2011

Role of Tyk2 in the Development of Beige Cells

Samantha Umali

Virginia Commonwealth University

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ROLE OF TYK2 IN THE DEVELOPMENT OF BEIGE CELLS

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

By

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B.S., University of Virginia, 2008

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Richmond, Virginia
August, 2011
ACKNOWLEDGEMENTS

I would like to express my utmost gratitude to the following people for their support and assistance during the past two years. The contributions of you all were instrumental in forging the strength and resolve needed to achieve this illustrious goal in my postgraduate career.

Dr. Andrew Larner, thank you for your personal guidance, support, and knowledge that helped to navigate me through my Master’s. Without your help, the success of my achievements would not have been as rewarding. Thank you for your incredible patience in explaining the research material and helping me understand how meaningful these studies are to the field of science.

Dr. Keith Baker and Dr. Daniel Conrad, thank you for your expertise, precious advice, and great insights as members of my committee.

I would like to thank all members of the Larner Lab—Marta Derecka, Jenny Sisler, Vidisha Raje, Qifang Zhang, Magdalena Szelag Morgan, Karol Szczepanek, and Catherine Koebel Stromberg—for your help, support, and feedback in assisting me in my research endeavors. Thank you for encouraging me and extending your helping hands whenever I needed it.
To my family and friends, thank you for all the love, support, and caring words that helped me realize my potential. Throughout all of this, I knew you were all rooting for me, and that reassurance helped out immensely.
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<table>
<thead>
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<th>Description</th>
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<tbody>
<tr>
<td>(^{18}\text{F-FDG})</td>
<td>(^{18}\text{F-fluorodeoxyglucose})</td>
</tr>
<tr>
<td>A-MuLV</td>
<td>Abelson murine leukemia virus</td>
</tr>
<tr>
<td>ADP</td>
<td>Adenosine diphosphate</td>
</tr>
<tr>
<td>aP2</td>
<td>Adipose protein 2</td>
</tr>
<tr>
<td>ATP</td>
<td>Adenosine triphosphate</td>
</tr>
<tr>
<td>BAT</td>
<td>Brown adipose tissue</td>
</tr>
<tr>
<td>BMI</td>
<td>Body mass index</td>
</tr>
<tr>
<td>cAMP</td>
<td>Cyclic adenosine monophosphate</td>
</tr>
<tr>
<td>CC</td>
<td>Coiled-coil</td>
</tr>
<tr>
<td>C/EBP</td>
<td>CCAAT/enhancer binding protein</td>
</tr>
<tr>
<td>Cidea</td>
<td>Cell death-inducing DNA fragmentation factor alpha-like effector A</td>
</tr>
<tr>
<td>COX2</td>
<td>Cyclooxygenase 2</td>
</tr>
<tr>
<td>Cox8b</td>
<td>Cytochrome c oxidase, subunit VIIIb</td>
</tr>
<tr>
<td>CRP</td>
<td>C-reactive protein</td>
</tr>
<tr>
<td>CT</td>
<td>Computed tomography</td>
</tr>
<tr>
<td>CtBP</td>
<td>C-terminal binding protein</td>
</tr>
<tr>
<td>C-terminus</td>
<td>Carboxyl-terminus</td>
</tr>
<tr>
<td>db</td>
<td>Diabetic</td>
</tr>
<tr>
<td>DBD</td>
<td>DNA-binding domain</td>
</tr>
<tr>
<td>Dio2</td>
<td>Type II iodothyronine deiodinase</td>
</tr>
<tr>
<td>DPT</td>
<td>Dermatopontin</td>
</tr>
<tr>
<td>Elovl3</td>
<td>Elongase enzyme of very long chain fatty acids</td>
</tr>
<tr>
<td>En1</td>
<td>Engrailed-1</td>
</tr>
<tr>
<td>epidWAT</td>
<td>Epidydimal white adipose tissue</td>
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<tr>
<td>FADH2</td>
<td>Flavin adenine dinucleotide</td>
</tr>
<tr>
<td>fa</td>
<td>Fatty</td>
</tr>
<tr>
<td>FFA</td>
<td>Free fatty acids</td>
</tr>
<tr>
<td>FoxC2</td>
<td>Forkhead box C2</td>
</tr>
<tr>
<td>GAS</td>
<td>Gamma interferon-activated site</td>
</tr>
<tr>
<td>GDP</td>
<td>Guanosine diphosphate</td>
</tr>
<tr>
<td>GLUT4</td>
<td>Glucose transporter 4</td>
</tr>
<tr>
<td>H&amp;E</td>
<td>Hematoxylin and eosin</td>
</tr>
<tr>
<td>HIES</td>
<td>Hyperimmunoglobulin E (HyperIgE) syndrome</td>
</tr>
<tr>
<td>HSL</td>
<td>Hormone-sensitive lipase</td>
</tr>
<tr>
<td>Hoxc9</td>
<td>Homeobox protein Hox-C9</td>
</tr>
<tr>
<td>iBAT</td>
<td>Interscapular brown adipose tissue</td>
</tr>
<tr>
<td>IFN</td>
<td>Interferon</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>Igfbp3</td>
<td>Insulin-like growth factor-binding protein 3</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IMM</td>
<td>Inner mitochondrial membrane</td>
</tr>
<tr>
<td>ingWAT</td>
<td>Inguinal white adipose tissue</td>
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<tr>
<td>ISRE</td>
<td>Interferon-gamma stimulated response element</td>
</tr>
<tr>
<td>JAK</td>
<td>Janus kinase</td>
</tr>
<tr>
<td>JH</td>
<td>JAK homology</td>
</tr>
<tr>
<td>LCMV</td>
<td>Lymphocytic choriomeningitis virus</td>
</tr>
<tr>
<td>LD</td>
<td>Linker domain</td>
</tr>
<tr>
<td>LDL</td>
<td>Low-density lipoprotein</td>
</tr>
<tr>
<td>Lhx8</td>
<td>LIM homeobox 8</td>
</tr>
<tr>
<td>LPL</td>
<td>Lipoprotein lipase</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LXRalpha</td>
<td>Liver X receptor alpha</td>
</tr>
<tr>
<td>MCK</td>
<td>Muscle creatine kinase</td>
</tr>
<tr>
<td>Meox2</td>
<td>Mesenchyme homeobox 2</td>
</tr>
<tr>
<td>MRI</td>
<td>Magnetic resonance imaging</td>
</tr>
<tr>
<td>MSC</td>
<td>Mesenchymal stem cell</td>
</tr>
<tr>
<td>mWAT</td>
<td>Mesenteric white adipose tissue</td>
</tr>
<tr>
<td>Myf5</td>
<td>Myogenic transcription factor 5</td>
</tr>
<tr>
<td>MyHC</td>
<td>Myosin heavy chain</td>
</tr>
<tr>
<td>MyoD</td>
<td>Myogenic factor D</td>
</tr>
<tr>
<td>MyoG</td>
<td>Myogenin</td>
</tr>
<tr>
<td>myomiR</td>
<td>microRNA</td>
</tr>
<tr>
<td>NADH</td>
<td>Nicotinamide adenine dinucleotide</td>
</tr>
<tr>
<td>NK</td>
<td>Natural killer</td>
</tr>
<tr>
<td>NTD</td>
<td>N-terminal domain</td>
</tr>
<tr>
<td>N-terminus</td>
<td>Amino-terminus</td>
</tr>
<tr>
<td>ob</td>
<td>Obese</td>
</tr>
<tr>
<td>p107</td>
<td>Cellular protein 107</td>
</tr>
<tr>
<td>PEPCK</td>
<td>Phosphoenolpyruvate carboxykinase</td>
</tr>
<tr>
<td>PET/CT</td>
<td>Positron emission tomography</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PGC1alpha</td>
<td>PPARgamma co-activator-1 alpha</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>pRb</td>
<td>Retinoblastoma protein</td>
</tr>
<tr>
<td>PRDM16</td>
<td>PRD1-BF-1-RIZ1 homologous domain-containing protein 16</td>
</tr>
<tr>
<td>qPCR</td>
<td>Quantitative polymerase chain reaction</td>
</tr>
<tr>
<td>RIP140</td>
<td>Receptor-interacting protein 140</td>
</tr>
<tr>
<td>rWAT</td>
<td>Retroperitoneal white adipose tissue</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
</tr>
<tr>
<td>---------</td>
<td>--------------------------------------------------</td>
</tr>
<tr>
<td>SH2</td>
<td>Src homology 2</td>
</tr>
<tr>
<td>SNS</td>
<td>Sympathetic nervous system</td>
</tr>
<tr>
<td>STAT</td>
<td>Signal transducer and activator of transcription</td>
</tr>
<tr>
<td>S/V</td>
<td>Surface area to volume</td>
</tr>
<tr>
<td>TAD</td>
<td>Transactivation domain</td>
</tr>
<tr>
<td>Tcf21</td>
<td>Transcription factor 21</td>
</tr>
<tr>
<td>Th</td>
<td>T helper</td>
</tr>
<tr>
<td>TNFalpha</td>
<td>Tumor necrosis factor alpha</td>
</tr>
<tr>
<td>Twist1</td>
<td>Homolog of <em>Drosophila</em> Transcription factor Twist 1</td>
</tr>
<tr>
<td>Tyk2</td>
<td>Tyrosine Kinase 2</td>
</tr>
<tr>
<td>UCP1</td>
<td>Uncoupling protein 1</td>
</tr>
<tr>
<td>WAT</td>
<td>White adipose tissue</td>
</tr>
<tr>
<td>WDNM1-like</td>
<td>Westmead DMBA8 non-metastatic cDNA 1-like</td>
</tr>
<tr>
<td>Zic1</td>
<td>Zinc finger protein of cerebellum 1</td>
</tr>
</tbody>
</table>
ABSTRACT

ROLE OF TYK2 IN THE DEVELOPMENT OF BEIGE CELLS

By Samantha Miranda Umali, M.S.

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2011

Major Director: Andrew C. Larner, M.D., PH.D.
Professor, Department of Biochemistry and Molecular Biology

Obesity results from an excess of adipose tissue and is a major risk factor for type 2 diabetes, cardiovascular disease, and cancer. Adipose tissue exists in two main forms: white adipose tissue (WAT), which stores energy as triglycerides, and brown adipose tissue (BAT), which dissipates stored energy as heat. White adipose tissue is composed of several subcutaneous and visceral depots, each possessing distinct molecular and functional characteristics. Brown-like adipocytes can emerge in WAT depots in response to cold or beta-adrenergic stimulation. These cells have been called “beige” or “brite” (brown-in-white) cells. The reduction of obesity in mice treated with beta-adrenergic agonists is correlated with the emergence of beige cells. Beige cell development occurs
most readily in subcutaneous depots, and to the least extent in visceral depots. Understanding the molecular mechanisms underlying beige cell development in different WAT depots may be important in discovering new therapies against obesity and related diseases.

Our lab has previously discovered that Tyrosine Kinase 2 (Tyk2), an important mediator of cytokine signaling, promotes the development of classical brown adipose tissue. Due to the lack of functional BAT, Tyk2-knockout (Tyk2-/-) mice become grossly obese with age and develop several symptoms of the metabolic syndrome. In the present study, we have found a potential role of Tyk2 in the development of beige cells. Here, we show that mRNA expression of BAT-selective genes (UCP1, Cidea, Cox8b, and Elovl3) is significantly reduced in subcutaneous WAT of Tyk2-knockout (Tyk2-/-) mice compared to wild-type mice. Surprisingly, BAT-selective genes are induced in Tyk2-/- subcutaneous WAT by acute starvation. These findings suggest that Tyk2 is required for the development of beige cells under ambient conditions, and that the need for Tyk2 in beige cell development is bypassed during nutritional stress, a stimulus of the sympathetic response.
CHAPTER 1: INTRODUCTION

1.1 The Obesity Epidemic

Over centuries, humans have evolved an enhanced ability to store energy as fat in order to survive periods of famine. However, in modern societies where energy-dense foods are abundant and available, this ability no longer provides a survival advantage but instead drives unprecedented weight gain. During the last 20 years, obesity has become a worldwide epidemic that continues to grow at an alarming rate. In 2008, 1.5 billion adults age 20 and older were overweight, 700 million of them being obese (World Health Organization, 2010). In 2010, 43 million children under the age of 5 were overweight (World Health Organization, 2010). In the United States, obesity among adults has doubled since 1980, while the number of overweight adolescents has tripled (U.S. Department of Health and Human Services, 2001).

The rising rates of obesity have increased the prevalence of chronic life-threatening diseases, including hypertension, dyslipidemia (high levels of total cholesterol and triglycerides), type 2 diabetes, coronary heart disease, stroke, gallbladder disease, osteoarthritis, sleep apnea, and some cancers (endometrial, breast, and colon) (National Heart, Lung, and Blood Institute, 1998). Obesity is the fifth leading risk for global deaths, causing 2.8 million deaths each year (World Health Organization, 2010). In the United States alone, obesity is responsible for about 300,000 deaths per year and is the second
leading cause of preventable deaths (U.S. Department of Health and Human Services, 2001). In addition to its health impacts, obesity has a significant impact on the economy. In 2008, overall medical costs for obesity-related diseases in the United States were $147 billion, approximately 10% of national health care expenditures (Finkelstein et al., 2009). Furthermore, obesity is often associated with lost work productivity and chronic absence from work (Ferrie et al., 2007).

1.2 Obesity

Obesity is a chronic metabolic disease that is characterized by excess body fat. It develops when energy intake exceeds energy expenditure over time. Obesity plays a significant role in the development of the metabolic syndrome. The metabolic syndrome, also referred to as insulin resistance syndrome or syndrome X, is a constellation of metabolic risk factors that increase the risk for coronary artery disease, stroke, and type 2 diabetes (Grundy, 2005). These risk factors include abdominal obesity, dyslipidemia (high levels of triglycerides, low levels of high-density lipoprotein (HDL) cholesterol), hypertension, insulin resistance, a pro-thrombotic state (elevated levels of clotting factors), and a pro-inflammatory state (increased levels of pro-inflammatory cytokines) (Grundy, 2005).

Obesity can be diagnosed in several ways. The most common measure of obesity is body mass index (BMI). BMI is an indirect measure of total body fat and is calculated by dividing weight by height squared (kg/m²). Individuals with a BMI over 25 kg/m² are considered overweight, while individuals with a BMI over 30 kg/m² are considered obese.
(Centers for Disease Control and Prevention, 2010). Other measures of overweight and obesity include: waist circumference, skinfold thickness, waist-to-hip circumference ratio, and more advanced techniques that directly measure body fat, such as ultrasound, computed tomography (CT), and magnetic resonance imaging (MRI) (Centers for Disease Control and Prevention, 2010). In lean adults, body fat accounts for about 8-18% of body weight in men and 14-28% in women (Wilmore and Costill, 1988). In obese adults, the amount of body fat can increase by fourfold to reach 60-70% of body weight.

The cause of obesity cannot be explained by a single factor alone. Genes, metabolism, behavior, and environment all contribute to the development of obesity (Centers for Disease Control and Prevention, 2010). Family, twin, and adoption studies show that heredity is a strong predictor of obesity (Andersson and Walley, 2011). However, the rapid increase in the prevalence of obesity is most likely due to environmental factors, such as high-fat diets and/or decreased physical activity (Schmidt, 2002). Not only does environment cause obesity, it can also aggravate obesity in individuals genetically predisposed to the disease (Schmidt, 2002). Nevertheless, obesity is a complex problem that results from an interplay between various factors. The complexity of obesity presents a challenge in developing therapies for this disease and demonstrates a clear need to better understand its underlying mechanisms.

1.3 Adipose Tissue

Adipose tissue plays a critical role in regulating the balance between energy intake and energy expenditure. Two main types of adipose tissue exist: white adipose tissue and
brown adipose tissue. White adipose tissue (WAT) stores energy in the form of triglycerides, which are then metabolized into a form that can be used when energy is needed. WAT also functions as an endocrine organ, secreting adipokines—hormones, pro-inflammatory cytokines, and other proteins produced by adipose tissue—that regulate overall metabolism. In contrast to WAT, brown adipose tissue (BAT) oxidizes stored lipids to produce heat, a process called thermogenesis.

WAT and BAT differ widely in their morphology, reflecting their different functional roles. White adipocytes consist of a single, large lipid droplet, minimal cytoplasm, and few small mitochondria with randomly oriented cristae. Brown adipocytes are smaller, contain fewer lipids, which are stored in multilocular rather than unilocular droplets, and possess many large mitochondria densely packed with laminar cristae. Macrocopically, WAT is white to yellowish in color, and BAT is brownish-red due to its abundance of iron-containing mitochondria. Compared to WAT, BAT is more highly vascularized with a dense network of capillaries, and more highly innervated by fibers of the sympathetic nervous system (SNS). BAT has a high oxidative capacity due to enhanced expression of genes involved in fatty acid oxidation and mitochondrial respiration. The most distinguishing feature of BAT is its unique expression of uncoupling protein 1 (UCP1), a 32-kDa protein found in the inner mitochondrial membrane (IMM). BAT thermogenesis is completely dependent on UCP1, which functions primarily in the uncoupling of mitochondrial respiration and ATP synthesis. This uncoupling dissipates the proton gradient across the IMM that is generated by the electron transport chain, releasing chemical energy as heat.
Adipose tissue is located in several discrete areas of the body, which differ somewhat between mouse and human. In rodents, BAT is found primarily between the shoulder blades (interscapular), in the neck (cervical), in the armpits (axillary), around the kidneys (retroperitoneal, or perirenal), and surrounding major arteries in the thoracic cavity (mediastinal, or para-aortic). In humans, BAT is found in the neck, above the clavicle (supraclavicular), adjacent to the vertebrae (paravertebral), around the kidneys, and in the mediastinal and para-aortic regions. In both mice and humans, WAT is found in the abdomen surrounding internal organs (visceral) and underneath the skin (subcutaneous). The main visceral depots are located adjacent to the stomach and spleen (omentum), the intestines (mesenteric), kidneys, epidydimal tubes (epidydimal) or ovaries (periovarian, or parametrial), and the heart (cardiac). Unlike mice, humans do not possess epidydimal WAT. The subcutaneous depots are located primarily in the lower body, with minor amounts found in the upper body. In mice, the lower subcutaneous depot surrounds the pelvis, from the back to the front of the hind legs (inguinal, or posterior), while the upper depots reside between the shoulder blades (interscapular, or anterior), in the armpits (axillary), and in the neck (cervical). In humans, subcutaneous fat exists mainly in the buttocks, hips, and thighs. The relative amount of each adipose depot is not fixed, but varies depending on several factors, including age, sex, environmental temperature, and nutritional status (Cinti, 2009).

Despite the differences in anatomical location, human and murine adipose tissues are morphologically and functionally similar. Based on gene expression, the inguinal depot in mice is considered the equivalent of subcutaneous WAT in humans, whereas the
epidydimal depot, referred to as the “purest” visceral WAT depot in mice, is considered the equivalent of visceral WAT in humans. The features that characterize WAT and BAT are well described in a review by Fruhbeck et al. (2009) and are summarized in Table 1.1.

<table>
<thead>
<tr>
<th>Function</th>
<th>White Adipose Tissue (WAT)</th>
<th>Brown Adipose Tissue (BAT)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Energy storage in the form of triglycerides, lipolysis and lipogenesis, high secretory activity of adipokines</td>
<td>Energy expenditure by heat production (thermogenesis), mitochondrial biogenesis and oxidative phosphorylation, lower fat storage capacity and low secretory activity of adipokines</td>
</tr>
<tr>
<td>Macroscopic features</td>
<td>Subcutaneous, omental, mesenteric, retroperitoneal, epidydimal, and ovarian location</td>
<td>Interscapular, paravertebral, axillary, and perirenal location</td>
</tr>
<tr>
<td></td>
<td>White to yellow in color, adequate vascularization (+++), mainly sympathetic innervation (+)</td>
<td>Brown in color, high vascularization (++++) and sympathetic innervation (++++)</td>
</tr>
<tr>
<td>Microscopic features</td>
<td>Unilocular adipocytes with one single large lipid droplet that occupies 90% of cell volume, variable shape and size (25-200 μm), few, small, elongated mitochondria, high presence of other cell types (e.g., fibroblasts, immune cells)</td>
<td>Multilocular adipocytes with abundant small lipid droplets, polygonal shape and small size (15-60 μm), abundant, large, round mitochondria, low presence of other cell types</td>
</tr>
<tr>
<td>Molecular features</td>
<td>Lack of UCP1 (-) and Cidea (-) expression, low UCP2 (+++), PGC1α (+), PRDM16 (+), Dio2 (+), cytochrome c (+), β3-AR (+), β1-AR (++), α1/2-AR (+) expression, High leptin (+++) and RIP140 (+++), expression</td>
<td>Low leptin (present at birth), RIP140, UCP2 (+), β1-AR (+), α1/2-AR (+) expression, High UCP1 (+++), β3-AR (+++), β1-AR (+), α1/2-AR (+), PGC1α (+), PRDM16 (+++), Cidea (+++), Elov3 (+++), Dio2 (+++), cytochrome c (+++) expression</td>
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Table 1: Characteristics of White and Brown Adipose Tissue (modified from Fruhbeck et al., 2009)

1.4 White Adipose Tissue

White adipose tissue plays a multifaceted role in energy homeostasis. WAT stores energy in the form of triglycerides during excess energy intake (i.e., feeding), releases energy as free fatty acids (FFAs) for other organs during energy need (i.e., fasting), and
secretes adipokines that communicate with other organs to regulate metabolism. White adipose tissue is composed of several depots, categorized as visceral WAT or subcutaneous WAT.

Although visceral and subcutaneous WAT are derived from the same origin, they are functionally different tissues. Visceral WAT and subcutaneous WAT differ by their cellular and molecular composition, lipolytic activity, endocrine function, and response to insulin and other hormones (Ibrahim, 2010). Differences in gene expression underlie these functional differences (Gesta et al., 2006). Compared to subcutaneous WAT, visceral WAT is more cellular, containing more immune cells, inflammatory cells (macrophages), and large adipocytes. Large adipocytes in visceral WAT are less efficient in absorbing and storing fatty acids than small adipocytes found in subcutaneous WAT (Marin et al., 1992). Visceral WAT is more vascularized than subcutaneous WAT, allowing for greater secretory activity of pro-inflammatory cytokines and growth factors, such as resistin, TNFalpha, IL-6, and CRP (Ibrahim, 2010; Manopolopoulos, 2010). Subcutaneous WAT, on the other hand, produces higher levels of adipokines associated with insulin sensitivity, such as leptin and adiponectin. Visceral WAT is more innervated by the SNS, possessing higher levels of beta-adrenergic receptor expression (Wahrenberg et al., 1989). Thus, visceral WAT is more sensitive to catecholamine-induced lipolysis and less sensitive to alpha2- and insulin-dependent lipogenesis (Wahrenberg et al., 1989; Arner et al., 1990; Hellmer et al., 1992; Calle et al., 1999). During noradrenaline stimulation, visceral adipocytes show a four- to fivefold increase in lipolysis when compared to subcutaneous adipocytes (Wahrenberg et al., 1989).
In contrast, subcutaneous WAT consists of smaller adipocytes, which have a greater capacity to absorb circulating fatty acids, a lower secretory activity of adipokines, and a greater sensitivity to insulin-dependent lipogenesis (i.e., low lipolytic activity) (Ibrahim, 2010).

1.5 White Adipose Tissue and Obesity

Obesity results largely from an excess of white adipose tissue. The different types of obesity are characterized by the regional distribution of excess WAT. Excess visceral WAT is referred to as visceral or “apple-shaped” obesity, and an excess of subcutaneous WAT is called subcutaneous or “pear-shaped” obesity. Visceral obesity is a major risk factor of the metabolic syndrome and its related diseases, such as insulin resistance, type 2 diabetes, hyperlipidemia, and cardiovascular disease (Wang et al., 2005; Klein et al., 2007; Fox et al., 2007; Despres et al., 1995; Ross et al., 1996; Rexrode et al., 1998). Individuals with visceral obesity have a metabolic profile characteristic of the metabolic syndrome: higher plasma levels of glucose, insulin, triglycerides, apolipoprotein B-containing lipoproteins, total cholesterol, and low-density lipoprotein (LDL) cholesterol (Despres et al., 1995). Some individuals also display chronic low-grade inflammation caused by an increased secretion of pro-inflammatory cytokines, such as IL-6, IL-1, and TNFalpha (Alvehus et al., 2010).

Unlike visceral obesity, subcutaneous obesity is associated with better overall metabolism and insulin sensitivity in both rodents and humans (Porter et al., 2009; Snijder et al., 2005; Miyazaki et al., 2002; Kim et al., 2007; Klein et al., 2004; Tanko et al., 2003).
Subcutaneous WAT is more responsive than visceral WAT to synthetic PPARgamma agonists, anti-diabetic drugs (Miyazaki et al., 2002). Removal of subcutaneous (inguinal) WAT in mice results in increased lipid accumulation in visceral (mesenteric) WAT, increased plasma insulin levels, decreased insulin sensitivity, and increased TNFalpha. These abnormalities can be corrected by re-implantation of subcutaneous WAT (Ishikawa et al., 2004). Moreover, implantation of subcutaneous WAT into the abdominal cavity of mice improves whole-body metabolism (Hocking et al., 2008, Tran et al., 2008). A clinical study in women showed that the most severe insulin resistance was found in individuals with the highest percentage of visceral WAT and lowest percentage of subcutaneous WAT. Furthermore, the most favorable metabolic profile was found in women with the lowest percentage of visceral WAT and highest percentage of subcutaneous WAT (Tanko et al., 2003).

1.6 Brown Adipose Tissue

Brown adipose tissue is a unique organ found exclusively in mammals. BAT is found abundantly in small mammals, hibernating animals, and human infants. It is estimated that BAT evolved about 150 million years ago, providing mammals with the ability to maintain body temperature. Heat production in response to environmental temperature or diet is referred to as adaptive thermogenesis (Enerback, 2010).

In small mammals, such as rodents, the major depot of BAT is located primarily in the interscapular region, whereas minor amounts exist in the axillary, cervical, mediastinal, and retroperitoneal regions. Interestingly, brown-like adipocytes are also found
interspersed in WAT of adult animals that have been exposed to cold or chronically treated with beta_3-adrernergic agonists. In human infants, BAT is located in the axillary, cervical, perirenal, and peridrenal depots (Cannon and Nedergaard, 2004). At birth, total BAT weighs about 150-250 g (2-5% of total body weight) and virtually disappears within the first years of childhood. This decrease in BAT mass is not a matter of age per se, but a matter of size. Compared to human adults, rodents and neonates have much higher surface-to-volume (S/V) ratios, making them more susceptible to cold. BAT thermogenesis provides smaller animals with a protective mechanism against hypothermia. In response to the cold, energy expenditure (i.e., oxygen consumption) in rodents is increased by two- to fourfold (Cannon and Nedergaard, 2004).

1.7 Beta-Adrenergic Stimulation of Brown Adipose Tissue

The amount and activity of brown adipose tissue are increased by prolonged exposure to cold or beta_3-adrenergic agonists. During periods of cold, temperature information is sensed by thermoceptors in the skin and transmitted to the hypothalamus. The hypothalamus then stimulates sympathetic nerves, which release catecholamines (e.g., norepinephrine) that bind to and activate G-protein coupled beta_3-adrenergic receptors in BAT. G_s alpha activates adenylyl cyclase, which catalyzes the conversion of ATP to cyclic AMP (cAMP). cAMP activates protein kinase A (PKA), which stimulates lipolysis by phosphorylating hormone-sensitive lipase (HSL) and perilipin A. Perilipin A, a lipid droplet-associated protein, coats lipid droplets and protects them from endogeneous lipases, such as HSL. When perilipin A is phosphorylated, it dissociates from lipid
droplets, allowing HSL to bind and begin lipolysis. Activation of HSL, the rate-limiting step of lipolysis, results in the breakdown of triglycerides into glycerol and free fatty acids.

Free fatty acids (FFAs) serve as substrates for thermogenesis. FFAs are converted to molecules of acyl-CoA, which are transported to the mitochondria by the carnitine shuttle. FFAs undergo beta-oxidation in the mitochondrial matrix, generating molecules of acetyl-CoA. Acetyl-CoA enters the tricarboxylic acid (TCA) cycle, leading to the formation of reduced electron carriers, NADH and FADH$_2$. NADH and FADH$_2$ are oxidized as they pass electrons onto components of the electron transport chain, which are subsequently reduced. The energy produced by these reduction-oxidation events is used to pump protons from the mitochondrial matrix to the intermembrane space. This proton motive force is used in most cells to drive the conversion of ADP to ATP by ATP synthase. However, in brown adipocytes, the mitochondrial respiratory chain is uncoupled from ATP synthesis by UCP1, dissipating the proton motive force as heat.

UCP1 is activated by fatty acids and inhibited by purines. Two models have been proposed to explain the mechanistic actions of UCP1. The first model proposes that UCP1 is a true proton transporter, whereas the second model proposes that UCP1 is a fatty acid anion transporter (Skulechev et al., 1991, Garlid et al., 1998). In the first model, UCP1 acts as a typical proton channel—an integral mitochondrial membrane protein that allows the transport of protons across the inner mitochondrial membrane. In the second model, UCP1 transports FFA anions out of the mitochondrial matrix, which become protonated in the acidic intermembrane space. Neutral FFA-H diffuses back across the IMM and dissociates again in the more basic mitochondrial matrix. The net effect is the return of
protons into the matrix. Both models result in the dissipation of the proton gradient and the loss of potential energy as heat.

In addition to lipolysis and heat production via UCP1 activation, cell proliferation and mitochondrial biogenesis are induced by beta-adrenergic stimulation of BAT. In rodents and humans, beta3-adrenoceptors are found abundantly in BAT and are responsible for driving thermogenesis. Figure 1 summarizes the steps required for the activation of BAT thermogenesis (reviewed by Cannon and Nedergaard, 2004).

Figure 1: Beta-Adrenergic Stimulation of BAT Thermogenesis (Cannon and Nedergaard, 2004)
1.8 Brown Adipose Tissue in Human Adults

Until recently, brown adipose tissue was considered to be virtually nonexistent in human adults. Because of size (e.g., smaller S/V ratio), indoor heating, and the use of warm clothing, human adults have a reduced need for BAT to protect themselves from the cold. However, recent studies using PET/CT scans, which monitor glucose uptake using a radiolabeled glucose analogue (\(^{18}\text{F}\)-fluorodeoxyglucose, or \(^{18}\text{F}\)-FDG), have demonstrated that human adults have significant amounts of metabolically active BAT. Adult patients with pheochromocytomas (catecholamine-producing tumors) showing high \(^{18}\text{F}\)-FDG uptake in areas of putative BAT provided the first evidence that humans can develop BAT during adulthood (English et al., 1973; Ricquier et al., 1982; Bouillaud et al., 1983; Lean et al., 1986; Bouillaud et al., 1988). Adult patients with rare BAT tumors called hibernomas have also been documented (Chatterton et al., 2002; Lin et al., 2005; Tsuchiya et al., 2006). More importantly, BAT has been found in healthy human adults (Hany et al., 2002; Cohade et al., 2003; Rousseau et al., 2006; Nedergaard et al., 2007; Cypress et al., 2009; van Marken Lichtenbelt et al., 2009; Virtanen et al., 2009; Saito et al., 2009; Lee et al., 2011). Human BAT is located in several discrete areas, including the supraclavicular, cervical, paravertebral, mediastinal, para-aortic, and perirenal regions. The presence of BAT in human adults has been verified histologically in the same anatomical regions (Saito et al., 2009, Virtanen et al., 2009, van Marken Lichtenbelt et al, 2009; Zingaretti et al., 2009; Lee et al., 2011).
Exposure to cold increases the amount of BAT in humans. Although active BAT is detectable only in 3-7% of adults under thermoneutral conditions, this percentage increases up to 96% when individuals are subjected to a 2-hour cold exposure (van Marken Lichtenbelt et al., 2009). One study showed that individuals during the winter months have greater amounts of BAT than the same individuals during the summer months (Saito et al., 2009; Au-Yong et al., 2009). Another study demonstrated that outdoor workers from Finland have a higher prevalence of BAT surrounding major neck arteries than indoor workers of the same age (Huttunen et al., 1981). These findings suggest that, although the amount of BAT is virtually undetectable in humans, it has a remarkable capacity to expand under certain stimuli, such as the cold.

1.9 Brown Adipose Tissue and Obesity

In recent years, brown adipose tissue has been recognized for its potential and demonstrated anti-obesity properties. In mice, BAT has been shown to protect against obesity, insulin resistance, and type 2 diabetes. In response to overfeeding, BAT burns excess caloric intake to produce heat, a process called diet-induced thermogenesis. Diet-induced thermogenesis was first observed in mice fed “cafeteria” (high-fat, high-sugar) diets (Rothwell and Stock, 1979). Cafeteria-fed mice consumed more calories, but gained less weight than expected from caloric intake. The cafeteria diet stimulated the expansion and activation of BAT, as observed by increased BAT mass, oxygen consumption, respiratory enzyme activity, and UCP1 expression (Rothwell and Stock, 1979; Rothwell and Stock, 1983; Rothwell and Stock, 1986).
Loss of BAT activity, due to the removal of BAT, UCP1 expression, or beta-adrenergic signaling, is associated with genetic and diet-induced obesity in rodents. Strains of genetically obese rodents, such as ob/ob and db/db mice and Zucker fa/fa rats, exhibit decreased sympathetic activity, UCP1 expression, and thermogenesis (Himms-Hagen, 1983). Surgical excision or denervation of BAT causes an accumulation of excess WAT and a reduction in energy expenditure (Dulloo and Miller, 1984). Genetic ablation of BAT via expression of a targeted toxigene results in reduced energy expenditure, diet-induced obesity, and other metabolic problems, such as hyperlipidemia, hyperinsulinemia, and insulin resistance (Lowell et al., 1993). Deletion of UCP1 in mice causes increased weight gain when mice are housed at thermoneutrality. The obesogenic effect observed in UCP1-knockout mice is accelerated under a high-fat diet due to impaired diet-induced thermogenesis (Liu et al., 2003). Like UCP1-knockout mice, mice lacking all three beta-adrenergic receptors (beta-less mice) develop massive obesity under a high-fat diet. Moreover, beta-less mice display a significant reduction in metabolic rate and have brown adipocytes that resemble white adipocytes (e.g., are unilocular and express leptin) (Bachman et al., 2002). In humans, polymorphisms of UCP1 or combined polymorphisms of UCP1 and the beta3-adrenoceptor are associated with obesity and type 2 diabetes (Jia et al., 2010; Nagai et al., 2010; Kim and Lee, 2010).

On the other hand, experimental increases of BAT, UCP1 expression, and adrenergic stimulation are associated with a lean and healthy phenotype in mice. Mice with higher amounts of BAT gain less weight, are more insulin-sensitive, have lower levels of FFAs, and are protected from diabetes and other metabolic dysfunction. Transgenic
mice overexpressing UCP1 are resistant to genetic and diet-induced obesity (Kopecky et al., 1995; Kopecky et al., 1996). Targeted disruption of cell death-inducing DFF45-like effector A (Cidea), a negative regulator of UCP1 activity, results in a lean phenotype (Zhou et al., 2003). Pharmacological treatment of obese rodents with beta$_3$-adrenergic agonists reduces obesity by preventing weight gain and stimulating weight loss (Yen et al., 1984; Munro et al., 1987; Holloway et al., 1992; Largis et al., 1994; Yoshida et al., 1994; Yoshida et al., 1994). These mice also display smaller white adipocytes, larger BAT mass, increased thermogenesis, and the induction of brown adipocytes in WAT (Munro et al., 1987; Holloway et al., 1992).

Altogether, these findings provide strong evidence that diet-induced thermogenesis is a critical mechanism underlying body weight homeostasis. The anti-obesity properties of BAT in rodents and the presence of active BAT in human adults have raised the possibility that increasing the amount and/or activity of BAT might be a useful strategy in treating human obesity. Whether or not human adults exhibit diet-induced thermogenesis is unknown. Nevertheless, recent studies have found that the amount of BAT is inversely correlated with body mass index (i.e., leaner individuals have more BAT than obese individuals) (van Marken Kichtenbelt et al., 2009). Scientists suggest that a relatively small amount of BAT could make a significant impact on energy balance. It is estimated that just 50 g of BAT can raise a person’s daily energy expenditure by 20% (Rothwell and Stock, 1983). Although the physiological role of BAT in humans is debated, the possibility to increase it artificially to treat obesity and related disorders cannot be excluded.
1.10 Development of Adipocytes

Mesenchymal stem cells (MSCs) give rise to a variety of cell types, including adipocytes, myocytes, chondrocytes, and osteoblasts. The exact number of intermediate stages between the MSC and the mature adipocyte is unknown. However, it is proposed that MSCs first give rise to early fat precursors called adipoblasts, which later turn into preadipocytes that are competent to differentiate into mature adipocytes (Cornelius et al., 1994). The development of adipose tissue is orchestrated by a number of transcription factors. The key transcription factors that promote WAT and BAT development are CCAAT/enhancer binding proteins (C/EBPs) and peroxisome proliferator-activated receptors (PPARs).

The C/EBPs are a family of basic-leucine zipper (bZIP) transcription factors, composed of six members C/EBPalpha to C/EBPzeta. C/EBPalpha, C/EBPbeta, and C/EBPdelta are the only isoforms involved in adipogenesis (Wu et al., 1999). C/EBPalpha regulates the transcription of several genes critical for adipose tissue function, such as adipose protein 2 (aP2), glucose transporter 4 (GLUT4), acetyl-CoA carboxylase, and stearyl-CoA desaturase (Darlington et al., 1995). C/EBPbeta and C/EBPgamma induce the expression of PPARgamma and C/EBPalpha, two factors found to be sufficient in inducing adipogenesis in vitro (Farmer, 2006).

The PPARs are a family of nuclear receptors with three members: PPARalpha, PPARbeta, and PPARgamma. PPARgamma is the isoform involved in adipocyte differentiation. PPARgamma is required for normal lipid accumulation and activates the transcription of genes involved in fatty acid binding, uptake, and storage, such as aP2,
lipoprotein lipase (LPL), acyl-CoA synthase, and phosphoenolpyruvate carboxykinase (PEPCK) (Imai et al., 2004). PPARgamma also promotes insulin-dependent glucose uptake by activating GLUT4. For this reason, synthetic PPARgamma agonists, such as thiazolidinediones, are clinically used in the treatment of type 2 diabetes. In brown adipocytes, PPARgamma induces the expression of UCP1 and mitochondrial genes, but only promotes thermogenesis when combined with adrenergic stimulation (Hansen and Kristiansen, 2006; Villarroya et al., 2007).

1.11 Development of Classical Brown Adipocytes

White and brown adipocytes have long been assumed to share a common developmental origin due to their common expression of genes involved in fat metabolism. However, recent studies have revealed that brown adipose tissue shares a common developmental origin with skeletal muscle, not white adipose tissue. The link between BAT and skeletal muscle was first observed by lineage tracing using the homeobox gene Engrailed-1 (En1) (Atit et al., 2006). Cells expressing En1, which are derived from the dermamyotome, give rise to dermis, skeletal muscle, and BAT (Atit et al., 2006). Microarray analyses have shown that muscle-specific mRNAs, such as myogenic factor D (myoD), myogenin (myoG), and muscle-specific microRNAs (myomiRs) are expressed in brown adipocytes (Timmons et al., 2007; Walden et al., 2009). In vivo fate mapping has demonstrated that myogenic transcription factor 5 (Myf5) is expressed in precursors that develop into skeletal muscle and BAT, but not WAT (Seale et al., 2008). These findings strongly suggest that BAT is more closely related to skeletal muscle than WAT.
PRD1-BF-1-RIZ1 homologous domain-containing protein 16 (PRDM16) has recently been described as the master regulator of BAT development (Seale et al., 2008). PRDM16 was first identified as a protein involved in human leukemic translocations (Moir et al., 1984; Bloomfield et al., 1985; Welborn et al., 1987; Secker-Walker et al., 1995). A genome-wide analysis of transcription factors revealed that PRDM16 is involved in the expression of the BAT phenotype in vivo and in vitro (Seale et al., 2007). Ectopic expression of PRDM16 in skeletal muscle progenitors induces the differentiation of brown adipocytes and simultaneously suppresses the development of skeletal muscle (Seale et al., 2008). Moreover, overexpression of PRDM16 induces mitochondrial biogenesis, activation of thermogenic genes, and cAMP-mediated uncoupled respiration (Seale et al., 2008). On the other hand, loss of PRDM16 from BAT precursors results in skeletal muscle development and reduced expression of BAT-selective genes (Seale et al., 2007; Seale et al., 2008). From these studies, it was concluded that PRDM16 controls a bidirectional switch between brown adipocytes and skeletal myocytes. However, recent studies have demonstrated that PRDM16 also regulates the development of brown-like adipocytes in white adipose tissue (Seale et al., 2007; Seale et al., 2010). Transgenic expression of PRDM16 induces the expression of BAT-selective genes and the development of UCP1-expressing multilocular and unilocular cells in subcutaneous WAT (Seale et al., 2010).

Bone morphogenic protein 7 (BMP7) also plays a key role in brown adipogenesis. BMP7 can activate the full thermogenic program of brown adipose tissue (Tseng et al., 2008; Schultz et al., 2011) by inducing the transcription of early regulators of BAT.
differentiation: PRDM16, PGC1alpha, UCP1, PPARgamma, and C/EBPs (Tseng et al., 2008). Moreover, BMP7 induces mitochondrial biogenesis (Tseng et al., 2008).

1.12 Development of Beige Cells

Clusters of brown-like adipocytes can be found interspersed in white adipose tissue (Enerback, 2009; Frontini and Cinti, 2010). Brown-like adipocytes in WAT, also known as “beige” or “brite” (brown-in-white) cells (Ishibashi and Seale, 2010; Petrovic et al., 2010), emerge in response to beta-adrenergic stimulation by cold or beta3-adrenoceptor agonists (Cousin et al., 1992; Collins et al., 1997; Ghorbani and Himms-Hagen, 1998; Guerra et al., 1998; Himms-Hagen et al., 2000; Xue et al., 2005; Seale et al., 2008) or by PPARgamma stimulation (Petrovic et al., 2010; Wilson-Fritch et al., 2004; Fukui et al., 2000; Puri et al., 2008; Vernochet et al., 2009). Beige cells contain the same hallmarks of classical brown adipocytes, including multilocular lipid storage, abundant mitochondria, expression of UCP1 and other thermogenic genes (Cidea, Elovl3, PGC1alpha, and PPARalpha), and thermogenic activity (Petrovic et al., 2010).

Despite their similarities to classical brown adipocytes, beige cells appear to derive from a different origin. Beige cells do not have the same gene expression profile as classical brown adipocytes, including the expression of transcription factors highly expressed in BAT, such as Zic1, Lhx8, Meox2, and PRDM161 (Timmons et al., 2007; Petrovic et al., 2010; Schulz et al., 2011), and muscle-specific genes, such as myogenin,

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1 PRDM16 and Zic1 were not expressed in beige cells from epidydimal WAT (Timmons et al., 2007; Schulz et al., 2011), but were expressed in inguinal WAT (Seale et al., 2011; Schulz et al., 2011).
myoD, and myomiRs (Timmons et al., 2007; Walden et al., 2009; Petrovic et al., 2010). Moreover, beige cells do not express Myf5, a molecular marker for cells of the skeletal muscle/brown adipose tissue lineage (Timmons et al., 2007; Seale et al., 2008). They do, however, express known classical WAT genes, such as Igfbp3, Tcf21, DPT, and Hoxc9 (Timmons et al., 2007; Petrovic et al., 2010; Schulz et al., 2011).

The increased development of beige cells appears to reverse or protect against obesity in rodents. Mouse strains resistant to diet-induced obesity, such as the A/J strain, exhibit increased expression of beige cells (Collins et al., 1997). Transgenic mice expressing PRDM16, which promotes beige cell development in subcutaneous WAT, are protected from obesity and metabolic dysfunction (Seale et al., 2011). The induction of beige cells is correlated with the reduction of obesity in mice treated with beta3-adrenergic agonists (Collins et al., 1997; Ghorbani and Himms-Hagen, 1998). In humans, the emergence of beige cells in subcutaneous WAT is correlated with increased insulin sensitivity (Yang et al., 2003; Timmons and Pedersen, 2009). For example, insulin-resistant patients exhibit reduced expression of BAT-selective genes in subcutaneous WAT (Yang et al., 2003). Beige cells are detected by a large induction of UCP1 at the mRNA level and a corresponding increase in UCP1 protein, as revealed by immunohistochemistry. In both lean and obese patients, UCP1 expression has been found in biopsies from visceral WAT (Oberkofler et al., 1997). It is estimated that WAT in humans contain about one brown-like adipocyte for every 100-200 white adipocytes (Oberkofler et al., 1997).

Inguinal WAT, a type of subcutaneous WAT, is more susceptible to “browning” than epidydimal WAT, a visceral depot. Immunohistochemistry shows that inguinal WAT
contains more clusters of UCP1-positive multilocular cells than epidydimal WAT, which is almost entirely made of UCP1-negative unilocular cells (Guerra et al., 1998; Petrovic et al., 2010; Seale et al., 2011). These morphological differences are likely due to differences in gene expression. BAT-selective genes, such as UCP1, PRDM16, Cidea, Cox8b, and Elovl3, are highly expressed in inguinal WAT and found at low or undetectable levels in epidydimal WAT (Seale et al., 2011).

Recently, several factors have been shown to influence the beige cell phenotype, including PGC1alpha, FoxC2, COX2, pRb, p107, RIP140, LXRalpha, and Twist1.

PPARgamma co-activator-1 alpha (PGC1alpha) is a PPARgamma-interacting protein found abundantly in highly oxidative tissues, including BAT, slow twitch muscle fibers, heart, brain, and liver. Exogenous addition of PGC1alpha via adenovirus in human white adipocytes from the subcutaneous depot induces UCP1 expression by increasing the transcriptional activity of PPARgamma on the UCP1 promoter (Tiraby et al., 2003). PGC1alpha also activates the expression of mitochondrial enzymes of the respiratory chain and increases mitochondrial biogenesis (Puigserver et al., 1998; Uldry et al., 2006). Interestingly, brown preadipocytes lacking PGC1alpha are still capable of differentiating (Uldry et al., 2006). Mice lacking PGC1alpha exhibit low UCP1 expression and cold sensitivity (Lin et al., 2004).

Forkhead box C2 (FoxC2) is a key regulator of adipocyte metabolism. In mice overexpressing FoxC2 in WAT and BAT, WAT was reduced and had acquired a BAT-like histology, whereas BAT was hypertrophic (Cederberg et al., 2001). Moreover, UCP1 was induced in WAT. These transgenic mice expressing FoxC2 were protected against diet-
induced obesity and insulin resistance. In wild-type mice fed a high-fat diet, FoxC2 mRNA levels were upregulated and worked to counteract diet-induced obesity (Cederberg et al., 2001). Mice expressing only one FoxC2 allele had decreased BAT mass (Cederberg et al., 2001). In insulin-resistant patients, the expression of FoxC2, along with BAT-selective genes, is reduced in subcutaneous WAT (Yang et al., 2003).

pRB is a member of the repressors of E2F (Rb) family of transcription factors, which play key roles in the regulation of the cell cycle and the differentiation of many tissues. pRB is differentially regulated during adipocyte differentiation (Hansen et al., 2004). Mouse fibroblasts lacking pRB differentiate into mitochondria-rich brown adipocytes with a gene expression pattern characteristic of BAT (Dali-Youcef et al., 2007).

p107, another member of the Rb family, has been implicated in the development of beige cells. Mice lacking p107, another member of the Rb family, have WAT depots that contain multilocular adipocytes expressing elevated levels of thermogenic genes, PGC1alpha and UCP1 (Scime et al., 2005). Upon cold exposure, pRB is inhibited via phosphorylation, which results in the induction of UCP1 expression in BAT (Scime et al., 2005). Treatment of white adipocytes with a beta3-adrenoceptor agonist downregulates pRB expression, resulting in the transformation of WAT to BAT (Scime et al., 2005).

RIP140, a transcriptional co-repressor for nuclear receptors, acts as a negative regulator of whole-body glucose tolerance and energy expenditure. Mice lacking RIP140 are lean, show resistance to diet-induced obesity and hepatic steatosis, and have increased oxygen consumption (Leonardsson et al., 2004). Expression of genes involved in energy
dissipation and mitochondrial uncoupling (UCP1) is markedly increased (Leonardsson et al., 2004).

Liver X receptor alpha (LXRalpha) is a direct transcriptional inhibitor of UCP1 expression. LXRalpha recruits RIP140, a transcriptional co-repressor for nuclear receptors, and together they bind to the PPARgamma/PGC1alpha response element of the UCP1 promoter (Wang et al., 2008).

Twist1 suppresses mitochondrial metabolism and uncoupling by inhibiting the binding of PGC1alpha to the promoters of its target genes. Mice expressing only one functional Twist1 allele are obesity-resistant and exhibit altered mitochondrial metabolism in BAT (Pan et al., 2009). Transgenic mice expressing Twist-1 in adipose tissue are prone to diet-induced obesity (Pan et al., 2009).

Cyclooxygenase 2 (COX2) is a rate-limiting enzyme in prostaglandin synthesis. COX2 has recently been identified as a downstream effector of beta-adrenergic signaling in WAT and is required for the induction of beige cells and UCP1 expression (Vegiopoulos et al., 2010; Madsen et al., 2010). Overexpression of COX2 in WAT results in beige cell development, increased energy expenditure, and protection against diet-induced obesity (Vegiopoulos et al., 2010). Moreover, increased prostaglandin levels in mesenchymal progenitors shift differentiation toward a brown adipocyte phenotype (Vegiopoulos et al., 2010).

Figure 2 summarizes the differentiation pathways of white, brown, and beige adipocytes from mesenchymal stem cells.
Figure 2: Differentiation of White, Brown, and Beige Adipocytes (modified from Fruhbeck et al., 2009)
1.13 Molecular Signature of Brown and Beige Adipose Tissue

The following genes are molecular markers of brown adipose tissue that possess either a structural or regulatory function required for fully differentiated BAT. It has previously been shown that these genes are highly expressed in subcutaneous WAT in mice (Seale et al., 2011). In the present study, we investigated the expression levels of these BAT-selective genes in WAT.

**UCP1**

Uncoupling protein 1 (UCP1), formerly known as thermogenin, belongs to a family of mitochondrial carrier proteins. UCP1 is predominantly expressed in brown adipocytes and is required for the thermogenic activity of brown adipose tissue. UCP1 is inhibited by purine nucleotides (e.g., GDP, ADP) and is activated by free fatty acids that are released following beta-adrenergic stimulation. UCP1 significantly reduces the rate of ATP production by uncoupling the respiratory chain, dissipating energy in the form of heat (reviewed in Cannon and Nedergaard, 2004). In white adipose tissue, UCP1 mRNA and protein are present at very low levels and increases in response to cold exposure or treatment with beta-adrenoceptor agonists (Cousin et al., 1992; Ghorbani and Himms-Hagen, 1997; Guerra et al., 1998; Himms-Hagen et al., 2000; Huttunen et al., 1981; Xue et al., 2005). Multilocular cells that express UCP1 in WAT are characterized as beige cells.

**PRDM16**

PRD1-BF-1-RIZ1 homologous domain-containing protein 16 (PRDM16) is the master regulator of brown adipocyte differentiation. PRDM16 interacts with co-activators PGC1alpha and PGC1beta to activate transcription of BAT-selective genes, such as UCP1,
PGC1alpha, and Dio2 and suppress the expression of muscle-specific genes, such as MyoD, MyoG, myosin heavy chain (MyHC), and muscle creatine kinase (MCK) (Seale et al., 2007). Moreover, PRDM16 interacts with co-repressors CtBP1 and CtBP2 to inhibit WAT-selective genes, such as angiotensinogen, resistin, and WDNM1-like (Kaijimura et al., 2008). Transgenic expression of PRDM16 in preadipocytes isolated from inguinal WAT induces the expression of UCP1 and other BAT-selective genes, such as Cidea, Elovl3, and Cox8b (Seale et al., 2011).

**Cidea**

Cell death-inducing DFF45-like effector A (Cidea) was first described as an apoptotic protein (Inohara et al., 1998). In adipose tissue, Cidea is a lipid droplet-associated protein that plays a role in fat storage (Puri et al., 2008). In mice, mRNA levels of Cidea are high in interscapular BAT but undetectable in epidydimal WAT (Zhou et al., 2003). Cidea is a negative regulator of lipolysis in WAT (Nordstrom et al., 2005) and UCP1 uncoupling activity in BAT (Zhou et al., 2003; Lin and Li, 2004). Mice treated with a PPARgamma agonist, rosiglitazone, have increased levels of Cidea and contain multilocular UCP1-expressing adipocytes (beige cells) in inguinal WAT (Petrovic et al., 2010).

**Cox8b**

Cytochrome c oxidase subunit VIIIb (Cox8b) is a component of the cytochrome c oxidase (COX) complex, which catalyzes the last step of mitochondrial respiration (i.e., the conversion of oxygen to water). Cox8b is induced by PRDM16 in brown and beige adipocytes (Wilson-Fritch et al., 2004; Kaijimura et al., 2008; Kaijimura et al., 2009;
Barbatelli et al., 2010; Seale et al., 2011). Although Cox8b is highly expressed in BAT, its role in BAT is poorly understood.

**Elovl3**

Elovl3 is an elongase enzyme of very long chain fatty acids. Elovl3 is more highly expressed in brown adipocytes than white adipocytes. Treatment with PPARgamma agonist rosiglitazone, together with norepinephrine, enhances Elovl3 mRNA levels (Jorgensen et al., 2007). Interestingly, rosiglitazone treatment elevates Elovl3 levels to higher levels in white adipocytes than in brown adipocytes. Cold exposure significantly increases Elovl3 expression in inguinal WAT, not epidymidal WAT, suggesting that inguinal WAT is more susceptible to “browning” (Jakobsson et al., 2005).
CHAPTER 2: BACKGROUND OF STUDY

Our lab has previously made the novel observation that Tyk2-knockout (Tyk2-/-) mice become spontaneously obese and develop many symptoms of the metabolic syndrome (Gornicka, 2009). We hypothesize that the obese phenotype observed in Tyk2-knockout mice is due to decreased energy expenditure, resulting from impaired brown adipocyte differentiation in classical brown adipose tissue. The major focus of this study is to determine if Tyk2 plays a role in the development of brown-like adipocytes (beige cells) in classical white adipose tissue.

2.1 Tyrosine Kinase 2 (Tyk2)

Until now, Tyk2 has not been previously associated with obesity. There are no published reports on a role of Tyk2 in the regulation of metabolic homeostasis. Tyrosine Kinase 2 (Tyk2) is a non-receptor tyrosine kinase of the Janus kinase (JAK) family. Tyk2 was the first member of the JAK family to be identified, with an important role in type I interferon (IFN) signaling (Krolewski et al., 1990). Mutant cell lines lacking Tyk2 have impaired IFNalpha/beta signaling, observed by the absence of Stat3 activation and a slight reduction in Stat1 and Stat2 activation. Type I IFN signaling is restored after reconstitution of Tyk2 (Karaghiosoff et al., 2000).
Tyk2 plays a critical role in cytokine signaling, particularly of IL-6, IL-10, IL-12, and IL-23 (reviewed by Watford and O’Shea, 2006; Figure 3). Tyk2, together with Jak2, is required for the differentiation of IFNγ-producing Th1 cells, mediated by IL-12. Tyk2 is also needed for Th17 cell differentiation, which is activated by IL-23.

Figure 3: Tyk2-Dependent Cytokine Signaling (Watford and O’Shea, 2006)

Tyk2-deficient mice are viable and fertile (Karaghiosoff et al., 2000). However, they display a number of defects when challenged with viral and bacterial pathogens. Impaired type I IFN signaling in Tyk2-/- mice causes resistance to lipopolysaccharide (LPS)-induced toxic shock and impaired LPS-induced NO production in macrophages (Karaghiosoff et al., 2000; Karaghiosoff et al., 2003; Kamezaki et al., 2004). Impaired IL-12 signaling in Tyk2-/- mice causes defects in the differentiation of IFNγ-producing Th1 cells (Muller et al., 1993; Watling et al., 1993) and the activation of natural killer (NK) cells (Shimoda et al., 2002). These defects result in an enhanced susceptibility to infection with pathogens, such as the lymphocytic choriomeningitis virus (LCMV).
(Karaghiosoff et al., 2000), *Leishmania* (Schleicher et al., 2004), and the Abelson murine leukemia virus (A-MuLV) (Stoiber et al., 2004). Moreover, the lack of Th1 response is accompanied by an enhanced Th2 response that accelerates allergic reactions, such as lung inflammation (Seto et al., 2003).

Only a single patient with Tyk2 deficiency has been identified so far (Minegishi et al., 2006). Similar to Tyk2-deficient mice, the patient lacking Tyk2 presented defects in cytokine signaling and developed multiple, opportunistic infections of various organs. However, defects in the Tyk2-/- patient were broader and more profound compared to the mice. This is likely due to the fact that type I IFN signaling was completely absent in the Tyk2-/- patient, whereas only a partial defect in type I IFN signaling was observed in Tyk2-/- mice. The reason for this discrepancy is unknown. The Tyk2-/- patient suffered from severe atopic dermatitis and was clinically diagnosed with hyperimmunoglobulin E (hyperIgE) syndrome (HIES), a primary immunodeficiency defined by the accumulation of IgE resulting from an enhanced Th2 response.

2.2 The JAK/STAT Signaling Pathway

The JAK/STAT pathway plays a critical role in the signaling of a wide variety of cytokines that regulate cell development and homeostasis (reviewed by Aaronson and Horvath, 2002; Igaz et al., 2001; Keisseleva et al., 2002; O’Shea et al., 2002; Shuai and Liu, 2003).

The Janus kinase (JAK) family consists of four members: Jak1, Jak2, Jak3, and Tyk2. Each JAK has a unique function and, with the exception of Jak3, all JAKs are
ubiquitously expressed in mammals. JAKs are relatively large proteins containing over 1000 amino acids with a molecular weight of 120-140 kDa. All JAKs have a similar domain structure consisting of seven JAK homology (JH) domains. A catalytically active kinase domain (JH1), located at the carboxyl-terminus, phosphorylates specific tyrosine residues of its substrates. Adjacent to the kinase domain is a catalytically inactive pseudokinase or kinase-like domain (JH2), which has been suggested to negatively regulate kinase activity. The tandem structure of these two kinase domains is a hallmark of JAKs. For this reason, JAKs were named after the two-faced Roman god, Janus. Following the pseudokinase domain is a Src homology 2 (SH2)-like domain (JH3-JH5) that plays a structural role for receptor interaction. At the amino-terminus of the protein is a 4.1, ezrin, radixin, moesin (FERM) domain (JH6-JH7), which mediates the binding between JAKs and cytokine receptors and also regulates kinase activity.

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**Figure 4: Domain Structure of JAKs and STATs**
Primary structure of (a) JAKs (Yamaoka et al., 2004) and (b) STATs (Levy and Darnell, 2002).
The major substrates for JAKs are the signal transducers and activators of transcription (STAT) proteins. STATs are latent cytoplasmic transcription factors that mediate a wide range of actions induced by cytokines. There are seven known STATs: Stat1, Stat2, Stat3, Stat4, Stat5a and Stat5b, and Stat6. STAT proteins contain 750-900 amino acids and are 80-95 kDa in molecular weight. STATs share structurally and functionally conserved domains: an N-terminal regulatory domain (NTD), a coiled-coil (CC) domain, a DNA-binding domain (DBD), a Src homology 2 (SH2) domain, a linker domain (LD), and a C-terminal transactivation domain (TAD). At the carboxyl-terminus, STAT proteins have a conserved tyrosine residue, whose phosphorylation is required for dimerization and DNA binding. Some STATs, such as Stat3, also contain a conserved serine residue, whose phosphorylation is required for maximal transactivation.

The JAK/STAT pathway consists of a series of tyrosine phosphorylation events (summarized in Figure 5). JAKs are loosely associated with cytokine receptors in the absence of ligands. When a cytokine binds to its receptor, homo- or heterodimerization of the cytokine receptor occurs. Dimerization of the receptor brings receptor-associated JAKs in close proximity to each other. The JAKs transphosphorylate each other at specific tyrosine residues, and in turn tyrosine phosphorylate the cytoplasmic domain of the receptor. The phosphorylated tyrosines on the receptor provide docking sites for SH2-containing STATs. STATs bind to the phosphorylated receptor and are then phosphorylated by activated JAKs. This causes the STATs to dissociate from the receptor and form homo- or heterodimers with each other. Activated STATs then translocate into the nucleus and bind to specific DNA sequences found in the promoter region of target
genes: an interferon-stimulated response element (ISRE) or a gamma-interferon activation site (GAS).

Cytokine signaling through the JAK/STAT pathway regulates multiple cellular responses, including cell growth, survival, and differentiation. Mutations in the JAK/STAT pathway have been implicated in several diseases, such as Hodgkin lymphoma, myeloproliferative disorders, and other cancers (Jatiani et al., 2010).

Figure 5: General Overview of the JAK/STAT Pathway (modified from Schindler, 2002)
2.3 Role of Tyk2 in Obesity

Our lab has previously found that Tyk2-knockout (Tyk2-/-) mice on a SV129 background become spontaneously obese (Figure 6a) and develop symptoms characteristic of the metabolic syndrome (Gornicka, 2009). Mice lacking Tyk2 presented impaired glucose tolerance and insulin resistance, which are major risk factors for type 2 diabetes. These phenotypic changes were accompanied by changes in mRNA expression of genes involved in glucose metabolism, lipid metabolism, and the coordination of metabolic responses in the hypothalamus (leptin and neuropeptides).

The obese phenotype in Tyk2-knockout mice was also demonstrated in two additional mouse strains: C57BL/6 and BALB/c (Derecka, 2011). Mice on a C57BL/6 background are described as an obesity-prone strain, whereas BALB/c mice are resistant to diet-induced obesity. These knockout strains developed high-fat diet-induced obesity and displayed abnormal glucose tolerance. However, they did not appear to be hyperphagic.

2.4 Role of Tyk2 in Brown Adipose Tissue Development

A lack of increased food intake with increasing body weight suggested that Tyk2-knockout mice have a defect in energy expenditure. mRNA expression of thermogenic genes (UCP1, PRDM16, and Cidea) was downregulated in Tyk2-knockout mice (C57BL/6 and BALB/c) (Derecka, 2011). Moreover, the expression of PPARalpha, which activates transcription of beta-oxidation enzymes, was also decreased. There were no significant changes in RNAs encoding common adipose genes, such as PPARgamma and PGC1alpha,
suggesting that Tyk2 functions selectively in the development and thermogenic activity of BAT.

Histological analysis of BAT revealed large differences in lipid accumulation between wild-type and Tyk2-knockout mice. Tyk2-/- BAT contained large adipocytes with unilocular lipid droplets, resembling WAT (Figure 6b). This altered morphology is likely a consequence of the observed downregulation of BAT-selective genes.

Brown preadipocytes were isolated from neonatal wild-type and Tyk2-knockout mice and subjected to in vitro differentiation with induction media consisting of dexamethasone, insulin, and 3-isobutyl-1-methylxanthine (IBMX). Oil Red O staining, which stains triglycerides and lipids, was performed to detect mature adipocytes. Tyk2-/- brown preadipocytes failed to differentiate in vitro, compared to wild-type preadipocytes (Figure 6c).
Figure 6: Tyk2 in Obesity and Brown Adipose Tissue Development
(a) Picture representation of body weight of 12-month-old WT and Tyk2-/- mice (Gornicka, 2009). (b) Hematoxylin and eosin (H&E) stain of BAT from WT and Tyk2-/- mice (Derecka, 2011). (c) Oil red O stain of WT and Tyk2-/- BAT preadipocytes differentiated in vitro (Derecka, 2011).
2.5 Research Aims

Tyk2 is required for the development of classical brown adipocytes. The focus of this study was to determine if Tyk2 also plays a role in the development of beige cells, brown-like adipocytes that can be found in classical white adipose tissue. In the present study, we focused on mRNA expression of BAT-selective genes in two types of white adipose tissue: epidydimal WAT (a visceral depot) and inguinal WAT (a subcutaneous depot). A recent study has shown that inguinal WAT, not epidydimal WAT, expresses BAT-selective genes UCP1, PRDM16, Cidea, Cox8b, and Elovl3 (Seale et al., 2011). The development of beige cells in mice expressing or lacking Tyk2 was assessed by the mRNA expression of these BAT-selective genes in subcutaneous WAT.

Specific Aims

1. To confirm the expression of BAT-selective genes in subcutaneous WAT of wild-type mice
2. To identify if there is a role of Tyk2 in the development of beige cells in subcutaneous WAT
CHAPTER 3: MATERIALS AND METHODS

3.1 Mice

All mice were bred and maintained in the animal facilities at Virginia Commonwealth University School of Medicine, according to regulations established by the Institutional Animal Care and Use Committee (IACUC). Tyk2-deficient mice on a C57BL/6 background were kindly provided by Dr. Ana Gamero from the Department of Biochemistry of Temple University School of Medicine in Philadelphia, PA. Tyk2 knockouts were generated by Dr. Kazuya Shimoda and colleagues from the Department of Internal Medicine of Kyushu University in Fukuoka, Japan. The disruption of Tyk2 expression was performed by electroporation of embryonic stem cells with a vector that carried a neomycin resistance cassette that replaced the first coding exon of the Tyk2 gene. 10-12 week old wild-type and Tyk2-deficient male mice (C57BL/6 background) were used for all experiments.

3.2 Dissections

The following dissections were made: interscapular BAT (iBAT) on the dorsal side of the mouse between the shoulder blades; inguinal WAT (ingWAT) underneath the skin surrounding the pelvis, from the back to the front of the thighs; epidydimal WAT (epidWAT) surrounding the epidydimal tubes above the testes; mesenteric WAT
(mWAT) attached to the intestines; and retroperitoneal WAT (rWAT) behind the kidneys. The tissues were flash frozen and stored at -80°C.

Figure 7: Adipose Depots Dissected in Study (modified from Cinti, 2009)

3.3 Isolation of total RNA and cDNA synthesis

50 g of each tissue were homogenized in 1 ml of TRI Reagent (Molecular Research, Cincinnati, OH) using a Glas-Col Homogenizer (Cole-Parmer, Vernon Hills, IL). Homogenates were transferred to 1.5 ml tubes, and 200 ul of chloroform was added. Tubes were vortexed for at least 30 seconds, incubated at room temperature for 5 minutes, and centrifuged at 12,000 x g for 15 minutes at 4°C. The upper aqueous phase was transferred to fresh 1.5 ml tubes, and an equal amount of 100% isopropanol (1:1) was added. Tubes were vortexed briefly and incubated at -20°C for at least one hour or overnight. The samples were centrifuged at 12,000 x g for 15 minutes at 4°C. The RNA pellets were washed once with 70% ethanol, air-dried for at least 15 minutes at 37°C, and
resuspended with DEPC-treated water. Extra chloroform extraction and ethanol wash steps were performed to eliminate possible phenol contamination and improve the efficiency of cDNA synthesis. Isolated RNA samples were treated with DNase (Promega, Madison, WI) for 1 hour at 37°C, followed by an addition of stop solution and incubation at 65°C for 10 minutes. Using a spectrophotometer, the concentration of RNA samples was determined by measuring absorbance at 260 nm, and the purity by the 260 nm/280 nm absorbance ratio. 2 ug of total RNA from each sample were reverse transcribed using the Tetro cDNA Synthesis Kit (Bioline, Taunton, MA).

### 3.4 Quantitative PCR

mRNA levels of the genes of interest were analyzed by real-time quantitative PCR (qPCR) using the SensiMix SYBR and Fluorescein Kit (Bioline, Taunton, MA) according to manufacturer’s instructions. The reaction mixtures were prepared as follows: for measuring TBP mRNA (internal control)—12.5 ul of SYBR and Fluorescein mix, 1 ul of 5 uM forward primer, 1 ul of 5 uM reverse primer, 5 ul of cDNA (final concentration 5 ng/ul), 5.5 ul of H2O (total volume 25 ul); for measuring mRNA levels of target genes—12.5 ul SYBR and Fluorescein mix, 2.5 ul of 5 uM forward primer, 2.5 ul of 5 uM reverse primer, 5 ul of cDNA (final concentration 5 ng/ul), 2.5 ul of H2O (total volume 25 ul). All samples were assayed in duplicates and analyzed using the CFX96 Real-Time PCR Detection System (Bio-Rad, Hercules, CA) using the following amplification conditions: 1 cycle of 95°C for 10 minutes, 40 cycles of 95°C for 15 seconds and 60°C for 1 minute. The results were analyzed according to the deltaC(t)
method using a reference gene (variation of Livak method). Briefly, to calculate relative mRNA expression, expression of the gene of interest (target) was first normalized to a reference gene (TBP mRNA): expression = $2^{\left[Ct(\text{TBP mRNA}) - Ct(\text{target})\right]}$. This was applied to each adipose tissue of all experimental groups of mice. The gene expression ratio of inguinal WAT to epidydimal WAT was calculated by setting the expression of the target gene in epidydimal WAT to 1. Gene expression was also measured by normalizing the mRNA levels of the epidydimal WAT and inguinal WAT of each data set to the mRNA levels of epidydimal WAT in wild-type mice under basal conditions.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward primer (5′-3′)</th>
<th>Reverse primer (5′-3′)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UCP1</td>
<td>CTG GCC TTA AGC GGT CCTC</td>
<td>CTG GGC TAG GTA GTG CCA GTG</td>
</tr>
<tr>
<td>Cidea</td>
<td>TGC TCT TCT GTA TCG CCC AGT</td>
<td>GCC GTG TTA AGG AAT CTG CTG</td>
</tr>
<tr>
<td>Cox8b</td>
<td>GAA CCA TGA AGC CAA CGA CT</td>
<td>GCG AAG TTC ACA GTG GTT CC</td>
</tr>
<tr>
<td>Elov13</td>
<td>TCC GCG TTC TCA TGT ACG TCT</td>
<td>GGA CCT GAT GCA ACC CTA TGA</td>
</tr>
<tr>
<td>PRDM16</td>
<td>CAG CAC GGT GAA GCC ATT C</td>
<td>GCG TGC ATC CGC TTG TG</td>
</tr>
<tr>
<td>TBP</td>
<td>GAA GCC GTG GTA CAA TTC CAG</td>
<td>CCC CTT GTA CCC TTC ACC AAT</td>
</tr>
</tbody>
</table>

**Table 2: Primers Used for Real-Time qPCR Analysis**

3.5 Statistical analysis

Results are presented as the mean ± standard error (SE). Statistical comparison was performed using the two-tailed Student’s t-test. A P-value less than 0.05 (P<0.05) was considered as statistically significant and annotated by *. Other statistically significant P-values are annotated as follows: **P<0.01 and ***P<0.001.
CHAPTER 4: RESULTS

4.1 BAT-selective genes are expressed in subcutaneous WAT of wild-type mice

Considering the differences between WAT depots in their propensity to develop beige cells, we wanted to determine which depots selectively express UCP1 and other molecular markers of fully differentiated BAT. We dissected five different types of adipose tissues—epidydimal WAT (epidWAT), mesenteric WAT (mWAT), retroperitoneal WAT (rWAT), inguinal WAT (ingWAT), and interscapular BAT (iBAT)—from 10-12 week old male mice on a C57BL/6 background. From these tissues, we isolated RNA and synthesized cDNA for gene expression analysis by real-time qPCR. We first looked at mRNA expression levels of UCP1 because of its unique expression in brown adipocytes. UCP1 levels of each depot were compared to those of epidydimal WAT, the “purest” visceral WAT depot. As expected, we found robust expression of UCP1 in interscapular BAT, a 600-fold enrichment relative to epidydimal WAT (P<0.001) (Figure 8a). The two visceral depots that were tested, mesenteric WAT and retroperitoneal WAT, did not express UCP1, whereas the subcutaneous depot, inguinal WAT, highly expressed UCP1 at a level 35-fold higher than epidydimal WAT (P<0.001) (Figure 8a). Inguinal WAT also exhibited large 20- to 100-fold increases in Cidea (20-fold), Cox8b (100-fold), and Elovl3 (20-fold) expression, and a slight but
significant increase in PRDM16 (1.3-fold) (Figure 8b). All increases were statistically significant (P<0.05).
Figure 8: BAT-selective genes are expressed in subcutaneous WAT of wild-type mice
Real-time qPCR analysis of (a) UCP1 mRNA levels in epidydimal WAT (epidWAT), mesenteric WAT (mWAT), retroperitoneal WAT (rWAT), inguinal WAT (ingWAT), and interscapular BAT (iBAT) of 10-12 week old male WT mice fed a regular chow diet and (b) mRNA levels of BAT-selective genes (UCP1, Cidea, Cox8b, Elov13, and PRDM16) in epidWAT and ingWAT of WT mice. Values are mean ± SE (n = 4-5 mice per group). *P<0.05, ***P<0.001.
4.2 Expression of BAT-selective genes is reduced in subcutaneous WAT of Tyk2-/- mice

Our lab has previously found that Tyk2-knockout (Tyk2-/-) mice become obese with age and have impaired brown adipose tissue. Because Tyk2 is involved in classical BAT development, we wanted to determine if Tyk2 is also involved in the development of beige cells. We focused our attention on epidydimal WAT and inguinal WAT, which showed clear differences in UCP1 expression in wild-type mice (Figure 8a). Using realtime qPCR, we measured fold enrichment of UCP1, Cidea, Cox8b, Elovl3, and PRDM16 in Tyk2-/- inguinal WAT relative to epidydimal WAT, and compared these values with those observed in wild-type mice (Figure 8b). We found that BAT-selective genes were significantly downregulated in subcutaneous WAT of Tyk2-/- mice compared to wild-type mice: Cidea by 7-fold, Cox8b by 10-fold, Elovl3 by 90-fold, and PRDM16 by 3-fold (all P-values <0.05) (Figure 9). The most notable change was demonstrated by a 175-fold decrease in UCP1 expression (P<0.01).
Figure 9: Expression of BAT-selective genes is reduced in subcutaneous WAT of Tyk2-/- mice
Real-time qPCR analysis of mRNA levels of BAT-selective genes (UCP1, Cidea, Cox8b, Elov3, and PRDM16) in epidWAT and ingWAT of 10-12 week old male WT and Tyk2-/- mice fed a regular chow diet. Values are mean ± SE (n = 4-5 mice per group).
*P<0.05, **P<0.01.
4.3 BAT-selective genes are induced in Tyk2-/ subcutaneous WAT by acute starvation

To verify the changes in expression that we observed in Figure 9, we repeated the experiment with another set of Tyk2-knockout mice. Much to our surprise, both experiments yielded different results. It was later realized that the second set of knockout mice was starved overnight for 12 hours in preparation for metabolic experiments. While the first group of mice (unstarved Tyk2-/-) showed significant decreases in BAT-selective gene expression (Figure 9), the second group of mice (starved Tyk2-/-) displayed high expression of BAT-selective genes in their subcutaneous WAT (Figure 10). UCP1 was increased by 110-fold, Cidea by 110-fold, Cox8b by 230-fold, Elov13 by 5-fold, and PRDM16 by 2-fold.

To determine if this starvation effect was due to the lack of Tyk2, we looked at gene expression in subcutaneous WAT of wild-type mice under starved conditions. We found that there were no significant differences in BAT-selective gene levels between unstarved and starved wild-type mice (except for Cidea) (Figure 10). Thus, acute starvation causes an induction of BAT-selective genes only in Tyk2-/- subcutaneous WAT.

One caveat about our measurements is that the mRNA levels of each mouse were normalized to the mRNA levels of its own epidydimal WAT, under the assumption that BAT-selective gene expression would be constantly low in this depot. When we compared mRNA levels of epidydimal WAT between wild-type and Tyk2-/- mice, we unexpectedly saw a 10-fold induction of UCP1 in Tyk2-/- epidydimal WAT compared to
wild-type epidydimal WAT (Figure 10b). Although statistically non-significant, this
induction of UCP1 in epidydimal WAT accounted for the large decrease in the
ingWAT/epidWAT expression ratio of UCP1 in Tyk2-/- mice, shown in Figure 9. After
correcting for this, the 175-fold decrease of UCP1 in Tyk2-/- inguinal WAT became a
35-fold decrease (Figure 10b). Interestingly, the level of UCP1 (27-fold enrichment) in
Tyk2-/- inguinal WAT after 12 hours of starvation was approximately equal to the UCP1
level observed in inguinal WAT of unstarved wild-type mice (35-fold enrichment)
(Figure 10b).
Figure 10: BAT-selective genes are induced in Tyk2-/− subcutaneous WAT by acute starvation
Real-time qPCR analysis of mRNA levels of BAT-selective genes: (a-b) UCP1, (c) Cidea, (d) Cox8b, (e) Elovl3, and (f) PRDM16 of 10-12 week old male WT mice fed a regular chow diet (unstarved) or fasted for 12 hours (starved). Values are mean ± SE (n = 4-5 mice per group). *P<0.05, **P<0.01, ***P<0.001.
CHAPTER 5: DISCUSSION

Our lab has previously discovered that Tyk2, an important mediator of cytokine signaling, promotes the development of classical brown adipose tissue (Gornicka, 2009; Derecka, 2011). The focus of this study was to determine if Tyk2 also plays a role in the development of beige cells, brown-like adipocytes that can be found in classical white adipose tissue.

In the present study, we focused on mRNA expression of BAT-selective genes in two types of white adipose tissue: epidydimal WAT (a visceral depot) and inguinal WAT (a subcutaneous depot). A recent study has shown that inguinal WAT, not epidydimal WAT, expresses BAT-selective genes UCP1, PRDM16, Cidea, Cox8b, and Elovl3 (Seale et al., 2011). The development of beige cells in mice expressing or lacking Tyk2 was assessed by the mRNA expression of these BAT-selective genes in subcutaneous WAT. UCP1 is uniquely expressed in brown adipose tissue and can therefore serve as a marker for brown-like adipocytes (beige cells) that are present in white adipose tissue. The other BAT-selective genes (Cidea, Cox8b, Elovl3, and PRDM16) were also evaluated to support any significant changes observed in UCP1 expression.

Our first aim was to confirm the expression of BAT-selective genes in subcutaneous WAT of wild-type mice. Using real-time qPCR, we found that, in mice housed at ambient temperature (22°C), UCP1 mRNA is enriched in subcutaneous WAT
(inguinal depot) compared to the levels observed in visceral WAT (epidydimal, mesenteric, and retroperitoneal depots) (Figure 8a). Moreover, Cidea, Cox8b, Elovl3, and PRDM16 were enriched in inguinal WAT relative to epidydimal WAT (Figure 8b). Our data mimicked the same expression patterns observed by Seale et al. (2011).

Our second aim was to identify if there is a role of Tyk2 in the development of beige cells in subcutaneous WAT. We examined BAT-selective mRNA levels in inguinal WAT of Tyk2-/- mice and found that they were downregulated compared to mRNA levels in epidydimal WAT (Figure 9). Surprisingly, we found that epidydimal WAT of Tyk2-/- mice displayed higher UCP1 expression than epidydimal WAT of WT mice (Figure 10b); however, it is unclear whether UCP1 is truly induced in Tyk2-/- epidydimal WAT. For this purpose, we have recently isolated tissues from WT and Tyk2-/- mice to repeat this experiment.

Unexpectedly, we found that a 12-hour starvation can induce beige cell development in Tyk2-/- subcutaneous WAT. In starved Tyk2-/- mice, mRNAs of UCP1, Cidea, Cox8b, Elovl3, and PRDM16 were enriched in inguinal WAT relative to their mRNA levels in epidydimal WAT (Figure 10). A similar induction of BAT-selective genes occurred with combined starvation and cold exposure (data not shown). In wild-type mice, however, there appeared to be no significant changes in gene expression in the inguinal depot upon starvation (Figure 10).

To our knowledge, there are no published reports on the effects of starvation on beige cell formation. In classical brown adipose tissue, it has been shown that starvation decreases sympathetic activity, resulting in decreased UCP1 expression and thermogenic
activity (Hayashi and Nagasaka, 1983; Tulp, 1983; Rothwell et al., 1984; Trayhurn and Jennings, 1986; Trayhurn and Jennings, 1988; Champigny and Ricquier, et al., 1990; Ohno et al., 1993). However, beige cells, which are derived from a different developmental origin, may respond to starvation entirely differently.

Indeed, starvation does not have the same effects on white adipose tissue as it does on brown adipose tissue. It has been shown that fasting and other stress stimuli, such as severe exercise and cold exposure, increase sympathetic activity in WAT, which then stimulates lipid breakdown and mobilization (Migliorini et al., 1997; Giordano et al., 2005). As a result, more free fatty acids are released which can activate UCP1. This, however, does not explain why starvation would cause beige cell development in the first place. It seems counterintuitive for an organism to increase its energy expenditure during times of energy need. We postulate that this induction of beige cells in Tyk2-/− mice may be a compensatory mechanism for the decrease in classical BAT function resulting from the combined effect of starvation and defective beta-oxidation already present in Tyk2-/− mice. However, more experiments need to be done to validate this speculation.

Overall, this thesis demonstrates that Tyk2 is involved in the development of beige cells under ambient conditions. Moreover, it demonstrates that the need for Tyk2 in beige cell development can be bypassed by acute starvation. In the future, it will be important to determine if the BAT-selective mRNA levels observed in this study are accompanied by the appearance (or lack thereof) of UCP1-expressing multilocular cells in subcutaneous WAT. For this purpose, we have recently fixed and paraffin-blocked different adipose tissues, epidydimal WAT, inguinal WAT, and interscapular BAT, for
immunohistochemistry using an anti-UCP1 antibody. It would also be interesting to see if the starvation-induced expression of beige cells in Tyk2/- mice would still be present after the stress stimulus is removed (i.e., after mice are re-fed). Since Tyk2 plays a role in the development of both brown and beige adipocytes, understanding the molecular mechanisms of Tyk2 in these two processes may be important in discovering new therapies against obesity and related diseases.
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VITA

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