# Identification of Genes with Altered Gene Expression in the Adipose Tissue of Mouse Models of Varied Growth Hormone Signaling

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## ABSTRACT

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Microarray analyses were performed comparing mRNA expression levels in subcutaneous adipose tissue between mice with disruption in the growth hormone (GH) receptor gene (GHR-/- mice) and littermate controls. The data revealed that 87 genes were significantly upregulated and 72 genes were significantly downregulated in GHR-/- mice versus littermate controls. Among the 159 altered genes, a subgroup of five genes was chosen for confirmation by a second mRNA quantification method, real time reverse transcriptase polymerase chain reaction (RT-RT PCR). RT-RT PCR studies confirmed the altered gene expression for only three genes, angiotensinogen (Agt), adiponutrin (Adpn) and angiopoietin-like protein 4 (Angptl4). To better understand the role of GH in adipose specific expression of these three genes (Adpn, Agt, Angptl4), RT-RT PCR analysis was expanded to include an additional genotype of altered GH function, the bovine GH transgenic mice, an additional diet treatment high-fat diet, as well as two other adipose depots, the epididymal and retroperitoneal adipose depots. This thesis discusses in detail all gene alterations observed in the microarray analyses and the RT-RT PCR analyses for Adpn, Agt, Angptl4 in adipose tissue.

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# CHAPTER 1: INTRODUCTION

## Overview of Adipose Tissue

Adipose tissue is a very complex tissue. Overall, the basic function of adipose tissue is to store fatty acids in times of energy excess and to release the stored lipids in times of energy deprivation. The fatty acids released by the lipid-filled cells (adipocytes) in adipose tissue are used by most organs for fuel when glucose is limited. Besides adipocytes, adipose tissue contains connective tissue matrix, nerve tissue, stromovascular cells and immune cells (Frayn, Karpe, Fielding, Macdonald, & Coppack, 2003).

In addition to its function in storing energy, adipose tissue is an extremely active tissue able to both respond to afferent signals from many hormone systems as well as generate its own endocrine signals, called adipokines. Adipokines participate in diverse metabolic processes including food intake, regulation of energy balance, insulin action, lipid and glucose metabolism, angiogenesis and vascular remodeling, regulation of blood pressure and coagulation (Ferroni et al., 2005; Lall, Tung, Ohlsson, Jansson, & Dickson, 2001; Lau, 2000; Mohamed-Ali, Pinkney, & Coppack, 1998). Adipokines synthesized by adipose tissue include tumor necrosis factor (TNF)-α, resistin, Interleukin-6, Interleukin-8, acylation-stimulating protein (ASP), angiotensinogen, plasminogen activator inhibitor-1, leptin, adiponectin and several factors related to proinflammatory and immune processes (Rondinone, 2006).

Adding to the complexity of adipose tissue in mammals is the finding that adipose tissue is metabolically distinct depending on its specific location in the body. For example, certain depots, like the fat pads in the heels, fingers, and toes and the periorbital

fat supporting the eyes, provide more mechanical support and contribute very little to energy homeostasis. The adipose tissue that surrounds organs and the abdominal mesentery are all collectively known as intra-abdominal or visceral adipose tissue while the adipose tissue found beneath the skin is referred to as subcutaneous adipose tissue. Each adipose depot is unique in its characteristics and metabolism, leading to the depot-specific differences of adipose tissue. Visceral adipose tissue is known to have higher cellularity, nerve innervations, and blood flow along with a higher density of cortisol and androgen receptors when compared to subcutaneous adipose tissue (Bjorntorp, 1996). This higher capacity to respond to lipid-accumulating hormones, such as cortisol and insulin, could help explain a greater accumulation of fat in the visceral regions. Because each adipose depot is metabolically distinct, it is essential to compare and contrast the unique features of each of the depots specifically.

## Overview of Growth Hormone

Growth hormone (GH), also known as somatotropin, is a protein hormone of about 191-amino acids that is synthesized and secreted by cells called somatotrophs in the anterior pituitary (Li, Evans, Simpson, 1945). GH is a major participant in the control of several complex and overlapping physiologic processes, including growth and metabolism. The metabolic actions of GH are numerous and affect almost all types of tissues and cell types. GH excess leads to overgrowth of various organs (Melmed, 1990). In contrast, GH deficiency (or absence of GH receptor) results in short stature and other phenotypic disturbances (Maheshwari, Silverman, Dupuis, & Baumann, 1998; Rosenfeld, Rosenbloom, & Guevara-Aguirre, 1994). GH-deficient adults before and after GH

treatment also reveal the importance of GH in a range of tissues or body compartments including muscle, bone, heart, and the vascular system (Waters & Thompson, 1999). This hormone is also of considerable interest as a drug used in both humans and animals.

Adipose tissue is a direct target for GH, because adipocytes express the GH receptor on their surface. GH, when bound to the receptors on adipocytes, promotes hydrolysis of the triglycerides in the adipose tissue to release free fatty acids into the blood stream (Johansen, Laurino, Barreca, & Malmlof, 2005). Thus, GH has a profound impact on adipose tissue and ultimately promotes a lean phenotype. Likewise, GH-deficient states are associated with obesity (Clasey et al., 2001). Several studies suggest that GH impacts adipose tissue differently depending on the location of the adipose tissue (Chen et al., 2001; Flint & Gardner, 1993; Kadim, McCutcheon, Purchas, & Wickham, 1996; Lall et al., 2001). The site-specific effects of GH are observed early when GH influences adipocyte development. For example, Flint and Gardner (1993) reported that GH is essential for the differentiation of adipocytes in wild-type rats. The study confirmed that administration of bovine GH to rats recovering from ratGH antiserum doubled the subcutaneous fat pads but not the visceral fat pads compared to controls (Flint & Gardner, 1993). Furthermore, Berryman et al. (2004) reported that epididymal fat pads remain proportional to body size in the absence or repression of growth hormone signaling, while subcutaneous pads are significantly larger. This suggests a depot-specific role of GH. Thus, the impact of GH on adipose tissue is not uniform and stresses the need to use whole animal models and various adipose depots as opposed to isolated adipocytes or cell culture systems when assessing GH's impact on adipose tissue.

## Statement of the Problem

Complex diseases, such as diabetes and obesity, are caused by changes in expression levels of many genes and are controlled and influenced by several hormones produced by the body, including GH. Because adipose tissue function and metabolism can be very distinct depending on the location of the tissue, and because GH is thought to impact adipose tissue in a depot-specific manner, whole animal models would be invaluable to study the relationship between altered expression of certain genes in the adipose tissue and GH. To that end, two mouse models are available with extreme variations in GH signaling. GH receptor knockout mice (GHR -/-) have no GH signaling, which is achieved by a disruption of the GH receptor gene in these mice. These mice are dwarfs with an increased body fat percentage (Berryman et al., 2004). The second transgenic model expresses the bovine GH (bGH) gene constitutively, has increased GH signaling, and has been shown to be large with a lower percent body fat (Berryman et al., 2004). Comparing these mouse lines to littermate control mice, one can compare and contrast the changes in gene expression as influenced by the GH signaling in various adipose depots. For this study, genome-wide comparisons (using microarray analysis) of all genes altered in the subcutaneous adipose tissue of GHR -/- mice compared to littermate controls were analyzed. Following this, RT-RT PCR was used to confirm the microarray data of the following five genes: adiponutrin (Adpn), angiotensinogen (Agt), angiopoietin-like 4 (Angptl4), insulin-induced gene 1 (Insig1) and Stearoyl-Coenzyme A desaturase 2 (Scd2). Next, RT-RT PCR was used to compare the gene expression patterns of the RT-RT PCR confirmed genes in the subcutaneous, epididymal and retroperitoneal

adipose depots of the GHR-/-, bGH, and control mice while on a high-fat (HF) or low-fat (LF) diet. The hypotheses of this study are that: (a) several genes will be up or downregulated in the subcutaneous fat pads of the GHR -/- compared to the littermate controls, (b) the five genes chosen for confirmation by RT-RT PCR will show similar results to the microarray, and (c) the expression patterns for the same gene will be opposite in the GHR -/- compared to the bGH mice. That is, the gene that is upregulated in the GHR-/- mice will be downregulated in the bGH mice and vice versa.

## Research Questions

Using GHR -/-, bGH and wild-type mice previously described (Berryman et al., 2006), the following questions were asked:

1. In the microarray data, how many total genes and which specific genes are up or downregulated in the subcutaneous fat pad of GHR-/- compared to the littermate controls?

2. Does the RT-RT PCR analysis of Adpn, Agt, Angptl4, Insig1 and Scd2 confirm the microarray results?

3. Do mRNA levels of the RT-RT PCR confirmed genes increase or decrease in subcutaneous, epididymal and retroperitoneal adipose depots of GHR-/-, bGH and WT mice while on a HF or LF diet?

## Significance of Study

Even though every cell of the body contains a full set of chromosomes and identical genes, only a fraction of these genes are turned on or expressed. The combined expression of a large number of genes is a critical component of normal growth and

development. Disruptions or changes in gene expression are responsible for many diseases. These disruptions can be caused by various hormones and signals. This study will provide information about the role played by the GH signal in the expression of genes found in adipose tissue. The up or downregulation of five genes (Adpn, Agt, Angptl4, Insig1 and Scd2) in animal models with varied GH signaling may be correlated with the increase or decrease in adipose tissue observed with the lack of GH signaling and excess GH signaling respectively. Also, these genes play a role in adipose tissue modeling and triglyceride storage and release. In addition, the results of this study will help explain the role of GH in preventing or promoting obesity and diabetes.

## Delimitations/Limitations

1. While this study will reveal the fluctuations in gene expression of the five select genes caused by variations in GH signaling, the molecular pathway by which these genes are altered will still remain unknown as other pathways are altered (i.e. insulin sensitivity) in these mouse models.

2. Effects seen within the mouse models used in this study cannot be fully generalized to the human population.

3. The hierarchal nature of mice has been shown to alter the food consumption of individual mice which could cause variations in adiposity, thus affecting individual gene expression levels.

4. GH responses vary among species.

5. Variations in the DNA length of the microarray samples along with possible synthesis and handling errors involved with shipping the samples to another facility for microarray analysis cannot be confirmed by sequencing.

6. A two-step real-time reverse transcriptase PCR (RT-RT PCR) procedure would be more sensitive compared to the single-step RT-RT PCR procedure used in this study.

## Definition of Terms

Adipocyte: fat cell.

Adipokines: hormones that signal changes in adipose tissue mass and energy status so as to control the storage and release of lipids in circulation.

Adipose depots: specific storage sites in the body where fat is deposited and accumulated.

Adipose hyperplasia: refers to an increase in adipose tissue mass through an increase in the number of new adipocytes.

Adipose hypertrophy: refers to an increase in adipose tissue mass through an increase in size of the existing adipocytes.

Aponeurosis: membranes which separate muscles from each other.

Hyperphagia: abnormally increased appetite for and consumption of food.

Hypophysectomy: removal of the pituitary gland.

Lipoatrophy: loss of fat tissue.

Lipodystrophy: abnormal distribution of fat tissue.

Lipogenesis: the pathway for fatty acid synthesis. During this process, the circulating free fatty acids are taken up by adipose cells and stored as lipid droplets.

Lipolysis: the hydrolysis of lipids (fats). During this process, free fatty acids are released into the bloodstream and circulate throughout the body.

Multilocular: having many compartments or cavities

PCR (polymerase chain reaction): a technique used to amplify a small DNA sequence in a short period of time by repeating the cycles of denaturation, annealing with a primer and extension with DNA polymerase.

Transgenic animals: an animal that carries a foreign gene that has been deliberately inserted into its genome.

Unilocular: having a single compartment or cavity.

# CHAPTER 2: REVIEW OF LITERATURE

## Adipose Tissue

The terms adipose tissue and fat are often used interchangeably, but there is a clear difference between the two. The term fat refers to the lipid in the form of triglycerides that are abundant in adipose tissue, but may be found in other tissues as well. Adipose tissue predominantly consists of fat (80%), with the remaining tissue (20%) being made of a combination of water, protein, and minerals (Shen et al., 2003). There are two distinct types of adipose tissue in mammals: white and brown adipose tissue. The primary function of adipose tissue is to store energy in the form of triglycerides in times of excess and to release energy in the form of free fatty acids in times of energy deprivation. While this is the primary function of white adipose tissue (WAT), brown adipose tissue (BAT) is primarily involved in thermogenesis. In addition to these distinct functions, the histology of the two types of tissues is very different. While white adipocytes are unilocular and filled with lipid, the brown adipocytes are multilocular and rich in mitochondria (Cinti, 2006). Because WAT is the focus of this thesis, the remaining literature review will focus on this tissue.

Adipose tissue can also be classified according to anatomical location. That is, adipose tissue has several subcutaneous and visceral depots (Cinti, 2005). The subcutaneous adipose tissue primarily refers to the layer found between the dermis and the aponeuroses and fasciae of the muscles and includes mammary adipose tissue (Shen et al., 2003). Among the subtle differences in adipose tissue locations in humans and rodents, rodents have two distinct subcutaneous depots: anterior and posterior depots

located around the forelimbs and dorsal region or the hindlimbs, respectively (Cinti, 2005). The presence of two distinct subcutaneous depots is not seen in humans.

Several definitions of visceral adipose tissue exist depending on the kind of imaging methodology or equipment used. The broadest definition of visceral adipose tissue (VAT) includes adipose tissue within the chest, abdomen and pelvis (Shen et al., 2003). For human studies that are performed using magnetic resonance imaging (MRI), VAT is defined as adipose tissue deposited around the internal organs of the abdomen, trunk, and pelvis (Demerath et al., 2007). However, VAT can also be defined more strictly to include only those adipose depots that are drained by the portal vein. In humans, this would include solely the mesenteric and omental adipose depots. In rodents, the broad definition of VAT could include fat pads such as retroperitoneal, perirenal, mesenteric and epididymal fat pads, whereas the narrow definition only allows mesenteric adipose tissue, because rodents do not possess omental fat (Miyazawa-Hoshimoto et al., 2005; Morton, Ramage, & Seckl, 2004). The morphological differences in the adipose tissue depots include the extent of nerve innervation, quantity of blood vessels and macrophages, as well as the size of the adipocyte (Hauner, 2004). These differences can result in metabolic and functional differences among the depots. The unique features of subcutaneous adipose tissue are discussed later in this chapter.

***Plasticity and Endocrine Function of Adipose Tissue***

Adipose tissue is to some degree plastic. That is, adipose tissue can be modified based on specific nutritional or environmental stimuli. For example, adipose tissue can expand with consistent overnutrition or with obesity, which occurs by the coordinated

interplay between adipocyte hypertrophy (increase in cell size), adipocyte hyperplasia (increase in cell number) and angiogenesis (Gregoire, 2001; Hausman, DiGirolamo, Bartness, Hausman, & Martin, 2001; Rosen & Spiegelman, 2006; Smas & Sul, 1995). In fact, adipocytes are the only cell whose size may vary dramatically under varying physiological conditions. Adipocyte cell size could modulate several signaling pathways by changing the relationships between the cell and the extracellular matrix (Farnier et al., 2003). The plasticity of adipose tissue is also evident in its ability to synthesize and secrete proteins under varying conditions, thus classifying adipose tissue as a true endocrine organ. The hormone-like proteins, also called adipokines, include molecules such as leptin, resistin, adiponectin, TNF-α, and angiotensinogen (Kratchmarova et al., 2002). These adipokines have many physiological effects on different organs, including immune cells, blood vessels, reproductive organs, skeletal muscle, bone, liver, and the brain (Rajala & Scherer, 2003). It is believed that adipokines are dysregulated in response to alterations of fat mass. Specifically, a decrease in serum levels of most of the adipokines, apart from adiponectin, occurs with weight loss (Kratchmarova et al., 2002). These adipokines also affect insulin action, glucose, and fat metabolism and consequently insulin resistance, ultimately impacting the risk of type-2 diabetes (Rondinone, 2006).

***Depot/Site Specific Differences in Adipose Tissue***

Adipose tissue exhibits depot-specific differences. This refers to the concept that different depots of fat are associated with different metabolic characteristics. For example, it is well accepted that android (upper body) obesity is more frequently associated with diabetes mellitus and coronary artery disease than gynoid (lower body)

obesity (Vague, 1956). Specifically, elevated VAT is associated with insulin resistance and dyslipidemia, as well as systemic inflammation (Cefalu et al., 1995; Miyazaki et al., 2002; Montague & O'Rahilly, 2000) and is an independent risk factor for type 2 diabetes (Boyko, Fujimoto, Leonetti, & Newell-Morris, 2000), myocardial infarction (Nicklas et al., 2004) and hypertension (Hayashi et al., 2004).

There are also well known gender-specific differences in the adipose tissue deposition in humans. While women have a higher percentage of total body fat, the distribution of the individual depots is different from men and the distinct pattern of adipose tissue distribution contributes to functional differences between the same adipose depots in different genders. For example, using lipolytic activity as a measure to quantify the gender differences in adipose tissue, it is clear that free fatty acid release by the upper-body subcutaneous fat depots is higher in men than in women, and that basal fat oxidation (adjusted for fat free mass) in subcutaneous fat depots is lower in females as compared to males, thereby contributing to a higher fat storage in women (Blaak, 2001). In vitro and in vivo data show greater lipolytic sensitivity of abdominal subcutaneous fat and lesser lipolytic sensitivity of femoral and gluteal subcutaneous fat in women than in men (Williams, 2004). The unique metabolic properties of adipose tissue help define the heterogeneity of this tissue and this is due, in part, to differences in proteins expressed among different adipose depots. For example, enzyme activities involved in triglyceride storage and release differ among the various depots, such as the enzyme LPL (lipoprotein lipase) involved in the uptake of fatty acids by adipose tissue and HSL (hormone sensitive lipase) involved in breakdown of fatty acids. Specifically, basal

activity of LPL is higher in adipose tissue isolated from the subcutaneous-femoral compartment than in that from the subcutaneous-abdominal compartment in women (Arner, Engfeldt, & Lithell, 1981). In men, LPL activity is shown to be either higher or unchanged in subcutaneous abdominal adipose tissue than that in gluteo-femoral adipose tissue (Fried & Kral, 1987). This may partly explain the gender-specific differences in the adipose tissue distribution.

Besides gender differences, there are inherent differences between depots. For example, there are fundamental differences in metabolism. LPL activity not only has gender differences within the same depot, as previously mentioned, but also is increased in both retroperitoneum and omentum adipose tissue, resulting in greater lipid uptake, as compared to that from the subcutaneous abdominal compartment (Marin et al., 1992). These differences suggest that intra-abdominal adipose tissue may play a more active role in fatty acid uptake compared to the subcutaneous adipose tissue. In contrast, the site-specific differences in lipolysis (HSL activity), specifically basal lipolytic activity, is higher in the subcutaneous regions than the omentum and mesentery (He, Engelson, Albu, Heymsfield, & Kotler, 2003; Koska et al., 2002). Furthermore, it is known that heterogeneity in lipolysis among the various depots is observed in response to various stimulating and inhibitory factors (Jaworski, Sarkadi-Nagy, Duncan, Ahmadian, & Sul, 2007).

Depot-specific differences are regulated by a combination of external stimuli, such as the amount of hormones, as well as internal stimuli. Cell culture studies confirm that human subcutaneous, mesenteric, and omental preadipocytes isolated from the same

subjects and cultured under identical conditions maintained fat depot-specific characteristics even after many population doublings ex vivo (Tchkonia et al., 2005; Tchkonia et al., 2006). This indicates the existence of inherent mechanisms contributing to depot-specific properties. Subcutaneous preadipocytes exhibit higher replicative potential, adipogenic transcription factor expression, lipid accumulation, and less TNFα-induced apoptosis than omental preadipocytes (Tchkonia et al., 2005). The same study also found that two subtypes of preadipocytes exist with different capacities for replication, differentiation, and susceptibility to TNFα-induced apoptosis (tchkonia et al., 2005). These subtypes can convert to the other and the ratio of the subtypes varies among fat depots (Tchkonia et al., 2005). Apart from the depot-specific dynamic properties of preadipocytes isolated from subcutaneous, mesenteric, and omental depots, these cells also exhibit unique patterns of gene expression, supporting the contention that depot-specific characteristics inherent to preadipocytes contribute to regional differences in function (Tchkonia et al., 2007).

Another means by which depot-specific differences are maintained is via the cell composition and available circulation to a particular depot. This in turn could contribute to the influence of external stimuli such as hormones on depot-specific differences. Thus, specific depots would be more prone to the effects of GH and other such lipolytic agents and could potentially have a higher lipolysis rate overall. Towards lipogenesis, the density of cortisol and androgen receptors seems to be higher in visceral adipose than other adipose tissue regions (Belanger et al., 2006; Tchernof et al., 2006). The endocrine perturbations found in visceral obesity with an abundance of lipid-accumulating

hormones (cortisol and insulin) and a relatively low secretion of the lipid-mobilizing hormone (sex steroid hormones and GH) would therefore be expected to result in visceral fat accumulation (Bjorntorp, 1996).

While increased fat content is seen in visceral adipose, there are several unique features to subcutaneous adipose tissue. For example, Goodpaster, Thaete, Simoneau, & Kelley (1997) showed that subcutaneous adipose is as strongly associated with insulin resistance as visceral adipose (Goodpaster, Thaete, Simoneau, & Kelley, 1997). Furthermore, the rate of lipid turnover is lower in subcutaneous adipose than visceral adipose in humans (Martin & Jensen, 1991). While subcutaneous adipocytes are less responsive to the lipolytic effects of catecholamines (Wahrenberg, Lonnqvist, & Arner, 1989), they are more responsive to the antilipolytic effects of insulin (Bolinder, Kager, Ostman, & Arner, 1983) when compared to omental adipocytes. Subcutaneous adipose distribution is also age- and gender-specific. Body weight gain in young and middle-aged females causes preferential fat accumulation in the subcutaneous region, while males of any age and females over 60 years of age show preferential accumulation in the visceral region (Enzi et al., 1986).

Collectively, these data suggest a delicate interplay between the stimuli available to the various depots and this creates a tissue that is constantly changing and remodeling and that varies tremendously among each adipose depot as well as between genders. Thus, a comparison of expression levels with the use of genome-wide arrays can serve as a useful tool in better understanding the depot-specific differences in adipose tissue.

## Growth Hormone

Growth hormone (GH) secreted by the anterior pituitary follows a receptor-mediated pathway to elicit action. The GH system includes GH, the GH receptor (GHR), and the GH binding protein (GHBP). GH was first recognized because of the critical role it played in postnatal linear growth in mammals (Daughaday & Salmon, 1999). Initial studies of GH indicated that the GH was solely made by the pituitary and targeted liver GHRs mainly, which was responsible for the production of IGF-1 (Le Roith, Bondy, Yakar, Liu, & Butler, 2001). IGF-1 was responsible for much of the growth-promoting properties of GH (Le Roith, Bondy, Yakar, Liu, & Butler, 2001). It is now understood that: (a) GH acts via GHRs expressed by a range of cells in addition to the liver (Barnard, Bundesen, Rylatt, & Waters, 1984; Edmondson et al., 1995; Pantaleon et al., 1997; Werther, Haynes, & Waters, 1993), (b) GH mRNA and protein are expressed in extrapituitary sites (Harvey, Johnson, Sharma, Sanders, & Hull, 1998; Lobie et al., 1993; Pantaleon et al., 1997; Weigent & Blalock, 1991), and (c) GH does not solely rely on IGF-I to mediate its action (Ohlsson, Bengtsson, Isaksson, Andreassen, & Slootweg, 1998; Slootweg, van Buul-Offers, Herrmann-Erlee, & Duursma, 1988; Zezulak & Green, 1986). Regardless, pituitary GH is the major contributor of circulating GH and this secretion occurs in a pulsatile fashion. GH secretion is modulated by multiple factors including GH-releasing hormone (GHRH), somatostatin, ghrelin, glucocorticoids, and sex hormones (Bertherat, Bluet-Pajot, & Epelbaum, 1995).

GH action begins with the binding of GH to the GHR on target tissues. Until recently, it was thought that biological activity of GH to GHR binding occurred by the

binding of GH to the GHR monomer, resulting in recruitment of the second receptor and subsequent signal transduction (Cunningham et al., 1991; de Vos, Ultsch, & Kossiakoff, 1992). It is now known that GHR exists as a preformed dimer in the target tissue plasma membrane, which, upon binding to GH, activates changes in certain sites of the GHR, followed by a subunit rotation within the receptor dimer and signal transduction (Brown et al., 2005; Gent, van den Eijnden, van Kerkhof, & Strous, 2003; Gent, van Kerkhof, Roza, Bu, & Strous, 2002). This signal transduction involves the activation of JAK-2 (janus kinase-2), and JAK-2 phosphorylation of multiple intracellular GHR tyrosine residues (Cunningham et al., 1991; de Vos et al., 1992). Several different signaling pathways may then be triggered, including signal transducers and activators of transcription (STAT), mitogen-activated protein (MAP), and Phosphoinositide 3 (PI-3) kinases (Herrington & Carter-Su, 2001).

The metabolic actions of GH are numerous and affect almost all types of tissues. This is illustrated by the fact that GH excess leads to overgrowth of various organs (Melmed, 1990), whereas GH deficiency (or absence of GHR) results in short stature and other phenotypic disturbances (Maheshwari et al., 1998; Rosenfeld et al., 1994). GH-deficient adults before and after GH treatment also reveal the importance of GH in a range of tissues or body compartments including muscle, bone, heart, and the vascular system (Waters & Thompson, 1999). The concentration of GH in circulation is generally regulated by control loops and negative feedback mechanisms to allow response to physiological needs. The number of receptors available for binding also controls the physiological impact of GH.

### *Growth Hormone Receptor*

The impact of GH on a tissue is, in part, related to the amount of GHR present on the plasma membrane. The highest levels of GHR are found in liver, adipose tissue, muscle, and kidney (Ballesteros, Leung, Ross, Iismaa, & Ho, 2000). GH receptors are found in many other tissues, though, such as the prostate, lung, heart, mammary glands, and lymphocytes (Hill, Riley, Bassett, & Waters, 1992; Mercado, DaVila, McLeod, & Baumann, 1994; Mertani & Morel, 1995; Nyberg & Burman, 1996; Rapaport et al., 1995). Receptor turnover is rapid with a half-life of about 60-minutes and hormones such as insulin, IGF-1, and dexamethazone can trigger downregulation of the receptor (Flores-Morales, Greenhalgh, Norstedt, & Rico-Bautista, 2006; Gorin & Goodman, 1985; Ilondo et al., 1986; Murphy & Lazarus, 1984). Ligand-induced endocytosis and proteolysis at the cell surface, which results in shedding of the growth hormone binding protein (GHBP) (see next section for explanation of GHBP), are among the mechanisms that contribute to receptor turnover (Baumann, 1994). The GHR life cycle is determined by three processes: (a) receptor biosynthesis and transport to the cell surface, (b) GHR internalization and lysosomal degradation, and (c) GHR proteolysis with shedding of its extracellular domain (van Kerkhof, Smeets, & Strous, 2002). Together, these processes determine the surface expression or availability of the receptor for its ligand, which ultimately dictates the impact of GH on a tissue.

### *Growth Hormone Binding Protein*

GH interaction with GHR is critical for the signal cascade that elicits GH function. Specifically for GH, a high-affinity binding protein called the Growth Hormone

Binding Protein (GHBP), was first described in rabbit and human serum, and has been found in every species studied thereafter (Fisker, 2006). The GHBP is essentially the extracellular domain of GHR, only in a circulating form (Fisker, 2006). GHBP is produced by proteolytic cleavage through a process known as receptor ectodomain shedding (Fisker, 2006). The enzyme, tumor necrosis factor-α converting enzyme (TACE) and other physiological factors such as platelet-derived growth factors and serum can induce the shedding of GHBP (Guan et al., 2001; Schantl, Roza, van Kerkhof, & Strous, 2004; Zhang, Jiang, Black, Baumann, & Frank, 2000). While receptor shedding is one of the ways GHBP is produced, in rats and mice GHBP is derived from the GH receptor gene by an alternative mRNA splicing. That is, in rodents, the transmembrane and intracellular domains of the GH receptor are replaced by a hydrophilic carboxyl terminus (Baumbach, Horner, & Logan, 1989; Edens, Southard, & Talamantes, 1994; Sadeghi, Wang, Lumanglas, Logan, & Baumbach, 1990; Smith, Kuniyoshi, & Talamantes, 1989). Although the exact function of GHBP remains elusive, GHBP is positively correlated with amounts of body fat (Fisker et al., 2004; Morrison, Weis, & Wittwer, 1998). It is theorized that GHBP controls GH bioactivity in two ways: (a) by prolonging GH's half-life, and (b) by restricting GH's availability to target tissues (Fisker, 2006; Hansen et al., 2002). Thus, the levels of GH, GHR turnover, post-receptor regulation of GHR, and plasma GHBP levels influence the biological effects of GH.

### *GH Function*

GH, as the name implies, has a major role in promoting growth by influencing macronutrient availability. GH exhibits distinct effects on macronutrient metabolism

depending on length of exposure. Short-term exposure to GH creates an acute insulin-like activity while chronic exposure has anti-insulin or diabetogenic activity. Acute insulin-like actions include increased glucose and amino acid metabolism (Batchelor & Mahler, 1972; Goodman, 1978), increased protein synthesis (Kostyo & Nutting, 1973) as well as increased glycogenesis (Newman, Armstrong, & Bornstein, 1978; Porterfield, 1979) and lipogenesis (Pandian, Gupta, & Talwar, 1971). Acutely, GH inhibits noradrenaline-stimulated lipolysis resulting in hypoglycemia (Swislocki, 1968a; Swislocki, 1968b). However, the chronic, delayed effects of GH include hyperglycemia (De Bodo & Altszuler, 1957), hyperinsulinemia (Altszuler, Steele, Rathgeb, & De Bodo, 1967), increased lipolysis (Fain, Kovacev, & Scow, 1965), decreased glucose metabolism (Altszuler, Rathgeb, Winkler, De Bodo, & Steele, 1968), and insulin resistance (Fraser, Joplin, Opie, & Rabinowitz, 1962; MacGorman, Rizza, & Gerich, 1981).

## GH Influence on Adipose Tissue

The effects of GH on adipose tissue and adipocytes are complex. Primarily, GH promotes lipolysis in adipose tissue leading to a decreased overall body fat mass. Accordingly, GH deficiency results in an increase in fat mass as has been observed in both humans and animals. For example, GH deficient (GHD) adults have increased VAT and abnormal lipid profiles and are at increased risk of developing cardiovascular disease. GH replacement with or without diet and exercise interventions effectively reduces VAT and improves lipid abnormalities in GHD adults (Bengtsson et al., 1993; Chrisoulidou et al., 2000; Gibney et al., 1999). Salomon, Cuneo, Hesp and Sonksen (1989) published one of the first placebo-controlled trials involving GH replacement in

GHD adults. After 6 months, GH-treated subjects experienced a significant decline in total body fat mass compared to subjects receiving placebo (Salomon, Cuneo, Hesp, & Sonksen, 1989). Using computed tomography (CT) scanning, Bengtsson et al. (1993) demonstrated that GH exerts its greatest impact on VAT depots. Lowered GH concentrations are also seen in obese states, specifically with abdominal (android) obesity (Clasey et al., 2001).

Animal studies show a similar trend in the influence of GH on adipose tissue. In other words, GH excess promotes a lean phenotype in animals and deficiency an obese phenotype. However, the effects of GH on adipose tissue are not always uniform among various depots. The study by Yang and colleagues found that GH increased lipolysis in hypophysectomized rats (Yang, Bjorntorp, Liu, & Eden, 1996). While Hausman, Hausman and Martin (1999) did not compare GH action among different adipose depots, they showed that there was a ten-fold decrease in lipogenesis in the subcutaneous adipose tissue of hypophysectomized pig fetuses upon treatment with GH (Hausman, Hausman, & Martin, 1999). In addition, a study by Flint and Gardner (1993) clearly demonstrated for the first time that GH is required for the differentiation of adipocytes in vivo (Flint & Gardner, 1993). This study also suggested that GH played a role in the differentiation of adipocytes within each depot (Flint & Gardner, 1993). A study that only looked at visceral fat in transgenic rabbits that express bGH in the liver and kidney showed a reduction in the visceral fat (Costa, Solanes, Visa, & Bosch, 1998). The lipid-lowering quality of GH has also been studied using transgenic pigs expressing the bGH gene

(Solomon, Pursel, Paroczay, & Bolt, 1994). These transgenic pigs exhibited 85% less carcass fat compared to controls at sacrifice (Solomon, Pursel, Paroczay, & Bolt, 1994).

Several studies in mice have confirmed these results and will be described in more detail in the subsequent sections. For example, Chen et al. (2001) showed that there is a reduction in the actual weights of retroperitoneal and epididymal fat pads in transgenic mice expressing porcine GH as well as lower body weights overall (Chen et al., 2001). The same study reported that there were increased levels of key transcription factors stimulated by insulin and involved in lipogenesis in wild-type mice but not the transgenic mice expressing porcine GH, suggesting that the adipose tissue in the transgenic mice was resistant to insulin-induced lipogenesis (Chen et al., 2001). Adiposity is also increased when there is a repression of GH signaling or a complete deficiency of GH signaling, with preferential accumulation in the subcutaneous region (Berryman et al., 2004). The same study reported a decrease in the percent fat mass and thus adiposity in mice over-expressing GH (Berryman et al., 2004). A recent study by Flint, Binart, Boumard, Kopchick and Kelly (2006) showed the stimulatory effect of GH on both adipocyte proliferation (cell numbers) and differentiation (adipocyte lipid content) in the parametrial adipose depot (Flint, Binart, Boumard, Kopchick, & Kelly, 2006). In this study, while the parametrial adipose depot of GHR-/- mice was dramatically impaired due to a decrease in the number of adipocytes and a significant reduction in adipocyte volume, this same impairment was not seen in the subcutaneous depot of the same mouse model (Flint et al., 2006). Thus, along with the adipocyte proliferation and differentiation in the subcutaneous adipose depot being independent of

GH, the absence of the GH receptors creates intrinsic changes in adipocytes from this depot and it persists for at least ten days in vitro (Flint et al., 2006). Collectively, the morphological data in both animal and human models confirm the lean phenotype and lipolysis promoted by GH either in vitro or in vivo.

## Growth Hormone Mouse Models

Because of the potential safety issues surrounding human studies and because in vitro systems fail to reveal the integrated impact of this pervasive hormone, whole animal studies offer valuable information regarding the influences and functions of GH in the body. Mouse models with all levels of GH signaling have been generated and partially characterized. For the most part, mouse models with altered GH signaling mimic the observed phenotype in humans and hence are good models to use (Berryman et al., 2004). There are numerous mouse lines that have altered GH signaling caused by mutations not only in the GH associated genes but other genes that are either indirectly or directly related to GH function. While the metallothionein-I-human GH releasing hormone (MT-hGHRH) mice (Hammer, Brinster, Rosenfeld, Evans, & Mayo, 1985) and the bGH mice (Kopchick, Bellush, & Coschigano, 1999) are examples of increased GH signaling, the Snell dwarf mice (Snell, 1929), Ames dwarf mice (Andersen et al., 1995), the GHR-/- (Kopchick et al., 1999), and “lit/lit” mice (Liang et al., 2003) are all examples of decreased GH signaling. The following section discusses the characteristics of two specific mouse lines used in this thesis: mice with a gene disruption in the GHR gene (GHR -/- mice) and mice transgenic for bovine GH (bGH mice).

***Growth Hormone Receptor Disrupted Gene Knockout Mice (GHR-/-)***

GHR-/- homozygous animals have a disruption in the GHR and GH-binding protein gene and have absolutely no GH signaling through the GH receptor. Hence, these animals are GH resistant and, as a consequence, have elevated serum GH concentrations but dramatically reduced IGF-I levels in circulation, resulting in severe postnatal growth retardation and dwarfism (Coschigano, Clemmons, Bellush, & Kopchick, 2000). These homozygous animals are significantly smaller than the wild-type and the GHR-/+ heterozygous mice, are long-lived, have reduced glucose and insulin levels, and have increased insulin sensitivity (Coschigano et al., 2000; Coschigano et al., 2003; Liu et al., 2004). Apart from being smaller, these mice have a slower growth rate compared to their littermate controls and reach their maximum weight at an earlier age than the homozygous wild-type mice and the heterozygous GHR-/+ mice (Coschigano et al., 2000).

An earlier study by Berryman et al. (2004) compared the total and depot-specific adipose stores, as well as adipocytokine levels in GHR-/- mice. Male GHR-/- mice showed a significant increase in percent fat mass compared to littermate wild-type controls (Berryman et al., 2004). This same study also suggested that the fat mass was not distributed equally among the various depots, but rather was deposited in a depot-specific manner (Berryman et al., 2004). More specifically, the subcutaneous and retroperitoneal fat pads were greater in the GHR -/- mice when the fat mass was normalized to body weight (Berryman et al., 2004). The subcutaneous depot in particular is enlarged and was also reported to be disproportionately enlarged when these same mice were fed a HF diet

(Berryman et al., 2006). Thus, the lack of GH signaling increases adiposity in mice as it does with humans, and the impact on adipose tissue is not uniform, with the subcutaneous depot more radically impacted.

***Bovine Growth Hormone (bGH) Expressing Mice***

As the name implies, bGH animals constitutively express the bovine GH transgene and have excessive amounts of circulating GH. The bGH mice have been characterized as having a large body size and lower percent body fat, specifically showing an inclination toward lower epididymal and retroperitoneal adipose tissues when expressed as a percent of total body weight (Berryman et al., 2004). These mice are normoglycemic but hyperinsulinemic and show slightly impaired or normal glucose tolerance when fed normal chow diet (Kopchick et al., 1999). The bGH mice also show several alterations in lipid and lipoprotein metabolism. These alterations include lower very-low-density lipoprotein (VLDL) levels, triglyceride levels, and hepatic triglyceride secretion rate and increased LPL activity (Frick et al., 2001). Furthermore, bGH mice also have higher high-density lipoprotein (HDL) and total cholesterol levels (Frick et al., 2001; Quaife et al., 1989).

These mice also seem to be somewhat protected from diet-induced obesity. Olsson et al. (2005) showed that these transgenic mice are resistant to diet-induced obesity, because they had no additional weight gain after exposure to a HF diet for eight weeks when compared with bGH mice on a normal chow diet, despite hyperphagia. This study also indicated that the HF diet-fed bGH mice displayed increased energy intake, energy expenditure, dyslipidemia and developed diabetes, indicating dietary fat is a

metabolic stimulator of these processes (Olsson et al., 2005). Using a different strain of bGH mice, Berryman et al. (2006) also showed that bGH mice were resistant to diet-induced obesity. They also compared the food intake and energy expenditure between the bGH and GHR -/- mice and showed that the energy intake was greatest with the bGH animals when fed a HF diet (Berryman et al., 2006). Overall, the over-expression of GH creates a lean phenotype confirming the lipolytic role of GH on adipose tissue.

## Methods to Quantify Gene Expression

Diabetes, obesity, hypertension, and several other complex diseases affecting mankind are likely to be caused by abnormal expression of multiple genes (Guillausseau, Tielmans, Virally-Monod, & Assayag, 1997; Shimkets & Lifton, 1996). In most complex diseases, the change in expression of one gene is often caused by interaction of a number of factors on the promoter region of the gene, resulting in coordinate changes in mRNA levels of a number of responsive genes. More specifically, alterations in GH signaling in the mouse models previously described can change the expression of many other genes simultaneously by affecting the promoter region of the target genes. Thus, it would be valuable to use a technology that can simultaneously measure coordinate changes in mRNA levels from many genes between two samples. One technology capable of genome-wide comparisons is microarray gene expression analysis.

### *Microarrays*

Microarray assays provide the opportunity to define the physiological patterns in which a gene is expressed. Several studies have been done using the complementary DNA (cDNA) microarray technology. After the pioneering work of Schena, Shalon,

Davis and Brown (1995), microarray technology has progressed through significant advancement and refinement (Venkatasubbarao, 2004). The use of microarray technology to assess gene expression levels is now widespread in biology and, particularly in the clinical setting, the applicability of the methodology is likely to broaden as the technology evolves, data analysis procedures improve, and costs decline (Howbrook et al., 2003; Jordan, 2002; Russo, Zegar, & Giordano, 2003). The two most common array platforms are based on collections of cDNA clones (Schena et al., 1995) or short (25 bases) oligonucelotides synthesized in situ by photolithographic methods (Lockhart et al., 1996). A third type of microarray, also considered a modification of the short oligonucleotide array, is the long (70 bases) oligonucleotide array and this version is also commonly used for expression profiling (Blanchard, Kaiser, & Hood, 1996; Hughes et al., 2001; Relogio, Schwager, Richter, Ansorge, & Valcarcel, 2002; Van't Veer et al., 2002).

***The Oligonucleotide Microarray Platform.*** This oligonucelotide microarray chip is produced by a combination of photolithography and solid phase DNA synthesis (Lipshutz, Fodor, Gingeras, & Lockhart, 1999). The very high information content and ability to represent whole genomes on a single chip are useful to perform genome expression surveys. The 20- to 90-mer oligonucleotides used in this platform are synthesized in situ on chips that are made of glass, a gel matrix or nylon surfaces using robotic contact printing with pins, photochemical techniques (developed by Affymetrix, Inc.), an ink-jet oligonucleotide synthesizer (developed by Agilent Technologies) or by other conventional synthesis methods followed by on-chip immobilization (Hughes et al.,

2001; Kane et al., 2000; Lipshutz et al., 1999). Commonly, this involves the spotting of presynthesized oligonucleotides. The spotted oligonucleotide formats are designed to accommodate two-color experiments where experimental and control samples are simultaneously hybridized to the same array (Lipshutz et al., 1999). The long oligonucleotide array platform, specifically made by Applied Biosystems (ABI), consists of 60-mers contact printed on filters. The ABI system relies on sensitive chemiluminescent detection, which renders the experiments single-channel where one sample is applied to each microarray (Kuo, Jenssen, Butte, Ohno-Machado, & Kohane, 2002).

The use of chemiluminescence offers significant advantages in terms of detecting a higher number of low copy-number transcripts, which are frequently the most interesting to study. Also, oligonucleotide arrays are synthesized requiring only DNA sequence data, making them less time consuming compared to the cDNA microarrays. Cross hybridization problems are avoided because probes can be designed to represent the most unique part of a given transcript. High costs involved with chip synthesis and insufficient sequence information for certain genes are the limitations of use for this platform when compared to cDNA arrays.

***The cDNA Microarray Platform.*** Probes for cDNA (500–5000 bases long), generated from cDNA libraries or clone collections using either vector-specific or gene-specific primers and are printed onto glass slides or nylon membranes as spots at defined locations (Schena et al., 1995). The advantages of cDNA arrays are relatively low production cost, high sensitivity, and flexibility. However, a prepreparation step

involving the synthesis of cDNA from RNA sequences and cross-hybridization of related or overlapping genes are two limitations of this platform (Kane et al., 2000; Li, Pankratz, & Johnson, 2002).

While microarrays are simple assays that measure the relative expression levels of tens of thousands of genes, insights into the limitations and pitfalls of microarrays have led to caution about data interpretation. Some of the limitations include long protocols for sample preparation that can cause synthesis or handling errors that cannot be confirmed by sequencing and variations in the DNA length in samples. While the relative merits of the two systems continue to be discussed (Moreau, Aerts, De Moor, De Strooper, & Dabrowski, 2003), the validation of microarray results using independent mRNA quantification techniques, including Northern blotting, ribonuclease protection, in situ hybridization, or real-time reverse transcriptase polymerase chain reaction (RT-RT PCR) remains a critical element of any microarray experiment (Brazma et al., 2001; Chuaqui et al., 2002). Also, microarrays can be very misleading because this highly developed process involves multiple steps, from sample selection to data analysis, each susceptible to potentially costly errors. Hence reconfirmation of the results using a different tool for quantification of gene expression is warranted. Using a second method to quantify gene expression is required if a more thorough analysis of individual gene expression is desired, as other methods are more cost-effective. Although there are many methods available to confirm the results of microarray analysis as mentioned earlier, this review will focus on the single method utilized in this study, a real-time reverse transcriptase polymerase chain reaction (RT-RT PCR).

***Real-time Reverse Transcriptase PCR (RT-RT PCR)***

Polymerase chain reaction (PCR) has the ability to amplify even small numbers of transcripts. RT-RT PCR takes advantage of this technique to perform gene expression analysis by employing primers specific to the gene of interest. It involves the use of a forward and reverse primer that can amplify the cDNA sequence between it and this amplification can be measured using photosensitive chemicals (the fluorescent reporter). A number of probes and dyes are available for this purpose and they are discussed in the following paragraph. The amount of product formed is seen through a fluorescent signal generated by the reporter. Initially, the signal is weak, but as the amount of product accumulates the signal increases exponentially. This is followed by a signal saturation which is due to the reduction of primer concentrations, the reporter, or the dNTPs (Kubista, Ståhlberg, & Bar, 2001). Also, because all response curves saturate at the same level in a RT-RT PCR, the end point measurements do not reveal the amounts of the template molecules present at the start of the reaction. As a result, the response curves, which are different for each sample during the growth phase of the reaction, are useful in determining the differences in initial amounts of the template molecules (Kubista et al., 2006). The number of amplification cycles required for the response to reach a particular threshold signal (referred to as the CT value) is used to quantify these differences in amounts of initial template. The amplification response curves are expected to be parallel in the growth phase of the reaction, and the setting of the threshold should therefore not be critical.

Fluorescent reporters used in RT-RT PCRs can be sequence specific probes or nonspecific labels. Among the nonspecific labels, asymmetric cyanine dyes such as SYBR Green I and BEBO (Bengtsson, Karlsson, Westman, & Kubista, 2003; Zipper, Brunner, Bernhagen, & Vitzthum, 2004) are popular for RT-RT PCR. While these dyes have virtually no fluorescence when they are free in solution, they become brightly fluorescent when they bind to DNA due to changes in their aromatic ring structure (Nygren, Svanvik, & Kubista, 1998). In RT-RT PCR, the fluorescence of these dyes increases with the amount of double-stranded product formed, but the dye fluorescence depends to some degree on the DNA sequence as well. However, a certain amount of a particular double-stranded DNA target, in the absence of significant amounts of other double-stranded DNA, gives rise to the same fluorescence every time (Kubista et al., 2006). Hence, these dyes are excellent for RT-RT PCR when samples are compared at the same level of fluorescence in absence of interfering DNA. On the other hand, probes that are specific to a particular sequence are based on nucleic acids or some of their synthetic analogues and give a fluorescent signal only when that message is amplified. Examples of probes with these specific dyes are the hydrolysis probes, popularly called Taqman probes (Holland, Abramson, Watson, & Gelfand, 1991), and Molecular Beacons (Tyagi & Kramer, 1996; Tyagi, Bratu, & Kramer, 1998).

There are a number of issues to consider when running RT-RT PCR reactions. Similar to conventional PCR, RT-RT PCR can produce an undesired primer-dimer product. This can be assessed by performing a melting curve analysis after completing the PCR. Primer–dimer products typically are shorter than the targeted product and they

melt at a lower temperature. As a result, their presence is easily recognized by a melting curve analysis. The reliable specificity, sensitivity, and accuracy of gene amplification are the strengths of the RT-RT PCR technique (Mackay, Arden, & Nitsche, 2002; Mocellin, Rossi, Pilati, Nitti, & Marincola, 2003). The use of SYBR Green chemistry, apart from its photo-physical properties and temperature stability (Zipper et al., 2004), confers additional benefits in terms of being able to conduct a melt curve analysis to verify specificity of product amplification. Also, the presence of a single, well-defined peak indicates the presence of a single product. Due to the fact that RNA quality is vital to producing high-quality cDNA, RNA extraction performed under optimal RNase-free conditions yields better PCR products.

The RT-RT PCR data analysis is crucial for correct interpretation of the results. Two approaches of data analysis, the comparative CT (cycle threshold) method (also known as ΔΔCT method (Livak & Schmittgen, 2001)) and the standard curve method (Morrison et al., 1998) are commonly used. After correct setup, run, and technical quality control of a RT-RT PCR, the two parameters for data analysis are determined for each well: the CT value and the PCR efficiency (E). The CT value is defined, according to the fit point method (Luu-The, Paquet, Calvo, & Cumps, 2005), as a fractional number of cycles, where the PCR kinetic-curve reaches a program-defined threshold amount of fluorescence. The PCR efficiency, E, is a major issue in RT-RT PCR data analysis. Even though identical reaction setups with identical templates are used, several factors, such as phenol, ethanol, hemoglobin, heparin and even the reverse transcriptase, are known to inhibit PCR efficiency (Bustin & Nolan, 2004; Suslov & Steindler, 2005). While there

are different approaches to determining the efficiency of the PCR, the prominent method is an external standard curve with a serial dilution series of a template (e.g. cDNA or plasmid DNA) measured in separate wells (Morrison et al., 1998). In this case, the dilutions are very susceptible to pipetting errors leading to incorrect determination of E, with a tendency to overestimate E (Peirson, Butler, & Foster, 2003). Moreover, screening for interwell variations concerning E is not possible. More practical disadvantages of the standard curve approach are the high consumption of reagents (and cDNA) and the occupancy of many wells during each PCR run.

Along with standardizing most of the procedures to control variations, internal reference genes, which are also known as housekeeping genes, are used in most experiments to normalize the results of gene expression. This strategy targets RNA encoded by housekeeping genes and benefits from the fact that all the steps required to obtain the final PCR measurement are controlled. The procedure is simplified as both the gene of interest and the reference gene is measured using RT-RT PCR. The most commonly known reference genes include β-actin (Actb), glyceraldehydes-3-phosphate dehydrogenase (Gapdh), hypoxantin-guanine phosphoribosyl transferase (Hgprt), ubiquitin (Ubc) and ribosomal protein s3 (Rps3). There are a number of programs that allow the assessment of multiple reference genes. The program geNorm allows the most appropriate reference gene to be chosen by using the geometric mean of the expression of the candidate cDNA (Vandesompele et al., 2002). The underlying principles of this software have been described previously (Vandesompele et al., 2002). Even though this is a robust method for providing accurate normalization, it is not always possible to

measure multiple reference genes due to limited sample availability and cost. Furthermore, even if multiple genes are chosen, the resolution of the particular assay remains dependent on the variability of the chosen reference genes.

In spite of the above mentioned disadvantages with respect to RT-RT PCR, this is the only method that offers the opportunity to observe the amplification kinetics of a PCR in "real-time" via accumulation and measurement of specific fluorescence signals with each cycle (Heid, Stevens, Livak, & Williams, 1996; Higuchi, Dollinger, Walsh, & Griffith, 1992; Wittwer, Herrmann, Moss, & Rasmussen, 1997). Thus, microarray data, which tends to be more exploratory and expensive, is commonly followed by independent confirmatory validation studies through RT-RT PCR.

## Summary

This review of literature confirms that abdominal/visceral distribution of adipose tissue is associated with endocrine disturbances including increased activity of the hypothalamic-pituitary-adrenal axis and a blunted secretion of GH. Theoretically, these endocrine perturbations can be a consequence of obesity, but the endocrine aberrations may have causal effects as well. The regulation of adipose tissue distribution is an important problem in view of the close epidemiological and metabolic associations between centralized fat accumulation and disease. With excess visceral fat accumulation, multiple endocrine perturbations are found, including elevated cortisol and androgens in women, as well as low GH and, in men, testosterone secretion. These abnormalities probably derive from a hypersensitive hypothalamic-pituitary-adrenal axis. These hormonal changes exert profound effects on adipose tissue metabolism and distribution

by altering the expression of multiple genes in the various adipose depots. Thus, a study of the expression profiles of these altered genes using microarrays and RT-RT PCR will provide valuable information about the various alterations in adipose tissue caused by GH, and bring us closer to understanding the depot-dependent role of GH in adipose tissue expression and function.

# CHAPTER 3: MATERIALS AND METHODS

Microarray analysis between the subcutaneous fat pads of GHR -/- and the WT mice served as the starting point for the methods used in this thesis. The microarray data indicated that 87 genes were significantly upregulated and 72 genes were significantly downregulated in GHR-/- versus littermate control (WT) subcutaneous fat pads. Following this, the up or downregulation of five of these genes, namely Agt, Adpn, Angptl4, Insig1 and Scd2, was confirmed using Real-time reverse transcriptase PCR (RT-RT PCR). These five genes were chosen for confirmation using RT-RT PCR based on the commonalities in their functions and the significant increase or decrease in their expression levels in the subcutaneous fat pads of the GHR-/- mice compared to the WT mice. Their functions are associated with adipose tissue remodeling and triglyceride storage and release. Details of the microarray analysis and the RT-RT PCR methods follow.

## Animals

All animals used in this study were bred at the mouse facility of Edison Biotechnology Institute, Ohio University (Athens, OH). The two transgenic mouse lines used in this study, the bGH and the GHR-/- mice, are in the C57BL/6J background. The bGH mice were obtained by the pronuclear microinjection in a pure C57BL/6J background, as described previously (Berryman et al., 2004). The GHR-/- mice were generated by homologous recombination also as previously described (Zhou, He, Baumann, & Kopchick, 1997) and backcrossed for eight generations into the C57BL/6J genetic background, as described previously (Coschigano et al., 2003). The control mice

to be used in this study were age-matched littermate controls from the GHR-/- mouse line. A maximum of 6 male mice in each of the mouse models, fed either a HF or LF diet, were used. The mice used in this study are a subset of the mice previously described (Berryman et al., 2006). All procedures were approved by the Ohio University Institutional Animal Care and Use Committee and fully comply with federal, state and local policies.

## Diets

The housing and diets used in this study were previously described (Berryman et al., 2006). The mice were weaned at 28 days of age onto a standard rodent chow diet, Purina Mills LabDiet®, ProLab RMH 3000 (St. Louis, Missouri), which provided 14% of kilocalories from fat, 16% from protein and 60% from carbohydrates. Along with food and water being fed ad libitum, the animals were exposed to a 14/10 hour light/dark cycle. At 10 weeks of age, the male mice were randomly assigned to either to a HF or LF diet such that there were five mice in the LF diet and six mice in the HF diet for each genotype. These diets have been used earlier in rodent models and have been described in detail previously (Woods, Seeley, Rushing, D'Alessio, & Tso, 2003). In brief, the HF diet contained 20 g of fat/100 g of diet (19 g of butter oil and 1 g of soybean oil to provide essential fatty acids) and provided 4.54 kcal/g, while the LF diet contained 3 g of butter oil and 1 g of soybean oil/100 g of diet and provided 3.81 kcal/g. The diets were purchased from Dyets, Inc.  (Bethlehem, PA).

## Adipose Tissue Collection

All animals were sacrificed by cervical dislocation after 11.5 weeks on the diet; thus, all mice were 21.5 weeks in age. Subcutaneous, epididymal and retroperitoneal adipose depots were dissected and the wet mass weighed and recorded as described previously (Berryman et al., 2006). Tissues from this study were immediately flash-frozen in liquid nitrogen and used for RNA analysis by microarray and RT-RT PCR described below.

## RNA Isolation and cDNA Synthesis

RNA isolation occurred through the use of one of two commercially available kits. The retroperitoneal adipose tissue was processed for total RNA isolation using the Qiagen RNeasy Lipid Tissue Mini Kit (Valencia, California), following the manufacturer's instructions. Due to the low yield with this kit, a second kit was used for the isolation of mRNA from the remaining fat pads. The relatively inexpensive Tel-Test STAT-60 Reagent (Friendswood, Texas) kit was used for epididymal and subcutaneous adipose tissue RNA isolation. This phenol-chloroform-isopropanol extraction and precipitation procedure was performed as per the manufacturer's instructions. Isolated RNA was stored in 50 µl diethylpyrocarbonate (DEPC)-treated water at -80ºC. Beckman Spectrophotometer DU® 640 (Fullerton, California) was used to determine the concentrations of the isolated RNA.

## Microarray Analysis

Microarrays were performed using Agilent mouse whole genome G4122A chips. Six chips were processed by Mogene, Inc., an Agilent approved microarray Analysis

Company. Seven subcutaneous fat pad samples were used; 3 from GHR-/-, 3 WT and 1 pooled control subcutaneous fat pad. Figure 1 explains the set up of each of the microarray chips as suggested by Mogene, Inc.

| | | |
|---|---|---|
| Pooled +/+ (control) sample versus GHR -/- pad from mouse 1 | Pooled +/+ (control) sample versus GHR -/- pad from mouse 2 | Pooled +/+ (control) sample versus GHR -/- pad from mouse 3 |
| Pooled +/+ (control) sample versus wild-type (control) pad from mouse 4 | Pooled +/+ (control) sample versus wild-type (control) pad from mouse 5 | Pooled +/+ (control) sample versus wild-type (control) pad from mouse 6 |

*Figure 1*. Processing order of microarray chips as suggested by Mogene, Inc.

All steps associated with the microarray analysis beginning with the processing of the sample RNA to the pathway analysis of the microarray data were completed by Mogene Inc. and are explained by their technicians in detail in Appendix A. The criteria used for identifying genes of interest were: (a) alteration in expression between the pooled sample and the GHR -/- pad greater than 2-fold, (b) a fold-change in expression that was determined to be statistically significant (all statistical analyses performed by Mogene and in Appendix A), and (c) the same gene was not significantly different in the

chip that compared the pooled control samples to the WT controls. Data were presented as fold-change in the GHR-/- compared to the WT.

## Real-Time Reverse Transcriptase PCR

The iScript cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, California) was used to create the complimentary DNA (cDNA). To achieve a standardized total RNA concentration, only 1 μg of the isolated RNA was used from each sample. A 1:4 dilution of the cDNA with sterile water was prepared and stored at -20ºC until the RT-RT PCR was performed. RT-RT PCR, as fully described in Appendix B, was used to amplify and determine the quantity of specific cDNAs in the samples. The specific amplification conditions for RT-RT PCR are listed in Table 1. Primer efficiencies were performed with the forward and reverse primers of the five genes of interest as well as the three housekeeping genes, Hgprt, Rps3 and Ubc. The sequences of all these primers are listed in Table 2. The specific gene primers for each of the five genes were used to amplify 5 μl of the 1:4 diluted cDNA samples using the iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, California) PCR Kit. SYBR Green has the unique property of only binding double-stranded sequences. Thus, a higher level of fluorescence indicated a higher amount of double-stranded sequence or higher level of the specific cDNA of interest in the sample. In order to normalize the experimental results, RT-RT PCR reactions were performed for three housekeeping genes in order to account for variability in the initial quantities of cDNA and stability during the experimental procedures. Of the three housekeeping genes, Hgprt and Rps3 were selected by the geNorm VBA applet Microsoft Excel program, based on gene stability.

Table 1

*RT-RT PCR Amplification Conditions*

| Cycle | Time in each cycle (in minutes) | Step | Number of repeats | Temperature (in $^{0}$C) |
|---|---|---|---|---|
| 1 | 3:00 | 1 | 1 | 95 |
| 2 | 0:15 | 1 | 40 | 94 |
| | 0:30 | 2 | | 60 |
| | 0:30 | 3 | | 72 |
| 3 | 1:00 | 1 | 1 | 95 |
| 4 | 1:00 | 1 | 1 | 55 |
| 5 | 0:10 | 1 | 80 | 55 |

Table 2

*Primers*

| | Forward | Reverse |
|---|---|---|
| Agt | 5’ TGTGACAGGGTGGAAGATGA 3’ | 5’ AGAACCTTGTCAGAGGTGCTT 3’ |
| Adpn | 5’ATGGCAAACTTGTGGGAGAC 3’ | 5’ GGAGGTTGCAGACTTTGCGC 3’ |
| Angptl4 | 5’ GGAAAAGATGCACCCTTCAA 3’ | 5’ TGCTGGATCTTGCTGTTTTG 3’ |
| Insig1 | 5’ ACATTTGATCGTCCCGAAG 3’ | 5’ AATCAGGGGACGTGTACTGG 3’ |
| Scd2 | 5’ CTGACCTGAAAGCCGAGAAG 3’ | 5’ CAAGAAGGTGCTAACGCACA 3’ |
| Hgprt | 5’ AAGCTTGCTGGTGAAAAGGA 3’ | 5’ TTGCGCTCATCTTAGGCTTT 3’ |
| Rps3 | 5’ ATCAGAGAGTTGACCGCAGTTG 3’ | 5’ AATGAACCGAAGCACACCATAG 3’ |
| Ubc | 5’ AGGTCAAACAGGAAGACAGAC 3’ | 5’ TCACACCCAAGAACAAGCACA 3’ |

## Statistical Analysis

Significant data reported for the microarray analysis were based on the ANOVA calculations that were performed by Mogene, Inc. and are fully explained in Appendix A. SPSS 14.0 was used to perform all statistical analysis of RT-RT PCR data. A 3 (genotype) * 2 (diet) factorial ANOVA was used to compare the differences in mRNA expression among the three adipose tissue pads in the three genotypes and their interaction with the diets. This was followed by a t-test of only the data that was significantly different in the ANOVA. Results were represented as mean ± standard error of the mean. P values $< 0.05$ were considered significant.

# CHAPTER 4: RESULTS

## Microarray Analysis

Microarray analysis was used to measure the mRNA differences between the subcutaneous fat pads of GHR -/- versus WT mice. In these assays, a total of 159 genes were found to be either up or downregulated by more than two-fold. Of these genes, 87 were significantly upregulated and 72 were significantly downregulated in the GHR-/- versus WT subcutaneous adipose tissue. The 25 genes that were most significantly altered are shown in Tables 3 and 4. A full listing of all genes is provided in Appendices C and D.

## Real-Time Reverse Transcriptase PCR (RT-RT PCR) Analysis

One of the objectives of performing RT-RT PCR was to confirm the alterations in gene expression seen in microarray analysis for genes of particular interest. The genes that were chosen for RT-RT PCR analysis are genes whose proteins are involved in adipose tissue remodeling and triglyceride storage. These processes were considered to be a priority based on present interests of the laboratory linking adipose tissue to GH function. Primers for RT-RT PCR were designed for the following genes: Perp, Glut5, Adpn, Agt, Angptl4, Elovl6, Ucp1, Insig1 and Scd2. Primers with acceptable efficiency levels were Adpn, Agt, Angptl4, Insig1, and Scd2. Several redesigns of new primers for Glut5, Perp and Ucp1 did not result in acceptable primer efficiencies and the altered gene expression could not be confirmed by RT-RT PCR analysis. Of the genes with acceptable primer efficiencies (Adpn, Agt, Angptl4, Insig1 and Scd2), subcutaneous expression of

Table 3

*Twenty Five Most Upregulated Genes in the GHR-/- Mice Compared to Littermate Controls.*

| Gene Name | Description | Basic Function | Fold Change in GHR-/- | Significance (Probt) |
|---|---|---|---|---|
| Perp | TP53 apoptosis effector | Facilitates apoptosis | 15.5 | 9.51E-03 |
| AW 125753 | Expressed sequence | Unknown | 12 | 8.92E-03 |
| Wisp2 | WNT1 inducible signaling pathway protein2 | Positive regulator of epithelial cells | 5.6 | 0.01 |
| Acta1 | Actin, alpha 1, skeletal muscle | Major constituent of the contractile apparatus of skeletal muscle | 4.8 | 0.02 |
| Edn 1 | Endothelin | Involved in the neuroactive ligand receptor pathway | 4.4 | 0.05 |
| Agt | Angiotensinogen | Renin-angiotensin system of blood pressure regulation | 3.8 | 0.01 |
| Angptl4 | Angiopoietin-like 4 | Alterations in lipid and cholesterol metabolism | 3.7 | 1.14E-04 |
| Aldh1a1 | Aldehyde dehydrogenase family 1 subfamily A1 | Act on aldehyde substrates using NADP as a cofactor for energy production and conversion | 3.6 | 1.29E-03 |
| Unc93a | Unc-93 homolog A (C.elegans) | unknown function | 3.6 | 0.02 |
| Chst1 | Carbohydrate sulfotransferase 1 | keratin sulfate biosynthesis pathway | 3.6 | 7.94E-04 |
| 2310020A2 | RIKEN cDNA 2310020121 gene | | 3.6 | 0.05 |
| Slc7a8 | Solute carrier family 7 (cationic amino acid transporter system), member 8 | Catalyze transfer of L-amino acid from one side of the membrane to the other side | 3.4 | 3.22E-03 |
| Gdpd1 | Glycerophospho-diester phosphodiesterase | Glycerol metabolizing enzyme | 3.2 | 0.01 |

Table 3 (continued).

| Gene Name | Description | Basic Function | Fold Change in GHR-/- | Significance (Probt) |
|---|---|---|---|---|
| Hpgd | Mus muscuclus hydroxyprostaglandin dehydrogenase 15 (NAD) | Electron carrier in the electron transport chain | 3.2 | 4.22E-05 |
| Cys1 | Mus musculus cystin 1 (Cys 1) | Molecular function is unknown | 3.1 | 0.01 |
| Alb1 | Albumin 1 | Interacts with specific metals and lipids for transport | 3 | 7.64E-03 |
| Tcfcp2l2 | Transcription factor CP2-like 2 | Transcription factor, binds to DNA to modulate transcription | 2.9 | 0.02 |
| AK042648 | | | 2.9 | 0.05 |
| Emp2 | Epithelial membrane protein 2 | Facilitates plasma membrane delivery of certain integrins | 2.9 | 0.02 |
| Maoa | Monoamine oxidase A | Catalyzes redox reactions | 2.8 | 5.93E-03 |
| Palmd | Palmdelphin | Involved in the regulation of cell shape | 2.8 | 9.23E-03 |
| Btc | Mus musculus betacellulin, epidermal growth factor family member (Btc) | Induction of cell growth and proliferation | 2.7 | 0.01 |
| Lgals6 | Mus musculus lectin, galactose binding soluble 6 | Interacts with mono, di or trisaccharide carbohydrates | 2.7 | 3.92E-05 |
| Mt2 | Metallothionein 2 | Binds to metals, specifically zinc | 2.7 | 1.22E-03 |
| Thbs2 | Mus musculus thrombospondin 2 | Cell adhesion molecule | 2.7 | 7.90E-03 |

*Note.* Genes are listed in descending order based on magnitude of change.

Table 4

*Twenty-Five Most Downregulated Genes in the GHR-/- Mice Compared to Littermate Controls.*

| Gene Name | Description | Basic Function | Fold Change in GHR-/- | Significance (Probt) |
|---|---|---|---|---|
| Slc2a5 | Solute carrier family 2 (facilitated glucose transporter), member 5 | Transmembrane transporter of fructose and glucose | 7.42 | 3.39E-04 |
| Ucp1 | Uncoupling protein 1, mitochondrial | Catalyzes transfer of protons, uncoupling electron transport from ATP synthesis | 6.415 | 1.42E-03 |
| Elovl6 | ELOVL family member 6, elongation of long chain fatty acids (yeast) | Catalyzes the transfer of all acyl groups away from amino-acyl groups | 5.735 | 1.28E-03 |
| Ghr | Growth hormone receptor | Cell surface receptor for GH | 5.246 | 6.03E-16 |
| Scd2 | Stearoyl-coenzyme A desturase 2 | Involved in redox reactions in the presence of iron compounds | 5.001 | 1.22E-03 |
| Thrsp | Thyroid hormone sensitive SPOT14 homolog (rattus) | Hepatic protein inducible by thyroid hormone | 4.403 | 9.19E-04 |
| Cox7a1 | Cytochrome C oxidase, subunit VIIa 1 | Involved in the electron transport chain | 4.24 | 7.31E-03 |
| Adpn | Adiponutrin | Phospholipase involved in the lipid metabolic process | 4.213 | 6.45E-03 |
| Cox8b | Cytochrome C oxidase, subunit VIIIb | Involved in the electron transport chain | 4.158 | 6.27E-03 |
| Gys2 | Glycogen synthase 2 | Catalyzes the biosynthesis of glycogen | 3.995 | 1.11E-03 |
| Mal2 | Mal, T-cell differentiation protein 2 | Lipid associated protein that aids in activities performed by phospholipid membranes | 3.724 | 2.31E-03 |
| Cspg3 | Mus musculus chondroitin sulfate proteoglycan 3 | Calcium binding domain present in several membrane bound and extra cellular proteins | 3.669 | 1.05E-04 |

Table 4 (continued).

| Gene Name | Description | Basic Function | Fold Change in GHR-/- | Significance (Probt) |
|---|---|---|---|---|
| Thea | Thioesterase, adipose associated | Supports transition of thermogenic brown adipose tissue to being more metabolically active. | 3.615 | 0.01 |
| Sult1e1 | Mus musculus sulfotransferase family 1E member | In presence of estrogen, catalyzes transfer of sulphur groups | 3.588 | 1.37E-03 |
| Mod1 | Mus musculus malic enzyme, supernatant | Involved in the malate metabolism of the TCA cycle | 3.343 | 1.18E-06 |
| BC024408 | | | 3.343 | 3.03E-06 |
| Ppp1r3b | Protein phosphatase 1, regulatory (inhibitor) subunit 3B | Molecular function is unknown | 3.289 | 5.45E-04 |
| AK053478 | | | 3.18 | 9.16E-04 |
| Insig1 | Mus musculus insulin induced gene 1 | Binds to insulin and is involved in adipose tissue remodeling | 3.153 | 8.69E-06 |
| Lactb2 | Lactamase, beta 2 | Molecular function is unknown | 3.098 | 9.27E-05 |
| Mup4 | Mus musculus major urinary protein 4 | Transporters of small hydrophobic molecules | 3.071 | 1.29E-03 |
| Mup1 | Mus musculus major urinary protein 1 | Transporters of small hydrophobic molecules | 3.044 | 3.69E-06 |
| Mid1ip1 | Mid1 interacting protein 1 (gastrulation specific G-12-like (zebra fish)) | Binds to C terminus of protein for negative regulation of microtubule depolymerization | 3.017 | 1.20E-03 |
| Agpat2 | O-acyltransferase 2 (lysophosphatidic acid acyltransferase, beta) | Involved in the biosynthesis of phospholipids | 2.962 | 1.46E-05 |
| Slc25a1 | Mus musculus solute carrier family 25 (mitochondrial carrier, citrate transporter), member 1 | Mitochondrial carrier protein | 2.935 | 2.66E-03 |

*Note.* Genes are listed in descending order based on magnitude of change.

only Adpn, Agt and Angptl4 were confirmed to be significantly different between GHR -/- mice and the control mice on a standard LF diet.

The direction of expression was similar to the microarray data. Specifically, Agt and Angptl4 were upregulated in both microarray analysis (see Table 3) and RT-RT PCR analysis (see Figure 2), while Adpn was downregulated by both analyses (see Table 4 and Figure 2). The significant difference in Scd2 and Insig 1 expression observed by microarray was not confirmed by RT-RT PCR; thus, these two genes were not pursued further.

To better understand the role of GH in adipose tissue-specific expression of the three genes (Adpn, Agt, Angptl4); RT-RT PCR analysis was expanded to include an additional genotype of altered GH function, an additional diet treatment and two other adipose depots. Specifically, expression of all three genes was assessed in subcutaneous, epididymal and retroperitoneal adipose samples from GHR -/-, bGH and WT mice fed either a HF or LF diet.

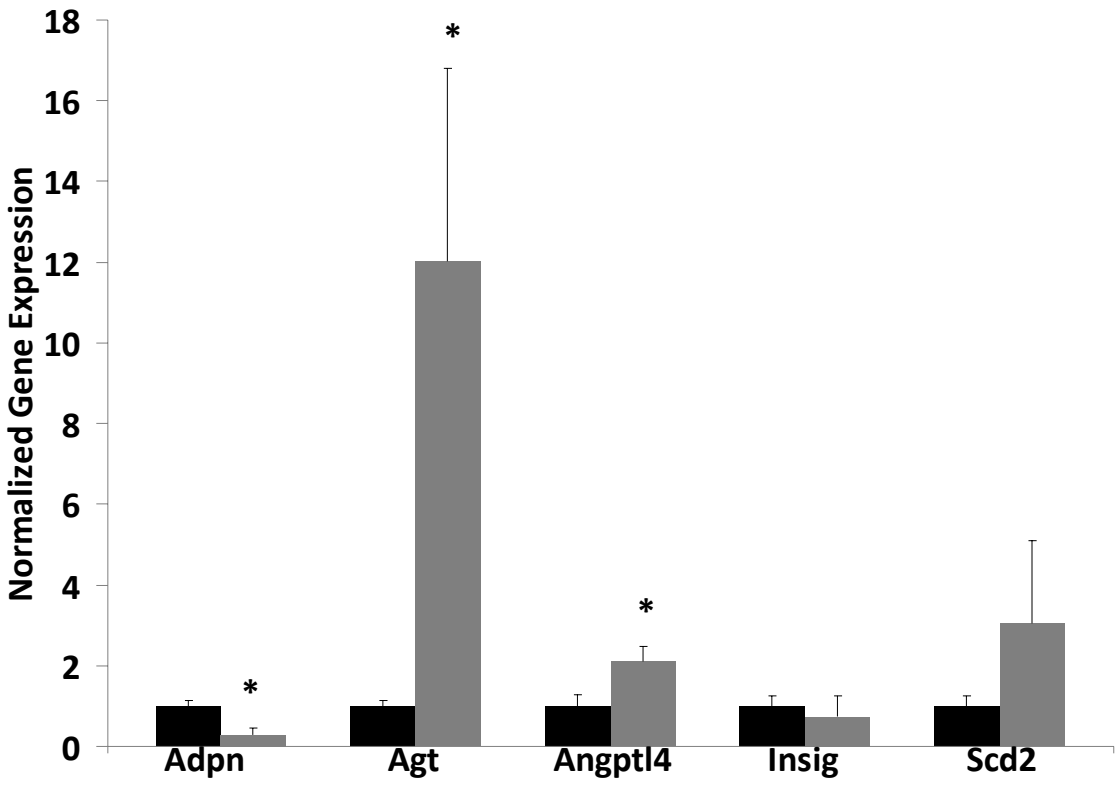
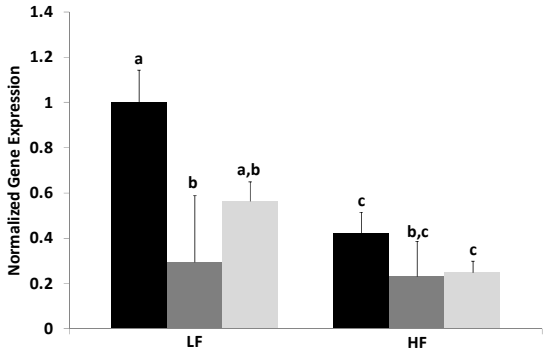



*Figure 2.* Relative expression of Adpn, Agt, Angptl4, Insig1 and Scd2 mRNA in the subcutaneous adipose tissue of GHR -/- ( grey bars) and littermate control ( black bars) mice fed a LF diet. Values are means + SEM, n=5 for the LF and n=6 for the HF. * denotes statistical significance at $p<0.05$. Comparisons are not made between genes.

***Adiponutrin (Adpn)***

As mentioned earlier, Adpn subcutaneous expression *(F(2,27) = 8.619, p>.05)* was significantly decreased in the LF GHR -/- versus LF WT mice (see Figure 3A). However, no significant differences were found between LF bGH mice and the other gentoypes. The significant difference in Adpn expression observed in the subcutaneous depot was not maintained in the other two depots analyzed. That is, there was no significant difference in Adpn expression in either the retroperitoneal or epididymal depots among LF WT, LF GHR-/- and LF bGH mice (see Figure 3B and Figure 3C). In addition, no significant difference was seen among the subcutaneous adipose depot of all three genotypes on a HF diet. In fact, there were no significant differences in Adpn expression among any of the groups compared in retroperitoneal adipose tissue (see Figure 3C). Alternatively, a significant increase in the expression of Adpn *(F(2,27) = 1.967, p>.05)* in the epididymal tissue was observed in the HF bGH mice as compared to the HF GHR -/- mice even though differences were not observed on the LF diets (see Figure 3B). When comparisons were made between the same genotype on different diets, significantly decreased expression was only found in the subcutaneous depot of the HF WT and HF bGH mice compared to the LF WT and LF bGH, respectively (see Figure 3A). Table 5 summarizes the significant differences found for Adpn gene expression using RT-RT PCR.

A

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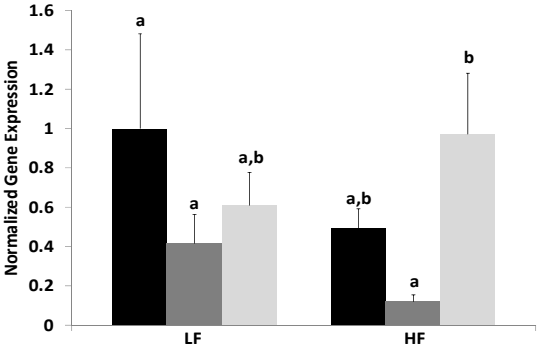


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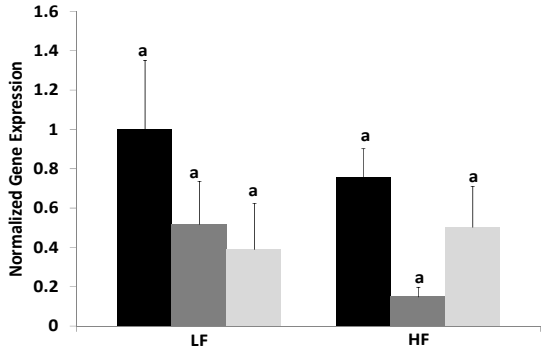



*Figure 3 A-C.* Adpn expression in subcutaneous (A), epididymal (B) and retroperitoneal (C) adipose tissue of GHR -/- (▭ grey bars), bGH(▭ pale bars) and littermate control (■ black bars) mice fed either a LF or HF diet. Values are shown as means + SEM, n=5

for the LF diet and n=6 for the HF diet. Statistical comparisons are made between different genotypes on the same diet and the same genotypes on different diets. Comparisons were not made between different genotypes on different diets. Means with a common letter do not differ, P>0.05.

Table 5

*Significant Differences Found for Adpn Gene Expression*

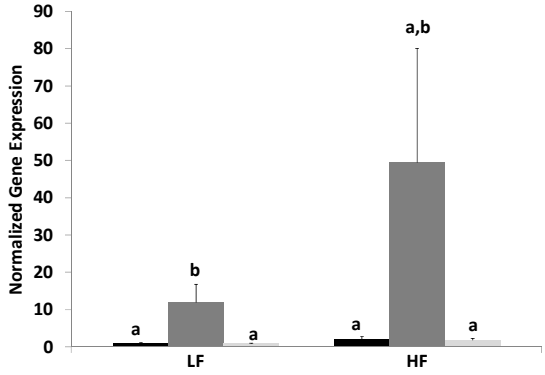
| Group | Adipose Tissue Depot | Significance | Significance value |
|---|---|---|---|
| LF-WT | Subcutaneous | > LF GHR-/- | 0.004 |
| | Subcutaneous | > HF WT | 0.004 |
| LF GHR-/- | Subcutaneous | < LF WT | 0.004 |
| LF bGH | Subcutaneous | > HF bGH | 0.006 |
| HF WT | Subcutaneous | < LF WT | 0.004 |
| HF GHR-/- | Epididymal | < HF bGH | 0.011 |
| HF bGH | Subcutaneous | < LF bGH | 0.006 |
| | Epididymal | > HF GHR-/- | 0.011 |

*Note.* Only significant differences at P<0.05 are reported. Statistical comparisons are made between different genotypes on the same diet and the same genotypes on different diets. Comparisons were not made between different genotypes on different diets.

***Angiotensinogen (Agt)***

Subcutaneous Agt expression was shown to be upregulated by microarray and RT-RT PCR analysis in LF GHR -/- versus LF WT mice *(F(2,27) = 3.492, p>.05).* Interestingly, subcutaneous Agt expression was also significantly increased in the LF GHR-/- mice compared to the LF bGH mice (see Figure 4A). In addition, no significant differences were observed between LF WT, LF GHR-/- and LF bGH mice in either epididymal (see Figure 4B) or retroperitoneal (see Figure 4C) depots. On a HF diet, there were no significant differences in the subcutaneous expression of Agt (see Figure 4A). The expression of Agt was significantly increased in the epididymal adipose depot *(F (2,27) =1.691, p>.05)* of the HF GHR -/- mice compared to the HF WT mice (see Figure 4B). Comparisons of the same genotypes across diets resulted in no significances for the subcutaneous and retroperitoneal depots. However, the HF WT mice had significantly decreased Agt expression compared to the LF WT mice in the epididymal depot (see Figure 4B). Table 6 summarizes the significant differences found for Agt gene expression using RT-RT PCR.

A

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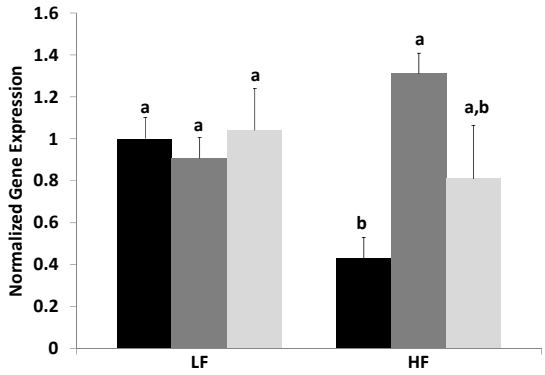



C

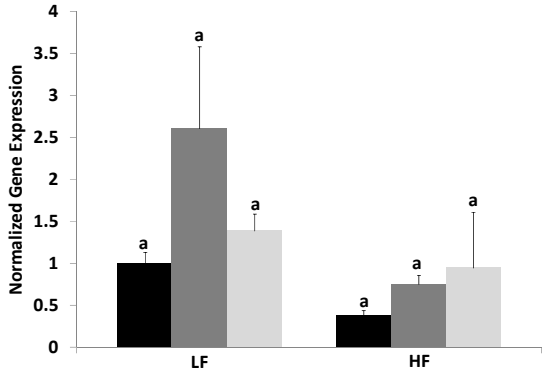



*Figure 4 A-C.* Agt expression in subcutaneous (A), epididymal (B) and retroperitoneal (C) adipose tissue of GHR -/- (▬ grey bars), bGH(▭ pale bars) and littermate control

(■ black bars) mice fed a LF or HF diet. Values are means + SEM, n=5-6. Comparisons are made between different genotypes on the same diet and the same genotypes on different diets. Comparisons were not made between different genotypes on different diets. Means with a common letter within the compared groups do not differ, P>0.05.

Table 6

*Significant Differences Found for Agt Gene Expression*

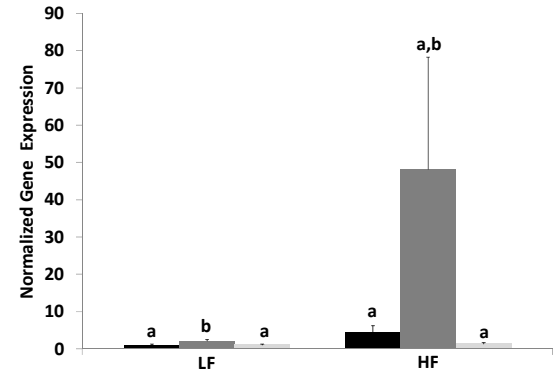
| Group | Adipose Tissue Depot | Significance | Significance Value |
|---|---|---|---|
| LF-WT | Subcutaneous | < LF GHR-/- | 0.021 |
| | Epididymal | > HF WT | 0.04 |
| LF GHR-/- | Subcutaneous | > LF WT | 0.021 |
| | | > LF bGH | 0.02 |
| LF bGH | Subcutaneous | < LF GHR-/- | 0.02 |
| HF-WT | Epididymal | < LF WT | 0.04 |
| | | < HF GHR-/- | 0.024 |
| HF GHR-/- | Epididymal | > HF WT | 0.024 |

*Note*. Only significant differences at P<0.05 are reported. Statistical comparisons are made between different genotypes on the same diet and the same genotypes on different diets. Comparisons were not made between different genotypes on different diets.

***Angiopoietin-like 4 (Angptl4)***

Subcutaneous Angptl4 ($F(2,27) = 2.299$, $p>.05$) expression was significantly increased between the LF GHR-/- and LF WT mice and between the LF GHR-/- and LF bGH mice (see Figure 5A). Epididymal Angptl4 expression did not show any significant differences among the genotypes or between diets (see Figure 5B). While there were no significant differences in the retroperitoneal expression of Angptl4 on the LF diet, significantly decreased expression was observed in the HF bGH mice compared to both HF GHR-/- mice and HF WT mice ($F(2,27) = 3.949$,$p>.05$) (see Figure 5C). There were no significant differences between the HF WT, HF GHR-/- and HF bGH mice in the subcutaneous depot (see Figure 5A). Comparisons of the genotypes across diets showed increased retroperitoneal Angptl4 expression of HF GHR -/- and HF WT compared to LF GHR-/- and LF WT, respectively (see Figure 5C). No other significant differences were observed when comparing the genotypes across diets for subcutaneous (see Figure 5A) and epididymal (see Figure 5C) expression of Angptl4. Table 7 summarizes the significant differences for Angptl4 gene expression using RT-RT PCR.

A


Normalized Gene Expression
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70
60
50
40
30
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a
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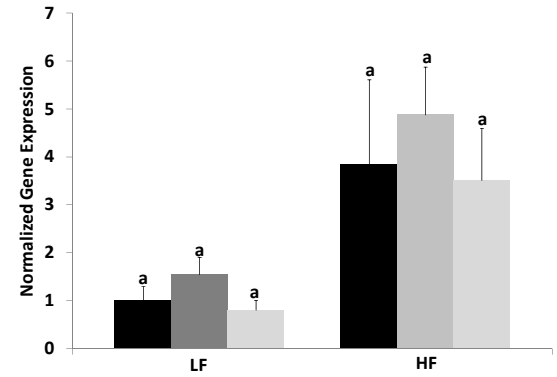
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6
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4
3
2
1
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a
LF
HF

C

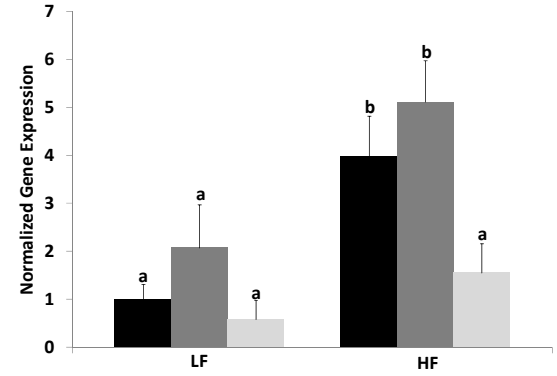
Normalized Gene Expression
7
6
5
4
3
2
1
0
a
a
a
b
b
a
LF
HF

*Figure 5 A-C.* Angptl4 expression in subcutaneous (A), epididymal (B) and retroperitoneal (C) adipose tissue of GHR -/- (▭ grey bars), bGH(▭ pale bars) and littermate control (■ black bars) mice fed a LF or HF diet. Values are means + SEM, n=5-6. Comparisons are made between different genotypes on the same diet and the same genotypes on different diets. Comparisons were not made between different genotypes on different diets. Means with a common letter do not differ, P>0.05.

Table 7

*Significant Differences Found for Angptl4 Gene Expression*.

| Group | Adipose Tissue Depot | Significance | Significance Value |
|---|---|---|---|
| LF WT | Subcutaneous | < LF GHR-/- | 0.032 |
| | Retroperitoneal | < HF WT | 0.01 |
| LF GHR-/- | Subcutaneous | > LF WT | 0.032 |
| | | > LF bGH | 0.048 |
| | Retroperitoneal | < HF GHR-/- | 0.025 |
| LF bGH | Subcutaneous | < LF GHR-/- | 0.048 |
| HF WT | Retroperitoneal | > LF WT | 0.01 |
| | | > HF bGH | 0.026 |
| HF GHR-/- | Retroperitoneal | > LF GHR-/- | 0.025 |
| | | > HF bGH | 0.003 |
| HF bGH | Retroperitoneal | < HF WT | 0.026 |
| | | < HF GHR-/- | 0.003 |

*Note*. Only significant differences at $P<0.05$ are reported. Statistical comparisons are made between different genotypes on the same diet and the same genotypes on different diets. Comparisons were not made between different genotypes on different diets.

# CHAPTER 5: DISCUSSION

The purpose of this thesis was to identify the altered mRNA expression of select genes in the subcutaneous, epididymal and retroperitoneal adipose depots of two mouse models that express opposite extremes of GH signaling, the GHR-/- and bGH mice. These two strains are in the C57BL/6J genetic background, making them susceptible to diet-induced obesity resulting in expanded adipose tissue (West, Boozer, Moody, & Atkinson, 1992). Microarrays were used to perform a global survey of all gene alterations in the subcutaneous adipose tissue of GHR-/- mice compared to control mice. The subcutaneous adipose depots were chosen for the initial microarray analysis based on a previous study that showed that while most fat pads remained proportional to body size in the absence of growth hormone signaling (GHR -/- mice), the subcutaneous pads were significantly larger than littermate controls (Berryman et al., 2004). This suggests that the subcutaneous adipose stores are more responsive to GH. Additionally, several studies have indicated that GH impacts adipose tissue differently depending on the location (Chen et al., 2001; Flint & Gardner, 1993; Kadim et al., 1996; Lall et al., 2001). The microarray results showed that 87 genes were significantly upregulated and 72 genes were significantly downregulated in the subcutaneous adipose depot of GHR -/- mice compared to littermate controls. A subgroup of 5 genes (Adpn, Agt, Angptl4, Insig1 and Scd2) was further analyzed using RT-RT PCR. While RT-RT PCR confirmed the microarray results for subcutaneous Adpn, Agt and Angptl4 expression, microarray results were not confirmed for subcutaneous Insig1 and Scd2 expression. Hence, Insig1 and Scd2 were not pursued further. The remaining genes (Adpn, Agt and Angptl4) were

further analyzed using RT-RT PCR in subcutaneous, retroperitoneal and epididymal adipose tissue from WT, GHR -/- and bGH mice fed either a LF or HF diet.

## Microarray Analysis and Confirmation by Real-Time Reverse Transcriptase PCR (RT-RT PCR)

Many microarray studies have been conducted assessing changes in gene expression in adipose tissue. Several studies have reported distinct gene expression differences between subcutaneous and visceral adipose tissue. For example, in studies performed on adipose tissue of extremely obese individuals, the genes leptin (Dusserre, Moulin, & Vidal, 2000; Hube et al., 1996; Montague et al., 1998) and perilipin (Arvidsson, Blomqvist, & Ryden, 2004) were shown to be more highly expressed in the subcutaneous depot while thrombospondin and carboxypeptidase E (Ramis et al., 2002) were found to be more highly expressed in the omental visceral depot. Another study compared the differences in gene expression in omental visceral adipose tissue of lean and obese human subjects and found that genes involved in lipolysis activation and several growth factors were downregulated in obese subjects compared to the lean subjects (Gomez-Ambrosi et al., 2004). A recent study of normal weight subjects found that genes were consistently differentially expressed between subcutaneous and omental adipose tissue (van Beek et al., 2007). The same study also compared differences in gene expression between subcutaneous and omental visceral adipose tissue of lean to mildly obese subjects and found that expression patterns in adipose tissue are highly homogeneous within each adipose depot (van Beek et al., 2007). Burton, Guan, Nagarajan and McGehee (2002) used microarrays to show that there is increased gene

expression of all genes involved in adipocyte differentiation during the first 24 hours of 3T3-L1 adipocyte differentiation (Burton, Guan, Nagarajan, & McGehee, 2002). Microarrays have also helped determine the differential gene expression between preadipocytes and adipocytes of subcutaneous human adipose tissue (Urs et al., 2004). For example, genes involved in lipid metabolism were expressed at higher levels in adipocytes, whereas genes expressed at higher levels in preadipocytes were associated with the formation of the extracellular matrix and were markers of early differentiation and cell commitment (Urs et al., 2004). Thus, microarray studies have proved informative at illustrating depot-specific differences, differences between obese and lean states, and differences due to differentiation in human and cell culture studies.

Microarray studies are also commonly used in obese or diabetic studies on adipose tissue in mice. For example, a study by Nadler et al. (2000) used DNA microarrays to assess the changes in epididymal adipose gene expression associated with obesity and diabetes of lean, obese, and diabetic mice. A study by Soukas, Cohen, Socci and Friedman (2000) also used microarrays to identify the altered gene expression in periuterine white adipose tissue caused by leptin in leptin deficient mice. These two studies showed that genes normally associated with adipocyte differentiation are downregulated in obesity. A study by Higami et al. (2004) used microarrays to compare gene expression profiles in epididymal adipose of C57Bl/6J mice exposed to fasting, short-term caloric restriction and long-term calorie restriction. This study reported that long-term caloric restriction had the highest number of gene expression alterations in the epididymal adipose depot (Higami et al., 2004). Another study also using caloric

restriction found that genes involved in lipid metabolism were downregulated with age in the retroperitoneal adipose depot and that this downregulation was prevented with caloric restriction (Linford et al., 2007). Even though the individual adipose depots were not compared, a global decrease in expression of genes associated with adipocyte differentiation and signaling molecules along with an increase in the expression of genes associated with inflammatory markers were seen in microarray analysis of abdominal white adipose of HF diet fed C57BL/6J mice compared to control mice (Moraes et al., 2003). While many studies have used adipose samples from humans and other species, as well as adipocyte cell lines for micorarray analysis, no studies have looked at adipose tissue gene expression changes due to GH.

The microarray results obtained in this thesis showed a total of 159 differentially altered transcripts, as shown in Appendices C and D. The differentially expressed transcripts were of genes involved in apoptosis (Perp, Mis1ip1), lipid metabolism (Adpn, Mal2, Thea, Cspg3) and adipose remodeling (Insig1, Agpat2, Agt) along with genes involved in a variety of other functions ranging from transport proteins (Slc2a5, Cspg3, Slc25a1) to hormone signaling molecules (Thrsp, Mod1, Sult1e1). Also, as expected with a GH receptor mutation in the GHR-/-mice, there was specific downregulation of GHR gene in the subcutaneous adipose of GHR-/- as compared to control tissue. In addition, the production of insulin-like growth factor-1 (IGF-1) is believed to be stimulated by GH and is retarded by the lack of GH receptors (Fleenor, Arumugam, & Freemark, 2006). The microarray data confirmed the downregulation of IGF-1 in GHR -/- as compared to

control mice. Thus, the specific downregulation of both GHR and IGF-1 in the subcutaneous adipose of GHR-/- mice further validates the microarray analysis.

While the microarray results were confirmed by RT-RT PCR for Adpn, Agt and Angptl4 in the subcutaneous adipose depot of GHR-/- mice, the results for Insig1 and Scd2 were not significantly different than the littermate controls. These conflicting results emphasize the need to confirm microarray data. Furthermore, the altered gene expression of Insig1 and Scd2 in the microarray could be a false positive caused either by: (a) the probes on the microarray cross-hybridizing to related but different genes in the mixed population of labeled oligonucleotides, or (b) the presence of splice variants for Insig1 and Scd2 which could have hybridized to the probe. The conflicting data from RT-RT PCR for Insig1 and Scd2 may be attributed to the inherent multiple enzymatic steps involved in RT-RT PCRs. Enzymatic manipulations are sequence and structure dependent and may result in artificial outcomes. Large amplification reactions in RT-RT PCRs can compound the error and introduce a nonlinear bias in the samples. It is also interesting to note that both Insig1 and Scd2 had lower expression levels than the GH receptor gene in the subcutaneous adipose of GHR-/- mice. This low level of expression could also be a reason why the RT-RT PCR and microarray data do not agree as genes with low expression levels can be problematic to quantify using either technique.

Because expression levels of Adpn, Agt and Angptl4 were consistent between microarray and RT-RT PCR analyses, the expression levels of these genes were compared in other adipose depots, between WT, bGH and GHR-/- mice and with HF and LF feeding. The phenotypes of the bGH and GHR -/- mice represent both extremes in GH

signaling with the bGH mice being lean, insulin resistant and short-lived while the GHR -/- mice are obese, insulin sensitive and long-lived (Berryman et al., 2004). The GHR -/- and the control mice are also more susceptible to diet-induced obesity with preferential fat deposition in the subcutaneous region while bGH mice are somewhat resistant to diet-induced obesity (Berryman et al., 2006). Because these genotypes have very different adiposities and respond differently to diet changes, alterations in gene expression of Adpn, Agt and Angptl4 in three distinct fat pads were studied when the bGH, GHR-/- and WT were fed either a LF or a HF diet.

## Adiponutrin (Adpn)

Adiponutrin (Adpn) is a nonsecreted adipocyte protein that was discovered in a preadipocyte cell line and was determined to be mainly expressed in adipose tissue (Baulande, Lasnier, Lucas, & Pairault, 2001). Adpn is a membrane protein, is sensitive to nutrient status, and appears to serve a role in membrane trafficking or vesicular transport of other secreted molecules (Baulande et al., 2001). In rodents, adiponutrin gene expression is regulated by changes in nutrition and energy balance and an increased expression of adiponutrin is observed in brown as well as white adipose tissues of obese Zucker (*fa/fa*) rats (Baulande et al., 2001). Further studies with this model have shown Adpn mRNA levels decrease upon fasting and rapidly increase with refeeding or feeding with a high-carbohydrate diet (Polson & Thompson, 2003). Another study by Polson and Thompson (2004) showed that this increase in the induction of Adpn mRNA in epididymal adipose tissue after a meal was not in response to the meal, but was determined by the macronutrient composition of the meal. That is, adiponutrin mRNA

levels were increased by both high-protein and high-sucrose diets but not by HF diets (Polson & Thompson, 2004). Bertile and Raclot (2004) showed that Adpn gene expression differentially decreased based on the duration of fasting. A 100% decrease in Adpn mRNA levels was seen in the epididymal and subcutaneous adipose of severely fasted rats and refeeding partially restored the Adpn mRNA levels in epididymal adipose only (Bertile and Raclot, 2004). Even though there are no studies examining Adpn in the adipose tissue of mice, all features of Adpn (based on rat studies) suggest a possible contribution of adiponutrin to energy homeostasis especially during periods of fasting and refeeding. This suggests that our HF and LF diet regimens may cause a difference in Adpn expression. To date, no studies have linked GH with Adpn mRNA or protein expression.

In the present study, the significant downregulation of Adpn in the subcutaneous adipose of the obese GHR -/- mice on a LF diet suggests that low-levels of adiponutrin expression may be unique to either an obese state or the unique, insulin sensitive obese state of GHR-/- mice. The literature does not support a downregulation of Adpn with obesity, at least in epididymal adipose tissue. That is, increased expression of this protein was reported in epididymal adipose of (*fa/fa*) obese Zucker rats (Baulande et al., 2001). This suggests that Adpn expression may be linked to insulin sensitivity. To that end, the same study also found that insulin alone appears to increase adiponutrin mRNA content in 3T3-L1 adipocytes (Baulande et al., 2001). This finding suggests that the insulin sensitivity of the GHR-/- mice might be more influential on Adpn than their obese state. The lack of any significant differences in the expression of Adpn in retroperitoneal or

epididymal adipose tissue of the three genotypes when fed a LF diet suggests that Adpn may contribute to the unique subcutaneous-specific enlargement observed in GHR-/- mice. With HF feeding, it is interesting that only Adpn expression in epididymal adipose was significantly different between the GHR-/- and the bGH mice. This suggests that while epididymal adipose Adpn expression is not influenced by short-term HF feeding (Polson & Thompson, 2004) prolonged exposure to HF diets increases Adpn expression, especially in the bGH mice.

**Angiotensinogen (Agt)**

Agiotensinogen (Agt) is a precursor of the protein angiotensin II, an important player in the renin-angiotensin system (RAS) of blood pressure regulation (Engeli et al., 1999). Adipose tissue is believed to be the second major source of Agt after the liver (Jonsson, Game, Head, & Frewin, 1994; Karlsson et al., 1998). Adipose-derived Agt has been suggested to be converted locally to angiotensin II and promotes adipocyte growth and differentiation as well as contributing to blood pressure regulation (Massiera et al., 2001). While it is known that GH can increase the circulating levels of liver Agt in blood (Karlsson et al., 1992), GH exposure did not cause any change in Agt expression levels in Ob1771 adipocyte cell lines (Aubert, Darimont, Safonova, Ailhaud, & Negrel, 1997). Huge variations are seen in the patterns of adipose Agt gene expression between different animal models of obesity. A study by Frederich, Kahn, Peach and Flier (1992) demonstrated that Agt mRNA was three-fold higher in the epididymal fat pad of genetically obese mice (ob/ob and db/db) than in lean controls. In contrast, another study found a significant decrease in adipose Agt gene expression in abdominal adipose tissue

of obese Zucker rats (Jones, Standridge, Taylor, & Moustaid, 1997). The same study also found that Agt gene expression was significantly lower in the abdominal adipose tissue of obese agouti yellow mice (Jones et al., 1997). Of note, this study did not clearly define what depot was studied and considered to be abdominal. A more recent study by Hainault et al. (2002) showed that the production of Agt by inguinal and retroperitoneal adipose tissue increases more dramatically with age in obese Zucker rats compared with lean controls. Rahmouni, Mark, Haynes and Sigmund (2004) showed that adipose Agt expression is increased in the visceral adipose but not the subcutaneous adipose of HF fed transgenic mice expressing the human angiotensinogen gene (hAGT). While there are diet associated differences in Agt expression, Bertile and Raclot (2004) showed that subcutaneous and epididymal Agt mRNA expression is not influenced by fasting or refeeding in these animal models. Therefore, it is clear that there are variations in adipose Agt expression between subcutaneous and intra-abdominal adipose depots under different dietary conditions. Apart from the study by Aubert et al. (1997) on the influence of GH on Agt expression in adipocyte cells, there have been no other studies addressing the effect of GH on adipose Agt expression.

The depot-specific differences reported by the above mentioned studies were also confirmed in the present study. That is, Agt expression was increased only in the subcutaneous depot of GHR -/- mice and not in the other depots or in the bGH and WT mice when fed a LF diet. However, this result contradicts other studies performed using human adipose samples that have reported a higher Agt gene expression in visceral adipose compared with subcutaneous adipose (Dusserre et al., 2000; van Harmelen et al.,

2000). While no specific explanation exists for the regional differences in adipose Agt expression, the anatomic and biochemical differences inherent to subcutaneous and visceral adipose tissues could contribute to the depot-specific expression of Agt. Interestingly, Agt epididymal adipose expression was significantly increased in the GHR-/- mice compared to control mice when fed a HF diet, but not in the LF diet. This is similar to a study by Rahmouni et al. (2004) that showed an increased adipose Agt expression only in the visceral adipose depots (perirenal, epididymal and parametrial adipose) of transgenic hAGT mice fed a HF diet in the C57BL/6J background, the same genetic background strain used in this study. The increased Agt expression in the subcutaneous adipose tissue of LF GHR-/- mice and in epididymal adipose of HF GHR -/- mice, could be contributing to the adipocyte growth and differentiation function of adipose Agt (Massiera et al., 2001) and to the obese phenotype that is characteristic of the GHR-/- mice. Further studies that specifically correlate the altered mRNA levels with the protein levels of adipose Agt would help to uncover the role of adipose Agt in adipose tissue growth and differentiation.

**Angiopoietin-like 4 (Angptl4)**

Angiopoietin-like 4 (Angptl4) is a secreted protein involved in the inhibition of the enzyme lipoprotein lipase (LPL) and is believed to be induced by fasting (Kersten et al., 2000) and during 3T3-L1 preadipocyte differentiation (Yoon et al., 2000). The gene is expressed predominantly in adipose tissue, liver and placenta, and is believed to be suppressed by leptin (Yoon et al., 2000). Angptl4 is also known as hepatic fibrinogen/angiopoietin-related protein (HFARP), fasting induced adipose factor (FIAF),

and peroxisome proliferator-activated receptor (PPAR γ-angiopoietin-related (PGAR). Angptl4 is also one target gene of the nuclear receptors PPARα and PPARγ, which govern lipid metabolism in white adipose tissue (Yoon et al., 2000). A study by Mandard et al. (2006) showed that in adipose tissue Angptl4 stimulates lipolysis, resulting in elevated plasma free fatty acids and glycerol levels, and may promote fatty acid oxidative metabolism and uncoupling. Overall, this increased lipolysis and fatty acid oxidation resulted in diminished fat stores (Mandard et al., 2006). To date, no studies have linked GH with alterations in Angptl4 expression.

The GHR -/- mice used in this study are characterized by their increased fat stores while their bGH counterparts are lean (Berryman et al., 2004). This also translates into decreased lipolysis in the GHR-/- and increased lipolysis in the bGH mice. Yet, the present study shows that with LF feeding, lipolytic Angptl4 gene expression is higher in the subcutaneous adipose of GHR -/- as compared to WT mice. In addition, Angptl4 expression was significantly decreased in the subcutaneous adipose of bGH mice compared to the GHR -/- mice on a LF diet. The combined results seen with the GHR-/- mice and the bGH mice suggests a possibly unique role played by GH in Angptl4 expression. This decreased expression of Angptl4 was also seen in the retroperitoneal depot of HF bGH mice compared to the HF GHR -/- and HF WT mice. This contrasts with the study by Kersten et al. (2000) on PPARα null mice which showed that while the abundance of Angptl4 in plasma decreased on a HF diet, there were no corresponding changes in the mRNA expression levels in white adipose tissue. However, this same study does not mention the specific adipose depot that contributed to the white adipose

tissue samples. The retroperitoneal adipose depot seems to be more active than the other depots for the expression of Angptl4 also because there is significantly increased expression of HF GHR-/- compared to the LF GHR-/- . Because the lipolytic activity of GH is suppressed in the GHR-/- mice, the increased expression of Angptl4 could be a possible contributor to basal lipolysis in this model.

## Future Studies

Microarray analysis and subsequent confirmation using RT-RT PCR analysis provided very valuable information regarding the alterations in gene expression caused by GH. While the altered mRNA expression levels of Adpn, Agt and Agptl4 were confirmed by RT-RT PCR, the mRNA expression levels of Scd2 and Insig1 seen in the microarrays were not confirmed by RT-RT PCR analysis. The hypothesis that extremes in GH signaling would cause opposite expression patterns for Adpn, Agt and Angptl4 was not completely supported by the data presented in this thesis. Although the subcutaneous adipose expressions of Agt and Angptl4 increased while that of Adpn decreased in the absence of GH signaling, the opposite genotype (bGH mice) did not usually result in an opposite effect on expression. While the trend between the GHR -/- and the bGH mice for all the three genes was opposite in all depots, differences were not statistically significant.

There are inherent limitations to the methods used in this thesis. Microarray data may not be accurate for each transcript due to variations in DNA lengths used for the microarray as well as possible error in sample preparation. Errors in sample preparation are a drawback for RT-RT PCR as well. While the quality of the cDNA generated from

the RNA samples was checked before the microarrays, the quality of the cDNA was not checked prior to use with RT-RT PCR. However, the quality of the RNA used for RT-RT PCR was checked using a spectrophotometer prior to cDNA synthesis. Errors in RT-RT PCR can also be created if primer-dimers are accidentally formed during the reaction. However, microarrays and their confirmation using RT-RT PCRs have still continued to provide valuable information regarding altered gene expression in the mouse genome. Furthermore, it would be very useful to perform microarray analysis of the epididymal and retroperitoneal adipose depots to identify common gene expression patterns in all three adipose depots.

Future studies with larger sample sizes would be necessary to draw conclusive evidence of altered gene expression. While this present study focused on five genes out of the 159 altered genes, it would be helpful to confirm the altered gene expressions of all remaining genes that were significant in the microarray analysis. Unfortunately, RT-RT PCR can be relatively expensive making analysis of all altered genes impractical. Notably, RT-RT PCR did not always confirm altered gene expression, as seen with Insig1 and Scd2. Northern blots could serve as the alternate method to identify such genes yet this method is problematic for genes with very low expression levels. Still, this method could offer another means to confirm microarray data.

Altered mRNA expression profiles based on microarrays and RT-RT PCR cannot provide conclusive evidence of functional alterations in gene expression. Hence, future studies examining the protein expression levels of Adpn, Agt and Angptl4 using Western blots is warranted. Because the mouse models have altered metabolism, cell culture

studies using 3T3-L1 adipocytes would help determine whether the differences observed are due to GH or due to another metabolic change in these animals.

## Conclusions

To summarize, a global survey of gene expression alterations in the subcutaneous adipose of GHR-/- mice compared to littermate controls using microarrays yielded a total of 159 gene alterations. Of the 159 genes with altered gene expression, 87 genes were upregulated and 72 genes were downregulated in the GHR-/- mice. Five genes, Adpn, Agt, Angptl4, Insig1 and Scd2, were studied by RT-RT PCR to confirm microarray results. Agt and Angptl4 were upregulated, Adpn was downregulated in both microarray and RT-RT PCR while RT-RT PCR did not confirm the downregulation of Insig1 and Scd2. For genes with gene expression levels confirmed by both microarray and RT-RT PCR, the altered gene expression profiles of Adpn, Agt and Angptl4 in subcutaneous, epididymal and retroperitoneal adipose of GHR-/- and bGH mice exposed to LF and HF diets were studied.

Adpn gene expression profiles were: (a) decreased in the subcutaneous adipose of LF GHR-/- mice compared to LF WT mice, (b) increased in subcutaneous adipose of LF-WT compared to HF-WT and LF bGH compared to HF bGH mice, and (c) increased in epididymal adipose of HF bGH mice compared to HF GHR-/- mice.

Agt gene expression profiles were: (a) increased in subcutaneous adipose of LF GHR-/- mice compared to LF WT mice and in LF GHR-/- mice compared to the LF bGH mice, (b) increased in epididymal adipose of HF GHR-/- mice compared to HF WT mice, and (c) decreased in epididymal adipose of HF WT mice compared to LF WT mice.

Angptl4 gene expression was: (a) increased in subcutaneous adipose of LF GHR-/- mice compared to LF WT and LF bGH mice, (b) increased in retroperitoneal adipose of HF GHR-/- compared to HF bGH mice, (c) decreased in retroperitoneal adipose of HF bGH mice compared to HF WT mice, and (d) increased in retroperitoneal adipose of HF WT and HF GHR-/- compared to LF WT and LF GHR-/- mice, respectively.

Further studies need to explore protein expression levels, confirm expression of other genes shown to be altered by microarray, and evaluate expression changes by microarray of other adipose depots.

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# APPENDIX A: MICROARRAY ANALYSIS PROTOCOL, AS PROVIDED BY MOGENE, INC.

Total RNA was amplified using Agilent low input linear amplification kit according to the process outlined by the manufacturer (Agilent Technologies). 1-5 ug of amplified target cRNA was labeled with either cy5 or cy3 using Micromax kit from Perkin Elmer. The labeled material was passed through zymo RNA Clean-up Kit-5 columns from Zymo Research Corporation, CA, to remove any un-incorporated label and eluted in 15-20 ul of RNAse-free water purchased from Ambion. Concentration of labeled cRNA and the label incorporation was determined by Nanodrop-1000 spectrophotometer. All of the labeling and post labeling procedures were conducted in ozone-free enclosure to ensure the integrity of the label. Labeled material were setup for fragmentation reaction according to Agilent protocol described in their processing manual and hybridized overnight in the rotating oven at 60 C in an ozone-free room. Wash conditions used were as outlined in the Agilent processing manual and the arrays were scanned using Agilent scanner. Agilents feature extraction software 7.5 version was used for extracting array data. Further analysis was done using Rosetta luminator software.

**ANOVA calculations of the microarray data, as provided by Mogene, Inc.**

The ANOVA calculations follow those outlined in by Wolfinger, et al. (2001). It consists of two steps: Normalization model and gene model. In the normalization model, log intensity is considered to be linearly related to dye, array, and dye*array. Dye is treated as fixed effect while array and dye*array random effects. In the gene model, residues of log (intensity) from the normalization model is considered to be linearly related to array, spot (replicated probes on the array), spot*array, and sample, where the first three factors are treated as random effects while the sample fixed effect. Variance components for the random effects are estimated using the method of restricted maximum likelihood, while the parameters of fixed effect are estimated using least square method.

**Pathway analysis, as provided by Mogene, Inc.**

Pathway information (which pathways involve which genes) is downloaded from the Kyoto Encyclopedia of Genes and Genomes (KEGG) website (ftp://ftp.genome.ad.jp/pub/kegg/pathways/). Given a profile of gene expression levels from microarray experiments, the hypothesis that the expression levels of the genes involved in a particular pathway can be obtained by randomly assigning expression levels to each gene from the expression levels available in the whole profile is tested. This is

equivalent to test that the distribution of the expression levels of genes in the particular pathway follows the same distribution of the expression levels of all the genes in the whole profile. Kolmogorov-Smirnov (two-sample) test is used to calculate the p-values.

# APPENDIX B: REAL-TIME REVERSE TRANSCRIPTASE PCR

Real-Time reverse transcriptase PCR (RT-RT PCR) is widely used to perform gene expression analysis by employing primers specific to the gene of interest. RT-RT PCR is a modification of another gene amplification method namely, the Polymerase chain reaction (PCR). This PCR amplifies short DNA sequences that are found within the chosen forward and reverse primers, using the enzyme polymerase. This process is repeated multiple times and in RT-RT PCR, the level of fluorescence is monitored throughout the reaction.

The cycle threshold (Ct) value obtained in RT-RT PCR are compared among samples to determine the expression levels of the DNA sequence that was amplified by the specific gene primers. Ct is the specific cycle number during the early exponential phase of PCR when the amount of fluorescence has reached a certain threshold where it is significantly higher/more intense than background levels. A lower Ct indicates a faster increase in the fluorescent signal and thus implies a higher quantity of the product of interest expressed in the tissue.

## APPENDIX C: TABLE OF UPREGULATED GENES IN MICROARRAY ANALYSIS

| Gene Name | Description | Fold Change in the GHR-/- | Significance (Probt) |
|---|---|---|---|
| Perp | TP53 apoptosis effector | 15.5 | 9.51E-03 |
| AW 125753 | Expressed sequence | 12.0 | 8.92E-03 |
| Wisp2 | WNT1 inducible signaling pathway protein2 | 5.6 | 0.01 |
| Acta1 | Actin, alpha 1, skeletal muscle | 4.8 | 0.02 |
| 0610041G09Rik | | 4.7 | 7.21E-03 |
| Edn 1 | Endothelin | 4.4 | 0.05 |
| Agt | Angiotensinogen | 3.8 | 0.01 |
| Angptl4 | Angiopoietin-like 4 | 3.7 | 1.14E-04 |
| Aldh1a1 | Aldehyde dehydrogenase family 1 subfamily A1 | 3.6 | 1.29E-03 |
| Unc93a | Unc-93 homolog A (C.elegans) | 3.6 | 0.02 |
| Chst1 | Carbohydrate (keratin sulfate Gal-6) sulfotransferase 1 | 3.6 | 7.94E-04 |

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|---|---|---|---|
| 2310020A2 | RIKEN cDNA 2310020121 gene | 3.6 | 0.05 |
| Slc7a8 | Solute carrier family 7 (cationic amino acid transporter system), member 8 | 3.4 | 3.22E-03 |
| B430320C24Rik | | 3.2 | 0.02 |
| Gdpd1 | Glycerophosphodiester phosphodiesterase | 3.2 | 0.01 |
| Hpgd | Mus muscuclus hydroxyprostaglandin dehydrogenase 15 (NAD) | 3.2 | 4.22E-05 |
| Cys1 | Mus musculus cystin 1 (Cys 1) | 3.1 | 0.01 |
| Alb1 | Albumin 1 | 3.0 | 7.64E-03 |
| Tcfcp2l2 | Transcription factor CP2-like 2 | 2.9 | 0.02 |
| AK042648 | | 2.9 | 0.05 |
| Emp2 | Epithelial membrane protein 2 | 2.9 | 0.02 |
| 1700110N18Rik | | 2.8 | 1.13E-03 |
| 1190005106Rik | | 2.8 | 1.25E-03 |

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|---|---|---|---|
| Maoa | Monoamine oxidase A | 2.8 | 5.93E-03 |
| Palmd | Palmdelphin | 2.8 | 9.23E-03 |
| Btc | Mus musculus betacellulin, epidermal growth factor family member (Btc) | 2.7 | 0.01 |
| Lgals6 | Mus musculus lectin, galactose binding soluble 6 | 2.7 | 3.92E-05 |
| Mt2 | Metallothionein 2 | 2.7 | 1.22E-03 |
| Thbs2 | Mus musculus thrombospondin 2 | 2.7 | 7.90E-03 |
| Acta2 | Actin, alpha 2, amooth muscle,aorta | 2.7 | 5.90E-04 |
| Anxa1 | Mus musculus annexin A1 | 2.7 | 0.01 |
| BC027232 | | 2.6 | 2.22E-03 |
| Dstn | Mus musculus destrin | 2.6 | 4.06E-03 |
| LOC434314 | Hpothetical gene supported by AK088091;AK088706 | 2.6 | 0.01 |
| Mlstd2 | Male sterility domain containing 2 | 2.6 | 7.83E-03 |
| Ndrg1 | Mus musculus N-myc downstream regulated gene 1 | 2.5 | 3.87E-03 |

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|---|---|---|---|
| 2310067E08Rik | | 2.5 | 0.02 |
| Anxa3 | Mus musculus annexin A3 | 2.5 | 0.01 |
| Gpr64 | Mus musculus G protein coupled receptor | 2.5 | 4.11E-03 |
| Mmp11 | Mus musculus matrix metalloproteinase 11 | 2.5 | 1.30E-03 |
| Phf16 | Mus musculus PHD finger protein 16 | 2.5 | 0.01 |
| Crip2 | Mus musculus cysteine rich protein 2 | 2.4 | 2.73E-03 |
| E030003E18Rik | | 2.4 | 3.47E-03 |
| Fbxo31 | F-box only protein 31 | 2.4 | 6.68E-04 |
| Myrip | Mus musculus myosin VIIA and Rab interacting protein | 2.4 | 4.02E-03 |
| Nnat | Neuronatin | 2.4 | 0.04 |
| Hmgcr | 3-hydroxy-3-methylglutaryl-Coenzyme A reductase | 2.4 | 0.02 |
| Prg4 | Proteoglycan 4(megakaryocyte stimulating factor, articular | 2.4 | 5.66E-03 |

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|---|---|---|---|
| | superficial zone protein) | | |
| Rbp7 | Retinol binding protein 7, cellular | 2.4 | 7.54E-04 |
| Kcns3 | Potassium voltage gated channel, delayed rectifier, subfamily S, member 3 | 2.3 | 1.78E-03 |
| Txnip | Thioredoxin interacting protein | 2.3 | 2.51E-05 |
| Efemp1 | Epidermal growth factor-containing fibulin like extracellular matrix protein 1 | 2.3 | 0.01 |
| Tmeff1 | Mus musculus transmembrane protein with EGF like and two follistatin like domains 1 | 2.3 | 8.78E-07 |
| 4632428N05Rik | | 2.3 | 2.64E-03 |
| Inmt | Indoethylamine N-methyltransferase | 2.3 | 3.32E-03 |
| Lox | Mus musculus lysyl oxidase (Lox) | 2.3 | 0.02 |
| E430002G05Rik | | 2.3 | 2.02E-04 |

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|---|---|---|---|
| Cd1d2 | CD1d2 antigen | 2.2 | 5.90E-03 |
| Csrp1 | Cysteine and glycine rich protein 1 | 2.2 | 6.87E-03 |
| Cyp4v3 | Cytochrome P450, family 4, subfamily V, ploypeptide 3 | 2.2 | 7.39E-03 |
| Emb | Mus musculus embigin | 2.2 | 0.05 |
| Sbsn | Mus musculus suprabasin | 2.2 | 7.48E-03 |
| Tgfb3 | Mus musculus transforming growth factor beta 3 | 2.2 | 6.61E-03 |
| 1110003O08Rik | | 2.1 | 4.74E-03 |
| Grm1 | Metabotropic glutamate receptor 1 | 2.1 | 0.05 |
| Lgals4 | Mus musculus lectin, galactose binding soluble 4 | 2.1 | 5.15E-08 |
| 1110007F12Rik | | 2.1 | 2.94E-03 |
| Chst11 | Mus musculus carbohydrate sulfotransferase 11 | 2.1 | 0.05 |
| Serpine1 | Mus musculus serine proteinase inhibitor, clade E, member 1 | 2.1 | 0.01 |
| Sh3yl1 | Mus musculus Sh3 domain | 2.1 | 0.04 |

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|---|---|---|---|
| | YSC-like 1 | | |
| Kctd15 | Potassium channel tetramerization domain containing 15 | 2.1 | 5.25E-04 |
| Sgpl1 | Mus musculus sphingosine phosphate lysate 1 | 2.1 | 2.88E-03 |
| TC1248463 | | 2.1 | 0.02 |
| Tmeff2 | Mus musculus transmembrane protein with EGF like and two follistatin like domains 2 | 2.1 | 1.98E-08 |
| AB023957 | | 2.0 | 0.01 |
| Slc40a1 | Mus musculus solute carrier family 40, member 1 | 2.0 | 7.78E-03 |
| Selenbp1 | Mus musculus selenium binding protein 1 | 2.0 | 7.11E-04 |
| Cd1d1 | Mus musculus CD1d1 antigen | 2.0 | 4.26E-04 |
| Ctdspl | Domain, RNA polymerase II, polypeptide A) small phosphatase like | 2.0 | 3.69E-06 |
| Fzd1 | Mus musculus frizzled | 2.0 | 3.28E-03 |

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|---|---|---|---|
| | homolog 1 | | |
| Galntl2 | UDP-Nacetyl-alpha-D-galactosaminyltransferase-like 2 | 2.0 | 5.12E-03 |
| Tpm4 | Mus musculus tropomyosin 4 | 2.0 | 0.01 |
| 5430416O09Rik | | 1.9 | 5.65E-03 |
| Cyp4b1 | Mus musculus cytochrome P450, family 4, subfamily b, polypeptide 1 | 1.9 | 0.01 |
| Osbpl11 | Oxysterol binding protein like 11 | 1.9 | 1.77E-03 |
| 2600010E01Rik | | 1.9 | 5.95E-4 |
| 6430556C10Rik | | 1.9 | 2.53E-03 |
| Hoxd8 | Homeo box D8 | 1.9 | 0.01 |
| 2310075E07Rik | | 1.9 | 0.01 |
| Crtac1 | Cartilage acidic protein 1 | 1.9 | 4.73E-03 |
| Gabarapl1 | Mus musculus gamma-aminobutyric acid receptor associated protein like 1 | 1.9 | 7.25E-06 |
| Myo1c | Myosin IC | 1.9 | 6.30E-03 |
| Anxa8 | Mus musculus annexin A8 | 1.9 | 4.64E-05 |

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|---|---|---|---|
| D430015B01Rik | | 1.9 | 4.52e-08 |
| Map3k6 | Mus musculus mitogen activated protein kinase kinase kinase 6 | 1.9 | 5.41E-05 |
| Npr3 | Mus musculus natriuretic peptide receptor 3 | 1.9 | 3.23E-03 |

## APPENDIX D: TABLE OF DOWNREGULATED GENES IN MICROARRAY ANALYSIS

| Gene Name | Description | Fold Change in GHR-/- | Significance (Probt) |
|---|---|---|---|
| Slc2a5 | Solute carrier family 2 (facilitated glucose transporter), member 5, | 7.4 | 3.39E-04 |
| Ucp1 | Uncoupling protein 1, mitochondrial, | 6.4 | 1.42E-03 |
| Elovl6 | ELOVL family member 6, elongation of long chain fatty acids (yeast), | 5.7 | 1.28E-03 |
| Ghr | Growth hormone receptor, | 5.2 | 6.03E-16 |
| Scd2 | Stearoyl-coenzyme A desturase 2, | 5.0 | 1.22E-03 |
| Thrsp | Thyroid hormone sensitive SPOT14 homolog (rattus) | 4.4 | 9.19E-04 |
| ENSMUST00000081396 | | 4.2 | 1.53E-03 |
| Cox7a1 | Cytochrome C oxidase, subunit VIIa 1 | 4.2 | 7.31E-03 |

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|---|---|---|---|
| Adpn | Adiponutrin | 4.2 | 6.45E-03 |
| Cox8b | Cytochrome C oxidase, subunit VIIIb | 4.2 | 6.27E-03 |
| Gys2 | Glycogen synthase 2 | 4.0 | 1.11E-03 |
| Mal2 | Mal, T-cell differentiation protein 2 | 3.7 | 2.31E-03 |
| Cspg3 | Mus musculus chondroitin sulfate proteoglycan 3 | 3.7 | 1.05E-04 |
| NAP102507-1 | | 3.6 | 2.61E-03 |
| Thea | Thioesterase, adipose associated | 3.6 | 0.01 |
| NAP112201-1 | | 3.6 | 1.17E-03 |
| Sult1e1 | Mus musculus sulfotransferase family 1E member | 3.6 | 1.37E-03 |
| Mod1 | Mus musculus malic enzyme, supernatant | 3.3 | 1.18E-06 |
| BC024408 | | 3.3 | 3.03E-06 |
| 9130213B05Rik | | 3.3 | 2.02E-07 |
| Ppp1r3b | Protein phosphatase 1, regulatory (inhibitor) subunit 3B | 3.3 | 5.45E-04 |

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|---|---|---|---|
| 2010003K11Rik | | 3.2 | 2.00E-03 |
| AK053478 | | 3.2 | 9.16E-04 |
| Insig1 | Mus musculus insulin induced gene 1 | 3.2 | 8.69E-06 |
| Lactb2 | Lactamase, beta 2 | 3.1 | 9.27E-05 |
| 4933404O19Rik | | 3.1 | 4.17E-03 |
| Mup4 | Mus musculus major urinary protein 4 | 3.1 | 1.29E-03 |
| Mup1 | Mus musculus major urinary protein 1 | 3.0 | 3.69E-06 |
| Mid1ip1 | Mid1 interacting protein 1 (gastrulation specific G-12-like (zebra fish)) | 3.0 | 1.20E-03 |
| Agpat2 | O-acyltransferase 2 (lysophosphatidic acid acyltransferase, beta) | 3.0 | 1.46E-05 |
| Slc25a1 | Mus musculus solute carrier family 25 (mitochondrial carrier, citrate transporter), member 1 | 2.9 | 2.66E-03 |
| Pygl | Mus musculus liver glycogen phosphorylase | 2.9 | 1.35E-06 |

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|---|---|---|---|
| Slc9a2 | Solute carrier family 9, member 2 | 2.8 | 7.00E-03 |
| Tinag | Mus Musculus tubulointerstitial nephritis antigen | 2.8 | 2.75E-03 |
| Acly | ATP citrate lysate | 2.8 | 1.23E-03 |
| Ppp2r5b | Protein phosphatase 2, regulatory subunit B, beta isoform | 2.7 | 7.23E-04 |
| Pdhb | Mus Musculus pyruvate deydrogenase (lipomide) beta | 2.7 | 2.23E-07 |
| Chsy1 | Carbohydrate synthase 1 | 2.7 | 2.51E-04 |
| Elovl3 | Mus Musculus elongation of very long chain fatty acids like 3 | 2.7 | 0.04 |
| Lctl | Lactase-like | 2.7 | 1.16E-03 |
| Gpd2 | Glycerol phosphate dehydrogenase 2, mitochondrial | 2.7 | 3.60E-06 |
| Cidea | Mus Musculus cell death inducing DNA fragmentation factor, alpha subunit-like | 2.7 | 0.01 |

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|---|---|---|---|
| | effector A | | |
| Aldh1a7 | Aldehyde dehydrogenase family 1, subfamily A7 | 2.7 | 6.18E-05 |
| AA674270 | | 2.7 | 7.49E-03 |
| Slc25a10 | Solute carrier family 25 (mitochondrial carrier, dicarboxylase transporter), member 10 | 2.7 | 2.80E-03 |
| Paqr9 | Progestin and adipoQ receptor family IX | 2.7 | 8.88E-04 |
| Ptges | Mus musculus prostaglandin E synthase | 2.6 | 3.49E-04 |
| Acas2 | Mus musculus acetyl-Coenzyme A synthetase 2 | 2.6 | 6.13E-06 |
| Fabp3 | Mus musculus fatty acid binding protein 3, muscle and heart | 2.6 | 0.03 |
| AK046951 | | 2.6 | 7.04E-04 |
| Aacs | Acetoacetyl-CoA synthetase | 2.6 | 4.72E-03 |
| A930016D02Rik | | 2.6 | 3.31E-03 |
| Gale | Galactose 4 epimerase, UDP | 2.5 | 5.59E-03 |
| 6330414G02Rik | | 2.5 | 1.54E-03 |

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|---|---|---|---|
| TC1323472 | | 2.5 | 1.42E-04 |
| 1700020C11Rik | | 2.5 | 6.27E-03 |
| Tst | Mus musculus thiosulfate sulfurtransferase | 2.5 | 5.96E-03 |
| Igf1 | Insulin like growth factor-1 | 2.5 | 1.24E-08 |
| AK046777 | | 2.5 | 6.83E-03 |
| Glycam1 | Mus musculus glycosylation dependent cell adhesion molecule 1 | 2.4 | 5.50E-03 |
| Otop1 | Otopetrin 1 | 2.4 | 1.91E-03 |
| Gbe1 | Mus musculus glucan (1,4-alpha-), branching enzyme 1 | 2.4 | 1.59E-03 |
| Syt12 | Mus musculus synaptotagmin 12 | 2.3 | 4.88E-03 |
| Ihj | | 2.3 | 6.32E-06 |
| 5830411E10Rik | | 2.3 | 4.93E-06 |
| 9130214F15Rik | | 2.3 | 1.39E-03 |
| Tnfrsf13c | Mus musculus tumor necrosis factor receptor superfamily, member 13c | 2.3 | 4.38E-03 |
| Kcnj16 | Mus musculus kcnj16 mRNA for inwardly rectifying | 2.3 | 4.77E-03 |

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|---|---|---|---|
| | potassium channel Kir5.1, partial cds | | |
| BB219290 | | 2.3 | 1.90E-03 |
| Nsdhl | Mus musculus NAD(P) dependent steroid dehydrogenase-like | 2.2 | 1.53E-07 |
| Dct | Mus musculus dopachrome tautomerase | 2.2 | 2.20E-04 |
| Cycs | Mus musculus cytochrome c, somatic | 2.2 | 8.01E-09 |
| Acac | | 2.2 | 8.29E-06 |
| Trfr2 | Mus musculus transferring receptor 2 | 2.2 | 5.95E-04 |
| Rgs7 | Mus musculus regulator of G protein signaling 7 | 2.2 | 9.98E-04 |
| 9630013D21Rik | | 2.2 | 4.88E-04 |
| Ptger3 | Mus musculus prostaglandin E receptor 3 | 2.2 | 6.99E-05 |
| Cpt1b | Mus musculus carnitine palmitoyltransferase 1b, muscle | 2.2 | 5.13E-04 |
| Tekt1 | Mus musculus tektin1 | 2.1 | 2.60E-03 |

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|---|---|---|---|
| Mmp3 | Mus musculus matrix metalloproteinase 3 | 2.1 | 5.27E-04 |
| D130058121Rik | | 2.1 | 3.09E-03 |
| Cd79b | Mus musculus CD79B antigen | 2.1 | 2.86E-03 |
| Bhlhb2 | Mus musculus basic helix-loop-helix domain containing, class B2 | 2.1 | 6.56E-03 |
| 2610205H19Rik | | 2.1 | 3.66E-03 |
| H2-Q8 | Mus musculus histocompatibility 2, Q region locus 8 | 2.1 | 4.45E-04 |
| Fasn | Fatty acid synthase | 2.1 | 2.32E-03 |
| V00827 | | 2.0 | 6.02E-05 |
| Slc2a4 | Mus musculus solute carrier family 2, member 4 | 2.0 | 3.48E-03 |
| Pmvk | Mus musculus phosphomevalonate kinase | 2.0 | 8.60E-06 |
| Tkt | Mus musculus transketolase like 1 | 2.0 | 8.38E-05 |
| Igh-6 | Immunoglobin heavy chain 6 | 2.0 | 1.62E-03 |
| BB212617 | | 2.0 | 2.03E-03 |

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|---|---|---|---|
| 2010309G21Rik | | 2.0 | 7.48E-03 |
| Pkm2 | Mus musculus pyruvate kinase, muscle | 2.0 | 1.83E-05 |
| Marco | Mus musculus macrophage receptor with collagenous structure | 2.0 | 1.59E-04 |
| ENSMUST00000077170 | | 2.0 | 4.40E-04 |
| AK041039 | | 2.0 | 1.03E-03 |
| AK035497 | | 2.0 | 8.47E-03 |
| Acp5 | Mus musculus acid phosphatase 5, tartrate resistant | 2.0 | 7.16E-04 |
| A530026G17 | | 2.0 | 6.22E-03 |
| Timd4 | T-cell immunoglobin and mucin domain containing 4 | 2.0 | 4.48E-04 |
| Srebf1 | Sterol regulatory element binding factor 1 | 2.0 | 0.01 |
| Ms4a1 | Mus musculus membrane-spanning 4-domains, subfamily A, member 1 | 2.0 | 6.90E-07 |
| Cxcl13 | Mus musculus chemokine ligand 13 | 2.0 | 1.93E-03 |

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|---|---|---|---|
| Blr1 | Burkitt lymphoma recepot 1 | 2.0 | 8.89E-03 |
| AI505012 | | 2.0 | 4.16E-03 |
| 1810037B05Rik | | 2.0 | 3.47E-03 |
| LOC243420 | | 1.9 | 0.02 |
| Dlat | Dihdrolipoamide S acetyltransferase (E2 component of pyruvate dehydrogenase complex) | 1.9 | 7.19E-06 |
| BC034068 | | 1.9 | 7.17E-03 |
| Alas1 | Aminolevulinic acid synthase 1 | 1.9 | 3.28E-07 |
| 3010033K07Rik | | 1.9 | 0.02 |
| 1110025G12Rik | | 1.9 | 6.70E-03 |
| Ndufab1 | Mus musculus NADH dehydrogenase 1, alpha/beta subcomplex, 1 | 1.9 | 2.64E-07 |
| TC1278514 | | 1.9 | 2.44E-03 |
| Serpinh1 | Mus musculus serine (or cysteine) protinase inhibitor, clade H, member 1 | 1.9 | 9.98E-04 |