\pm$ 0.131 ^{ab}
35	20.26 $\pm$ 0.10 ^b	5.70 $\pm$ 0.33 ^{bc}	1.285 $\pm$ 0.134 ^b
40	20.46 $\pm$ 0.10 ^b	4.85 $\pm$ 0.32 ^c	1.158 $\pm$ 0.132 ^{ab}

Values within columns with different superscripts are significantly different from each other.

The real-time PCR results for GnRH transcription were inconclusive. Although the means were similar between fertile and nonfertile females ($P > 0.95$), the resulting large standard errors were so large as to prevent any meaningful comparisons.

In situ hybridization The NPY *in situ* results confirmed the real-time PCR data. In the hypothalamus, NPY expression was confined to the arcuate nucleus. Arcuate hybridization was specific, as the signal disappeared with the addition of excess unlabeled oligonucleotide to the radiolabeled probe mixture. Hybridization to the arcuate nucleus was greater in infertile oMt1a-oGH transgenic females ($n=4$) (average hybridization area = $1.29 \times 10^{-2} \text{ mm}^2$) than in fertile WT animals ($n=2$) (average hybridization area = $1.27 \times 10^{-2} \text{ mm}^2$) (Fig. 4), although this difference did not achieve statistical significance, probably because of the small number of animals in this experiment.

As in the real-time PCR results, GnRH hybridization was much more difficult to identify in the preoptic area, and given the small number of animals analyzed, meaningful results could not be obtained (data not shown).

Discussion

The purpose of these experiments was to characterize elevated circulating GH as it affected body composition and reproductive performance. More specifically, they aimed to document the changes in leptin concentrations induced by transgene expression, and to demonstrate a relationship between these differences and fertility. In addition, hypothalamic NPY expression was assessed as a possible intermediary molecule involved in transducing some of the effects of leptin and GH on the reproductive endocrine axis.

This study confirms previous reports of GH-induced changes in body composition in oMt1a-oGH mice (Pomp *et al.* 1992, Thomas *et al.* 2001). The lean phenotype of the TG ON mice is the result of improved feed efficiency and increased lean tissue deposition (Pomp *et al.* 1992). The development of obesity in TG ON/OFF mice is also in agreement with previous work from our laboratory (Pomp *et al.* 1996, Oberbauer *et al.* 1997, Oberbauer & Murray

Figure 3 Neuropeptide Y (NPY) gene expression in fertile (F) and infertile (IF) oMt1a-oGH transgenic (TG) and WT mice. Transcription is relative to WT F animals. Unlike superscripts are different ($P<0.05$). Numbers in parentheses indicate number of animals in each treatment.

1998). GH early in development, that is, beginning at 3 weeks of age, stimulates differentiation and proliferation of preadipocytes, resulting in more adipocytes in oMt1a-oGH than WT animals (Oberbauer *et al.* 1997, Oberbauer & Murray 1998). Once the GH transgene stimulus is removed, filling of adipocytes occurs,

promoting adipose deposition and subsequent obesity (Oberbauer *et al.* 1997, Oberbauer & Murray 1998). TG ON/OFF mice possessed higher circulating leptin levels than did TG ON mice, reflecting the elevated fat mass in animals of this treatment group. The close correlation between plasma leptin concentration and fat

Figure 4 Neuropeptide Y expression in the hypothalamus of (a) an infertile oMt1a-oGH transgenic mouse and (b) a fertile WT cohort. Arrows indicate specific hybridization to the arcuate nucleus. Localization of mRNA expression was performed on 14 μ m coronal sections hybridized with 33 P-labeled oligonucleotide probe.

mass has been documented elsewhere as well (Rosenbaum *et al.* 1996).

Infertile TG ON animals exhibited more copulatory plugs than did fertile counterparts, indicating that these females are unable to establish pregnancy, and thus continue to cycle and remate. While this suggests inadequate prolactin secretion after the mating stimulus, prolactin supplementation in these mice does not improve pregnancy rates (Pomp *et al.* 1995). Estrous cycle length is not different between TG and WT treatment groups (Thomas *et al.* 2001), although over 40% of the infertile TG ON females in this experiment exhibited irregular estrous cycling, which was also present to a lesser degree in WT

mice. Menstrual abnormalities are common in acromegalic women (Jadresic *et al.* 1982), and transgenic mice expressing bovine or human GH constructs often demonstrate alterations in gonadotropin secretion (Chandrashekar *et al.* 1992, Chandrashekar & Bartke 1993, Tang *et al.* 1993), implying that 'excess' circulating GH alters gonadotropin secretion, leading to an abnormal reproductive cycle. However, since gonadotropin concentrations were not measured in oMt1a-oGH mice, we are unable to confirm or refute this concept at this time. Alternatively, reproductive aging may also have contributed to the reduced reproductive performance documented here. Vaginal smears with either chronic cornification or leukocytic cells are indicative of declining reproductive function (Nelson *et al.* 1982). However, it seems unlikely that reproductive aging is a major cause of infertility in transgenic animals, as a majority of the infertile transgenic females did not display a constant presence of vaginal cornification or leukocytic cells. In addition, reproductive aging in rodents is associated with reduced hypothalamic NPY message (Sahu *et al.* 1988, Chua *et al.* 1991, Sahu & Kalra 1998), which was not observed in this study. NPY concentrations did not vary by age within treatment groups, and infertility was associated with an increase, rather than a decrease, in NPY expression.

The reduced pregnancy rate in TG ON animals is in agreement with observations from other GH transgenic mouse lines (Cecim *et al.* 1995) and may be a result of reduced leptin signaling. Yu *et al.* (1997) observed that low leptin concentrations (10^{-10} and 10^{-12}) stimulated GnRH release from hypothalamic explants of adult male rats, but higher concentrations of leptin significantly depressed GnRH. Baskin *et al.* (1998) hypothesized that binding of leptin to its receptor at the surface of the arcuate nucleus may in fact result in downregulation of receptor transcription and reduce leptin signaling capacity. Leptin administration, in the presence of adrenal steroids, activates leptin receptor (Ob Rb) signal transduction and subsequent STAT-3 phosphorylation, which stimulates transcription of SOCS-3. SOCS-3 expression then negatively regulates leptin signal transduction by downregulation of the JAK-STAT pathway (Bjorbaek *et al.* 1999, Madiehe *et al.* 2001, Wang & Campbell 2002). In addition, both GH (Ram & Waxman 2000) and glucocorticoids (Madiehe *et al.* 2001), which are also elevated in oMt1a-oGH females actively expressing the transgene (Thomas *et al.* 2001), are also capable of activating members of the SOCS family to reduce cytokine signaling.

NPY is important for generation of the GnRH surge during proestrus (Bauer-Dantoin *et al.* 1992, Besecke & Levine 1994, Khorram *et al.* 1998), although chronically elevated NPY expression inhibits reproductive function (Pierroz *et al.* 1995) through suppression of GnRH secretion (Catzeffis *et al.* 1993). Leptin attenuates NPY expression both *in vitro* (Yu *et al.* 1997) and *in vivo*

(Schwartz *et al.* 1996), although in animals with relatively elevated leptin concentrations, leptin may be less able to downregulate NPY expression due to reduced signaling capacity, resulting in suppression of GnRH and reduced reproductive competence. This is in agreement with the elevated circulating leptin concentrations and NPY expression observed in the hypothalami of infertile transgenic females. Because our GnRH data were inconclusive, we were unable to determine how changes in NPY expression would affect GnRH message. However, elevated hypothalamic NPY expression and reduced reproductive performance suggest alterations in the hypothalamic-pituitary-ovarian axis.

Previous studies in our laboratory have documented a reduced pregnancy rate in TG ON/OFF females (Thomas *et al.* 2001), which was not seen here. This is surprising, as obesity is associated with menstrual abnormalities (Rogers & Mitchell 1952, Hartz *et al.* 1979, Bray *et al.* 1997) and pregnancy complications (Kalkhoff 1992) in women. Furthermore, hyperleptinemia and adiposity are negatively correlated with gonadotropin secretion in pubertal girls (Bouvattier *et al.* 1998). However, the TG ON/OFF females used in the Thomas *et al.* (2001) study were examined immediately following transgene inactivation, and residual GH effects may have contributed to the reduced fertility observed. In addition, the differences in body composition and plasma leptin concentrations in animals between GH treatment groups are not pronounced until later in life (this study). In this report, infertile TG ON, TG ON/OFF and WT ON/OFF animals displayed significantly elevated plasma leptin levels compared with fertile counterparts, a finding reflective of a larger fat mass.

The increased interval required by TG ON females to generate pups may be in part due to a reduced ability of the reproductive tract to support fetal development. Transgenic females are often observed to have small uterine horns; in addition, many are avascular, even after pregnancy (A Thomas, personal observation). Although TG ON females displayed the longest gestation length, litter size was not different between treatment groups, in agreement with previous studies from our laboratory (Murray & Pomp 1995, Pomp *et al.* 1995, Thomas *et al.* 2001). Pomp *et al.* (1995) reported lower average pup weights for transgenic mice when compared with WT controls. In a similar study using mice expressing a variety of GH constructs, Naar *et al.* (1991) observed that fetal size was a function of maternal genotype, not fetal genotype. The combination of increasing numbers of dead and reduced numbers of live offspring with increasing age of the dam may be due to poor maternal behavior. Both failure to feed pups and cannibalism are common problems among oMt1a-oGH transgenic mothers (A Thomas, personal observation).

Previous reports have characterized the reproductive deficits in oMt1a-oGH transgenic mice (Murray & Pomp

1995, Pomp *et al.* 1995, Thomas *et al.* 2001), although the mechanisms by which GH suppresses fertility have remained elusive. In this study, chronic expression of the oMt1a-oGH transgene resulted in decreased reproductive performance, and elevated leptin levels were associated with infertility in transgenic females with chronically elevated GH levels. This suggests that, in addition to high GH concentrations, elevated circulating levels of leptin also contribute to the reduction in fertility observed in oGH transgenic females, especially in those animals with increased adipose accrual. High circulating concentrations of leptin may reduce leptin receptor signaling due to induction of SOCS-3 transcription, resulting in a reduced ability of leptin to downregulate NPY expression and subsequent suppression of reproductive performance.

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