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THE ROLE OF THE EXTRACELLULAR MATRIX PROTEIN LAMININ-α4 IN OBESITY AND ADIPOCYTE BEIGING

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DEDICATION PAGE

To Dr. Cohen –

I am greatly appreciative of your support, mentorship, and belief in me over the past six years. You challenged me and helped me grow as a scientist and leader in your lab. As I take the next step in my career, I am so thankful for all you have taught me.

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ABSTRACT

During the past several decades, the prevalence of obesity and related metabolic diseases has reached epidemic scale across the world. While obesity alone heavily impacts the healthcare system, it also increases the risk for developing co-occurring health conditions including heart disease, stroke, and certain forms of cancer. Moreover, those of lower socioeconomic status and from minority racial and ethnic populations are disproportionately burdened by these diseases. There is a clear and imminent need for non-invasive therapies to effectively treat obesity. Recently, the field of adipocyte thermogenesis has gained increasing momentum towards the development of such therapies. However, the complexity of thermogenic regulation in adipocytes has made it difficult to isolate drug targets that are successful *in vivo* and in clinical stages. Through increasing our knowledge of thermogenic regulators in adipose tissue, we can build a more comprehensive picture of the drivers of phenotypic shifts in adipocytes to identify more appropriate targets for drug discovery pipelines.

One such potential regulator of adipocyte behavior and thermogenesis is the extracellular matrix protein laminin-α4 (LAMA4). The work presented in this dissertation illuminates the role of laminin-α4 in human obesity and in suppressing adipocyte beiging and contributes novel findings to the field of adipocyte thermogenesis and metabolic research. Chapter 1 provides background on metabolic diseases, adipose tissue, thermogenesis, and the extracellular matrix, including an introduction to laminin proteins and an overview of their current applications in biomedicine and tissue engineering. Chapter 2 details the findings of a comparative study of LAMA4 expression patterns in white adipose tissue in humans and mice with obesity and following weight loss. This investigation uncovered a parallel upregulation of LAMA4 expression on both the mRNA and protein levels in the subcutaneous white adipose tissue of

mice and human subjects with obesity. However, LAMA4 expression remained unchanged following short-term weight loss in both models. Chapter 3 of this dissertation describes a mechanistic investigation into the regulation of adipocyte thermogenesis by LAMA4 in human and murine models. *In vivo*, the absence of LAMA4 in mice led to elevated mitochondrial marker and peroxisome proliferator-activated receptor γ coactivator-1 α (PGC-1α) expression. Murine beige adipocytes transfected with siRNA targeting LAMA4 exhibited enhanced uncoupling protein 1 (UCP1) and AMP-activated protein kinase α (AMPKα) expression. When thermogenic adipocytes derived from human induced pluripotent stem cells were treated with recombinant human laminin-411 during differentiation, UCP1 and AMPKα expression were suppressed, suggesting that LAMA4 negatively regulates adipocyte thermogenesis via the AMPK-PGC-1α pathway. The observed extracellular-to-intracellular signaling transduction may be mediated through integrins and integrin linked kinase (ILK), as silencing of ILK in murine adipocytes mirrored the results of the LAMA4 silencing experiments. Overall, the results of this dissertation describe a regulatory function of LAMA4 in maintaining a white adipocyte phenotype through the inhibition of adipocyte beiging. Additionally, this work validates the relevance of these findings in human adipocyte models and obesity, laying the foundation for future examination into the therapeutic benefit of targeting LAMA4 in humans.

CHAPTER 1: INTRODUCTION

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1.1 Metabolism

In order to function, all living organisms require biochemical processes that supply energy and convert substances to useable forms [1]. Metabolism is the sum of these biochemical reactions, including catabolism and anabolism, and takes place in all cells. Cells utilize glucose, fatty acids, and amino acids to generate substrates for cellular respiration and energy generation through glycolysis, fatty acid β-oxidation (FAO), and transamination or deamination, respectively [2,3]. The pathways that regulate the conversion, storage, breakdown, and biosynthesis of molecules utilized in these processes are encompassed within cellular metabolism. When disruptions to these key pathways arise, it can lead to abnormal metabolic functioning and, eventually, the development of metabolic diseases such as obesity and diabetes.

1.1.1 Metabolic Processes

Cellular respiration is a critical process generating ATP from the breakdown of glucose molecules. ATP is a highly negatively charged molecule capable of storing large amounts of energy which is released upon hydrolysis [1]. During glycolysis, the first step of cellular respiration, glucose is broken down to form pyruvate in addition to generating ATP and NADH [4]. Glycolysis can be performed under both aerobic or anaerobic conditions, where in the latter pyruvate is then converted to lactate [4]. In aerobic respiration, pyruvate is oxidized to acetyl-CoA which enters the citric acid cycle in the mitochondrial matrix, leading to the formation of ATP and

carbon dioxide, and the reduction of electron carrier molecules NADH and $FADH_2$. Lastly, these electron carriers deposit electrons into the Electron Transport Chain (ETC) for the final step of cellular respiration, oxidative phosphorylation. As electrons move down the chain of proteins in the mitochondrial inner membrane, proton-motive force is generated, driving protons out of the matrix [5]. Protons then flow back down their concentration gradient into the matrix through ATP synthase, during which large amounts of ATP are produced [5].

This process is reliant upon sufficient levels of pyruvate, which can be generated not only from glucose via glycolysis, but also fatty acids via mitochondrial β-oxidation (FAO). FAO also serves as a means of creating ketone bodies in times of carbohydrate scarcity. Fatty acids are derived from larger triglyceride (TG) molecules and are typically stored as lipid droplets in adipose tissue. TG molecules are broken down to fatty acids (FFAs) which can then circulate systemically and be taken up by other cells [1]. FFAs are converted to acyl-CoA in the cytoplasm then shuttled into the mitochondria by the carnitine shuttle where they undergo degradation [3]. Each cycle of the β-oxidation pathway yields acetyl-CoA, NADH, and $FADH_2$ which can then enter downstream steps of cellular respiration [3].

Most tissues are flexible in their utilization of starting substrates for cellular respiration but prefer to use glucose when it is readily available. Cells can switch to the use of FFAs as substrates for oxidation during fasting and starvation to minimize their use of glucose which is needed for the brain [3]. The heart is unique in that it predominantly uses FFAs as a source of energy even in the fed state [1]. The various functions that certain tissues, such as skeletal muscle, play in systemic metabolic homeostasis determines their preference for different fuel types and illustrates the importance of tissue cross talk.

1.1.2 Hormonal Regulation

Achieving and maintaining metabolic homeostasis requires the ability to respond to changes in energy availability and integrate action across multiple metabolic tissues. Hormones are critical regulators of metabolic action in various cell types and allow the transmission of a signal from one tissue to another. They can inform and regulate processes involving satiety and hunger (leptin, ghrelin), glucose homeostasis (insulin, glucagon), inflammation, lipid distribution, and more through modulating the expression of key enzymes required in metabolic pathways.

Hormones regulating metabolic processes are produced and secreted by both classical endocrine organs and also by metabolic tissues themselves. They are released into the circulatory system to act upon tissues containing complementary receptors. Peptide hormones will bind cell-surface receptors to mediate rapid action. Binding to their receptor stimulates signaling cascades via cyclic adenosine 3,5-monophosphate (cAMP) and phosphatidylinositol (3,4,5) trisphosphate (PIP3) which will regulate downstream enzyme activity [6]. Other hydrophobic hormones may instead pass through the plasma membrane to bind nuclear receptors in the cytosol, translocate to the nucleus, and directly alter the expression of key enzymes [6].

1.1.2.1 Peptide Hormones

Leptin and ghrelin regulate energy balance through their role in hunger and satiety. Adipose tissue is the primary source of production and secretion of the adipokine leptin, which increases as adipose mass increases. Leptin acts on the leptin receptor in the hypothalamus to decrease food intake and increase energy expenditure through the activity of orexigenic and anorexigenic neuropeptides [7]. Ghrelin is a gastric peptide hormone that acts opposingly to leptin to increase hunger and initiate food intake, in addition to increasing the secretion of growth hormone. Ghrelin is predominantly produced in the stomach, with a small amount of additional ghrelin being

produced in the hypothalamus, and exerts its effects on the hypothalamus and vagus nerve through binding to the growth hormone secretagogue receptor [8].

Insulin and glucagon are two major peptide hormones secreted by the pancreas that regulate glucose homeostasis, having antagonistic effects on glucose uptake and storage. Insulin is produced by β cells in the Islets of Langerhans and secreted in response to blood glucose elevation. Gut-derived incretin hormones such as glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic peptide (GIP) are also known to increase insulin secretion [9]. Insulin receptors (IR) are located in many tissue types including skeletal muscle, adipose, liver, and brain. Insulin binds to the IR, a receptor tyrosine kinase, to activate insulin receptor substrate (IRS). This stimulates translocation of the insulin-regulated glucose transporter 4 (GLUT4) to the cell membrane for glucose uptake in muscle and fat cells via the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) pathway [10]. In the liver, GLUT4 is present in very low levels whereas the GLUT2 transporter, which is not regulated by insulin, is more predominant. This transporter is unique and allows the entry and exit of glucose, hinting at the larger role the liver plays in glucose production to maintain glucose homeostasis. In the liver, insulin promotes glucose storage instead through the activation of pathways involved in glycolysis and glycogen synthesis, and the inhibition of glucose synthesis. This occurs through the activation of AKT, which inhibits glycogen synthase kinase (GSK)-3 to relieve the inhibition on glycogen synthase (GS). Additionally, AKT inhibits forkhead box O1 (FoxO1), thus suppressing glucose-6-phosphatase and phosphoenolpyruvate carboxykinase-1, two important regulators of gluconeogenesis [11].

Glucagon is produced in the α-cells of the pancreas in response to hypoglycemia and mediates action by binding to the glucagon receptor, primarily expressed in liver. Downstream of this receptor activation, cAMP levels rise and activate protein kinase A (PKA) which activates

glycogen phosphorylase, phosphofructokinase (PFK)-2, pyruvate kinase (PK), and cAMP response element-binding protein (CREB). The resulting effects are the promotion of glucose formation pathways (glycogenolysis and gluconeogenesis), and the inhibition of glucose consumption or storage pathways (glycolysis and glycogenesis), with the overall outcome of raising blood glucose levels [12].

Another major adipokine, adiponectin, is involved in the regulation of a multitude of metabolic processes including insulin sensitization and the healthy expansion of adipose tissue, and additionally possesses anti-inflammatory properties. Adiponectin is secreted from adipose tissue and can bind to two receptor isoforms, AdipoR1, predominantly expressed in skeletal muscle, and AdipoR2, predominantly expressed in liver [13]. A major pathway through which adiponectin mediates its effects is AMP-activated protein kinase (AMPK) and peroxisome proliferator-activated receptor alpha (PPARα). In skeletal muscle, adiponectin stimulates p38 mitogen-activated protein kinase (MAPK) and PPARα activity which results in the upregulation of lipid oxidation and glucose utilization [14]. In the liver, adiponectin can reduce lipogenesis and gluconeogenesis and increase mitochondrial β-oxidation via AMPK activation [14]. In adipocytes, adiponectin inhibits lipolysis and promotes lipid storage as well as enhances GLUT4-mediated glucose uptake [14]. Overall, adiponectin is a critical mediator of appropriate lipid storage and works across a number of tissue types to modulate metabolic processes and enhance insulin sensitivity.

1.1.2.2 Steroid Hormones

In addition to peptide hormones, steroid hormones, such as glucocorticoids, estrogens, androgens, and mineralocorticoids, are highly involved in the regulation and control of endocrine and metabolic processes. These hydrophobic and fat-soluble molecules are derived from

cholesterol and are able to pass through the membrane and bind to intracellular receptors. Many of these belong to a larger receptor family called nuclear receptor transcription factors which can alter gene expression. Following the binding of ligand in the cytoplasm, nuclear receptors undergo a conformational change allowing them to translocate to the nucleus. There they bind to specific response elements in the DNA to modulate gene transcription, either up or down.

Cortisol is a steroid hormone that binds to the glucocorticoid receptor to regulate the stress response, metabolism, and the inflammatory response. Cortisol is released from the adrenal gland in response to stress under the control of the hypothalamus-pituitary-adrenal (HPA) axis. A main action of cortisol, in relation to metabolic health, is to increase blood glucose levels. This is accomplished through the elevation of gluconeogenesis in the liver and decrease of glucose uptake and storage in other tissues. In the pancreas, cortisol also suppresses insulin release and enhances glucagon production [15]. Inappropriate production of cortisol, in either direction, can lead to endocrine dysfunction. High levels of cortisol for long periods of time can result in the development of Cushing syndrome, whose symptoms include those commonly associated with metabolic disease, such as obesity and insulin resistance. Insufficient production of cortisol can lead to the development of Addison disease.

Sex steroid hormones also play important roles in metabolic regulation in addition to their roles regulating reproduction. These include estrogens, progestins, and androgens, which are released from the gonads. Estrogen and progesterone are released from the ovaries and are known to regulate metabolic factors. Estrogens have been well documented to modulate food intake, blood glucose levels, energy expenditure, and lipid storage. Defects or reduced activity in the estrogen receptor-α in humans leads to many of the symptoms associated with metabolic disease and obesity [16]. The role of progesterone in metabolism and glucose homeostasis is less well understood, but

studies have shown that progesterone increases blood glucose levels through both a reduction in glucose uptake and an increase in liver gluconeogenesis [17]. Androgens act in a number of different metabolic tissues to alter glucose uptake, lipid metabolism, adipose deposition, and energy expenditure. In those with XY chromosomes, androgens such as testosterone decrease visceral adiposity, improve muscle and liver insulin sensitivity, and increase insulin secretion [18]. Conversely, in those with XX chromosomes, an excess of androgens can actually promote visceral adiposity and insulin resistance, such as in the case of polycystic ovarian syndrome (PCOS). PCOS affects around 4–10% of women of reproductive age and its diagnosis carries a heightened risk for developing obesity and type 2 diabetes [19]. As such, sex steroid hormones evidently play an important role in regulating metabolic function and their dysregulation can promote metabolic disease.

1.2 Metabolic Disease

The prevalence of metabolic diseases is on the rise worldwide in both adults and children and they are increasingly reaching epidemic proportions. The predominant metabolic diseases today include diabetes mellitus, obesity, and non-alcoholic fatty liver disease (NAFLD). These are highly related illnesses that involve the dysregulation of several key metabolic processes in endocrine tissues such as adipose, skeletal muscle, liver, and pancreatic β-cells (Fig. 1.1). They are furthermore associated with the development of additional conditions that heighten the risk of death, including cancer, cardiovascular disease, and liver cirrhosis.

1.2.1 Prevalence

Diabetes mellitus is characterized by long-term hyperglycemia arising from either the inability to produce sufficient insulin (type 1, T1D) or the inability of peripheral tissues to accurately sense and respond to insulin (type 2, T2D). The 2020 CDC National Diabetes Statistics

Report estimates that 13% of adults in the United States have diabetes, and one-third of the population is pre-diabetic [20]. T1D most commonly arises from autoimmune disorders whereby immune cells attack and destroy the insulin-producing β cells in the pancreas, and has been associated with specific genetic and environmental factors [21]. It accounts for the majority of childhood diabetes cases (around 80-90%), while T2D accounts for the vast majority of adult diabetes cases (around 90-95%) [21]. T2D arises from insulin resistance in periphery tissues and is strongly associated with high adiposity and obesity. Recently, the incidence of T2D in children and adolescents has increased rapidly, likely owing to the rise in childhood obesity levels. In addition to T1D and T2D, other forms of diabetes exist, though contribute to a much smaller proportion of overall cases. These include monogenic diabetes, caused by mutations in genes controlling β cell function or differentiation, and gestational diabetes, which occurs during pregnancy.

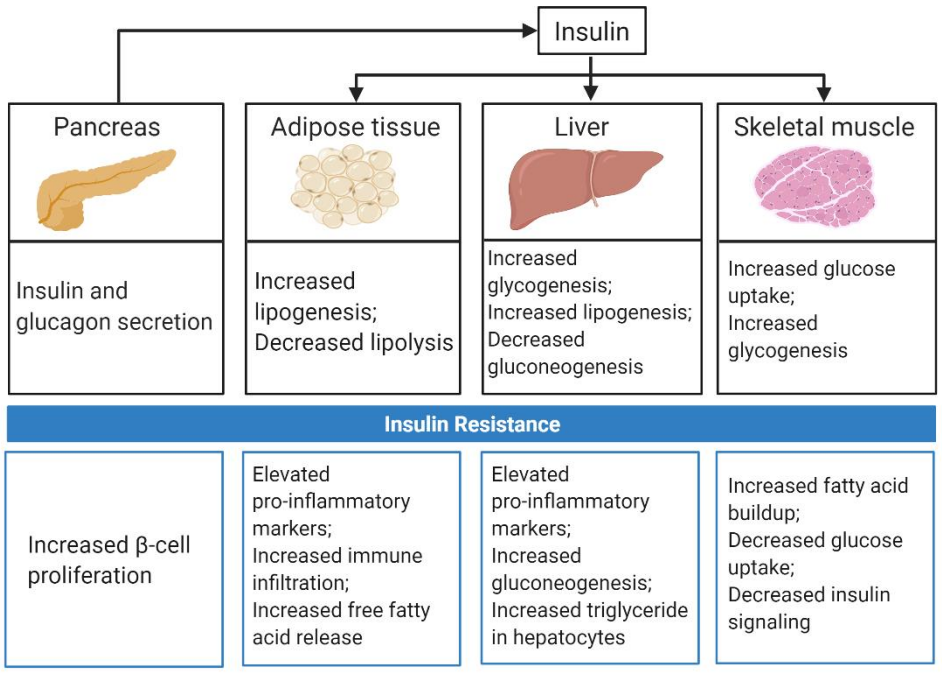

Figure 1.1 Metabolic tissues respond to insulin and insulin resistance

Metabolic tissues include the pancreas, adipose tissue, liver, and skeletal muscle. Insulin signaling results in upregulation or downregulation of various physiologic processes in these tissues. In the setting of insulin resistance, however, each tissue undergoes metabolic changes that can be detrimental to organ function and perpetuate the development of metabolic disease.

Obesity is characterized by excessive adiposity leading to a heightened risk for other diseases including hypertension, heart disease, and diabetes. It is classified by a Body Mass Index (BMI) $\geq$ 30.0 kg/m^2 for most populations (in Asia-Pacific populations, BMI $\geq$ 25.0 kg/m^2 indicates obesity), or a BMI $\geq$ 40.0 kg/m^2 for severe obesity [22]. A BMI between 25.0 kg/m^2 30.0 kg/m^2 is classified as overweight. The rates of obesity among adults have dramatically risen in the United States across the past fifty years, doubling from 13% in 1976-1980 to 27% just two decades later in 1999, and now to nearly 43% as of 2018 [23,24]. Severe obesity, also known as class III obesity, has risen to 9.2%. Childhood obesity has seen a steady increase as well, with nearly 20% of children ages 2-19 years old being considered obese in 2018, up from 17% in 2012 [25,26]. Racial

and ethnic minority groups, in addition to those of lower socioeconomic status, are disproportionally affected by obesity and associated metabolic diseases. The prevalence of obesity in 2018 was 17.4% among non-Hispanic Asian adults, 42.2% among non-Hispanic White adults, 49.6% among non-Hispanic Black adults, and 44.8% among Hispanic adults [24]. Obesity is a major risk factor for the development of T2D and the epidemiological patterns of the two are closely related.

Another metabolic disease closely associated with both obesity and T2D is NAFLD, which develops when excess triglyceride becomes inappropriately stored in the liver. The incidence of NAFLD is also rising along with the increases in obesity and T2D, and affects close to 33% of adults in Western countries [27]. According to the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK), around 66% of patients with T2D and 75% of people who are overweight also exhibit signs of NAFLD [28]. Over time, NAFLD can progress to non-alcoholic steatohepatitis (NASH) in about one-third of patients which can lead to liver disease, hepatocellular cancer, and death [29].

The growing prevalence of metabolic diseases is associated with complex behavioral, genetic, and environmental risk factors. This can include sedentary lifestyle, a diet high in fat and processed foods, parental obesity, smoking, and exposure to endocrine disrupting chemicals (EDCs) [30]. Patients with obesity or who are overweight have an increased risk for developing T2D, cardiovascular disease, mental illness, and experiencing a low quality of life [31].

1.2.2 Pathophysiology

The criteria for diagnosing metabolic syndrome have expanded in the past decade from an emphasis on insulin resistance to the inclusion of factors such as central obesity, high blood pressure, high fasting TG, and low high-density lipoprotein (HDL) cholesterol levels. However,

the predominant link among the pathophysiology of these prevalent metabolic diseases is the accumulation of excess fat which drives adverse metabolic functioning and insulin resistance. In obesity, energy intake exceeds energy expenditure, resulting in fat mass gain through the expansion of existing adipocytes (hypertrophy) or the increase in number of adipocytes (hyperplasia). The location, or fat depot, in which major expansion occurs is extremely important in the downstream development of associated diseases. The systemic insulin resistance and chronic inflammation commonly associated with obesity are thought to arise from the ectopic storage of fat in the liver, derived from overburdened visceral adipose. Thus, intra-abdominal or visceral fat accumulation in particular, as measured by waist-to-hip ratio, is correlated with increasing insulin resistance. Subcutaneous adipose mass, on the other hand, is negatively correlated with insulin resistance risk. A subset of obesity cases do not lead to insulin resistance, however, and this is attributed to the differing expandability of subcutaneous adipose tissue [32,33]. This ability to expand and remodel is highly influenced by the extracellular matrix, as will be discussed in depth later on in this chapter.

Systemic insulin resistance results in hyperglycemia, as skeletal muscle cells are unable to respond to insulin and remove glucose from the bloodstream. Although the early stages of T2D caused by obesity are characterized by sufficient insulin production, the feedback cycle created through chronic hyperglycemia leads to eventual decreases in insulin production capacity in addition to insulin resistance. Pancreatic β cells respond to initial hyperglycemia by increasing the production of insulin to very high circulating levels, termed hyperinsulinemia. This prolonged pancreatic β cell hyperfunction can cause β cell exhaustion and death, resulting in an inability to produce sufficient amounts of insulin to meet the demand [34].

In addition to causing chronic hyperglycemia, insulin resistance alters adipocyte and liver function leading to hyperlipidemia. In healthy adipocytes, insulin promotes the uptake and storage of FFAs as lipid droplets, termed lipogenesis. When insulin is not present, TG can be broken down into FFAs which are then released, through the process of lipolysis [35]. Thus, when adipose tissue is overburdened or insulin resistant and exhibiting a high rate of inappropriate lipolysis, large amounts of FFAs are released into circulation, known as hyperlipidemia. These may be taken up and stored ectopically by other tissues including the liver, further exacerbating the insulin resistance. In the liver, insulin resistance can enhance hepatic *de novo* lipogenesis, the biochemical process where FFAs are synthesized from acetyl-coA, through changes in expression of transcription factors associated with this pathway [35]. The expression of sterol regulatory element binding protein 1c (SREBP-1c) and carbohydrate response element binding protein (ChREBP), which both activate the transcription of the rate-limiting enzymes involved in hepatic *de novo* lipogenesis, is stimulated by high insulin levels and dietary saturated fatty acids [35]. While insulin also promotes the secretion of TG from the liver via regulation of very low density lipoprotein (VLDL), the rate of lipogenesis during insulin resistance outweighs the rate of secretion, leading to increasing liver TG levels [35]. When the burden becomes too high, however, inappropriate lipid storage in the liver can lead to adverse health outcomes such as heightened inflammation and fibrosis.

Lastly, there is a known link between obesity, insulin resistance, and chronic inflammation of adipose tissue. Subjects with obesity and insulin resistance exhibit elevated levels of the inflammatory markers C-reactive protein (CRP), tumor necrosis factor (TNF)-α, and interleukin 6 (IL-6), and low levels of the anti-inflammatory adipokine adiponectin. As adipose tissue expands and adipocytes become hypertrophic, triggers like hypoxia and lipid release stimulate pro-

inflammatory signaling cascades mediated by the nuclear factor kappa B (NF-κB) and c-Jun N-terminal protein kinases (JNK) pathways. This results in the secretion of chemokines that recruit cytokine-releasing immune cells to adipose tissue. These cytokines further promote pro-inflammatory signaling and dampen insulin signaling capacity, as the stimulation of the JNK pathway inhibits insulin signaling by phosphorylating the serine/threonine residues of IRS-1. This inhibits IRS-1 and diminishes PI3K/AKT signaling, leading to insulin insensitivity [36,37].

1.2.3 Current Treatment Options and Challenges

Although metabolic diseases like obesity and T2D have been growing public health concerns for several decades, few effective and long-term treatments exist. Weight reduction remains one of the best methods of preventing pre-diabetes from developing into T2D. In patients with obesity, reductions in weight by 5-10% can significantly improve glucose control, blood pressure, and cholesterol [38]. Weight loss and metabolic normalization in these patient groups is typically achieved through a combination of lifestyle intervention, bariatric surgery, and pharmacological agents. Nevertheless, only a small subset of the population is able to achieve and maintain weight loss long-term.

1.2.3.1 Lifestyle Intervention

A key initial strategy for treating and preventing obesity and T2D is modulating lifestyle and diet in addition to behavioral therapy. A multitude of cohort studies from different countries have illustrated the significant effect of lifestyle intervention, either dietary, physical activity, or both, in reducing T2D prevalence or preventing its onset [39]. Resultant weight loss during lifestyle intervention is described as one of the most important factors for diabetes prevention. For instance, the European Diabetes Prevention Study (EDIPS) found that T2D risk was 89% lower in subjects who sustained a 5% or greater weight loss over three years compared to subjects that did

not lose weight during the intervention [40]. However, the maintenance of this weight loss long-term can become increasingly difficult and almost 80% of those that lose weight will end up returning to their previous weight percentiles [41]. This is partially due to the body's adaptation to weight loss through a decline in 24-hour energy expenditure, creating a larger physiological drive for weight regain. Strategies for preventing weight regain, including weight loss maintenance sessions, standard behavioral therapy, and more recently, personalized cognitive-behavioral therapy, can support weight loss maintenance long-term [42,43].

1.2.3.2 Bariatric Surgery

Weight loss can also be effectively achieved with bariatric surgery, though this treatment method is predominantly reserved for patients with class III obesity or class II obesity with associated comorbidities. The most common surgical techniques performed are the sleeve gastrectomy (SG), where around 80% of the stomach is resected, and the Roux-en-Y gastric bypass (RYGB), whereby the size of the stomach is resected and then re-attached to the GI tract, bypassing the duodenum and reducing absorption. Both result in significant reductions in weight over time, with RYGB reported to lead to decreases of 30-35% in body weight after 3 years and improved remission rates in patients with T2D [44]. Although bariatric surgery leads to greater weight loss on average than other methods, these surgical techniques can be both costly and complicated [45,46]. The main issues that arise following bariatric surgery are related to surgical complications or nutritional deficiencies [44]. The less invasive laparoscopic methods are associated with fewer complications and reduced mortality in gastric bypass compared to open gastric procedures, and as technology evolves these procedures will become safer [44].

1.2.3.3 Pharmacological Approaches

Pharmacological treatment approaches in combination with insulin therapy are extremely common for patients with T2D and target issues both with insulin secretion and insulin resistance. Insulin secretion can be elevated through use of anti-diabetic drugs like sulfonylureas, which increase β cell insulin secretion, and, more recently, incretin-like drugs [47]. Drugs that target insulin resistance include thiazolidinediones (TZDs) and biguanides like metformin [48,49]. TZDs improve insulin sensitivity by activating PPARγ to promote adipogenesis and regulate lipid uptake and metabolism, but are becoming less commonly used due to concerns about increased risk for heart failure [48]. Metformin can reduce hepatic glucose production and improve hepatic and peripheral tissue insulin sensitivity, though many patients with T2D cannot use metformin due to contraindications or poor tolerance of side effects [38]. Additionally, many of these drugs, apart from metformin and incretins, can lead to weight gain.

The development of weight loss and weight management drugs for patients with obesity has been a much more difficult task with few successful candidates currently on the market. Historically, weight loss drugs focused on appetite control, intestinal absorption of fats, and central nervous system activation to increase energy expenditure, but many have been discontinued due to negative side effects [50]. Until more recently, only a few drugs, such as orlistat, which acts by decreasing the intestinal absorption of fats, and phentermine/topiramate, which is only approved for short-term use, were approved specifically for weight loss in the United States [50]. In the summer of 2021, the FDA approved Wegovy (semaglutide), a GLP-1 receptor agonist, which is administered by injection once a week for weight-management. It is indicated for patients with a BMI > 30 kg/m2, or BMI>27 kg/m2 with a weight-related ailment, and has shown an average of 15% weight loss over 16 months in study subjects [51]. It was previously approved for patients

with T2D, marketed under the names Ozempic (subcutaneous injection) and Rybelsus (tablet form). The drugs indicate warnings of the possible formation of thyroid tumors and side effects mainly include gastrointestinal ailments. While this new approval represents a step forward for pharmacological treatment of obesity, there are many additional promising routes of treatment that are currently under investigation.

Induction of beiging in white adipose depots or mitochondrial uncoupling to enhance energy expenditure is one area of interest at present. Although thermogenic induction of adipose tissue via β3 receptor activation was unsuccessful in human clinical studies, new avenues are being pursued including cold stimulation and novel browning agents. For instance, the phosphodiesterase 5 (PDE5) inhibitor sildenafil, which is currently approved for treating diabetes and erectile dysfunction, has shown efficacy in increasing browning of WAT in humans and thus gained more interest in the past few years as a potential anti-obesity drug [52]. Recently there has also been avid interest in developing drugs to target the endocannabinoid receptor. While the cannabinoid CB1 receptor antagonist/inverse agonist rimonabant was pulled from the market due to adverse psychological effects more than a decade ago, recent studies are investigating the use of CB1 neutral antagonists and partial agonists to reduce weight [50]. Pharmacological treatment, while potentially one of the more accessible methods for weight management, is not widely applicable at the current moment due to the lack of effective drugs and negative side effects accompanying those that are available, although this is steadily changing. More research is needed to identify candidate drugs for effective and long-term weight reduction, especially in target tissues like adipose.

1.3 Adipose Tissue and Thermogenesis

Adipose tissue is a key endocrine organ responsible for maintaining whole-body energy homeostasis through the regulation of glucose levels, free fatty acid (FFA) flux, and energy expenditure. It is a heterogenous tissue containing mature adipocytes, adipocyte precursors, vascular endothelial cells, immune cells, and fibroblasts [53]. Adipocytes can be broken into three distinct types: white, brown, and beige (brite), which have distinct origins, localizations, and functions that contribute to the maintenance of overall metabolic health.

1.3.1 Adipose Tissue Types and Origins

1.3.1.1 White Adipose Tissue

White adipose tissue (WAT) is dispersed throughout the body and performs several key physiologic functions including energy storage in the form of a large unilocular lipid droplet, insulation, and endocrine signaling through hormone and adipokine release. White adipocytes perform two main functions in regulating energy storage and release: lipogenesis and lipolysis.

In the fed state, insulin promotes glucose uptake and conversion into acetyl-CoA and glycerol-3-phosphate (for use as the backbone of TG molecules). Acetyl-CoA is formed through the action of several enzymes, including ATP citrate lyase (ACLY), acetyl-CoA carboxylase (ACC1), and fatty acid synthase (FASN). Insulin also promotes the uptake and esterification of fatty acids to be stored as TG. This conversion process occurs though the action of several enzymatic steps by acyl-CoA synthetase (ACS), glycerol-3-phosphate acyltransferase (GPAT), 1-acyl-glycerol-3-phosphate acyltransferase (AGPAT), phosphatidic acid phosphatase (PAP), and diacylglycerol acyltransferase (DGAT) [54]. TG molecules are predominantly formed from circulating FFAs and, to a smaller extent, from acetyl-CoA derived from *de novo* lipogenesis. In

contrast, lipolysis promotes the mobilization of energy stores from within adipocytes when energy is required. In this process, the breakdown of lipid and release of FFAs and glycerol occurs through the deactivation of perilipin-1 (PLIN1) and the activation of hormone sensitive lipase (HSL), adipose triglyceride lipase (ATGL), and monoglyceride lipase (MGL) [55]. These processes are highly regulated by insulin and β3-adrenergic receptor (β3-AR) activity.

WAT is dispersed throughout the body and exists in two major classifications. The first, subcutaneous adipose (sWAT), is found beneath the skin and the second, visceral adipose (vWAT), is intra-abdominal and surrounds the internal organs. Although somewhat similar, these two classes of WAT exhibit differing behaviors tied to their distinct biological roles. sWAT is considered protective and its expansion is associated with improved insulin sensitivity. Conversely, vWAT is associated with worsening metabolic effects likely due to the fact that it drains directly to the liver through the portal vein [56].

The origins of the different classes of adipocytes have been highly debated in the past decade, with many new findings illustrating the likelihood of subpopulations allowing for distinct function. Previously, white adipocytes were believed to originate only from myf5-negative mesenchymal precursors, however more recent lineage tracing suggests that a subpopulation of white adipocytes may originate from myf5-positive precursors as well [57]. vWAT and sWAT have also been shown to originate from distinct populations of precursors at different timepoints in gestation. vWAT uniquely originates from cells expressing Wilms' tumor 1 (wt1), while only sWAT precursors can be labelled with paired related homeobox transcription factor 1 (prx1)-cre, suggesting distinct pools of precursors for these two depots [58].

1.3.1.2 Brown Adipose Tissue

Brown adipose tissue (BAT) is predominantly located in the interscapular space and perirenal region in mice and is characterized by multilocular lipid droplets and a high content of cristae-dense mitochondria. The primary function of BAT is the regulation of non-shivering thermogenesis, although it is also an important regulator of glucose and lipid metabolism. Non-shivering thermogenesis is the process by which energy is dissipated as heat which can result from uncoupling protein 1 (UCP1)-dependent and UCP1-independent mechanisms. UCP1 acts by uncoupling the proton gradient from ATP synthesis on the mitochondrial inner membrane, whereas UCP1-independent thermogenesis occurs by futile cycling of creatine-substrate and Ca^{2+} [59]. Although previously thought to play only a minor role in human physiology and metabolism after infancy, several studies have supported the existence of more widespread BAT depots in adults near the supraclavicular region using 18F-Fluorodeoxyglucose positron emission tomography/computed tomography (18F-FDG-PET/CT), and thus the study of BAT for therapeutic purpose has gained interest in recent years [59].

BAT thermogenic activation occurs in response to cold exposure, β3-AR agonists, thiazolidinediones, and exercise. In classical UCP1-dependent BAT activation, catecholamines such as norepinephrine stimulate β3-AR activity which elevates the production of cAMP by adenylate cyclase. cAMP activates PKA which then acts on a number of downstream proteins to propagate the thermogenic response. This includes the activation of CREB, which upregulates the transcription of UCP1, and of the lipolytic pathway to elevate FFA levels [60]. Although β3-AR signaling stimulates lipolysis in brown adipocytes, this represents a small source of FFAs utilized. BAT is actually highly reliant on circulating FFAs released from white adipocytes for the process of thermogenesis [61]. FFAs are shuttled into the mitochondria where they are broken down by β-

oxidation, eventually leading to the pumping of protons out of the mitochondrial matrix. Due to the high levels of UCP1 and low levels of ATP synthase in brown adipocytes, protons flow back into the matrix without the production of ATP, reducing the membrane potential and generating heat. FFAs can also bind directly to UCP1 to support the shuttling of protons across the membrane [61].

Classical brown adipocytes share origins with myocytes, which both develop from myogenic factor 5 (myf5)-positive cell lines. Brown adipocytes arise from myf5-positive precursors expressing PR domain containing 16 (PRDM16) and early B cell factor 2 (EBF2) [61]. There are two identified subpopulations of brown adipocytes within interscapular BAT: $ADIPOQ^{high}$ and $ADIPOQ^{low}$ adipocytes. $ADIPOQ^{high}$ brown adipocytes express high levels of adiponectin (ADIPOQ) and UCP1 and are highly thermogenic, whereas $ADIPOQ^{low}$ brown adipocytes exhibit lower thermogenesis and greater lipid storage functions [62]. As lineage tracing methods become more advanced and more groups work to unravel the origins of specific adipocytes, it may become clearer how these subpopulations arise.

1.3.1.3 Beige Adipocytes

In addition to white and brown adipocytes there is a third intermediary form of adipocyte, termed beige or brite. The lineage of beige adipocytes is more closely aligned with white adipocytes and they are located in WAT depots [56]. However, they can be induced to display a brown-like phenotype under cold stimulation and β3-AR activation. Although there is controversy surrounding the existence or physiological relevance of beige adipocytes in humans, studies have identified the presence of inducible beige adipose precursors within human sWAT [62]. This has initiated interest in developing therapies that could promote beiging of WAT depots for the treatment of obesity.

The two main avenues that give rise to beige adipocytes are (1) through *de novo* adipogenesis from distinct precursors located in WAT and (2) through the conversion, or transdifferentiation, of white adipocytes into beige adipocytes [63]. While the former process is thought to be the predominant method through which beiging occurs, the area is still under investigation. Following the removal of the beiging cue, such as cold stimulation, beige adipocytes will revert to a dormant form wherein they exhibit a white adipocyte-like phenotype that can become reactivated in the future [62].

Beige adipocytes are known to arise from both myf5-negative and myf5-positive precursors. So far, several distinct subtypes of beige adipocyte precursors have been identified. One main beige adipocyte precursor population that has been characterized expresses α smooth muscle actin (αSMA), stem cell antigen (SCA1), target of anti-proliferative antibody-1 (TAPA-1 or CD81), and platelet-derived growth factor receptor α (PDGFRα). Another distinct subtype of beige adipocytes that has been identified originates from myogenic precursors expressing PDGFRα and myogenic differentiation factor (MYOD) and are β-AR signaling-independent. These display unique metabolic functioning where glucose, rather than FFAs, is the primary source of fuel, and they are induced by acetylcholine produced from nearby immune cells [62]. More work is needed to elucidate the different populations of beige adipocyte precursors and their origins to provide a clearer picture of how beige adipocyte recruitment can be targeted therapeutically.

1.3.2 Adipose Plasticity

Adipose plasticity under conditions of high caloric consumption is an important attribute which helps to deter the onset of metabolic disease. This plasticity can arise through tissue expansion, remodeling, and transdifferentiation in order to adapt to metabolic stressors and

changes in the cell environment. Adipose expansion can occur via hyperplasia (increased adipocyte number), which preferentially occurs in sWAT, or hypertrophy (increased adipocyte size), which preferentially occurs in vWAT [58]. The benefits of adipose expandability lie in the removal of excess fatty acids from circulation and limitation of their storage ectopically in the liver by providing a larger storage site. However, when maximal expansion has been reached, adipocytes begin to die and the tissue becomes fibro-inflamed, leading to metabolic dysfunction [58].

Remodeling of the adipocyte microenvironment, namely the vasculature and extracellular matrix (ECM), is another key component of adipose plasticity and its ability to respond to environmental change. Adipogenesis is accompanied by angiogenesis and ECM remodeling to ensure proper delivery of oxygen and nutrients and to allow for morphological changes that occur during differentiation. Adipose angiogenesis is supported by the secretion of pro-angiogenic factors from surrounding cells and the release of sequestered factors from the ECM [58]. Dysfunctional adipose tissue, which is maximally expanded, is characterized by hypoxia that can in turn enhance fibrosis and inflammation [64]. The fibrosis of adipose tissue limits the ability of adipocytes to continue expanding through both physical restriction and the impairment of pre-adipocyte differentiation, further solidifying the dysfunctional state [58].

Lastly, phenotypic and functional changes in adipocytes contribute to plasticity and metabolic flexibility. This includes the transdifferentiation or dedifferentiation of white adipocytes and alterations in mitochondrial dynamics to support metabolic homeostasis. For instance, adipocytes in mammary glands can undergo dedifferentiation to pre-adipocytes during lactation, and intradermal adipocytes can transdifferentiate to myofibroblasts following tissue injury, illustrating the adaptive capabilities of adipocyte differentiation trajectories [65]. Interestingly, the

ECM may play an important role in inhibiting or maintaining the dedifferentiated or transdifferentiated state. In sWAT, beiging, both through recruitment of precursors and transdifferentiation, can occur in response to environmental stimuli such as exercise and cold exposure [66]. These changes occur through regulation of mitochondrial remodeling, both biogenesis and autophagy, to modify energy dissipation and heat production in adipose depots.

1.4 The Extracellular Matrix

Cell microenvironments are composed of an array of proteins that support cell adhesion and maintain specific rigidities essential for sustaining specialized morphologies [67]. These proteins form the extracellular matrix (ECM), a network of connective tissue comprised of fibrous proteins and proteoglycans, divided into the interstitial matrix and the basement membrane (BM) [68]. ECM composition varies by tissue, as unique combinations of ECM proteins can engage with different classes of receptors and promote select differentiation pathways [69,70]. There has been increasing interest in studying the specific roles and intracellular signaling cascades associated with particular ECM proteins as they become increasingly implicated in disease. Most notable in recent years is the important role of the microenvironment in tumor growth and survival. Additionally, excess ECM deposition has been observed in in metabolic diseases like NAFLD.

1.4.1 Extracellular Matrix Proteins

1.4.1.1 Fibrous Proteins

The fibrous proteins class form the largest proportion of the ECM and contain the collagen, elastin, fibronectin, and laminin families. Collagens, in particular, are the most abundant protein family found in the ECM, with 28 different collagen species identified to date. Collagens contain three polypeptide α chains which form a rod-shaped triple helix displaying Gly-X-Y triplet repeats

[71]. Interruptions in these repeats can serve to add flexibility and plasticity to a collagen subunit, as are found in fibril-associated collagens [68]. Collagen can engage with receptors in addition to providing mechanical strength and stability. Elastin, another key ECM protein, plays an important role in tissue elasticity and is a major component of lung tissue, arteries, and skin [72]. Fibronectin and laminin are glycoproteins located in distinct regions of the ECM. Fibronectin is located in the interstitial provisional matrix while laminin proteins are found in the basement membrane. While fibronectin and laminin play important structural roles, they also interact with cell surface receptors and other proteins in the ECM to mediate cell-matrix communication [67].

1.4.1.2 Proteoglycans

Proteoglycans are highly diverse proteins made up of glycosaminoglycans (GAGs) attached to a variety of protein cores [67]. GAGs are unbranched polysaccharide chains that form hydrogels of varying pore sizes and adopt expansive conformations to fill excess space. Several significant glycosaminoglycans include hyaluronic acid, heparin, and heparan sulfate. Secreted proteoglycans play a number of roles in the ECM. They serve a function in force-resistance and act as regulators of trafficked molecules based on size and charge [68]. Proteoglycans can also modulate the activity of secreted growth factors, proteases, and protease inhibitors by binding to these molecules or acting as a reservoir.

In addition to secreted proteoglycans, proteins in this category can be located on the cell surface if the core protein component is an integral membrane protein or contains a glycosylphosphatidylinositol (GPI) anchor. Examples of these include syndecans, which bind fibronectin, and betaglycan, which binds the cytokine transforming growth factor beta (TGF-β) [68]. Typically, cell-surface proteoglycans will act as co-receptors with another receptor type, like integrins, whereby the proteoglycan will present a signaling ligand to the main receptor. Thus, the

proteoglycan family renders a range of critical functions in the cell microenvironment, particularly owing to the vast array of GAGs and protein cores that can combine to form a proteoglycan structure.

1.4.2 Signal Transduction

As well as serving as structural support and connective components, ECM proteins can communicate with cells to relay information about the environment and modulate intracellular signaling. This can occur through interactions with cell surface receptors and a process known as mechano-sensation. The main class of ECM receptors are integrins, composed of an α and β subunit, which can be divided into RGD, collagen, laminin, and leukocyte-specific receptor groups. Other receptor types include the discoidin domain receptors (DDR), CD44, and cell-surface proteoglycans [73]. Integrins mediate intracellular signaling through several key pathways to regulate cell migration, adhesion, and differentiation. These include the phosphatidylinositol 3-kinase (PI3K)/protein kinase B (AKT) pathway, focal adhesion kinase (FAK)/Src kinase pathway, mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) pathway, and Rho-associated protein kinases (ROCK)/ Rho GTPase pathway [74–76].

ECM proteins contain recognition sequences which allow them to bind these distinct receptors. One of the most well recognized integrin binding sequences is the RGD motif found in collagen, fibronectin, vitronectin, fibrinogen, and platelet endothelial cell adhesion molecule (PECAM) [77]. Other collagen species may contain GFOGER-like sequences to bind α1β1 and α2β1 integrins, a GVMGFO motif to bind the discoidin domain receptors, or a GPO motif to bind glycoprotein VI receptor and leukocyte-associated immunoglobulin-like receptor-1 [71]. In laminins, IKVAV is a key motif for interaction with integrins α3β1, α4β1, and α6β1 [78]. Additional sequences are still being uncovered which show binding activity with other receptors

and integrin subunits. Identifying these sequences is key for therapeutic targeting of specific receptors, functional blocking of ECM interaction sites, and in creating functional peptides to promote particular differentiation pathways in tissue engineering applications.

1.4.3 ECM Assembly & Degradation

Following secretion from the cell, ECM proteins are processed to their final form and assembled into higher order structures. Many families of ECM proteins are synthesized and secreted as larger precursors (procollagen, tropoelastin, precursor laminin) which serve to inhibit intracellular assembly into structures [68,72,79]. Proteolytic enzymes will then cleave these in the extracellular space, creating significantly less soluble molecules which are driven to self-assemble into fibrils and networks. Collagen and elastin fibrils can then be covalently crosslinked by lysyl oxidase between lysine residues, imparting tensile strength [68]. Alternatively, fibronectin is not synthesized as a precursor as it requires interactions with the actin cytoskeleton via integrin engagement to assemble [68]. The variety of structures that can be formed allow for the fine-tuning of biochemical and mechanical properties of distinct cellular microenvironments.

The remodeling and turnover of the ECM is a highly regulated and constant process. Cells maintain mechanical homeostasis through consistent degradation and replacement of ECM proteins but can also quickly remodel the ECM in response to environmental changes or injury. Two classes of extracellular proteases are secreted to initiate degradation: (1) metalloproteases, which include both matrix metalloproteinase (MMP) and disintegrin and metalloproteinase with thrombospondin motifs (ADAMTS) families, and (2) serine proteases, which include plasmin and cathepsin G [80]. Some proteases are highly specific while others can cleave multiple types of proteins. Protease activity can be regulated through several actions. Proteases can be secreted as active proteins or inactivate precursors that require cleavage to become functional. Certain

proteases may also bind cell surface receptors or associate with the membrane, limiting the region of proteins they can act upon. Lastly, cells can release protease inhibitors, such as tissue inhibitors of metalloproteases (TIMPs) and serpins, to halt protease activity [68]. Tight transcriptional control of ECM and protease synthesis therefore allows for the creation of compositionally and mechanically distinct microenvironments surrounding different cell types and during the process of differentiation which helps to reinforce differentiation trajectory.

1.5 Laminins

Laminins are large glycoprotein heterotrimer ECM proteins comprised of α-, β-, and γ-polypeptide chains that form an independent network in the BM of most tissue types [81]. Heterotrimers are indicated as laminin-αβγ (laminin-111 or simply LN-111). When discussing singular α-, β-, or γ chains, such as laminin-α1 or laminin-β2, it is abbreviated as LAMA1 and LAMB2, respectively.

First described in 1979, there have now been at least 16 laminin isoforms identified in mammals, composed of combinations of 5 α-chains, 4 β-chains, and 3 γ-chains [82]. Individual chains contain a short N-terminus, coiled-coil region, and C-terminus. The N-terminus of laminin chains is composed of a globular N-terminal (LN) domain and varying numbers of cysteine-rich laminin-type epidermal growth factor-like (LE) domains interspersed between globular laminin-IV (L4/LF) domains [83] (Fig. 1.2). The α-chain C-terminus contains five globular laminin G-like (LG) sub-domains that interact with ECM receptors, leading researchers to believe that this chain confers the observed tissue and developmental stage specific expression patterns [84–86].

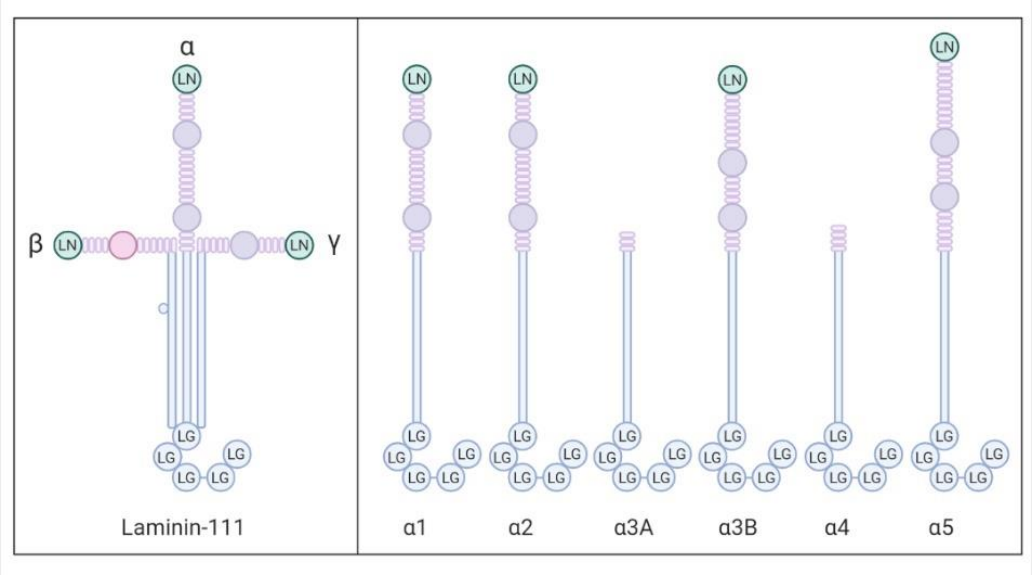

Figure 1.2 Laminin structure

(Left) The laminin-111 (α1, β1, γ1) heterotrimer. Green "LN" circles represent the laminin N-terminal domains. Purple ellipsoids represent the laminin-type epidermal growth factor-like (LE) domains. Purple and red circles represent the globular laminin-IV (L4/LF) domains. Blue "LG" circles represent the globular laminin G-like (LG) sub-domains at the C-terminal that interact with cell surface receptors. (Right) The laminin α-chains. α3A and α3B and are alternative splice variants of the laminin-α3 gene. Structural information for the creation of the figure derived from *The Laminin Family* [83].

1.5.1 Laminin Receptors

Laminins interact with cells through integrin and non-integrin receptors. The integrin receptor isoforms -α1β1, -α2β1, -α3β1, -α6β1, -α6β4, and -α7β1 are known to interact with the laminin-α chain in the C-terminal region LG1-3 domains [74]. Other receptors include non-integrin receptors such as syndecans, Lutheran/basal cell adhesion molecule, melanoma cell adhesion molecule, the 67 kDa laminin receptor, and dystroglycan, most of which bind to the LG4-5 domains of laminin-α chains [74,75]. Through receptor engagement laminins regulate a number

of canonical integrin intracellular signaling pathways. These include PI3K/AKT, FAK/Src, MAPK/ERK, and Rho GTPases [74–76].

1.5.2 Laminin Deposition

Laminins can be locally deposited or secreted by nearby cells [87–89]. In this manner, the environment surrounding a cell impacts function and may alter differentiation trajectory. In addition to the origin of the laminin subunits, the method of assembly can influence function. When deposited into the extracellular space, laminins self-assemble into a variety of network types. Various isoforms have been observed to form mesh-like networks, fibrils, linear arrays, or even plaques [90]. These diverse structures can differentially influence signaling through changes in receptor engagement or alterations in mechano-sensing cues [90–92]. The tissue-specific expression of laminin isoforms, as outlined in figure 1.3 (Fig. 1.3), and the variety of patterned networks they establish once secreted demonstrate a uniquely modular system that forms a foundation to instruct cellular responses by environmental signals. It is therefore important to understand the isoforms expressed in healthy metabolic tissues, the signaling pathways they influence, and any changes in expression that occur during metabolic disease.

1.5.3 Tissue Specific Expression Patterns of Laminins

1.5.3.1 Pancreatic Islets

In the unique formation process of the pancreatic islet microenvironment, islets secrete high levels of vascular endothelial growth factor A (VEGF-A) to attract vascular endothelial growth factor receptor 2 (VEGFR2) expressing endothelial cells that deposit BM proteins around β-cells [89]. Human islets possess a double BM between the endothelial cells and β-cells, each with a distinct composition of proteins that sustain the specialized function of the cell type in

contact [93]. Of the BM proteins that change throughout organogenesis and differentiation, laminin isoforms have been shown to portray distinct developmental and spatial expression patterns [94].

In the pancreas laminins are localized to exocrine cells, the vasculature, and the capsule surrounding the islets, however expression patterns differ between mice and humans. The heterotrimer laminin-α1,β1,γ1 (LN-111) is detected near fetal pancreatic islets, however is absent in adult islets and expressed at low levels in adult pancreatic endothelial cells, suggesting that its main role is supporting organogenesis [95]. LN-211 is located near murine pancreatic exocrine cells [96] and murine peri-islet BM [97]. LN-332 is found in both human and mouse BM near α-cells [98]. LN-411 and 421 are located in the vasculature of mouse and human islets and near exocrine cells [96,99]. Korpos et al. also identified the laminin-α4 chain (LAMA4) in murine peri-islet BM. The predominant isoform in the adult human peri-islet BM is LN-511, which suggests that the LAMA5 chain may support a specialized endocrine function [99,100].

1.5.3.2 Liver

The adult liver contains lobules formed by bile ducts and branches of the hepatic artery and portal vein surrounding the central vein. Liver capillaries, known as sinusoids, are lined with fenestrated endothelial cells that separate it from the Space of Disse [101]. Located in this space are stellate cells which, typically quiescent in healthy liver tissue, secrete ECM proteins when liver injury occurs [102]. The distinct regions of the liver support the differentiation of specialized cell types which is reflected by laminin α-chain patterns. LAMA1 is predominantly expressed in the developing liver and is not found in healthy adult liver [103]. The hepatic artery contains LAMA2, LAMA3, LAMA4, and LAMA5, while the portal vein expresses LAMA2, LAMA4, and LAMA5, and the central vein expresses LAMA4 and LAMA5. BECs express LAMA2, LAMA3, LAMA5,

and at low levels LAMA4 [103,104]. The Space of Disse contains LAMA1, produced by activated stellate cells, and, weakly, LAMA4 [103].

1.5.3.3 Skeletal Muscle

Skeletal muscle plays an integral role in metabolism and glucose homeostasis, acting as the main site of insulin-mediated glucose disposal. During primary myogenesis, cells from the dermomyotome delaminate to form the myotome. Muscle progenitor cells then become muscle stem cells that give rise to skeletal muscle [105]. Throughout these stages of tissue development, distinct patterns of laminin expression are observed. LN-111 and LN-511 are present in the BM of the dermomyotome and myotome of mice, however LN-111 is expressed by sclerotome cells and LN-511 by epithelial cells [106,107]. As myogenic precursors begin to differentiate during embryogenesis, LAMA1 chain expression is downregulated and LAMA2 chain expression is upregulated [108,109]. Throughout initial skeletal muscle development, LAMA2, LAMA4, and LAMA5 are expressed [110]. In adult skeletal muscle only LAMA2 remains to be expressed, while LAMA4 is located only in capillaries [111]. LN-211 is the predominant laminin isoform found in the BM of adult skeletal muscle [112].

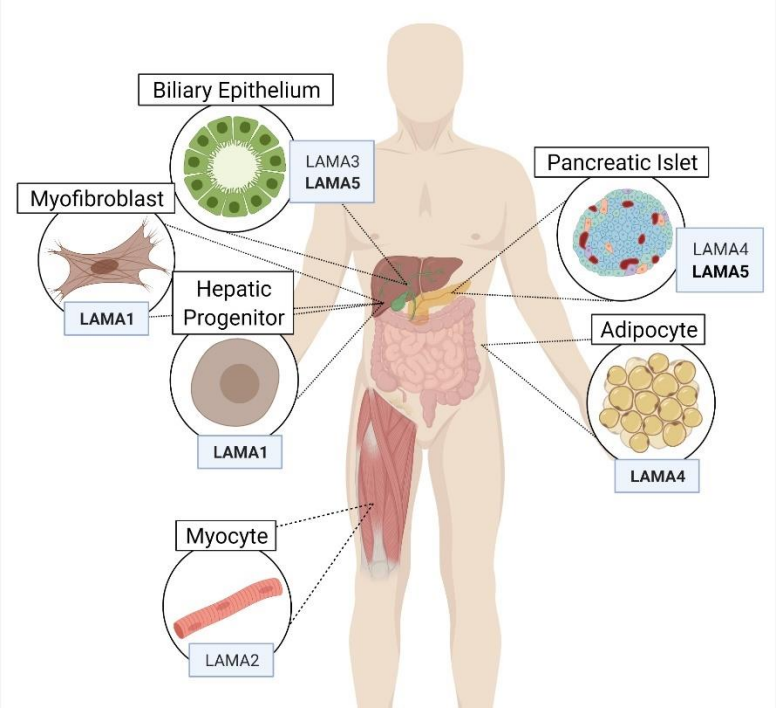

Figure 1.3 Laminin-α chain expression patterns in metabolic tissues

Pancreatic Islets: The basement membrane surrounding islets is deposited by pancreatic endothelial cells and contains LAMA1 during organogenesis, then largely LAMA5 and some LAMA4 in adults. **Liver**: Biliary epithelial cells (cholangiocytes) predominantly express LAMA3 and LAMA5. Myofibroblasts (activated stellate cells) secrete high levels of LAMA1 into the Space of Disse. Hepatic progenitor cells primarily express LAMA1. **Skeletal Muscle:** The main laminin-α chain expressed in myocytes is LAMA2, which promotes myocyte survival. **Adipose:** During white adipogenesis there is a dramatic increase in LAMA4 expression.

1.5.3.4 Adipose Tissue

During adipogenesis the ECM is remodeled and laminin isoform expression shifts. There is a decrease in expression of LAMA1 and LAMC2, a modest increase in LAMA2, LAMA3, LAMA5, LAMB2, and LAMC1, and a stark increase in LAMA4 and LAMB1 [113]. Niimi et al. report an upregulation of LN-411 in 3T3-L1 cells by 2.5 fold during differentiation, while Noro et al. report a 3.9 fold increase in LAMA4 expression, a 5.8 fold increase in LAMB1 expression, and

a 2.8 fold increase in LAMC1 expression after 28 days of differentiating human bone marrow derived MSC [113,114].

1.5.4 Roles and Functions of Laminins

1.5.4.1 Proliferation & Differentiation

Laminins play an important role in promoting proliferation and regulating differentiation in many metabolic tissue types. In the liver, laminin is essential for the proliferation and stemness of oval cells (in mice) and hepatic progenitor cells (in humans) located in the biliary epithelium. LAMA1 is required for the commitment of progenitor cells to BECs [115]. Shortly thereafter expression switches to LAMA5 which supports formation of mature bile duct structures [115]. In humans, laminin is also essential in the regulation and differentiation of hepatic progenitors. Lorenzini et al. found that hepatic progenitor cells grown on laminin display increased expression for progenitor and BEC markers and decreased expression for early hepatocyte markers [116]. Furthermore it has been shown that progenitor cells exposed to notch signaling will form BECs and maintain the expression of laminin, while cells that exit the laminin environment will differentiate to hepatocytes [117]. Moreover, inhibition of laminin signaling results in the early differentiation of progenitors to hepatocytes, suggesting laminins play an important role in determining cell fate [117].

Laminin also plays a key role in guiding muscle precursor differentiation. The peptide IKVAV (Ile-Lys-Val-ala-Val) corresponding to the active site in the LAMA1 chain is sufficient to support the process of myogenesis in murine myogenic precursor cells. The functionalization of hydrogel scaffolds with this peptide promotes cell proliferation, migration, and the expression of myogenic markers [118]. Studies of laminin surrounding pericytes located in muscle tissue, which contain myogenic and adipogenic potential, report a crucial functional role in determining cell fate.

While laminin inhibits the differentiation of type-1 pericytes to adipocytes, it promotes type-2 pericyte myogenic differentiation [119].

1.5.4.2 Immune Infiltration

Laminins also appear to play a role in immune infiltration and inflammation. Indeed, studies show that LN-411 and LN-511 mediate infiltration in non-metabolic tissues and could factor into the invasion of immune cells in T1D [120]. Korpos et al. found that laminin staining is reduced in the peri-islet BM of non-obese diabetic (NOD) mice compared to controls and that the sites of greatest loss co-localize with leukocyte invasion indicating the important barrier function of laminins [97]. Once inflammation subsides the BM is seen to reappear around α-cells in both NOD mouse and T1D human samples [97]. This may occur in response to inflammatory cytokine release, as interleukin 1β (IL-1β) can stimulate LN-332 expression in rat islets via the PI3K pathway [98]. Additionally, islets encapsulated in alginate containing laminin sequences (RGD, LRE, or PDSGR) show improved survival during exposure to inflammatory cytokines [121].

1.5.4.3 Metabolic Processes and Insulin Signaling

In pancreatic islets, laminins are associated with promoting β-cell insulin secretion via β1-integrin engagement. Nikolova et al. show that LN-111, LN-411, or LN-511 upregulate insulin expression (Ins1 and Ins2) in MIN6 cells and that β1-integrin blocking abrogates this effect [89]. More recently, Hadavi et al. demonstrate that LN-332 stimulates greater insulin secretion in human islets compared to LN-111 [122]. The mechanism of this phenomenon may be due to polarization created by laminins. A study by Gan et al. determined that LN-511 triggers clustering of β1-integrins and enrichment of scaffold structures toward the vasculature, which directs the spatial targeting of insulin granule fusion [123]. Islets from subjects with T2D portray similar

polarization, however this investigation was qualitative and did not study changes in β1-integrins or insulin granule fusion, so the relationship between laminin and insulin secretion coordination in T2D remains unclear [124]. Nevertheless, T2D risk has been genetically associated with laminin. A stretch enhancer at LAMA1, which selectively regulates LAMA1 expression in pancreatic progenitors, has been significantly associated with genetic pre-disposition to T2D in lean individuals, however further studies are required to understand the mechanism behind this association [125,126].

Laminin isoforms may also play a role in insulin signaling in skeletal muscle. The LAMA2 chain has been shown to interact with α-dystroglycan to activate the intracellular PI3K/AKT pathway, while interactions with integrins lead to FAK activation [127]. Disruption of LAMA2 and α-dystroglycan interactions result in reduced AKT and glycogen synthase kinase β (GSK3B) phosphorylation [128]. Furthermore, recent work has discovered an association between LAMA1, the dystrophin glycoprotein complex (DGC), γ-catenin, and the insulin receptor which promotes insulin signaling in murine muscle tissue [129]. Dissociation of this complex can lead to insulin resistance and muscle atrophy through down regulation of the PI3K/AKT pathway. In addition to insulin signaling, laminin may influence other metabolic processes in skeletal muscle. Studies of LAMA2 -/- mouse skeletal muscle tissue report altered proteomic profiles in proteins involved in metabolic processes such as glycolysis, fatty acid oxidation, and oxidative phosphorylation [130].

Lastly, while laminins have not been studied in depth in the context of human obesity, research suggests a role for specific laminin chains in adipose tissue function. Our group has shown that LAMA4-/- mice are protected from diet-induced obesity and liver steatosis and exhibit improved insulin sensitivity on high fat diet compared to WT mice [131,132]. This arises as a result of enhanced UCP1 expression in the subcutaneous WAT (sWAT) of LAMA4 -/- mice

leading to an overall enhancement in energy expenditure [132] (Fig. 1.4). These changes may occur through alterations in integrin-α7β1, which is downregulated in adipocytes in the absence of LAMA4. Silencing of the integrin-α7 chain leads to enhanced UCP1 expression in adipocytes [133]. Another study found that homozygosity for the LAMA5 gene SNP rs659822 C allele was associated with lower mean body weight, lower average total fat mass, and higher mean HDL cholesterol in European American women but higher average body weight in African American women [134]. More research is needed to elucidate how LAMA5 polymorphisms are associated with body weight and metabolic processes and what other factors may be at play.

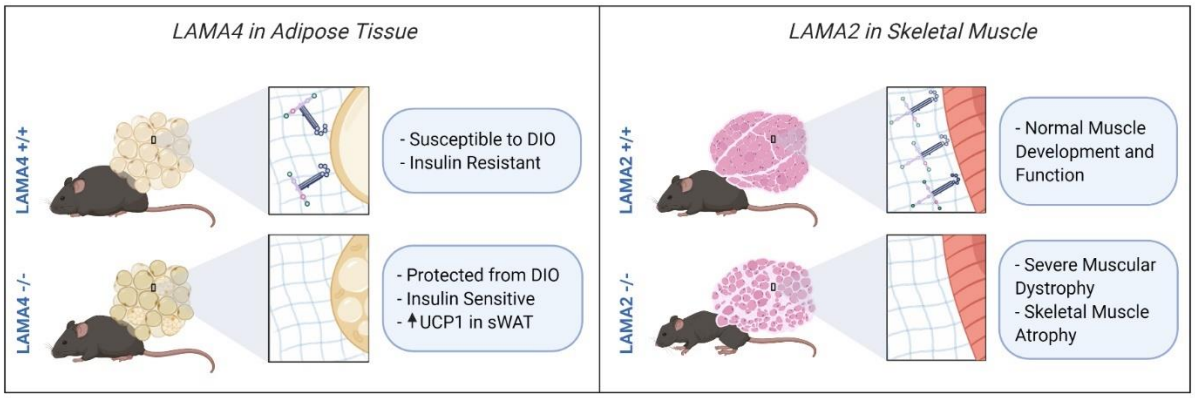

Figure 1.4 Mouse models of laminin-α chain deficiency

(**A**) Laminin-α4 (LAMA4) knockout in mice leads to phenotypic changes in adipose tissue. LAMA4 -/- mice are resistant to diet-induced obesity (DIO) and remain insulin sensitive due to enhanced energy expenditure driven by increased uncoupling protein 1 (UCP1) expression in the subcutaneous white adipose tissue (sWAT) as compared to WT mice. (**B**) Laminin-α2 (LAMA2) knockout mice display a reduction in skeletal muscle mass compared to WT mice, leading to severe muscular dystrophy.

1.5.5 Biomedical Applications of Laminins

Investigation of laminins has extended into clinical contexts, where these ECM components and their genetic underpinnings have been explored for the purposes of prognostication and therapeutic intervention. For instance, laminins have been identified as

markers of tumor invasiveness in hepatocellular carcinoma and extrahepatic cholangiocarcinoma [135,136]. With regard to therapeutics, repletion of laminins via intraperitoneal or retroorbital injection has been studied for inherited laminin deficiencies such as merosin-deficient congenital muscular dystrophy [137] and Pierson syndrome [138]. In the realm of metabolic disease, medical applications of laminins to date have primarily included genomic analysis of laminin-encoding sequences to assess for disease risk, modulation of laminin quantity for therapeutic purposes, and use of laminins to improve the efficacy of tissue engineering techniques (Table 1.1).

1.5.5.1 Genetic Risk

Sequencing of laminin-encoding regions of the genome has been performed in order to identify genetic risk variants. Via whole-exome sequencing of morbidly obese patients, Jiao et al. pinpointed a variant in the LAMB3 gene entitled rs2076349 (V527M) that is strongly associated with morbid obesity, conferring an odds ratio (OR) of 1.84 [139]. Genome-wide studies of patients with T2D completed by Perry et al. showed that a variant of LAMA1, rs8090011, was associated with T2D in lean patients (OR = 1.13) but not in obese patients (OR = 1.03) [126]. Taken together, these studies indicate that analysis of laminin-encoding sequences may assist in understanding genetic susceptibility for metabolic disease.

1.5.5.2 Concentration Modulation

Due to laminins' role in adipose tissue structure and support, it has been hypothesized that modulation of laminin quantity might be used to mitigate some complications of metabolic disease. One method investigated for this purpose is laminin knockdown with small interfering RNA (siRNA). Jiao et al. found that siRNA against LAMB3 inhibited human adipocyte differentiation *in vitro*, providing a promising target for obesity therapy [139]. Another group explored

supplementing cell cultures with LN-411 to induce differentiation of MSC into IPCs. These IPCs improved insulin secretion, fasting blood glucose, HbA1c, survival, and symptoms (polyuria/polydipsia) when transfused into T1D mice, suggesting potential therapeutic effect of laminin-induced IPCs in the setting of T1D [140].

1.5.5.3 Tissue Engineering

Laminins have also been studied within the burgeoning field of tissue engineering and regenerative medicine. The sequelae of both T1D and T2D have been identified as targets for laminin-based technologies. Patients with type T1D may require islet cell transplantation for long-term glycemic control, but transplantation of donor pancreas is complicated by limited supply of donor tissue and potential for rejection [141]. Consequently, attention has been directed toward creation of pancreatic neo-islet sheets *in vitro* for purposes of autologous transplant. Yamashita et al. described that growing rat islet cells on a graft culture framework with LN-332 coating greatly enhanced growth [142]. Advancing these findings, Sigmundsson et al. reported success in culturing mouse and human islets on α5-containing laminins, and subsequent transplantation of the islets into diabetic mice normalized blood glucose within four days [143]. Other studies propose scaffolds coated with laminins to enhance islet insulin secretion [122] and laminin-containing hydrogels to optimize delivery of neo-islets [144]. Another diabetes-related application is incorporation of laminins containing specific peptide sequences into fibrin networks to promote wound healing in a mouse model of T2D. Ishihara et al. discovered that when laminin heparin-binding domains (HBDs) were incorporated into fibrin matrices, growth factors were retained for longer time periods with increased adhesion of fibroblasts and endothelial cells. This suggests that laminin HBDs can facilitate wound healing via both potentiation of growth factor effects and adhesion of angiogenic cells [145].

Laminins have also been investigated for their utility in liver disease. They have been shown to improve human hepatocyte differentiation and culture with the goal of autologous transplant; in the context of metabolic disease, this could be useful for patients suffering from end-stage liver disease secondary to NAFLD. Human recombinant laminin isoforms have been employed to generate xeno-free primary human hepatocytes [146], and laminin-containing gelatin cryogel scaffolds have been shown to promote differentiation of stem cells into hepatocytes [147]. In addition to methods aimed toward generating new hepatocytes, there has also been exploration into prevention of fibrosis. One study tested the functionalization of a biospun membrane with a laminin β1-fragment and found that this membrane inhibited expression of matrix metalloproteinase 2, which degrades the basement membrane and contributes to fibrosis. This functionalized membrane curtailed fibrosis in a mouse model of peritoneal fibrosis, but the findings have relevance for application to liver fibrosis as well [148]. Though the regenerative medicine efforts described here are still in their infancy, laminins possess potential within the field of tissue engineering for treatment of metabolism-associated disease.

As our understanding of laminins evolves, we will become better equipped to develop treatments for metabolic disease. One promising endeavor is development of pancreatic neo-islets and hepatocyte culture for transplantation. Genetic approaches are being investigated for risk stratification and modulation of laminin expression. Our group in particular has explored the targeting of LAMA4 for treatment of obesity. Mice deficient in this laminin are resistant to developing obesity on high-fat diet; therefore, LAMA4 modulation may serve as a potential mechanism of action for future therapeutics [132]. Clearly, there is immense necessity for further elucidation of mechanisms underlying metabolic disease, especially the role of the laminins, with

the goal of developing efficacious therapeutics for metabolic conditions and their sequelae that affect millions worldwide.

Table 1.1 Biomedical applications of laminins

Applications	Technique	Laminin Type	Metabolic Disease
Regenerative Medicine	Culture of murine and human pancreatic neo-islets on laminin-coated scaffolds	Laminin-332, Laminin-α5	T1D
	Enhancement of human pancreatic islet insulin secretion with laminin-coated scaffolds	Laminin-111	T1D
	Laminin-containing hydrogels as delivery vehicle for porcine islets and human circulating angiogenic cells for autologous transplant	Not specified	T1D
	Xeno-free culture of primary human hepatocytes on recombinant laminins	Laminin-111, -211, -221, -332, -411, -421, -511, and -521	NAFLD
	Use of laminin-containing cryogel scaffolds to promote differentiation of human mesenchymal stem cells into hepatocyte-like cells	Not specified	NAFLD
	Functionalizing membranes with laminin for inhibition of peritoneal fibrosis in a murine model	Laminin-β1	NAFLD
	Incorporation of laminin heparin-binding domains into fibrin matrices to facilitate wound healing	Laminin-α3, -α4, and -α5	T2D

Table 1.1 continued:

Genetic Risk Stratification	Exome sequencing of laminin gene to detect variant associated with morbid obesity	Laminin-β3	Obesity
	Sequencing of laminin gene to detect variant associated with development of Type 2 Diabetes in lean patients	Laminin-α1	T2D
Therapeutics	Laminin knockdown with siRNA for inhibition of adipogenesis	Laminin-β3	Obesity
	Culture supplementation with laminin to promote differentiation of mesenchymal stem cells to insulin-producing cells	Laminin-411	T1D

CHAPTER 2: ASSOCIATION BETWEEN LAMININ-α4 EXPRESSION AND OBESITY IN HUMAN AND MURINE MODELS

This article was published in Frontiers in Endocrinology, Vol 12, Goddi et al., Laminin-α4 Is Upregulated in Both Human and Murine Models of Obesity, 698621, CC-BY 4.0 Copyright License (2021). Minor adaptations have been made to the original article for this dissertation.

2.1 Introduction

Obesity is an epidemic affecting 13% of people worldwide and contributing to more than $2 trillion in global economic impact [149,150]. Moreover, obesity can lead to the development of serious health conditions including cancer, hypertension, heart disease, and type 2 diabetes [151–153]. A clear necessity arises for the development of adipose targeted therapies to ameliorate metabolic dysfunction and reduce overall adiposity. In recent years, much has been learned about adipocyte biology, in particular the intracellular signaling pathways and transcription factors that mediate its function. However, less attention has been paid to the adipose microenvironment, with the exception of significant focus on macrophage infiltration that occurs in the setting of obesity. Recent work suggests that the extracellular matrix (ECM) itself considerably affects adipocyte biology.

The ECM is a network of macromolecules that contributes to cell support, migration, and signaling [67]. Three major classes of ECM biomolecules have been identified including structural proteins (collagen, elastin), specialized proteins (laminin, fibronectin), and proteoglycans [154]. Of these proteins, most emphasis in the field of adipocyte biology and obesity has been placed on general fibrosis and collagen which accounts for the largest proportion of the stromal ECM. Type 4 and type 6 collagen are essential components of the adipocyte microenvironment but have also

been implicated in obesity and related inflammatory phenotypes. In humans, collagen type IV alpha 1 (COL4A1) expression is significantly elevated in adipocytes from obese sWAT and furthermore, this expression diminishes 6 months after bariatric surgery [155]. Collagen type VI alpha 3 (COL6A3) is enriched around adipocytes in mice, however there is conflicting evidence as to its association with obesity in humans. Khan et al. 2009 report that COL6A3 expression is elevated during states of metabolic stress and adipose dysregulation in mice and humans [156]. Conversely, McCulloch et al. 2015 observed reduced COL6A3 expression in adipose of humans with obesity and suggest that COL6A3 is not the predominant collagen in human adipose tissue, indicating that such ECM proteins may serve different roles in murine and human adipose [157]. It is also evident that the composition of the ECM can be detrimental to adipocyte function, yet very few studies have looked in depth at the relationship between non-collagen basement membrane proteins and obesity, especially in human adipose tissue.

In particular, laminins are heterotrimeric basement membrane proteins each composed of an α, β, and γ chain. There are currently sixteen isoforms identified and which are distributed in a tissue-specific manner [83]. The laminin-α chains, which determine tissue specific expression patterns, contain differing numbers of laminin G-like (LG) domains in the C-terminus allowing for interaction with different types of receptors, such as integrins, syndecans, and dystroglycans [158]. The LAMA4 chain differs from other laminin-α chains in that it contains no laminin N-terminal (LN) globular domain or laminin IV (LF) domain, and merely four laminin-type epidermal growth (EGF) factor-like (LE) domains [83]. Laminin-α3A, a splice variant of laminin-α3, is the only other laminin-α chain with a similarly truncated N-terminus. This N-terminus short arm of laminin-α chains has been previously shown to play a role in basement membrane assembly and organization through interactions with other ECM components [83].

The LAMA4 chain is known to be highly upregulated during adipogenesis and has recently been suggested to engage integrin α7 on the adipocyte surface [113,114,133]. As with other laminin chains, LAMA4 is implicated in ECM remodeling in several tissue types including muscle and various tumor types, however it is only just beginning to be associated with adipose tissue remodeling and dysfunction in obesity [159,160]. Moest et al. 2013 show LAMA4 deposition at the adipocyte surface is increased in both diet-induced-obesity (DIO) and genetic ob/ob mouse models compared to controls [161]. Our group has demonstrated that mice deficient in *Lama4* are protected from DIO and exhibit improved insulin sensitivity [131]. In non-diabetic humans with obesity, LAMA4 levels were found to be increased in the secretome of visceral WAT (vWAT) adipocytes compared to sWAT, however the opposite trend has been reported in mice [161,162].

While these results suggest that LAMA4 may be an important regulator of adipocyte function, its clinical relevance to various models of obesity remains poorly characterized. In this study we aim to describe the relationship between LAMA4 and obesity in both mouse and human models and determine the effect of weight loss on LAMA4 expression. We analyzed laminin and collagen expression in the sWAT of mice following high fat diet (HFD) feeding and found that the expression of *Lama2*, *Lama4*, and several collagen subunits were significantly upregulated in the HFD fed mice. Of the laminin-α chains, only *Lama4* expression strongly correlated with weight. We then investigated laminin expression in humans using sWAT samples from female control subjects (BMI<30) and female subjects with obesity (BMI>35) undergoing bariatric surgery (baseline vs. 3 months post-surgery) and discovered that the expression of LAMA4 was significantly upregulated in the obese sWAT at both the mRNA and protein level. Interestingly, short-term weight-loss in both humans and mice did not result in a significant change in LAMA4

expression. We highlight an important relationship between LAMA4 and obesity in both mice and humans, suggesting that laminins play a critical role in obesity development in human subjects.

2.2 Materials and Methods

Animal Care and Diet Studies:

Animal procedures and numbers were approved by the University of Chicago Institutional Animal Care and Use Committee. WT male and female C57BL/6J mice were fed either regular chow diet (RCD, Teklad 2918; Harlan Laboratories) or 45% high fat diet (HFD, Teklad custom diet TD.06415; Harlan Laboratories) ad libitum and housed at room temperature. Beginning at 7 weeks of age, mice were weighed weekly until completion of the study. Two separate dietary studies were performed: RCD vs HFD for 8 weeks and HFD vs "weight loss" group where mice were fed HFD for 8 weeks and then switched to RCD for another 6 weeks (14 weeks total of dietary study). Mice were humanely killed at 16 or 22 weeks of age, respectively. Tissues were collected and snap frozen in liquid nitrogen and stored at -80°C.

Human Samples and Clinical Parameters:

10 non-diabetic female subjects with obesity undergoing laparoscopic sleeve gastrectomy (BMI>35) and 3 female control subjects (BMI<30) between the ages of 20 and 55 were voluntarily enrolled by the Center for the Surgical Treatment of Obesity at the University of Chicago. Eligibility requirements restricted subjects with diabetes or on medications for diabetes or that would alter glucose metabolism from participating in the study. Subcutaneous fat samples were collected via a needle biopsy 2 weeks prior to and 12-13 weeks post bariatric surgery according to a protocol modified from that of Carswell et al. [163,164].

RNA Extraction:

Tissue was homogenized using a Bullet Blender® at 4°F (Next Advance PINKE1, BBX24B) or a dispersion-based homogenizer (VWR VDI 12) for human and mouse samples, respectively. RNA was isolated using the E.Z.N.A Total RNA Kit II (Omega Biotek; R6934) following the manufacturer's instructions.

Quantitative Real-Time PCR:

The RNA samples were reverse transcribed using Quanta QScript Master Mix (VWR; 95048) and 500 ng RNA per 20μL sample reaction volume. Quantitative Real-Time PCR was performed with SYBR green using a Bio-Rad CFX Connect Real-Time PCR Detection System. Primers were purchased from IDT or Qiagen and can be found in Supplemental Table 1. For murine samples, Gapdh was used as a reference gene. For human samples the composite of GAPDH, YWHAZ, and RPL13A was used to control for total mRNA as previously described (22). Gene expression was evaluated by ddCT methods.

Immunoblotting

Samples were lysed using cold 1X RIPA buffer (EMD Millipore; 20-188) containing 1X phosphatase and protease inhibitor cocktail tablets (Roche) and a dispersion-based homogenizer (VWR VDI 12). After incubating on ice for 30 minutes, samples were briefly sonicated (Sonics Vibra-cell) and spun at 1,000xg for 10 minutes at 4°C to separate the lipid layer. The supernatant was collected and spun again at 12,000xg for 10 minutes at 4°C and supernatant from this spin was collected and stored at -80°C. Protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific; 23227). Samples were diluted in water and 4X Laemmli Sample Buffer (Bio-Rad; 1610747) and run on 4-15% SDS-PAGE Mini-PROTEAN

TGX gels (Bio-Rad) then transferred to Immobilon-P PVDF membranes (EMD Millipore). Membranes were blocked with 5% PhosphoBLOCKER™ Blocking Reagent (Cell Biolabs, Inc; AKR-103) in TBST for LAMA4 probing, or 5% Non-fat dry milk (LabScientific; M0841) in TBST for other antibodies for 1 hr. Blots were incubated overnight at 4°C in 1% blocking solution with LAMA4 antibody (Invitrogen; mouse mAb, MA5-24650) or β-Actin (CST; Rabbit mAb, 4970). Membranes were then incubated with IRDye secondary antibodies (LI-COR) for 1 hr. Immunodetection was performed using near-infrared Odyssey CLx System (LI-COR). Analysis was performed issuing the Image Studio Software (LI-COR).

Immunofluorescence:

Tissue was fixed in 10% formalin, paraffin embedded, and sectioned at 5μm thickness by a microtome at the University of Chicago Human Tissue Resources Center. Sections were baked for 60 minutes at 60°C, deparaffinized, and rehydrated in xylenes and alcohol. Heat induced epitope retrieval was performed with citric acid-EDTA buffer pH 6.2 as recommended by the manufacturer of the LAMA4 antibody. Sections were blocked in 10% donkey serum (Abcam ab7475) and incubated with primary antibody (anti-laminin alpha 4, Novus NBP2-42393, 1:300 dilution) overnight at 4°C. An Alexa Fluor-488 conjugated secondary antibody was added for 1 hour at room temperature (Abcam ab150113). Following incubation, sections were washed, stained with DAPI (Invitrogen S36939), and sealed. Images were taken using Fixed-DSU Confocal at the University of Chicago Integrated Light Microscopy Core and quantification of fluorescent signal was determined using ImageJ. All images were processed equally and in an unbiased manner to remove intracellular LAMA4 signal and background noise before signal quantification and analysis. Macro code for ImageJ analysis can be found in the Supplementary Materials file.

Adipose-Derived Stem Cell Isolation and Differentiation:

Primary adipose-derived stem cells were isolated from 14-17 week old WT male mice as previously described [132]. Cells were expanded and plated into 6-well plates for experiments, and subsequently differentiated to white adipocytes following a previously published protocol [165]. Induction medium containing complete medium supplemented with 17nM insulin, 60uM indomethacin, 0.1uM dexamethasone, and 250uM isobutylmethylxanthine was added to cells to initiate differentiation. On day 2 cells were given maintenance medium containing complete medium with 17nM insulin. Following this refeeding occurred every other day with complete medium (10% fetal bovine serum, 1% pen/strep, DMEM/F-12).

LN411 Coating:

6-well plates were coated with 0ug or 10ug of recombinant laminin-411 protein (BioLamina LN411) in DPBS per well according to manufacturer instructions. Plates were incubated at 37°C for 2 hours and the solution was aspirated before cell seeding. For all experiments involving LN411 coating, primary adipose derived stem cells were differentiated to white adipocytes as described. Adipocytes reached maturity by day 8 and were collected for RNA.

Fatty Acid Treatment:

On day 6 and 8 of differentiation, adipocytes were treated with 1.5mM BSA (Sigma A8806) in DPBS as the vehicle, 0.25mM Oleic Acid (Sigma O3008), or 0.25mM Palmitic Acid (Caymen Chemical; 29558). Cells were collected on day 10 of differentiation. The majority of studies utilized oleic acid to stimulate lipid loading as palmitic acid did not lead to enhanced lipid loading or increases of adipogenic gene expression.

Lipolysis:

Basal lipolysis of cells cultured on LN411 was quantified using the Abcam lipolysis assay kit (ab185433) with volumes adjusted to fit a 24-well plate format. Cells were washed in lipolysis wash buffer and then incubated with lipolysis assay buffer for 2.5 hours. Glycerol concentration in the assay buffer was determined by absorbance readings at 570 nm after incubation with the kit Reaction Mix for 30 minutes. Glycerol was then normalized to cellular protein content using the Pierce BCA Protein Assay Kit (Thermo Fisher Scientific; 23227).

Statistics:

Statistical tests for the different studies were computed as follows: for mouse studies where number of mice was essentially equal between groups and variance was expected to be similar, a student's t-test was performed for all data. In all human sample RNA analyses, statistical comparisons between control (n=3) and obese pre-surgery (n=10) were assessed with a Welch's unequal variances t-test, while comparisons between obese pre-surgery (n=9) and post-surgery (n=9) were assessed with a paired sample t-test, as RNA was obtained for both groups from the same set of subjects. In experiments involving LAMA4 staining of fixed human sWAT tissues where both pre (n=9) and post-surgery (n=6) samples could not be obtained from all subjects, a student's t-test was performed. In all cases, $p<0.05$ was considered significant.

2.3 Results

2.3.1 Laminin and collagen expression in a mouse model of obesity

To study the relationship between *Lama4* and obesity, we placed 8-week-old WT male mice on regular chow diet (RCD) or 45% high fat diet (HFD) for 8 weeks. Preliminary dietary studies showed that female mice placed on HFD did not gain significant weight during the dietary study timeline and therefore were not a good model to study the association of *Lama4* and obesity

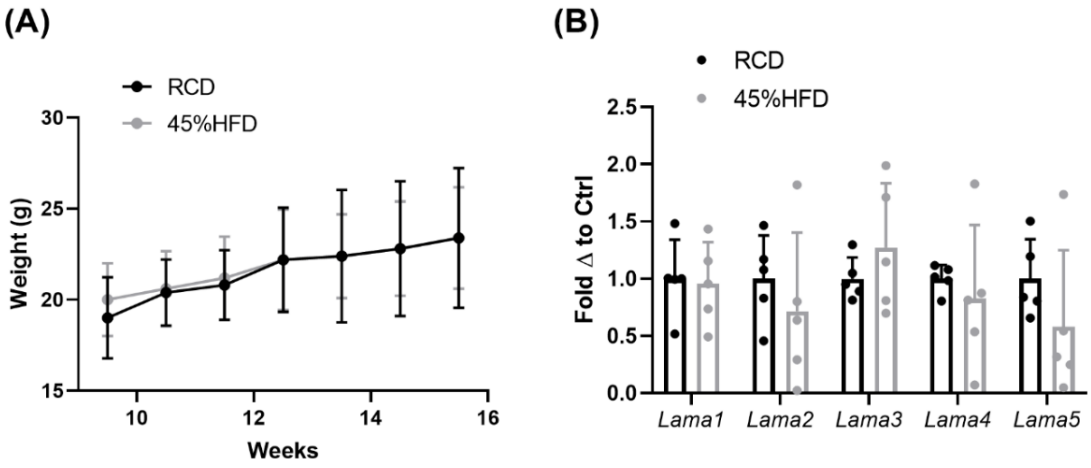

Figure 2.1 Laminin chain expression dietary study in adipose of female mice

(A) Weights of female mice placed on 45% HFD or chow (RCD) diet for 8 weeks. HFD (n=5), RCD (n=5). **(B)** Laminin alpha chain mRNA expression in sWAT from female mice on 45% HFD or RCD for 8 weeks. HFD (n=5), RCD (n=5).

(Fig. 2.1). The male mice on HFD gained considerably more weight than the RCD group (Fig. 2.2A). At 16 weeks of age we assessed the mRNA expression of laminin-α chains in adipose depots by quantitative real-time PCR (qrtPCR). Only subcutaneous WAT showed significant differences in laminin-α chain expression, while epididymal WAT and brown adipose tissue showed no significant differences, which informed our decision to focus on sWAT for the duration of the murine study (Fig. 2.3).

The expression of both *Lama2* and *Lama4* was significantly higher in sWAT of the HFD group compared to the RCD group, by 5.9 and 4.0-fold respectively (Fig. 2.2B). *Lama2*, while predominantly expressed in muscle tissue and implicated in diseases of muscular dystrophy has also recently been shown to inhibit osteogenesis and promote adipogenesis of mesenchymal stem cells (MSCs) via the hedgehog signaling pathway [166]. When relative *Lama2* expression was

compared to individual mouse weights at 16 weeks we found only a weak correlation ($R^2 = 0.38$) suggesting that *Lama2* expression may not directly tie to adipose tissue mass in mice (Fig. 2.2C). We did however identify a strong positive correlation between relative *Lama4* expression and weight ($R^2 = 0.70$) and between relative *Lama4* expression and adipocyte area ($R^2 = 0.77$) (Fig. 2.2D-F).

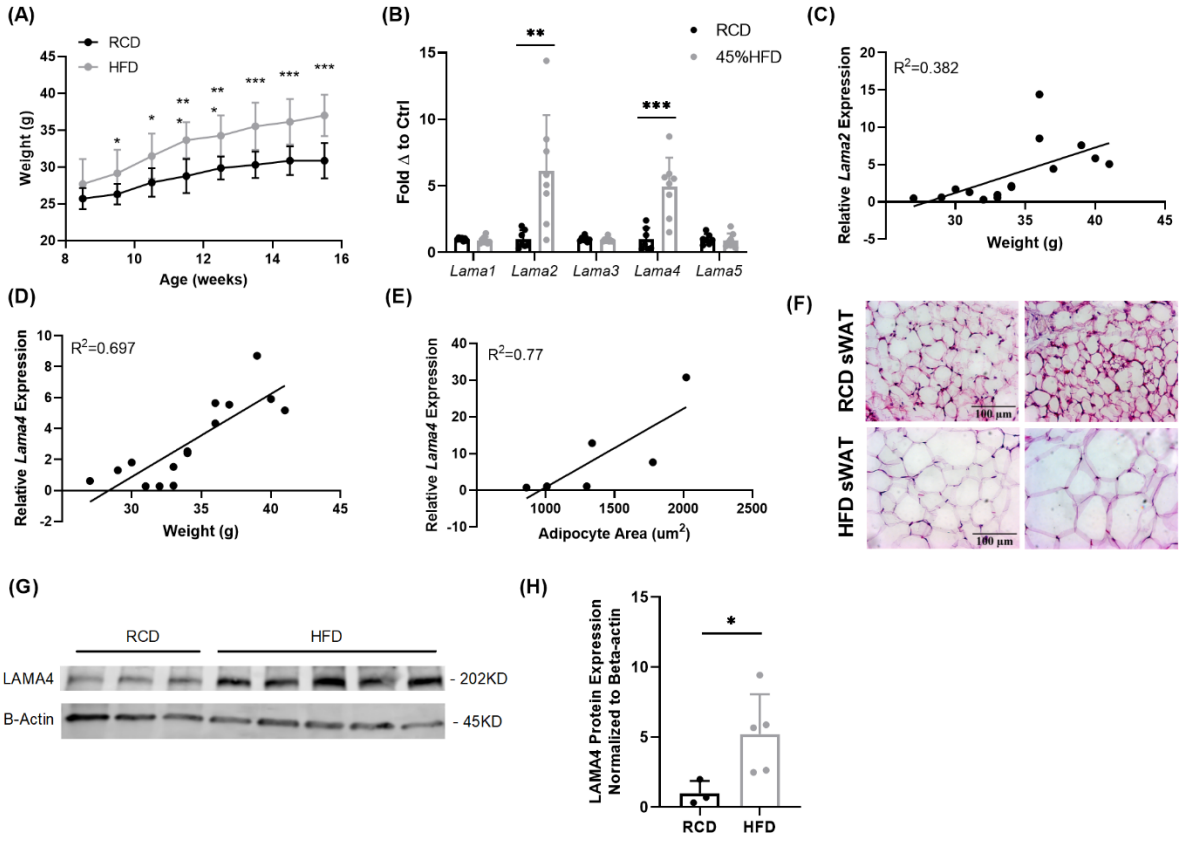

Figure 2.2 Gene expression of laminin-α chains in sWAT of DIO male mice

(A) Average weekly weights of mice on regular chow diet (RCD) and 45% High Fat Diet (HFD) for 8 weeks. RCD (n=7), HFD (n=8). (*,**,***) indicates $p < 0.05$, 0.01, 0.001, respectively. Data are Means ± SD. **(B)** Laminin-α chain mRNA expression in sWAT of mice after 8 weeks of dietary study normalized to the average of RCD control group. RCD (n=7), HFD (n=8). **(C)** Relative *Lama2* mRNA expression normalized to RCD group graphed against weight (grams) for each mouse in 8-week dietary study (n=15). Simple linear regression analysis. **(D)** Relative *Lama4* mRNA expression graphed against weight (grams) for each mouse in 8-week dietary study (n=15). Simple linear regression analysis. **(E)** Relative *Lama4* mRNA expression graphed against adipocyte area (um^2) for one cohort of HFD and RCD mice (n=6). Simple linear regression analysis. **(F)** Representative images of H&E stained sWAT tissue sections of HFD and RCD mice after 8 weeks of dietary study. All images are 40X magnification. **(G)** Protein expression of LAMA4 and loading control B-actin in sWAT of mice placed on RCD or 45% HFD for 8 weeks was assessed by western blot. Original blot images can be found in the supplementary file. **(H)** LAMA4 signal from the western blot was quantified and normalized to the loading control signal. Values are shown as fold changes in comparison to the average of the RCD group. RCD (n=3), HFD (n=5).

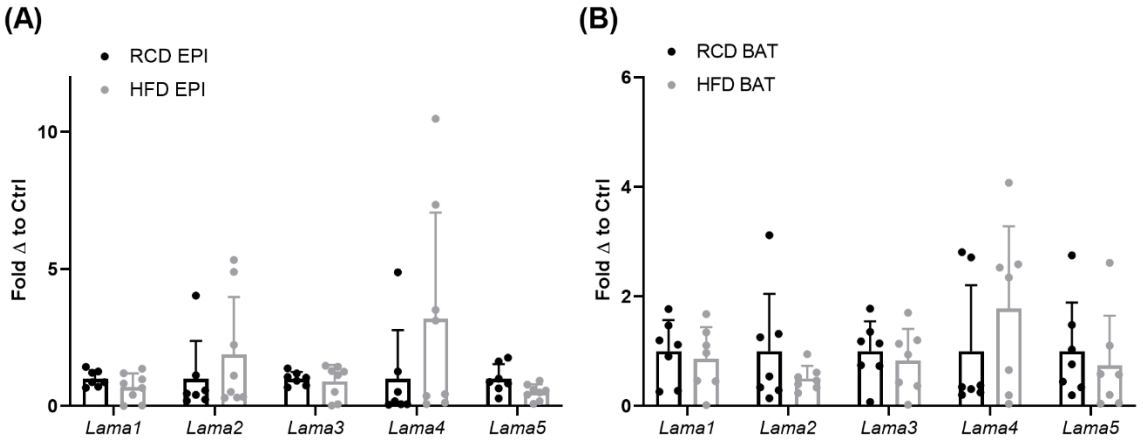

Figure 2.3 Laminin-α chain expression across adipose depots

(A) Laminin alpha chain mRNA expression in Epididymal WAT (EPI) from male mice on 45% HFD or RCD for 8 weeks. HFD (n=8), RCD (n=7). **(B)** Laminin alpha chain mRNA expression in Brown Adipose Tissue (BAT) from male mice on 45% HFD or RCD for 8 weeks. HFD (n=7), RCD (n=7).

The observed upregulation in *Lama4* was confirmed at the protein level by western blotting. Protein lysates from sWAT of HFD mice showed a 5-fold significant elevation of LAMA4 protein in comparison to RCD samples (Fig. 2.2G-H). These results indicate that, of all laminin-α chains, LAMA4 appears to be strongly related to subcutaneous white adipose accumulation in male mice.

In addition to laminin chains we also assessed the expression of several collagen subunits known to be associated with obesity in mice. We found that all collagen subunits tested (*Col1a1*, *Col3a1*, *Col4a1*, and *Col6a3*) were significantly upregulated in the sWAT of HFD mice compared to controls (Fig. 2.4A). *Col1a1* and *Col3a1* showed the greatest positive correlation with weight (R^2 = 0.625 and 0.689, respectively) (Fig. 2.4B-E).

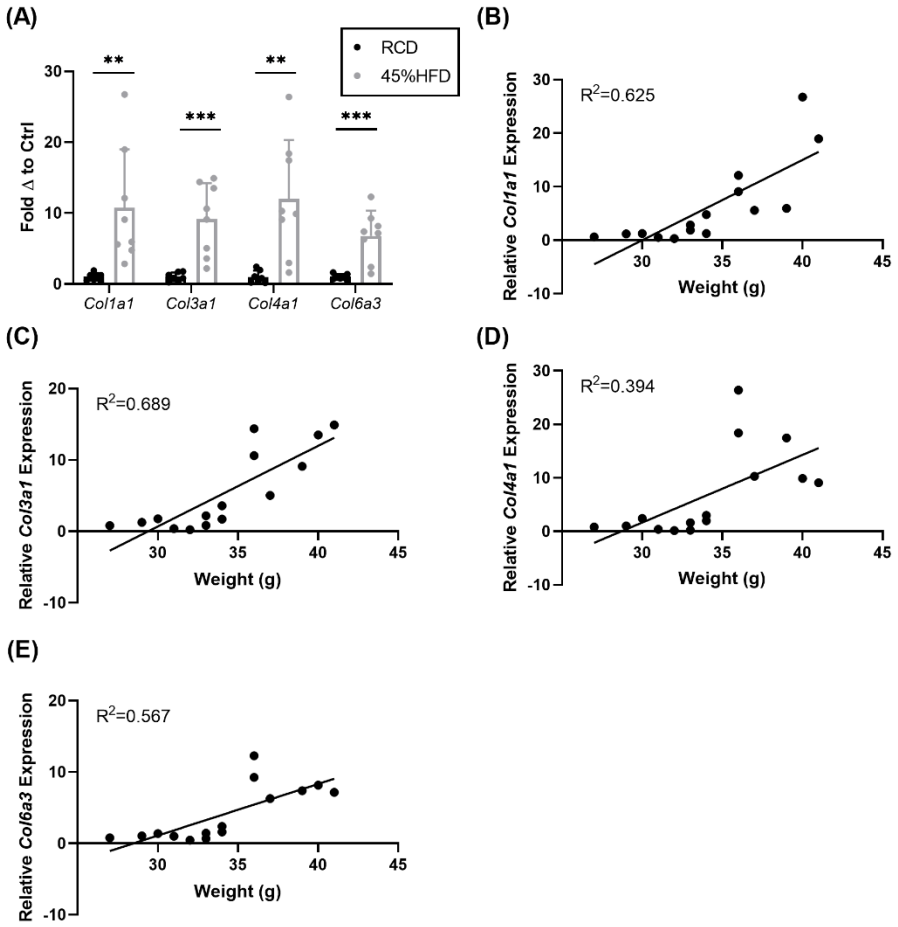

Figure 2.4 Gene expression of collagen species in sWAT of DIO mice

(A) Collagen species mRNA expression in sWAT of mice after 8 weeks of dietary study normalized to the average of RCD control group. RCD (n=7), HFD (n=8). (*,**) indicates $p < 0.05$, 0.01, respectively. Data are Means $\pm$ SD. **(B-E)** Relative *Col1a1* mRNA expression normalized to RCD group graphed against weight (grams) for each mouse in 8-week dietary study (n=15). Simple linear regression analysis.

2.3.2 In vitro relationship of Lama4 and adipocyte function

In vitro adipocyte studies were performed assessing the effect of heightened lipid loading via oleic acid treatment on *Lama4* expression levels. Previous studies show that treatment of 3T3-L1 adipocytes with oleic but not palmitic or stearic acid induces significant lipid loading and expression of adipogenic genes [167]. Primary murine adipose derived stem cells (ADSCs)

isolated from WT male mice were differentiated to mature white adipocytes and treated with 0.25mM fatty acid or vehicle for the last 4 days of the 10-day differentiation timeline. Expression of adipogenic markers such as peroxisome proliferator activated receptor gamma (*Pparg*), adiponectin (*Adipoq*), fatty acid binding protein 4 (*Fabp4*), and perilipin 1 (*Plin1*) were significantly upregulated in the oleic acid treated group (Fig. 2.5A). Heightened lipid loading, as measured by semi-quantitative Oil Red O staining, was observed in the oleic acid treated group as well, but not in the palmitic acid treated group (Fig. 2.6B-C). There was around a 2-fold increase in expression of laminin-α chains in the cells treated with the oleic acid, with *Lama2* and *Lama4* being the most statistically significant (Fig. 2.5B). We observed no elevation in laminin-α chain mRNA expression in the cells treated with palmitic acid (Fig. 2.6A).

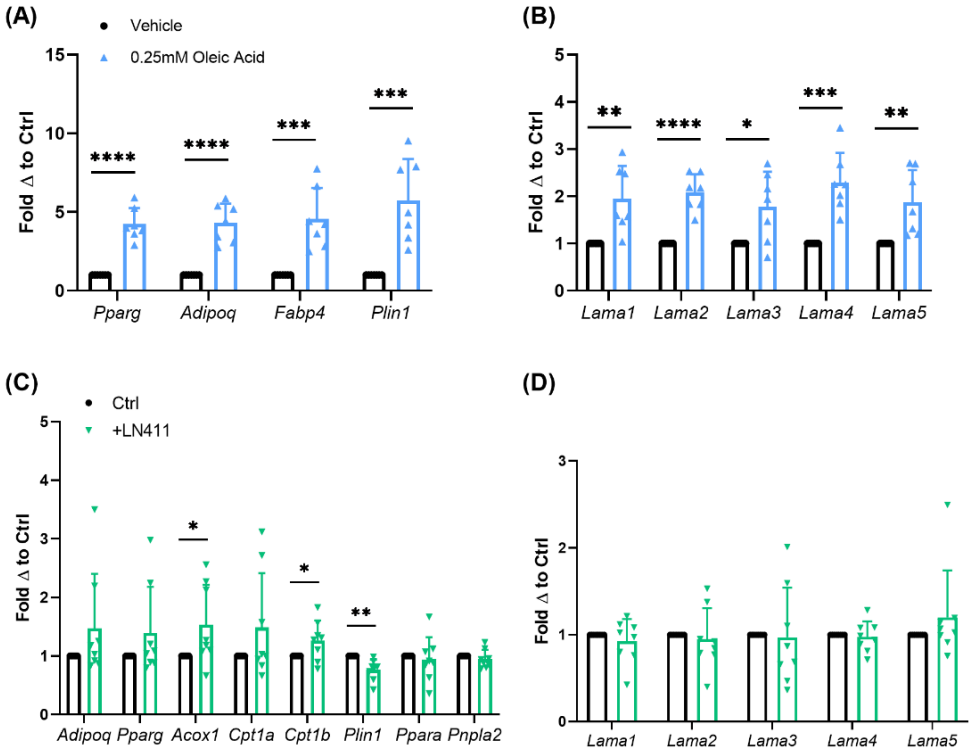

Figure 2.5 *In vitro* study of LAMA4 and lipid storage and metabolism in adipocytes

(A) Gene expression of adipogenic markers in primary murine pre-adipocytes treated with vehicle or 0.25mM Oleic Acid during differentiation. Vehicle (n=7), Oleic Acid (n=7). (*,**,***,***)

Figure 2.5 continued: indicates $p < 0.05, 0.01, 0.001, 0.0001$ respectively. Data are Means + SD. **(B)** Laminin-α chain gene expression in primary differentiated murine adipocytes treated with vehicle or 0.25mM Oleic Acid during differentiation. Vehicle (n=7), Oleic Acid (n=7). **(C)** Expression of genes involved in lipid metabolism, oxidation, and adipogenesis in primary murine differentiated adipocytes grown on culture plates coated with 0ug (n=8) or 10ug of LN411 (n=8). **(D)** Laminin-α chain gene expression in primary murine differentiated adipocytes grown on culture plates coated with 0ug (n=8) or 10ug of LN411 (n=8).

We next studied changes in lipid metabolism and fatty acid β-oxidation in response to growth on recombinant laminin-411 (LN411) coated wells. The LN411 trimer contains the LAMA4, laminin beta 1 (LAMB1), and laminin gamma 1 (LAMC1) chains. Primary murine ADSCs differentiated to white adipocytes on plates coated with 10ug of LN411 displayed significantly decreased gene expression of *Plin1* and increased expression of acyl-CoA oxidase 1, palmitoyl (*Acox1*) and carnitine palmitoyltransferase 1b (*Cpt1b*) compared to those grown on uncoated wells (Fig. 2.5C). Expression of related genes such as patatin-like phospholipase domain containing 2 (*Pnpla2*, previously known as adipose triglyceride lipase, *Atgl*) and peroxisome proliferator activated receptor alpha (*Ppara*) showed no significant changes while *Adipoq* and *Pparg* expression trended upwards in the LN411 treated group. No changes in laminin-α chain expression were observed, suggesting that the results are most likely due to LN411 mediated changes and not resultant from endogenous ECM compositional alterations (Fig. 2.5D).

To further investigate changes in lipid metabolism based on the previous results we assessed lipolysis rate in cells grown on 10ug of LN411. The LN411 group displayed slightly elevated basal lipolysis rate compared to control cells ($p=0.05$) (Fig. 2.7A). Interestingly, lipid loading as measured by semi-quantitative oil red o staining revealed a small but significant elevation in the LN411 group as well (Fig. 2.7B-C).

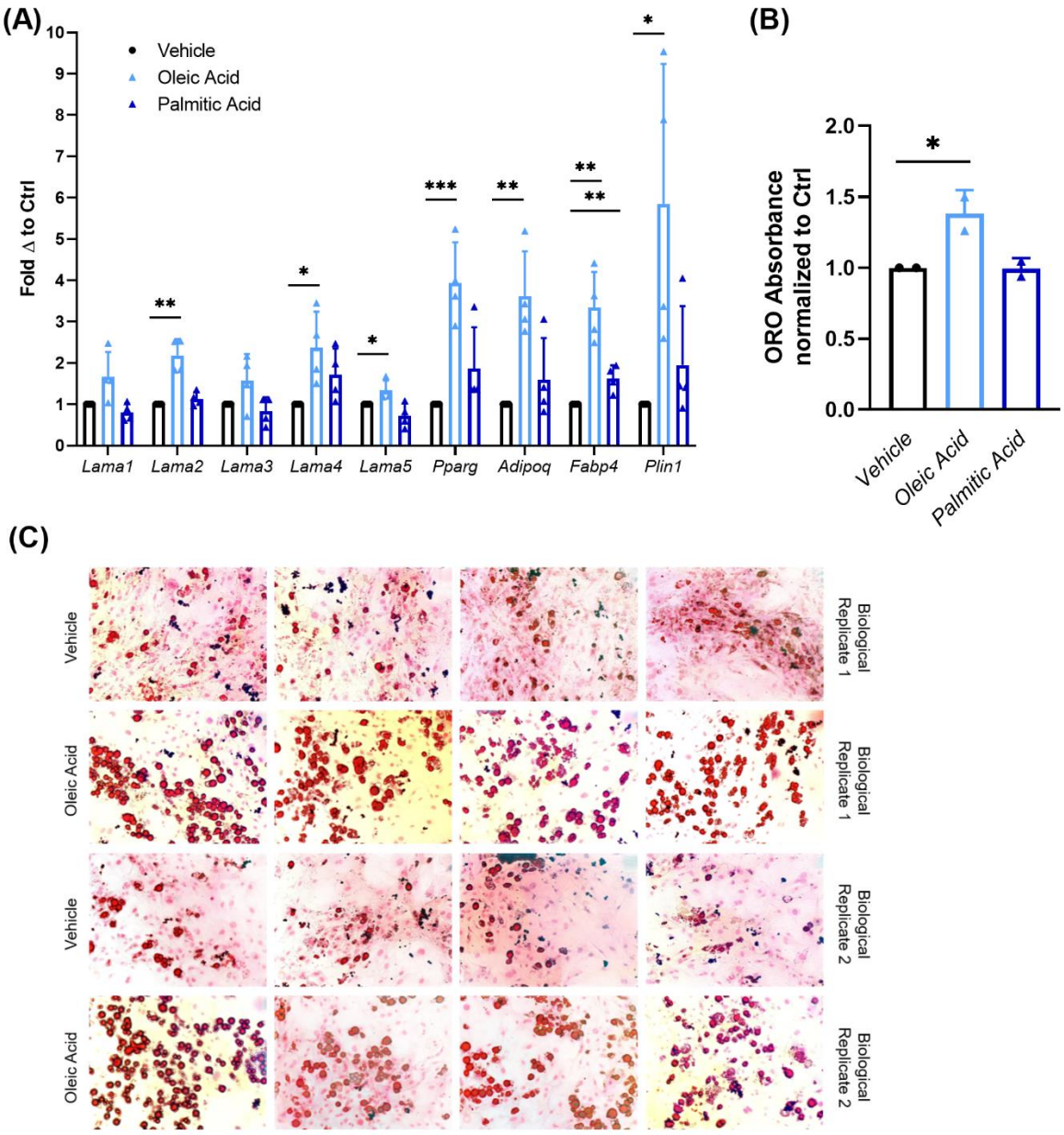

Figure 2.6 Effect of fatty acid treatments during adipocyte differentiation

(A) Laminin alpha chain mRNA expression and adipogenic gene expression in differentiated murine primary adipocytes treated with Vehicle, 0.25 mM oleic acid, or 0.25 mM palmitic acid for four days. Veh (n=4), Oleic Acid (n=4), Palmitic Acid (n=4). **(B)** Semi-quantitative measurement of lipid content by ORO absorbance at 492 nm, normalized to Vehicle. Veh (n=2), Oleic Acid (n=2), Palmitic Acid (n=2). **(C)** Images of ORO stained adipocytes, 40X. Veh (n=2), Oleic Acid (n=2).

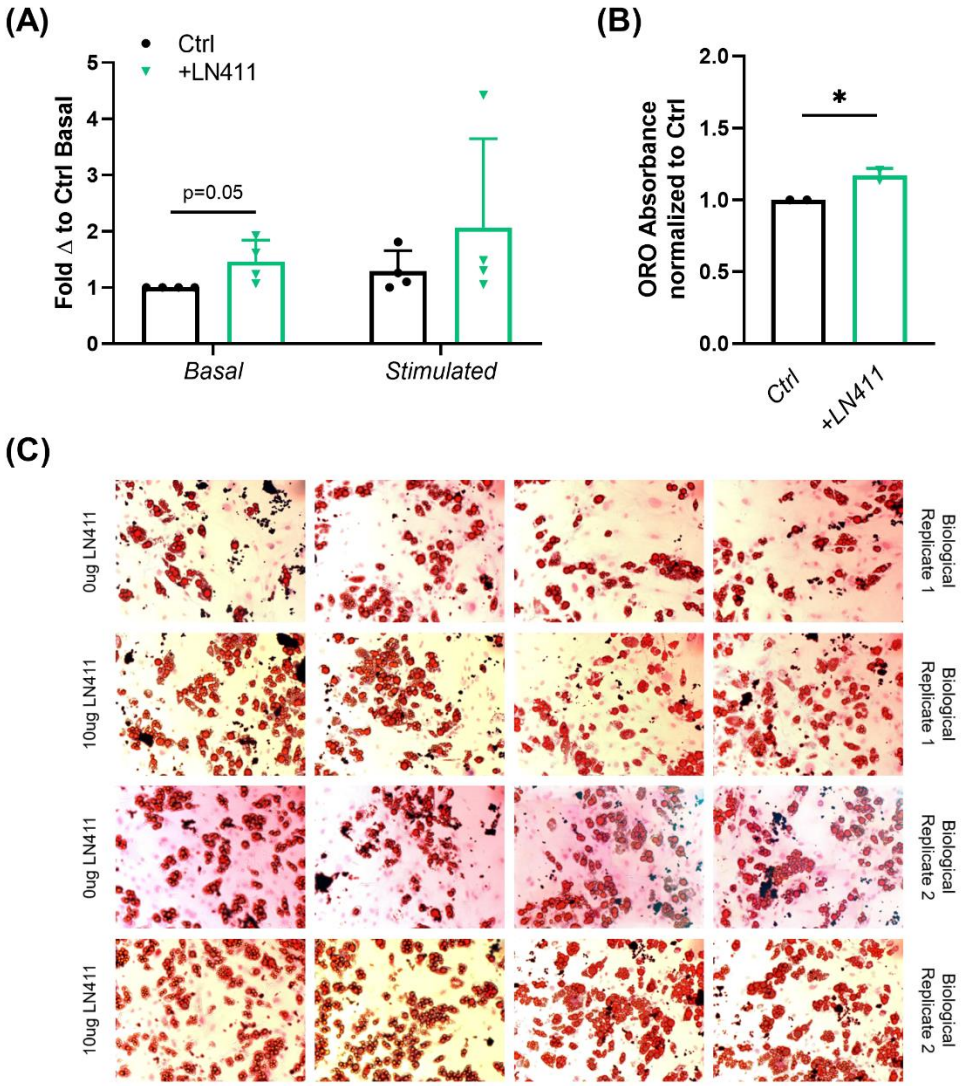

Figure 2.7 Lipolysis and lipid accumulation in response to LN411 treatment

(A) Relative lipolysis activity, as measured by glycerol content, in differentiated murine primary adipocytes grown on culture plate coated with or without LN411. Basal lipolysis indicates wells where no isoproterenol was added, while stimulated lipolysis indicates wells where isoproterenol was added. Basal (n=4), Stimulated (n=4). **(B)** Semi-quantitative measurement of lipid content by ORO absorbance at 492 nm, normalized to ctrl. Ctrl (n=2), +LN411 (n=2). **(C)** Images of ORO stained adipocytes, 40X. Ctrl (n=2), 10ug LN411 (n=2).

2.3.3 Laminin and collagen expression in human obesity

As *Lama4* expression was significantly augmented in HFD fed mice, we wanted to understand if this trend applied to human obesity. We were able to obtain a set of sWAT RNA

samples from female control subjects (BMI<30) and non-diabetic subjects with obesity (BMI>35) undergoing bariatric surgery (subject characteristics listed in **Table 2.1**) from an ongoing study at the University of Chicago investigating bariatric surgery and circadian rhythms in females. We measured the gene expression of several laminin-α chains known to be expressed in adult adipose tissue. The sWAT samples from subjects with obesity pre-surgery displayed 4-fold greater *LAMA4* expression than the control subjects ($p<0.01$) (Fig. 2.8A). We found no change in the mRNA expression levels of *LAMA2* or *LAMA5* (Fig. 2.4A). In contrast to our results of the mouse studies, we were unable to detect a significant difference in the expression of *COL1A1*, *COL3A1*, *COL4A1*, and *COL6A3* between the two groups (Fig. 2.8B). This may arise from the control group containing some overweight but not obese subjects (25<BMI<30) where collagen species may already be highly expressed.

Table 2.1 Deidentified subject characteristics

Note: IVGTTs were not performed or could not be safely completed for some subjects in the study. For obese pre-surgery, fasting glucose and insulin is shown for 7/10 subjects, and for obese post-surgery it is shown to 6/10 subjects.

	Control	**Obese Pre-Surgery**	**Obese Post-Surgery**
Subjects	N=3	N=10	N=10
Weight (kg)	64.13 (± 8.12)	124.95 (± 13.33)	103.48 (+ 12.19)
Height (cm)	161.76 (± 7.35)	166.85 (+ 6.34)	166.53 (+ 6.50)
BMI (kg/m^2)	24.53 (± 2.59)	44.95 (+ 5.12)	37.41 (+ 4.86)
Age	39 (± 16.52)	36 (+ 8.96)	36.2 (+ 8.66)
HbA1c (%) at Screening	5.43 (± 0.30)	5.54 (± 0.36)	5.54 (± 0.36)
Fasting Glucose (mg/dL)	87.55 (± 21.61)	87.38 (± 5.11)	75.55 (± 6.23)
Fasting Insulin (pmol/L)	56.52 (± 9.57)	121.57 (± 78.65)	29.37 (± 16.12)

Additionally, we obtained fixed adipose tissue sections from a portion of the control subjects and subjects with obesity. We verified by immunofluorescence staining that extracellular

LAMA4 protein was also significantly increased in the subjects with obesity pre-surgery compared to the controls ($p<0.05$) (Fig. 2.8C-D). The density of LAMA4 in the ECM of obese adipose was about 30% greater than in control adipose, suggesting that the adipocytes in sWAT of subjects with obesity do in fact produce and deposit more LAMA4 than adipocytes from subjects without obesity.

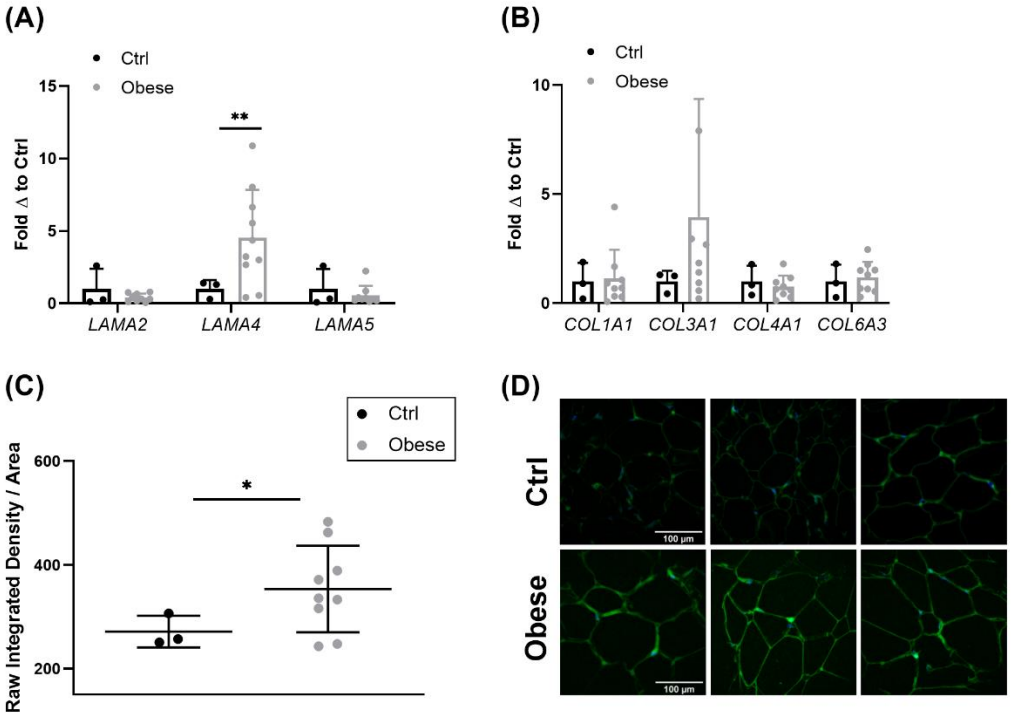

Figure 2.8 Expression of laminin and collagen-α chains in the sWAT of human subjects

(A) Laminin-α chain mRNA expression in sWAT biopsies of female control (BMI<30) and obese (BMI>35) subjects normalized to average of control subjects. Control (n=3), Obese (*LAMA2*, *LAMA5* n=9, *LAMA4* n=10). (*,**,***) indicates $p < 0.05$, 0.01, 0.001, respectively. Data are Means $\pm$ SD. **(B)** Collagen species mRNA expression in sWAT biopsies of human subjects normalized to average of control subjects. Control (n=3), Obese (n=9). **(C)** Density of extracellular LAMA4 as determined by raw integrated density signal over total area of signal in fixed sections of sWAT from control and obese subjects. Control (n=3), Obese (n=9). **(D)** Representative images of extracellular LAMA4 immunofluorescence staining from control and obese sWAT. LAMA4 (in green) and DAPI (in blue). All images are 40X magnification.

2.3.4 LAMA4 expression does not change following weight loss

Next, we compared the expression of LAMA4 in the subjects with obesity 3 months after bariatric surgery to determine if short term weight loss could reverse the observed upregulation of LAMA4. At this stage following bariatric surgery the subjects with obesity had lost a small but statistically significant percentage of weight compared to pre-surgery, averaging around 17% weight loss (Fig. 2.5A). However, we found that sWAT post-surgery samples had similar levels of *LAMA4* gene expression on average to the samples taken pre-surgery (Fig. 2.9B). We observed no difference in density of extracellular LAMA4 between pre-surgery and post-surgery fixed adipose sections as determined by immunofluorescence staining (Fig. 2.9C-D). Our findings indicate that short term weight loss in humans does not lead to consistent decreases in levels of LAMA4 expression.

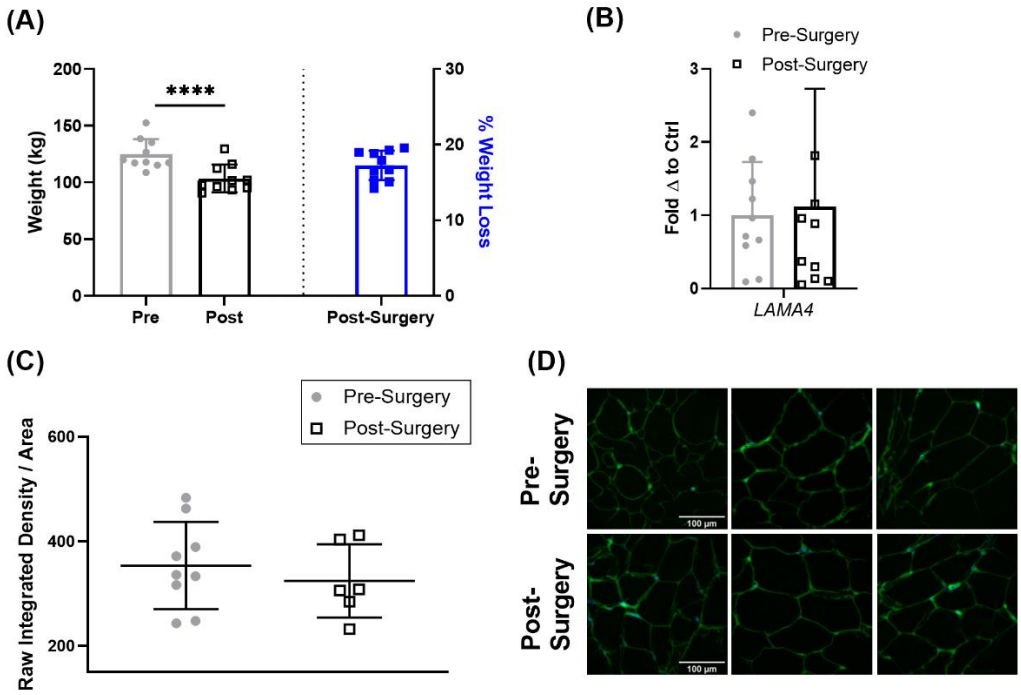

Figure 2.9 LAMA4 expression does not change following short-term weight loss in humans with obesity

Figure 2.9 continued: (A) Weight (kg) of human subjects before and 3 months post-surgery shown on left y-axis ($p<0.0001$) and % of weight loss 3 months post-surgery is shown on right y-axis. Data are Means $\pm$ SD. **(B)** *LAMA4* mRNA expression in sWAT biopsies of obese subjects pre-surgery and 3 months post-surgery, normalized to average of pre-surgery subjects. Pre-surgery (n=9), Post-surgery (n=9). **(C)** Density of extracellular LAMA4 as determined by raw integrated density signal over total area of signal in fixed sections of sWAT from obese subjects pre-surgery and 3 months post-surgery. Pre-surgery (n=9), Post-surgery (n=6). **(D)** Representative images of extracellular LAMA4 immunofluorescence staining from obese pre-surgery and post-surgery subjects. LAMA4 (in green) and DAPI (in blue). All images are 40X magnification.

Following these results, we were interested to investigate *Lama4* expression following weight loss in a more controlled manner in mice. We placed two groups of 8-week -old WT mice on 45% HFD for 8 weeks and then switched one group to RCD for another 6 weeks while the other continued to receive HFD. The difference between the Weight Loss group (switched back to RCD) and HFD group became larger over the course of the 6-week diet period and by 22 weeks the Weight Loss group was significantly lower in weight than the group that remained on HFD (Fig. 2.10A). From the time at which the diet was switched until the end of the study, the Weight Loss group lost on average around 5% of their body weight while the HFD group gained roughly 15% more weight (Fig. 2.10B). We observed no difference in sWAT *Lama4* expression between the groups, indicating that reversal of HFD feeding and a small amount of weight loss, at least in a short timeframe, does not result in reduced *Lama4* expression levels (Fig. 2.10C). In comparing the HFD and Weight Loss group, *Lama4* expression levels did not correlate strongly to adipocyte area, although the Weight Loss group did display significantly smaller adipocyte area in sWAT than the HFD group (Fig. 2.10D-F).

2.4 Discussion

The adipocyte microenvironment is an integral part of adipocyte functioning and in recent years has been further implicated in adipose dysfunction occurring in the obese state. However,

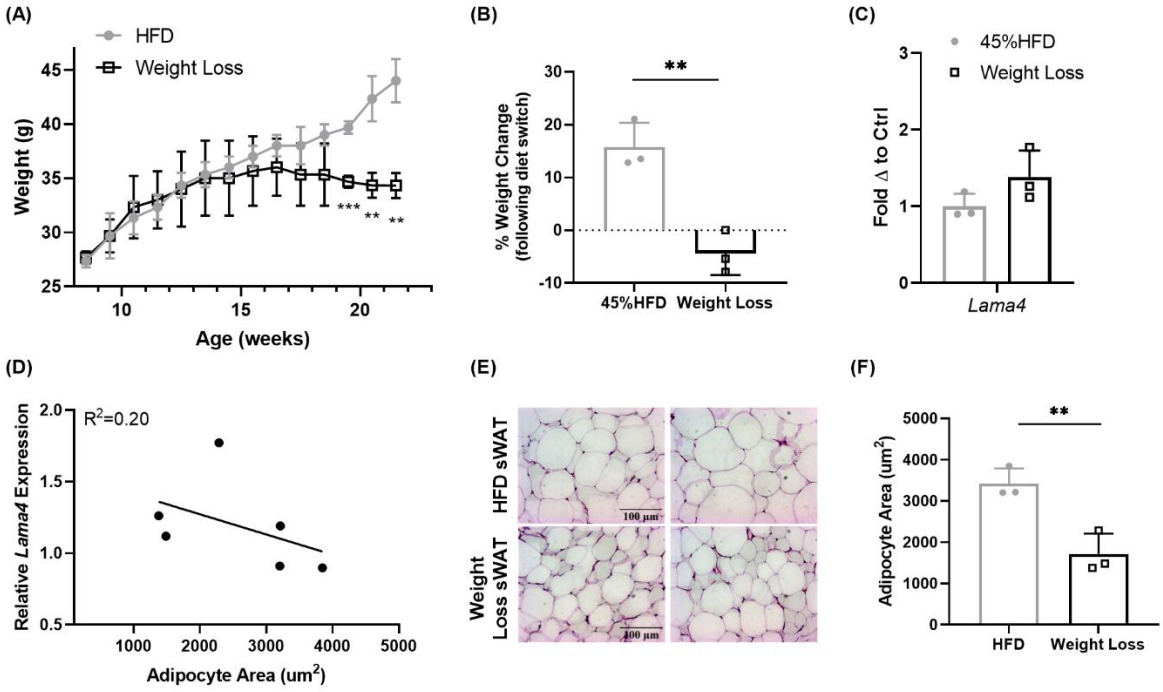

Figure 2.10 LAMA4 expression does not change following HFD feeding reversal in mice

(A) Average weekly weights of mice on 45% HFD for 14 weeks (HFD) or 45% HFD for 8 weeks and then RCD for 6 weeks. HFD (n=3), Weight Loss (n=3). **(B)** Weight change shown as percentage from timepoint of diet switch (16 weeks of age) until end of dietary study (22 weeks of age). Data are Means $\pm$ SD. **(C)** *Lama4* mRNA expression in sWAT of mice in HFD (n=3) or Weight Loss (n=3) group. **(D)** Relative *Lama4* mRNA expression graphed against adipocyte area (um^2) (n=6). Simple linear regression analysis. **(E)** Representative images of H&E stained sWAT tissue sections of HFD and Weight Loss group mice after 14 weeks of dietary study. All images are 40X magnification. **(F)** Adipocyte area (um^2) in sWAT of mice in HFD (n=3) and Weight Loss (n=3) group.

how non-collagen ECM components such as laminins contribute to adipocyte signaling and response in this context remains poorly studied. In this investigation we characterize the relationship between obesity and LAMA4 in both mice and humans and illustrate the important role of this ECM protein in adipose tissue across models. We demonstrate a parallel upregulation of *Lama4* in male mice fed HFD and *LAMA4* in female human subjects with obesity compared to lean controls and verify that this upregulation is reflected at both the mRNA and protein level. We

also conclude that short-term weight loss in DIO mice and human subjects with obesity does not downregulate LAMA4 expression. Overall, these findings describe a significant association between LAMA4 and obesity in humans.

LAMA4 expression is significantly upregulated during adipogenesis and has been linked to adiposity in previous studies. Moest et al. 2013 have established that LAMA4 is elevated in the sWAT and vWAT of DIO and ob/ob mouse models [161]. Furthermore, our group has shown that the silencing of *Lama4* in mice leads to the reduced adipose mass accumulation and protection from obesity on a HFD [131]. However, the applicability of this trend to humans was undetermined. In human obesity, LAMA4 has only been investigated to the extent of depot specific comparisons. Roca-Rivada et al. 2015 report heightened LAMA4 in secretomes of adipocytes from vWAT compared to sWAT in non-diabetic human subjects with obesity, however no control subjects were included for comparison [162]. The results from our study demonstrate that sWAT *Lama4* expression is elevated by 4-fold in a DIO mouse model and positively correlates with weight and adipocyte area. Similarly, in humans, *LAMA4* expression increased by 4-fold in obese sWAT samples compared to controls. While *Lama2* was also upregulated in HFD fed mice, the expression was not found to correlate strongly with weight and was not upregulated in the sWAT of human subjects with obesity. While interesting and deserving of further investigation in mice, there appears to be no obvious association at present between *LAMA2* and obesity in humans. The unique elevation of *LAMA4* in obese sWAT suggests that this chain assumes a specialized role in the adipocyte microenvironment during obesity.

It should be noted that direct sex comparisons were not made in the course of this study. In line with previous studies showing that sexual dimorphisms exist with regards to DIO in mice, we found that female mice placed on HFD did not gain significant weight or become obese during

the 8 week study period [168]. Therefore, LAMA4 could not be studied in the context of DIO. Additionally, we obtained our human adipose samples from an ongoing bariatric surgery study examining outcomes in female subjects, and so we were not able to investigate this association in male subjects. However, it is noteworthy that we observe parallel findings in both species, although different sexes. Future investigations should compare LAMA4 expression in male and female subjects with obesity to determine if there are any differences based on sex.

Additionally, while we focused our investigation on subcutaneous adipose tissue in this study, it is not yet clear how transferrable insights from murine sWAT are to human abdominal sWAT function [169]. Some studies suggest that gene expression for certain adipose functions in murine sWAT is more similar to human vWAT [170]. Our results indicate promise that LAMA4 may serve similar roles in murine sWAT and human sWAT, however further verification may be needed to assess therapeutic potential of regulating laminins in human adipose depots. Other limitations of this study included the housing of mice at room temperature conditions rather than thermoneutrality. While this could have contributed to heightened energy metabolism compared to conditions at thermoneutrality, both dietary groups were housed at this same condition and thus could be considered comparable.

While it is not fully understood what role LAMA4 may play in aggravating obesity in humans, it is likely that LAMA4 is both deposited in elevated levels in response to obesity and can influence adipocyte function and pathways relating to lipid metabolism. Our results indicate that in a simulation of HFD feeding *in vitro*, laminin-α chains are significantly upregulated, suggesting that a heightened lipid burden stimulates laminin expression in adipocytes. Furthermore, cells differentiated to white adipocytes in the presence of LN411 showed diminished *Plin1* expression. PLIN1, located on lipid droplets, inhibits lipolysis and is downregulated in adipose of subjects

with obesity [171]. Our results suggest some involvement of LAMA4 in regulating PLIN1 levels, and thus excessive LAMA4 may contribute to elevated lipolysis and circulating non-esterified fatty acids which is detrimental to systemic insulin sensitivity. The results of the lipolysis assay showing enhanced lipolysis rates in LN411 cells support these findings. However, it should be noted that this study utilized recombinant human LN411 applied to murine cells due to the limited availability of affordable murine LAMA4 sources. Thus, these results may not illustrate a complete picture of how LAMA4 may regulate PLIN1 activity in murine adipocytes. Future studies should focus on testing murine LAMA4 sources on murine adipocytes, and human LAMA4 on human adipocytes, when resources are more widely accessible.

We noted that genes related to β-oxidation were altered in response to LN411 treatment. The expression of multiple CPT1 isoforms trended upward in cells differentiated on LN411, although variable. sWAT *CPT1A* expression has previously been positively correlated with BMI in humans, however neither isoform is very abundant in white adipose tissue so it would be interesting to understand what role LAMA4 might play in CPT1 regulation in other tissues such as muscle [172]. *Acox1*, which codes for the first enzyme in the peroxisomal β-oxidation pathway, was also found to be significantly upregulated in the LN411 samples. *Acox1* has been previously studied in the context of obesity as mice deficient in *Acox1* exhibit resistance to DIO through sustained *Ppara* activation [173]. It is possible that LAMA4 may influence energy balance through a mix of genes involved in metabolism. While outside of the scope of this study, future investigations should focus on identifying specific relationships between LAMA4 and these metabolic pathways in adipocytes.

Lastly, in addition to investigating LAMA4 in obesity, we studied the effect of weight loss on expression of LAMA4 in sWAT. We found that short-term weight loss was not sufficient to

reduce LAMA4 expression from the high levels seen in HFD fed mice or human subjects with obesity. This could be due in part to the duration of weight loss investigated in this study, and it is possible that longer periods of weight loss could eventually lead to a downregulation of LAMA4. As LAMA4 is implicated in worsening metabolic behavior, it will be important for future studies to assess if LAMA4 remains unchanged following other types and durations of weight loss.

2.5 Conclusion

Although preliminary murine studies have suggested that obesity correlates with increased LAMA4 expression, it was undetermined whether this finding is true in lean and obese humans. Additionally, a more extensive evaluation of laminin-α chain expression had not been performed in mouse models of obesity. In this study, we confirm the correlation between obesity and increased LAMA4 in a mouse model and show that human sWAT displays a similar trend. Interestingly, we also note that weight loss does not appear to downregulate LAMA4 expression in humans or mice, at least in the short-term. *In vitro* findings demonstrated an increase in laminin-α chain expression in response to lipid loading and a potential association between a laminin-411 rich culture environment and lipid metabolism. Taken together, the weight loss and *in vitro* data suggest that the LAMA4-obesity connection is complex and is not modulated rapidly.

While the results of this study suggest that LAMA4 is significantly associated with obesity, we still do not fully understand the mechanisms underlying this observation. Prior publications have reported a role for LAMA4 in the inhibition of the thermogenic program in adipocytes, in regulating angiogenesis, and even in facilitating immune infiltration [132,174,175]. There is a strong need to elucidate the role of LAMA4 in each of these processes in greater depth in human adipose, and to explore other pathways that may implicate LAMA4. In doing so, we hope to uncover how *LAMA4* may be targeted effectively in therapies for obesity.

CHAPTER 3: LAMININ-α4 REGULATES THERMOGENESIS AND MITOCHONDRIAL BIOGENESIS IN ADIPOCYTES

3.1 Introduction

In recent years, there has been increasing evidence to support the beneficial metabolic effects of beige adipocytes, including improved insulin sensitivity, increased fatty acid oxidation, and reduced adiposity [176]. This has driven an extraordinary interest into the study of thermogenic regulators that could be targeted for therapeutic application in metabolic diseases like obesity and type 2 diabetes mellitus (T2D). While a number of pathways involved in adipocyte thermogenesis have been identified to date and more are discovered every year, there is still much remaining to be understood about extracellular factors within adipose tissue that regulate the thermogenic program and adipocyte differentiation.

Adipose tissue exists in three forms: white, brown, and beige. White adipose tissue is known for being energy storing, while brown adipose is rich in mitochondria and expends energy through the process of thermogenesis to generate heat. The third type, beige or brite, is found in white adipose depots but exhibits a higher mitochondrial density and a capacity for thermogenesis. Mitochondria are essential organelles which produce ATP through oxidative phosphorylation [177]. In beige or brown adipose, compared to other cell types, there is a greater amount of uncoupling protein 1 (UCP1). UCP1 resides in the inner membrane of mitochondria and facilitates proton leak which diffuses the proton gradient required for ATP generation, leading to heat production instead [178]. This process is known as thermogenesis and results in increased energy expenditure. For this reason, promoting thermogenesis in white adipose through beige adipocyte induction and enhanced mitochondrial content could be applied to treat obesity. Although many

drugs have been proposed to stimulate beiging, there is increasing interest in how adipocyte fate and function may be modulated through extracellular matrix (ECM) dynamics.

The cellular microenvironment plays a pivotal role in directing differentiation in all tissue types. This modulation can occur through environmental stiffness and mechanosensation, through direct contact of specific extracellular proteins with cell surface receptors, and through the sequestration or release of signaling molecules and factors within the extracellular space [67]. The differing environmental requirements of specific cell types is reflected in the diverse composition of extracellular niches. Recent publications have even illustrated the important effects of the extracellular matrix (ECM) composition and density on mitochondrial dynamics and bioenergetics in human mesenchymal stem cells and cancer cells, particularly through integrin-mediated signaling [179,180]. While ECM proteins, such as collagens, have been studied in great depth in their relation to adipose tissue and metabolic disease, there are many types of proteins beyond collagens that contribute to adipocyte differentiation and behavior [67]. As research in this field expands, other ECM protein families, such as laminins, have come into focus as important regulators of adipocyte thermogenesis.

Laminins are a family of ECM proteins located in the cellular basement membrane that contribute to cell migration, support, and communication of extracellular cues [83]. The many isoforms of laminin each contain an α-, β-, and γ- chain assembled into a cross-like structure. The α-chain is especially important as it contains domains in the C-terminus which interact with numerous cell surface receptors [86]. During white adipocyte differentiation, cells express and secrete increasing levels of the laminin-α4 (LAMA4) chain, suggesting a role for this protein unique to white adipogenesis [113,114]. Our group has shown that mice deficient in LAMA4 display elevated uncoupling protein 1 (UCP1) expression in subcutaneous white adipose tissue

(sWAT) compared to WT mice, and are protected from diet-induced obesity (DIO) [131,132]. More recently, we have published findings showing that differentiation of murine adipose-derived stem cells (ADSCs) to beige adipocytes on LAMA4 coated surfaces impairs *Ucp1* expression [133]. LAMA4 expression is also significantly elevated in the sWAT of humans with obesity and mice with DIO (45% high fat diet) [181]. Our results thus far indicate a correlation between LAMA4 and adiposity by proposing that LAMA4 can suppress thermogenic signaling pathways or differentiation towards a beige adipocyte phenotype.

For this study, we sought to identify the effects of LAMA4 on adipocyte thermogenesis and mitochondrial biogenesis and to understand how this regulation may occur. First, we studied the influence of LAMA4 on mitochondrial biogenesis and found that the deficiency of LAMA4 *in vivo* and acute silencing *in vitro* leads to an upregulation of PGC-1α expression in adipocytes. We next show that the acute silencing of LAMA4 *in vitro* is sufficient to enhance thermogenic gene expression in ADSCs during differentiation to beige adipocytes. Our investigation of associated pathways uncovered a major signaling protein that appears to be involved in this LAMA4 signal transduction: AMP-activated protein kinase (AMPK). We also show that LAMA4 negatively regulates thermogenesis in a human adipocyte model using human induced pluripotent stem cells (hiPSCs) differentiated to thermogenic adipocytes and treated with human recombinant laminin-411 during differentiation. These results denote a critical function for LAMA4 in negatively regulating adipocyte thermogenesis through AMPK which could be targeted in the future for therapeutic purposes.

3.2 Methods

Animal Model, Care, and Housing

Animal procedures and numbers were approved by the University of Chicago Institutional Animal Care and Use Committee. All experiments were conducted in accordance with accepted standards of humane animal care. The generation of *Lama4-/-* mice was previously described [174]. WT and *Lama4-/-* male C57BL/6J mice were given regular chow diet (Teklad 2918; Harlan Laboratories) *ad libitum* and housed at room temperature. Female mice were not used in this particular study as female *Lama4-/-* mice do not exhibit the same degree of enhanced sWAT thermogenesis as do male *Lama4-/-* mice. Mice were humanely sacrificed between 14-17 weeks of age for tissue collection and cell isolation.

Murine Adipose-Derived Stem Cell Culture and Differentiation

Primary adipose derived stem cells were isolated from 14-17 week old male mice as previously described [132]. Cells were expanded and plated into 6-well plates for experiments, and subsequently differentiated to beige adipocytes following a previously published protocol [182]. Induction medium containing complete medium (10% Fetal Bovine Serum, 1% Pen/Strep, DMEM/F-12) supplemented with 5 uM Dexamethasone, 125 nM Indomethacin, 0.5 mM Isobutylmethylxanthine, 850 nM Insulin, 1nM T3, 0.5 uM Rosiglitazone was added to cells to initiate differentiation. On Day 2 cells were given maintenance medium containing complete medium with 850 nM Insulin, 1nM T3, 0.5 uM Rosiglitazone. Refeeding then occurred every other day with maintenance medium until Day 16.

Human Induced Pluripotent Stem Cell Culture and Differentiation

An iPSC cell line (Coriell Cat# GM25256, RRID:CVCL_Y803, Origin: Human, Male) was obtained from The Coriell Institute for Medical Research (Camden, NJ) and cultured in feeder-free conditions on matrigel (Corning; 354277) in mTeSR1 (Stem Cell Technologies (SCT);

85850). Cells were routinely tested for pluripotency using STEMdiff Trilineage Differentiation Kit (SCT; 05230) and for mycoplasma (PromoKine; PK-CA20-700-20).

We generated beige adipocytes by modifying a previously published protocol by Guenantin et al. [183]. hiPSCs were cultured on Matrigel and dissociated and passaged as aggregates when cells were 80% confluent using ReLeSR (SCT; 05872). Differentiation was performed on 6-well tissue culture plates. Mesoderm induction was started on Day 0 when cells were 90% confluent by changing media to STEMPro34 (Gibco; 10639011), 1:100 Glutamax (Gibco; 35050061), 50 µg/ml L-ascorbic acid (Sigma; A4544), 10 ng/ml BMP4 (R&D; 314-BP-010/CF), 25 ng/ml Activin A (R&D; 338-AC-050/CF). Mesoderm induction media was replaced every other day. On Day 4, adipocyte differentiation was induced by replacing mesoderm induction media with DMEM/F-12, HEPES (Gibco; 11330032) media containing 10% FBS-D (Hyclone; SH30070.03), 10 µg/ml Insulin (Sigma; I9278), 500 µM IBMX (Sigma; I5879), 1 µM dexamethasone (Sigma; A4902), and 50 µM Indomethacin (Sigma; I7378). Media was replaced every 2 days. On Day 10 adipocyte maturation was started by switching cells to DMEM/F12, 10% FBS-D, 850 nM Insulin, 1 nM T3 (Sigma; T6397), and 0.5 µM Rosiglitazone (Cayman; 71740). Recombinant human laminin-411 (BioLamina; LN411) was included in the media for treated cells from Day 4 through Day 16 of differentiation. Cells were collected on Day 16 of differentiation.

siRNA Transfection

Murine adipose-derived stem cells were transfected on Day 10 and 14 of differentiation. Preparation of lipofectamine RNAiMAX with siRNA was carried out following the manufacturer's instructions for a 6-well volume (Thermo Fisher Scientific; 13778075). Per well: 9 ul of lipofectamine RNAiMAX was diluted in 150 ul of Opti-MEM (Thermo Fisher; 31985062) and 3 ul (ILK siRNA, Santa Cruz Biotechnology (SCBT); sc-35667) or 10 ul (Lama4 siRNA,

SCBT; sc-43148) of 10uM siRNA was diluted in 150 ul of Opti-MEM. Control siRNA A (SCBT; sc-37007) was also used at the respective concentrations per condition. The diluted lipofectamine RNAiMAX and diluted siRNA were then mixed in equal parts and incubated at room temperature for 15 minutes. 250 ul of the lipofectamine-siRNA mix was pipetted onto the cells and then they were placed for 20 minutes at 37C in a cell culture incubator, following which 2.5 ml of maintenance media was added per well. Cells were collected on Day 16 of differentiation.

Immunoblotting

Samples were lysed using cold 1X RIPA buffer (Sigma; 20-188) containing 1X phosphatase and protease inhibitor cocktail tablets (Sigma; 4906845001 and 05892970001) and a dispersion-based homogenizer (VWR VDI 12). After incubating on ice for 30 minutes, samples were briefly sonicated (Sonics Vibra-cell) and spun at 1,000xg for 10 minutes at 4°C to separate the lipid layer. The supernatant, excluding lipid layer, was collected and spun again at 10,000xg for 10 minutes at 4°C and supernatant from this spin was collected and stored at -80°C. Protein concentration was determined using the Pierce BCA Protein Assay Kit (Thermo Fisher; 23227).

Samples were diluted in water and 4X Laemmli Sample Buffer (Bio-Rad; 1610747) and run on 4-15% or 10% SDS-PAGE Criterion TGX gels (Bio-Rad), then transferred to Immobilon-P PVDF membranes (Sigma; IPVH00010). Membranes were blocked with 5% PhosphoBLOCKER™ Blocking Reagent (Cell Biolabs, Inc; AKR-103) or 5% Non-fat dry milk (LabScientific; M0841) in TBST for 1 hr. Blots were incubated overnight at 4°C in 1% blocking solution with antibody. Membranes were then incubated with secondary antibody (LI-COR Biosciences Cat# 926-68171, RRID:AB_10956389) for 1 hr. Immunodetection was performed using near-infrared Odyssey CLx System (LI-COR). Analysis was performed using LI-COR Image Studio Lite (RRID:SCR_013715). All samples were normalized to their paired control or,

for *in vivo* studies, the average of group/littermate controls in order to account for variation between mice.

Antibodies used in this study are as follows: Cell Signaling Technology: GAPDH (Cat# 2118, RRID:AB_561053), βActin (Cat# 4970, RRID:AB_2223172), Vinculin (Cat# 13901, RRID:AB_2728768), AMPKα (Cat# 2532, RRID:AB_330331); VDAC (Cat# 4661, RRID:AB_10557420), ILK (Cat# 3862, RRID:AB_2127050); Thermo Fisher Scientific: Phospho-AMPK alpha-1,2 (Thr183, Thr172) (Cat# 44-1150G, RRID:AB_2533585); Abcam: ATP5A (Cat# ab176569, RRID:AB_2801536), PGC1 alpha + beta (Cat# ab72230, RRID:AB_1640773); R&D Systems: UCP1 (Cat# MAB6158, RRID:AB_10572490); Millipore Sigma: LAMA4 (Cat# SAB4501719, RRID:AB_10744529), Complex 1-75kD (Cat# ABN302, RRID:AB_2915902).

RNA Extraction and Quantitative Real-Time PCR

RNA was isolated using the E.Z.N.A Total RNA Kit II (Omega Biotek; R6934) for murine studies or RNeasy Mini Kit (Qiagen; 74104) for hiPSC studies following the manufacturer's instructions. Tissue samples were disrupted using a dispersion-based homogenizer (VWR VDI 12). The RNA samples were reverse transcribed using Quanta QScript Master Mix (VWR; 95048) and 500 ng RNA per 20μL sample reaction volume. Quantitative Real-Time PCR was performed with SYBR green using a Bio-Rad CFX Connect Real-Time PCR Detection System. Primers were purchased from IDT or Qiagen; IDT primer sequences can be found in **Table 1**. For all samples, GAPDH was used as the housekeeping gene. Gene expression was evaluated by ddCT methods.

Statistics

Statistical tests for all *in vitro* studies where n was equal between groups and samples were paired, a student's paired t-test was performed for all data. In studies where samples were paired,

data was normalized to the paired control, which was assigned a value of 1.0, to account for variability in differentiation efficiency between experiments, and in expression of LAMA4 between primary cells derived from different mice. In all studies involving whole tissue from mice, comparisons between WT and *Lama4-/-* mice were assessed with a Welch's unequal variances t-test as variance between mice was expected to be high among the WT due to differences in LAMA4 expression and low among the *Lama4-/-* mice. In all cases, $p<0.05$ was considered significant.

3.3 Results

3.3.1 LAMA4 deficiency promotes mitochondrial biogenesis

Previously, we had shown that LAMA4 deficiency *in vivo* leads to elevated thermogenesis through increased expression of UCP1 in sWAT of male mice [132]. Following these results, we wished to better understand how these observed changes in thermogenesis may extend to changes in mitochondrial biogenesis. Initially, we determined that the protein expression of PGC-1, a master regulator of mitochondrial biogenesis, was significantly upregulated in the sWAT of *Lama4-/-* male mice compared to WT (Fig. 3.1a,b). This led us to look at the expression of mitochondrial complex 1 subunit 1 and 2 (*mt-Nd1* and *mt-Nd2*), commonly used to assess mitochondrial content. We found the mRNA levels of both genes to be significantly elevated in *Lama4-/-* sWAT (Fig. 3.1c). Complex 1-75kD protein expression, in addition to voltage-dependent anion channel (VDAC) and ATP synthase F1 subunit alpha (ATP5A) protein expression, were all significantly increased in *Lama4-/-* sWAT compared to controls (Fig. 3.1d). The increased expression of multiple mitochondrial content markers indicates a larger mitochondrial load and is suggestive of enhanced mitochondrial biogenesis in sWAT lacking LAMA4, as driven by elevated PGC-1 expression.

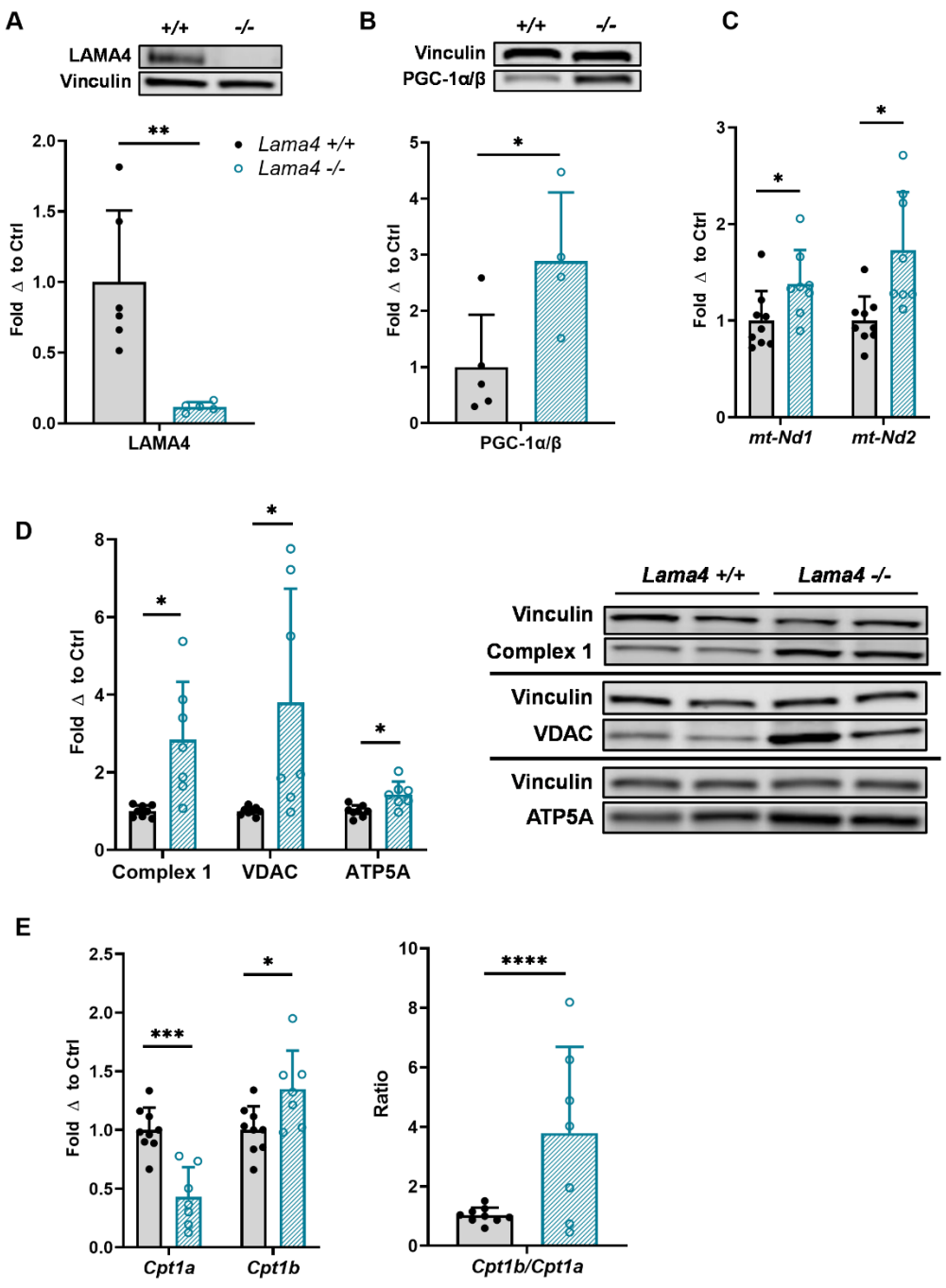

Figure 3.1 LAMA4 deficiency elevates mitochondrial biogenesis

(A) Protein expression of LAMA4 in sWAT of WT (n=6) and *Lama4-/-* (n=5) male mice was assessed by western blot. Reported as fold change in respect to average of WT mice after normalization to loading control (Vinculin) signal. (*,**,***) indicates p < 0.05, 0.01, 0.001,

Figure 3.1 continued: respectively. Data are Means + SD. **(B)** Protein expression of PGC-1α/β in sWAT of WT (n=5) and *Lama4-/-* (n=4) male mice was assessed by western blot. Vinculin was used as the loading control for normalization. **(C)** Relative *mt-Nd1* and *mt-Nd2* mRNA expression in sWAT of WT (n=9) and *Lama4-/-* (n=8) male mice. **(D)** Protein expression of Complex 1-75kD, VDAC, and ATP5A in sWAT of WT (n=8) and *Lama4-/-* (n=7) male mice was assessed by western blot. Vinculin was used as the loading control for normalization. **(E)** Relative *Cpt1a* and *Cpt1b* mRNA expression (left) or *Cpt1b/Cpt1a* ratio (right) in sWAT of WT (n=9) and *Lama4-/-* (n=7) male mice.

In addition to mitochondrial content markers, we identified a change in carnitine palmitoyltransferase 1 (CPT1) isoform expression. CPT1 shuttles long-chain fatty acids across the mitochondrial membrane to supply substrate for the rate-limiting step in fatty acid oxidation. The CPT1A isoform is more abundantly expressed in murine and human WAT, although expression of CPT1 overall is quite low in WAT. CPT1B is the isoform expressed in energetic tissues like muscle and brown adipose tissue (BAT). We found that the expression *Cpt1a* was reduced, while the expression of *Cpt1b* was elevated in *Lama4-/-* sWAT compared to controls, and overall, the ratio of *Cpt1b/Cpt1a* expression was significantly increased (Fig. 3.1e). This shift towards the *Cpt1b* isoform indicates a more brown-like adipose depot in the absence of LAMA4.

3.3.2 Acute silencing of LAMA4 in adipocytes enhances thermogenic gene expression

During our *in vivo* investigation, we noted variability in our results, particularly with WT mice, that seemed to be due to the wide range of LAMA4 expression that can occur in adipose of WT mice, a phenomenon described in our most recent publication [181]. In order to study the effect of LAMA4 signal transduction in a more controlled manner, we therefore next pursued *in vitro* silencing experiments. This would also aid us in isolating intracellular signaling changes occurring as a result of an acute downregulation of LAMA4 rather than a complete genetic knockout model, which may be more therapeutically insightful. We used WT murine adipose-derived stem cells (ADSCs) differentiated to beige adipocytes for this study as they lipid loaded

robustly and show an improved capacity for thermogenesis compared to 3T3-L1 lines. We transfected the cells with siRNA targeting *Lama4* on Day 10 and 14 of the 16 day differentiation program and yielded an average of 60-70% silencing efficiency (Fig. 3.2a).

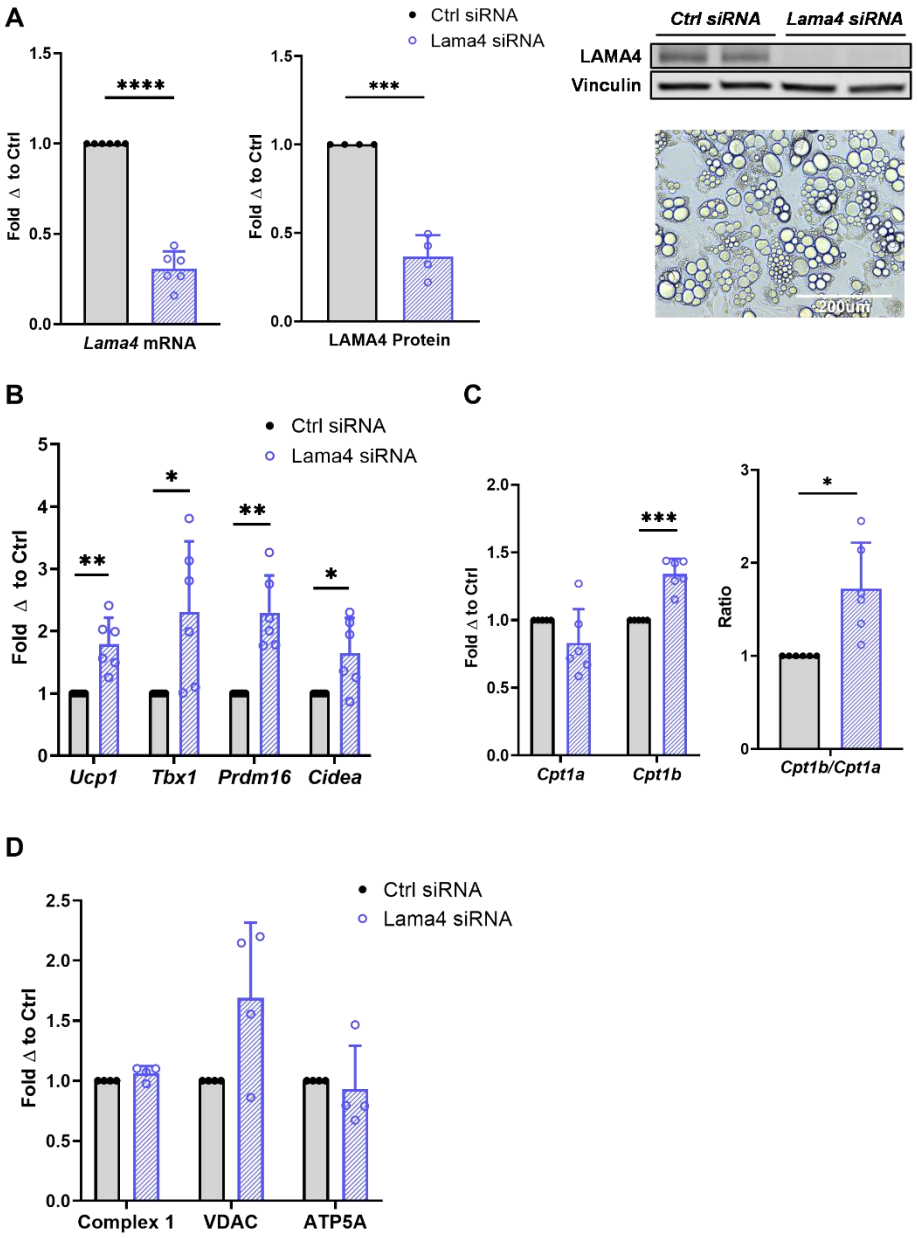

Figure 3.2 Acute silencing of LAMA4 in murine adipocytes enhances thermogenic gene expression

Figure 3.2 continued: (A) Relative *Lama4* mRNA expression and LAMA4 protein expression in adipocytes treated with control siRNA (n=6) and Lama4 siRNA (n=6). Reported as fold change in respect to paired control. (*,**,***,****) indicates $p < 0.05$, 0.01, 0.001, 0.0001, respectively. Data are Means + SD. Includes representative image at 20X magnification of differentiated adipocytes at time of collection. **(B)** Relative *Ucp1, Tbx1, Prdm16,* and *Cidea* mRNA expression in adipocytes treated with control siRNA (n=6) and Lama4 siRNA (n=6). **(C)** Relative *Cpt1a* and *Cpt1b* mRNA expression (left) or *Cpt1b/Cpt1a* ratio (right) in adipocytes treated with control siRNA (n=6) and Lama4 siRNA (n=6). **(D)** Protein expression of Complex 1-75kD, VDAC, and ATP5A in adipocytes treated with control siRNA (n=6) and Lama4 siRNA (n=6) was assessed by western blot. Vinculin was used as the loading control for normalization.

The beige adipocyte markers *Ucp1*, T-box 1 (*Tbx1*), PR domain containing 16 (*Prdm16*), and cell death activator CIDE-A (*Cidea*) were all significantly upregulated in the LAMA4 siRNA transfected adipocytes compared to control siRNA transfected adipocytes (Fig. 3.2b). Similar to the *in vivo* results, we also found a significant increase in the ratio of *Cpt1b/Cpt1a* expression upon LAMA4 silencing *in vitro* (Fig. 3.2c).

We noted that mitochondrial protein expression (Complex 1-75kD, VDAC, ATP5A) was not increased in these samples, perhaps due to the acute nature of the silencing, or the fact that some LAMA4 protein expression remained (approximately 30%) (Fig. 3.2d). Extracellular matrix proteins are more stable and less frequently replaced than intracellular proteins, which could feasibly limit the true window of silencing at the protein level to less than 6 days [184]. This acute silencing illustrates that, although enhanced beige marker expression can be achieved within a short timeframe, changes in mitochondrial content may require a longer period of silencing to be visualized, or the complete absence of LAMA4.

3.3.3 LAMA4 silencing upregulates expression of the AMPK-PGC-1α pathway

Following these results, we chose to assess intracellular signaling pathways associated with beiging and mitochondrial biogenesis that might be altered. Although we did not observe

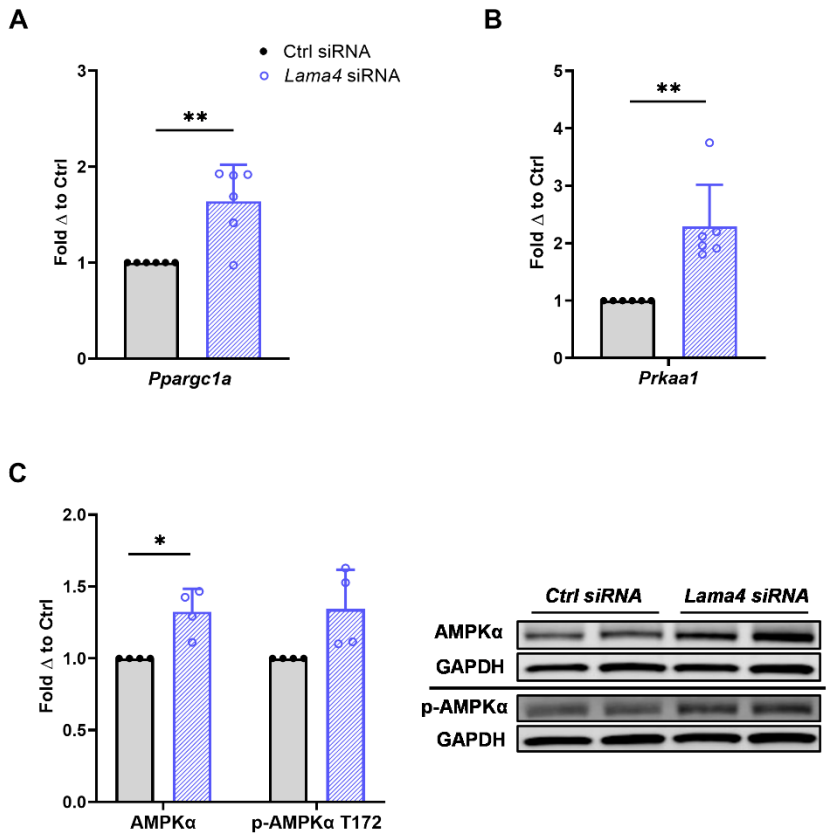

Figure 3.3 The AMPK-PGC-1α pathway is upregulated when LAMA4 is silenced

(A) Relative *Ppargc1a* mRNA expression (PGC-1α) in adipocytes treated with control siRNA (n=6) and Lama4 siRNA (n=6). Reported as fold change in respect to paired control. (*,**) indicates $p < 0.05$, 0.01, respectively. Data are Means + SD. **(B)** Relative *Prkaa1* mRNA expression (AMPKα) in adipocytes treated with control siRNA (n=6) and Lama4 siRNA (n=6). **(C)** Protein expression of AMPKα and p-AMPKα T172 in adipocytes treated with control siRNA (n=4) and Lama4 siRNA (n=4) was assessed by western blot. GAPDH was used as the loading control for normalization.

significant upregulation in mitochondrial content, we did find that LAMA4 silencing resulted in significantly elevated expression of the PGC-1α gene, *Ppargc1α* (Fig. 3.3a). In our prior publication, we reported that differentiation of murine ADSCs to beige adipocytes on LAMA4 coated surfaces leads to diminished PGC-1α expression [133]. Considering this relationship across multiple studies and models, we elected to investigate AMP-activated protein kinase (AMPK), a

serine/threonine kinase that has been implicated in adipocyte beiging and mitochondrial biogenesis [185]. AMPK lies upstream of PGC-1α activation and has also been associated with the regulation of the cell surface proteome, specifically β1-integrins [186]. Previous research has shown that silencing of AMPKα in the adipose tissue of mice produces diminished thermogenic capacity, while activation of AMPK in adipose enhances thermogenic gene expression [185].

We discovered a significant increase in the mRNA levels of the AMPKα gene, *Prkaa1*, when LAMA4 was silenced (Fig. 3.3b). Immunoblotting revealed that total protein levels of AMPKα were also elevated, as was the phosphorylation of AMPKα at T172, suggesting that the downregulation of LAMA4 leads to an elevation in AMPK activity, predominantly through an upregulation in AMPKα transcription (Fig. 3.3c). Our results suggest that, under LAMA4 silencing, the AMPK – PGC-1α pathway is upregulated and drives thermogenic gene expression.

3.3.4 LAMA4 suppresses thermogenic gene expression and AMPK in human adipocytes

Having shown that the acute silencing of LAMA4 could elevate thermogenic markers and AMPKα expression, we wanted to perform the converse experiment to determine if LAMA4 could directly inhibit thermogenesis and AMPKα expression. In past studies, we have demonstrated that the culture of murine *Lama4-/-* adipocytes on *Lama4+/+* derived ECM could quell the observed increases in *Ucp1* expression, suggesting that LAMA4 may be a direct negative regulator of adipocyte beiging [132]. Furthermore, we have shown more recently that differentiation of murine ADSCs to beige adipocytes in the presence of LAMA4 diminishes *Ucp1* expression [133]. However, these experiments did not investigate signaling intermediates such as AMPKα and were limited to murine thermogenic adipocyte models. It was not yet understood if this potential regulatory role would be applicable to human adipocyte models.

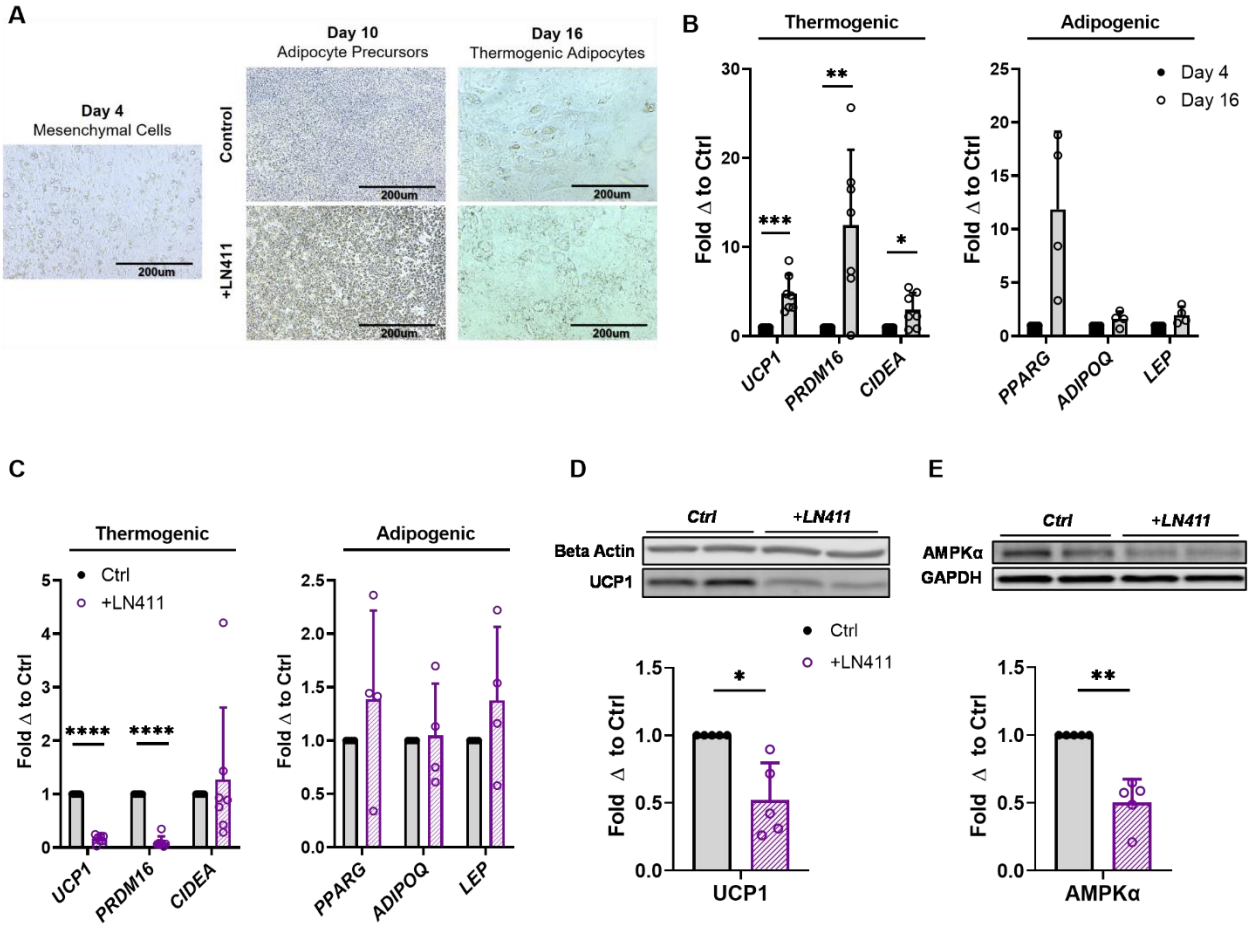

Figure 3.4 LAMA4 negatively regulates thermogenic gene expression in human adipocytes

(A) Representative images outlining differentiation and treatment trajectory of human induced pluripotent stem cells (hiPSCs) to beige adipocytes. Images were taken at 20X magnification. **(B)** Relative mRNA expression of thermogenic genes UCP1, PRDM16, CIDEA (n=7) and adipogenic genes PPARG, ADIPOQ and LEP (n=4) in hiPSCs on Day 4 and Day 16 of beige adipocyte differentiation. Reported as fold change in respect to paired control. (*,**,***,****) indicates $p < 0.05, 0.01, 0.001, 0.0001$, respectively. Data are Means + SD. **(C)** Relative mRNA expression of thermogenic genes UCP1, PRDM16, CIDEA (n=7) and adipogenic genes PPARG, ADIPOQ and LEP (n=4) in human beige adipocytes treated with or without LN411 during differentiation. **(D)** Protein expression of UCP1 in human beige adipocytes treated with or without LN411 during differentiation (n=5). Beta Actin was used as the loading control for normalization. **(E)** Protein expression of AMPKα in human beige adipocytes treated with or without LN411 during differentiation (n=5). GAPDH was used as the loading control for normalization.

To explore this possibility , we took advantage of direct differentiation of thermogenic adipocytes derived from hiPSCs (Fig. 3.4a). hiPSCs were successfully differentiated to beige adipocytes by Day 16 as shown through the elevation of beige markers *UCP1*, *PRDM16,* and *CIDEA,* and adipocyte markers peroxisome proliferator activated receptor gamma (*PPARG)* and adiponectin *(ADIPOQ)* (Fig. 3.4b). When human recombinant laminin-411 (LN411), which is comprised of the laminin-α4, laminin-β1, and laminin-γ1 chain, was included during differentiation, significant reductions in mRNA levels of thermogenic markers *UCP1* and *PRDM16* were observed (Fig. 3.4c). In addition to the reduction in *UCP1* mRNA levels, a significant decrease in UCP1 protein expression is detected in the LN411 treated adipocytes (Fig. 3.4d). Lastly, we found that LN411 treatment resulted in a decrease in total AMPKα levels, further supporting the idea that LAMA4 regulates AMPKα signaling (Fig. 3.4e). These results suggested that LAMA4 directly inhibits a thermogenic phenotype in human adipocytes through the suppression of AMPKα levels.

3.3.5 Silencing of Integrin Linked Kinase mimics the effects of LAMA4 silencing in adipocytes

Our data suggested a direct link between LAMA4 and the negative regulation of thermogenesis and the AMPK-PGC-1α pathway, therefore, we expanded our investigation towards identifying downstream targets of LAMA4. We previously established that integrin signaling is reduced in LAMA4 deficient beige adipose, specifically through the downregulation of integrin alpha 7 (ITGA7) and integrin beta 1 (ITGB1) [133]. Moreover, silencing of ITGA7 resulted in enhanced thermogenic gene expression, suggesting that the LAMA4-ITGA7 interaction could mediate the suppression of thermogenesis in adipocytes [133]. Integrins, a major class of transmembrane receptors that operate via heterodimers of an α and β subunit, are heavily implicated in ECM binding and cell signaling. Adaptor proteins downstream of integrins

participate in outside-in signaling cascades to alter cellular function [187]. During our study, we identified one such adaptor protein, integrin-linked kinase (ILK), which we hypothesized to be involved in adipocyte regulation via LAMA4.

ILK is a serine-threonine kinase that transduces extracellular signals through its interactions with the β1-subunit of integrins and the formation of signaling complexes. It is not fully understood how ILK is activated under different circumstances and its activity as a kinase or pseudokinase is highly debated due to unusual aspects of the kinase domain [188]. It has also been suggested that even if ILK does possess kinase capacity, the kinase and signaling activities may be independent of one another [189]. In the literature, ILK has been linked to laminin signaling in numerous cell types including muscle and neurons, and even directly to LAMA4 in human diseases and human endothelial cell differentiation [188,190]. This connection led us to study the potential role of ILK in linking LAMA4 signaling and adipocyte thermogenesis.

We began by assessing ILK expression in our *in vivo* mouse model. We found that the expression of ILK was significantly diminished on both the mRNA and protein level in the sWAT of *Lama4-/-* mice compared to controls (Fig. 3.5a). This suggested that, in the complete and long-term absence of LAMA4, ILK expression was downregulated in adipocytes. Surprisingly, when we measured ILK expression following the acute silencing of LAMA4 *in vitro*, we found a small but significant upregulation in expression (Fig. 3.5b). However, due to the numerous post-translational modifications that can regulate ILK function, these results were not necessarily indicative of ILK activity.

In order to more concretely determine if ILK could regulate thermogenic gene expression in adipocytes, we decided to proceed with direct silencing. We transfected adipocytes with siRNA targeting *Ilk* in the same manner as previously described and achieved efficient silencing on the

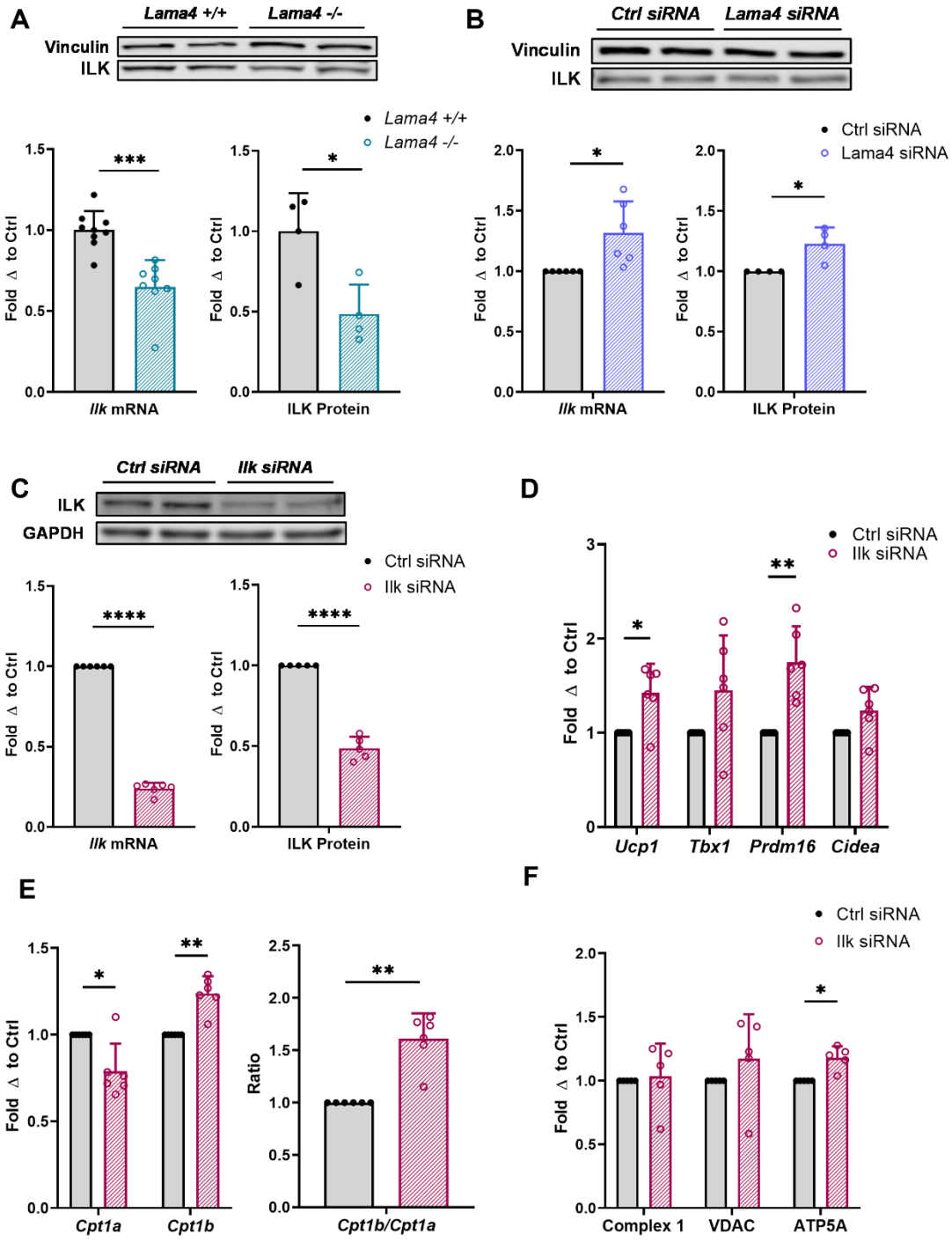

Figure 3.5 Silencing of ILK in murine adipocytes elevates thermogenic gene expression

(A) Relative *Ilk* mRNA expression (WT n=9, *Lama4-/-* , n=8) and ILK protein expression (WT n=4, *Lama4-/-* , n=4) in sWAT of male mice. Reported as fold change in respect to average of WT mice after normalization. (*,**,***,****) indicates $p < 0.05, 0.01, 0.001, 0.0001$, respectively.

Figure 3.5 continued: Data are Means + SD. **(B)** Relative *Ilk* mRNA expression and ILK protein expression in adipocytes treated with control siRNA (n=6) and Lama4 siRNA (n=6). Reported as fold change in respect to paired control. **(C)** Relative *Ilk* mRNA expression and ILK protein expression in adipocytes treated with control siRNA (n=5) and Ilk siRNA (n=5). Reported as fold change in respect to paired control. **(D)** Relative *Ucp1, Tbx1, Prdm16,* and *Cidea* mRNA expression in adipocytes treated with control siRNA (n=5) and Ilk siRNA (n=5). **(E)** Relative *Cpt1a* and *Cpt1b* mRNA expression (left) or *Cpt1b/Cpt1a* ratio (right) in adipocytes treated with control siRNA (n=5) and Ilk siRNA (n=5).

mRNA and protein level (Fig. 3.5c). Markers of beige adipocytes were again upregulated compared to the control siRNA condition, although not to the same magnitude as during LAMA4 silencing (Fig. 3.5d). We also observed the same CPT1 isoform shift as previously seen, a significant downregulation of *Cpt1a* and upregulation of *Cpt1b* expression, when ILK was silenced (Fig. 3.5e). Although there was no significant elevation in the expression most mitochondrial proteins in the ILK silenced samples, we did observe a small but significant increase in the expression of the mitochondrial protein ATP5A (Fig. 3.5f).

Lastly, ILK silencing led to a slight upregulation in *Ppargc1a* expression (P=0.055) and significant upregulation in AMPKα expression on the mRNA and protein levels, just as was observed when LAMA4 was silenced (Fig. 3.6a-c). These results suggest that ILK is capable of regulating thermogenic gene expression and the AMPK-PGC-1α pathway in adipocytes in a similar manner to LAMA4.

3.4 Discussion

During the course of this study, we investigated the inhibitory effect of the extracellular matrix protein LAMA4 on adipocyte thermogenesis and mitochondrial biogenesis in multiple models. We showed that the acute silencing of LAMA4 during beige adipocyte differentiation allowed for a greater elevation in thermogenic gene expression, and that the treatment of human

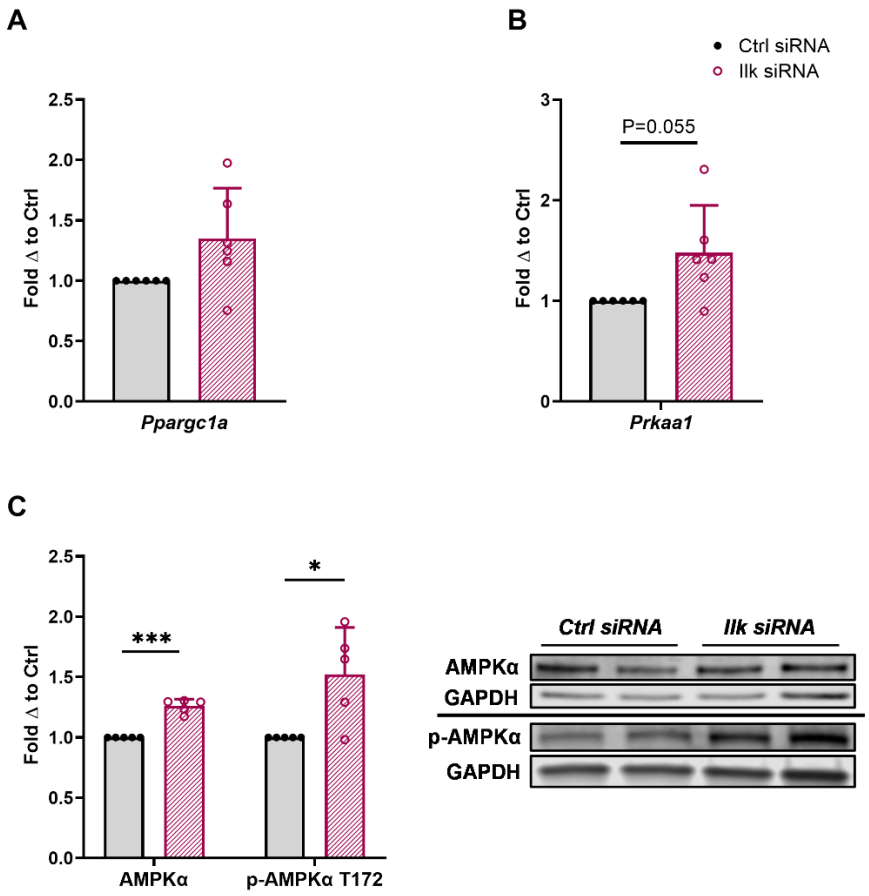

Figure 3.6 AMPKα expression increases when ILK is silenced

(A) Relative *Ppargc1a* mRNA expression (PGC-1α) in adipocytes treated with control siRNA (n=5) and Ilk siRNA (n=5). Reported as fold change in respect to paired control. (*,**,***) indicates $p < 0.05$, 0.01, 0.001, respectively. Data are Means + SD. **(B)** Relative *Prkaa1* mRNA expression (AMPKα) in adipocytes treated with control siRNA (n=5) and Ilk siRNA (n=5). **(C)** Protein expression of AMPKα and p-AMPKα T172 in adipocytes treated with control siRNA (n=5) and Ilk siRNA (n=5) was assessed by western blot. GAPDH was used as the loading control for normalization.

adipocytes with LN411 during beige differentiation inhibited UCP1 expression. We also found that both the complete genetic knockout and acute silencing of LAMA4 led to elevations in adipocyte expression of PGC-1α, a master regulator of mitochondrial biogenesis. In studying mechanisms by which this regulation may occur, we found that ILK expression was downregulated

in sWAT of *Lama4-/-* mice, and that silencing of ILK in adipocytes resulted in elevated thermogenic gene expression. Interestingly, both LAMA4 and ILK silencing led to the elevation of AMPKα expression, while treatment of human beige adipocytes with LN411 suppressed AMPKα expression. Overall, these results indicate that LAMA4 negatively regulates the AMPK-PGC-1α pathway to inhibit adipocyte beiging, and that this regulation may occur in part through integrin signaling and ILK.

One of the main outcomes of this study was the identification of LAMA4 as a negative regulator of AMPKα levels. AMPK is a serine/threonine kinase involved in maintaining cellular energy levels and is known to negatively regulate adipogenesis and promote a brown adipocyte phenotype [191]. AMPK becomes active when AMP and ADP levels are high in comparison to ATP, and functions to minimize energy intensive processes while increasing ATP production. When activated, AMPK increases fatty acid uptake and oxidation in addition to increasing the expression and activity of PGC-1α, leading to elevations in mitochondrial biogenesis [192]. Previous studies have shown that the direct activation of AMPKα in murine sWAT promoted beiging and improved metabolic parameters, while silencing AMPKα in sWAT compromised adaptive thermogenesis [185]. Our results suggest that LAMA4 acts to suppress AMPKα levels, likely for the promotion of white adipogenesis, while the silencing of LAMA4 relieves this inhibition and allows for the development of a more thermogenic phenotype.

Interestingly, AMPK also enhances CPT1 activity by phosphorylating and inhibiting acetyl-CoA carboxylases (ACC) [185]. The majority of work studying shifts in CPT1 isoform expression has taken place in cardiomyocytes and suggests that shifting isoform expression can lead to changes in long chain fatty acid (LCFA) oxidation rates. Specifically, that overexpression of CPT1a leads to reductions in LCFA oxidation rates [193]. As we saw an increase in the ratio of

Cpt1b to *Cpt1a* expression with LAMA4 and ILK silencing, it would be interesting to understand what the basis for this shift is, and how the change may interact with elevated AMPKα expression. While it is as yet unclear what mechanisms may be at play in this expression shift, the parallel changes in expression are suggestive of a link between LAMA4 and ILK signaling related to enhanced fatty acid oxidation.

We also identified integrin linked kinase as a protein of interest involved in LAMA4 signal transduction in adipocytes. ILK possesses several means of activation, few of which are well understood. It can execute downstream functions through enzymatic activity, protein-protein interactions, or localization to specific regions of the cell [194]. Historically, ILK's function as a kinase or a pseudokinase has been controversial, including how its various behaviors are stimulated. ILK has been proposed to be auto-phosphorylated at Ser-343, but it is unknown exactly how this contributes to its activity. It can also be phosphorylated at Thr-173 and Ser-246, but again, little is known about how these phosphorylation states or other post-translational modifications alter activity [188,194]. In addition, multiple isoforms of ILK exist and purportedly display differing characteristics, although ILK1 is the most commonly studied and typically the isoform that most commercial ILK antibodies recognize [188]. As interest in ILK grows, and tools to study its activity and various isoforms are more available, we may develop an understanding of how LAMA4 modulates ILK function and signaling in the future.

Within the scope of this study, we showed that ILK expression is downregulated in sWAT *in vivo* when LAMA4 is absent and that silencing of ILK leads to many of the same downstream changes as observed when LAMA4 is silenced, including elevations in thermogenic gene expression, PGC-1α expression, and AMPKα expression, in addition to a shift in CPT1 isoform expression. Unfortunately, we were unable to assess direct changes in ILK activity or signaling in

response to LAMA4 silencing at this time. More studies are needed in the future to determine if ILK is definitively linked to the regulation of adipocyte thermogenesis and mitochondrial biogenesis by LAMA4.

Lastly, another main conclusion we drew from this study was the applicability of this LAMA4 regulatory function in human adipocytes. Our results established that LAMA4 acts to negatively regulate the thermogenic program in human adipocytes, which is a significant finding for therapeutic and tissue engineering related applications. As laminin is becoming more frequently incorporated into 3D scaffolds for tissue growth, it is important to be conscious of which α chains may be more functional in supporting specific outcomes, either in stem cell differentiation, or therapeutics [195–197]. Additionally, functional blocking of the LAMA4 chain may be a viable future therapeutic target to promote the beiging of white adipose tissue and combat obesity. Further work is needed to assess how the downregulation of LAMA4 signaling might impact adipose *in vivo*, and to determine ideal silencing timeframes to achieve effective enhancements in energy expenditure.

CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS

In conclusion, the results of the two studies described in this dissertation illustrate how laminin-α4 (LAMA4) functions to maintain a white adipocyte phenotype and aid in adipose expansion, while inhibiting adipocyte beiging in both murine and human adipose models. Firstly, a significant association was shown between adiposity and elevated LAMA4 expression *in vivo* during the course of a human bariatric surgery study and a murine dietary study. Secondly, the mechanism by which LAMA4 negatively regulates adipocyte thermogenesis and mitochondrial biogenesis was investigated in murine and human beige adipocytes. This final chapter will provide a summary of the two studies and discuss future directions to further our understanding of LAMA4 action in adipocytes and determine the potential for its therapeutic targeting in metabolic disease.

4.1 LAMA4 is Associated with Obesity and White Adipose Expansion

Early efforts to profile the extracellular matrix composition during adipogenesis revealed that 3T3-L1 cells predominantly express the laminin-411 isoform, and that this expression increases by nearly 2.5-fold during differentiation [114]. Later work uncovered that human mesenchymal stromal cells induced to become adipocytes also demonstrated increasing expression of LAMA4 during differentiation [113]. There is an evident tissue-specific expression pattern linking LAMA4 to white adipocyte differentiation from multiple precursor cell types of human and murine origin. Nevertheless, the majority of research efforts pushing to shape an understanding of ECM dynamics during adipose tissue expansion, particularly in the context of obesity or metabolic disease, have focused on collagen isoforms.

In this investigation, we demonstrated a significant elevation in LAMA4 mRNA and protein expression in the sWAT of human subjects with obesity and mice with diet-induced obesity

compared to controls, which was unaltered following weight-loss. Furthermore, the culture of murine adipocytes on laminin-411 led to a small but significant increase in lipid loading (as measured by ORO) and a decrease in perilipin-1 mRNA expression. This suggests that increasing levels of LAMA4 may promote lipid uptake and/or storage in white adipocytes. However, is not yet fully clear which element arises first. The uptick in LAMA4 expression could result from an increased number of differentiating adipocytes or could be a purposeful response mechanism to adipocytes under the duress of excessive lipid storage to promote the differentiation of surrounding cells towards white adipocytes. Indeed, it is now known that the transcription factor zinc finger E-box-binding homeobox 1 (ZEB1), a central regulator of adipogenesis and white adipocyte differentiation, can induce the transcription of the *Lama4* gene [198,199]. Future experiments could determine if silencing LAMA4 during excessive lipid loading in white adipocytes *in vitro*, such as in the case of oleic acid treatment, could diminish overall adipogenesis and lipid loading.

In summary, our work comprehensively demonstrates that the significant upregulation in LAMA4 surrounding subcutaneous white adipocytes resulting from adipose expansion is unique among laminin-α chains and parallel between humans and mice. This finding solidifies the clinical relevance of LAMA4 to the study of endocrine diseases and supports our dual-species *in vitro* approach to interrogating the mechanism through which LAMA4 mediates intracellular signaling in our next study.

4.2 LAMA4 Negatively Regulates Adipocyte Thermogenesis

Following our prior, more observational study of LAMA4 and obesity, we endeavored to directly demonstrate the negative regulatory role of LAMA4 on adipocyte thermogenesis and identify key signaling intermediates. The majority of our previous work had focused on a complete knockout model to study the effects of LAMA4 in adipose tissue, which limited the therapeutic

application of any findings. Complete deficiency or genetic knockout of a protein from birth is rarely a viable approach for any human therapy. It would be much more worthwhile to study the acute knockdown of LAMA4 following adipocyte differentiation to determine, in a more clinically relevant manner, if the inhibition of LAMA4 signaling had therapeutic merit.

Based on this approach, we identified a few key areas and intracellular proteins of interest in the *Lama4-/-* model, such as mitochondrial biogenesis driven by PGC-1α and integrin linked kinase (ILK), to study in an *in vitro* acute silencing model. We found that in only six days of silencing the quantity of LAMA4 protein could be reduced by more than 50% and result in a significant elevation in thermogenic gene expression. These results strengthened our argument that LAMA4 could be targeted therapeutically to enhance adipocyte thermogenesis.

Our most intriguing mechanistic findings centered around the AMPK-PGC-1 pathway. Beige adipocytes in which LAMA4 was silenced displayed increased expression of PGC-1α and AMPKα. AMPKα was similarly elevated when ILK was silenced. Conversely, treating human beige adipocytes (derived from iPSCs) with recombinant LN411 during differentiation drastically suppressed AMPKα expression. AMPK functions as an anti-adipogenic regulator and favors a brown over white adipocyte phenotype, while LAMA4 functions as a positive regulator of adipogenesis [191]. Thus, LAMA4 may act to suppress AMPK expression and activity in order to promote adipogenesis, and in relieving this suppression, we allow for AMPK activity to proceed. This would lead to the many downstream activities associated with AMPK, namely activation of PGC-1α, mitochondrial biogenesis, increased fatty acid oxidation, and thermogenic gene expression.

To support this hypothesis, further studies could explore the mechanism by which *Prkaa1* gene transcription is regulated by LAMA4 and ILK. Online tools are now available to help model

and predict what transcription factors may bind to a particular promotor and could be used to identify candidate proteins. Once narrowed down, ChIP-seq could be used to determine if these transcription factors bind the *Prkaa1* promotor. Any successful candidates could then be investigated for differences in activity or expression in adipocytes when LAMA4 is silenced or absent. In this manner, one could identify specifically how LAMA4 regulates AMPKα mRNA and protein levels.

Lastly, although this study identified ILK as a likely signaling intermediate of this regulation, more work is needed to verify that ILK is responsible for the LAMA4 mediated inhibition of thermogenesis. While we observed many parallel results when LAMA4 and ILK were silenced, the expression of PGC-1α was upregulated only in the LAMA4 silencing conditions, suggesting mechanisms aside from or in addition to ILK that may regulate mitochondrial biogenesis. This study focused on integrin-based laminin signaling, but laminins are known to interact with many other receptor types, as described in Chapter 1. Future research should investigate the contribution of these other cell-surface receptor signaling pathways to thermogenic regulation in adipocytes.

4.3 Future Directions

The majority of our group's work up until this point has been building toward the notion that LAMA4 can be targeted for therapeutic purposes in diseases like obesity and T2D to enhance energy expenditure in adipose depots. We have direct evidence to support that the acute silencing of LAMA4 in mature adipocytes is sufficient to elevate thermogenic gene expression, however it remains to be discovered what specific domains of LAMA4 contribute to the observed regulation, and whether we can effectively block LAMA4 signaling in adipose tissue *in vivo*. Furthermore, other modes of inquiry could involve investigating the role of LAMA4 in mediating immune

infiltration in white adipose tissue during obesity, considering previous links between laminins and inflammation in other tissue types.

4.3.1 Determining Functional Domains

All laminin-α chains contain five LG domains (LG1-5) which are known to function as binding sites for cell-surface receptors, to bind growth factors and/or calcium, and interact with other ECM proteins to facilitate network formation. The laminin-α1 (LAMA1) LG domains have been studied in great detail through mutagenesis studies and the use of proteolytic laminin fragments. These studies have resulted in a greater understanding of the structure-function relationship between LAMA1 LG sequences and signaling events. For instance, the E3 fragment (α1LG4–5) contains bindings sites for heparin, the ECM protein fibulin, and the α-dystroglycan receptor, while the E8 fragment (α1LG1-3) has been shown to bind α3β1, α6β1, α6β4, α7β1 and α9β1 integrins [112,145]. Evidently, particular LG domains, and even specific sequences within these domains, contribute to distinct binding effects.

Although research concentrated on mapping the LAMA4 LG domains has been more limited than for the LAMA1 and LAMA2 chains, there are some studies which help to illustrate explicit sequence-function relationships within the LAMA4 LG domains. In 2002, Gonzalez et al. found that a particular sequence spanning the LG1-2 domains of LAMA4 (residues 919 – 1207) was involved in angiogenic signaling in endothelial cells [200]. Using an antibody against this sequence, they were able to block cell adhesion and interaction with αvβ3, α3β1, and α6β1 integrins to limit blood vessel development. Around the same time, Okazaki et al. performed a large-scale screen of synthetic peptide fragments corresponding to sequences across the entire LAMA4 G domain and discovered 20 biologically active peptides which could promote cell attachment [201]. They also found that several of these could bind heparin and/or the syndecan-4

receptor. However, these studies were limited by the knowledge available at the time, when cell adhesion was the most prominent perceived role of laminins. Now that laminins are more heavily implicated in guiding cell differentiation and utilized in tissue engineering applications, a more detailed study should be performed to determine the regions of the LAMA4 LG domains that correspond to specific cellular reactions across various cell types.

In the context of this dissertation, and for the potential therapeutic application of LAMA4 inhibition in adipose, the LG domain and sequence within it that regulates thermogenesis and AMPKα expression in adipocytes should be determined. This project would involve two phases of work: (1) utilizing peptide fragments of the LG domains to determine regions capable of inhibiting adipocyte thermogenesis, and (2) creation and testing of antibodies against target regions discovered in phase 1 for ability to enhance thermogenesis. It is likely, based on the findings of Gonzalez et al., that this target region may lie within the LG1-2 domains. This is because ILK, which may be involved in the LAMA4 signal transduction regulating thermogenesis, is also known to be involved in angiogenesis [188]. Unfortunately, very few peptides or blocking antibodies corresponding to the LAMA4 LG domains are commercially available at the moment, so this prospective study would require the design and synthesis of various overlapping synthetic fragments of the LG1 and LG2 domains. Once produced, these fragments could be coated onto cell culture plates or added to differentiation media as treatments onto beige adipocytes. Those, if any, that elicit reductions in thermogenic gene expression would be candidates for antibody design. Finally, these functional blocking antibodies could be assessed for their capacity to heighten thermogenic gene expression and AMPKα expression in cultured white adipocytes.

4.3.2 Therapeutic Targeting of LAMA4 in vivo

The final component to be evaluated is the viability of targeting LAMA4 for therapeutic application *in vivo.* Although our group has shown that the genetic knockout of *Lama4* in a mouse model leads to elevated thermogenesis in sWAT, it still remains to be tested whether incomplete silencing located only to white adipose tissue depots would be sufficient to cause heightened thermogenesis leading to adipose mass reduction. This could be investigated through many means, such as the use of functional blocking antibodies, *in vivo* RNAi, or creation of a tamoxifen-inducible *Lama4* knockout mouse model, all involving administration by injection into white adipose depots. Depending on the methodology and approach, a minimal threshold level of LAMA4 silencing sufficient to achieve a significant thermogenic response could be determined.

Assuming that this could successfully be achieved in mice, multiple parameters could be assessed following administration to ascertain the therapeutic benefit. For instance, once could investigate how the silencing or blocking of LAMA4 in WAT in mice with diet-induced obesity alters metabolic outcomes such as weight loss, insulin sensitivity, and inflammation. If these outcomes could be achieved, the next aspect of the investigation could focus on identifying effective treatment durations and measuring how long the after-effects could be sustained after ending treatment. This would shape our understanding of how feasible LAMA4 targeting would be *in vivo.*

4.3.3 Regulation of immune infiltration in WAT by LAMA4

Accumulating evidence suggests that the cell microenvironment, particularly the structure and composition of the ECM, plays a role in the ability of immune cells to infiltrate the surrounding regions. Much of this research has been completed in the context of cancer and tumor microenvironments. Studies have reported that ECM composition can modulate the

movement of immune cells, the phenotype of immune cells, and even alter immune cell metabolism. Although collagens are likely the largest contributors to physical barriers to immune infiltration, laminins have been implicated in immune cell movement as well.

As the expression of LAMA4 has been implicated in many cancer types, including gastric cancer, renal cell carcinoma, and hepatocellular carcinoma, it has been studied in depth in relation to tumor inflammation. Several of these studies have shown strong correlations between LAMA4 expression and immune infiltration, and suggest that LAMA4 regulates tumor-associated macrophage polarization [202]. Interestingly, studies of laminin expression and immune migration have describe LAMA5 and LN-511 as a barrier to T cell extravasation, whereas LAMA4 and LN-411 are permissive to immune infiltration and help to recruit T cells to certain tissues, particularly at the vasculature [203–206]. Furthermore, the literature would suggest that this function is likely to arise from receptor engagement rather than physical blockade. It has been established that LAMA4 binds strongly to the melanoma cell adhesion molecule (MCAM) receptor, which is found on many immune cells, such as T cells. The inhibition of this interaction through functional blocking antibodies against MCAM was shown to reduce T cell recruitment in the central nervous system and improve MS disease markers *in vivo* in one study [207].

Taken together, these findings suggest that LAMA4 may play a role in immune cell infiltration in WAT under the context of obesity and adipose expansion. As described in Chapter 1, adipose tissue inflammation in obesity is a key driver of the development of insulin resistance. Many researchers are actively pursuing anti-inflammatory drugs that could suppress this response, in the hopes of improving insulin sensitivity in WAT. Considering the evident association between LAMA4 expression in WAT and obesity as illustrated in Chapter 2, it may

be possible that high LAMA4 levels mediate increased immune infiltration and contribute to heightened inflammation in obesity. If such a link is discovered, and found to be dependent on receptor engagement, it could build a greater case for targeting LAMA4 in WAT, not only to increase beige adipocyte induction and thermogenesis, but also to minimize immune cell infiltration.

To investigate this proposed function, immune cell subtypes within the sWAT of *Lama4*-/- and WT mice placed on HFD should be profiled and compared initially. This could be accomplished through the use of CIBERSORT, an algorithm used to quantify and profile cell fractions, particularly immune cell populations, in bulk tissue gene expression profiles [208]. Subsequently, this could be followed by assessing the ability of immune cells to infiltrate adipose depot explants treated with chemokines, from both Lama4-/- and WT mice. If LAMA4 deficiency is found to improve inflammatory phenotypes and reduce immune infiltration, further work should focus on investigating whether MCAM is the primary receptor by which LAMA4 interacts with immune cells to mediate this movement, and a determination of whether functional blocking could inhibit this action in WAT. The overall and eventual goal of this study would be to determine if LAMA4 blocking could be used therapeutically in WAT in patients with obesity to reduce inflammation and promote insulin sensitization.

4.4 Concluding Remarks

In summary, the laminin-α4 chain possesses unique properties that enable and promote a white adipocyte phenotype. LAMA4 expression on both the mRNA and protein level is associated with obesity in human and murine disease models. This observation supports the assertion that LAMA4 plays a role in adipose expansion in the obese state and confirms the relevance of this ECM protein in human adipose tissue. Likewise, LAMA4 maintains a white adipocyte phenotype

through the negative regulation of thermogenic gene expression and mitochondrial biogenesis. By suppressing AMPKα transcription, LAMA4 and ILK signaling inhibit adipocyte beiging.

The studies described in this project have utilized *in vivo* and *in vitro* models, and examined each aspect in both mice and humans, which strengthens the findings of this work. Nevertheless, several limitations remain. Due to availability of resources and subjects, not all studies were performed in both male and female mice or humans. For instance, female mice were not used in any part of the study, as they did not portray any phenotype of adipocyte beiging and also did not become significantly obese during the dietary studies. Conversely, our obesity and LAMA4 study in humans involved only female subjects. We now know that androgens and estrogens can contribute to differential regulation of adipose tissue, so when possible, adipocyte signaling mechanisms should be studied in both sexes [209]. Furthermore, due to the well documented racial and ethnic disparities in the prevalence of metabolic diseases such as obesity, a diverse subject pool is principle. The study presented in Chapter 2 included female subjects of non-Hispanic Black and non-Hispanic White backgrounds, while the study in Chapter 3 utilized an hiPSC line derived from a non-Hispanic Asian male donor. Now that LAMA4 has been identified as an important protein of interest in human obesity, future studies could more robustly investigate LAMA4 expression and function across subjects and cell lines of both sexes and multiple racial and ethnic backgrounds.

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