The Good, The Brown, and The Healthy: Understanding Non-Thermogenic Brown Adipose Function in Obese Mice

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Division of Biology and Biomedical Sciences
Molecular Genetics and Genomics

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The Good, The Brown, and The Healthy:
Understanding Non-Thermogenic Brown Adipose Function in Obese Mice
by
Caryn Nicole Carson

A dissertation presented to
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of Washington University in
partial fulfillment of the
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of Doctor of Philosophy
Table of Contents

List of Figures .......................................................................................................................... v
List of Tables ............................................................................................................................. vii
Acknowledgements .................................................................................................................... viii
Abstract of the Dissertation ........................................................................................................ ix

Chapter 1: Introduction .................................................................................................................. 1
  1.1 Overview .............................................................................................................................. 2
  1.2 Obesity and Metabolic Syndrome ....................................................................................... 3
    1.2.1 Mouse Models of Obesity .................................................................................... 4
    1.2.2 LG/J and SM/J Inbred Mice .................................................................................. 4
  1.3 Brown Adipose as a Metabolic Organ ............................................................................... 5
    1.3.1 Insulin and Adrenergic Signaling ......................................................................... 6
  1.4 Brown Adipose as an Endocrine Organ .......................................................................... 7
    1.4.1 General Adipokines ............................................................................................. 7
    1.4.2 Inflammation ......................................................................................................... 9
  1.5 Brown Adipocyte Identity ............................................................................................... 10
  1.6 Gene by Environment Interactions in Brown Adipose ................................................... 11
  1.7 Scope of Thesis ................................................................................................................. 12

Chapter 2: Physiological Characterization of Healthy Obesity in SM/J Mice ......................... 14
  2.1 Abstract ............................................................................................................................ 15
  2.2 Introduction ....................................................................................................................... 15
  2.3 Results .............................................................................................................................. 17
    2.3.1 SM/J Mice Improve Glucose Parameters Without Weight Loss ....................... 17
    2.3.2 High Fat-Fed SM/J Mice Expand Their Interscapular Brown Adipose Tissue Depots ......................................................................................................................... 19
    2.3.3 Expanded Brown Adipose Tissue Serves as an Insulin-Stimulated Glucose Sink... 25
  2.4 Discussion ......................................................................................................................... 29
  2.5 Materials and Methods ................................................................................................. 30
2.5.1 Animal Husbandry and Phenotyping .................................................. 30
2.5.2 Blood Plasma Assays ........................................................................ 31
2.5.3 Adipose Histology ............................................................................ 31
2.5.4 H&E Staining .................................................................................... 32
2.5.5 Immunofluorescence ........................................................................ 32
2.5.6 Quantitative rt-PCR .......................................................................... 33
2.5.7 Mitochondrial DNA Quantification .................................................... 34
2.5.8 Brown Adipose Excision .................................................................... 34
2.5.9 Statistics ............................................................................................ 35

Chapter 3: Identification of Brown Adipose Gene Co-Expression Clusters Correlating with Metabolic Phenotypes ................................................................. 36

3.1 Abstract ................................................................................................. 37
3.2 Introduction ............................................................................................ 38
3.3 Results .................................................................................................... 40
  3.3.1 Brown Adipose Expression and Metabolic Traits Vary Among Strain and Diet Contexts ................................................................. 40
  3.3.2 Modules Enriched for Immune/Cytokine Response and Cell Division Correlate with Glucose Parameters and Brown Adipose to Body Weight Ratio ........ 42
  3.3.3 Gene Expression within Network Modules Varies Across Both Diet and Strain Contexts ............................................................... 43
  3.3.4 Strain-Specific Variation Drives Differential Expression and Differential Connectivity within Brown Adipose Gene Modules ........................................... 45

3.4 Discussion ............................................................................................... 52
3.5 Materials and Methods ....................................................................... 55
  3.5.1 Sample Collection and Sequencing ................................................... 55
  3.5.2 Gene Co-Expression and Phenotype Associations .......................... 56
  3.5.3 Differential Connectivity ................................................................ 57

Chapter 4: Transcriptional Changes in Brown Adipose Tissue of Healthy Obese SM/J Mice 59

4.1 Abstract ................................................................................................. 60
4.2 Introduction ............................................................................................ 60
4.3 Results .................................................................................................... 62
4.3.1 RNA sequencing reveals altered expression of cytokine and extracellular matrix genes in SM/J brown adipose at 30 weeks................................................................. 62
4.3.2 Increased Sfrp1 expression in expanded brown adipose tissue correlates with improved glucose tolerance ................................................................. 64
4.4 Discussion ............................................................................................................. 67
4.5 Materials and Methods ..................................................................................... 69
  4.5.1 RNA-sequencing ......................................................................................... 69
  4.5.2 Functional Enrichment Analysis ................................................................. 69
  4.5.3 Quantitative rt-PCR ................................................................................... 70
  4.5.4 Statistics ...................................................................................................... 70
Chapter 5: Conclusions ............................................................................................ 71
  5.1 Summary .......................................................................................................... 72
    5.1.1 Increase in Brown Adipose Mass Improves Insulin Sensitivity in Obesity ...... 72
    5.1.2 Transcriptional Co-Expression Networks in Brown Adipose Tissue are Heavily Dependent on Diet and Genetic Background ........................................ 75
    5.1.3 Unique transcriptional changes accompany expansion of brown adipose tissue ......................................................................................................................... 76
  5.2 Future Directions .............................................................................................. 78
  5.3 Final Thoughts .................................................................................................. 80
Chapter 6: References .............................................................................................. 81
Appendix A: Supplementary Tables ........................................................................ 126
List of Figures

Figure 2.1 Obese SM/J mice improve glucose parameters between 20 and 30 weeks of age... 18
Figure 2.2 Circulating cytokine levels in SM/J mice ................................................................. 19
Figure 2.3 Physiological parameters of the SM/J inbred mouse strain across age .............. 20
Figure 2.4 Physiological parameters of the LG/J inbred mouse strain .................................. 21
Figure 2.5 High fat-fed SM/J mice develop unique expansion of brown adipose tissue .... 22
Figure 2.6 Increased brown adipose tissue is likely due to increased number, not size, of cells .. .................................................................................................................. 23
Figure 2.7 Histochemistry reveals no morphological changes in expanded brown adipose tissue .................................................................................................................. 24
Figure 2.8 Thermogenic genes do not change between 20 and 30 week adipose tissue in high fat SM/J animals ................................................................. 26
Figure 2.9 SM/J thermogenic parameters .............................................................................. 27
Figure 2.10 Brown adipose expansion is required for improved insulin tolerance ............ 28
Figure 3.1 Brown adipose gene expression clustering by strain and diet ......................... 41
Figure 3.2 Gene network modules correlate with variation in metabolic traits ................. 42
Figure 3.3 Gene network modules correlated with one or more phenotypic trait in individual diet and strain cohorts ............................................................. 44
Figure 3.4 Principal component analysis of each individual module shows variation in diet and strain interactions ......................................................... 46
Figure 3.5 Genes that have increased expression in one diet show increased connectivity in the same diet ......................................................................................... 48
Figure 3.6 Genes that have increased expression in one strain show increased connectivity in the same strain ......................................................................................... 50
Figure 3.7 Expression by cohort for four candidate genes .................................................. 51
Figure 4.1 Genes differentially expressed in high fat-fed SM/J brown adipose tissue between 20 and 30 weeks are enriched for cytokine and extracellular region functions .......... 62
Figure 4.2 Expression of cytokine and extracellular proteins changes to a healthier profile with brown adipose expansion ................................................................. 63
Figure 4.3 Expression of Sfrp1 increases from 20 to 30 weeks only in the SM/J brown adipose depot........................................................................................................................................ 65

Figure 4.4 Brown adipose expression of Sfrp1 correlates with brown adipose mass and improved glucose tolerance only in SM/J mice........................................................................................................ 66

Figure 4.5 Genetic variation in Sfrp1 locus of humans and mice.................................................. 67

Figure 4.6 Proposed mechanism for action of Sfrp1 in SM/J brown adipose tissue ............... 68
List of Tables

Table 2.1 qPCR Primers .............................................................................................................. 33
Table 3.1 Co-Expression Module Statistics.............................................................................. 47
Table 3.2 Differentially Connected Genes Falling in Metabolic QTL ....................................... 52
Table A.1 Genes with high differential expression and connectivity by diet and strain .......... 128
Table A.2 Genes differentially expressed in SM/J high fat-fed brown adipose between 20 and 30 weeks................................................................................................................................. 129
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With gratitude,

Caryn Nicole Carson

Washington University in St. Louis

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ABSTRACT OF THE DISSERTATION

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By

Caryn Nicole Carson

Doctor of Philosophy in Biology and Biomedical Sciences

Molecular Genetics and Genomics

Washington University in St. Louis, 2020

Heather A. Lawson, Chair

Genetic and environmental factors heavily intertwine to affect metabolic homeostasis. To tease out the exact interactions between these two realms of influence, researchers often compare how one or multiple different inbred mouse strains react to various diets. An observation consistently seen across multiple strains on the same diet can reasonably be considered a general dietary effect, whereas an observation seen only in one strain of mice is more likely to result from a genetic cause or gene-by-environment interaction. Similarly to humans, a high fat diet causes many mouse strains to develop obesity and poor metabolic health, with varying degrees of hyperglycemia, hyperinsulinemia, systemic insulin resistance, hyperlipidemia, ectopic lipid deposition, and inflammation and dysfunction of metabolic tissues.

The LG/J and SM/J inbred mouse strains show disparate responses to long-term exposure to a high fat diet. While 20 weeks of high fat diet causes both strains to develop obesity, hyperglycemia and impaired glucose tolerance, the glycemic effect in SM/J mice is stronger
than in LG/J mice, and SM/J mice also develop hyperinsulinemia and impaired insulin sensitivity. Interestingly, an additional 10 weeks of high fat diet exposure results in reversion of unhealthy glycemic levels in SM/J mice, while LG/J mice maintain their elevated glucose parameters. This diabetic remission in SM/J mice is not the result of weight loss; the mice continue to gain weight, and in particular show a dramatic increase in the mass of their interscapular brown adipose tissue. Brown adipose tissue is best known for its function in non-shivering thermogenesis, the release of energy in the form of heat, however it has also emerged as a potent source of cytokines that can coordinate whole-body metabolic homeostasis. Based on the concurrent timing of the two phenomena, I hypothesized that the expansion of brown adipose tissue in the high fat-fed SM/J mice directly contributes to the glycemic normalization through secretion of pro-health cytokines and by serving as a more efficient glucose sink.

Though brown adipose is primarily associated with metabolic improvement through thermogenic action, I found no evidence that the expansion of brown adipose tissue in high fat-fed SM/J mice leads to increased thermogenesis. There is no change in the expression of the thermogenic genes *Ucp1, Cidea, and Eva1*, nor is there any change in mitochondrial DNA content, brown adipocyte morphology and Ucp1 staining, core body temperature, or circulating levels of thermogenesis-activating catecholamines. Instead, I found that the expression levels of *Irs1* and *Glut4*, two key members of the insulin-stimulated glucose uptake pathway, increase significantly with the brown adipose expansion. This suggested that the brown adipose expansion can act as an insulin-responsive sink for excess blood glucose, and indeed removal of the brown adipose depot before or after expansion prevents the improvement in whole-body insulin sensitivity.

To further explore the gene-by-environment interactions that cause the uniqueness of the SM/J brown adipose expansion and its association with metabolically healthy obesity, I ana-
lyzed the transcriptomic profile of the brown and white adipose tissues of high and low fat-fed LG/J and SM/J mice at 20 and 30 weeks. I performed weighted gene co-expression network analysis to identify clusters of genes whose expression in brown adipose tissue correlate with metabolic phenotypes. I identified four clusters that are enriched for genes in cell division, immune and cytokine response, organic molecule metabolism, and peroxisome function, as well as four clusters that have no significant enrichment. While other modules were identified in all cohorts, the cell division cluster was only found when the high fat-fed SM/J mice were included in the analysis. Principal components analysis of the expression of genes in this cluster shows a distinct grouping of the high fat-fed SM/J samples away from the other cohorts.

Twenty-nine genes in the cell division cluster also show significant differential expression between 20 and 30 weeks in high fat-fed SM/J brown adipose. In particular, expression of Sfrp1 (secreted frizzled-related protein 1) positively correlates with brown adipose mass across all SM/J cohorts and with improved glucose tolerance in the high fat-fed SM/J mice. Sfrp1 has previously been identified as a pro-adipogenic cytokine in white adipose that positively correlates with insulin sensitivity and declines in obesity. The Sfrp1 locus contains variants between LG/J and SM/J mice and is located within QTL for adiposity and glucose tolerance. The human SFRP1 genomic region contains a variant (rs973441) that is significantly associated with type 2 diabetes adjusted for BMI.

Overall, my dissertation robustly characterizes a novel mouse model of insulin-sensitive obesity dependent on natural brown adipose tissue expansion. I have defined the first brown adipose gene co-expression clusters and their relationship to metabolic phenotypes and identified the cytokine Sfrp1 as a novel stimulator of brown adipogenesis and insulin sensitivity. Together, these studies expand our knowledge of the potent non-thermogenic ability of brown adipose tissue to promote healthy metabolism in an obese state.
Chapter 1

Introduction

by
Caryn Carson
1.1 Overview

Adipose is an incredibly complex tissue. Commonly thought of in terms of energy storage, adipocytes also secrete a wide variety of cytokines to coordinate systemic health with various other metabolic organs. The inability of adipose tissue to keep up with excess nutrient levels is a harbinger of impending metabolic disorders, causing adipocyte hypertrophy, inflammation, insulin resistance, and ectopic lipid deposition into other organs. There are two primary types of adipose, “white” and “brown”. White adipocytes are characterized by one large lipid droplet that occupies almost all of the cell mass. Brown adipocytes contain multiple smaller lipid droplets as well as many mitochondria that cause the signature brown color of the cell. Both types of adipose store energy in the form of lipid droplets and secrete cytokines to influence systemic metabolism, however brown adipose can also undergo non-shivering thermogenesis to release energy as heat.

Identifying differences in adipose function between healthy and unhealthy metabolic states is critical for understanding how to prevent or ameliorate adipose dysfunction that leads to metabolic disorders. My thesis focuses on characterizing the role of brown adipose tissue in a mouse model of healthy obesity. Here I present current knowledge on obesity and metabolic dysfunction in humans and mouse models. I then detail metabolic and endocrine functions of brown adipose tissue in particular, and how these functions can change in obesity or metabolic disorders. I also present the transcriptional regulation of brown versus white adipocyte identity and what little is known about gene-by-environment effects in brown adipose tissue. Altogether, this information provides context for my research on the physiology, morphology, and transcriptional networks of brown adipose tissue in healthy obesity.
1.2 Obesity and Metabolic Dysfunction

Obesity and metabolic disorders are a public health epidemic in the United States and across the world at large. Recent estimates place prevalence of obesity at 35-40%, type II diabetes at 9.3% prevalence, and general metabolic syndrome at 34% prevalence in American adults (Aguilar, 2016; Flegal, 2016; McGinnis & Zoske, 2008), with increased risk among older populations (Ford, 2002). Though many people with obesity have no metabolic complications (Blüher, 2010; Brochu, 2001; Denis & Obin, 2013; Karelis, 2008; Primeau, 2011; Wildman, 2017), excess body weight is a predominant risk factor for type II diabetes and altered glucose homeostasis (Colditz, 1995; De Ycaza, 2018; Smith, 2019). Excess body weight can lead to development of additional metabolic complications due to the inability of existing adipose depots to keep up with blood nutrient levels (Gustafson, 2015). Adipose tissue serves as a storage organ, taking in glucose and lipids and maintaining them in lipid droplets as insurance against future energy deficits (Chouchani & Kajimura, 2019). Prolonged nutritional excess and subsequent adiposity leads to systemic insulin resistance and hyperinsulinemia (Friedman & Fehr, 2002; Kahn, 2003; J. Kim, 2007; Shulman, 2014) through adipocyte hypertrophy, ectopic lipid deposition (Chouchani & Kajimura, 2019; Gray & Vidal-Puig, 2007; Grundy, 2015; Sethi & Vidal-Puig, 2007), and inflammation and fibrosis of adipose tissue (Coletta, 2008; Ferrante, 2007; Grant & Dixit, 2015; Guilherme, 2019; S. Klein & Romijn, 2016; Lumeng, 2007; Mehran, 2012; Ouchi, 2011; Škopková, 2007; Spoto, 2014; Sun, 2013; Weisberg, 2003).

Obesity, diabetes, and other metabolic diseases have strong genetic and epigenetic components (Barroso & McCarthy, 2019; Pigeyre, 2016). There have been several studies identifying monoallelic drivers of obesity (Snyder, 2004; Stutzmann, 2008) and many gene-by-environment (Franks, 2011; Li & Qi, 2019; Llewellyn & Wardle, 2015; Nagpal, 2018; Wang, 2019) and genome-wide association studies (GWAS) of obesity and type II diabetes in
particular (Fall & Ingelsson, 2014; Heid, 2010; Turcot, 2018; Williams, 2014). Patients with obesity and type II diabetes have altered DNA methylation profiles causing altered gene expression that likely contributes to unhealthy adipose function (Agha, 2015; Crocker, 2020; Nilsson, 2014).

1.2.1 Mouse Models of Genetic and Diet-Induced Obesity

With the knowledge that genetics plays an important role in obesity, as well as the fact that humans are genetically heterogeneous, it is not surprising that the effects of diet and obesity in mice is highly strain-specific (Fontaine & Davis, 2016; Montgomery, 2013). Even closely related sub-strains of the oft-used C57Bl6 inbred mouse strain show differences in metabolic response to unhealthy diets (Attané, 2016; Sims, 2013). Many studies have compared various inbred mouse strains when exposed to a high fat diet, classifying mice as either obesity-prone or obesity-resistant based on weight gain, glucose and insulin levels, altered adipose function, and even diet-induced infertility (Alexander, 2006; Andrikopoulos, 2005; Chu, 2017; Collins, 1997; Eberhart, 1994; Leiter & Reifsnyder, 2004; Smith, 2000; Surwit, 1995; Surwit, 1998; Tortoriello, 2004; West, 1992; West, 1995). The C57Bl6 strain is particularly susceptible to diet-induced obesity, reliably developing glucose intolerance and insulin resistance on either a high fat-diet or “Western” cafeteria diet (Lang, 2019), and has been used as the background strain for many genetic models of obesity like the classic ob/ob leptin and db/db leptin receptor mutant mouse lines (Brockmann & Bevova, 2002).

1.2.2 LG/J and SM/J Inbred Mouse Strains

The LG/J and SM/J inbred mouse strains were originally bred for large and small body size, respectively (Brockmann & Bevova, 2002; Chai, 1956; Goodale, 1941). These two strains have distinct responses to high fat diet exposure: LG/J mice develop higher serum lipid levels,
but SM/J mice develop higher fasting glucose and a greater increase in body and fat pad weight (Cheverud, 1999; Ehrich, 2003). Recently, these strains have been used to interrogate the genetic architecture behind metabolic traits. Various levels of an advanced intercross between the LG/J and SM/J mice have led to the identification of quantitative trait loci underlying obesity and type II diabetes-related metabolic traits (Cheverud, 2001; Cheverud, 2011; Kraja, 2012; Lawson, 2011a; Lawson, 2011b; Lawson, 2010; Nikolskiy, 2015). The differential response to high fat diet between the LG/J and SM/J strains make them an interesting model to study how genetic background can affect adipose tissue and related metabolic dysfunction.

1.3 Brown Adipose Tissue as a Metabolic Organ

Brown adipose has been extensively studied as a thermogenic organ. The ability to release energy in the form of heat, known as non-shivering thermogenesis, was first characterized as a way for newborn mammals to regulate their body temperature. Thermogenesis has since been identified as a potent regulator of metabolic homeostasis (Cannon & Nedergaard, 2004; Chechi, 2013) of particular interest since the confirmation of active brown adipose depots in the supraclavicular region of adult humans just over ten years ago (Cypess, 2009; Nedergaard, 2007; Saito, 2009; van Marken Lichtenbelt, 2009; Virtanen, 2009). Thermogenic activation results in the intake of glucose and lipids from the blood into brown adipocytes, and can thus be utilized to normalize metabolite levels (Bartelt, 2011; Chondronikola, 2014; Chondronikola, 2016; Fromme & Klingenspor, 2011; Fu, 2013; Matsushita, 2014; McGlashon, 2015; Ouellet, 2012; Weir, 2018). Indeed, increasing the amount of brown adipose tissue through transplantation has been shown to improve glucose homeostasis and insulin sensitivity (Stanford, 2013), increase whole-body sympathetic activity to resist diet-induced obesity (Zhu, 2014), reverse type I diabetes (Gunawardana & Piston, 2012), and reverse diet-induced weight gain, insulin resistance, and adipose inflammation
(Shankar, 2019) in mouse models. In contrast, removal of brown adipose tissue through genetic or surgical methods results in obesity, hyperglycemia, hyperinsulinemia, and hyperlipidemia (Hamann, 1996; Lowell, 1993; Shankar, 2019).

1.3.1 Insulin and Adrenergic Signaling

Non-shivering thermogenesis in brown adipocytes occurs through the action of uncoupling protein 1 (UCP1), which also serves as the primary transcriptional and proteomic marker of adipocyte thermogenic capacity. UCP1 acts to increase proton leakage across the inner mitochondrial membrane, causing futile energy release as heat instead of ATP synthesis (Klingenspor, 2003). Activation of thermogenic activity is generally achieved by beta-adrenergic signaling stimulated through neuronal release of the catecholamine norepinephrine in response to cold or dietary stimuli (Bartness, 2010; Bronnikov, 1992; Himms-Hagen, 1985; Pulinilkunnil, 2011). While norepinephrine signaling also induces glucose uptake into brown adipocytes, it is unclear whether this is dependent on UCP1 activity or not (Hankir, 2017; Inokuma, 2005). Additionally, the necessity of each component of the norepinephrine – UCP1 signaling pathway for thermogenesis and resulting metabolic improvement seems to be dependent on age (Araiz, 2019), ambient temperature (Feldmann, 2009; Liu, 2003), and genetic background used (de Jong, 2017; Enerbäck, 1997; Ukropec, 2006).

Both norepinephrine and insulin signaling cause brown adipocytes to take up glucose from the blood (Chondronikola, 2020; Peirce & Vidal-Puig, 2013), processes that are blunted in obesity (Orava, 2013) and diabetes (Lapa, 2017). These processes are independent from each other (Marette & Bukowiecki, 1989; Olsen, 2019; Orava, 2011; Shimizu, 1996) and antagonistic. Norepinephrine inhibits insulin-stimulated glucose uptake in brown adipocytes (Klein, 1999) while loss of insulin signaling leads to increased expression of Ucp1 (Botezelli, 2020; Guerra, 2001). Deletion of the gene Mfn2, which is required for thermogenesis, leads to
improved whole-body insulin sensitivity, suggesting that the contribution of brown adipose tissue to prevention of insulin resistance can occur completely independently of thermogenesis (Mahdaviani, 2017). Exploiting insulin signaling in brown adipose tissue is one avenue to explore weight loss-independent regulation of glucose homeostasis (Marlatt, 2018), particularly since increasing the expression of the insulin-responsive glucose transporter *Glut4* in all adipose tissue leads to drastically improved glucose tolerance (Chadt & Al-Hasani, 2020; Shepherd, 1993) and brown adipose tissue has higher glucose uptake than white adipose tissue even under thermoneutral conditions (Weir, 2018).

### 1.4 Brown Adipose Tissue as an Endocrine Organ

Though brown adipose tissue has been primarily studied for its thermogenic capacity, it is also a critical endocrine organ that secretes numerous proteins to coordinate metabolism amongst various different organs (Deshmukh, 2018; Poekes, 2015; Villarroya, 2019; Villarroya, 2013; Wang, 2015; Whittle, 2011). Many of these adipokines are expressed and secreted by both brown and white adipose tissue, but there are a few that are preferentially secreted by brown adipose tissue. Here I will highlight several adipokines that have pronounced effects on metabolism and brown adipose function.

#### 1.4.1 General Adipokines

Adiponectin and leptin are two classic adipokines that dramatically affect whole-body metabolism (Funcke & Scherer, 2019; Houde, 2015). Adiponectin is a pro-health adipokine first identified in 1996 and downregulated in obesity (Hu, 1996). Adiponectin improves lipid homeostasis (Asterholm & Scherer, 2010) in obese mouse models, causing a reduction in ectopic lipids that leads to improved insulin sensitivity (Yamauchi, 2001; Yamauchi, 2002). Conversely, reduction in adiponectin signaling leads to insulin resistance and inflammation.
Adiponectin also acts through the brain to promote weight loss (Qi, 2004), while leptin acts on the brain to signal satiety (Caron, 2018). Leptin also acts in a paracrine matter to increase the amount of brown adipose tissue mass through adipogenesis (Wagoner, 2006), thus leading to increased insulin-stimulated glucose uptake (Wang, 1999) and improved whole-body glucose tolerance and insulin sensitivity (D’souza, 2017; DiSilvestro, 2016).

Neuregulin 4 (Nrg4) was the first adipokine identified as brown fat-enriched. Expression of Nrg4 increases during brown adipocyte differentiation and is reduced in both human and rodent obesity (Wang, 2014). Nrg4 reduces hepatic steatosis through activation of hepatic fatty acid oxidation and improves whole-body insulin sensitivity by increasing insulin-stimulated glucose uptake in skeletal muscle (Chen, 2017; Wang, 2014). In addition to these endocrine functions, within brown adipose tissue Nrg4 acts to promote angiogenesis, with a conditional knockout mouse model showing reduced blood vessels and increased adiposity and poor glucose tolerance on normal chow diet (Nugroho, 2018).

Fibroblast growth factor 21 (Fgf21) secreted by brown adipose tissue affects systemic metabolism through a wide range of organs (Poekes, 2015). Fgf21 acts upon the brain to increase liver insulin sensitivity in diet-induced obesity (Sarruf, 2010) and upon the heart to attenuate cardiac remodeling during hypertension (Ruan, 2018). Though its secretion is activated by thermogenesis (Hondaes, 2011), Fgf21 can induce weight loss in obese mice (Coskun, 2008) independently from Ucp1 activity (Kwon, 2015; Samms, 2015). It is generally accepted that Fgf21 administration also improves whole-body glucose clearance, however two independent studies came to opposite conclusions on whether this action is Ucp1-dependent or independent (Kwon, 2015; Samms, 2015). The mouse models used in these two studies had slightly different genetic backgrounds and were kept at slightly different ambient temperatures,
which may have caused the discrepancy in results and further highlights the necessity of considering gene-by-environment interactions when interpreting physiological data.

As a heavily vascularized tissue, proper brown adipose expansion and function relies upon proper angiogenesis (Sun, 2012). The adipokine vascular endothelial growth factor A (Vegfa) increases vascularization as well as promoting a brown adipocyte identity through increasing expression of Ucp1 and Pgc1a (Park, 2017; Sun, 2014). Vegfa expression increases during brown adipocyte differentiation, supporting adipocyte survival and inhibiting adipocyte apoptosis (Bagchi, 2013).

1.4.2 Fibrosis and Inflammation

Adipose tissue under metabolic dysfunction exhibits increased secretion of pro-fibrotic and pro-inflammatory cytokines, though brown adipose tissue seems to be more resistant to such obesity-related insults than white adipose tissue (Alcalá, 2017; Omran & Christian, 2020; Villarroya, 2018). Fibrosis occurs through altered extracellular matrix composition, particularly increased collagen deposition (Khan, 2009; Sun, 2013). Several adipokines that have increased expression in obesity promote fibrotic extracellular matrix composition, such as matrix metalloproteases (Chavey, 2003; Lee, 2014; Martinez-Santibanez, 2015) and semaphorins (Mejhert, 2013; Shimizu, 2013). This alteration of the extracellular matrix can both inhibit adipose expansion (leading to hypoxia and ectopic lipids) and cause insulin resistance within the adipose depot (Halberg, 2009; Kim, 2007; Sun, 2011).

Multigenerational exposure to a high fat diet causes hypomethylation and corresponding increased expression of pro-inflammatory genes in adipose tissue (Ding, 2014). Inflammation of adipose depots leads to impaired insulin sensitivity (Samuel & Shulman, 2016; Villarroya, 2018). This can occur through the actions of cytokines such as Angptl2, which is more highly
expressed in brown adipose than in white adipose (Fu, 2013; Tabata, 2009), and Cxcl12, which both recruits macrophages and is an autocrine insulin-desensitizing agent that causes lower \( Irs1 \) expression and lower glucose uptake (Kim, 2014; Shin, 2018). Other pro-inflammatory cytokines have broader impacts on systemic metabolism (Chida, 2008) or inhibit thermogenesis that would improve metabolic health (Pazos, 2016). Overall, though brown adipose is more resistant to diet-induced inflammation than white adipose tissue, obesity can still cause brown adipose malfunction leading to poor systemic metabolism.

1.5 Brown Adipocyte Identity

Brown and white adipocytes generally exist in distinct depots. However under certain conditions, such as thermogenic stimuli, brown-like adipocytes can appear in white adipose depots (Berry, 2017). These adipocytes are called beige adipocytes, and various studies have determined that beiging is caused by white to brown adipocyte transdifferentiation (Barbatelli, 2010; Cinti, 2009; Himms-Hagen, 2000; Rosenwald, 2013). Brown and beige adipocyte identity is epigenetically regulated (Brunmeir, 2016; Shapira, 2017) by histone deacetylases (Bagchi, 2018; Emmett, 2017; Rajan, 2018), long noncoding RNAs (Alvarez-Dominguez, 2015; Li, 2017), and microRNAs (Fu, 2014; He, 2018; Mori, 2012; Mori, 2014; Sun, 2011; Trajkovski, 2012; Trajkovski & Lodish, 2013; Xu, 2015).

All types of adipocytes originate from mesenchymal stem cells and require the action of the transcription factor peroxisome proliferator-activated receptor-\( \gamma \) (Pparg) (Harms & Seale, 2013; Kajimura, 2010; Loft, 2017; Rosen & MacDougald, 2006; Seale, 2010; Wang & Seale, 2016). Brown adipocyte lineage has also been identified as having a shared progenitor with smooth muscle cells (Hepler, 2017; Timmons, 2007), but different adipose depots may consist of adipocytes from other origins (Sanchez-Gurmaches & Guertin, 2014). Pgc1a (peroxisome
proliferator-activated receptor-γ coactivator 1α) is a cofactor of Pparg that has been identified as a brown adipose transcriptional marker, coordinating various processes such as mitochondrial biogenesis, oxidative metabolism, and angiogenesis (Chang, 2018; Puigserver, 1998; Uldry, 2006). Another brown adipose transcriptional marker, PR domain zinc finger protein 16 (Prdm16), controls chromatin architecture to determine brown over white transcriptional program (Harms, 2015; Kajimura, 2008; Seale, 2011) and is also a brown fat/skeletal muscle switch (Seale, 2008). Pparg agonists induce white to brown fat conversion through Prdm16 (Cohen, 2014; Ohno, 2012). Insulin signaling is also critical for brown adipogenesis, as Irs1 is required for brown adipocyte differentiation and acts upstream of Pparg (Fasshauer, 2001). A genetic knockout of Irs1 results in strong increase in expression of Wnt10b, which inhibits adipogenesis (Tseng, 2005), while genetic knockout of insulin leads to smaller brown adipose depots (Templeman, 2015; Templeman, 2016).

1.6 Gene-by-Environment Interactions in Brown Adipose Tissue

Though gene-by-environment interactions have been characterized in metabolism and obesity, very few studies have looked at gene-by-environment interactions in brown adipose tissue. In 1997, Collins et al. identified differences between the C57Bl6/J and A/J inbred mouse strains in high fat diet-induced reduction of brown adipose thermogenesis (Collins, 1997). This study formed the foundation for our understanding that genetic variation controls the ability of white adipose depots to undergo beiging in response to dietary fat (Coulter, 2003) or cold-exposure (Xue, 2007). Investigations into gene-by-environment interactions in specifically brown adipose tissue have almost exclusively been limited to studies on specific, already identified brown adipocyte identity genes (Da Fonseca, 2020; Pravednikova, 2020; Yoneshiro, 2013) or diet-induced obesity in mouse models with manipulated expression of one or two genes (Bale, 2003; de Jong, 2017; Enerbäck, 1997; Fu, 2014; Grünberg, 2017; Mahdaviani,
2017; Mercer & Trayhurn, 1987; Pazos, 2016; Shepherd, 1993; Syamsunarno, 2014; Templeman, 2015; Templeman, 2016). Given the genetic variation inherent in human populations, the lack of information on how genetic background affects brown adipose tissue function in obesity is a fairly large hole in our current knowledge, one that must be addressed if brown adipose is to ever become a successful therapeutic target.

1.7 Scope of Thesis

All previous studies of metabolism in the SM/J inbred mouse strain used mice at the age of 20 weeks or younger. A fortuitous accident shortly prior to the start of my project led to some high fat-fed SM/J mice aging out to 30 weeks and the discovery of a curious phenomenon: these mice that had previously shown poor metabolic health developed a healthy glycemic profile indistinguishable from low fat-fed counterparts. Though it might be expected that these mice improved their glycemic control through weight loss, they instead continued to gain weight and fat mass and showed a particularly dramatic expansion of the interscapular brown adipose depot that has never before been described. In the following chapters, I will present my research aimed at understanding and characterizing this expansion of brown adipose tissue and how it affects systemic metabolism in the healthy obese SM/J mice.

In Chapter 2, I report detailed physiological characterization of high and low fat-fed SM/J mice at 20 and 30 weeks. High fat-fed SM/J mice show significant improvements in basal glucose, glucose tolerance, and insulin tolerance between 20 and 30 weeks. The unique increase in brown adipose tissue as a proportion of total adipose mass is likely due to increased number of cells and is not accompanied by any morphological or transcriptional whitening of the depot. The expanded brown adipose tissue primarily affects systemic metabolism through improvements in insulin sensitivity, with upregulated expression of key genes in the insulin-
stimulated glucose uptake pathway. In Chapter 3, I identify multiple brown adipose gene co-expression clusters that correlate with metabolic traits or brown adipose mass. Expression of genes in these clusters is heavily dependent on diet and genetic background, in particular genes enriched for cell division function have high expression in high fat-fed SM/J mice. Finally, in Chapter 4, I characterize the transcriptional changes that accompany the brown adipose expansion in high fat-fed SM/J mice. This expanded brown adipose has a healthier transcriptional profile of inflammatory cytokines and extracellular proteins. One gene in particular, Sfrp1, has previously been identified as encoding a pro-adipogenic cytokine in white adipose tissue. The expression of Sfrp1 shows a significant correlation with brown adipose mass and with improved glucose tolerance in high fat-fed SM/J mice. Together, this body of work provides exciting new knowledge on non-thermogenic brown adipose function that could be utilized and manipulated to improve glycemic control in obesity.
Chapter 2

Physiological Characterization of Healthy Obesity in SM/J Mice

by

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2.1 Abstract

Disruption of glucose homeostasis increases the risk of type II diabetes, cardiovascular disease, stroke, and cancer. We leverage a novel rodent model, the SM/J mouse, to understand glycemic control in obesity. On a high fat diet, obese SM/J mice initially develop impaired glucose tolerance and elevated fasting glucose relative to low fat-fed controls. Strikingly, their glycemic dysfunction resolves by 30 weeks of age despite persistence of obesity. A prominent phenotype is that the mice dramatically expand their brown adipose depots as they resolve glycemic dysfunction. This occurs naturally and spontaneously on a high fat diet, with no temperature or genetic manipulation. Removal of the brown adipose depot significantly reduces insulin sensitivity indicating that the expanded tissue contributes to metabolic health by functioning as an insulin-stimulated glucose sink. We describe morphological and physiological changes that occur during the unique brown adipose expansion and remission of glycemic dysfunction in obese SM/J mice. Understanding how the expanded brown adipose contributes to glycemic control in SM/J mice will open the door for innovative therapies aimed at improving metabolic complications in obesity.

2.2 Introduction

An estimated 10-30% of obese individuals maintain glycemic control and some longitudinal studies suggest their risk of developing type II diabetes is no greater than matched lean individuals (Meigs, 2006). No causative factors underlying glycemic control in obesity have been discovered, however the strongest predictors of impaired glycemic control in obesity are increased visceral fat mass and adipose tissue dysfunction (Goossens, 2017; Klöting, 2010). Thus, research efforts have focused on understanding the genetic and physiological mechanisms of action of adipose. Recent research reveals that brown adipose activity is associated with anti-
diabetic properties. Cold exposure in both obese and lean individuals causes increased uptake of fatty acids and glucose into brown adipose tissue (Saito, 2009). Further, increased brown adipose activity has been shown to improve glucose homeostasis and insulin sensitivity in adults (Chondronikola, 2014). Transplantation of brown adipose tissue into mouse models of diabetes greatly improves glucose parameters, including fasting glucose levels and response to a glucose challenge (Gunawardana & Piston, 2012). While there are a variety of obese and diabetic mouse models, there are no mouse models for understanding the relationship between brown adipose and glycemic control in obesity.

The SM/J inbred mouse strain has long been used for studying interactions between diet and metabolism, and recently has started to help uncover the genetic architecture underlying diet-induced obesity and glucose homeostasis. It has previously been shown that fed a high fat diet, SM/J mice display many of the characteristics of a diabetic-obese mouse: obesity, hyperglycemia and glucose intolerance at 20 weeks of age (Cheverud, 2011; Ehrich, 2003; Lawson, 2010; Lawson, 2011a; Lawson, 2011b). We discovered that SM/J mice undergo a remarkable transformation between 20 and 30 weeks of age. Despite persistence of the obese state, these mice enter into diabetic remission: returning to normoglycemia and reestablishing glucose tolerance and improving insulin sensitivity. Contemporary with this remission of glycemic parameters is a dramatic expansion of the intrascapular brown adipose depot. Our results strongly suggest that SM/J’s brown adipose contributes to diabetic remission through non-thermogenic mechanisms. This study describes the morphological and physiological changes that occur during this transition, and establishes the SM/J mouse as a unique model for understanding the relationship between brown adipose and glycemic control in obesity. Understanding this relationship in a genetic model of glycemic resolution will set the stage for identifying novel, potentially therapeutic targets for the improvement of glycemic control.
2.3 Results

2.3.1 SM/J mice improve glucose parameters without weight loss

When fed a high fat diet from 3 weeks of age, SM/J mice develop obesity, hyperglycemia, and impaired glucose tolerance by 20 weeks (Ehrich, 2003). By 30 weeks, despite the persistence of obesity, high fat-fed SM/J’s resolve their hyperglycemia and impaired glucose tolerance to levels indistinguishable from low fat-fed controls (Figure 2.1A-D). Thirty-week high fat-fed SM/J mice have dramatically increased serum and pancreatic insulin levels compared to 20 week animals (Miranda, 2020), and insulin sensitivity improves along with improved glycemic parameters (Figure 2.1E and F). Notably, metabolic hormones such as adiponectin, glucagon, IGF1, and leptin do not show any changes in circulating levels between 20 and 30 weeks (Figure 2.2). Further, the changes in glucose phenotypes are not transient effects, rather high fat-fed animals maintain improved basal glucose, glucose tolerance, and insulin tolerance as they age (Figure 2.3).

High fat-fed C57BL/6J mice also show a reduction in fasting glucose that is accompanied by increased insulin with age (Ahren, 2004). In contrast to SM/J, the difference in circulating glucose between the high fat-fed and low fat-fed C57BL/6J remain significantly different over time. Moreover, high fat-fed C57BL/6J mice show marked glucose intolerance that does not resolve with age. We observe a similar trend in the LG/J strain of mice, where high fat-fed animals maintain higher fasting glucose levels and impaired glucose tolerance relative to low fat-fed controls as they age (Figure 2.4). The unique remission of hyperglycemia and improved glucose tolerance observed in the high fat-fed SM/J strain indicates a genetic basis.
Figure 2.1 Obese SM/J mice improve glucose parameters between 20 and 30 weeks of age. SM/J mice gain weight between 20 and 30 weeks of age on both high and low fat diets (A), n = 140, 106, 78, and 48 mice in HF20, HF30, LF20, and LF30 cohorts. 30 week-old high fat-fed mice have significantly lower fasting glucose levels than at 20 weeks (B). 30 week-old high fat-fed mice have improved glucose tolerance relative to 20 weeks (C-D). Lower fasting glucose and improved glucose tolerance corresponds with improved insulin sensitivity (E-F). Equal numbers of males and females represented; n = 39-49 mice per 30 week cohort, 51-71 mice per 20 week cohort. * p<0.05, ** p<0.01, *** p<0.001
2.3.2 High fat-fed SM/J mice expand their interscapular brown adipose tissue depots

Contemporary with the resolution of glycemic parameters, high fat-fed SM/J mice dramatically expand their interscapular brown adipose depots, which is not seen in low fat-fed control mice (Figure 2.5A-C). This has never been described in another mouse strain, and we do not observe the phenomenon in the LG/J strain of mice on the same diets at any age (Figure 2.5D). To understand whether the tissue mass expansion is due to increased size of individual cells or to increased number of total cells, we quantified adipocyte cell size and the mitotic index. There are no significant differences in average cell size in high fat-fed mice between 20

![Figure 2.2 Circulating cytokine levels in SM/J mice. Plasma levels of IGF1 (A), glucagon (B), adiponectin (C), and leptin (D) in high and low fat-fed SM/J mice at 20 and 30 weeks. No cohorts showed any significant differences in levels. Equal numbers of males and females represented, n = 8-16 animals per cohort.](image-url)
Figure 2.3 Physiological parameters of the SM/J inbred mouse strain across age. Basal glucose (A) and glucose tolerance (B) of high and low fat-fed SM/J mice at 10, 20, 30, 40, and 50 weeks. Insulin (C) and insulin tolerance (D) of high and low fat-fed SM/J mice at 20, 30, 40, and 50 weeks. Interscapular brown adipose depot weight in grams (E) and as a percentage of total body weight (F) of SM/J mice at 10, 20, 30, 40, and 50 weeks. Equal numbers of males and females represented, n=21-73 (A-C) or 16-25 (D-E) for 10-40 week animals, and n = 6-7 (A-E) for 50 week animals.
Figure 2.4 Physiological parameters of the LG/J inbred mouse strain. Body weight of high and low fat-fed LG/J mice at 20 and 30 weeks of age (A). Basal glucose (B) and glucose tolerance (C-D) of high fat-fed LG/J mice are higher than low fat-fed controls at 30 weeks. Insulin sensitivity (E-F) is not different among the LG/J cohorts. n = 30-60. Equal numbers of males and females represented. All measurements taken identically to SM/J mice as described in main Methods. *p<0.05, **p<0.01, ***p<0.0001
and 30 weeks, or relative to low fat-fed controls (Figure 2.6A). Mice on both diets undergo altered adipocyte area profiles between 20 and 30 weeks of age, however the low fat tissue develops a profile significantly trending towards larger adipocytes at 30 weeks (p=6.4^{0.07}) whereas the high fat tissue develops a profile significantly trending towards smaller adipocytes at 30 weeks (p=2.2^{16}) (Figure 2.6B-C). This suggests that the expansion of the brown adipose depot in high fat-fed mice is not the result of increased lipid uptake into already existing adipocytes. Quantification of brown adipose cells stained positive for the mitotic marker phosphohistone H3 showed a trend towards a higher mitotic index in the brown adipose of high

Figure 2.5 High fat-fed SM/J mice develop unique expansion of brown adipose tissue.
Representative pictures of 20 (A) and 30 (B) week-old high fat-fed female mice with insets showing larger brown adipose lobes in 30 week animals. Quantification of interscapular brown adipose depot as a proportion of total fat mass in SM/J mice (C) or as a percent of total body weight in LG/J mice (D) shows significant increase in only SM/J high fat animals. n = 16-25 mice per cohort.
fat-fed animals (Figure 2.6D), suggesting that the increased mass may be due to increased number of cells.

Because obesity has been associated with structural and functional “whitening” of brown adipose depots in rodents (Lapa, 2017; Roberts-Toler, 2015; Shimizu, 2014; Shimizu & Walsh, 2015), we confirmed that the tissue expansion in SM/J mice has the expected properties of brown fat. Histological analysis of the fat depot taken from high fat-fed SM/J mice at 30

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**Figure 2.6** Increased brown adipose tissue is likely due to increased number, not size, of cells. Brown adipose sections from high and low fat-fed mice at 20 and 30 weeks were stained with hematoxylin and eosin (H&E) (A-C) or DAPI to mark nuclei and a phosphohistone H3 (pHH3) antibody to mark mitotic nuclei (D). Average cell area (A) and cell area density graphs for high fat (B) and low fat-fed (C) cohorts. Data plotted on a log10 scale for visualization. n= 4 images each of 4 mice per cohort.
Figure 2.7 Histochemistry reveals no morphological changes in expanded brown adipose tissue. Representative images of brown (left panels) and white (right panels) adipose from (A-B) high fat-fed mice at 20 and (C-D) 30 weeks of age and from (E-F) low fat-fed mice at 20 and (G-H) 30 weeks of age. Stained for H&E (left picture) or UCP1 (yellow) and DAPI (blue) (right picture). Histology procedures described in main Methods section.
weeks of age confirms the adipocytes in this expansion are brown adipocytes, with small
multilocular lipid droplets and high UCP1 staining (Figure 2.7). Expression of canonical brown
adipose genes (Ucp1, Cidea, Eva1, Prdm16, Pgc1a) do not change between 20 and 30 weeks
(Figure 2.8), nor is there any evidence of a general “beiging” phenomenon in white adipose
tissue (Wu, 2012). There is no significant difference in brown adipose tissue mitochondrial
content, (Figure 2.8J), core body temperature, or circulating free fatty acids between high and
low fat-fed cohorts or between 20 and 30 weeks of age (Figure 2.9A-B). Additionally, while
there are diet-dependent differences in the catecholamines norepinephrine and epinephrine,
which activate UCP1-mediated leak respiration and non-shivering thermogenesis, there is no
change in levels between ages in the high fat-fed mice (Figure 2.9C-D). Thus, the interscapular
adipose depot in high fat-fed SM/J mice maintains a brown adipose identity after expansion that
is not dependent on whole-animal beiging, and is also not associated with altered
thermogenesis.

2.3.3 Expanded brown adipose tissue serves as an insulin-stimulated glucose sink

If the brown adipose expansion is directly related to the glycemic resolution of the high
fat-fed SM/J mice, preventing or removing that expansion should revert their glucose
parameters to an unhealthy state. To test these predictions, we removed the interscapular brown
adipose depots from hyperglycemic 20 and normoglycemic 30 week-old mice. Interestingly,
removal of the brown adipose depot at 20 weeks does not affect serum glucose or insulin levels,
or the glucose tolerance of the animals, indicating that the expanded brown adipose is
downstream of the primary signal (Figure 2.10A-C) (Miranda, 2020). However, removal of the
brown adipose depot before expansion prevents the natural improvement in insulin tolerance
and removal of the expanded tissue at 30 weeks results in a reversion to 20 week-old
measurements (Figure 2.10D). These results strongly suggest that the expanding brown adipose
Figure 2.8 Thermogenic genes do not change between 20 and 30 week adipose tissue in high fat SM/J animals. Gene expression quantified in three adipose depots of high fat-fed mice: intrascapular brown adipose (iBAT), inguinal white adipose (ingWAT), and reproductive white adipose (repWAT), n = 6-10 mice per cohort and tissue. Canonical brown adipose genes (A) Ucp1, (B) Cidea, (C) Eva1, (D) Pgc1a, and (E) Prdm16 show high expression in iBAT and no difference between 20 and 30 week-old mice. Beige adipose marker (F) Tbx1 is not expressed in any depot. All adipose depots had roughly equal expression of general adipose markers (G) Adipoq, (H) Fabp4/AP2, and (I) Pparg. (J) Mitochondrial copy number was significantly higher in brown adipose tissue than in inguinal white adipose tissue at both 20 and 30 week time points with no significant difference between high or low fat-fed mice, n = 6-7 mice per cohort and tissue. Statistical differences calculated between 20 and 30 week cohorts within individual depots, * p<0.05, ** p< 0.01.
Figure 2.9 SM/J thermogenic parameters. Body temperature (A) and plasma cholesterol (B), free fatty acids (C), triglyceride (D), norepinephrine (E) and epinephrine (F) concentrations do not change between 20 and 30 weeks in high fat-fed mice. n = 8-16 (A, E, F) or 15-21 (B-D) mice, equal numbers of males and females represented. *p<0.05, **p<0.01.
Figure 2.10 Brown adipose expansion is required for improved insulin tolerance. Fasting glucose (A) and insulin (B) and glucose tolerance (C) were not significantly altered by removal of the brown adipose depot before (20 week excision) or after (30 week excision) expansion. Insulin tolerance (D) was significantly worse after BAT ablation, n = 8 mice per excision cohort. Expression of Irs1 (E) and Glut4 (F) increases significantly in expanded brown adipose depot, n = 8 mice per cohort. Equal numbers of males and females represented; * p<0.05, ** p< 0.01, *** p <0.001
tissue of obese SM/J mice serves as an insulin-stimulated glucose sink. This is supported by increased expression of both *Irs1* and *Glut4* (Figure 2.10E-F), key members of the insulin-stimulated glucose pathway (Pessin & Saltiel, 2000).

2.4 Discussion

Brown adipose has anti-diabetic properties that are conserved between mice and humans (Chechi, 2013; Cypess, 2009; Hanssen, 2016; Saito, 2009; Saito, 2013; Stanford, 2013; van Marken Lichtenbelt, 2009; Virtanen , 2009). Most brown adipose tissue research focuses on the effects of non-shivering thermogenesis on metabolic health. However, brown adipose coordinates a diverse array of physiological processes through the action of secreted factors associated with glucose homeostasis (e.g. FGF21, BMPs, VEGFA, IL-6, NRG4) (Poekes, 2015; Villarroya, 2017; Villarroya, 2019). Further, the mechanism of insulin-stimulated glucose uptake into brown adipose tissue was well established before the discovery of beta-adrenergic stimulation of thermogenesis (Marette & Bukowiecki, 1989). This mechanism is independent of UCP1 activity (Inokuma, 2005; Olsen, 2019; Orava, 2011). Insulin-stimulated glucose uptake increases in animals with brown adipose transplants (Stanford, 2013) and is blunted under obese (Orava, 2013) and diabetic conditions (Lapa, 2017). Here we report a natural expansion of brown adipose tissue in obese mice that coincides not with increased thermogenesis, but with improved insulin sensitivity, likely through increased insulin-stimulated glucose uptake into the brown adipose tissue. We have shown corresponding increased expression of two critical members of the insulin signaling pathway: *Glut4*, which mediates insulin-stimulated but not norepinephrine-stimulated glucose uptake (Shimizu, 1996), and *Irs1*, which is also necessary for brown adipose differentiation (Fasshauer, 2001). It is possible that brown adipose expansion in high fat fed SM/J mice is a protective mechanism to improve insulin sensitivity before the hyperglycemia gets out of control.
There is great interest in harnessing the potential of brown adipose to treat obesity and diabetes, either through the calorie burning action of non-shivering thermogenesis or the endocrine action of adipokines. Research into the effects of brown adipose on systemic metabolism is in its infancy, and the community needs appropriate animal models to interrogate its physiological roles and identify potentially druggable targets. We present the SM/J mouse strain as a unique model to address this need. The SM/J mouse provides a tractable, genetic system in which to understand the relationship between brown adipose and glycemic control in obesity. Understanding this relationship in the SM/J mouse will open doors for identifying novel, potentially druggable targets for the improvement of glycemic control in humans.

2.5 Methods

2.5.1 Animal Husbandry and Phenotyping

SM/J (RRID:IMS R_JAX:000687) and LG/J (RRID:IMS R_JAX:000675) mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Experimental animals were generated at the Washington University School of Medicine and all experiments were approved by the Institutional Animal Care and Use Committee in accordance with the National Institutes of Health guidelines for the care and use of laboratory animals. Pups were weaned at 3 weeks and reared in same-sex cages of 3-5 animals until necropsy. At weaning, mice were randomly placed on a high fat diet (42% kcal from fat; Teklad TD88137) or an isocaloric low fat diet (15% kcal from fat; Research Diets D12284). Feeding was *ad libitum*. The animal facility operates on a 12 hour light/dark cycle with a constant ambient temperature of 21°C. Animals were weighed weekly until sacrifice. At 18 and 28 weeks of age, animals were subject to an intraperitoneal glucose tolerance test after a 4 hour fast. At 19 and 29 weeks of age animals were subject to an intraperitoneal insulin tolerance test. At 20 or 30 weeks of age, body
composition was determined by MRI and temperature was measured with a rectal thermometer. After a 4 hour fast, at 20 or 30 weeks of age, animals were given an overdose of sodium pentobarbital and blood was collected via cardiac puncture. Euthanasia was achieved by cardiac perfusion with phosphate-buffered saline. After cardiac perfusion, tissues were collected and flash frozen in liquid nitrogen and stored at -80°C, or processed according to protocols for histology and other assays.

2.5.2 Blood Plasma Assays

Fasting blood glucose was measured using a GLUCOCARD Vital glucometer (Arkay, MN USA). ELISAs measuring plasma levels of free fatty acids (Wako Life Sciences 995-34693), leptin (Crystal Chem #90030), adiponectin (Crystal Chem #80569), IGF1 (Crystal Chem #80574), and glucagon (Crystal Chem #81518) were quantified according to manufacturer’s protocol. Catecholamines were assayed through the Vanderbilt University Medical Center’s Hormone Assay and Analytical Services Core (www.vumc.org/hormone/assays; NIH grants DK059637 (MMPC) and DK020593 (DRTC)).

2.5.3 Brown Adipose Histology

At the time of tissue collection, small portions of interscapular brown and reproductive white adipose tissues were placed in 1 mL of neutral buffered formalin. These samples were incubated at 4°C while gently shaking for 24 hours. Immediately afterwards, samples were placed into plastic cages and processed into paraffin blocks using a Leica tissue processor with the following protocol: 70% EtOH for 1 hour x 2, 85% EtOH for 1 hour, 95% EtOH for 1 hour x 2, 100% EtOH for 1 hour x 2, Xylenes for 1 hour x 2, paraffin wax. Adipose blocks were sectioned into 6 µm sections, with 2-4 slices on each slide.
2.5.4 H&E Staining

Slides were incubated at 60°C for 1 hour, then placed in xylenes to remove remaining paraffin wax. Slides were then rehydrated using successive decreasing EtOH concentrations (xylenes x 2, 100% EtOH x 2, 95% EtOH, 70% EtOH, H2O). Slides were incubated in hematoxylin (Leica Surgipath 3801570), Define (3803590), Blue Buffer 8 (3802915), and eosin (3801616), and dehydrated (95% EtOH, 100% EtOH, xylene x 2). Imaging was performed using the Zeiss AxioPlan2 microscope and Olympus DP software. Analysis of adipocyte size was performed using ImageJ. Images were converted to black and white and skeletonized to reveal only the cell wall outlines. Cell area was calculated from outlines with a lower limit of 50 um and upper limit of 700 um to reduce noise. All cells from a cohort (4-7 images each from 4 animals per cohort, equal numbers of males and females) were pooled for cell area density analysis. A Welch's unequal variances t-test was performed between ages in each diet to determine significant differences.

2.5.5 Immunofluorescence

Slides were incubated at 60°C for 1 hour, then placed in xylenes to remove remaining paraffin wax. Slides were then rehydrated using successive decreasing EtOH concentrations (xylenes x 2, 50% EtOH in xylenes, 100% EtOH x 2, 95% EtOH, 70% EtOH, 50% EtOH, 0.3% H2O2 in MeOH, H2O). Slides were washed with TBS and blocked in 10% normal donkey serum (Abcam ab7475) for 1 hour, followed by incubation with primary antibody overnight at 4°C. [Primary antibodies: rabbit anti-Ucp1 (1:100, Sigma U6382) and mouse anti-PHH3 (1:100, Invitrogen MA5-15220)]. After an additional wash, secondary antibody was applied for 1 hour at room temperature [Secondary antibodies: donkey anti-rabbit 488 (1:1000, Abcam ab150061) and donkey anti-mouse 647 (1:200, Abcam ab150107)]. Fluoroshield Mounting
Medium with DAPI (Abcam) was applied to seal the coverslip and slides were stored at 4°C. Imaging was performed using the Zeiss Confocal microscope and Zen Lite imaging program. PHH3 analysis was performed using the CellProfiler program. Background was subtracted from DAPI and PHH3 channels using ImageJ. DAPI channel was used to identify total nuclei in CellProfiler. Adipose nuclei images were overlaid with PHH3 stain to identify mitotic adipose nuclei. Mitotic nuclei were summed across all 4 slides for each individual. Mitotic adipose index is reported as mitotic adipose nuclei divided by adipose nuclei multiplied by 100%.

2.5.6 Quantitative rt-PCR

Total RNA was extracted from brown, subcutaneous inguinal, and visceral reproductive adipose samples using the Qiagen RNeasy Lipid Kit. High-Capacity cDNA Reverse Transcription Kit (Thermofisher) was used for reverse transcription. Quantitative-rtPCR was performed to assess expression levels of target genes with an Applied Biosystems (USA) QuantStudio 6 Flex instrument using SYBR Green reagent. Results were normalized to L32 expression, which was experimentally determined to not be differentially expressed across diet and age cohorts. cDNA products were analyzed by the ΔCₜ method. Primers listed in Table 2.1.

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<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<td>GGGATTGTGACTCTGATGG</td>
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<tr>
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<td>ACGTCATCTTGGCATGACT</td>
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<td>h19</td>
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<td>AAGGTTTAGAGAGGGGCGC</td>
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2.5.7 Mitochondrial DNA Quantification

DNA was extracted from brown and inguinal adipose tissues using the Qiagen DNeasy Blood and Tissue Kit. Briefly, 40mg of tissue was homogenized in 10% proteinase K through vortexing and incubation at 56°C. DNA was precipitated with ethanol, collected in a spin column, and eluted in 150mL of buffer. DNA concentration was quantified on a Nanodrop, and 50ng was used in a qPCR reaction to quantify the amount of h19 (nuclear gene) and CytB (mitochondrial gene). Mitochondrial content was calculated as the ratio of mtDNA to nucDNA. Primers listed in Table 2.1.

2.5.8 Brown Adipose Excision

Interscapular brown adipose tissue depots were removed from 20 or 30 week-old high fat-fed SM/J mice. A small longitudinal incision was made between the shoulder blades. All interscapular adipose tissue was carefully removed, and a cauterizing wand used to stop excessive bleeding when necessary. Surgeries were performed under general anesthesia by IP injection of ketamine/xylazine (100/200 mg/Kg) and mice were maintained in the surgical plane by isofluorane/oxygen for the duration of the procedure. Incisions were closed with 5-0 nonabsorbable sutures. Ketoprofen (2 mg/Kg) was provided post-procedure and topical antibiotic was applied to the incision for up to 3 days as necessary. Animal health and well-being was monitored daily. Sutures were removed at 10 days post-surgery. Mice were allowed to recover for four weeks after surgery or until they reached 30 weeks of age, then underwent a glucose tolerance test and an insulin tolerance test one week later. After an additional week of recovery, animals were sacrificed and plasma and multiple tissues harvested (reproductive and inguinal adipose depots, liver, heart, soleus, pancreas, hypothalamus) as described above.
2.5.9 Statistics

Data within individual cohorts were assessed for normality using a Wilks-Shapiro test. Outliers were identified by a Grubbs test (p < 0.05) and removed. Data were tested for significant differences among cohorts by ANOVA with a Tukey’s post-hoc correction. The sex X diet X age term was not significant for any phenotype so males and females were pooled for analyses. P-values <0.05 were considered significant. All statistical analyses were performed using the R software package.
Chapter 3

Identification of Brown Adipose Gene Co-Expression Clusters Correlating with Metabolic Phenotypes

by
Caryn Carson and Heather A. Lawson

Adapted for dissertation from the published manuscript:

3.1 Abstract

Adipose is a dynamic endocrine organ that is critical for regulating metabolism and is highly responsive to nutritional environment. Brown adipose tissue is an exciting potential therapeutic target, however there are no systematic studies of gene-by-environment interactions affecting function of this organ. We leveraged a weighted gene co-expression network analysis to identify transcriptional networks in brown adipose tissue from LG/J and SM/J inbred mice fed high or low fat diets, and correlate these networks with metabolic phenotypes. We identified 8 primary gene network modules associated with variation in obesity and diabetes-related traits. Four modules were enriched for metabolically relevant processes such as immune and cytokine response, cell division, peroxisome functions, and organic molecule metabolic processes. The relative expression of genes in these modules is highly dependent on both genetic background and dietary environment. Genes in the immune/cytokine response and cell division modules are particularly highly expressed in high fat-fed SM/J mice, which show unique brown adipose-dependent remission of diabetes. The interconnectivity of genes in these modules is also heavily dependent on diet and strain, with most genes showing both higher expression and co-expression under the same context. We highlight several genes of interest, \textit{Col28a1}, \textit{Cyp26b1}, \textit{Bmp8b}, and \textit{Ngef}, that have distinct expression patterns among strain-by-diet contexts and fall under metabolic QTL previously mapped in an F\textsubscript{16} generation of an advanced intercross between LG/J and SM/J. Each of these genes have some connection to obesity and diabetes-related traits, but have not been studied in brown adipose tissue. In summary, our results provide important insights into the relationship between brown adipose and systemic metabolism by being the first gene-by-environment study of brown adipose transcriptional networks.
3.2 Introduction

Obesity and associated metabolic disorders are reaching epidemic prevalence worldwide. While some of this prevalence is the result of increasingly inactive lifestyles and changing dietary norms, the hundreds of genome-wide association study (GWAS) ‘hits’ for obesity and metabolic diseases indicate there is also a strong genetic component (Cheng, 2018; Fall & Ingelsson, 2014). Thus it is critical to understand how gene-by-environment interactions are contributing to metabolic dysfunction. Such interactions have been shown to underlie variation in obesity and diabetes risk (Abadi, 2017; Franks, 2011; Li & Qi, 2019; Llewellyn & Wardle, 2015; Nagpal, 2018; Schrempft, 2018), and many individual genes have been identified with natural variants in human populations affecting metabolic response to environmental perturbations (Benson, 2019; Pigeyre, 2016; Snyder, 2004; Stutzmann, 2008; Wang, 2019; Williams, 2014). Research in animal models, in particular mouse models, has been used to manipulate dietary intake and environment in order to better understand the gene-by-environment interactions most relevant to human metabolism (Brockmann & Bevova, 2002; Eberhart, 1994; Lawson & Cheverud, 2010; Wayhart & Lawson, 2017; West, 1995). Frequently, studies of obesity in mice look at adipose tissue as a primary metabolic organ, with relatively recent focus on brown adipose tissue as a pro-health therapeutic target for obesity (Cannon & Nedergaard, 2004).

Brown adipose tissue is distinct from white adipose tissue and has mostly been studied for its role in non-shivering thermogenesis, the release of energy as heat through the activity of UCP1 (Feldmann, 2009; Rothwell & Stock, 1979). Brown adipose tissue is found in adult humans (Cypess, 2009; Virtanen, 2009) and increased brown adipose activity is associated with a healthier metabolic profile (Chechi, 2013) and lower body fat percentage (van Marken Lichtenbelt, 2009). It is also associated with amelioration of elevated plasma lipid levels in a
hyperlipidemic mouse model (Bartelt, 2011) and remission of the metabolic dysfunction associated with impaired pancreatic islet function in a mouse model of type I diabetes (Gunawardana & Piston, 2012). Mice lacking brown adipose tissue develop obesity and metabolic dysfunction (Hamann, 1996) that is independent from the loss of thermogenic UCP1 activity (Enerbäck, 1997; Feldmann, 2009; Liu, 2003), indicating that brown adipose contributes to healthy metabolism through thermogenesis-independent mechanisms. Several studies have sought to identify potential metabolically-relevant brown adipose cytokines, or “batokines” underlying these mechanisms (Deshmukh, 2018; Villarroya, 2019; Wang, 2015). However, very little research has focused on understanding the potential regulation of these batokines, and most studies regarding transcriptional networks in brown adipose tissue have focused exclusively on identifying regulators and effectors of nonshivering thermogenesis and brown adipocyte identity (Cheng, 2018; Cui, 2016; Emmett, 2017; Harms & Seale, 2013; He, 2018; Loft, 2017; Mori, 2012; Mori, 2014; Pazos, 2016; Rajan, 2018; Seale, 2011; Siersbaek, 2012; Sun, 2011; Trajkovski, 2012; Yao, 2014).

We wanted to more broadly understand the transcriptional networks existing within brown adipose tissue, to investigate how these networks are affected by genetic background and dietary environment, and determine how they associate with metabolic variation. To do this, we chose to study SM/J and LG/J inbred mice fed high and low fat diets. These strains were derived separately for small (SM/J) or large (LG/J) body size (Chai, 1956; Goodale, 1941), and both strains respond to a high fat diet with obesity, elevated fasting glucose, and impaired glucose tolerance at 20 weeks of age (Ehrlich, 2003). However, by 30 weeks, high fat-fed SM/J mice resolve their glycemic dysfunction. This occurs concurrently with a dramatic expansion of their interscapular brown adipose depots, making SM/J mice a unique and intriguing model system in which to investigate brown adipose transcriptional networks, how they correlate with
metabolic traits, and how they are affected by dietary environment. In this study, we employed a weighted gene co-expression network analysis (WGCNA) (Langfelder & Horvath, 2008; Zhang & Horvath, 2005) and identified eight primary gene modules within brown adipose tissue that correlate with one or more obesity and diabetes-related phenotypes. The expression profile of genes within these modules is dependent on both strain and dietary contexts, indicating gene-by-environment interactions that contribute significantly to variation in brown adipose tissue function. This study is an important first step in elucidating metabolically-relevant brown adipose transcriptional networks, how they are affected by genetic background and diet, and how they contribute to systemic metabolism through mechanisms beyond thermogenesis.

3.3 Results

3.3.1 Brown adipose expression and metabolic traits vary among strain and diet contexts

To understand how brown adipose gene expression in different genetic backgrounds contributes to metabolic variation in different environmental contexts, we raised LG/J and SM/J mice on isocaloric high and low fat diets. The mice were extensively phenotyped (Chapter 2) and RNA sequencing of brown adipose was used to assess both mRNA and noncoding RNA transcript levels. To understand how genetic background and diet interact to affect the brown adipose transcriptome, samples were clustered based on gene expression (Figure 3.1, top). Strain was a much better predictor of overall clustering than diet, with the LG/J samples clustering into a single group encompassing both diets. The SM/J samples separated into two main clusters, one for each diet, indicating that the SM/J’s brown adipose was more responsive to dietary environment. A heatmap was used to visualize the metabolic variation among the strains and diets (Figure 3.1, bottom). Glucose and insulin parameters consistently showed
higher values in the high fat-fed SM/J’s relative to low fat-fed mice or LG/J mice on either diet. Lipid levels were more mixed and variable throughout the entire population. LG/J mice had lower brown adipose to body weight ratios than SM/J mice. Within the SM/J’s, high fat-fed mice had both higher body weight and higher brown adipose to body weight ratios than low fat-fed mice.

**Figure 3.1 Brown adipose gene expression clustering by strain and diet.** Dendrogram of samples clustering based on brown adipose transcriptome (top). All diet by strain cohorts consist of 16 samples (8 male and 8 female) except the low fat LG cohort (8 male and 6 female). Sample demographics are indicated visually below the dendrogram (sex, diet, strain, and age at necropsy). Phenotypic values for each sample: red = high value, white = low value, and grey = missing value (bottom).
3.3.2 Modules enriched for immune/cytokine response and cell division correlate with glucose parameters and brown adipose to body weight ratio

To identify gene network modules in brown adipose tissue that associate with the observed variation in metabolic phenotypes, we performed WGCNA (Langfelder & Horvath, 2008) on the brown adipose gene expression profiles of all cohorts. An unsigned Topological Overlap Matrix assigned genes to 15 discrete modules and eigenvalues were calculated for each module. We then correlated the module eigenvalues with each metabolic phenotype, and found that 8 of 15 modules showed significant correlation with at least one phenotype (Figure 3.2). Four modules showed significant correlation with at least three of the 4 glucose and insulin parameters (magenta, blue, brown, and pink) and three modules showed significant correlations.

![Module–Trait Relationships](image)

**Figure 3.2** Gene network modules correlate with variation in metabolic traits. Correlation of gene module eigenvalues with metabolic trait values. Enriched GO Terms included in module name. Boxes labeled “NS” showed no significant correlation, while those labeled “LC” had a significant correlation but the strength was between -0.5 and 0.5. Boxes are color coded with strength of the correlation: red = high positive correlation, white = no correlation, blue = high negative correlation.
with both body weight and brown adipose to body weight ratio (midnight blue, turquoise and yellow). The blue and brown modules in particular had significant correlations with both glucose phenotypes and brown adipose to body weight ratio.

To determine if the modules were enriched for particular classes of genes, we employed Gene Ontology (GO) term enrichment within each module. Three modules had multiple enriched GO terms and were assigned a general descriptor term for the top 10 enriched terms: blue = immune/cytokine response, brown = cell division and red = organic molecule metabolic processes. The pink module had only one enriched GO term (peroxisome) while the remaining four modules had no significantly enriched GO terms (at an FDR = 0.05). To focus the remainder of our results, we primarily discuss the four modules with enriched GO terms, however all analyses were performed on the modules without enriched GO terms as well.

3.3.3 Gene expression within network modules varies across both diet and strain contexts

To determine how similar the module-trait relationships are across diets and strains, we analyzed each strain and each diet individually through the WGCNA pipeline (Figure 3.3). At least one immune or immune/cytokine response module was present in both strains and both diets, however the cell division module only appeared in the high fat-fed SM/J brown adipose. This led us to hypothesize that some modules may be driven by expression within a particular cohort. To test this, we performed principal components analysis on the genes within each module (Figure 3.4).

Variation in the blue immune/ cytokine response module was driven mainly by strain, which then further separated by diet within strain. It is well-established that white adipose tissue is an immunologically active organ that, in obesity, displays both active and adaptive immune responses that affect systemic metabolism (Grant & Dixit, 2015; Zeyda, 2013). However the
immunological role of brown adipose tissue is relatively understudied (Villarroya, 2018). Our data indicate that genetic background strongly modifies brown adipose tissue’s immunological and cellular signaling processes in response to nutritional environment.

The brown cell division module showed remarkable clustering of the high fat-fed SM/J cohort, which is consistent with our result that enrichment of this term is driven by high fat-fed SM/J mice. Further, high fat-fed SM/J mice had the highest brown adipose to body weight ratios and our previous work showed that the brown adipose expansion observed in these mice is the result of hyperplasia (Chapter 2). Both pink peroxisome and red organic molecule

Figure 3.3 Gene network modules correlated with one or more phenotypic trait in individual diet and strain cohorts. Raw expression counts were split into high fat (A), low fat (B), LG (C), and SM (D) cohorts and TMM-normalized in edgeR. The WGCNA package was applied to each individual cohort, providing module correlation with phenotypic trait, GO term enrichment of each module. Gene module-phenotypic trait correlation maps are provided for each diet and strain cohort.
processes showed moderate diet-by-strain clustering.

3.3.4 Strain-specific variation drives differential expression and differential connectivity within brown adipose gene modules

To identify the genes that were most differentially expressed between the diets and strains we calculated both differential expression and connectivity. Combining these metrics allowed us to investigate individual genes in transcriptional networks that are particularly susceptible to differences in diets or genetic backgrounds. To our knowledge this has never been explored in brown adipose tissue. Genes passing an FDR threshold of 0.05 were considered differentially expressed, regardless of fold change. Connectivity was calculated as the degree of co-expression of each gene with all other genes in that module. Genes with differences in connectivity greater than 0.5 between diets or strains were considered differentially connected.

Genes in the immune/cytokine response (blue), cell division (brown), and peroxisome (pink) modules had increased connectivity in high fat-fed mice regardless of genetic background (Figure 3.5). This indicates that genes in these modules were more tightly co-expressed under nutritional excess. In contrast, the organic molecule metabolic processes (red) module had a more even distribution of connectivity, with a trend towards higher connectivity in low fat-fed mice. While overall there are roughly even numbers of genes upregulated in high or low fat-fed mice, the distribution of differential expression in individual modules shows increased expression in one diet over the other (Figure 3.5, Table 3.1). Genes with the highest differential expression also tended to have differential connectivity, with the majority showing increased expression and increased connectivity in animals fed the same diet.

Analyzing differential expression and connectivity by strain revealed much stronger
connectivity in the SM/J mice compared to the LG/J mice in all four of our primary modules and half of our secondary modules (Figure 3.6, Table 3.1). Similar to the connectivity-by-diet analysis, genes with high differential expression also had increased connectivity in the same genetic background. However, this may be skewed by the overall quantity of genes that are differentially expressed by strain (Table 3.1). To break down connectivity and expression patterns between diets and between strains, we classified genes that showed both differential connectivity and significant differential expression between diets or between strains as potential hub genes. In total, this resulted in 2617 potential hub genes: 250 that are differentially

<table>
<thead>
<tr>
<th>Module (Color)</th>
<th># Genes</th>
<th>DE Diet (HF, LF)</th>
<th>DC Diet (HF, LF)</th>
<th>DE Strain (SM, LG)</th>
<th>DC Strain (SM, LG)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blue</td>
<td>1202</td>
<td>236 (170, 66)</td>
<td>537 (517, 20)</td>
<td>895 (757, 138)</td>
<td>1055 (1051, 4)</td>
</tr>
<tr>
<td>Brown</td>
<td>1060</td>
<td>148 (132, 16)</td>
<td>789 (786, 3)</td>
<td>934 (811, 123)</td>
<td>463 (424, 39)</td>
</tr>
<tr>
<td>Pink</td>
<td>107</td>
<td>35 (19, 16)</td>
<td>61 (61, 0)</td>
<td>60 (25, 35)</td>
<td>28 (22, 6)</td>
</tr>
<tr>
<td>Red</td>
<td>339</td>
<td>223 (60, 163)</td>
<td>141 (4, 137)</td>
<td>96 (65, 31)</td>
<td>227 (224, 3)</td>
</tr>
<tr>
<td>Magenta</td>
<td>103</td>
<td>93 (78, 15)</td>
<td>78 (78, 0)</td>
<td>44 (36, 8)</td>
<td>56 (56, 0)</td>
</tr>
<tr>
<td>Midnight Blue</td>
<td>43</td>
<td>5 (5, 0)</td>
<td>4 (0, 4)</td>
<td>42 (42, 0)</td>
<td>16 (0, 16)</td>
</tr>
<tr>
<td>Turquoise</td>
<td>1795</td>
<td>122 (47, 75)</td>
<td>425 (250, 175)</td>
<td>1789 (1175, 614)</td>
<td>594 (307, 287)</td>
</tr>
<tr>
<td>Yellow</td>
<td>553</td>
<td>177 (40, 137)</td>
<td>434 (2, 432)</td>
<td>544 (372, 172)</td>
<td>473 (471, 2)</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>5202</td>
<td>1039 (551, 488)</td>
<td>2469 (1698, 771)</td>
<td>4404 (2680, 1724)</td>
<td>2912 (2555, 357)</td>
</tr>
</tbody>
</table>

Table 3.1 Co-Expression Module Statistics. Columns include total number of genes in module, number of genes differentially expressed (DE) by diet (number in each direction), number of genes differentially connected (DC) by diet (number in each direction), number of genes differentially expressed by strain (number in each direction), and number of genes differentially connected by strain (number in each direction).

Figure 3.4 (previous page) Principal component analysis of each individual module shows variation in diet and strain interactions. Principal component analysis of normalized gene expression counts for each module. Variation in the immune/cytokine response module (A) is driven mainly by strain, which further separates into diet. High fat-fed SM/J mice stand out in the cell division module (B). Moderate clustering in the peroxisome (C) and organic molecule processes modules (D) as well as the unenriched magenta (E) and midnight blue modules (F). Variation in the unenriched turquoise (G) and yellow (H) modules is heavily driven by strain. Samples are color-coded based on strain (LG/J = blue, SM/J = red) and diet (low fat = light, high fat = dark). Plots are labeled with module color and enriched biological process.
expressed and connected by diet, 1977 by strain, and 390 for diet-by-strain.

To further refine these lists and identify candidates that are likely to be contributing to metabolic variation, we filtered for genes that are differentially connected and differentially expressed with a fold change ≥ 2. This produced a list of 20 genes, 10 of which belonged to the immune/cytokine (blue) or cell division (brown) modules (Table A.1). Interestingly, these 10 genes were all upregulated and have higher connectivity in the high fat-fed SM/J cohort, indicating that the brown adipose of SM/J mice is particularly responsive to nutritional excess.

This is consistent with our previous work showing brown adipose tissue-dependent resolution of diabetes in high fat-fed SM/J mice (Chapter 2). Nineteen of these hub genes contained small nucleotide variants between the LG/J and SM/J strains (Nikolskiy, 2015) (Table A.1), which could be contributing to the gene-by-environmental differential expression patterns we observe. Further, four of these genes, *Col28a1*, *Bmp8b*, *Cyp26b1*, and *Ngef*, fall within the support intervals of metabolic quantitative trait loci (QTL) mapped in an F16 advanced intercross of the SM/J and LG/J strains (these QTL encompass 62, 68, 163, and 143

Figure 3.5 (previous page) Genes that have increased expression in one diet show increased connectivity in the same diet. Differential expression is plotted along the y-axis as log2 foldchange between diets (yellow color indicates FDR-corrected p-value < 0.05). Connectivity is plotted along the x-axis as kdiff between diets. Genes with a difference in connectivity above 0.5 or below -0.5 were considered differentially connected (blue), and vertical grey dashed lines are provided to visualize these cut-offs. Genes with both differential expression and differential connectivity are colored in green. Horizontal grey dashed lines are also provided to visualize log2 foldchange values above 1 and below -1. Positive values indicate higher expression or connectivity in high fat-fed cohort, negative values indicate higher expression or connectivity in low fat-fed cohort.

Figure 3.6 (next page) Genes that have increased expression in one strain show increased connectivity in the same strain. Differential expression is plotted along the y-axis as log2 foldchange between diets (yellow color indicates FDR-corrected p-value < 0.05). Connectivity is plotted along the x-axis as kdiff between strains. Genes with a difference in connectivity above 0.5 or below -0.5 were considered differentially connected (blue), and vertical grey dashed lines are provided to visualize these cut-offs. Genes with both differential expression and differential connectivity are colored in green. Horizontal grey dashed lines are also provided to visualize log2 foldchange values above 1 and below -1. Positive values indicate higher expression or connectivity in SM/J cohort, negative values indicate higher expression or connectivity in LG/J cohort.
genes, respectively) (Table 3.2; Figure 3.7) (Lawson, 2010; Lawson, 2011a, Lawson, 2011b). These genes represent actionable candidates that can be tested for their function in brown adipose tissue and effects on obesity and systemic metabolism.

3.4 Discussion

Adipose is a dynamic endocrine organ that is critical for regulating systemic metabolism. Further, adipose tissue function displays a high degree of plasticity under different conditions.

**Figure 3.7 Expression by cohort for four candidate genes.** Normalized expression data are provided for Col28a1 (A), Bmp8b (B), Cyp26b1 (C), and Ngef (D). A one-way ANOVA with Tukey’s post hoc correction was performed among cohorts to identify significant differences. *p<0.05, **p<0.01, ***p<0.0001.
nutritional conditions (Haugen & Drevon, 2007; Rosen & Spiegelman, 2014; Sethi & Vidal-Puig, 2007). Though brown adipose has high therapeutic potential for obesity and related metabolic disorders, research on this tissue is in its infancy and most genetic studies focus on identifying the factors involved in its thermogenic function and in determining brown adipocyte identity. Yet recent research reveals that brown adipose is a source of endocrine signals with both anti-diabetic and anti-obesogenic properties (Lowell, 1993; Stanford, 2013). High fat diet has been shown to alter brown adipose activity and blunt its positive effects on systemic metabolism (Ohtomo, 2017; Shankar, 2019). Yet, as illustrated by numerous studies, dietary response is heavily dependent on genetic background (Alexander, 2006; Andrikopoulos, 2005; Chu, 2017; Eberhart, 1994; Ehrich, 2003; Montgomery, 2013; Sims, 2013; Smith, 2000; Survit, 1998; Walkley, 1978; West, 1992; West, 1995). Here we present the first study on the effects of genetic background and diet on brown adipose transcriptional networks associating with

<table>
<thead>
<tr>
<th>Gene</th>
<th>Col28a1</th>
<th>Ngefl</th>
<th>Cyp26b1</th>
<th>Bmp8b</th>
</tr>
</thead>
<tbody>
<tr>
<td>Coordinates</td>
<td>Chr6: 7997808-8192617</td>
<td>Chr1: 87476829-87573870</td>
<td>Chr6: 84571944-84593908</td>
<td>Chr4: 123105351-123124537</td>
</tr>
<tr>
<td>Upstream Variants</td>
<td>2</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Gene Body Variants</td>
<td>982</td>
<td>124</td>
<td>4</td>
<td>6</td>
</tr>
<tr>
<td>Downstream Variants</td>
<td>8</td>
<td>0</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>QTL (Trait)</td>
<td>Dserum6a (Triglycerides)</td>
<td>Dserum1b (Cholesterol)</td>
<td>Ddiab6c (AUC 20wks)</td>
<td>Ddiab4b (AUC 20wks)</td>
</tr>
<tr>
<td>Module (Traits)</td>
<td>brown (Glucose, GTT AUC, ITT AUC, Insulin, BAT:Body Weight)</td>
<td>yellow (Cholesterol, Body Weight, BAT:Body Weight)</td>
<td>blue (Glucose, GTT AUC, ITT AUC, BAT:Body Weight)</td>
<td>red (Cholesterol, Body Weight)</td>
</tr>
</tbody>
</table>

**Table 3.2 Differentially Connected Genes Falling in Metabolic QTL.** Provided for each gene of interest are genomic coordinates (GRC38.72-mm10), the number of variants less than 1kb upstream from transcription start site, within the gene body, and less than 1kb downstream from transcription stop site, the metabolic QTL that contains the gene, the co-expression module the gene belongs to, and the metabolic phenotypes significantly correlated with that module. * brown = Cell Division; blue = Immune/Cytokine response; red = Organic metabolic processes; yellow = No Enrichment. GTT = glucose tolerance test; ITT = insulin tolerance test; AUC = Area Under the Curve

52
metabolic variation.

We illustrate that the SM/J brown adipose transcriptome is more susceptible to dietary perturbations in comparison with the LG/J strain (Figure 3.1). The LG/J and SM/J strains are frequently used in metabolic studies because they vary in their metabolic response to dietary fat (Cheverud, 2011; Ehrich, 2003; Lawson, 2010; Lawson, 2011a; Lawson, 2011b). We recently demonstrated that high fat-fed SM/J mice dramatically expand their interscapular brown adipose depots and that contemporary with this expansion, mice enter diabetic remission (Chapter 2). Understanding the genetic underpinnings of this phenomenon could open new avenues for understanding novel biology and highlight therapeutic targets for obesity-related metabolic dysfunction.

We identified eight primary gene co-expression modules that were highly correlated with obesity and diabetes traits (Figure 3.2). Four of these modules showed significant over-representation of genes belonging to biological categories that affect adipose function and systemic metabolism: immune/cytokine response, peroxisomes, organic metabolic processes, and cell division. Genes involved in immune and cytokine response showed a conserved network correlating with glucose and insulin traits (Figure 3.3-4). This is consistent with previous work relating hyperglycemia, hyperinsulinemia, and other diabetes-related traits with inflammatory markers and immune infiltration of adipose (Coletta, 2008; Gao, 2015; Škopková, 2007; Zeyda, 2013). Peroxisome genes are essential to lipid metabolism, and have been shown to regulate the thermogenic function of both brown and beige adipocytes (Park, 2019). Genes composing the organic metabolic processes category include those that perform essential functions in glucose and lipid uptake. Genes involved in cell division form a network specific to high fat-fed SM/J mice, and strongly correlate with glucose and insulin traits as well as with brown adipose to body weight ratio (Figure 3.3-4). The gene-by-environmental specificity of
this module, its association with glycemic parameters, and the unique characteristics of brown adipose in the SM/J strain, indicate that the genes in this module are compelling candidates for further studies of biological mechanisms of action of brown adipose and systemic metabolism.

We highlight 4 genes that fall in QTL previously mapped in an F_{16} generation of a LG/J x SM/J advanced intercross population (Table 3.2) (Cheverud, 2001; Lawson, 2010). \textit{Cyp26b1}, cytochrome P450 family 26 subfamily B member 1, is part of the blue immune/cytokine response module. It is a retinoic acid hydroxylase that regulates cellular concentrations of all-trans-retinoic acid. Retinoic acid is a vitamin A derivative that is essential for cell growth and differentiation. Early studies show that retinoids, including retinoic acid, play an essential role in adipose differentiation (Kuri-Harcuch, 1982; Safonova, 1994), and a recent study found that retinoic acid mediates adipogenic defects in human white adipose-derived stem cells (Takeda, 2016). \textit{Col28a1}, collagen type XXVIII alpha 1, is part of the brown cell division module. It belongs to a class of collagens involved in extracellular matrix (ECM). The ECM is a critical component in cellular signaling, either through direct interaction with cell-surface receptors or through the ability to regulate growth factor bioavailability (Frantz, 2010). Collagen is highly enriched in adipocytes, and its depletion is associated with metabolic dysfunction (Khan, 2009). \textit{Ngef}, neuronal guanine nucleotide exchange factor, is part of the yellow (no enrichment) module, which is highly correlated with body weight. Variants in \textit{Ngef} has been associated with adiposity in two separate genome-wide association studies in humans (Kim, 2015; Norris, 2009). \textit{Bmp8b}, bone morphogenic protein 8b, is part of the red organic metabolic processes module. Of these four candidates, only \textit{Bmp8b} has been studied in brown adipose. It is secreted by brown adipocytes and amplifies the thermogenic response of cells by increasing sensitivity to adrenergic input (Pellegrinelli, 2018; Whittle, 2012).

Gene-by-environment interactions are critical for understanding the intricacies and
nuances of obesity and metabolic dysfunction, and for identifying potential therapeutic targets. Though there is increasing interest in brown adipose tissue as a potential therapeutic target for such diseases, there have been no studies on gene-by-environment interactions in brown adipose tissue, and few studies on brown adipose in mouse strains other than C57BL/6J. Our results indicate that gene-by-environment interactions significantly contribute to variation in brown adipose transcriptional networks. Understanding how genetic variation mediates brown adipose tissue’s response to an obesogenic diet will be key to harnessing its therapeutic potential. Further, the unique transcriptomic profile of high fat-fed SM/J brown adipose tissue and its correlation with diabetic remission highlight compelling candidates for understanding brown adipose tissue’s endocrine function and biological mechanisms of action beyond thermogenesis.

3.5 Materials and Methods
3.5.1 Sample Collection and Sequencing

Animal generation and phenotyping were described in section 2.5.1. Sixty-four LG/J and SM/J mice were used for sequencing analysis, representing 4 males and 4 females from each diet (high and low fat) and age (20 and 30 weeks). Total RNA was isolated from interscapular brown and reproductive white adipose tissues using the RNeasy Lipid Tissue Kit (QIAGen). RNA concentration was measured via Nanodrop and RNA quality/integrity was assessed with a BioAnalyzer (Agilent). RNAseq libraries were constructed using the RiboZero kit (Illumina) from total RNA samples with RIN scores >7.5. Libraries were checked for quality and concentration using the DNA 1000LabChip assay (Agilent) and quantitative PCR, according to manufacturer’s protocol. Libraries were sequenced at 2x100 paired end reads on an Illumina HiSeq 4000. After sequencing, reads were de-multiplexed and assigned to
individual samples.

FASTQ files were filtered to remove low quality reads and aligned against LG/J and SM/J custom genomes using STAR (Dobin, 2013; Nikolskiy, 2015) . Briefly, LG/J and SM/J indels and SNVs were leveraged to construct strain-specific genomes using the GRC38.72-mm10 reference as a template. This was done by replacing reference bases with alternative LG/J and SM/J bases using custom python scripts. Ensembl R72 annotations were adjusted for indel-induced indexing differences for both genomes. Read counts were normalized via upper quartile normalization and a minimum normalized read depth of 10 was required.

3.5.2 Gene Co-expression and Phenotype Associations

The Weighted Gene Co-Expression Network Analysis (WGCNA) R package was used to determine gene co-expression modules and their correlation with metabolic traits (Langfelder & Horvath, 2008). Briefly, edgeR-normalized counts for each gene were converted to a standard normal. Genes with standard deviation of at least 0.25 were deemed biologically variable and used in the subsequent analysis (7740 genes total) (Kogelman, 2014). Samples were clustered based on expression of all genes and two low fat female LG/J samples were removed as outliers before continuing with the analysis. The adjacency matrix was created from biweight midcorrelations calculated between all genes and raised to a power β of 12, chosen based on a scale-free topology index above 0.9. Raising the absolute value of the correlation by this power is done to emphasize high correlations at the expense of low correlations (Fuller, 2007).

The blockwiseModules function was used to create an unsigned Topological Overlap Measure using the adjacency matrix and to identify modules of highly interconnected genes. Each module was assigned a color for identification. Module eigengenes were calculated as the first principal component for each module, and Pearson’s correlations were calculated between
each module eigengene and each phenotype to estimate module-trait relationships. Module-trait
correlations were considered significant at an FDR-corrected p-value < 0.05 and an absolute
correlation of at least 0.5. Gene Ontology term enrichment was calculated for individual
modules from a background of all expressed genes in the dataset. Modules were considered
enriched for a term at a Bonferroni-corrected p-value < 0.05. Modules with no significantly
enriched terms were designated as “No Enrichment” and modules with multiple enriched terms
were classified with an overarching description of the top ten significantly enriched terms. Four
subnetworks were also created (HF, LF, LG/J, SM/J) with the same 7740 genes as in the full
network analysis. Within each subnetwork, we assigned genes to modules and defined module-
trait relationships. Subnetworks were also created for female and male samples, to ensure sex-
effects were not driving module assignment. Though the sex subnetworks varied in the total
number of modules assigned, both showed a module related to the original “blue” immune/
cytokine response, “brown” cell division, and “red” organic molecule metabolic processes
modules. The “pink” peroxisome module did not replicate, but this is likely due to the
enrichment for that original module being relatively weaker (Bonferroni-corrected p=0.04 for a
single term, vs multiple significantly enriched terms in the blue, brown, and red modules).
Based on these results, we pooled both sexes for the analyses in this paper.

3.5.3 Differential Connectivity

Differential connectivity is a measure of the differences in gene interactions between
high and low fat-fed mice or between SM/J and LG/J mice. The connectivity of each gene
within each cohort was calculated with an adjacency matrix as a measure of how correlated the
gene is with all other genes in its module. Differential connectivity was calculated between
diets or strains for each gene

\[ k_{Diff} = \frac{HF - LF}{HF + LF} \quad \text{or} \quad k_{Diff} = \frac{SM - LG}{SM + LG} \]

to provide values between -1 and 1. Genes were considered differentially connected when one cohort had three times the
connectivity of the other cohort, or an absolute $k\text{Diff} > 0.5$. Genes with positive differential connectivity are more highly connected in the HF or SM cohorts than in the LF or LG cohorts. To further narrow down genes into those most likely to be biologically impactful hub genes, differential expression between diets and strains was calculated for all genes using the exactTest function in edgeR. Genes with an FDR-corrected p-value < 0.05 are considered to be significantly differentially expressed. Hub genes in diet or strain contexts were called as those with both an absolute differential connectivity value > 0.5 and significant differential expression in the same comparison.
Chapter 4

Transcriptional Changes in Brown Adipose Tissue of Healthy Obese SM/J Mice

by

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4.1 Abstract

Understanding the genetic regulation of healthy brown adipose function could lead to new and innovative therapies for glycemic control irrespective of weight loss. We have identified 267 genes that uniquely change expression in high fat-fed SM/J brown adipose tissue between 20 and 30 weeks. These expression changes correspond with a natural expansion of brown adipose tissue that corresponds with improved glucose parameters in obesity. The genes are enriched for cytokine and extracellular functions with an overall healthier transcriptional profile, particularly through decreased expression of inflammatory genes. We highlight Sfrp1 (secreted frizzled-related protein 1) as a compelling candidate gene that is likely driving the increase in brown adipose tissue and related improvement in glucose tolerance. This gene encodes a cytokine that has never directly been studied in brown adipose tissue, but is known to promote white adipogenesis leading to improved glucose uptake and decreased inflammation. Expression of Sfrp1 significantly correlates with brown adipose mass in SM/J mice and with improved glucose tolerance in high-fat fed SM/J mice. Further characterization of this cytokine could reveal it as a potent activator of brown adipogenesis and promoter of healthy glycemic control in an obese state.

4.2 Introduction

Brown adipose tissue has gained a great deal of attention over the past decade due to its potential to improve health under obese and diabetic conditions. Numerous studies have defined non-shivering thermogenesis, glucose and lipid uptake, and endocrine signaling functions for brown adipose tissue that affect whole-body metabolism (Cannon & Nedergaard, 2004; Poekes, 2015; Stanford, 2013). There have also been several studies that looked at how brown adipose tissue is affected by aging, obesity, and their interaction. Aging results in the
“whitening” of brown adipose, with tissue remodeling and loss of thermogenic activity, (Goncalves, 2017; Lecoultre & Ravussin, 2011; Pfannenberg, 2010; Yoneshiro, 2011). Obesity causes a similar whitening of brown adipose tissue, accompanied by increased inflammation, oxidative stress, impaired insulin signaling, and even brown adipocyte hypertrophy and death (Alcalá, 2017; Alcalá, 2019; Briscini, 1998; Gao, 2015; Kotzbeck, 2018; Lapa, 2017; Roberts-Toler, 2015; Shimizu, 2013; Shimizu, 2014).

Having characterized the brown adipose expansion in SM/J mice as a genetic phenomenon promoting healthy glycemic control in obesity, we wanted to determine if there are unique transcriptional changes in the brown adipose tissue that accompany the unique physiological changes. To do this, we performed RNA-sequencing on the interscapular brown and reproductive white adipose tissue of high and low-fat fed SM/J and LG/J mice at 20 and 30 weeks. We identified 267 genes that are uniquely differentially expressed between the brown adipose tissue of high fat-fed SM/J mice at 20 and 30 weeks. This set of genes is enriched for cytokine and extracellular function, and the direction of expression change is consistent with a healthier transcriptional profile in the expanded brown adipose tissue. We further characterize Secreted frizzled-related protein 1 (Sfrp1) as a compelling candidate gene underlying the dramatic brown adipose expansion and related glycemic resolution. This study is the first identification of brown adipose tissue in obesity that maintains a healthy transcriptional profile with reduced expression of inflammatory genes in particular. Further investigation of Sfrp1 as a promoter of brown adipogenesis and healthy glucose tolerance could lead to new therapeutics for glycemic control in obesity.
4.3 Results

4.3.1 RNA sequencing reveals altered expression of cytokine and extracellular matrix genes in SM/J brown adipose at 30 weeks

Since the brown adipose tissue expansion is unique to high fat-fed SM/J mice, we anticipated that there would be corresponding unique transcriptomic changes in the brown adipose. Indeed, we identified 267 genes whose expression significantly and uniquely changes between 20 and 30 weeks of age in high fat-fed SM/J brown adipose tissue (at a 5% FDR, out of 13,253 total genes expressed; Table A.2). These expression changes occur when the mice resolve their glycemic dysfunction and expand their brown adipose depots. These genes are not differentially expressed in white adipose tissue taken from the same animals or in low fat-fed

![Genes differentially expressed in high fat-fed SM/J brown adipose tissue between 20 and 30 weeks are enriched for cytokine and extracellular region functions.](image)

**Figure 4.1** Genes differentially expressed in high fat-fed SM/J brown adipose tissue between 20 and 30 weeks are enriched for cytokine and extracellular region functions. Venn diagrams showing number of genes differentially expressed by age in SM/J (A) and LG/J (B) tissues and the lack of overlap between cohorts. Gene set enrichment analysis on the 267 genes differentially expressed in high fat SM/J brown adipose tissue (C).
Figure 4.2 Expression of cytokine and extracellular proteins changes to a healthier profile with brown adipose expansion. Heatmap of differentially expressed brown adipose tissue genes between high fat-fed 20 and 30 week-old mice belonging to cytokine, extracellular matrix, or both gene ontologies.

SM/J controls (Figure 4.1A). Additionally, they are not differentially expressed in the LG/J strain of mouse, once again underscoring the genetic basis of the phenomenon (Figure 4.1B). Over-representation analysis indicates these genes are enriched for those involved in cytokine-cytokine receptor interactions ($p=3.23e^{-06}$), signaling receptor activity ($p = 5.70e^{-06}$), cell surface receptor signaling ($p=2.04e^{-07}$), and extracellular matrix components ($p = 7.93e^{-13}$) (Figure 4.1C).

Several genes belonging to these biological categories have evidence for their involvement in glucose homeostasis and change expression in a direction that is associated with improved metabolic health in high fat-fed SM/J mice between 20 and 30 weeks of age (Figure 4.2). These are intriguing results because brown adipose has been identified as a source of cytokines that influence glucose homeostasis, and extracellular matrix changes are essential for tissue expansion, cellular signaling, and regulation of growth factor bioavailability (Lin, 2017; Wang, 2015). However, extreme changes in ECM protein levels are associated with
adipose dysfunction in obesity; thus a fine balance between tissue remodeling and excessive accumulation of ECM proteins must be achieved to maintain adipose tissue homeostasis (Sun, 2013). The direction of expression change in high fat-fed SM/J mice reveals that their brown adipose expansion is associated with decreased expression of inflammatory (e.g. interleukin 7 receptor, Il7r) (Kim, 2014) and fibrotic markers (e.g. collagen type VIII alpha 1 chain, Col8a1; semaphorin 3C, Sema3c) (Mejher, 2013; Sun, 2013), and changes in extracellular matrix components (e.g. matrix metallopeptidase 12, Mmp12; procollagen c-endopeptidase enhancer, Pcolce) (Huang, 2011; Lee, 2014) and cytokines (e.g. coagulation factor VII, F7; leptin, Lep; secreted frizzled-related protein 1, Sfrp1) (D’souza, 2017; Edén, 2015; Gauger, 2013) associated with metabolic health. Other mouse models of diet-induced obesity develop unhealthy brown adipose transcriptomes characterized by increased expression of pro-inflammatory genes and fibrotic markers (Alcalá, 2017). The direction of expression change in SM/J brown adipose tissue supports the uniqueness of this mouse model.

4.3.2 Increased Sfrp1 expression in expanded brown adipose tissue correlates with improved glucose tolerance

We focused on Sfrp1 (secreted frizzled-related protein 1) as a candidate gene that may underlie the increased brown adipose mass in SM/J high fat-fed mice. Sfrp1 is a cytokine that inhibits Wnt/β-catenin signaling. Wnt/β-catenin signaling promotes the fibroblast identity and Sfrp1 blocks Wnt signaling to promote adipogenesis (Gauger, 2013; Lagathu, 2010; Wang, 2018). Its expression significantly increases in high fat-fed SM/J brown adipose between 20 and 30 weeks, when the mice increase the size of their brown adipose depots and enter diabetic remission, and does not increase in white adipose of these same animals, or in the white or brown adipose of LG/J mice (Figure 4.3). Further, Sfrp1 expression significantly correlates with brown adipose depot mass and with improved glucose tolerance in high fat-fed SM/J mice.
but does not correlate with these parameters in LG/J mice (Figure 4.4). We performed mediation analysis and found that the effects of Sfrp1 expression on glucose tolerance in high fat-fed SM/J’s is significantly mediated by brown adipose mass (p=0.004).

Data from human GWAS revealed several variants in the SFRP1 locus, including one variant (rs9734441) that is significantly associated with type II diabetes adjusted for BMI (Figure 4.5A) (Mahajan, 2018). There are several variants in the Sfrp1 locus between SM/J and LG/J genetic backgrounds, and the gene is located within the support intervals of QTL

Figure 4.3 Expression of Sfrp1 increases from 20 to 30 weeks only in the SM/J brown adipose depot. Expression of Sfrp1 in brown (A) and white (B) adipose tissue of SM/J mice, n = 8-16 animals per cohort. Expression of Sfrp1 in brown (C) and white (D) adipose tissue of LG/J mice, n = 8 animals per cohort. *p<0.05, **p<0.01, ***p<0.0001.
associated with adiposity and glucose tolerance that were mapped in an F16 LGxSM intercross (Figure 4.5B) (Lawson, 2010; Lawson, 2011b). These data further underscore that the SM/J genetic background has unique variants that contribute to its brown adipose expansion and diabetic remission. We hypothesize that DNA variants in SM/J mice increase Sfrp1, which in turn promotes brown adipogenesis by inhibiting Wnt/β-catenin signaling, and this in turn may contribute to the tissue’s role as a glucose sink (Figure 4.6).

4.4 Discussion

Figure 4.4 Brown adipose expression of Sfrp1 correlates with brown adipose mass and improved glucose tolerance only in SM/J mice. Relative expression of Sfrp1 correlates with brown adipose mass in SM/J (A) but not LG/J (B) mice. Sfrp1 also correlates with improved glucose tolerance in high fat-fed SM/J mice (C) but not high fat-fed LG/J (D) mice.
Brown adipose tissue has traditionally been studied for the effect of non-shivering thermogenesis on whole-body metabolism (Cannon & Nedergaard, 2004). Though recent studies have also interrogated the endocrine role of brown adipose tissue (Poekes, 2015), transcriptional analysis still primarily focuses on the control of brown adipose identity as defined through thermogenic genes (Kajimura, 2010). Understanding brown adipose transcription outside of the regulation of non-shivering thermogenesis could reveal novel brown adipose tissue functions in metabolism.

**Figure 4.5 Genetic variation in Sfrp1 locus of humans and mice.** Screenshot of variants in human adipose tissue in the Sfrp1 locus region (A). Rs973441 is significantly linked with type II diabetes adjusted for BMI. The Sfrp1 locus region contains multiple variants between SM/J and LG/J strains and is located in QTL associated with adipose weight and glucose tolerance mapped in an F16 intercross of the strains (B).
We analyzed the transcriptome of expanded brown adipose tissue in obese SM/J mice with glycemic control and found that genes associated with cytokine activity are enriched in the expanded tissue and diabetic remission. Cytokine secretion likely contributes to the obesity-independent ability of SM/J’s brown adipose tissue to affect systemic glucose metabolism (Matsushita, 2014; Samms, 2015). We highlight Sfrp1 as a particularly intriguing cytokine that may underlie this phenomenon. Sfrp1 inhibits Wnt signaling, thus promoting adipogenesis (Ackers & Malgor, 2018; Bennett, 2002; Lagathu, 2010). Expression of Sfrp1 increases as adipocytes differentiate, proportional to the strength of adipogenic stimulation, and also increases during treatment with thiazolidinedione drugs, which generally act through improving insulin-mediated glucose metabolism (Lagathu, 2009). Though Sfrp1 has never been directly studied in brown adipose tissue, it is a member of a brown adipose coexpression module of genes enriched for cell division, with its expression strongly correlated with brown adipose mass in particular (Chapter 3).

In human obesity, SFRP1 expression is downregulated in subcutaneous white adipose tissue, correlating with impaired insulin sensitivity (Ehrlund, 2013). Further, its expression was

![Diagram](image)

**Figure 4.6 Proposed mechanism for action of Sfrp1 in SM/J brown adipose tissue.** Sfrp1 sequesters Wnt away from its receptor, leading to phosphorylation and degradation of beta-catenin and thus allowing an adipogenic transcriptional program.
found to be deficient in obese patients with glycemic dysfunction (Chen & Wang, 2018; Ehrlund, 2013; Lagathu, 2010). Finally, high fat-fed Sfrp1−/− mice show dysregulation of glucose metabolism, including downregulation of Glut4 in white adipose tissue (Gauger, 2013). We show increased Sfrp1 expression in an expanding brown adipose depot that corresponds with increased Glut4 expression underlying potential improvement in insulin-stimulated glucose uptake. Though previous studies of Sfrp1 have only been conducted in white adipose tissue, the data we present fit a model where brown adipocyte-secreted SFRP1 may act in an autocrine manner to stimulate brown adipose proliferation, leading to its role as an insulin-stimulated glucose sink and resulting in improved glucose tolerance and insulin sensitivity.

4.5 Materials and Methods

4.5.1 RNA-Sequencing

Sample collection and RNA-sequencing were described in Sections 2.5 and 3.5. Library complexity was assessed and differential expression between each age cohort for each strain-by-diet cohort was determined after TMM normalization in edgeR (Chen, 2015). Genes were considered differentially expressed with an fdr-corrected p-value < 0.05, regardless of log fold change.

4.5.2 Functional Enrichment Analysis

Functional enrichment of differentially expressed genes was tested by over-representation analysis in the WEB-based Gene Set Analysis Toolkit v2019 (Zhang, 2005). We performed analyses of gene ontologies (biological process, cellular component, molecular function), pathway (KEGG), and phenotype (Mammalian Phenotype Ontology). For each tissue, the list of all unique differentially expressed genes was analyzed against the background of all unique genes expressed in that tissue. A Benjamini-Hochberg FDR-corrected p-value ≤
0.05 was considered significant.

4.5.3 Quantitative rt-PCR

Confirmation of Sfrp1 expression levels in white and brown adipose tissue was performed as described in section 2.5.6. Forward primer: TACTGGCCCGAGATGCTCAA; Reverse primer: GAGGCTTCCGTGGTATTGGG.

4.5.4 Statistics

As previously described (2.5.9), data were tested for significant differences among cohorts by ANOVA with a Tukey’s post-hoc correction. P-values <0.05 were considered significant. Mediation analysis was performed using the ‘mediation’ package in R. All statistical analyses were performed using the R software package.
Chapter 5

Conclusions

by

Caryn Carson
5.1 Summary

Adipose tissue is a central organ in metabolism. To better treat obesity-related metabolic diseases, we must first understand how adipose functions in obesity with and without accompanying metabolic dysfunction. Brown adipose tissue is a particularly attractive area of research because it promotes healthy metabolism in several different ways: taking up glucose and lipids from the blood, expending excess energy through non-shivering thermogenesis, and secreting cytokines to coordinate with other tissues. Generally, brown adipose loses its function in obesity, with reduced thermogenic activity, altered cytokine secretion, and adipocyte hypertrophy. In the Lawson lab, we have identified a mouse model of diet-induced obesity that develops healthy glycemic control concurrent with an expansion of brown adipose mass. My thesis characterizes the role of brown adipose tissue in this mouse model of healthy obesity. To do this I focused on the following questions: 1) Is the expanded brown adipose required for the development of healthy obesity or is it a secondary phenotype? 2) Does the brown adipose tissue function and morphology change during the expansion? 3) Are there transcriptional changes that accompany the expansion and any changes in function?

In this chapter, I summarize the findings of my graduate research in answering these questions, discuss the broader significance, and propose future directions. Together, my work furthers our understanding of brown adipose function and transcription in obesity and glycemic control.

5.1.1 Increase in Brown Adipose Mass Improves Insulin Sensitivity in Obesity

In Chapter 2, I report my findings on the physiology of healthy obese SM/J mice. At 20 weeks of age, high fat-fed SM/J mice have worse basal glucose, glucose tolerance, and insulin sensitivity than low fat-fed counterparts. By 30 weeks, the high fat-fed SM/J mice resolve their
glycemic parameters to levels equivalent to low fat-fed mice, a resolution that is sustained through at least 50 weeks of age and is not observed in the control LG/J strain of mice. I assessed serum levels of the metabolic hormones adiponectin, leptin, glucagon, and IGF1 to see if changes in these molecules could be driving the metabolic phenotypes, but none showed changes between 20 and 30 weeks in the high fat-fed mice. Instead I found the glycemic improvement was accompanied by a unique and dramatic expansion of the interscapular brown adipose depot. I hypothesized that increased tissue mass must occur either through increased number of cells, increased size of existing cells, or a combination of the two. I found no increase in the size of adipocytes, but I did find a trend towards increased presence of the mitotic marker phospho-histone H3 in the brown adipose tissue of high fat-fed mice, suggesting that the increase in brown adipose mass is caused by increased number of cells.

Brown adipose is known to affect glucose homeostasis in both humans and mice, and most research into this ability focuses on the effects of non-shivering thermogenesis (Chechi, 2013; Cypess, 2009; Hanssen, 2016; Saito, 2009; Saito, 2013; Stanford, 2013; van Marken Lichtenbelt, 2009; Virtanen, 2009). To determine whether the expansion of brown adipose in the high fat-fed SM/J mice was promoting glycemic control through thermogenesis, I quantified the expression of several genes known to be markers for this process: Ucp1, Cidea, Eva1, Pgc1a, and Prdm16, as well as Tbx1, a marker gene specific to beiging in white adipose tissue (de Jong, 2015; Seale, 2009). None of these genes showed differential expression between 20 and 30 weeks in brown or white adipose depots, suggesting that there is no increase in thermogenesis on a per cell basis. However it is possible that simply having more thermogenic cells in the brown adipose depot could lead to increased thermogenesis by the depot, even if individual cells do not show increased thermogenesis. To test this, I quantified core body temperature, which I would expect to increase if there is increased thermogenesis, serum lipid
levels, which I would expect to decrease if there was increased lipid uptake into the brown adipose depot for use as thermogenic fuel, and serum levels of the catecholamines norepinephrine and epinephrine, which stimulate thermogenesis. None of these parameters showed any changes in high fat-fed SM/J mice between 20 and 30 weeks, therefore I concluded that the brown adipose expansion is not causing glycemic control through increased thermogenesis.

Though the co-occurrence of glycemic resolution and brown adipose expansion is striking, it was possible that the phenomena were unrelated, or that the brown adipose expansion was not directly contributing to the glycemic resolution. To test the necessity of the brown adipose depot for glycemic improvements in the high fat-fed SM/J mice, I surgically removed the depot from mice before (20 weeks) and after (30 weeks) they showed glycemic improvements. Removal of the brown adipose depot did not affect the resolution of basal glucose levels or glucose tolerance, but it did result in loss of the improvement in insulin tolerance that we observed in mice that did not undergo surgery. This suggested that the brown adipose expansion in high fat-fed SM/J mice primarily contributes to systemic metabolic improvements through better insulin-stimulated glucose uptake, which was an established brown adipose function even before the discovery of adrenergic stimulation of thermogenesis (Marette & Bukowiecki, 1989). The expanded brown adipose tissue shows increased expression of two key members of the insulin signaling pathway: Glut4, which mediates insulin-stimulated but not norepinephrine-stimulated glucose uptake (Shimizu, 1996), and Irs1, which is also necessary for brown adipose differentiation (Fasshauer, 2001). Insulin-stimulated glucose uptake is usually blunted in obesity and diabetic-like conditions (Lapa, 2017; Orava, 2013), but transplantation of brown adipose ameliorates this blunting (Stanford, 2013). It is possible that brown adipose expansion in high fat fed SM/J mice is a protective mechanism to improve
insulin sensitivity before hyperglycemia gets out of control.

5.1.2 Transcriptional Co-Expression Networks in Brown Adipose Tissue are Heavily Dependent on Diet and Genetic Background

In Chapter 3, I describe my results from analyzing transcriptional co-expression networks in brown adipose tissue. I used the mice phenotyped in Chapter 2 and obtained RNA-sequencing data for the brown adipose tissue from four mice in each sex (female or male) by diet (high fat or low fat-fed) by age (20 or 30 weeks) by strain (LG/J or SM/J) cohort. Transcriptome clustering revealed that LG/J brown adipose is much more susceptible to diet-induced changes than is SM/J brown adipose, highlighting the importance of accounting for genetic background when performing metabolic research. I identified eight gene co-expression clusters that correlated significantly with one or more phenotypic trait. Of these eight clusters, four had significant enrichment for at least one gene ontology term: immune and cytokine response, cell division, peroxisome, and organic molecule metabolic processes. The immune and cytokine response and organic molecular metabolic processes clusters were conserved in multiple strain and diet sub-cohorts, suggesting that these gene networks are important for adipose function regardless of diet or genetic background. Genes involved in immune and cytokine response correlated with insulin and glucose traits, consistent with the association between diabetes-related traits and inflammation of adipose tissue (Coletta, 2008; Gao, 2015; Škopková, 2007; Zeyda, 2013). Genes in the organic metabolic processes category include those that perform essential functions in glucose and lipid uptake, which explains their correlation with serum lipid traits.

Genes involved in cell division form a network strongly correlated with glucose and insulin traits and brown adipose to body weight ratio. This cluster is only present when the high fat-fed SM/J cohort is included in the analysis, it does not appear in a low fat-fed only or LG/J
only analysis. Principal components analysis revealed a distinct clustering of the high fat-fed SM/J samples due to increased expression of the genes involved in cell division. These genes also show increased connectivity, or strength of co-expression, on a high fat diet and in the SM/J genetic background. The gene-by-environmental specificity of this cluster, its association with glycemic parameters, and the unique expansion of brown adipose in high fat-fed SM/J mice, indicate that the genes in this cluster are compelling candidates for further studies of the interaction between brown adipose and systemic metabolism.

Gene-by-environment interactions are critical for understanding the intricacies and nuances of obesity and metabolic dysfunction. Though there is interest in brown adipose tissue as a potential therapeutic target for metabolic diseases, there have been no studies on gene-by-environment interactions in brown adipose tissue, and few studies on brown adipose in any mouse strains other than C57BL/6J. My results indicate that gene-by-environment interactions significantly contribute to variation in transcriptional networks of brown adipose tissue. Understanding how genetic variation affects the function of brown adipose tissue is critical before it can be successfully targeted for therapeutic treatment of diabetes or obesity.

5.1.3 Unique Transcriptional Changes Accompany Expansion of Brown Adipose Tissue

In Chapter 4, I detail results from my analysis of transcriptional changes in brown adipose tissue between 20 and 30 weeks of age. High fat-fed SM/J brown adipose, which undergoes a dramatic expansion corresponding with improved whole-body glucose homeostasis, is the only tissue to show distinct differential expression between these two ages. Very few genes were differentially expressed in brown adipose tissue from LG/J and low fat-fed SM/J mice or in white adipose tissue from any cohorts. The 267 genes differentially expressed in brown adipose of high fat-fed SM/J mice were enriched for cytokine and
extracellular region functions, with expression levels at 30 weeks consistent with healthier adipose function, particularly through reduced expression of inflammatory genes.

I focused on Sfrp1 (Secreted frizzled-related protein 1) as a gene encoding a particularly interesting cytokine. Sfrp1 is an inhibitor of Wnt signaling and thus promotes adipogenesis (Ackers & Malgor, 2018; Bennett, 2002; Huang, 2017; Kennell & MacDougald, 2005; Lagathu, 2010; Lin, 1997; Ross, 2000). Overexpression of Wnt molecules results in mice with decreased adipose, with a 70% decrease in brown adipose tissue (Wright, 2007). Expression of Sfrp1 increases proportional to the strength of adipogenic stimulation during adipogenesis, and also increases during treatment with thiazolidinedione drugs, which generally act through improving insulin-mediated glucose metabolism (Lagathu, 2009). High fat-fed Sfrp1−/− mice develop decreased expression of Glut4 in white adipose tissue, further implicating the role of Sfrp1 in glucose homeostasis (Gauger, 2013). Though Sfrp1 has never been directly studied in brown adipose tissue, it is a member of the brown adipose coexpression module of genes enriched for cell division, and its expression is strongly correlated with brown adipose mass in particular (Chapter 3). My results show increased Sfrp1 expression in high fat-fed SM/J brown adipose tissue between 20 and 30 weeks. This significantly correlates with increased brown adipose mass and improved glucose tolerance, correlations that are not observed in LG/J mice.

In human obesity, SFRP1 expression is downregulated in subcutaneous white adipose tissue, correlating with impaired insulin sensitivity (Ehrlund, 2013). Further, its expression is decreased in obese patients with glycemic dysfunction (Chen & Wang, 2018; Ehrlund, 2013; Lagathu, 2010). There are multiple genetic variants in the SFRP1 locus in humans, including one that is significantly associated with type II diabetes adjusted for BMI (rs973441) (Mahajan, 2018), as well as multiple variants between LG/J and SM/J genetic sequences in the Sfrp1
region, suggesting that genetic background plays an important role in the effect of Sfrp1 on systemic metabolism.

Overall, I have identified increased Sfrp1 expression in an expanding brown adipose depot that corresponds with increased Glut4 expression underlying improvement in insulin-stimulated glucose uptake. Though previous studies of Sfrp1 have only been conducted in white adipose tissue, the data I present fit a model where secretion of SFRP1 by brown adipocytes stimulates brown adipose proliferation, leading to its role as an insulin-stimulated glucose sink and resulting in improved glucose tolerance and insulin sensitivity.

5.2 Future Directions

My dissertation research has established a model for healthy, non-thermogenic brown adipose function in obesity, transcriptional co-expression networks in brown adipose correlating with metabolic traits, and the role of the cytokine Sfrp1 as a promoter of adipogenesis in brown adipose. However, there is still a great deal to uncover in the model itself, its application to human obesity, and the action of Sfrp1 in particular.

First, I have established that the expansion of the interscapular brown adipose depot affects insulin sensitivity, but is not required for the general improvement in basal glucose and glucose tolerance. Instead, that improvement is likely a result of increased serum insulin and improved islet function (Miranda, 2020). The specific mechanism by which a high fat diet in the SM/J background leads to brown adipogenesis is still unclear. I hypothesize that the elevated serum insulin levels are the primary adipogenic signal, which could be tested with a brown adipose-specific knockout of insulin receptors (Templeman, 2015; Templeman, 2016).

Second, this model of non-thermogenic brown adipose function improving glycemic
control in obesity raises questions on the potential of brown adipose to improve systemic metabolism in human obesity. Identification of brown adipose in humans requires thermogenic activity, however this activity is decreased in obesity (Cypess, 2009; van Marken Lichtenbelt, 2009; Virtanen, 2009; Yoneshiro, 2011). It is possible that non-thermogenic functions of brown adipose tissue are therefore overlooked in human obesity. One theory on people with metabolically healthy and unhealthy obesity is that differences in adipose tissue determine whether additional metabolic disorders are developed (Blüher, 2010; Denis & Obin, 2013; Karelis, 2008; Primeau, 2011). Yet this theory only discusses the distribution of white adipose between subcutaneous and visceral adipose depots. It would be interesting to determine if people with obesity that have higher brown adipose mass, but not increased thermogenic activity, are more likely to maintain a metabolically healthy state.

Finally, Sfrp1 is an exciting candidate gene that likely contributes to the adipogenesis necessary for the brown adipose expansion. The mechanism by which high fat-fed SM/J mice upregulate Sfrp1 expression only in brown adipose must be determined to fully understand this process, however. If elevated serum insulin is indeed the initiating adipogenic signal, then the pathway from insulin receptors to Sfrp1 transcription would need to be elucidated. Additionally, I would need to verify that Sfrp1 function in brown adipose is similar to its function in white adipose. I am currently analyzing how Sfrp1 expression changes throughout the differentiation of fibroblasts into brown adipocytes. We have also started the process of generating a brown adipose-specific knockout of Sfrp1 in SM/J mice. This will allow determination of whether Sfrp1 is necessary for the brown adipose expansion and associated metabolic improvements. To complement this experiment, overexpressing Sfrp1 in a different mouse model of diet-induced obesity would determine whether it is sufficient to increase brown adipose mass and improve metabolic health independently.
5.3 Final Thoughts

My dissertation work provides new data on brown adipose in a novel mouse model of healthy obesity. I have established the importance of insulin-stimulated glucose uptake as a non-thermogenic mechanism by which brown adipose can positively affect systemic glucose metabolism. This function could be utilized in obesity where thermogenic activity has been blunted.

I have identified brown adipose transcriptional co-expression networks that correlate with metabolic phenotypes. These networks highlight genes related to immune and cytokine response and organic molecule metabolic processes that could be studied in brown adipose tissue for the first time. Further, I identified genes related to cell division that correlate with the increased brown adipose mass in high fat-fed SM/J mice. This includes the pro-adipogenic cytokine Sfrp1, which has never before been studied in brown adipose tissue.

These exciting results ultimately come from a phenomenon observed completely by accident, and serve as a reminder that many of the best scientific discoveries are made by finding something weird and poking it with a stick to see what happens.
Chapter 6

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118


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Appendix A

Supplementary Tables
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**Table A.1 Genes with high differential expression and connectivity by diet and strain.** Columns include direction of differential expression (DE) and differential connectivity (DC) by diet and strain, whether the gene locus has SNPs between LG/J and SM/J mice, whether those SNPs are predicted to have functional consequences, and what metabolic QTL the locus falls into, if any.
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Table A.2 Genes differentially expressed in SM/J high fat-fed brown adipose between 20 and 30 weeks. Columns include Ensembl IDs, gene names, average normalized expression at 20 and 30 weeks, log fold change from 20 to 30 weeks, log counts per million, p-value, fdr-corrected p-value, and co-expression module that the gene belongs to (blue = midnight blue module; turq = turquoise module).