RNA Expression of Receptors for Growth Hormone, Insulin-like Growth Factor 1, and Insulin in Mouse Whole Adipose Tissue, Stromal Vascular Fraction, and Adipocytes

A thesis presented to
the faculty of
the College of Arts and Sciences of Ohio University

In partial fulfillment
of the requirements for the degree
Master of Science

Vivian A. Lesende
December 2015

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This thesis titled
RNA Expression of Receptors for Growth Hormone, Insulin-like Growth Factor 1, and
Insulin in Mouse Whole Adipose Tissue, Stromal Vascular Fraction, and Adipocytes

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ABSTRACT

LESENDE, VIVIAN A., M.S., December 2015, Biological Sciences

RNA Expression of Receptors for Growth Hormone, Insulin-like Growth Factor 1, and Insulin in Mouse Whole Adipose Tissue, Stromal Vascular Fraction, and Adipocytes

Director of Thesis: Darlene E. Berryman

Increasing rates of obesity and associated complications worldwide have increased research interest in the complex interplay of hormones, metabolism, and adiposity. There is much evidence indicating that hormones such as growth hormone (GH), insulin-like growth factor 1 (IGF-1), and insulin affect white adipose tissue (WAT) in a depot-specific manner. This suggests that hormone-induced intracellular signaling is more active in some WAT depots compared to others, as evidenced by hormone receptor expression levels. To add another layer of complexity, WAT is made up of many cell types with distinct functions, including adipocytes and cells of the stromal vascular fraction (SVF), which can differentially express these receptors. The purpose of this thesis is to further develop our understanding of how GH-, IGF-1-, and insulin-induced signaling affect adiposity in a WAT depot-specific manner. The original aims of this thesis were to: 1) use quantitative polymerase chain reaction (qPCR) to compare the expression of the receptors of the above hormones (GH, IGF-1, and insulin) in the SVF and adipocyte portions of the most commonly studied WAT depots in wildtype (WT) mice (subcutaneous, epididymal, retroperitoneal, and mesenteric), and 2) compare the expression of these receptors in the WAT depots of growth hormone antagonist (GHA) and WT mice. Unfortunately, due to low adipocyte RNA concentrations and purity, the first aim was adjusted to examine receptor RNA expression in whole WAT (as opposed...
to isolated adipocytes) and SVF. No significant differences in receptor expression were observed in the first aim, but a trend toward greater insulin receptor (IR) RNA expression in the SVF of the epididymal depot compared to the SVF of the mesenteric depot was seen. The main significant finding of the second aim was that growth hormone receptor (GHR) was more strongly expressed in GHA mice than in WT mice in the mesenteric depot, a result that could be explained by differences in SVF cell populations in GHA mesenteric WAT. Thus, future studies should focus on examining differences in WAT immune cell populations in the two genotypes, as well as optimizing the process of RNA isolation from adipocytes to allow for a more clear comparison of the two portions.
DEDICATION

To my mother and Chase Alexander
ACKNOWLEDGMENTS

Thank you to my advisor, Dr. Darlene E. Berryman, as well as my committee members, Dr. John Kopchick, Dr. Shigeru (Nick) Okada, and Dr. Karen Coschigano for all the guidance I have received in completing this project. Also, the assistance I have received from all the members of the Kopchick lab has been invaluable.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Abstract</td>
<td>3</td>
</tr>
<tr>
<td>Acknowledgments</td>
<td>6</td>
</tr>
<tr>
<td>List of Tables</td>
<td>9</td>
</tr>
<tr>
<td>List of Figures</td>
<td>10</td>
</tr>
<tr>
<td>Chapter 1: Introduction</td>
<td>12</td>
</tr>
<tr>
<td>1.1: Metabolic functions of WAT</td>
<td>12</td>
</tr>
<tr>
<td>1.2: Mouse and human WAT depots</td>
<td>15</td>
</tr>
<tr>
<td>1.2.1: Depot locations and classifications</td>
<td>15</td>
</tr>
<tr>
<td>1.2.2: Depot-specific differences</td>
<td>19</td>
</tr>
<tr>
<td>1.3: WAT cell types</td>
<td>22</td>
</tr>
<tr>
<td>1.4: Comparison of adipocytes and SVF</td>
<td>25</td>
</tr>
<tr>
<td>1.5: Growth Hormone Receptor</td>
<td>27</td>
</tr>
<tr>
<td>1.5.2: GHR-induced signaling</td>
<td>28</td>
</tr>
<tr>
<td>1.5.3: GHR expression in WAT</td>
<td>29</td>
</tr>
<tr>
<td>1.6: IGF-1R and IR</td>
<td>29</td>
</tr>
<tr>
<td>1.6.1: IGF-1R and IR structure</td>
<td>29</td>
</tr>
<tr>
<td>1.6.2: IGF-1R- and IR-induced signaling</td>
<td>30</td>
</tr>
<tr>
<td>1.6.3: IGF-1R and IR expression in WAT</td>
<td>33</td>
</tr>
<tr>
<td>1.7: Summary</td>
<td>34</td>
</tr>
<tr>
<td>Chapter 2: Background Information and Experiments</td>
<td>35</td>
</tr>
<tr>
<td>2.1.: Evidence for a depot-specific impact of GH on WAT</td>
<td>35</td>
</tr>
<tr>
<td>2.2: GHR mRNA expression in WAT</td>
<td>38</td>
</tr>
<tr>
<td>2.3: IGF-1R mRNA expression in WAT</td>
<td>39</td>
</tr>
<tr>
<td>2.4: IR mRNA expression in WAT</td>
<td>40</td>
</tr>
<tr>
<td>2.5.: Aims and Hypotheses</td>
<td>42</td>
</tr>
<tr>
<td>2.6.: Aim 2 preliminary data for GHA mice</td>
<td>44</td>
</tr>
<tr>
<td>Chapter 3: Materials and methods</td>
<td>50</td>
</tr>
<tr>
<td>3.1: Animals</td>
<td>50</td>
</tr>
<tr>
<td>3.2: Body composition analysis</td>
<td>50</td>
</tr>
</tbody>
</table>
Table 1: A comparison of general characteristics of subcutaneous and visceral white adipose tissue in humans and various rodent models .................................................................20
Table 2: A comparison of gene and protein expression in subcutaneous and visceral WAT depots in humans and various rodent models ........................................................................21
Table 3: Differences in immune cell populations in lean and obese adipose tissue ..........24
Table 4: A comparison of stromal vascular fraction and adipocyte portions of WAT depots in humans ................................................................................................................26
Table 5. Summary of characteristics of GHR -/- and GHA mice .........................................37
Table 6. Sequences of qPCR primers used ........................................................................56
Table 7. A comparison of SVF and adipocyte RNA concentrations ..................................60
Table 8. Comparison of metabolic parameters of 12 month-old male WTMice ...............61
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>TNF-α’s inhibition of insulin-induced signaling</td>
<td>15</td>
</tr>
<tr>
<td>2</td>
<td>Locations of most commonly studied WAT depots in male mice</td>
<td>17</td>
</tr>
<tr>
<td>3</td>
<td>Locations of WAT depots in humans</td>
<td>18</td>
</tr>
<tr>
<td>4</td>
<td>Cellular components of WAT</td>
<td>23</td>
</tr>
<tr>
<td>5</td>
<td>Overview of GH-, insulin-, and IGF-1-induced signaling</td>
<td>32</td>
</tr>
<tr>
<td>6</td>
<td>mRNA expression of receptor genes in WAT in three month old mice</td>
<td>41</td>
</tr>
<tr>
<td>7</td>
<td>mRNA expression of receptor genes in WAT in nine month old mice</td>
<td>42</td>
</tr>
<tr>
<td>8</td>
<td>Body composition data for 12 month-old male GHA and WT mice</td>
<td>45</td>
</tr>
<tr>
<td>9</td>
<td>ITT (A) and GTT (B) for 12 month-old male GHA and WT mice</td>
<td>46</td>
</tr>
<tr>
<td>10</td>
<td>WAT depot weights (A), WAT depot weights normalized to body weight (B), organ weights (C), and lengths (D) of 12 month-old male GHA and WT mice</td>
<td>47</td>
</tr>
<tr>
<td>11</td>
<td>Serum insulin concentration of 12 month-old male GHA and WT mice</td>
<td>48</td>
</tr>
<tr>
<td>12</td>
<td>Body composition data for 12 month-old male WT mice</td>
<td>58</td>
</tr>
<tr>
<td>13</td>
<td>WAT weights of 12 month-old male WT mice</td>
<td>59</td>
</tr>
<tr>
<td>14</td>
<td>Serum insulin and WAT mass as a percentage of body weight in 12 month-old WT mice</td>
<td>62</td>
</tr>
<tr>
<td>15</td>
<td>RNA expression of GHR (A), IGF-1R (B), and IR (C) in SVF of adipose tissue depots of 12 month-old male WT mice</td>
<td>64</td>
</tr>
<tr>
<td>16</td>
<td>RNA expression of GHR (A) and IR (B) of subcutaneous, epididymal, retroperitoneal, and mesenteric WAT depots of 12 month-old male WT mice</td>
<td>65</td>
</tr>
<tr>
<td>17</td>
<td>RNA expression of GHR, and IGF-1R, and IR of whole subcutaneous (A), epididymal (B), retroperitoneal (C), and mesenteric (D) WAT depots of 12 month-old male WT mice</td>
<td>66</td>
</tr>
</tbody>
</table>
Figure 18: RNA expression of receptors in subcutaneous (A), epididymal (B), retroperitoneal (C), and mesenteric (D) WAT depots of 12 month-old male GHA and WT mice.
CHAPTER 1: INTRODUCTION

Due to the rise in the rates of obesity and associated complications in recent decades, interest in adipose tissue (AT) research has increased. Once considered an organ whose sole functions were energy storage and the cushioning of organs, results presented in recent years have revealed that AT is much more complicated than initially thought. For example, AT is now considered to possess potent endocrine function. White (W) AT, the most abundant type of AT and the subject of this thesis, communicates with other cells to maintain energy balance through adipokines (hormones secreted by WAT) such as leptin (Zhang, Proenca et al. 1994), adiponectin (Nakano, Tobe et al. 1996), and resistin (Steppan, Bailey et al. 2001). In addition to its ability to produce and secrete hormones of its own, WAT is also affected by hormones including insulin (Dimitriadis, Mitrou et al. 2011), insulin-like growth factor-1 (IGF-1) and growth hormone (GH) (Ottosson, Vikman-Adolfsson et al. 1995) through cell surface receptors specific for these hormones on the cells within the tissue. Furthermore, as revealed by transgenic mouselines, hormones can act on WAT in a depot-dependent manner (Berryman, List et al. 2011), adding another layer of complexity to WAT. As a result, it is necessary to study WAT and obesity in terms of these hormonal interactions and anatomical differences. To that end, the goal of this thesis is to better understand the expression levels of several hormone receptors known to influence WAT mass and composition in a depot-specific manner.

1.1: Metabolic functions of WAT

The classical function of WAT, an energy reservoir, is carried out by the adipocyte, a cell with a unilocular, large lipid droplet within WAT that maintains energy
balance of the organism through the storage and release of fatty acids (Cinti 2005). During times of excess energy intake, the size of the lipid droplet within the adipocyte and the overall WAT mass increases; during times when energy intake is inadequate, the WAT stores decrease. The storage of dietary fats occurs through the activity of the enzyme lipoprotein lipase, which hydrolyzes lipoprotein triglycerides, allowing for the uptake of fatty acids and the esterification of glycerol 3-phosphate (Frayn, Karpe et al. 2003). De novo lipogenesis also occurs in WAT to convert excess carbohydrate to lipids (Acheson, Schutz et al. 1988). Through lipolysis, which relies on hormone sensitive lipase (HSL) (Fredrikson, Stralfors et al. 1981) and adipose triglyceride lipase (ATGL) (Zimmermann, Strauss et al. 2004), stored triglycerides in WAT are broken down to provide energy (Hales, Luzio et al. 1978).

The discovery of leptin in 1994 brought the discovery that AT is an endocrine organ that exerts its effects on other tissues through the secretion of adipokines (Zhang, Proenca et al. 1994). Leptin is produced primarily in WAT (but also in other tissues to a lesser extent), with its serum concentrations shown to be positively correlated to the amount of AT (Considine, Sinha et al. 1996; Ostlund, Yang et al. 1996). Leptin has many functions, with the primary one being its ability to inhibit food intake through its targeting of areas of the brain involved in appetite (Stephens, Basinski et al. 1995). Since the discovery of leptin, many other adipokines with widely varying functions have been discovered, providing evidence that WAT is a much more interactive organ than previously thought. Another well-studied adipokine is adiponectin, which, in contrast to leptin, negatively correlates with adiposity (Yamamoto, Hirose et al. 2002). Adiponectin is thought to protect against the development of obesity, diabetes, and cardiovascular
disease through its anti-inflammatory properties (Ouchi, Kihara et al. 1999; Yokota, Oritani et al. 2000; Ryo, Nakamura et al. 2004). Importantly, adiponectin is insulin-sensitizing through its suppression of hepatic glucose production (Berg, Combs et al. 2001).

Another more recent role of WAT is its ability to alter the body’s inflammatory state. That is, inflammation plays a role in the metabolic processes of WAT, particularly in obese individuals. This chronic, low-grade inflammation results from increased populations of immune cells in WAT, particularly infiltrating inflammatory macrophages (Weisberg, McCann et al. 2003). The presence of inflammation in obese WAT was first reported in 1993, when it was noted that TNF-α expression is increased in the WAT of obese mice (Hotamisligil, Shargill et al. 1993). The inhibition of TNF-α resulted in an improvement in insulin resistance, demonstrating inflammation’s link to glucose homeostasis (Hotamisligil, Arner et al. 1995). It has been found that ~59% of mRNA transcripts in obese WAT are transcripts from inflammation-related genes; furthermore, it has been shown that these changes in expression positively correlate with the development of insulin resistance (Xu, Barnes et al. 2003). Inflammation is thought to be linked to insulin resistance in part through inflammatory cytokines’ activation of c-Jun N-terminal kinase (JNK), which phosphorylates and inhibits IRS-1 (insulin receptor substrate 1), inhibiting the tyrosine kinase activity of the insulin receptor (IR) (Hotamisligil, Peraldi et al. 1996; Hirosumi, Tuncman et al. 2002) (see figure 1 for a summary of how TNF-α inhibits insulin-induced signaling). Thus, the inflammatory status of WAT is an important feature of the tissue that impacts the overall health of the
organism, and the resultant metabolic dysfunction caused by WAT inflammation is an area that requires further investigation.

Figure 1. TNF-α’s inhibition of insulin-induced signaling. Binding of TNF-α to the TNF-α receptor results in the phosphorylation of JNK and of IRS-1, thus inhibiting insulin’s action. Furthermore, TNF-α-induced signaling results in activation of inhibitor kappa beta kinase beta (IKK), followed by translocation of activator protein 1 (AP-1) and nuclear factor κβ (NF-κβ) to the nucleus, resulting in an increase in inflammatory cytokine production. Used with permission (Carter-Kent, Zein et al. 2008).

1.2: Mouse and human WAT depots

1.2.1: Depot locations and classifications

The distribution of WAT is organized into several distinct areas or “depots”. These depots are considered either “subcutaneous” or “visceral”, depending on their
anatomical location (Cinti 2012). In mice, the primary subcutaneous depots are located
directly under the skin both anteriorly or posteriorly, which includes the inguinal depot,
the most commonly studied subcutaneous depot (Cinti 2005). In addition to inguinal,
other depots of the posterior subcutaneous adipose include dorso-lumbar and gluteal;
depots of the anterior subcutaneous adipose include the interscapular, subscapular,
axillary, and cervical depots (Cinti 2012). The main visceral depots in mice are located
intraabdominally and include the mesenteric (surrounding the intestines), retroperitoneal
(behind the kidneys), and perigonadal (surrounding the testes-“epididymal”-or ovaries-
“periovarian”) depots (Cinti 2005) (See Figure 2 for an overview of adipose depots in
mice). However, the mesenteric depot is considered the only “true” visceral depot
because it drains into the portal vein (Catalano, Stefanovski et al. 2010). These four
depots - inguinal, perigonadal, retroperitoneal, and mesenteric - are the depots most
commonly studied in mice.
Figure 2. Locations of most commonly studied WAT depots in male mice. The inguinal depot (SUBQ) is the subcutaneous depot primarily studied and is located beneath the skin in the groin region. While mesenteric, retroperitoneal, and epididymal adipose depots are all considered intraabdominal (IA), only one, mesenteric, is considered “true visceral” due to its drainage into the portal vein to the liver. The location of the kidney is designated with a “K”. Used with permission (Sackmann-Sala 2010; Berryman, List et al. 2011)

In humans, the subcutaneous adipose depots include the abdominal, gluteal, and femoral depots (Wronska and Kmiec 2012). The intraabdominal depots in humans are the gonadal, retroperitoneal, mesenteric, pericardial, and omental depots (Cinti 2005; Bjorndal, Burri et al. 2011). Similarly to mice, only certain intraabdominal depots may actually be classified as visceral due to their drainage into the portal vein; in humans, these are the mesenteric and omental depots (Foster and Pagliassotti 2012). Much of the metabolic dysregulation seen in visceral obesity is attributed to the effects on hepatocytes
of fatty acids and adipokines secreted from these visceral depots (Hotamisligil, Peraldi et al. 1996). See Figure 3 for an overview of adipose depots in humans.

![Figure 3](image)

**Figure 3.** Locations of WAT depots in humans. Subcutaneous depots include the subcutaneous abdominal, gluteal, and femoral. Visceral depots include the epicardial, mesenteric, omental, retroperitoneal, and gonadal depots. However, due to their drainage into the portal vein, only the epicardial, omental, and mesenteric depots are considered true visceral depots. Modified and used with permission (Awada, Parimisetty et al. 2013)

There are several additional differences between human and rodent AT depots. For example, the pericardial depot, which is composed of paracardial and epicardial fat, is associated with the heart (Bjorndal, Burri et al. 2011; Fitzgibbons and Czech 2014);
there is evidence to suggest that this depot exhibits some characteristics of brown fat (Cheung, Gertow et al. 2013; Sacks, Fain et al. 2013). Previously, it was thought that rodents lack this depot (Marchington, Mattacks et al. 1989); however, recently epicardial AT has been identified in mice, particularly in those that are high-fat diet-fed (Yamaguchi 2015). The omental depot, which begins at the stomach and spleen and extends into the abdomen, is much less developed in rodents (Cinti 2005; Bjorndal, Burri et al. 2011).

1.2.2: Depot-specific differences

WAT depots differ in many aspects, leading to their classification as subcutaneous or visceral. As mentioned previously, while intraabdominal depots are frequently referred to as “visceral”, only those depots that drain into the portal vein may be considered “true” visceral. For purposes of this thesis, only these ‘true’ depots will be referred to as visceral. Many studies regarding depot-specific differences have been conducted in a variety of models, including obese and lean human and rodent models. For example, adipokine output is depot-dependent. As expected, visceral AT shows higher expression of proteins linked to the development of insulin resistance, such as resistin (Steppan, Bailey et al. 2001; Milan, Granzotto et al. 2002). In contrast, subcutaneous AT seems to be more involved in the production of adiponectin (Lihn, Bruun et al. 2004), which promotes insulin sensitivity and is associated with leanness (Berg, Combs et al. 2001; Fruebis, Tsao et al. 2001; Yamauchi, Kamon et al. 2001). Levels of insulin-induced intracellular signaling proteins seem to suggest that visceral AT is more metabolically active than subcutaneous AT (Lefebvre, Laville et al. 1998; Laviola, Perrini et al. 2006; Veilleux, Blouin et al. 2009); however, there is conflicting
literature regarding mitochondrial respiratory capacity (Deveaud, Beauvoit et al. 2004). Conflicting reports also exist regarding TNF-α expression in visceral and subcutaneous depots (Montague, Prins et al. 1998; Hube, Birgel et al. 1999; Mohamed-Ali, Goodrick et al. 1999; Winkler, Kiss et al. 2003). There are many other ways in which depots differ. Table 1 and Table 2 provide additional comparisons of the general characteristics of subcutaneous and visceral AT and depot-specific mRNA and protein expression levels, respectively.

*Table 1.* A comparison of general characteristics of subcutaneous as compared to visceral WAT depots in humans and various rodent models.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Subcutaneous (as compared to visceral)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adipocyte susceptibility to apoptosis</td>
<td>↓ (human subjects)</td>
<td>(Niesler, Siddle et al. 1998)</td>
</tr>
<tr>
<td>Adipocyte size</td>
<td>↑ (human subjects)</td>
<td>(Lundgren, Buren et al. 2004)</td>
</tr>
<tr>
<td>Inflammatory cell populations</td>
<td>↓ (mast cells in obese C57BL/6 mice)</td>
<td>(Altintas, Azad et al. 2011)</td>
</tr>
<tr>
<td></td>
<td>↓ (macrophages in human subjects)</td>
<td>(Curat, Wegner et al. 2006)</td>
</tr>
<tr>
<td>Capillary density per adipocyte</td>
<td>↑ (human subjects)</td>
<td>(Gealekman, Guseva et al. 2011)</td>
</tr>
<tr>
<td>Angiogenesis</td>
<td>↑ (human subjects)</td>
<td>(Gealekman, Guseva et al. 2011)</td>
</tr>
<tr>
<td>Presence of crown-like structures (CLS)*</td>
<td>↓ (C57BL/6 mice)</td>
<td>(Altintas, Azad et al. 2011)-</td>
</tr>
<tr>
<td>Mitochondrial respiratory capacity</td>
<td>↑ (C57BL/6 mice)</td>
<td>(Schottl, Kappler et al. 2014)-</td>
</tr>
<tr>
<td></td>
<td>↓ (Wistar rats)</td>
<td>(Deveaud, Beauvoit et al. 2004)-</td>
</tr>
</tbody>
</table>

*a*clusters of macrophages around an adipocyte
Table 2. A comparison of gene and protein expression in subcutaneous as compared to visceral WAT depots in humans and various rodent models.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Subcutaneous (as compared to visceral)</th>
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</tr>
</thead>
<tbody>
<tr>
<td>PPARγ</td>
<td>↓ (mRNA, human subjects)</td>
<td>(Lefebvre, Laville et al. 1998)</td>
</tr>
<tr>
<td>Interleukin-6</td>
<td>↓ (protein in incubations of adipocytes and AT, human subjects)</td>
<td>(Fried, Bunkin et al. 1998) (Fain, Madan et al. 2004)</td>
</tr>
<tr>
<td>Resistin</td>
<td>↓ (mRNA, Zucker rats)</td>
<td>(Milan, Granzotto et al. 2002)</td>
</tr>
<tr>
<td>Adiponectin</td>
<td>↑ (mRNA, human subjects)</td>
<td>(Lihn, Bruun et al. 2004)</td>
</tr>
<tr>
<td>Growth hormone receptor</td>
<td>↓ (mRNA, human subjects)</td>
<td>(Erman, Veilleux et al. 2011)</td>
</tr>
<tr>
<td>Insulin receptor</td>
<td>↓ (mRNA, human subjects)</td>
<td>(Lefebvre, Laville et al. 1998)</td>
</tr>
<tr>
<td>Collagen alpha-3(VI) (COL6A3)</td>
<td>↓ (mRNA, human subjects)</td>
<td>(Pasarica, Gowronska-Kozak et al. 2009)</td>
</tr>
<tr>
<td>GLUT4</td>
<td>↓ (mRNA, human subjects)</td>
<td>(Veilleux, Blouin et al. 2009)</td>
</tr>
<tr>
<td>TNF-α</td>
<td>↑ (mRNA, human subjects)</td>
<td>(Hube, Birgel et al. 1999)</td>
</tr>
<tr>
<td></td>
<td>↑ (protein, human subjects)</td>
<td>(Winkler, Kiss et al. 2003)</td>
</tr>
<tr>
<td></td>
<td>↑/↓ (mRNA in cultured adipocytes)</td>
<td>(Montague, Prins et al. 1998)</td>
</tr>
<tr>
<td></td>
<td>Undetectable (protein in subcutaneous AT in vivo human subjects)</td>
<td>( Mohamed-Ali, Goodrick et al. 1999)</td>
</tr>
</tbody>
</table>
1.3: WAT cell types

In addition to the aforementioned depot differences, the cellular composition of WAT depots varies greatly depending on the anatomical location of the specific depot. The cells that make up WAT depots are divided into two categories: mature adipocytes and cells of the stromal vascular fraction (SVF). It has been estimated that white adipocytes make up only 50% of WAT, with the remaining being cells of the SVF (Bukowiecki, Collet et al. 1982). The adipocyte and SVF portions may be separated and isolated through the use of collagenase to digest AT (See figure 4 for an overview of how the SVF is isolated and the cellular components that constitute the SVF.) As mentioned previously, white adipocytes function to store and release triacylglyceride, depending on the organism’s energy state, as well as secrete adipokines (Hales, Luzio et al. 1978). The SVF is a group of diverse cells that includes mesenchymal stem cells, endothelial cells, endothelial precursor cells, fibroblasts, and a variety of immune cells (Cancello and Clement 2006; Riordan, Ichim et al. 2009). The SVF portion has been shown to secrete adipokines of different varieties and amounts compared to adipocytes (Fain, Madan et al. 2004). Mesenchymal stem cells are the precursors to pre-adipocytes that eventually become functional mature adipocytes, but are considered pluripotent and can also be induced to differentiate into chondrogenic, myogenic, and osteogenic cell lines (Riordan, Ichim et al. 2009). The CD34+/CD31- endothelial precursor cells in the SVF differentiate in vitro to endothelial cells and participate in revascularization of an ischemic hind limb in nude mice (Miranville, Heeschen et al. 2004). Among the many cell types in the SVF, perhaps the most well studied are the immune cells due to their involvement in the
development of insulin resistance. The following section will explore the association between obesity and immune cells.

Figure 4. Isolation of cellular components of WAT. Excised adipose tissue may be broken down into adipocytes and stromal vascular cells by incubating the tissue with collagenase. Following centrifugation, mature adipocytes float to the top and the stromal vascular cells form a pellet. The stromal vascular fraction (SVF) is composed of a variety of cell types, including mesenchymal stem cells, endothelial cells, endothelial precursor cells, fibroblasts, and various immune cells.

It has been shown that immune cells play many roles in metabolism, including adipose tissue expansion, lipolysis, and non-shivering thermogenesis (Ferrante 2013). Immune cells present in adipose tissue include cells of both the innate and adaptive immune system: adipose tissue macrophages (ATMs), natural killer T (NKT) cells,
invariant NKT (iNKT) cells, neutrophils, dendritic cells, mast cells, B cells, and CD3+, CD4+, CD8+, and regulatory (Treg) T cells (Ferrante 2013). All of these cell populations exhibit an increase or decrease in number during the AT expansion that occurs during obesity (Ferrante 2013). See Table 3 for an overview of the changes in immune cell populations that occur during AT expansion.

Table 3. Differences in immune cell populations in obese as compared to lean WAT

<table>
<thead>
<tr>
<th>Immune cell</th>
<th>Obese (as compared to lean)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mast cells</td>
<td>↑ (C57BL/6 mice, increases primarily in visceral)</td>
<td>(Altintas, Azad et al. 2011)</td>
</tr>
<tr>
<td>Dendritic cells</td>
<td>↑ (humans, positively correlates with AT mass)</td>
<td>(Bertola, Ciucci et al. 2012)</td>
</tr>
<tr>
<td>Eosinophils</td>
<td>↓ (C57BL/6 mice, correlates negatively with adiposity)</td>
<td>(Wu, Molofsky et al. 2011)</td>
</tr>
<tr>
<td>Neutrophils</td>
<td>↑ (human subjects, positively correlates with adiposity)</td>
<td>(Dixon and O'Brien 2006)</td>
</tr>
<tr>
<td>M1:M2 macrophage ratio</td>
<td>↑ (C57BL/6 mice, M1 and M2 macrophages increase--M1 increases most)</td>
<td>(Fujisaka, Usui et al. 2009)</td>
</tr>
<tr>
<td>CD8+ T cells</td>
<td>↑ (C57BL/6 mice)</td>
<td>(Rausch, Weisberg et al. 2008; Nishimura, Manabe et al. 2009)</td>
</tr>
<tr>
<td>CD4+ T cells</td>
<td>↓ (C57BL/6 mice)</td>
<td>(Nishimura, Manabe et al. 2009)</td>
</tr>
<tr>
<td>Regulatory T cells</td>
<td>↓ (C57BL/6 mice)</td>
<td>(Nishimura, Manabe et al. 2009)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Feuerer, Herrero et al. 2009)</td>
</tr>
</tbody>
</table>
1.4: Comparison of adipocytes and SVF

As would be expected, the adipocytes and cells that constitute the SVF fraction differ in terms of protein expression. For example, it has been shown that both the adipocyte and SVF portions of AT secrete a variety of adipokines but differ in the type and amount (Fain, Madan et al. 2004). In general, the SVF secretes a higher concentration of inflammatory cytokines, such as tumor necrosis factor α (TNF-α) and interleukin 6 (IL-6) (Fain, Madan et al. 2004), than the adipocyte portion (Zeyda, Farmer et al. 2007). Interestingly, expression of nuclear envelope proteins Lamins A & C, which are linked with inherited partial lipodystrophies (Garg and Agarwal 2009), is elevated in the SVF of only subcutaneous AT (Peinado, Jimenez-Gomez et al. 2010). This is particularly of interest because it demonstrates how proteins may be differentially expressed in not only depots, but also in the two cellular fractions of AT.

Since expression levels of a variety of genes can vary depending on depot as well as the SVF and adipocyte fractions, it is important to evaluate different depots and the different fractions in order to improve our understanding of the function of adipose tissue components. For example, a study by Peinado et al. suggests that the primary differences in subcutaneous and visceral depots and the resultant association of excess visceral adipose to metabolic disorders are due to the secretion of SVF proteins (Peinado, Jimenez-Gomez et al. 2010). It should be noted that in this study, the expression of the adipokines, omentin and visfatin, are elevated in the SVF of visceral AT compared to the SVF of subcutaneous AT (Peinado, Jimenez-Gomez et al. 2010). However, when the expression of these adipokines in whole tissue subcutaneous and visceral samples is
compared, no differences are observed (Peinado, Jimenez-Gomez et al. 2010).

Furthermore, the visceral and subcutaneous AT adipocytes from lean individuals show highly similar protein expression in contrast to the SVF portion, which show significant differences (Peinado, Jimenez-Gomez et al. 2010). This study emphasizes the importance of comparing the SVF and adipocyte portions in addition to whole tissue when comparing protein expression between two depots. Refer to Table 4 for additional comparisons of SVF and adipocyte characteristics.

*Table 4. A comparison of adipocyte and SVF portions of WAT depots in humans.*

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Adipocytes (as compared to SVF)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Leptin</td>
<td>↑ (protein, human subjects)</td>
<td>(Fain, Madan et al. 2004)</td>
</tr>
<tr>
<td></td>
<td>↑ (mRNA, human subjects)</td>
<td>(Masuzaki, Ogawa et al. 1995)</td>
</tr>
<tr>
<td>Adiponectin</td>
<td>↑ (protein, human subjects)</td>
<td>(Fain, Madan et al. 2004)</td>
</tr>
<tr>
<td>Tumour necrosis factor-α</td>
<td>↓ (protein, human subjects)</td>
<td>(Fain, Madan et al. 2004)</td>
</tr>
<tr>
<td>Interleukin-6</td>
<td>↓ (protein, human subjects)</td>
<td>(Fain, Madan et al. 2004)</td>
</tr>
<tr>
<td>Lamin A &amp; C</td>
<td>↓ (protein, human subjects)</td>
<td>(Peinado, Jimenez-Gomez et al. 2010)</td>
</tr>
</tbody>
</table>

In addition to the differences in expression shown in Table 4, adipocytes and cells of the SVF differentially express receptors for hormones related to growth and metabolism. For example, adipocytes as well as some of the cells of the SVF, such as AT macrophages, are known to express growth hormone receptor (GHR) (Gavin, Saltman et al. 1982; Smith, Benghuzzi et al. 2000). GH’s interaction with the GHR on macrophages has been shown to inhibit the cells’ expression and secretion of IL-1β, thus inhibiting the
differentiation of pre-adipocytes (Lu, Kumar et al. 2010). Likewise, adipocytes are known to possess the insulin-like growth factor 1 receptor (IGF-1R) and IR (Berhanu, Kolterman et al. 1983; Kloting, Koch et al. 2008); however, there is little available data to suggest the presence or absence of these receptors in SVF cells. Additional information related to these hormones and their receptors will be discussed in the subsequent section 1.5: Growth Hormone Receptor

1.5.1: GHR structure

GH is a 22,000 kDa polypeptide hormone necessary for the induction of postnatal growth in mammals (Edens and Talamantes 1998). GH is secreted from the anterior pituitary in a pulsatile manner, with the largest pulses occurring at night (Khan, Sane et al. 2002). GH exerts its effects on target tissues through specific interactions with the GHR on the surface of cells to induce an intracellular signaling cascade, as will be described in detail below (Edens and Talamantes 1998). The GHR weighs approximately 100-130 kDa depending on the species of origin and is ~620 amino acids in length (Edens and Talamantes 1998). The mature receptor is composed of an extracellular domain at the N terminus, where the GH molecule binds, of ~245 amino acids, a transmembrane domain of 24 amino acids, and an intracellular domain of ~350 amino acids at the C terminus, where signaling occurs (Edens and Talamantes 1998). The GHR is considered a class 1 cytokine receptor in that it utilizes the Janus-kinase signal transducers and activators of transcription (JAK-STAT) signaling pathway ((Brooks and Waters 2010).
1.5.2: GHR-induced signaling

GHR exists in a pre-dimerized form (Brown, Adams et al. 2005) that becomes activated by binding GH at its two GHR binding sites (Argetsinger, Campbell et al. 1993; Brooks and Waters 2010). Upon GH binding, the intracellular domains of the GHR rotate, activating Janus kinase 2 (JAK2) and Src family kinases associated with the intracellular domain of the GHR (Brown, Adams et al. 2005; Brooks and Waters 2010; Brooks, Dai et al. 2014). As a result of this activation, cross-phosphorylation of the tyrosine residues of the GHR cytoplasmic tails occurs (Brown, Adams et al. 2005). Following the recruitment of additional signaling molecules, STAT5 α and β become phosphorylated and dimerize, allowing them to enter the nucleus to bind to STAT responsive elements in the enhancers of targeted genes that influence growth and metabolism. Activation, synthesis, and secretion of IGF-1 also occurs in most cells as a result of GH action (Herrington, Smit et al. 2000). See figure 5 (on page 32 of this thesis) for an overview of GH-induced signaling. Important to this thesis is the fact that, WAT is one of many tissues that are well established to possess cells that express GHR, which is described more fully in the subsequent section. GH-induced signaling in the adipocyte increases basal lipolysis and improves AT’s response to lipolytic catecholamines (Ottosson, Lonroth et al. 2000). However, despite actions promoting lipolysis and reduction of AT, GH-induced signaling has a diabetogenic effect (Bornstein, Taylor et al. 1969) induced by its suppression of insulin signaling (Adamson and Efendic 1979). One of the mechanisms by which GH-induced signaling suppresses insulin sensitivity is elucidated in a publication by del Rincon et al. (del Rincon, Iida et al. 2007) In brief, GH-induced signaling upregulates mRNA and protein expression of the p85α regulatory
subunit of phosphatidyl inositol 3’ kinase (PI3K), a kinase crucial to the insulin signaling pathway (del Rincon, Iida et al. 2007). Other mechanisms include reduction of insulin receptor, insulin receptor substrate 1 (IRS-1), and PI3K levels (Thirone, Carvalho et al. 1997; Johansen, Laurino et al. 2005), as well as reduction in insulin-stimulated phosphorylation of many insulin substrates (Thirone, Carvalho et al. 1997).

1.5.3: GHR expression in WAT

GH exerts its metabolic effects by interacting with the GHR in many tissues, with the primary ones being skeletal muscle, liver, and AT. GHR in AT has been shown to be expressed particularly in isolated adipocytes (Vikman, Carlsson et al. 1991) and pre-adipocytes (Gerfault, Louveau et al. 1999). However, GHR is also expressed in AT macrophages (Lu, Kumar et al. 2013), but other stromal vascular cell types isolated from AT, to the best of my knowledge, have not been specifically investigated. Like many molecules, GHR is expressed differentially in visceral and subcutaneous adipose depots. Studies have shown that rats have higher GHR expression in adipocytes from epididymal depots than in adipocytes from retroperitoneal or subcutaneous depots (LaFranchi, Hanna et al. 1985). Similarly, in pigs, adipocytes from the omental depot have higher GHR expression than adipocytes from perirenal and subcutaneous fat (Brameld, Atkinson et al. 1996). Furthermore, a study in humans showed greater RNA expression in the abdominal depot compared to the gluteal (subcutaneous) depot (Karastergiou 2013).

1.6: IGF-1R and IR

1.6.1: IGF-1R and IR structure

IGF-1 is a 70 amino acid polypeptide that mediates at least some of the anabolic and mitogenic activities of GH (Laron 1999). IGF-1 secretion from the liver as well as
paracrine secretion from other tissues is dependent on GH-induced signaling (Laron 2001). Insulin is a 55 amino acid dipeptide hormone produced by the beta cells of the pancreas that stimulates the translocation of the insulin-sensitive glucose transporter 4 (GLUT4) to the cell membrane of fat and muscle cells, allowing for the uptake of glucose (Birnbaum 1989; Wilcox 2005). As IGF-1’s name implies, the protein has a very similar structure to that of proinsulin, the precursor to insulin; indeed, amino acids 1 to 29 of IGF-1 share homology with insulin’s B chain and amino acids 42-62 share homology with insulin’s A chain (Rinderknecht and Humbel 1978).

The receptors utilized by IGF-1 and insulin, IGF-1R and IR, respectively, have high structural similarity; in fact, it is often difficult to discern the biological effects mediated by each specifically (Kim and Accili 2002). Both receptors have beta-alpha-alpha-beta subunit structure with alpha and beta subunits of 88 kDa and 67 kDa, respectively; they also have similar amino acid composition, with sequences 41-84% alike depending on domain (Fujita-Yamaguchi, LeBon et al. 1986; De Meyts and Whittaker 2002). Furthermore, the two receptors are known to form heterodimers between each other that bind IGF-1 with higher affinity and insulin with a lower affinity (Soos, Field et al. 1993), as seen in figure 5. Both the IGF-1R and IR belong to the receptor tyrosine kinase family; however, unlike other receptor tyrosine kinases, they exist on the cell in a pre-dimerized state rather than dimerizing in response to ligand binding (Heldin and Ostman 1996; Hubbard and Till 2000).

1.6.2: IGF-1R- and IR-induced signaling

Similarly to GH-induced signaling, IGF-1 binding results in cross-phosphorylation of the IGF1-R, leading to a signaling cascade initiated by tyrosine
phosphorylation of substrates including the IRS 1-4 (Sun, Rothenberg et al. 1991; Pelicci, Lanfrancone et al. 1992; Patti, Sun et al. 1995; Lavan, Lane et al. 1997) (for simplicity, only IRS 1 is shown in figure 5). IRS 1-4 then interact with proteins possessing SH2 domains, for example, the p85 regulatory subunit of PI3K, growth factor receptor binding protein 2 (Grb2), and SHP-2/Syp, a protein tyrosine phosphatase (Dupont and Holzenberger 2003). Tyrosine phosphorylation of each of these substrates results in the induction of signaling cascades associated with cell proliferation, differentiation, glucose transport, and the prevention of apoptosis (Dupont and Holzenberger 2003).

Activation of the IR by insulin modulates the uptake of glucose into cells by insulin-responsive GLUT4 (Birnbaum 1989). Insulin’s binding to the IR induces cross-phosphorylation of residues on proteins associated with the intracellular domains of the IR (IRS 1-4), followed by recruitment of proteins such as the p85 regulatory subunit of PI3K (Summers, Whiteman et al. 2000). Activation of the Akt/PKB signaling pathway then results in GLUT4 translocation to the cell membrane (Ishiki, Randhawa et al. 2005). As discussed previously, insulin’s actions can be inhibited by GH signaling; thus, chronically elevated levels of GH have a diabetogenic or anti-insulin effect known to be induced through several mechanisms (Thirone, Carvalho et al. 1997; Johansen, Laurino et al. 2005; del Rincon, Iida et al. 2007). See Figure 5 for an overview of insulin- and IGF-1-induced signaling.
Figure 5. Overview of GH-, insulin-, and IGF-1-induced signaling. The insulin and IGF-1 receptors are highly similar, allowing for some cross-reactivity in binding. Binding of either receptor results in cross-phosphorylation of the receptor, followed by phosphorylation of IRS, allowing IRS to bind to the p85 regulatory subunit of PI3K. The final outcome of this pathway is the translocation of GLUT4 to the cell surface and the upregulation of genes related to glycogen synthesis and inhibition of lipolysis. The binding of GH to the GHR results in cross-phosphorylation of the receptor, inducing rotation of the intracellular domains and activation of JAK2 and Shc. Phosphorylation of transcription factor STAT5 results in translocation of this molecule to the nucleus, where it regulates genes related to cell proliferation and growth, as well as production of IGF-1. Chronically elevated GH is known to induce diabetogenic effects through the inhibition of insulin signaling (not shown). Used with permission (Trobec, von Haehling et al. 2011).
1.6.3: IGF-1R and IR expression in WAT

As with GHR, IGF-1R has been detected in isolated adipocytes and pre-adipocytes from pigs (Gerfault, Louveau et al. 1999) as well as in human adipocytes in culture (Kern, Svoboda et al. 1989). However, several studies have failed to detect IGF-1R in both pig (Richardson, Hausman et al. 1994) and human adipocytes (DiGirolamo, Eden et al. 1986; Bolinder, Lindblad et al. 1987). Regarding the functions of IGF-1-induced signaling in AT, IGF-1 has been shown to be involved in pre-adipocyte differentiation into adipocytes (Smith, Wise et al. 1988). Furthermore, IGF-1R knockout mouse studies have shown that IGF-1 plays a role in regulating adiposity (Kloting, Koch et al. 2008). Specifically, in IGF-1R knockout mice, adipose mass is increased and the mean adipocyte size is increased compared to controls. These differences are exacerbated in perigonadal depots compared to subcutaneous depots (Kloting, Koch et al. 2008), which implies potential depot differences in the expression of this receptor.

It is well known that IR is expressed in adipocytes, due its role in the stimulation of GLUT4 translocation to the cell membrane; however, IR protein has also been shown to be expressed in 3T3-L1 preadipocytes (Reed, Kaufmann et al. 1977). Depot-specific differences in insulin signaling molecules have been noted as well (Laviola, Perrini et al. 2006); that is, in non-obese, non-diabetic humans, expression of insulin signaling molecules is higher in the omental depot than in the subcutaneous depot (Laviola, Perrini et al. 2006). From my literature search, it appears that neither IGF-1R nor IR expression levels have been investigated in SVF cells, which suggests that this area requires further investigation.
1.7: Summary

Taken together, the topics discussed in this chapter emphasize the importance of investigating not only depot-specific differences, but also differences observed in SVF of WAT. This chapter summarized specific characteristics of subcutaneous and visceral WAT depots as well as how they differentially express many proteins, in addition to providing an introduction to GH-, IGF-1-, and insulin-induced signaling in WAT. The following chapter will provide further background on the depot-specific impact GH-, IGF-1-, and insulin-induced signaling have on WAT using data generated from mouse lines, focusing on unpublished data that have been generated in our laboratory.
CHAPTER 2: BACKGROUND INFORMATION AND EXPERIMENTS

2.1: Evidence for a depot-specific impact of GH on WAT

Many experiments conducted in our laboratory using GH transgenic lines suggest a depot-specific effect of GH on WAT. Two transgenic models in particular have been instrumental in deriving the depot-specific effects of GH on adiposity: 1) the GH receptor null (GHR -/-) mice, which lack functional GHR and are dwarf, obese, insulin-sensitive, and long-lived (List, Sackmann-Sala et al. 2011); and 2) the GH receptor antagonist (GHA) mice, which produce a GH antagonist that functions to limit GH-induced signaling, resulting in obesity, but retains normal insulin levels and lifespan (Coschigano, Holland et al. 2003). These mouse lines allow us to investigate the effects of adiposity seen when GH signaling is reduced (GHA mice) or removed (GHR -/- mice).

GHR -/- mice were first produced in 1997 through disruption of the GHR/BP gene by homologous recombination of a DNA fragment into exon 4 of the mouse GHR gene (Zhou, Xu et al. 1997). Subsequent investigation has revealed that WAT depots are affected differentially due to the ablation of GHR. That is, while GHR -/- mice have increased adiposity, not all fat depots are equally affected. Specifically, the subcutaneous depots are enlarged preferentially compared to intraabdominal depots (Berryman, List et al. 2011), suggesting that GH affects WAT to a greater extent in the subcutaneous depot.

The preferential enlargement of the subcutaneous depot in GHR -/- mice and other mouse models of GH disruption has been noted by numerous other studies (Berryman, List et al. 2004; Meyer, Korthaus et al. 2004; Berryman, List et al. 2010). This trend (an enlargement of the subcutaneous depot and impairment in development of the paraovarian depot) has also been shown for female GHR-/- mice as compared to WT
Subsequent in vitro experiments demonstrate that adipocytes from the parametrial depot are significantly impaired in terms of proliferation and differentiation, while adipocytes from the inguinal depot show no impairment (Flint, Binart et al. 2006). This suggests that the ablation of GH action has a greater effect on the parametrial depot. GHR -/- mice also show reduced expression of GLUT4 protein in the inguinal depot compared to wild-type mice, indicating that the removal of GH signaling affects insulin-induced translocation of GLUT4 (Sackmann-Sala, Berryman et al. 2014). Furthermore, data have suggested that visceral fat in GHR -/- mice may not have the negative effects observed in wild-type mice, as the removal of visceral fat does not result in improvement in insulin-sensitivity as observed in wild-type controls (Masternak, Bartke et al. 2012). Thus, GHR -/- mice show several important differences in AT distribution and function compared to WT mice.

GHA mice, which are used in Aim 2 of this thesis, were first produced in 1991 through pronuclear microinjection of a bovine GH minigene with a mutation of a codon in the third alpha helix of the bovine GH (resulting in glycine 119 being replaced by lysine) (Chen, White et al. 1991). Thus, the introduced antagonist competes with pituitary or endogenous GH for GHR binding sites, reducing, but not eliminating, GH-induced signaling. Similarly to GHR -/- mice, GHA mice possess a larger inguinal depot compared to WT mice (Berryman, List et al. 2004). A recent study examined the effects of high-fat feeding on WAT expansion in these mice (Yang, Householder et al. 2015). The GHA mice show a more dramatic expansion of WAT, particularly in the subcutaneous and perigonadal depots, but remain insulin sensitive and glucose tolerant with only slight increases in serum glucose levels. However, despite having decreased
GH signaling, GHA mice do not exhibit the same improvements in glucose homeostasis and lifespan extension seen in GHR -/- mice (Coschigano, Holland et al. 2003; Berryman, Lubbers et al. 2014). This difference has been attributed primarily to the fact that GHA mice possess only moderately reduced GH action, while GHR -/- mice possess total ablation of the GH action via the GHR (Berryman, Lubbers et al. 2014). Another important feature of the GHA mice in comparison to non-transgenic littermate controls is that GH-specific binding in liver is elevated in GHA mice, while GH-specific binding in kidney is reduced (Chen, Chen et al. 1997). Thus, not all tissues respond in a similar fashion in GHA mice with respect to GHR tissue levels. For a summary of characteristics of GHR -/- and GHA mice, see Table 5.

**Table 5. Summary of characteristics of GHR -/- and GHA mice.**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>GHR -/-</th>
<th>GHA</th>
</tr>
</thead>
<tbody>
<tr>
<td>% fat mass</td>
<td>↑ 6 weeks of age - 2 years of age (Berryman, List et al. 2004) (Egecioglu, Bjursell et al. 2006)</td>
<td>↑ 6 months of age (Berryman, List et al. 2004)</td>
</tr>
<tr>
<td>Serum adiponectin</td>
<td>↑ (Berryman, List et al. 2004)</td>
<td>↑ (Berryman, List et al. 2004)</td>
</tr>
<tr>
<td>Serum leptin</td>
<td>unchanged (Berryman, List et al. 2004), unchanged (Egecioglu, Bjursell et al. 2006)</td>
<td>↑ (Berryman, List et al. 2004)</td>
</tr>
<tr>
<td>Serum IGF-1</td>
<td>20% WT level (Coschigano, Holland et al. 2003)</td>
<td>75-80% WT level (Coschigano, Holland et al. 2003)</td>
</tr>
<tr>
<td>Fasting serum insulin levels</td>
<td>↓ (Coschigano, Holland et al. 2003)</td>
<td>unchanged (Coschigano, Holland et al. 2003)</td>
</tr>
<tr>
<td>Fasting serum glucose levels</td>
<td>↓ (Coschigano, Holland et al. 2003)</td>
<td>unchanged (Coschigano, Holland et al. 2003)</td>
</tr>
<tr>
<td>Lifespan</td>
<td>↑ (Coschigano, Holland et al. 2003)</td>
<td>unchanged (Coschigano, Holland et al. 2003; Berryman, Lubbers et al. 2014)</td>
</tr>
</tbody>
</table>

*versus WT littermate controls*
In addition to data generated from these genetically engineered mouse lines, several studies, including an honors thesis project by D. Bailey Miles from our laboratory, have attempted to directly compare adipose depot expression of GHR, IGF-1R, and IR in normal C57Bl/6J mice. The findings from these preliminary studies will be discussed in the subsequent sections.

2.2: GHR mRNA expression in WAT

A recent study in our laboratory compared the mRNA expression of GHR, IGF-1R, and IR in whole AT depots of 3 month-old and 9 month-old C57Bl/6J male mice (Miles 2009). Both age-related and depot-specific differences were observed in this study. In the mesenteric depot, GHR had a higher relative expression in the 9 month-old group compared to the 3-month old group (figures 6 and 7), either suggesting that GH-induced intracellular signaling becomes more important in this depot as mice age or that the level of GHR increases with age to compensate for reduced GH levels.

Depot-specific differences in GHR expression were also noted in the thesis. The 3 month-old group had higher GHR expression in the epididymal and retroperitoneal depots than in the subcutaneous and mesenteric depots (figure 6). In the 9 month-old group, the epididymal depot also showed higher GHR expression than the subcutaneous depot (figure 7). These results suggest that GH-induced intracellular signaling is more active in the epididymal depot, which is contrary to conclusions drawn from studies in GHR -/- and GHA mice, which show that subcutaneous adipose is most greatly affected by the reduction in GH signaling (Berryman, List et al. 2004). However, these results do match the proliferation and differentiation studies that have been performed by Flint et al (Flint, Binart et al. 2006). Unfortunately, these studies only assessed GHR expression
levels in whole tissue; thus, it is impossible to determine the adipocyte versus SVF contribution to this expression, which may explain the disconnect between the depot expression levels of GHR and the WAT phenotypes in these mice. Further studies regarding the expression of GHR in wild type mice have been discussed extensively in chapter 1.

2.3: IGF-1R mRNA expression in WAT

In the thesis of D. Bailey Miles, expression of IGF-1R exhibited age-related differences as well (Miles 2009). IGF-1R mRNA had a relatively higher expression in the subcutaneous depot of the 9 month-old group compared to the 3 month-old group; conversely, the epididymal depot showed a relatively higher expression in the 3 month-old group compared to the 9 month-old group (figures 6 and 7). These data are supported by a study using 8 month-old IGF-1R knockout mice, in which epididymal fat mass increased preferentially in these mice (Kloting, Koch et al. 2008).

WAT depot-specific differences in IGF-1R expression were observed in both age groups. In the 3 month-old group, IGF-1R was more strongly expressed in the epididymal depot compared to the subcutaneous depot (figure 6). In contrast, the 9 month-old group showed a heightened expression in the mesenteric depot compared to the retroperitoneal depot (figure 7). Positive correlations were also observed between IGF-1R and IR expression, supporting IGF-1R’s contribution to insulin signaling. Further studies regarding the expression of IGF-1R in wild type mice have been discussed extensively in chapter 1.
2.4: IR mRNA expression in WAT

Depot-specific differences in IR expression showed trends similar to those of the IGF-1R expression (Miles 2009). The 3 month-old mice had greater IR expression in the epididymal depot than in the subcutaneous and mesenteric depots. Also, the retroperitoneal depot had higher expression than the subcutaneous depot (figure 6). The 9 month-old mice showed higher IR expression in the mesenteric depot compared to the subcutaneous and retroperitoneal depots (figure 7).

In summary, it was found that visceral depots have elevated IR expression compared to subcutaneous depots, which supports previous findings by Lefebvre et al. (Lefebvre, Laville et al. 1998), but contradicts a study by Bolinder et al. (Bolinder, Kager et al. 1983), which concluded that there was no significant difference between numbers of receptors in omental and subcutaneous AT. Further studies regarding the expression of IR in wild type mice have been discussed extensively in chapter 1.
Figure 6. mRNA expression of receptor genes in WAT in three month old mice. Real-time PCR analysis of GHR (A), IGFIR (B), IR (C). Shown are means ± SEM, n=13 for Epi, n=12 for Mes, n=7 for Retro, n=14 for Subq. Means that share a common letter are not significantly different, p>0.05. Modified and used with permission (Miles 2009)
Figure 7. mRNA expression of receptor genes in WAT in nine month old mice. Real-time PCR analysis of GHR (A), IGFIR (B), IR (C), and IR-B (D). Shown are means ± SEM, n=5 for Epi, n=6 for Mes, n=5 for Retro, n=7 for Subq. Means that share a common letter are not significantly different, p>0.05. Modified and used with permission (Miles 2009)

2.5.: Aims and hypotheses

This thesis builds on research completed by D. Bailey Miles detailed in the Ohio University Honors Tutorial College Thesis, “Depot specific differences in GH, IGF-1 and insulin receptors in adipose tissue”. This work will further develop the knowledge base regarding depot-specific differences in wild type mice by elucidating which cellular components of WAT are primarily responsible for the expression of GHR, IGF-1R, and IR. Furthermore, this project will be the first to directly compare the expression of these receptors in the adipose depots of GHA mice. Based on previous studies regarding depot-
specific differences and the effects of the ablation of these receptors, I originally proposed these aims and hypotheses:

Aims

1.) To determine the relative RNA expression of GHR, IGF-1R, and IR in the adipocyte and SVF portions of WT mouse subcutaneous, epididymal, retroperitoneal, and mesenteric WAT depots.

2.) To determine the difference in RNA expression of the above receptors in the whole WAT depots of GHA mice compared to WT mice.

Hypotheses

1) Aim 1 hypothesis: WT mice will exhibit the highest expression of GHR in SVF (in visceral depots only due to elevated macrophage populations), but the highest expression of IGF-1R and IR in adipocytes (due to the importance of both these receptors in insulin signaling in adipocytes). Furthermore, due to increased populations of immune cells present in visceral depots, GHR expression will be highest in these depots compared to subcutaneous depots.

2) Aim 2 hypothesis: The GHA mice will have elevated expression of IGF-1R and IR in the visceral adipose depots compared to the subcutaneous depots. Contrary conclusions have been drawn regarding the importance of GHR-mediated GH signaling in the subcutaneous depot, so it is difficult to propose how the RNA expression will be affected in this depot. However, most of the evidence derived from mouse models of reduced GH signaling seems to suggest that GHR-mediated signaling is important in this depot; therefore, I
hypothesize that GHR mRNA will be elevated in subcutaneous WAT compared to the other depots.

Note that Aim 1 was modified due to problematic isolation of RNA from adipocytes; receptor expression was examined in RNA from SVF of one group of WT mice (Aim 1 mice) and in RNA from whole WAT depots of a second group of WT mice (Aim 2 WT controls).

2.6.: Aim 2 preliminary data for GHA mice

There are preliminary data available for the GHA and wild type control mice used in Aim 2 that were generated by others in the laboratory. I was not involved in that data collection, however, I want to show these data here. Prior to sacrificing the WT and GHA mice utilized in Aim 2, body composition analyses and glucose and insulin tolerance tests (GTTs & ITTs) were performed. At dissection, the body lengths of the mice were recorded as well as the weights of WAT depots and several other tissues. These data are shown in figures 8, 9, and 10 below and overall are in accordance with previous studies with these mice (Coschigano, Holland et al. 2003; Berryman, List et al. 2004).
Figure 8. Body composition data for 12 month-old male GHA and WT mice. Data are reported as (A) mass in grams and as (B) percentage of body mass. Shown are means ± SEM, n=8 for each group. Means designated with an asterisk are significantly different, p<0.05.
Figure 9. ITT (A) and GTT (B) for 12 month-old male GHA and WT mice. Shown are means ± SEM, n=8 for each group. Means designated with an asterisk are significantly different, p<0.05.
Figure 10. WAT depot weights (A), WAT depot weights normalized to body weight (B), organ weights (C), and lengths (D) of 12 month-old male GHA and WT mice. Shown are means ± SEM, n=8 for each group. Means designated with an asterisk are significantly different, p<0.05.
Figure 11. Serum insulin concentration of 12 month-old male GHA and WT mice. Shown are means ± SEM, n=8 for each group.

The body composition analyses, ITTs and GTTs, and analyses of serum insulin and tissue weights provide important background information that emphasizes the differences in GHA and WT mice. Body composition analyses showed that the GHA and WT mice have a significant difference in weights and percentages of all body components (fat, lean, fluid); however, their total body weights showed no significant difference (figure 8). This indicates that while the two groups of mice have similar body weights, GHA mice have a greater percentage of fat compared to lean mass, while WT mice have a greater percentage of lean mass compared to fat. This agrees with the body composition analysis of GHA mice reported previously (Berryman, Lubbers et al. 2014). That is, male GHA mice tend to have significantly smaller body weights compared to littermate controls until approximately one year of age when the body weights are no longer significantly different.
Data obtained from ITTs and GTTs revealed that this cohort of GHA mice exhibit a stronger response to insulin and were thus more insulin sensitive at the 45, 60, and 90 min time points compared to WT mice (figure 9A), but were less glucose tolerant at the 15 and 30 min time points (figure 9B). Previously, GTT data collected from 72 week-old GHA and WT mice showed no significant difference in area under the curve in the two groups (Berryman, Lubbers et al. 2014). A statistical comparison of serum insulin levels (figure 11) in the two groups showed no significant difference. As a previous study using 16- to 18-week-old male mice showed GHA mice have significantly less serum insulin compared to controls, this most likely indicates an age-related difference (Yakar, Setser et al. 2004). Additionally, even when GHA mice are kept on a high-fat diet, they do not develop hyperinsulinemia (Yang, Householder et al. 2015).

The analysis of tissue weights obtained from the dissection of the GHA and WT mice tissues showed that GHA mice have significantly smaller liver, kidney, and soleus muscle, but increased brown adipose tissue (BAT) mass (figure 10C). Before normalization to body weight, there were no significant differences in WAT depot masses in the two groups (figure 10A), but after normalization, GHA showed relative enlargement of the subcutaneous depot (figure 10B), which correlates with previous studies showing enlargement of this depot in these mice (Berryman, List et al. 2004). These data, combined with the body composition data, provide further evidence that while GHA mice have less lean tissue mass, their WAT makes up a larger percent of body weight than in WT mice.
CHAPTER 3: MATERIALS AND METHODS

3.1: Animals

Two separate cohorts of male, 12 month-old mice of the C57BL/6J genetic background were used for the specific aims of this thesis. For Aim 1, only C57BL/6J wild type mice were used (n=8). For Aim 2, WAT depots were obtained from GHA mice produced as previously described (Chen, White et al. 1991). In brief, to create the mouse line, microinjection was utilized to insert a bovine GH minigene containing an amino acid substitution in the third alpha helix into the germline; this results in the replacement of glycine 119 by lysine. Mice were genotyped using PCR utilizing DNA isolated from a tail, as previously described (Berryman, List et al. 2004). Eight mice were used from each group, wild type (WT) and GHA. For both aims, mice were caged two to four per cage in rooms with controlled light cycles (12 hours light and dark), with temperature of 22 ± 2°C and were provided food and water ad libitum. Preliminary data including body composition, tissue weights, glucose and insulin tolerance tests, and serum insulin levels for this cohort of GHA and WT mice are shown in section 2.6 of this thesis. All procedures were approved by the Ohio University Institutional Care and Utilization Committee.

3.2: Body composition analysis

The day before dissection, a quantitative NMR machine (Minispec, Bruker Optics, Billerica, MA or Echo MRI whole body composition analyzer; Echo Medical Systems, Houston, TX) was used to analyze body composition of mice used in Aim 1 as previously described (Palmer, et al. 2009).
3.3: Tissue collection

For Aims 1 & 2, the mice were anesthetized with CO₂, bled (blood was collected via retro-orbital bleed) and euthanized via cervical dislocation. Blood samples were maintained at room temperature for 20 minutes, then centrifuged at 4000g for 10 minutes at 4 °C to collect the serum. Serum samples were stored at -80°C. Subcutaneous, epididymal, retroperitoneal, and mesenteric adipose depots were dissected and weighed. For Aim 1, the WT WAT depots were placed in 50 mL conical tubes containing 15 mL Hank’s Balanced Salt Solution (HBSS) (HyClone, HBSS/Modified, no calcium, magnesium) kept on ice for further processing as described in the subsequent section. For Aim 2, the GHA adipose depots were flash frozen in liquid nitrogen and stored at -80 °C until further processing.

3.4: SVF isolation (Aim 1)

Tubes with adipose depots in Hank’s HBSS were inverted several times to wash the tissues. The tissues and HBSS were poured into a petri dish where the tissues were minced into small pieces using dissecting scissors. The minced tissue was transferred to conical tubes on ice. A solution of HBSS with 2 mg/mL collagenase (Sigma Life Science, Collagenase from Clostridium histolyticum, ≥ 125 CDU/mg solid, Lot # SLBL6040V) was prepared and added to each depot in an equal volume-to-volume ratio. The tubes were removed from ice and placed in a shaking incubator set at 37° C and 150 rpm. The tissues were allowed to digest approximately 30 minutes. The tubes were removed and placed on ice immediately once all the tissue appeared to be digested.

Following complete digestion of tissues, floating adipocytes were skimmed from the surface using a plastic pipette and transferred to 1.5 mL microcentrifuge tubes on ice.
The HBSS solution in the tubes contain the SVF cells. This solution was poured through a 100 µm cell strainer (Fisher Scientific, Falcon, catalog #08-771-19) into a clean 50 mL conical tube. The conical tubes were then centrifuged at 4°C, at approximately 4,500 x g for 10 minutes to pellet the SVF cells at the bottom. While waiting for centrifugation of the SVF, Trizol reagent (Thermo Fisher Scientific, Life Technologies) was added to the adipocytes in a 3:1 (Trizol:cells) ratio. The tubes were then vortexed briefly and frozen at -80°C.

The tubes containing the SVF were removed from the centrifuge. The supernatant was decanted, being careful not to disturb the pellet of cells collected in the bottom. The tubes were then tapped gently to dislodge the pellet and resuspend the cells. The small amount of supernatant remaining (along with the suspended cells) was transferred to 1.5 mL microcentrifuge tubes. To remove any excess cells attached to the bottom of the conical tube, 600 µL of Trizol reagent was added to the conical tubes, followed by brief pipetting up and down. This solution was added to the microcentrifuge tubes containing the stromal vascular cells. Finally, the tubes were vortexed briefly and then frozen at -80°C. For more information on the SVF isolation protocol used, see Appendix A.

3.5: Whole tissue preparation (Aim 2)

To prepare whole tissue for RNA isolation, collection and homogenization steps were performed as follows. Approximately 150 mg sections of frozen GHA mouse WAT from subcutaneous, epididymal, retroperitoneal, and mesenteric depots was collected and submerged in 200 µL Trizol reagent in 1.5 mL microcentrifuge tubes (Life Technologies, Fisher Scientific) to prepare them for RNA isolation.
3.6. RNA isolation

To prevent contamination by RNases, the entire work area, gloves, and all utensils were sprayed consistently with RNase AWAY (Molecular BioProducts, Fisher Scientific). Isolating the RNA from the adipocytes and SVF cells (Aim 1) as well as the tissue samples (Aim 2) was done in a similar manner. For Aim 1, the frozen adipocytes and SVF in Trizol was utilized. For Aim 2, 150 mg portions of the frozen GHA AT were used for RNA isolation.

For Aim 2, the tissues in microcentrifuge tubes with 200 µL Trizol (as described in the previous section) were homogenized using a handheld homogenizer (Kontes, Pellet Pestle Motor) with detachable tips. From this point, the RNA isolation procedure was the same for Aims 1 and 2 with the exception that for Aim 1 samples were first thawed before proceeding.

Tubes were vortexed at maximum speed for about 5 min, and then were centrifuged for 5 min at 17,000 x g at 4º C. These settings were maintained throughout the procedure. Following centrifugation, a 1 mL syringe with a 22G x 1 ½ (0.7 mm x 40 mm) needle was used to remove the Trizol layer, being careful to avoid touching the clear/white lipid layer floating on top (this lipid layer is present in the whole tissue samples used in Aim 2, as well as the adipocyte samples from Aim 1, but not in the SVF samples). This layer was then transferred to a new tube. Two hundred microliters of chloroform was added, the samples were vortexed, and finally centrifuged for 15 min. Using a 200 µL micropipette, the clear aqueous layer on top of the samples was removed, being careful not to touch the protein layer (white and cloudy) or the Trizol layer. It was then transferred to a new tube. To the new tubes, 500 µL isopropanol was added, which
was followed by vortexing. The samples were kept at room temperature for 10 min, followed by 10 min centrifugation. The supernatant was then poured off into a new tube, being careful not to pour out the pellet. Ice cold 70% ethanol was then used to complete a series of three washes. During each wash, 1 mL ethanol was pipetted into the tubes, followed by a brief vortex, then a 15 min centrifugation. The supernatant was again poured off. This process was completed twice more. Following this, the supernatant was poured off a final time, and the tubes were tapped gently on an RNase AWAY-sprayed paper towel to remove excess ethanol. The tubes were then allowed to dry upside-down for at least one hour. Finally, the RNA pellet was dissolved in 25 µL molecular grade water (DNAase-, RNAase, and proteinase-free water, Sigma-Aldrich) by gently pipetting up and down. RNA quantity and quality were analyzed using the Nanodrop 2000 UV/Vis spectrophotometer (Thermo Fisher Scientific). RNA samples utilized for cDNA synthesis had minimum concentration of 100 ng/µL and 260/280 and 260/230 ratios of approximately 1.80 to 2.00. For more details on the RNA isolation protocol used, see Appendix B.

3.7: Synthesis of cDNA from isolated RNA

RNA isolated from SVF cells, adipocytes, and whole AT was converted to cDNA using the Thermo Scientific Maxima First Strand cDNA Synthesis Kit for RT-qPCR. In brief, 4 µL 5X Reaction Mix and 2 µL Maxima enzyme were combined with a volume of RNA normalized to concentration for each sample. Components were mixed in microcentrifuge tubes on ice, followed by the addition of molecular grade water to bring the total volume to 25 µL. The tubes were incubated for 10 min at 25°C, then for 15 min at 50°C. The reaction was then terminated by heating at 85°C for 5 min.
3.8: qPCR analysis

qPCR was performed using the Thermo Scientific Maxima SYBR Green/Fluorescein qPCR Master Mix (2x) kit. The Bio-Rad iCycler iQ Real Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA) was utilized to run the reaction. In brief, the reaction master mix was first prepared by combining 12.5 µL of the Maxima SYBR Green/Fluorescein qPCR Master Mix (2X), 0.3 µM of both the forward and reverse primers, and nuclease-free water to bring the reaction volume to 25 µL. These components were mixed thoroughly then added to the PCR plate wells, followed by 5 µL of 1:15 diluted template cDNA. The thermal cycling conditions were as follows: initial denaturation (50°C, 2 min) and then 40 cycles of denaturation (95°C, 15 s), annealing (60°C, 30 s), and extension (72°C, 30 s). Primers were utilized to measure the expression of GHR, IGF-1R, and IR, as well as the reference genes eukaryotic translation elongation factor 2 (EEF2) and beta-2-microglobulin (B2M). For Aim 1, primers for F4/80 and adiponectin (ADIPOQ) were used to ensure adequate separation of the SVF and adipocyte portions. All qPCR primers were purchased from Sigma-Aldrich. qPCR primer sequences are given in table 6. Threshold count (Ct) values were obtained from the iCycler instrument and converted to relative quantities using the equation $2^{(\text{minimum Ct value of all samples})-(\text{Ct value of specific sample})}$. The normalization factor was then calculated based on the geometric mean of the housekeeping genes.
Table 6. Sequences of qPCR primers used.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Forward Sequence (5’ to 3’)</th>
<th>Reverse Sequence (5’ to 3’)</th>
</tr>
</thead>
<tbody>
<tr>
<td>GHR</td>
<td>GCCTGGGGACAAGTTCTTCTGGA</td>
<td>TGCAGCTTGTGCTTTGCTTTCCC</td>
</tr>
<tr>
<td>IGF-1R</td>
<td>AGAACCGAATCATCATAACG</td>
<td>TTTTAAATGGTGCCCTCTTG</td>
</tr>
<tr>
<td>IR</td>
<td>CAAACAGATGCCACTAATCC</td>
<td>CTTTGAGACAATAATCCAGCTC</td>
</tr>
<tr>
<td>B2M</td>
<td>CTGGTCTTTCTATATCCTGGCT</td>
<td>CATGTCTCGATCCCAGTAGAC</td>
</tr>
<tr>
<td>EEF2</td>
<td>CGGTACTTTTGATCCCGGCA</td>
<td>TGATGCGTCGAACCACCTTG</td>
</tr>
<tr>
<td>F4/80</td>
<td>CTTTGCTATGGGGCTTCCAGTC</td>
<td>GCAAGGAGGACAGTTTATCGTG</td>
</tr>
<tr>
<td>ADIPOQ</td>
<td>CTCTCCTGGTCTCTTAATCCT</td>
<td>ACCAAGAAGACCTGCATCTC</td>
</tr>
</tbody>
</table>

3.9: ELISA quantification of plasma insulin (Aim 1)

Plasma insulin in 12-month old male WT mouse plasma was quantified in duplicate using an Ultra-Sensitive Mouse Insulin ELISA Kit (Crystal Chem Inc., Downers Grove, IL, catalog #90082) according to the manufacturer’s instructions.
3.10: Statistical measures

All data were reported as mean ± standard error of the mean (SEM). For Aim 1, significant differences in receptor RNA expression among depots and significant differences in receptor expression were determined using a one-way ANOVA followed by a Tukey’s post-hoc test. This statistical test was also used to determine significant differences in adipose tissue depot mass and body composition. For Aim 2, significant differences in expression between GHA and WT were determined using a two-tailed t-test, and where necessary, the Mann-Whitney U test. Statistical analyses were performed using RStudio (Boston, MA). Significant differences were designated by a p value < 0.05.
CHAPTER 4: RESULTS

4.1: Aim 1

The results of the body composition analysis of the 8 male 12-month old mice are shown in figure 12. As expected, the mice possessed more lean mass than fat and fluid mass.

Figure 12. Body composition data for 12 month-old male WT mice. Data are reported as (A) mass in grams and as (B) percentage of body mass. Shown are means ± SEM, n=8 for each group. Means that share a common letter are not significantly different, p>0.05.
Analysis of WAT depot mass showed that the epididymal depot was significantly larger than the subcutaneous, retroperitoneal, and mesenteric depots (figure 13).

![Graph showing WAT depot weights of 12 month-old male WT mice.](image)

**Figure 13.** WAT depot weights of 12 month-old male WT mice. Shown are means ± SEM, n=8 for each group. Means that share a common letter are not significantly different, p>0.05.

While the initial Aim was to compare receptor expression between adipocyte and SVF portions, despite many attempts to isolate quality RNA from adipocyte fractions, RNA sample concentrations remained poor in three out of four of the depots (table 7). Furthermore, many adipocyte samples possessed 260:280 and 260:230 ratios below the cutoff of 1.80. This could be attributed to the high lipid content and fragility of adipocytes, two traits which makes isolating high quality RNA difficult. However, high quality RNA was isolated from the SVF portions. For this reason, a comparison of SVF and whole WAT receptor expression was performed instead using whole WAT from the WT cohort from Aim 2.
Table 7. A comparison of SVF and adipocyte RNA concentrations.

<table>
<thead>
<tr>
<th>Depot</th>
<th>Adipocyte RNA concentration</th>
<th>SVF RNA concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Subcutaneous</td>
<td>48.5±15.1</td>
<td>209.3±12.5</td>
</tr>
<tr>
<td>Epididymal</td>
<td>273.6±35.1</td>
<td>426.4±51.6</td>
</tr>
<tr>
<td>Retroperitoneal</td>
<td>41.7±11.1</td>
<td>177.3±38.1</td>
</tr>
<tr>
<td>Mesenteric</td>
<td>37.5±14.7</td>
<td>247.3±29.1</td>
</tr>
</tbody>
</table>

A comparison of the body composition, WAT weight data, and serum insulin levels determined by ELISA indicate that the group of 12 month-old male WT mice used in this Aim show no significant differences in these parameters from the group of 12 month-old male WT mice used in Aim 2 (these data for mice used in Aim 2 are shown in figures 8, 10, and 11, comparison data shown in table 8).
**Table 8.** Comparison of metabolic parameters of 12 month-old male WT.

<table>
<thead>
<tr>
<th></th>
<th>Aim 1 WT</th>
<th>Aim 2 WT</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fat (g)</td>
<td>7.7±1.9</td>
<td>8.7±1.0</td>
<td>0.35</td>
</tr>
<tr>
<td>Lean (g)</td>
<td>28.0±0.9</td>
<td>26.0±0.7</td>
<td>0.09</td>
</tr>
<tr>
<td>Fluid (g)</td>
<td>6.5±0.23</td>
<td>6.1±0.2</td>
<td>0.22</td>
</tr>
<tr>
<td>Fat (% of body weight)</td>
<td>18.0±3.9</td>
<td>21.0±1.8</td>
<td>0.21</td>
</tr>
<tr>
<td>Lean (% of body weight)</td>
<td>69.0±3.4</td>
<td>65.3±1.8</td>
<td>0.35</td>
</tr>
<tr>
<td>Fluid (% of body weight)</td>
<td>16.0±0.4</td>
<td>15.0±0.1</td>
<td>0.08</td>
</tr>
<tr>
<td>Subcutaneous (g)</td>
<td>0.71±0.2</td>
<td>1.0±0.3</td>
<td>0.88</td>
</tr>
<tr>
<td>Epididymal (g)</td>
<td>1.4±0.2</td>
<td>1.6±0.2</td>
<td>0.52</td>
</tr>
<tr>
<td>Retroperitoneal (g)</td>
<td>0.33±0.1</td>
<td>0.42±0.1</td>
<td>0.28</td>
</tr>
<tr>
<td>Mesenteric (g)</td>
<td>0.59±0.2</td>
<td>0.53±0.1</td>
<td>0.76</td>
</tr>
<tr>
<td>Serum insulin (ng/mL)</td>
<td>1.3±0.7</td>
<td>2.1±0.4</td>
<td>0.25</td>
</tr>
</tbody>
</table>

This group of WT mice used in this aim had a mean serum insulin level (determined by ELISA) of 1.3±0.7 ng/ml. Although the mice showed a large variation in serum insulin, the serum insulin level showed a strong positive correlation with WAT mass as a percentage of body weight ($R^2=0.850$) (figure 14). As it was not possible to do a direct comparison of expression in WAT and SVF from the same mice (due to the large amount of WAT necessary for SVF isolation), this comparison ensured that the two groups of mice utilized were phenotypically similar.
Figure 14. Serum insulin and adipose tissue mass as a percentage of body weight in 12 month-old WT mice.

To ensure adequate separation of adipocytes from SVF, an attempt was made to compare RNA expression of ADIPOQ and F4/80 in these samples, which are considered to be expressed mainly in adipocytes and the SVF respectively. However, too few adipocyte samples possessed sufficient RNA concentration or purity to provide an appropriate comparison of RNA expression of these genes in adipocyte and SVF samples.

The qPCR layout utilized allowed for the comparison of GHR, IGF-1R, and IR RNA among depots (figure 15) and for the direct comparison of RNA expression levels of the three receptors (figure 16). No significant differences were observed in the RNA expression of the receptors among the SVF of the WAT depots. However, IR RNA expression in the SVF of the epididymal depot trended toward being greater than the expression in the SVF of the mesenteric depot (P=0.0834)(figure 15). Note that \( n \) of qPCR analyses do not match \( n \) values of mice, as in some cases it was necessary to
eliminate samples with outlying reference gene RNA expression levels in order to meet reference gene stability requirements. Additional variation in the $n$ of the IGF-1R analyses is due to the IGF-1R RNA being undetectable in some samples.
Figure 15. RNA expression of GHR (A), IGF-1R (B), and IR (C) in SVF of WAT depots of 12 month-old male WT mice. Real-time PCR analysis of GHR (n=6-7), IGF-1R (n=3-5), and IR (n=4-6). Shown are means ± SEM.
The comparison of levels of GHR, IGF-1R, and IR in each depot showed no significant differences (figures 16 and 17).

*Figure 16.* RNA expression of GHR, IGF-1R, and IR in SVF of subcutaneous (A), epididymal (B), retroperitoneal (C), and mesenteric (D) WAT depots of 12 month-old male WT mice. Real-time PCR analysis of GHR (n=6-7), IGF-1R (n=3-5), and IR (n=4-6)). Shown are means ± SEM.
Figure 17. RNA expression of GHRIGF-1R, and IR of whole subcutaneous (A), epididymal (B), retroperitoneal (C), and mesenteric (D) WAT depots of 12 month-old male WT mice. Real-time PCR analysis of GHR (n=5-7), IGF-1R (5-7), and IR (n=5-7). Shown are means ± SEM.

4.2: Aim 2

The background data (including data for body composition, WAT depot weight, plasma insulin, and GTT and ITT) for the groups of GHA and WT mice used in Aim 2
are detailed in section 2.6. The results of the qPCR analysis of GHR, IGF-1R, and IR in subcutaneous, epididymal, retroperitoneal, and mesenteric WAT depots indicated that GHR RNA is expressed significantly more in mesenteric WAT of GHA mice than in WT mice. While this was the only significant difference observed when receptor RNA expression was compared between genotypes, IGF-1R in the subcutaneous depot of GHA mice was trending toward being significantly higher than in WT mice (p = 0.096), as was the case with IR in the mesenteric depot (p = 0.074) (figure 18).
Figure 18. RNA expression of receptors in subcutaneous (A), epididymal (B), retroperitoneal (C), and mesenteric (D) WAT depots of 12 month-old male GHA and WT mice. Real-time PCR analysis of GHR (n=6-8), IGF-1R (n=5-8), and IR (n=6-8), comparison of expression in GHA and WT WAT depots. Shown are means ± SEM. Significant differences in GHA and WT receptor expression are designated with an asterisk, p<0.05.
CHAPTER 5: DISCUSSION AND CONCLUSIONS

Differential hormone receptor expression may contribute to the many depot differences that have been reported in WAT. Therefore, the purpose of this study was to better understand the expression of GH, IGF-1, and insulin receptors in various WAT depots. In Aim 1, we attempted to compare the expression in the SVF versus adipocytes in wild-type animals; however, due to quality issues with the RNA isolated from adipocytes, this Aim instead became a comparison of receptor expression among the various depots in the SVF only. In Aim 2, we hoped to help explain the well-documented differences in depot expansion in GHA transgenic mice. These mice show preferential weight gain in the subcutaneous depots. Below summarizes the results, discusses limitations and describes future studies based on my findings.

5.1: Aim 1

While no significant differences were found in the comparison of the RNA expression of GHR, IGF-1R, and IR in whole WAT of WT mice, a trend toward significance was observed in the SVF expression of IR. More specifically, IR tended to be more highly expressed in the SVF of the epididymal depot than in the SVF of the mesenteric depot. As IR is expressed in several types of SVF cells, including monocytes, macrophages, and neutrophils (Liang, Han et al. 2007; Mauer, Chaurasia et al. 2010); differing cell populations in these depots could account for differences in IR expression. Contrary to this hypothesis are results from a study in younger WT mice (5 month) which examined immune cell populations and found no significant differences in immune cell populations among depots, or in the total number of SVF cells per gram of tissue (Harshman 2012). However, the mice used in this thesis were much older. We know
based on many longitudinal measures of body composition over lifespan in mice of the
C57Bl/6J strain (Berryman, List et al. 2004; List, Palmer et al. 2009; Palmer, Chung et al.
2009; Berryman, List et al. 2010) that adiposity continues to significantly increase with
advancing age in mice from this strain. Therefore, it is not unexpected that age-related
changes in expression of hormone levels will shift with advancing age. Furthermore,
evidence for age-related changes in SVF, specifically in the epididymal depot, is
provided by a study examining SVF populations of old (18-22 month) and young
C57BL/6 mice (3-4 month) (Lumeng, Liu et al. 2011). This study found an increase in
the total number of SVF cells per epididymal depot (that persisted after normalization for
the weight of the depot), as well as a two-fold increase in lymphocytes in the older mice
in this depot (Lumeng, Liu et al. 2011). Thus, the Lumeng et al. study provides evidence
that dramatic changes in the composition of SVF occur as mice age. As SVF populations
change over time, it is likely that the certain SVF cell populations possessing these
receptors are altered over time as well.

In addition to the comparison of receptor RNA expression in SVF, a comparison
of expression in whole WAT was made; however, no significant differences in the
expression among depots were observed. These results differ from previous receptor
RNA expression data in whole WAT in 3 and 9 month-old mice (Miles 2009). In that
previous study with 9 month-old mice, RNA expression of GHR in whole tissue from the
mesenteric and epididymal depots did not differ significantly, but in 3 month-old mice,
GHR was expressed more highly in the epididymal depot compared to the mesenteric and
subcutaneous depots. Furthermore, in the 9 month-old mice, IGF-IR was expressed more
highly in the mesenteric depot compared to the retroperitoneal depot, while IR was
expressed more highly in the mesenteric depot compared to the retroperitoneal and subcutaneous depots. As I did not detect any significant differences in receptor expression among depots in whole WAT of 12 month-old mice, this could suggest that WAT depots' functional differences are a result of proteins besides GHR, IGF-1R, and IR.

5.2: Aim 2

Overall, qPCR analysis of receptor expression between the genotypes revealed few significant differences. In fact, the only significant difference was the higher expression of GHR in the mesenteric depot of the 12 month-old male GHA mice compared to the WT mice. Recall, that mesenteric is the only true visceral depot studied in this thesis; thus, it may respond differently to the reduction in GH-induced signaling. This difference could suggest that, in GHA mice, the competition for GHR binding by the GHA molecule functions to upregulate GHR expression in this WAT depot. Contrary to this hypothesis, a previous study has shown that addition of pegvisomant, the GH antagonist, has no effect on GHR expression when administered to a human hepatoma cell line (Deladoey, Gex et al. 2002). However, it is important to note that this study examined the in vitro effects of the GH antagonist only, allowing for the possibility that it may behave differently in vivo. Furthermore, the lack of differences in GHR RNA expression in all the remaining depots in this thesis suggest that this explanation is, at minimum, tissue dependent. GHR expression has been shown to be regulated differently in different tissues. More specifically, GHR mRNA expression in the kidney of GHA mice is reduced compared to non-transgenic littermates, while it is elevated in the liver (Chen, Chen et al. 1997), indicating that different tissues respond differently to GHA.
An alternative explanation for this result could lie in the differing SVF cell populations of WAT depots. In humans, visceral depots are known to have higher numbers of immune cells, more crown-like structures and secrete more inflammatory cytokines (Fried, Bunkin et al. 1998; Milan, Granzotto et al. 2002; Altintas, Azad et al. 2011). In mice, the mesenteric depot is known to express high levels of monocyte chemoattractant protein-1 (MCP-1), a molecule responsible for the activation and migration of WAT macrophages (Yu, Kim et al. 2006). Thus, the mesenteric depot is likely to be more responsive to the inflammatory state. WAT tissue macrophages, in addition to adipocytes, express the GHR (Lu, Kumar et al. 2010). It is known that 5 month-old WT mice show no significant differences in WAT macrophage numbers in the subcutaneous, epididymal, and mesenteric depots (Harshman 2012). While WAT macrophage populations in GHA mice have not been determined, nor how populations differ between GHA WAT depots, one explanation for the change in GHR expression in this depot is that GHR-expressing SVF cells are more prevalent in this depot in GHA mice.

IR in the mesenteric depot, while not quite reaching significance, was expressed more in GHA than in WT mice. This trend could also be attributed to excess immune cells, such as monocytes, macrophages, and neutrophils in the mesenteric depot, as it is known that these cells undergo IR-induced signaling (Liang, Han et al. 2007; Mauer, Chaurasia et al. 2010). Showing a similar pattern to GHR and IR in the mesenteric depot, IGF-1R expression in the subcutaneous depot approached significance, with GHA mice expressing the receptor more strongly than the WT mice. Male GHA mice have been shown to have decreased expression of IGF-1 compared to WT controls (Coschigano,
Holland et al. 2003); thus, the lack of IGF-1 in GHA mice could be upregulating the IGF-1R in this depot. In addition to the differences in receptor RNA observed between the genotypes in some depots, the depots and receptors that do not show expression differences reveal important characteristics of these mice as well. The lack of significant differences observed might underlie the fact that GHA mice, with reduced levels of GH-induced signaling, do not possess extended lifespans or significant improvements in glucose metabolism as is observed in GHR-/- mice that exhibit ablation of the GHR (Berryman, Lubbers et al. 2014). Lending support to this hypothesis is the fact that in none of the depots did the reduction in GH-induced intracellular signaling in the GHA mice result in significantly altered IR expression levels. This lack of change in IR is supported by work in which it was shown that GHA mice do not exhibit the change in insulin levels as seen in GHR-/- mice (Coschigano, Holland et al. 2003). As reduction in insulin-induced signaling is linked to lifespan extension (Bluher, Kahn et al. 2003), this important difference in GHA and GHR-/- mice may explain why GHA mice do not possess the lifespan extension seen in GHR-/- mice. Considering these points, the data acquired from my work suggest that GHA mice may have metabolic characteristics more similar to WT mice than to GHR-/- and thus the results of inhibition of GH-induced signaling are not adequate to result in lifespan extension and improvements in glucose metabolism in later life.

5.3: Limitations

For Aim 1, it would be of value to verify that the SVF isolation procedure was efficient at separating the SVF cells from the adipocytes. To do this, the expression of markers of adipocytes versus the SVF could be performed. An attempt was made to
gather expression data of several adipocyte- and SVF-specific genes, including ADIPOQ (an adipocyte marker) and F4/80 (a macrophage marker) for purposes of ensuring adequate separation of the SVF and adipocyte portions. Unfortunately, the number of adipocyte samples of adequate concentration and quality did not allow for a direct comparison of the expression level of ADIPOQ and F4/80 in SVF and adipocytes. Although the RNA expression level of these genes was highly variable in the SVF portions, a direct comparison of the expression in corresponding adipocyte and SVF samples would have provided clearer, more convincing results.

The qPCR analysis of IGF-1R was particularly problematic, as the IGF-1R RNA was undetected in many samples. As might be expected, this resulted in small n values and large variation in the RNA expression levels of that gene. As a result, the data generated with IGF-1R, in particular, are questionable. It is not clear why this particular primer set/gene resulted in greater problems as the primer set has been used successfully by others in our laboratory; however, many primer sets can be used with greater success in certain tissues and cells compared to others. Thus, testing of alternative primer sets for IGF-1R should be designed and tested.

As the SVF isolation process required such a large amount of WAT, it was unfortunately not possible to perform a direct comparison of SVF and whole WAT from the control mice. Directly comparing receptor expression in the SVF versus whole tissue would have been valuable in this study although a comparison of SVF to adipocytes, as originally planned, would have been more useful.

Although the control mice were of the same genetic background and possessed no significant differences in several parameters, it is important to recognize that there are
differences among individual mice, even in those of the same age and genetic background. Therefore, it is suggested to interpret this preliminary data as a first step in examining receptor expression in SVF and adipocytes.

5.4: Future studies

As this thesis focused on a comparison of SVF and whole WAT depots in two separate but similar groups of mice, further studies could attempt to optimize the RNA isolation process from adipocytes, or optimize the SVF isolation process to allow for use of less WAT, thus allowing for a direct comparison of whole tissue and SVF. Also, as this thesis examined the RNA expression of receptors and found limited significant differences, it is possible that the same comparison when made at the protein level could produce different results. While it has not been determined how closely GHR, IGF-R, and IR RNA expression correlates with the expression of their corresponding proteins, some studies provide evidence that many genes can have largely varying RNA and protein levels (Tian, Stepaniants et al. 2004; Lundberg, Fagerberg et al. 2010). This comparison would only be feasible for IGF-1R and IR; this would be challenging for GHR as antibodies to mouse GHR are not commercially available.

Further studies could examine how SVF cell populations differ among GHA and WT WAT depots. Using antibodies specific to SVF cell surface markers, fluorescence activated cell sorting (FACS) could be used to count and sort SVF cell populations. This method has been used effectively in 5 month-old WT and bovine growth hormone (bGH) transgenic mice to reveal differences in SVF cell populations in the two genotypes and has shown significant depot and genotype effects (Harshman 2012). Thus, the SVF composition likely varies in the GHA line as well. This method, applied to GHA mice,
could give important insight into why GHA mice show differences in receptor expression in the mesenteric depot.

Age is another variable that is likely important. The GHA and WT mice have no difference in lifespan (Coschigano, Holland et al. 2003). However, there is evidence that many of the variables tested longitudinally in these mice change with advancing age. For example, leptin and glucose tolerance increase with advancing age in GHA male mice relative to controls (Berryman, Lubbers et al. 2014). Therefore, it is likely that the age of the mice will impact the tissue results presented in Aim 2. In this study, we used 1 year old mice. One year old male GHA mice are severely obese by this age but have no major changes yet to glucose homeostasis (Berryman, Lubbers et al. 2014).
REFERENCES


epicardium induced by mesenchymal transformation and PPARγ activation."

Proceedings of the National Academy of Sciences 112(7): 2070-2075.


APPENDIX A: SVF ISOLATION

Protocol adapted from (Harshman 2012)

Materials:

• Hank’s Balanced Salt Solution (HBSS) (HyClone, HBSS/Modified, no calcium, magnesium)
• Collagenase (Sigma Life Science, Collagenase from Clostridium histolyticum, > 125 CDU/mg solid, Lot # SLBL6040V)
• 50 mL conical tubes
• 1.5 mL Eppendorf tubes
• 100 µm cell strainers (Falcon Cell Strainers, Fisher Scientific)

Procedure:

1. The day of the dissection, prepare a stock of 4 mg/mL collagenase/HBSS solution—keep on ice.
2. Dissect and weigh adipose tissue depots and place in 10 mL cold HBSS.
3. Pour tissue into petri dish (avoiding pouring out too much solution with it) and cut into small pieces with dissection scissors before putting into 15 mL tubes containing HBSS & collagenase (3 mL/1.2 g adipose tissue).
4. Wrap with parafilm and place in incubator set at 37 °C at 150 rpm. Incubate for 20-30 min, and remove immediately when no visible tissue pieces remain. Place on ice to stop reaction.
5. Skim floating adipocytes using plastic pipette, place in Eppendorf tubes on ice to allow for further separation.
6. Filter remaining SVF portion through 100 µm strainers into 50 mL conical tubes.
7. Use 200 µL pipet to remove SVF solution separated from adipocytes on ice. Filter this SVF through the 100 µm filter into the 50 mL tubes.
8. Spin 50 mL tubes containing SVF for 10 minutes at 4 C, 260 rcf.
9. While SVF is in centrifuge, add 1 mL Trizol to adipocytes in Eppendorf tubes.
10. Remove SVF tubes from centrifuge, pour off supernatant, and tap to resuspend cells. Using 1000 µL pipet, transfer to Eppendorf tubes. Add 1 mL of Trizol to the 50 mL conical tubes, pipet up and down to collect any cells remaining in tube, then transfer this to the Eppendorf tubes and freeze at -80°C.
APPENDIX B: RNA ISOLATION

Protocol adapted from (Lubbers 2012)

Note: This protocol describes RNA isolation from both whole adipose tissue and adipocytes. It is noted where the two procedures differ.

Materials:
- Cells frozen in Trizol or whole adipose tissue frozen at -80ºC
- Trizol reagent (Thermo Fisher Scientific, Life Technologies) (at 4ºC)
- Chloroform (at RT)
- Isopropyl alcohol (at RT)
- Ice cold ethanol (at -20ºC)
- Molecular grade water (at RT)
- Syringes and needles
- RNAse/DNAse-free pipette tips
- Eppendorf tubes (RNAse/DNAse-free)
- RNAse Away Decontamination Reagent (Thermo Fisher Scientific, Life Technologies)

To prevent degradation of RNA by RNAses, thoroughly clean work area and all tools (including gloves, homogenizer tips, pipettes, and reagent tubes) with RNAse Away.

Procedure:
1. Add a 100-150 mg piece of frozen adipose tissue to Eppendorf tubes containing 200 µL Trizol reagent. Be careful not to allow the tissue to thaw.
2. Using a handheld homogenizer and homogenizer tips, homogenize adipose tissue in Trizol reagent until the contents are uniform.
3. Add an additional 800 µL Trizol reagent to the Eppendorf tubes and briefly vortex.
4. At this point, the procedures for isolating RNA from whole adipose tissue and adipose tissue cells become the same. After thawing the Eppendorf tubes containing adipose tissue cells and Trizol on ice, briefly vortex and centrifuge for 5 min at 4ºC, 17xg (these settings will be maintained throughout the procedure).
5. Following centrifugation, a lipid layer should float at the top of the Eppendorf tubes (NOTE: This is not present when isolating from stromal vascular cells). Use a syringe and needle to remove the pink, bottom layer, avoiding touching the floating lipid layer.
6. Add 200 µL chloroform and vortex. Centrifuge again for 15 min.
7. Using a 200 µL pipette, transfer the clear, top layer to a new, labelled tube, being careful not to touch the bottom Trizol layer. The Trizol in the first set of tubes may then be discarded appropriately.
8. To the new set of tubes, add 500 µL isopropyl alcohol and vortex. Allow tubes to remain at room temperature for 5-10 min, then centrifuge for 10 min. During this centrifugation step, it is helpful to place all the tubes in the centrifuge in the same orientation, so it is easier to find the location of the RNA pellet.
9. At this point, an RNA pellet will have formed at the bottom of the Eppendorf tube. Pour out the supernatant, being careful not to disturb the RNA pellet.

10. Add 1 mL ice-cold ethanol to the Eppendorf tubes and vortex. Centrifuge for 15 min. Repeat this step two more times to clean the RNA pellet.

11. After the final ethanol centrifugation, pour out the supernatant, tap gently on a paper towel sprayed with RNase Away, and orient upside down, leaning against a pipette tip box so as to allow for airflow. Allow to dry for 1 hour.

12. Suspend in 25 µL water by gently pipetting up and down. Freeze at -20ºC.