THE ROLE OF SEX STEROID HORMONES IN THE REGULATION OF THE OBESE GENE AND LEPTIN SECRETION

By

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ABSTRACT

Leptin, the hormone product of the *Obese (Ob)* gene, is secreted from adipocytes to communicate energy status, reduce food consumption and increase metabolism of fat stores. Interestingly, females have higher leptin levels than males in humans, rodents, and other mammals, even when adjusted for fat mass. We therefore tested whether the hormones estrogens and androgens control leptin synthesis in cultured cells. A mouse adipocyte cell line (3T3-L1) was treated with 17β-estradiol and dihydrotestosterone and RNA was harvested for analysis by qRT-PCR. Media extracts were also collected for leptin ELISA. The present study suggests a weak stimulation of *Obese* mRNA accumulation by estrogen that parallels the accumulation of *Resistin* mRNA, a known estrogen responsive gene. In contrast, dihydrotestosterone strongly reduces the *Ob* mRNA levels in 3T3-L1 adipocytes with effects detected at 0.1 nM, with maximal and significant effects at 10 nM following 12 hours of treatment. The DHT effect is accentuated if *Ob* mRNA accumulation is normalized relative to mRNA of the adipocytes differentiation marker, *PPARγ*. Treatment with estrogen and androgen receptor antagonists prevent estrogen enhanced *Ob* message accumulation and DHT decreased *Ob* mRNA levels, respectively. No differences in leptin secretion from 3T3-L1 cells were detected 12 hours after DHT treatment.
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INTRODUCTION

*Obesity*

Obesity is an increasing problem in the United States and throughout the world. Across the globe approximately one billion adults are overweight and 300 million of those are obese (Friedman, 2003). In the U.S. alone, half the country’s population is either overweight or obese (Qi et al., 2008). With the marked increase in the incidence of obesity over the past ten years, diseases associated with this condition have also increased including: diabetes, heart disease, and hypertension (Friedman, 2003). The battle to fight this condition and associated diseases requires understanding of the factors that contribute to obesity including social, medical, and genetic components.

Understanding the causes of obesity in humans have been a focus of research since the late 20th century. There is a lot of evidence that supports the idea that obesity is simply linked to an excess in calorie consumption and a decrease in energy expenditure (Hill et al., 2003). The social environment in which western societies are immersed encourages this pattern by making enticing, inexpensive, and calorie-rich foods easily available to individuals who, as a group are reducing their physical activity (Hill et al., 2003). This reduction is partly due to technology which decreases the need for jobs with extended physical labor and also to an increase in activities with limited energy expenditure such as; watching TV, playing video games, or using the computer (Hill et al., 2003).

Perhaps an equally compelling argument is the idea that the tendency to become obese is at least in part controlled by genetics (Friedman, 2003). “Obesity genes” are those involved in energy balance which should allow food intake to match calorie
expenditure. These genes encode signaling molecules that tell the body to either burn energy stores, as well as when to eat and when to stop (the feelings of hunger and satiety). Studies have provided evidence that these genes do play a role in weight regulation (Friedman, 2003). Support for the theory that genetics drives the increase in obesity, comes from the uneven distribution of weight gain in the past 10 years among U.S. adults. While on average, the body mass index (BMI) has gone up across all groups, the lower percentile BMI classes have not changed as dramatically as those in higher percentiles. The percent of the population with low BMIs (15-20) has not changed on average between 1991 and 2000, while the average number of individuals with BMI >20 has increased in the past 9 years (Flegal and Troiano, 2000). This suggests that a group of individuals and not the entire population have had a marked increase in BMI. The data from the past ten years strongly suggests that there is a group within the population that is more genetically predisposed to obesity and a separate group that is less likely to become obese.

If there are indeed two separate sub-populations of individuals, one with obesity-related gene defects and one without, they may have diverged due to evolution and inheritance. Some human populations evolved in environments with plentiful food resources while other populations were established in regions with limited or variable food resources (Neel, 1962). The first scenario would have been common for western societies that kept domesticated animals or those that had access to fertile croplands earlier than other societies. The second group would not have had easy access to sustenance and perhaps needed more energy stores for periods when they could not find food. The idea that individuals who did not have immediate access to food would need to
increase energy storage mechanisms, is known as the “thrifty gene hypothesis” (Neel, 1962). This idea is supported by the fact that certain populations which were prone to starvation throughout history have been shown to be the most obese groups when given a more western diet (Douse and Mitchell, 1990). To investigate the potential role of genetics in the incidence of obesity, and to more specifically look at “obesity genes” scientists have begun to utilize rodent models, using both naturally occurring mutants or knockout mice engineered to ablate potential regulatory genes.

*Rodent Models of Obesity*

Two strains of obese mutant mice arose from two separate spontaneous mutations at Jackson Laboratories: *Obese (Ob)* (Ingalls et al., 1950) and *Diabetic (Db)* (Hummel et al., 1966). These mice became increasingly obese at approximately double the weight of their wildtype littermates. *Ob/ob* mice had life spans comparable to their lean littermates, however they were sterile. In a study with mice heterozygous for this trait, obese individuals were found at a ratio of 1:3 in each litter suggesting that this mutation was inherited in a recessive pattern (Ingalls et al., 1950). *Db/db* mice have abnormally high blood sugar concentrations and a shortened lifespan. Through mating experiments, this mutation was determined to be inherited through alleles independent of *Ob* (Hummel et al., 1966).

Parabiotic studies, in which two mice are joined together surgically, have also provided some insight into how the products from these two genes may work together. When *ob/ob* mice were surgically joined with wild type mice they gained significantly less weight and exhibited reduced food intake (Coleman, 1973). When *db/db* mice were
joined through parabiosis with normal mice, the previously mentioned symptoms were not cured and wild-type mice decreased food intake to the point that they died from starvation (Coleman and Hummel, 1969). This indicated that the \( db/db \) mice were insensitive to an unknown satiety signal, which they were capable of producing at high levels and which were sensed by the wild type. It also suggested that the product of the \( Db \) gene served as the missing sensor for satiety, in that products from the wild type mice were unable to correct the Diabetic phenotype.

Uniting an \( ob/ob \) mouse with a \( db/db \) mouse caused the weight loss and eventual death of the \( ob/ob \) mouse while the \( db/db \) mouse remained unchanged. This indicated that the \( db/db \) mouse (similar to the wild type mouse) was producing an unknown satiety factor which acted to suppress appetite in the \( ob/ob \) mice. This suggests that \( ob/ob \) mice have normal “satiety centres” to sense this signal. \( Db/db \) mice however, were unable to sense this satiety factor, which they clearly produce, suggesting that the product of this gene mutation may be downstream of that unknown satiety factor (Coleman, 1973).

**Cloning the Ob Gene and Understanding the Ob Gene Product**

Although these two mutant phenotypes were established in the 1950’s and 1960’s, the actual genetic identity of these mutations were not uncovered until the 1990’s when genetic tools were developed. The mouse \( Ob \) gene was isolated through the use of positional cloning in which the mutant allele was segregated through mouse breeding crosses (Friedman, 1998). Mice with the \( ob \) mutation are infertile, so in these experiments ovaries from \( ob/ob \) females were transplanted into wild-type females. Mating these animals with \( ob/ob \) males resulted in live births of mutant progeny (Friedman, 1998).
DNA from the progeny was then digested by restriction endonucleases for analysis by Southern blot (Friedman, 1998). The Ob mutation was identified to be a single base pair substitution immediately following leucine 104 (Zhang et al., 1994). This mutation, a substitution of C\(\rightarrow\)T, changes arginine to a stop codon, creating an abnormally short transcript and thus misshapen protein product (Zhang et al., 1994). In humans, the Ob gene maintains 85% sequence similarity to the mouse gene product and is also well conserved in many vertebrate species (Zhang et al., 1994).

The 16 kD Ob gene product has been found in the plasma of both mice and humans, but not in the plasma of ob/ob mice (Halaas et al., 1995). Leptin was determined to have sequence similarity to other previously established cytokine signaling molecules (Halaas et al., 1995). Cytokines, which are molecules that aid in cellular communication, signal through very specific tyrosine kinase-associated receptors located on the plasma membrane of their target cells. Cytokines are involved in many diverse processes in the body including immune response and neural function (Kishimoto et al., 1994).

Following isolation of the Ob protein, it could then be administered to further elucidate its effects. Daily injections of the Ob gene protein in Ob deficient mice and humans (Halaas et al., 1995) significantly reduced body weight and food intake (Halaas et al., 1995). The protein was subsequently named “leptin” after the Greek word “leptos” meaning “thin” (Halaas et al., 1995). Leptin administration also increased oxygen consumption and body temperature in these mice suggesting the involvement of leptin in the signaling for β-oxidation of fat stores (Pelleymounter et al., 1995).

The identification of the leptin protein allowed studies of the mechanisms that control its synthesis and secretion. When energy stores are plentiful, leptin production is
increased to signal satiety (Friedman and Halaas, 1998). In contrast, during fasting, leptin production is decreased. Obesity due to leptin signaling malfunction occurs as a result of increased feeding and decreased exploitation of energy stores. Leptin injections in \textit{ob/ob} mice have been shown to decrease body weight, with that decrease restricted to adipose tissue loss (Friedman, 1998). Plasma leptin levels in humans are directly correlated to body mass index (BMI), total body fat, and food intake (Maffei et al., 1995b). \textit{Ob} mRNA decreased in the majority of obese human patients following diet and consequent weight loss. \textit{Ob} mRNA levels in mice are directly related to fat levels (Maffei et al., 1995a).

\textbf{Isolation of the Leptin Receptor}

Once the leptin protein was identified and isolated, it could be used to identify some of its downstream targets. Upon further examination of \textit{Ob} mRNA expression, increased levels of \textit{Ob} transcript were found in the adipocytes of \textit{db/db} mice when compared to wild type mice (Maffei et al., 1995a). These studies indicate that the deficiencies in weight maintenance and energy homeostasis in \textit{db/db} mice are not due to any shortage of leptin, in fact, these mice have higher \textit{Ob} transcript levels and leptin protein levels than wild-type mice (Maffei et al., 1995a). This study supported the idea that the \textit{Db} gene product is downstream of leptin in the signaling pathway. Researchers wanted to test whether this was true, but first they had to establish a biochemical link between the product of the \textit{Db} gene and leptin.

Using a leptin-alkaline phosphatase (AP) fusion protein, researchers were able to identify specific regions of the brain where leptin binds \textit{in situ} (Tartaglia et al., 1995).
Binding sites in the choroid plexus, the part of the brain that acts as a barrier between molecules and the cerebrospinal fluid, led researchers to isolate RNA from this particular section of the brain and to make a cDNA library. Clones from this cDNA library were transfected into COS cells for screening with AP-Ob. This library was then screened for clones that encoded a protein that demonstrated specific and irreversible binding to AP-Ob. This protein was identified and named “leptin receptor” (Ob-R) (Tartaglia et al., 1995). The sequence encoding Ob-R was determined to have similarity to class I cytokine receptors, with a hydrophobic signaling sequence, mature extracellular domain, and potential interleukin-6 (IL-6) signal-transducing capabilities (Tartaglia et al., 1995).

Primers were then designed for the newly discovered leptin receptor to target sections of genomic DNA, which mapped the gene to chromosome 4. By backcrossing mice from various genetic backgrounds, and examining genes which were previously mapped, the Ob-R gene was indeed found to be encoded by the specific chromosomal location that is known to be associated with the Db mutation (Tartaglia et al., 1995).

Five leptin receptor isoforms were then identified by screening a mouse cDNA library with various fragments of the Ob-R gene (Lee et al., 1996). A number of cDNA clones matched the fragments, but they contained differences in the DNA of the coding sequence following the codon for Lysine 889. These isoforms were designated Ob-Ra through Ob-Re with Ob-Rb acting as the most common biologically active form of the leptin receptor found in vivo (Lee et al., 1996). Each of these isoforms has an identical transmembrane region followed by a distinct cytosolic region (Lee et al., 1996). Leptin receptor isoforms are found in several regions of the brain, but are the most concentrated
in the arcuate nucleus (ARC) and periventricular nucleus (PVN) of the hypothalamus (Mercer et al., 1996).

**Figure 1: Schematic of the various leptin receptor isoforms.**

All 5 of the receptor isoforms contain sequence similarity in the ligand binding region. Ob-Ra through Ob-Rd also contain the same transmembrane (TM) region, while Ob-Re is a shortened soluble product. The membrane bound receptors (a-d) are made through differential splicing and thus have differing intracellular regions. Receptors Ob-Ra and Ob-Rb contain Box 1, a Jak recruitment site and only the long form contains a STAT-3 recruitment site also called Box 2.
All leptin receptor isoforms were shown to bind leptin in a 1:1 ratio (Devos et al., 1997). One potential difference between the leptin receptor and most other members of the cytokine receptor family is that certain forms including Ob-Rb and Ob-Ra may exhibit ligand independent dimerization. Where most receptors require binding of their ligand (cytokine) to dimerize and activate their signal transduction cascade, leptin receptors may exist in the dimeric form in the absence of leptin (Devos et al., 1997). A more recent study has shown that the amount of dimerized leptin receptors increases upon leptin treatment in culture as measured by fluorescence resonance energy transfer (Biener et al., 2005).

**Ob-Rb and Traditional Leptin Signaling**

The full length leptin receptor (Ob-Rb) was shown to function as an IL-6-type cytokine receptor (Baumann et al., 1996). Most traditional cytokine receptors have an extracellular region that consists of approximately 200 base pairs and signaling occurs through homodimerization, upon binding with its cytokine ligand. Through this dimerization, the receptor is then able to stimulate intracellular signaling via activation of Janus associated kinase (JAK) protein kinases, which allows for phosphorylation of various tyrosine residues along the receptor. These phosphorylated tyrosines can then in turn interact with signal transducer and activator of transcription (STAT) proteins and other growth factors (Kishimoto et al., 1994), which then enhance expression of downstream target genes.
Similarly, signaling by leptin through Ob-Rb stimulates transcription of STAT proteins, demonstrating the ability to up-regulate this signaling cascade (Baumann et al., 1996). A plasmid containing a fully functional Ob-Rb sequence was designed to investigate the signaling capacity of Ob-Rb. This plasmid was co-transfected with reporter gene constructs for various STAT molecules in COS cells. Transcription of these genes and corresponding protein levels were measured via luciferase assay and Western blot respectively. The long form increased transcription of STAT-3 and STAT-5B, and this effect was also seen in the Western blots (Baumann et al., 1996). This work confirmed that upon the binding of leptin to the long form of its receptor, the JAK/STAT pathway is activated to stimulate transcription of various genes in the nucleus.

Mutant constructs of Ob-Rb demonstrate that there is a specific region (known as Box 3) that is important for STAT-3 activation, but not STAT-5B. Studies demonstrated that the effects elicited by leptin signaling through its receptor in inducing gene expression were severely decreased with a mutation to Box 3 (Baumann et al., 1996). Analysis of hypothalamic nuclear extracts from various rats demonstrated that leptin activates STAT-3 in the hypothalamus (Vaisse et al., 1996). STAT-3 was the only STAT protein in the hypothalamus to be activated through leptin treatments (Vaisse et al., 1996). This result indicates the singular pathway for direct leptin signaling in this region is through STAT-3 and thus the probable mechanism for the regulation of food intake.

Recent work suggests that leptin may also be able to stimulate STAT-3 independently of JAK2 phosphorylation. In JAK2-null cells, leptin was shown to stimulate STAT-3 phosphorylation but when the product of the Sarcoma gene (Src) was inhibited, phosphorylation of STAT-3 was severely reduced. This suggests that leptin
signaling may lead to the phosphorylation of STAT-3 by way of Src, a protein-tyrosine kinase, independent of JAK2, in addition to the traditional JAK/STAT mechanism (Jiang et al., 2008). This newer result could indicate either a redundancy in the leptin signaling pathway or perhaps a cell-specific modifier of this signal.

Additional studies have shown the precise site of Ob-R interaction with STAT3. Mice with mutations in TYR\textsubscript{1138}, the STAT-3 recruitment site, (known as s/s mutants) have a similar phenotype to \textit{db/db} mice, confirming the importance of STAT-3 in maintenance of energy homeostasis. Additionally, this mutant demonstrated the potential importance of multiple signaling pathways in the regulation of reproduction by leptin. While both \textit{ob/ob} and \textit{db/db} mice are infertile, s/s mice exhibit increased fertility (Bates et al., 2003). Similarly, mice created with a neural knockout for STAT-3 in Ob-Rb expressing neurons are severely obese but exhibit completely normal fertility (Piper et al., 2008). These studies imply that the STAT-3 signaling pathway is not the key regulator of leptin’s role in reproduction.

\textit{Alternative Signaling Pathways Associated with Ob-Rb}

Although leptin receptor signaling through STAT-3 is a well accepted mechanism to stimulate transcription, other signaling molecules have been associated with various regions of the long form of the receptor. The long form of the leptin receptor has four intracellular tyrosine residues which serve as binding sites for intracellular signaling molecules. These residues fall at amino acids 974, 985, 1077 and 1138 and all seem to interact with different molecules once phosphorylated (Fruhbeck, 2006). Targeted mutations at each of these sites have provided insight into what happens within the cell.
upon leptin binding to its receptor. TYR\textsuperscript{1138} is the site at which the receptor signals through STAT-3, as previously mentioned. Ob-Rb has also been shown to signal through STAT-5, which interacts with TYR\textsuperscript{1077} (Fruhbeck, 2006). TYR\textsuperscript{985} interacts with protein tyrosine-phosphatase SHP-2, which in turn phosphorylates GRB-2, to up-regulate the MAPK pathway (Banks et al., 2000). TYR\textsuperscript{985} has also been shown to be a docking site for suppressor of cytokine signaling 3 (SOCS3) which shuts down leptin signaling through its receptor (Bjorbak et al., 2000). Other molecules which are recruited to TYR\textsuperscript{985} are insulin receptor substrates (IRS) 1 and 2, indicating that leptin and insulin may have direct signaling crosstalk (Fruhbeck, 2006).

Indirectly, insulin treatments stimulate leptin production. Ob mRNA transcript levels increased following an insulin injection in fasted rats (Saladin et al., 1995). This study was supported by cell culture experiments in primary adipocytes where insulin treatments also stimulated Ob gene expression (Saladin et al., 1995). Interestingly, insulin has been shown to elicit its effects on glucose homeostasis through insulin receptor-substrate and phosphatidylinositol 3-kinase, a pathway through which leptin also signals (Niswender et al., 2003), indicating that leptin and insulin signaling are linked (Niswender et al., 2003).

\textit{Leptin Signaling in the Brain}

To identify the brain regions targeted by leptin, Maffei et al. (1995) made lesions to the hypothalamus, a region of the brain known to be important in nutritional homeostasis, which had been shown to express leptin receptors (Maffei et al., 1995a). By disrupting this part of the brain, researchers could compare these altered mice to \textit{db/db}
mutants, and they found that these two groups were extremely similar (Maffei et al., 1995a). This suggested that the brain disruptions affected leptin receptor activity, confirming this part of the brain as a potential downstream target for leptin signaling (Maffei et al., 1995a). These lesions also caused increased leptin mRNA expression in adipocytes (Maffei et al., 1995a), indicating that when leptin receptors are not active, leptin gene expression increases to compensate for the missing signal. This work suggests that leptin, its receptor, the hypothalamus, and adipocytes are all part of a complex regulatory loop.

To further investigate the effects of leptin on the hypothalamus, microinjections of recombinant leptin were administered into the various regions of the brain including the arcuate nucleus (ARC), ventromedial hypothalamus (VMH) and lateral hypothalamus (LH). These studies showed that although leptin injections in all of these regions decreased food intake, the decrease was much higher with injections to the ARC, naming this as the primary site of the action of leptin in the control of food intake (Satoh et al., 1997).

Ob-Rb signaling via STAT3 mediates the effects of leptin on melanocortin production, a group of hormones involved in many regulatory processes in the body including pigmentation, sexual function, exocrine secretion, and energy homeostasis (Bates et al., 2005). Importantly, Ob-Rb signaling inhibits feeding through the down-regulation of both NPY (neuropeptide Y), one of the main neurotransmitters involved in energy balance, as well as agouti-related peptide (AgRP), a neuropeptide which stimulates appetite (Myers et al., 2008). Not only has NPY been shown to regulate metabolism, but it is also involved in the control of fertility and growth (Bates and Myers,
Leptin signaling through Ob-Rb also stimulates the action of pro-opiomelanocortin (POMC) which is the precursor for a number of different signaling proteins including α-MSH, which regulates appetite and reproductive behavior, as well as adrenocorticotrophic hormone (ACTH), which regulates glucocorticoids secretion (Bates and Myers, 2003).

Signaling capacity of the Ob-R Short Forms

In db/db mice the inability to control metabolism is due to a point mutation in the leptin receptor, which results in a donor splice site and subsequent translation of a 106 bp insertion. This insertion in the gene transcript includes a stop codon that creates an abnormally shortened protein. The db/db product thus contains the conserved transmembrane region common to all isoforms of the leptin receptor, as well as a significantly shorted cytoplasmic region, which is very similar to Ob-Ra (Chen et al., 1996). In both wild-type and ob/ob mice, leptin injections stimulate STAT3 in hypothalamus demonstrating that ob/ob mice have normal leptin receptors, however, in db/db mice the same activation is not seen (Vaisse et al., 1996). This indicates that the shortened cytoplasmic region of the db/db Ob-R product is inadequate for leptin signaling through STAT3 (Vaisse et al., 1996). Furthermore, the db/db receptor product does not activate any STAT proteins (Ghilardi et al., 1996), which is consistent with the idea that db/db mice are insensitive to leptin signaling and further demonstrates the importance of STAT proteins in the leptin signaling cascade.

Similarly, in humans, leptin receptor mutations may stem from shortened proteins. One homozygous mutation produces leptin receptors which lack not only the intracellular
domain, but the transmembrane domain as well (Clement et al., 1998). These patients are morbidly obese and infertile (Clement et al., 1998). This mutant form of the leptin receptor is a short 170 bp when compared to the normal human leptin receptor long-form transcripts which are approximately 276 bp (Clement et al., 1998). This mutated protein product is caused by a single base-pair substitution that leads to the ablation of a splice donor site, meaning that the mutated transcript is lacking one exon (number 16) (Clement et al., 1998). Six frameshift and missense mutations in this gene were recently isolated in humans with severe early onset obesity (Kimber et al., 2008). Four of these mutations occur on the extracellular region of the receptor, but seem to affect downstream signaling and not leptin binding (Kimber et al., 2008). All four of these mutants have a diminished ability to phosphorylate JAK2, and exhibit either completely ablated or severely diminished abilities to phosphorylate STAT3 and ERK1/2 (Kimber et al., 2008). These data suggests that these regions are important for protein folding and most likely conformational changes required for full leptin signaling.

The function of the naturally occurring short receptor isoforms, which are produced through differential splicing (Ob-Ra, -Rc, and –Rd) (Lee et al., 1996) or proteolysis (Ob-Re) (Ge et al., 2002), is not yet clear. Alternate splicing results in various leptin receptor isoforms immediately after lysine 889 (Lee et al., 1996). This means that the short forms lack a number of binding sites, limiting the interaction of the receptor with intracellular signaling molecules.

Ob-Re is the soluble form of the leptin receptor (Fei et al., 1997). Recent studies have shown that the soluble receptor may also be produced through a process known as ectodomain shedding (Ge et al., 2002). It has been shown that the cleaving of the
membrane bound receptors, on the extracellular face of the membrane can give rise to the soluble receptor (Ge et al., 2002). Studies with human embryonic kidney (HEK) cells which stably express Ob-Rb demonstrate a reduction in leptin binding with this receptor when cells are incubated with the soluble receptor (Yang et al., 2004). This data suggests that Ob-Re may be involved in the binding of leptin and may reduce free plasma leptin levels, reducing leptin responses.

Ob-Ra is the most common of the short forms (Fei et al., 1997) and has been shown to stimulate weak signal transduction \textit{in vitro} (Murakami et al., 1997). Similar to Ob-Rb, Ob-Ra is able to stimulate phosphorylation of Jak2, as Ob-Ra retains the Box-1 region of the intracellular portion of the receptor. Using Chinese hamster ovary cells with stable Ob-Ra expression, mRNA expression of leptin inducible genes \textit{c-fos}, \textit{c-jun} and \textit{jun-B} was increased upon leptin treatment (Murakami et al., 1997). Since Ob-Ra lacks box 2 and box 3, this signaling must occur through the box 1 region of the receptor. Ob-Ra is also able to phosphorylate ERK1 (a MAP kinase) in culture suggesting that Ob-Ra may have some signaling capacity independent of the traditional mechanisms of the long form (Bjorbaek et al., 1997). Ob-Ra may signal through these mechanisms in various regions of the body, and may connect leptin with some of its other roles potentially including reproduction.

Ob-Ra may also transport leptin from circulation into various tissues (Hileman et al., 2000). Madin-Darby Canine Kidney (MDCK) cells with stable expression of Ob-Ra, were grown in a monolayer on small filter membrane which fit in special dishes to create an upper and a lower chamber (above and below the cells). These cells were then treated with radioactive leptin and the amount of radioactivity in the media on the lower side was
measured with a $\gamma$ counter. Scientists were able to show that leptin bound to the apical cell surface was transported through the cell toward the basolateral membrane (Hileman et al., 2000). If Ob-Ra indeed does facilitate leptin transmembrane movement, it may serve to transport it into its various target tissues. The endocytic mechanism which makes this possible is as yet unknown. Ob-Ra may also be a mechanism for the degradation of leptin. Interestingly, while a significant amount of radioactive leptin was transported through these cells via Ob-Ra, the majority was degraded inside the cell (Hileman et al., 2000).

**Leptin & Reproduction**

Leptin also plays a role in reproductive competence to communicate information on energy homeostasis and food availability to regulate reproductive ability. *ob/ob* mice are infertile, suggesting leptin may play a key role in the communication of stored energy to the reproductive machinery. Starved mice exhibit decreased reproductive function, but when supplemented with leptin, the detrimental starvation effects on gonadal function are reversed. The starvation-induced delay in ovulation is also eliminated by these treatments (Ahima et al., 1996). Leptin-treated *ob/ob* female mice exhibit significantly elevated luteinizing hormone (LH) as well as increased ovarian and uterine weights (Barash et al., 1996). *Ob/ob* females treated with leptin were also able to copulate with wild-type males and produce offspring, although all of these pups were dead within two days of birth. The offspring death has been attributed to the inability of *ob/ob* leptin-treated mothers to lactate (Chehab et al., 1996).
Leptin treatment also has an effect on reproduction in wild-type female mice (Chehab et al., 1997). In a pre-pubertal population, leptin administration led to stunted growth in these individuals due to the reduction in food intake (Chehab et al., 1997). Leptin also caused early maturation of reproductive organs including vaginal openings, uteri, ovaries, and oviducts. Leptin treatment also accelerated the onset of the first estrous cycle and these mice produced viable offspring up to 9 days earlier than their paired controls (Chehab et al., 1997). When leptin is given to fasted wild-type female mice, these mice continue to ovulate normally, whereas fasted mice without leptin treatment exhibit a delay in ovulation (Schneider, 2006). These data suggests that leptin may act as a signal of nutritional status for the onset of puberty.

Leptin is also directly correlated to the estrous cycle in humans. In a study with thirteen women, results showed that leptin levels changed throughout the menstrual cycle (Stock et al., 1999). Leptin levels were also studied in twenty-nine pregnant women, in which leptin levels peaked at approximately week 28. Maternal weight gain was also positively correlated with leptin levels (Stock et al., 1999).

In males, the relationship between leptin and reproduction is less clear. Ob/ob male mice showed elevated follicle-stimulating hormone (FSH) and increased seminal and testicular weights following leptin treatment (Barash et al., 1996). In rats, experimental calorie restriction seems to alter male fertility as well as attractiveness to females. In moderately calorie restricted groups, the sexual behavior of males remained the same, but these individuals had decreased attractiveness in mate preference tests. In severely calorie restricted individuals, males exhibit impotence and significantly reduced leptin levels (Govic et al., 2008). A separate study using adult male rats showed that
leptin treatments also cause changes in reproductive hormones including gonadotropins, prolactin and testosterone. Chronic administration of leptin in fed rats increased plasma prolactin and decreased testosterone when compared to untreated controls (Sirotkin et al., 2008).

**Sexual Dimorphism of Leptin**

Interestingly, weight gain seems to be sexually dimorphic. Obesity is more prevalent in women than men (Ogden et al., 2006) and in 2000, women of ages 40-59 were shown to have a higher BMI on average than males in the same age group (Flegal and Troiano, 2000). Males and females also store their fat in different places, with men having a higher proportion of visceral fat and women having more subcutaneous fat stores (Ogden et al., 2006). Visceral fat is correlated with a higher risk for heart disease, while subcutaneous fat is not directly linked to this health issue (Ogden et al., 2006). In a study using rats, castrated males had significantly more subcutaneous fat stores than control males, and ovariectomized (OVX) females had significantly more visceral fat than control females (Clegg et al., 2006). The effects on subcutaneous fat stores in OVX females were reversed with estrogen replacement therapy (ERT) (Clegg et al., 2006).

One hypothesis for the difference in fat stores may be linked through evolution to reproduction. Reproduction requires an immense amount of energy and thus metabolism and efficient energy balance is crucial to this process. Pregnant females of many mammalian species significantly increase food intake and store this excess as lipids which can later be oxidized for energy during costly activities, such as late stage fetal growth and lactation (Schneider et al., 2000). Physiologically, the stimuli and neural
circuitry for eating and foraging in animals are also involved in the facilitation of certain facets of reproduction and thus promote reproduction when energy is abundant (Schneider, 2004).

Human females have approximately 40% higher leptin levels than men even after compensating for BMI, adiposity, and age (Saad et al., 1997). At puberty, leptin levels increase in females but decrease in males (Ahmed et al., 1999). The sexual dimorphism begins at the early stages of pubertal development and is well defined by the later stages (Ahmed et al., 1999). Leptin receptor isoforms may also be sexually dimorphic, but the results from a number of studies are contradictory. In one study, levels of the soluble receptor in humans are significantly higher in males than females (Chan et al., 2002), but in another, women had higher levels of bound leptin (McConway et al., 2000). In skeletal muscle tissue, females have significantly higher levels of 3 classes of leptin receptor isoforms (secreted, short, and long) when compared to muscle biopsy tissue obtained from men (Guerra et al., 2008).

*Sex Steroid Hormones*

Testosterone (T), a member of the group of steroid hormones collectively named androgens, is produced in the testes in males and the ovaries in females. This hormone can be converted to other steroid hormones including estrogen (E) or dihydrotestosterone (DHT) (Mayes and Watson, 2004). Once T is converted to DHT, it can no longer be used to produce estrogen. DHT is responsible for the masculization of many phenotypic characteristics in male mammalian development (Mayes and Watson, 2004). Estrogen, a hormone important in the estrous cycle, is produced primarily in the ovaries where
testosterone (T) is converted to this compound by the enzyme aromatase (Mayes and Watson, 2004). E, T, and DHT are all steroid hormones that cross the cell membrane and act mostly through their receptors: estrogen receptor (ER) or androgen receptor (AR), all of which act as transcription factors in the nucleus (Mayes and Watson, 2004).

Estrogen elicits its effects in the body through two main receptors, ER-α and ER-β (Hall et al., 2001). These two receptors have some overlapping tissue distribution, but are also found in separate tissues and thus potentially regulate different functions. The receptors can form homodimers with themselves or heterodimers with each other to act as transcription factors (Hall et al., 2001). ER-α is responsible for the majority of the well known feminizing effects of estrogen in mammals, especially in the brain. ER-β has been shown to regulate various estrogenic effects in the periphery (Sanchez et al., 2002). ERα & β are able to signal through STAT-3 and STAT-5 to stimulate transcription. This signaling involves MAPK, Src-kinase and PI-3 kinase, indicating a mechanism similar to that of leptin (Bjornstrom and Sjoberg, 2002). Both forms of the estrogen receptor have been found to be present in adipose tissue (Pedersen et al., 1995) and preadipocytes (Joyner et al., 2001).

Estrogen has been demonstrated to elicit anorectic effects in mammals. When mice were ovariectomized (OVX) and given estrogen injections, both food intake and body weight gain decreased in comparison to OVX mice treated with the vehicle (Bonavera et al., 1994). This hormone also targets NPY-producing neurons in the hypothalamus, specifically the PVN. In vitro experiments showed that estrogen treatment significantly decreased NPY secretion into incubation medium when compared to untreated rat brain sections (Bonavera et al., 1994). Estrogen has also been demonstrated
to inhibit the increase of NPY mRNA in the ARC which occurs when ovariectomized rats lose weight. Estrogen replacement halved the amount of NPY mRNA that is normally produced in fasted rats (Baskin et al., 1995). Estrogen increases the excitation of POMC neurons in the ARC of wild type, $ob/ob$ and $db/db$ mice. This indicates that the effects on these neurons via estrogen occur through a mechanism that is possibly parallel to leptin (Gao et al., 2007). Thus, the anorexic effects of estrogen were established prior to the discovery of leptin and provide an interesting avenue for comparison and study.

Leptin was first linked to estrogen in human subjects by Shimizu et al. (1997). They found that serum leptin concentrations were the highest in premenopausal women followed by postmenopausal women and males exhibited the lowest serum levels (Shimizu et al., 1997a). Human cross-sectional studies show that leptin levels are positively correlated to estrogen and negatively correlated to testosterone (Chan et al., 2002).

Estrogen has been shown to increase serum leptin in both intact and ovariectomized rats (Alonso et al., 2007). These studies showed that Ob-R expression decreased as estrogen treatments increased in adipose tissue (Alonso et al., 2007). Estrogen-induced estrous in ovariectomized rats stimulated serum leptin levels significantly when compared to the control (Tanaka et al., 2001). Studies show that leptin sensitivity is affected by estrogen. Mice with relatively high estrogen levels exhibited increased leptin sensitivity in the brain, as indicated by a significant decrease in food intake and the subsequent increase in weight loss. Neither ovariectomized non-treated females nor control males showed this trend (Clegg et al., 2006).
The mouse *Ob* gene also contains an estrogen-response element within its promoter and thus may be regulated by estrogen at the level of transcription (Shimizu et al., 1997b). Treatment with estrogen on primary cultures of rat adipocytes has shown that *Ob* mRNA levels increase after a 24-hour treatment with 10 nM 17β-estradiol (Machinal et al., 1999). Leptin secretion is stimulated by dexamethasone and estrogen, but not progesterone, in tissue sections from women (Casabiell et al., 1998). These effects are not observed in similar tissue sections from men (Casabiell et al., 1998). Aromatase knock-out (ArKO) mice accumulate central adiposity at an increased rate and exhibit elevated leptin levels when compared to control mice indicating the importance of estrogen in fat storage and maintenance of normal leptin levels (Jones et al., 2000).

Androgens also influence fat distribution in mammals. Interestingly, androgen receptor null male mice are obese and leptin resistant (Fan et al., 2008). This alone suggests a link between androgen signaling and the effects of leptin in mice. This relationship was examined in wild type male mice, in which the androgen receptor (AR) was coexpressed with Ob-Rb in the ARC. *In vitro* studies using HEK cells showed that leptin signaling via STAT3 to target genes such as POMC and SOCS2 was enhanced by the addition of AR constructs. AR also increased the STAT3 nuclear localization stimulated by leptin in these cell culture experiments (Fan et al., 2008).

DHT has been shown to inhibit the differentiation of preadipocytes via their receptor *in vitro* (Gupta et al., 2008). Fluorescently labeled free fatty acid was used to measure the amount of fatty acids incorporated into triglycerides. This experiment showed that DHT inhibited the integration of fatty acids into triglycerides (Gupta et al., 2008).
Transcription of the *Ob* gene has been shown to be affected by androgen treatment and these effects can be ablated by the addition of generic AR antagonists (Machinal et al., 1999). *Ob* mRNA levels are decreased in rat adipocytes by about 20% after *in vitro* exposure to 10 nM DHT and no effect from this treatment was seen on leptin secretion. Effects elicited by DHT were ablated with the addition of pharmaceutical antiandrogen, cyproterone acetate, which serves as an AR antagonist *in vitro* (Machinal et al., 1999). A more recent study with human primary subcutaneous adipose tissue demonstrated a pronounced increase in *Ob* mRNA in response to estrogen treatment, whereas only an extremely high dose of DHT showed any inhibition (Machinal-Quelin et al., 2002). This study also demonstrated the ability of both estrogen and testosterone treatments to stimulate leptin release into the media. This effect by testosterone was prevented through the use of an aromatase inhibitor treatment and the effects of estrogen were ablated using ER antagonists (Machinal-Quelin et al., 2002).

These observations suggest that there may be regulation of *Ob* gene expression primarily by estrogen, and that this regulation may act through a complex circular feedback mechanism. It may be possible that this regulation is a function of temporal and environmental factors. For example, the presence of leptin may indicate a nutritionally stable time for females to enter their estrous cycle, and then estrogen may in turn, serve to promote leptin production and adipose tissue formation. These regulatory effects are as yet, undetermined.

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*3T3-L1 Cells as a model for study*
A number of studies have used 3T3-L1 cells as a model for leptin production and regulation in adipocytes. 3T3-L1 preadipocyte cells, derived from a murine fibroblast line, were shown to spontaneously accumulate fat droplets (Green and Kehinde, 1974) and differentiate into adipocytes after prolonged periods of confluence (Green and Kehinde, 1975). The differentiation process consists of a change in cellular morphology involving a retraction of fibroblast-like processes and a rounding of the cell. These morphological changes are preceded by increased triglyceride synthesis after growth arrest. Differentiation was observed using Oil Red O stain following formalin fixation (Green and Kehinde, 1976).

Differentiation can be induced with a combination known as MDI: Methylisobutyl-xanthine, Dexamethasone, and Insulin (Sadowski et al., 1992). The differentiation of 3T3-L1 cells from preadipose to adipose cells occurs in approximately 6-8 days after the addition of MDI to cell culture plates. As preadipocytes, 3T3-L1 cells secrete little to no leptin protein (MacDougald et al., 1995) which is similar to primary cultures from rats. However, when the cells are fully differentiated, they do produce leptin and secrete the protein into the media (MacDougald et al., 1995).

In one particular study, when 3T3-L1 cells were differentiated into adipocytes, leptin mRNA and adipocyte marker protein mRNAs were shown to be expressed simultaneously (MacDougald et al., 1995). During this study leptin expression was maximized at approximately 8 days after the addition of MDI which is consistent with the full differentiation of these cells into adipocytes (MacDougald et al., 1995). 3T3-L1 cells have also been used to examine the role of various transcription factors in the regulation of the Obese gene. Ob gene expression is preceded by the expression of
CCAAT/enhancer binding protein (C/EBPα) and this gene contains at least one functional C/EBPα site in the promoter. Mutation at this site ablated Ob activation in 3T3-L1 cells, implicating C/EBPα as an important transcription factor in promoting Ob gene expression as measured through a luciferase reporter assay.

A separate study demonstrated that estrogen can stimulate Resistin gene expression in 3T3-L1 adipocytes that have been differentiated from preadipocytes. Investigators found an almost linear dose-dependence of estrogen for both resistin mRNA production as well as resistin protein release from the cell (Chen et al., 2006). This demonstrates the ability of these cells to respond to estrogen treatments and their suitability as a system to investigate estrogen-responsive genes. In contrast, a recent study using 3T3-L1 cells demonstrated no significant induction of Ob gene expression by estrogen at any dose (Yi et al., 2008).

The same study also confirmed the ability to manipulate Ob gene expression by changing the activity of the two estrogen receptors in these cells (Yi et al., 2008). ERα and ERβ specific antagonists were used to examine the control of various adipokines by estrogen in 3T3-L1 cells. Total RNA was extracted and changes in cytokine mRNA was measured using qRT-PCR. When ERα antagonists were added, leptin mRNA decreased, indicating that leptin was positively correlated to ERα. They also found that addition of ERβ antagonists increased leptin mRNA, suggesting that leptin is negatively correlated to ERβ (Yi et al., 2008). This suggests that some ratio of these two receptor subtypes may be important in the regulation of leptin expression and production in adipocytes.

The androgen receptor (AR) was also found in 3T3-L1 cells (Singh et al., 2006). Both testosterone and dihydrotestosterone were shown to inhibit adipogenic
differentiation in a dose-dependent manner in these cells. This action was mediated through the inhibition of C/EBPα and peroxisome proliferator-activated receptor (PPARγ) protein \textit{in vitro} (Singh et al., 2006).

PPARs are nuclear receptors which function as transcription factors. Specifically, PPARγ is expressed in 3T3-L1 adipocytes upon differentiation (Diradourian et al., 2005). This receptor is activated by binding to its ligand, and stimulates the transcription of a number of cell specific genes (Diradourian et al., 2005). It is also becomes deactivated by the phosphorylation of its Activating Function (AF)-1 domain by AMP-activated Kinase (AMPK). This phosphorylation targets the receptor for degradation by the ubiquitin-proteasome pathway (Diradourian et al., 2005). Since PPARγ is expressed specifically in adipocytes and not the pre-adipocyte precursor cells (Hwang et al., 1997), it serves as a marker of differentiation in 3T3-L1 cells.

\textit{Summary of Planned Experiments}

The mechanisms behind the sexual dimorphism of leptin levels in humans provide an appealing area to be examined. Sex steroid hormones estrogen and androgens may play a role, but the current data is so far inconclusive. \textit{In vivo}, cells and tissue do not experience the effect of one hormone in isolation, but in tandem with other hormones, all of which have their own effects. Cell culture experiments can be used to further elucidate the relationship between sex steroid hormones and leptin synthesis and secretion. 3T3-L1 cells provide an excellent model for study as they can be maintained relatively easily as preadipocytes and can then be differentiated into adipocytes.
Leptin levels in females are higher than those in males, and this may be due to estrogenic stimulation or androgen repression of leptin synthesis or secretion from adipocytes. Since females have higher levels of leptin than males, the effects may be due to an inhibitory effect of androgens in males rather than an enhancement from estrogen in females. Once it is established whether estrogen enhances leptin synthesis or secretion, and whether androgens have inhibitory effects, the effects of ratios of these two types of hormones in 3T3-L1 cells can be examined. The experiments described in this thesis will address these two main hypotheses and also examine the balance of these two kinds of steroid hormones.
MATERIALS AND METHODS

Chemical Reagents

All materials (17β-estradiol, testosterone, dihydrotestosterone, etc.) were purchased from Sigma Chemical unless otherwise mentioned. Dulbecco’s minimal essential medium (DMEM), penicillin-streptomycin, fetal bovine serum (FBS) gold, calf serum (CS) and trypsin were all purchased from Gibco. Trizol and the Superscript III kit were purchased from Invitrogen and SYBR green was purchased from Applied Biosystems. Leptin ELISA kits were ordered from Linco and Microcon Ultracel YM-10 Centrifugal Filter Devices from Millipore.

Cell Culture

3T3-L1 preadipocyte cell cultures, generously provided by Ron Morrison of UNCG, were maintained in Dulbecco’s Modified Eagle’s Medium (DMEM) with 10% calf serum (CS) and 1% penicillin-streptomycin. The cells were maintained in flasks in an incubator at 37°C and 5% CO₂ and split when they reached ~70% confluency, or approximately every 2-3 days. To differentiate the cells into adipocytes, cells were plated in DMEM supplemented with CS and maintained until approximately 2 days past confluence. The media was then changed to DMEM with 10% Fetal Bovine Serum (FBS), 0.5mM 1-methyl-3-isobutylxanthine, 1μM dexamethasone, 1.7 μM insulin (MDI). At 2 days post MDI treatment, media was changed to DMEM with 10% FBS and 0.4 μM insulin. After 2 additional days, the media was changed to DMEM with 10% FBS, with additional changes every 48 hours until the adipocytes were collected.
Hormone Treatments

Adipocytes were treated with β-17-estradiol, testosterone or dihydrotestosterone at 7 days post MDI treatment for dose-response experiments. Stocks of these steroid hormones were prepared in 95% ethanol at 1 mM. Serial dilutions were made in ethanol until a 1 uM stock was used to create hormone treatments in DMEM. Twelve hours prior to each hormone treatment, the cell media was changed to serum-free DMEM with L-glutamine and 1% penicillin-streptomycin. In dose-response experiments, cells were incubated with hormones following the previously mentioned serum-starvation for 12 hours. The following concentrations of hormones were administered to cells in fresh serum-free DMEM containing 17β-estradiol at 0nM, 0.01nM, 0.1 nM, 1 nM and 10 nM and testosterone and dihydrotestosterone at 0 nM, 0.1 nM, 1 nM and 10 nM. During time course experiments, cells were harvested at 0h, 3h, 6h and 12h following hormone treatments. For each experiment, the media from the period of hormone treatment was saved for quantification of secreted leptin via ELISA. At the end of the hormonal incubation, the media was collected and frozen for ELISA assays.

RNA Extraction

RNA was harvested using one of two methods: Trizol or by using the RNAeasy Kit (Qiagen). The kit procedure gave higher quality RNA so it was used for the extraction of all samples collected for this thesis. For the original sample set, RNA extraction via Trizol was used. As RNA is stable in Trizol, these samples could be stored at 4°C until the RNA was extracted. Chloroform was added to the cells in Trizol and they were centrifuged at room temperature for 30 minutes at 13,200 rpm. The RNA was then
stored in isopropanol at -20 °C overnight. The RNA was then spun for 45 minutes at 13,200 rpm, the pellet was washed with 70% ethanol in DEPC-treated water, resuspended with DNase I and DNase buffer (Promega). Following a 30-minute incubation at 37°C, the DNase was heat deactivated at 65°C for 10 minutes. RNA extracted using RNeasy was either immediately converted to cDNA through a first strand synthesis reaction or kept at -80°C. The concentration of each RNA sample was determined by measuring the concentrations of 2μL of sample on a Nanodrop (Thermo Scientific) spectrophotometer.

*First Strand Synthesis*

First strand synthesis reactions were run in accordance with SuperScriptIII (Invitrogen) protocol. The following components were mixed together and incubated at 65°C for 5 minutes and then put on an ice block for at least 1 minute: 5μg RNA, 1μL 10nM dNTP mix, 1μL 5ng/μL random hexamers and DEPC treated water to bring the reaction up to 10μL. 10μL of a cDNA mix were then added to the RNA and primer mixture; cDNA mix included 10X RT buffer, 25nM MgCl2, 0.1M DTT, RNaseOUT (40U/μL) and SuperScript III RT (200U/μL). The reverse transcription reaction was then run on a Perkin Elmer 0.5 Thermocycler (Thermo Electron Corporation) under the following conditions, with a single cycle of: 25°C for 10 minutes, 50 °C for 50 minutes, 85 °C for 5 minutes and then held at 4 °C. Following the thermal cycle, each sample was centrifuged and RNase H was added to each tube and incubated at 37 °C for 20 minutes. These samples were stored in the freezer and brought up to a total volume of either 50 or 100 μL prior to qRT-PCR.
Quantitative Real-Time PCR

Quantitative Real-Time PCR for Actin, Resistin (Retn), Peroxisome proliferator-activated receptor gamma (PPARγ) and Obese (Ob) transcripts was performed on each cDNA sample using Power SYBR Green PCR Master Mix and the primers shown in Table 1. Power SYBR Green Master Mix is a combination of normal PCR reagents and the SYBR green dye which fluoresces upon binding to double stranded DNA. Primers were designed using PrimerExpress software (Applied Biosystems) or Beacon Designer 7 (Premier Biosoft). The total qRT-PCR reaction volume for each gene was 20µL per well and each sample was run in triplicate. 40 cycles were run at 95°C for 15 seconds and 60°C for 1 minute, preceded by a 1 minute denaturing step at 95°C using the Applied Biosystems 7500 Fast Real-Time PCR system. The instrument reports the amount of copies of the gene of interest at each cycle and specifically at which cycle the reaction becomes exponential. Relative changes in expression were calculated using the standard curve method.
Transcript Primer | Sequence (5’ → 3’)
--- | ---
Actin forward | ACGGCCAGGTCACTACATTG
Actin reverse | CAAGAAGGAAGGCTGGAAAAGA
Leptin (Ob) Forward | TCGGAAACCCGACAGAGTCT
Leptin (Ob) Reverse | ACCCTCAAAGGTCGACACACA
PPARγ Forward | GCTCCTGTAGGTTCTGTGTTG
PPARγ Reverse | ACACTGCTAGACGGACTC
Resistin Forward | CCACCTACTTCTACCTACC
Resistin Reverse | GTCGGACCTGATATACTCTC

Table 1 Primer sequences used for qRT-PCR listed with the indicated mouse transcripts

Calculation of Relative Changes in Expression

All primers were tested for specificity through the examination of melt curves which provide information about amplicon specificity. Any primer set which yielded melt curves with multiple peaks was eliminated from use. Primer sets were also tested for Efficiency (E). E is a measure of how well a primer set responds to serial dilutions of template. It can be calculated using the slope of the best-fit line of the relationship between the log of the template concentration using 4 dilutions separated by 5- or 6-fold and C_t. C_t is the cycle number at which the reaction begins to be exponential. Through the use of the standard curve, relative abundances for each gene of interest were calculated. Standard curves need to span the range of C_T values within each sample and should be run on the same qRT-PCR place as the experimental samples. Once the standard curve
equations are determined, \( C_T \) values from each of the samples can be entered which gives
the log of the relative concentration. Taking the anti-log of this value will yield a
concentration for each gene of interest. Ratios of target genes to normalization genes can
then be taken, and all values are adjusted to be relative to the control sample values.

**Protein Quantification: Bradford and ELISA Assays**

Media collected from the cells was concentrated using Microcon Ultracel YM-10
Centrifugal Filter Devices (Millipore) by approximately 30-fold in order for leptin levels
to be detected using a Mouse Leptin ELISA kit (Linco). Total protein in each sample
was measured, following concentration, via Bradford Assay. Standard curves for the
Bradford assays were created using bovine serum albumin (BSA) (Sigma) ranging from
0-7 µg. 10 µL of each experimental sample following concentration was compared to
these standard curves to estimate total protein.

The “sandwich” ELISA assay method was used, where an antigen specifically
binder to the leptin antibody and attaches to the 96-well plate. After all other proteins are
washed away, a detection antibody was added to bind to the leptin-antigen complex.
Adding a substrate allows the entire complex to be measured through the production of
visible light which represents the amount of leptin bound to the plate. Adsorption values
at 450nm were measured and the concentrations of secreted leptin, in ng/mL, from the
concentrated media samples were calculated using a standard curve. Each concentrated
sample was run in duplicate. Secreted leptin levels were normalized to either total protein
(from the Bradford Assay) or to the original volume of the supernatant considering the
fold concentration in each sample.
RESULTS

Optimization of qRT-PCR to measure changes in target mRNA abundance

Females of many species have higher leptin levels than males (Clegg et al., 2006; Di Carlo et al., 2002; Mayes and Watson, 2004; McConway et al., 2000). Therefore we tested two hypotheses to explain this observation, by asking whether estrogen induces leptin synthesis or if androgens inhibit leptin synthesis. We used the 3T3-L1 preadipocyte cell line to test this hypothesis. These cells were differentiated to form fully-developed adipocytes, as previously described (MacDougald et al., 1995), and the experimental design targeted these hypotheses in a two pronged approach. We measured changes in Ob gene expression and leptin secretion (protein in the cell culture media) in response to hormone treatments in these cells.

Changes in mRNA levels were measured accurately using quantitative real-time polymerase chain reaction (qRT-PCR). All target genes were normalized to Actin, a housekeeping gene, which is representative of the total relative amount of mRNA in each sample. Primers were designed for Actin and each of 3 target genes: Peroxisome proliferator-activated receptor gamma (PPARγ), an adipocyte-specific marker (Zhang et al., 2008) to provide an indicator of differentiation; Resistin (Retn), a pre-established estrogen-responsive gene in adipocytes (Chen et al., 2006) to verify that estrogen-treatments were successfully inducing gene expression and Obese (Ob), the gene which encodes leptin.

The efficiency of the amplification of these target genes was determined by 5- or 6-fold serial dilutions of template. C_T, or the threshold (cycle number) at which the PCR reaction becomes exponential could then be plotted vs. the log of fold-change in template
concentration, as shown in Figure 2, for these four primer sets. Efficiency for each primer set is determined using the slope of each best-fit line. As demonstrated in Figure 2, Actin and the 3 target genes had different efficiencies. Actin, which has the steepest slope, has the highest efficiency, followed by Resistin, Ob and PPARγ respectively. Once primers were designed, the relative abundance of each target gene transcript could be measured. The abundance of a given target transcript is inversely related to the C_T number, with a higher C_T being associated with lower abundance of target. Standard curves created from the previously described 5- and 6-fold dilutions were used to calculate the relative abundance of mRNA transcripts for a given gene. Resistin was present at the lowest abundance of all genes measured (Fig.2), while Actin was the most abundant transcript in these samples.

Effects of 17β-estradiol on Ob mRNA Expression

To examine the potential effects of estrogen on Ob gene expression we chose to use treatment doses that had been previously shown to induce an estrogen responsive gene in 3T3-L1 cells (Chen et al., 2006). An initial experiment, performed by Clare Hector, suggested that estrogen treatment increased levels of Ob mRNA in a concentration-dependent manner, as measured via semi-quantitative PCR. These samples were then re-analyzed for this thesis project using qRT-PCR (with primer optimization described above), as shown in Figure 3. In this original sample set, Ob mRNA message accumulation is increased at all estrogen doses by at least 3.9-fold with the greatest response at 1 nM in which there is an approximate 13-fold increase in Ob message level.
Statistical analysis can not be performed with these samples as each bar represents the mean and standard error of 3 technical replicates, not biological replicates.

As these values were from single biological replicates, we repeated and extended these experiments. As the Ob expression at the highest dose appeared to saturate and was supra-physiological, we removed that dose and added a lower dose of 0.01 nM to identify the lowest concentration at which effects became apparent. The effect of these treatments on Ob mRNA accumulation in response to 0.01, 0.1, 1, and 10 nM of estrogen for 6 biological replicates from 3 separate experiments is shown in Figure 4. Unfortunately this effect is not significant due to large variation between experimental replicates. This is reflected in a number of samples, some of which have C_T values which are off of the standard curve for both Ob and Actin. In Figure 4A, where the Ob message levels are reported relative to Actin, the greatest effect is found at 10 nM estrogen, where there is an approximate 4-fold increase in Ob mRNA relative to the untreated control. Although there is not a simple dose-dependent relationship between estrogen concentration and Ob mRNA accumulation (Figure 4), estrogen does increase Ob mRNA accumulation at 0.01, 0.1 and 1 nM doses, however no significant values are reported.

**Accounting for Variation in Experimental Samples**

To ask if the variation in magnitude of the estrogen effect on Ob message accumulation in Figure 4 was due to variation in biological replicates or technical variation, we examined the technical replicates from estrogen treated samples. C_T levels were highly consistent among technical replicates, as demonstrated in Figure 5, which compares the average technical replicates for both Actin and Ob in eight vehicle treated
biological replicates from four separate experiments. The small error bars demonstrate the consistency of this method based on the ability of the designed primers and other technical aspects of the procedure to accurately measure the abundance of target in a given sample. This result indicates that qRT-PCR is very efficient in measuring the levels of transcripts. The variation in Figure 4 therefore must result from biological variation between samples. This biological variation may be the result of variation in the amount of estrogen induced gene expression due to stability, solubility, or uptake of estrogen or may be due to variation in the degree of adipocyte differentiation. We therefore examined two additional genes which were previously shown to be estrogen induced in this cell line (Chen et al., 2006) or induced during adipocyte differentiation (Zhang et al., 2008), as markers of variability of these two processes.

Since leptin is only produced in fully differentiated adipocytes, we then wanted to examine the possibility that variation in the expression of this adipokine was influenced by variable differentiation in these samples. We measured the accumulation of PPARγ mRNA in these samples and the levels of the PPARγ message relative to actin is shown in Figure 6. These values vary between treatments and biological replicates. There are very large non-significant increases at both 0.01 and 1 nM doses of estrogen (45-fold and 20-fold respectively). Therefore the large degree of variability of PPARγ in these samples makes it difficult to observe specific effects when this transcript is used as a control.

We also normalized the Ob mRNA relative to the PPARγ mRNA, as shown in Figure 4B, for the newer estrogen treated samples. This graph suggests that when the degree of differentiation of these samples is considered, there are no significant changes in the expression of the Ob gene induced by estrogen treatment, and the pattern of the
degree of changes in response to each dose of estrogen is changed. Once the data is normalized to PPARγ the only dose at which there is a substantial response is 1 nM which is a non-significant ~5-fold induction.

Since we observed differences in PPARγ in both our biological replicates and between our estrogen treatments in the newer sample set, we wanted to ask whether this variation in differentiation was present in the original sample set. As shown in Figure 3, there is also variation in the PPARγ mRNA levels, with a maximal, 5.7-fold increase in the 10 nM dose. When Ob is normalized to PPARγ in original samples, as shown in Figure 7A, the effects of estrogen are not as substantial. In fact, when Ob mRNA is normalized to PPARγ, only the 10 and 100 nM estrogen doses have substantial effects on Ob gene expression, with approximate 8- and 5-fold increases respectively (Figure 7A). Statistical analysis could not be completed with one biological replicate.

Effects of 17β-estradiol on Resistin mRNA Expression

Since we did not observe a consistent estrogen dose response curve in Ob mRNA accumulation, we asked whether estrogen treatments were successfully stimulating estrogen-responsive genes. Resistin gene expression has been examined in 3T3-L1 cells and is induced by estrogen (Chen et al., 2006), thus relative transcript levels of this gene can be measured as a positive control to demonstrate the effectiveness of our estrogen treatments. We measured changes in Resistin mRNA abundance in the newer estrogen treated samples. The Retn message level was at lower levels than Actin, Ob, and PPARγ transcripts, which resulted in much more variable qRT-PCR results. Therefore expression in individual biological replicates, as well as the average values normalized relative to
actin, are reported in Table 2. This table indicates that Retn mRNA accumulation was extremely variable in these estrogen treated samples. At all estrogen doses some samples exhibited non-significant increases, with the largest at 0.01 and 1 nM estrogen, where there were ~2.2- and 3.8- fold increases relative to the untreated control. Resistin gene expression was still variable when normalized to PPARγ as demonstrated in Table 2, so the Retn variation is not simply due to differences in differentiation.

In the original sample set, analyzed in Figure 3, estrogen treatment increased levels of Retn mRNA in a concentration-dependent manner, although there is substantial technical variation in these samples. The concentration which began to stimulate increased Retn mRNA was 0.01 nM after approximately 12 hours of treatment, eliciting an approximate 1.3-fold increase. Maximal increase was in response to 100 nM estrogen, which was an 125-fold increase in Retn mRNA transcripts. This result suggests that estrogen treatments were stimulating transcription of estrogen-responsive genes in these samples, with a similar level to that in the more recent samples, but with a more consistent dose dependent pattern. Again, statistical analysis could not be performed on this sample set as each treatment only was represented by 1 biological replicate. Normalization of Resistin to PPARγ does not change the estrogen responsive pattern, but rather the magnitudes of the changes. Estrogen-induction in Retn gene expression begins at 10 nM and is maximal at 100 nM with approximate 6.5- and 180-fold inductions respectively (Figure 7B).

The variation in these samples, even after attempting to correct for changes in differentiation suggests that estrogen treatments may not be reaching the cells consistently. Estrogen was dissolved in 95% ethanol rather than the standard 100%
ethanol. This could mean that estrogen was not consistently dissolved in some of the estrogen treatments. Some of this variation may also be due to the fact that Resistin is present at very low levels in these samples and it is possible that the primers had trouble detecting changes accurately. However, the trend in the original sample set is consistent with previously published data (Chen et al., 2006).

Together these analyses of Retn gene expression suggest that estrogen may be stimulating Retn transcription in some of the newer samples, but not in others, limiting our ability to make confident conclusions about estrogen’s stimulation of transcription in these samples. Normalization of Retn in the original sample set minimized some of the variation, but did not eliminate the large estrogen-induced increases in Retn message abundance. The data from the original sample set is supported by previously published data, in which estrogen elicited large increases in Resistin mRNA abundance (Chen et al., 2006). This result indicates that Resistin can serve as a positive control for estrogen treatments in 3T3-L1 cells, and that in our larger sample set, estrogen treatments are variable.

**Effects of ICI 182780 on Ob mRNA Accumulation**

A second approach was utilized to examine the effects of estrogen signaling on Ob transcription. 3T3-L1 adipocytes were treated with an estrogen receptor antagonist (ICI 182780). This compound inhibits α and β estrogen receptor (ER) isoforms (Chen et al., 2006). Cells were treated with estrogen and the inhibitor, and the Ob message accumulation is compared to untreated controls, as shown in Figure 8. Although no induction by estrogen was observed in this experiment, there was a slight reduction in Ob
mRNA expression in response to the inhibitor (Figure 8). This reduction, may signify a regulatory aspect of the estrogen receptor in transcription that is non-traditional, in other words, this regulation may be independent of estrogen binding to its receptor. Alternatively, this result may suggest that there is some residual estrogen in the serum-free media or within cells, which is stimulating estrogen-responsive gene expression, and this stimulation is eliminated in the presence of the ER inhibitor.

**Effects of Testosterone on Ob mRNA Accumulation**

We also tested the alternate hypothesis that the sexual dimorphism of leptin is due to an inhibitory effect of androgens on leptin synthesis. Testosterone (T) treatments were administered to 3T3-L1 cells at 3 different doses (0.1, 1 and 10 nM) as these doses had been used to alter gene expression in previous cell culture studies (Machinal-Quelin et al., 2002; Machinal et al., 1999). The effect of T on Ob message accumulation relative to Actin is shown in Figure 9. These testosterone treatments had no significant effect on Ob mRNA levels at any dose in 3T3-L1 cells (Figure 9). 0.1 nM testosterone lead to a non-significant ~1.8-fold induction in Ob mRNA.

T can be converted to other steroid hormones including estrogen (E) or the androgen, dihydrotestosterone (DHT) (Mayes and Watson, 2004). Thus the lack of an effect of T on Ob mRNA levels (Figure 9) may be due to its conversion to E or DHT, by the enzymes aromatase and 5α-reductase, respectively. Since both enzymes are present in adipocytes it may be that testosterone is converted to either E or DHT with a net ratio that does not lead to significant changes in Ob expression.
The effects of Dihydrotestosterone on Ob, Retn, and PPARγ mRNA accumulation

Once T is converted to DHT, it can no longer be used to produce estrogen. To truly elucidate whether androgens inhibit leptin synthesis or secretion, DHT was used. Cells were treated with 0, 0.1, 1, and 10 nM DHT (Gupta et al., 2008; Machinal-Quelin et al., 2002; Machinal et al., 1999) for 12 hours at which time RNA was harvested and the abundance of Ob mRNA was determined by qRT-PCR, as shown in Figure 10. DHT treatment significantly decreased levels of Ob mRNA normalized to Actin, in a concentration-dependent trend at doses equal to or greater than 1 nM with a maximal and significant reduction of 60% at the 10 nM dose (p<0.05) (Fig. 10A).

Since we observed some variation in the levels of differentiation in the estrogen treated samples as judged by PPARγ message levels, we wanted to ensure that the changes seen in DHT samples were in fact due to a DHT response, and not differing levels of differentiation. After quantifying PPARγ relative changes in mRNA abundance after DHT treatment (Figure 13), we see a trend for increasing PPARγ message with increasing DHT, although large variation in this message complicates this analysis. Actin mRNA levels may simply represent the total amount of mRNA in a sample, not the amount of differentiated adipocytes (Figure 11). When Ob mRNA levels are normalized relative to PPARγ, three doses (0.1, 1, and 10 nM) elicit reductions in Ob mRNA, although no significant differences were detected (Figure 11). This experiment was run twice, once with PPARγ and Ob on separate plates (Fig.11A), and once with both targets on the same plate (Fig. 11B). In the DHT dose-response experimental samples, one of the control biological replicates (0 nM DHT) contained particularly high PPARγ transcript levels which leads to high variability, and weakens statistical analysis of these samples.
We also measured Resistin message accumulation in response to DHT treatment. Figure 12A demonstrates that although Resistin levels are normalized to Actin, the mRNA accumulation is increased in all DHT treated samples, but there is quite a bit of variation, and no significant changes are reported. When the Retn mRNA accumulation is normalized to PPARγ (Fig. 12B) we see an opposite trend for decreasing Retn transcript abundance in response to DHT treatment although no significant differences were detected.

Effects of Cyproterone Acetate on Ob mRNA accumulation

To further understand how the regulation by DHT occurs, 3T3-L1 adipocytes were treated with an androgen receptor antagonist, cyproterone acetate, and the effect of this inhibitor alone and in the presence of DHT was compared to controls. This inhibitor alone, administered as a 1 µM treatment as described previously (Machinal-Quelin et al., 2002), had no significant effects on Ob mRNA expression. However the reduction in Ob mRNA levels by DHT, although non-significant, was partially prevented by a 30-minute pre-incubation with the antagonist prior to the 12 hour DHT treatment (Fig. 14). This result suggests that the reduction in Ob mRNA transcripts may occur through DHT binding to the androgen receptor to reduce transcription of the Ob gene.

Effects of dihydrotestosterone on Ob gene expression over time

The inhibitory effect of DHT on Ob mRNA levels was examined in a time-course experiment and the results are reported in Figure 14. Cells were treated with 1 nM DHT, as it is a physiologically relevant dose (Gupta et al., 2008), and it elicited a decrease in
Ob mRNA when normalized to both Actin and PPARγ. Cells were then harvested at 0, 3, 6 and 12 hours following this treatment. There is an approximate 3.5-fold induction of Ob mRNA at 3 hours in DHT treated samples, however this is not significantly different from Ob mRNA abundance at time zero, and may be due to variation between biological replicates. In fact, the DHT-treated biological replicates at this time point had relative expression values of 5.25, 5.13 and 0.46 which suggests that Ob mRNA may in fact be increasing in response to DHT at 3 hours post-treatment, although this result was not significantly different from the control. This result requires further examination. The inhibitory effect of DHT treatment was not evident until 12 hours following treatment (~10% reduction). In one of the 3 biological replicates for the 12-hour time point, Ob mRNA transcript abundance was relatively high while the others were quite low (1.98, 0.34 and 0.47). This suggests that although no significant results could be reported, DHT treatment may in fact be decreasing Ob gene expression by 12 hours. These results are consistent with the earlier experiments indicating a decreasing trend in Ob gene expression at 12 hours with 1 nM DHT.

Effects of dihydrotestosterone on leptin secretion

Since we were able to detect significant differences in Ob mRNA in response to DHT, we wanted to examine how these effects translated into leptin secretion. To do this we collected media extracts from the hormone treated 3T3-L1 cells, concentrated them and measured total protein (Bradford assay), fold-concentration and total leptin (ELISA) in each sample. We were unable to detect any differences in leptin secretion in response to DHT as shown in Figure 15 panels A and B which report leptin normalized to total protein.
protein and to the volume of the cell culture supernatant, respectively. These results come from three separate experiments which all had different trends in response to the hormone, but with no significant changes in any experiment. In one experiment secreted leptin decreased over time, in another it remained the same and in a third experiment secreted leptin increased. Similar trends were found when values were normalized to secreted protein and culture volume. The three distinct experimental trends are the reason why so much variation is seen in these treatments and suggest that the secretion of leptin does not directly parallel the Ob message accumulation.

We also examined changes in leptin secretion from the androgen receptor antagonist experiment and those results are reported in Figure 16. In Figure 16A there is an increase in secreted leptin in response to 1 µM cyproterone acetate, which is unexpected based on the lack of changes in Ob mRNA levels by this treatment (Figure 13). However, when leptin is normalized to the volume of the culture supernatant, there are no significant changes for any samples relative to the untreated control (Figure 16B).

Analysis of the leptin secretion normalized to both total protein and supernatant volume allows us to ask if there are effects of the hormones which may be masked in either of the normalizations. Normalizing to protein concentration, which is measured by Bradford assay, allows us to relate the amount of leptin in the media to the total amount of secreted protein. However, in some cases, the Bradford assay was not sensitive enough to measure small changes in total protein, which is at low levels even after concentration, and thus, the total protein may be either over or under-represented in various samples. Normalizing to culture media volume eliminates the need for comparison to values from the Bradford assay, and eliminates any other potential changes in protein levels which
may be attributed to the hormone treatments. In this specific experiment, we would not expect to see an increase in secreted leptin in response to the AR antagonist alone, thus the graph normalized to supernatant volume makes more biological sense.

Changes in secreted leptin over time were also measured via ELISA as shown in Figure 17. Here we see that the samples which were DHT treated had significantly less leptin in them at zero hours in comparison to the vehicle treated controls (Figure 17A). Differences in these samples stabilize at 3 and 6 hours, and then a significant reduction in response to DHT is seen at 12 hours (Figure 17A). No significant differences could be reported when the data is normalized to supernatant volume (Figure 17B). Figure 17B is consistent with results shown in Figure 15, in that DHT has no significant effect on leptin secretion compared to the control at 12 hours. This comparison again suggests that normalizing to supernatant volume is more appropriate, as in Figure 17A the control (Time 0) leptin levels are vastly different from each other. Normalizing to supernatant volume eliminates this difference, which makes the most biological sense, as these values should not be different at time zero.
Figure 2: Standard curves for quantitative real-time PCR primers.

Primers recognize 5- or 6-fold changes in template in a linear manner. Slopes for Actin, Ob, PPARγ and Retn are 3.8859, 2.0181, 1.2138 and 2.2265 respectively.
Figure 3: Estrogen increases both Ob and Retn mRNA transcripts in the original preliminary data set as normalized to Actin.

Estrogen increases both Ob and Retn mRNA transcripts in the original preliminary data set as normalized to Actin. The relative mRNA expression for 1 biological replicate per treatment is reported. Bars represent the average and standard error of the technical replicates.
Figure 4: Estrogen has variable effect on Ob mRNA transcription

Ob mRNA levels are (A) normalized to actin or (B) normalized to PPARγ. The average and standard error of 6 biological replicates are reported. Relative changes in mRNA levels were compared to the control using Student’s t test. No significant changes were detected.
Figure 5: Variation between qRT-PCR technical replicates in untreated control samples.

The average and standard error of 8 untreated control samples from 4 separate experiments are reported.
Figure 6: Estrogen has no significant effect on \textit{PPARγ} mRNA transcription when normalized to \textit{Actin}.

The average and standard error of 4-5 biological replicates per treatment are reported. Relative changes in mRNA levels normalized to \textit{Actin} were compared to the control using Student’s t test. No significant differences were detected between samples.
Figure 7: Estrogen increases *Ob* and *Retn* mRNA transcripts in the original preliminary data set as normalized to *PPARγ*.

(A) *Ob* mRNA normalized to *PPARγ* and (B) *Retn* mRNA normalized to *PPARγ*. Bars represent the average and standard error of technical replicates from 1 biological replicate.
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Table 2: Estrogen has variable effects on *Retn* mRNA transcription as normalized to *Actin* and *PPARγ*.

Individual replicates for each treatment are reported as well as the average and standard error. In total, 7 biological replicates had no amplified *Resistin* transcripts and thus are omitted from this table. Relative changes in mRNA levels were compared to the control using Student’s t test. No significant results are reported.
Figure 8: ICI has no significant effect on *Ob* gene expression normalized to *Actin*.

The averages and standard errors from 3 biological replicates are reported. Relative changes in mRNA levels were compared via ANOVA. No statistically significant changes are reported.
Figure 9: Testosterone (T) has no significant effect on Ob transcript abundance normalized to Actin.

The averages and standard errors of 3 biological replicates are reported. Relative changes in mRNA levels were compared to the control using Student’s t test but no significant differences were detected.
Figure 10: Dihydrotestosterone (DHT) significantly inhibits Ob mRNA accumulation.

*Ob* mRNA levels were normalized to *Actin*. The average and standard error of 3 biological replicates are reported. Relative changes in mRNA levels relative to *Actin* were compared to the control using Student’s t test. * Indicates a p value of <0.05.
Figure 11: Dihydrotestosterone (DHT) has no significant effect on Ob mRNA abundance when normalized to PPARγ

Ob mRNA levels were normalized to PPARγ. The average and standard error of (A) 2-3 biological replicates of an experiment run without a standard curve on the plate and (B) 3 biological replicates with a standard curve run on the plate are reported. Relative changes in mRNA levels relative to PPARγ were compared to the control using Student’s t test. No significant differences were detected.
Figure 12: Dihydrotestosterone affects *Retn* message with opposite trends when normalized to *Actin* or *PPARγ*

(A) *Retn* mRNA abundance normalized to *Actin* and (B) *PPARγ*. The average and standard error of 2-5 biological replicates are reported. Relative changes in mRNA levels were compared to the control using Student’s t test. No significant differences were detected.
Figure 13: PPARγ levels are variable in DHT-treated samples

The average and standard error of 4-5 biological replicates are reported. Changes in mRNA levels normalized to Actin were compared to the control using Student’s t test. No significant values were detected.
Figure 14: The androgen receptor antagonist, cyproterone acetate (CA), has no significant effect on *Ob* gene expression.

The averages and standard errors of 3 biological replicates are reported with values normalized to *Actin*. Relative changes in mRNA levels were analyzed via ANOVA. No significant changes were detected.
Figure 15: The effects of 1 nM dihydrotestosterone on Ob mRNA transcript abundance over time normalized to Actin.

DHT has no significant effect at any time point. The averages and standard errors of 2-3 biological replicates are reported. Relative changes in mRNA levels were compared to the control using Student’s t test. No significant values are reported.
Figure 16: DHT has no significant effect on leptin secretion.

Leptin secretion was normalized to (A) total secreted protein or (B) mL culture media as measured via ELISA. The averages and standard errors of 9 biological replicates per dose are reported. Leptin levels were compared to the control using Student’s t test. No significant differences were detected.
Figure 17: The effects of cyproterone acetate (CA), androgen receptor antagonist on leptin secretion from 3T3-L1 cells

(A) Leptin secretion was normalized relative to total secreted protein or (B) mL culture media. The averages and standard errors of 3 biological replicates per treatment are reported. Values were compared via one-way ANOVA and no statistically significant changes are reported.
Figure 18: The effects of dihydrotestosterone treatment over time as normalized to (A) total protein and (B) mL media

The averages and standard errors of 3 biological replicates per treatment at each time point are reported. Changes in leptin levels were normalized to total protein and then compared to the control using Student’s t test. * Indicates a p value of <0.05.
DISCUSSION

Females of many species have higher leptin levels than males (Clegg et al., 2006; Di Carlo et al., 2002; Mayes and Watson, 2004). Therefore we tested the hypothesis that estrogen increases Ob mRNA transcription in 3T3-L1 cells. Ob transcript abundance was examined in response to a number of different doses of estrogen. Preliminary data suggested that estrogen enhanced the accumulation of Ob mRNA by approximately 13-fold as compared to an untreated control, when normalized to Actin mRNA levels. Because this initial data set included only 1 biological replicate for each dose, it was not possible to judge the statistical significance of this effect. A larger data set yielded a smaller induction (approximately 4-fold) by estrogen, with non-significant increases in transcript abundance at 3 doses. The increase in Ob message was dramatic in some of the samples, but large variation in Ob message level between biological replicates negatively impacted the statistical significance. This estrogen-induced increase in Ob mRNA transcript abundance is in agreement with previously published data indicating an estrogen induced expression of the Ob gene in primary adipocytes (Machinal-Quelin et al., 2002; Machinal et al., 1999). It also suggests that estrogen-induced changes in Ob transcript abundance can be evaluated using qRT-PCR in 3T3-L1 cells.

Since we were able to observe some induction of the Ob gene in response to a number of estrogen doses (although the effect was not statistically significant), we hypothesized that this increased expression in these cells may be due to the transcriptional stimulation of estrogen binding to its receptor and acting as a transcription factor in the nucleus. This hypothesis is partially supported by the results presented in Figure 8, which demonstrates a reduction in Ob mRNA in response to estrogen receptor
inhibitor, ICI182780. This inhibitor, which reduces the activity of ER-α and ER-β (Hall et al., 2001), seems to be having some negative effect on *Ob* transcription which suggests that estrogen signaling is playing a role.

The trend for decreasing *Ob* mRNA abundance in response to treatment with an estrogen receptor inhibitor, although non-significant, is consistent with previously published data in other systems (Machinal-Quelin et al., 2002; Machinal et al., 1999). However, one recent study (Yi et al., 2008) was unable to show an induction in *Ob* mRNA in response to estrogen. They were however, able to show that both forms of the estrogen receptor (ERα and ERβ) may be involved in the regulation of *Ob* gene expression (Yi et al., 2008). By inhibiting the individual ERs pharmaceutically, they found that ERα was positively correlated with *Ob* mRNA levels in 3T3-L1 cells while ERβ was negatively correlated with these levels (Yi et al., 2008).

We tested our second hypothesis, that androgens may inhibit *Ob* gene expression and/or leptin secretion, using the approaches described previously. Our examination of the effects of androgens on *Ob* gene expression is the most comprehensive study as of yet, as it includes testosterone (T) dose-response, as well as dihydrotestosterone (DHT) dose-response, time-course and androgen receptor antagonist data. The present study demonstrates that DHT inhibits *Ob* gene expression in a dose-dependent manner in 3T3-L1 adipocytes (Figure 10). Figure 10 shows that DHT inhibits *Ob* mRNA abundance normalized to *Actin* starting at 0.1 nM doses, with a significant, maximal reduction (60%) at the 10 nM dose.

These significant results are likely due to the robust response of *Ob* expression to DHT and to a stronger experimental protocol used for the analysis of DHT effects. All
biological replicates were prepared in parallel, and both *Ob* and *Actin* were analyzed along with standard curves in the same qRT-PCR analysis. This experiment yielded very little technical variation and all C_T values for both *Actin* and *Ob* were on the standard curve. Experience with both experimental setup and data analysis, strengthen this conclusion and make it the most compelling piece of data in this thesis.

Since we were able to observe a significant decrease in *Ob* mRNA transcripts in response to DHT (Fig.10), we wanted to ask whether this reduction was due to DHT binding to the androgen receptor to regulate transcription. This hypothesis is supported by the results showing that these inhibitory effects were partially prevented by treatment with the androgen receptor antagonist, cyproterone acetate (Fig. 13). Here the *Ob* abundance is decreased by treatment with 1 nM DHT and this effect is partially restored by cyproterone acetate treatment, although these differences are not significant. These trends are in agreement with previously published data from other systems (Machinal-Quelin et al., 2002; Machinal et al., 1999) in which the inhibitory effects of DHT were prevented through treatment with this receptor antagonist. This experiment was performed with 1 nM DHT which has a weaker effect on *Ob* expression then the 10 nM dose. Now that the dose response curve is complete, this suggests that a 10 nM DHT dose would be a more appropriate dose to combine with the inhibitor treatment.

The inhibitory effect of DHT on *Ob* mRNA transcript abundance has been shown in other systems, including primary rat adipocytes (Machinal et al., 1999) and human primary adipocytes (Machinal-Quelin et al., 2002; Wabitsch et al., 1997). In the first study, a single dose (10 nM) of DHT for 24 hours inhibited *Ob* mRNA by approximately 20%, and this effect was ablated by treatment with the same AR antagonist used in these
thesis experiments. In human primary cultures, DHT treatment was used on adipose tissue taken from men (Machinal-Quelin et al., 2002). These experiments found that DHT only elicited effects at higher doses of 100 nM (Machinal-Quelin et al., 2002). These thesis experiments are the first to show changes in \( Ob \) expression with multiple doses of DHT (Fig.10) at multiple time points (Fig.14) on \( Ob \) mRNA accumulation, with significant reductions at 12 hours with a 10 nM dose of DHT.

Interestingly, when examining the effects of DHT on \( Ob \) transcripts over time the inhibitory trend of the effects of the hormone can be seen at both 6 and 12 hours. The reduction is maximal at the latest time point, albeit non-significant (Fig.15). This timing probably indicates that these effects are at the level of gene expression (Michels and Hoppe, 2008), or an example of “classical” signaling which involves DHT binding to androgen receptors. The receptors will then dimerize and bind to their response element on the promoter of various genes. The AR has been shown to bind to two types of sequences: generic hormone response elements (HREs) with the sequence 5’- AGA/TACA/TGCA/GT/AGTTCT-3’ and direct repeats, sequences 5’- A/GGCTCTNNNA/TGTTCT/C-3’ (McEwan, 2004). Whether the decreases in \( Ob \) mRNA are due to direct AR binding to the \( Ob \) gene or are the result of a second transcription factor whose synthesis is regulated by AR is an interesting question that awaits additional experimentation.

Paired together these three experiments, especially the DHT dose-response curve (Figure 10) in which there is a significant reduction in \( Ob \) mRNA in response to 10 nM DHT, suggest that DHT plays a major role in the regulation of \( Ob \) gene expression. It is likely that DHT binds to its receptor to stimulate transcription in the nucleus, as is
evidenced by both the inhibitor data as well as the time course data, both of which show trends for decreased Ob gene expression at 12 hours, which is partially ablated by treatment with the androgen receptor antagonist.

One factor that can complicate the analysis of estrogen- and DHT-related gene expression in 3T3-L1 cells is uneven differentiation between samples. Differentiation was measured through the use of Peroxisome proliferator-activated receptor gamma (PPARγ), which encodes an adipocyte-specific gene product (Zhang et al., 2008). In the original data set, PPARγ mRNA abundance was variable between samples, with fairly large inductions at all doses (2-, 3.4-, 5.7- and 1.26-fold) when compared to the control (Fig.3). In the larger data set, PPARγ transcript abundance was up-regulated by approximately 40-fold at 0.01nM and by approximately 20-fold at 1 nM doses of estrogen, but this data set was also negatively impacted by large variation in PPARγ message levels (Figure 6).

In DHT treated samples (Figure 13), a non-significant trend for increased PPARγ mRNA in relation to DHT dose is seen. These results are consistent with previously published data in which estrogen significantly up-regulated PPARγ2 expression, and DHT had no significant effect (Dieudonne et al., 2000). The study by Dieudonne et al. (2000) supports our results for the effects of E and DHT on Ob mRNA expression.

In one recent study, DHT has been shown to inhibit the differentiation of preadipocytes by signaling through the androgen receptor in vitro (Gupta et al., 2008). In these thesis experiments, 3T3-L1 cells were fully differentiated at the time of DHT treatment. Our data does not suggest that DHT has any effect on differentiation, as demonstrated by no significant changes in PPARγ mRNA abundance in DHT-treated
cells. It would be interesting to look at other markers, mainly Preadipocyte factor-1 (Pref-1) (Zhang et al., 2008) as well as some adipogenesis genes throughout the differentiation process to see if DHT treatment may induce or inhibit these factors in 3T3-L1 cells. It may be that DHT also has some effect on the amount of differentiated adipocytes in a given depot in the body, which would also change the amount of total circulating leptin.

Analysis of the degree of differentiation in a sample is essential when dealing with adipokines since they are only secreted from fully differentiated adipocytes. Once Ob mRNA transcript abundance was normalized to PPARγ expression, most of the large inductions by estrogen were eliminated. In fact, in the original data set (Figure 7A), Ob mRNA abundance at each dose was lowered, but the dose-dependent trend remained in tact, while in the larger sample set, normalization to PPARγ changed the shape of the estrogen dose-response curve. Once the data was normalized to PPARγ, there was a non-significant 5.4-fold induction of Ob mRNA transcript abundance in response to the 1 nM dose of estrogen. This analysis of the data indicates that changes observed in Ob mRNA transcript abundance may be due simply to variable differentiation levels and may not be due to estrogen enhanced Ob gene expression.

Alternatively, if PPARγ is induced by estrogen, and Ob gene expression is also induced, normalizing to PPARγ could potentially mask the estrogenic effects. Normalizing the Ob mRNA levels to this differentiation-induced transcript (Fig.4B) suggested a very different response to estrogen, with almost no induction in Ob message levels. Also, we were able to show that although treatments were controlled as much as possible, some variation in differentiation was still seen in this system, which is not
considered in other studies that have contradictory conclusions about estrogen effects on $Ob$ expression (Chen et al., 2006; Yi et al., 2008).

Since we did observe some variation in the levels of differentiation in our samples, we also normalized our DHT data to changes in $PPAR\gamma$ expression. In this analysis, shown in Figure 11, we see that DHT-induced reductions remain, although they are non-significant at all doses. This analysis is complicated by the variation in $PPAR\gamma$ among biological replicates, thus further investigation is required. If it is true that DHT has no effect on $PPAR\gamma$ as found in this study and as previously demonstrated (Dieudonne et al., 2000), it strengthens our finding that DHT most likely directly inhibits $Ob$ mRNA accumulation.

Since our estrogen treatments elicited some variation and no significant inductions in $Ob$ gene expression, we wanted to ensure that estrogen was stimulating expression of known estrogen responsive genes in 3T3-L1 cells. A previous study in this system used similar differentiation methods and similar hormone doses to investigate the effects of estrogen on Resistin ($Retn$) gene expression (Chen et al., 2006). We measured $Retn$ transcript abundance in our samples as a positive control for our estrogen treatments. Our data shows that $Resistin$ levels vary in response to our estrogen treatments (Table 2), and each biological replicate is very different from other treatment-paired replicates. The extremely variable biological replicates indicate that estrogen may be stimulating $Retn$ transcription in some samples but not in others, limiting our ability to make confident conclusions about estrogen’s stimulation of transcription in our samples. It does suggest that variable estrogen effects may account for the limited estrogen enhanced $Ob$ mRNA levels.
The inability to obtain consistent estrogen induction of either *Ob* or *Retn* in any given experiment severely limited our ability to analyze the effects of receptor inhibitors in preventing estrogenic stimulation, as well as moving forward with a time course analysis of *Ob* mRNA accumulation. These experiments require further replication and further inquiry into the difficulty of obtaining replicable estrogen dose-response data. Previous studies have shown that DMEM, the base media used in this experiment, may elicit estrogen-like effects on cells in culture (Berthois et al., 1986). This media contains phenol red, a pH indicator which has been demonstrated to mimic estrogen (Berthois et al., 1986; Bukovsky et al., 2003) and may have been providing a high basal stimulation of *Ob* gene expression. This could be preventing us from measuring the effects of exogenous estrogen on *Ob* transcription accurately.

Another possibility for the variability that we have seen in the estrogen-treated samples is that estrogen has specific solubility requirements (Stecher, 1960). Estrogen stock solutions must be made in absolute (200 proof) ethanol (personal communication with Russell C. Hovey, Ph.D.) before diluting into the cell culture media for treatments. This is a problem because the experiments in this thesis were completed with estrogen stocks made in 95% (190 proof) ethanol. Inconsistent estrogen treatments could result from the inability of the molecule to go into solution when preparing estrogen stocks and to remain in solution when the stock is diluted. This inconsistency would carry over to the final hormone treatment which is prepared in cell culture media.

In one recent study, researchers observed no induction of *Ob* by estrogen at $10^{-5}$, $10^{-6}$, $10^{-7}$, $10^{-8}$ and $10^{-9}$ M in 3T3-L1 cells (Yi et al., 2008). This data was normalized to the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GADPH) instead of
Actin (Yi et al., 2008). This study gave no indication of whether GADPH stays constant over time or in response to estrogen treatments. In our study Actin levels remained relatively constant and were not significantly different between hormone treatments. The Yi et al. study also examined the expression of Resistin, a known estrogen responsive gene (Chen et al. 2006). They were unable to show any induction in this gene expression in response to estrogen, suggesting that their estrogen treatments were not effective. Studies from other systems including: female rats with (Alonso et al., 2007) and without ovaries (Tanaka et al., 2001), as well as rat primary adipocytes (Machinal et al., 1999), all report estrogen enhanced Ob mRNA levels.

Since we knew that estrogen enhances Resistin gene expression from a previously published study (Chen et al., 2006), we also decided to ask whether or not DHT had any effect on Retn transcript abundance. When the data was normalized to Actin (Figure 12) we saw no significant trend in Retn mRNA abundance in relation to DHT treatment. However, when the data was normalized to PPARγ we saw a dose-dependent trend for reductions in Retn mRNA in response to all DHT doses.

To our knowledge, this is the first study to examine the direct effects of androgens on Resistin gene expression in adipocyte cell cultures. One previous study examined the effects of testosterone on Resistin mRNA levels in intact mice and found that Retn mRNA transcript abundance was increased in response to testosterone treatment (Ling et al., 2001). The differences in the Resistin-DHT dose-response data when normalized to both Actin and PPARγ provide insight into the 3T3-L1 adipocyte system. Data normalized to Actin simply describes the levels of a target in relation to the standard amount of mRNA in a given sample. Normalizing the data to PPARγ considers changes
in the amount of differentiation in a given sample. As we have demonstrated through the measurement of PPARγ mRNA transcript abundance relative to Actin, levels of differentiation vary between samples. In the analysis of adipokines, which are only synthesized in fully differentiated adipocytes, it is vital to have consistent levels, or these changes must be accounted for in data analysis. For these reasons, normalizing to PPARγ may provide the most accurate information concerning the effects of hormones on adipokines.

Having collected evidence that DHT plays a role in Ob transcription, we also investigated the effects of DHT on leptin secretion from 3T3-L1 cells in dose-response, AR antagonist, and time-course experiments. This data, as analyzed by ELISA, shows no significant effect of DHT on leptin secretion (Figs. 16-18). Interestingly, this is in agreement with one previous study which used primary rat adipocyte cultures to investigate the effects of sex steroid hormones on leptin synthesis and secretion. They found that estrogen enhances Ob mRNA abundance and that DHT decreases this abundance after 24 hours of exposure to high doses (both at 100 nM) of hormone treatment. (Machinal et al., 1999). Leptin secretion from these adipocyte cultures, was not effected by DHT treatment in this study, even though DHT decreased the level of Ob gene expression (Machinal et al., 1999).

It may be that the effects of DHT on Ob mRNA transcription take more time to translate to differences in secreted protein. Perhaps examining these effects over a longer period of time would demonstrate differences in secretion between vehicle treated and DHT treated 3T3-L1 cells. Previous studies with human primary subcutaneous adipose cultures, treated with DHT for up to 12 days found significant reductions in leptin
concentrations at 3 days following treatment, with maximal reductions at 6 days (Wabitsch et al., 1997).

These thesis experiments were essential in establishing 3T3-L1 cells as an excellent model for the investigation of transcriptional regulation of the Ob gene, especially in relation to steroid hormone signaling. We also have shown that evaluating target genes in relation to a simple housekeeping gene may not be enough in this system. By measuring levels of PPARγ to account for differences in differentiation, we have shown that levels of differentiation in biological replicates should be quantified, especially in the examination of leptin, which is only produced by differentiated adipocytes and not their precursor cells (MacDougald et al., 1995). Small changes in the amount of differentiated cells may yield changes in Ob mRNA or secreted leptin which are not attributed to the treatment(s) of interest.

It may make sense in the future to use a different gene for the quantification of differentiation in a given sample. PPARγ is controlled by a number of factors (including estrogen) and it may be that this transcript is not expressed at high enough levels or stable enough to use for this sort of analysis. Although our results had quite a bit of variation, we were able to show that there is variation in the level of differentiation and that these differences may effect the relative quantification of cytokines.

We also verified in one of our samples, that Resistin is estrogen-responsive as previously published (Chen et al., 2006) and showed that this gene may be used as a positive control for estrogen treatments in these cells. It may be that although the idea of using Retn to evaluate the effectiveness of estrogen treatments was well founded, that another gene with higher abundance in our system, or perhaps different primers for Retn
transcripts, would yield more consistent, robust results. Our data also shows that DHT may inhibit Retn mRNA abundance when normalized to PPARγ expression for changes in differentiation. However these results are not significant and further analysis is necessary, perhaps with different primer sets for these genes.

In conclusion, this study examined the role of sex steroid hormones in the regulation of the Ob gene and leptin secretion. Parallel stimulation and inhibition by estrogen and androgens respectively suggests that the regulation of this gene may be controlled by both hormones directly. However, the stimulation by estrogen is still in question since variable Retn and Ob expression suggests that treatments were not consistently stimulating estrogen-responsive gene expression, thus no significant differences could be detected for these treatments. Also, normalization of Ob mRNA in relation to PPARγ minimized the effects of estrogen. The data in this thesis strongly supports the conclusion that DHT inhibits Ob mRNA gene expression as evidenced by a significant reduction in response to a 10 nM dose. This conclusion is supported by the trends shown in DHT time-course and androgen receptor antagonist experiments. Future experiments examining the expression of the leptin receptor in response to sex steroid hormones may provide further insight into the mechanisms by which females and males differentially control their leptin signaling. These combined results provide a potential mechanism for the sexual dimorphism of leptin levels in mammals.
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