THE METABOLIC EFFECTS OF LEPTIN THERAPY AND GLUCAGON SUPPRESSION THERAPY IN MOUSE MODELS OF DIABETES

by

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ABSTRACT

It has long been thought that the only hormone capable of reversing the catabolic consequences of diabetes is insulin. However, various studies have demonstrated that the adipocyte-derived hormone leptin can potently lower blood glucose levels in rodent models of insulin-deficient diabetes. In addition, the hormone glucagon is elevated in type 1 and type 2 diabetes, and glucagon suppression therapy has shown promise as an agent to treat diabetes in mice. Given the interest in both of these therapies, leptin treatment and glucagon antagonism were propelled into clinical trials for patients with type 1 and type 2 diabetes respectively. The overarching goal of this thesis was to perform preclinical studies to investigate the mechanism of the glucose lowering actions of leptin and the effects of glucagon suppression therapy in mouse models of diabetes. To achieve this, we probed the role of increased leptin action as a result of insulin therapy, determined the function of insulin-like growth factor binding protein-2 (IGFBP2) in the glucose lowering actions of leptin, investigated the necessity of insulin for leptin treatment and glucagon suppression therapy, and explored the potential of glucagon suppression therapy via glucagon receptor (Gcgr) small interfering ribonucleic acid (siRNA) delivered by lipid nanoparticle (LNP) technology. This thesis reveals that elevated leptin levels may contribute to the glucose lowering effect of insulin therapy in insulin-deficient diabetes. In addition, we demonstrate that physiological levels of IGFBP2 are neither sufficient nor required for the action of leptin on glucose homeostasis. Moreover, leptin can normalize many metabolic parameters in the complete absence of insulin, but blood glucose levels are volatile and the length of survival is finite. Furthermore, Gcgr siRNA can improve many diabetic symptoms in mouse models of type 1 and type 2 diabetes. Finally, we report that the metabolic manifestations associated with a complete lack of insulin cannot be overcome by *Gcgr* gene inactivation. Collectively the findings in this thesis contribute insight into the mechanism of action, and the therapeutic potential of leptin administration and glucagon suppression therapy as a treatment for diabetes.

PREFACE

All studies presented in this dissertation were principally conceived, designed, performed, and analyzed by the author UH Neumann, with assistance as described below. This thesis was written by UH Neumann with editing provided by WT Gibson, SM Clee, SD Covey and TJ Kieffer.

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Data from Chapter 3 will be submitted for publication. UHN and TJK designed the experiments. RKB designed and generated the leptin antagonist plasmid. MMK performed the hydrodynamic tail-vein injections. UHN performed experiments and analysed data.

All data in Chapter 4, except for Figure 12, are published in the following article: UH Neumann, S Chen, YYC Tam, RK Baker, SD Covey, PR Cullis, and TJ Kieffer, 2014. IGFBP2 is neither sufficient nor necessary for the physiological actions of leptin on glucose homeostasis in male *ob/ob* mice. *Endocrinology* 155:716-725. UHN, SDC, and TJK designed the experiments. UHN performed the experiments. SC, YYCT, and PRC provided the LNP-siRNA. RKB designed and generated the construct for the adenovirus expressing murine IGFBP2 (Ad-IGFBP2). UHN analysed data and wrote the initial manuscript. All authors were involved in the discussion and revision of the manuscript.

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LIST OF ABBREVIATIONS

α-MSH	α-melanocyte stimulating hormone
Actb	beta-actin
Ad-β-gal	adenovirus expressing β-galactosidase
Ad-IGFBP2	adenovirus expressing murine IGFBP2
ADP	adenosine diphosphate
AgRP	agouti-related peptide
ANOVA	analysis of variance
ARC	arcuate nucleus of the hypothalamus
ARRIVE	animals in research: reporting *in vivo* experiments
AS160	Akt substrate of 160 kDa
ATP	adenosine triphosphate
AUC	area under the curve
B2m	beta-2-microglobulin
BAT	brown adipose tissue
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
cMaf	v-maf avian musculoaponeurotic fibrosarcoma oncogene homolog
CMV	cytomegalovirus
CNS	central nervous system
CoA	coenzyme A
CPT1	carnitine palmitoyltransferase I
CREB	cAMP response element-binding protein
CTLA4	cytotoxic T-lymphocyte associated protein 4
DLin-KC2-DMA	2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane
DMH	dorsomedial hypothalamic nucleus
DNA	deoxyribonucleic acid
DSPC	1,2-distearoyl-sn-glycero-3-phosphocholine
DT	diphtheria toxin

DTR	diphtheria toxin receptor
ELISA	enzyme-linked immunosorbent assay
Foxa1	forkhead box a1
Foxa2	forkhead box a2
FOXO1	transcription factor forkhead box O1
FVII	factor VII
GABA	γ-aminobutyric acid
Gapdh	glyceraldehyde 3-phosphate dehydrogenase
Gcgr	glucagon receptor
GIP	glucose-dependent insulinotropic polypeptide
GLP-1	glucagon-like peptide 1
GLP-2	glucagon-like peptide 2
GLUT	glucose transporter
GSK3	glycogen synthase kinase-3
HbA1c	hemoglobin A1c
HBSS	Hank's balanced salt solution
HEPES	2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid
Het	heterozygous
HFD	high fat diet
HLA	human leukocyte antigen
HSL	hormone-sensitive lipase
ICV	intracerebroventricular
IGF	insulin-like growth factor
IGFBP	insulin-like growth factor binding protein
IL2RA	interleukin 2 receptor subunit alpha
INS	human insulin gene
Ins1	mouse insulin 1 gene
Ins2	mouse insulin 2 gene
InsKO	insulin knockout
i.p.	intraperitoneal

IRS	insulin receptor substrate
Jak	janus kinase
K_{ATP} channels	ATP-sensitive potassium channels
KO	knockout
LDL-C	low-density lipoprotein cholesterol
Lep	leptin
LepR	leptin receptor
LFD	low fat diet
LHA	lateral hypothalamic area
LNP	lipid nanoparticle
LPL	lipoprotein lipase
MafA	v-maf Avian musculoaponeurotic fibrosarcoma oncogene homolog A
MafB	v-maf Avian musculoaponeurotic fibrosarcoma oncogene homolog B
MAPK	mitogen-activated protein kinase
MBH	medial basal hypothalamus
MC4R	melanocortin-4 receptors
MODY	maturity onset diabetes of the young
mTOR	mechanistic target of rapamycin
NeuroD1	neurogenic differentiation 1
NOD	non-obese diabetic
NPY	neuropeptide Y
P	post-natal day
Pax6	paired-box 6
PBS	phosphate buffered saline
PC	prohormone convertase
PCR	polymerase chain reaction
Pdx-1	pancreatic/duodenal homeobox-1
PEG	polyethylene glycol
PEG-c-DMA	N-[(methoxy polyethylene glycol 2000 carbamyl]-1,2-dimyristyloxlpropyl-3-amine

pEmpty	empty plasmid
pfu	plaque forming units
Pgk1	phosphoglycerate kinase
PI3K	phosphatidylinositol 3-kinase
pLA	plasmid encoding leptin antagonist
POMC	pro-opiomelanocortin
Ppia	peptidylprolyl isomerase A
PTP1B	protein tyrosine phosphatase 1B
PTPN2	protein tyrosine phosphatase
PVN	paraventricular nucleus of the hypothalamus
RNA	ribonucleic acid
RT-qPCR	reverse transcriptase quantitative polymerase chain reaction
s.c.	subcutaneous
SEM	standard error of the mean
SF1	steroidogenic factor-1
SGLT2	sodium-glucose co-transporter 2
SHP2	SH2-containing tyrosine phosphatase-2
siRNA	small interfering ribonucleic acid
SOCS3	suppressor of cytokine signalling 3
SREBP-1c	sterol regulatory binding protein-1c
STAT	signal transducers and activators of transcription
STZ	streptozotocin
TAG	triacylglycerol
Tyr	tyrosine
UCP1	uncoupling protein 1
VLDL	very low density lipoprotein
VMH	ventromedial nucleus of the hypothalamus
WAT	white adipose tissue
WT	wildtype

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CHAPTER 1 - INTRODUCTION

1.1 Diabetes Mellitus

The burden of diabetes

Diabetes is a chronic condition characterized by high blood glucose levels due to insufficient insulin levels or an inability to respond appropriately to insulin. Globally there are 415 million adults with diabetes and this number is expected to rise to 642 million by 2040 [1]. Strikingly, one in two of these cases is undiagnosed, leaving individuals unaware that they may be developing serious long-term complications [1]. Furthermore, 218 million adults are glucose intolerant putting them at risk of developing diabetes [1]. Despite current treatments, the inability to achieve constant euglycemia can result in secondary complications including retinopathy, neuropathy and nephropathy [1-4]. These complications can lead to cardiovascular disease, kidney disease, blindness, lower-limb amputations and pregnancy-related complications, which decrease both quality of life and life expectancy [1, 5]. In 2015 alone it was estimated that diabetes caused 5 million deaths, equivalent to one death every 6 seconds [1]. This imposes a substantial economic burden with an estimated 673 - 1197 billion USD being spent in 2015, approximately 12% of global healthcare costs [1]. If treatments are not improved these negative consequences on human health and the economy will become unsustainable.

Type 1 and type 2 diabetes

The most common form of diabetes is type 2 diabetes, which accounts for ~90% of the total cases [1]. The disease is most often diagnosed in adulthood; however, the incidence is rising in children likely due to unhealthy lifestyles. Type 2 diabetes is highly correlated with obesity, and is characterized by hyperglycemia, progressive insulin resistance and relatively inadequate insulin secretion [1]. The pathophysiology of type 2 diabetes is complex resulting from interactions between genetics and the environment [6, 7]. Genome wide association studies have identified genetic variants that increase the risk of type 2 diabetes [6, 8, 9], which may increase susceptibility to environmental factors such as stress, poor diet, and lack of

physical activity. If individuals with type 2 diabetes are unable to manage their diabetes through healthy eating and exercise, they may be treated with glucose-lowering agents. The most commonly prescribed medications are metformin, which increases insulin sensitivity, and sulfonylureas, which increase insulin secretion [7]. Alternatives include sodium-glucose co-transporter 2 (SGLT2) inhibitors, which increase glucose excretion in the urine, dipeptidyl peptidase-4 inhibitors, which increase the levels of the active forms of the intestinal hormones glucose-dependent insulinotropic polypeptide (GIP) and glucagon-like peptide-1 (GLP-1) resulting in increased insulin secretion, as well as GLP-1 analogues [7]. In late-stage type 2 diabetes, β-cell destruction can occur, necessitating the requirement for insulin therapy [7].

Type 1 diabetes accounts for approximately 10% of the total cases of diabetes and the number of cases of type 1 diabetes is increasing at an alarming rate of 3% per year [1, 10, 11]. Onset of disease most often occurs in childhood and results from the autoimmune destruction of the insulin-producing pancreatic β-cells [12]. However, while the etiology of the disease remains unclear, genetic and environmental factors appear to be involved [12, 13]. The risk of developing type 1 diabetes increases if a parent or sibling has the disease, and many genetic loci have been found to be associated with type 1 diabetes (including human leukocyte antigen class II (*HLA-DQB1*), insulin (*INS*), protein tyrosine phosphatase (*PTPN2*), cytotoxic T-lymphocyte associated protein 4 (*CTLA4*), and interleukin 2 receptor subunit alpha (*IL2RA*)) [12]. It is hypothesized that genetically susceptible individuals experience a precipitating event (such as consumption of milk and wheat proteins, vitamin D deficiency, viral infection, or exposure to chemicals), which triggers immune cascades resulting in autoimmune attack of β-cells [12, 13]. Type 1 diabetes, which is more severe than type 2 diabetes, leads to cachexia, ketoacidosis, and hyperglycemia, and if left untreated, is a uniformly fatal disease [14]. Thus, patients afflicted with the disease must be treated with insulin for the rest of their lives by multiple daily injection or pump administration. In rare cases of brittle diabetes, islet transplantation may be performed; however, patients require immunosuppressive therapy and insulin independence is not sustained long-term [15].

1.2 The Hormone Insulin

Discovery of insulin and insulin therapy

The impact of the discovery of insulin was sensational and considered a miracle for patients with type 1 diabetes. In 1921 at The University of Toronto, Banting and Best isolated the internal secretion of pancreatic islets, which was capable of reversing diabetes in pancreatectomized dogs [16, 17]. In 1922, these effects were repeated in humans resulting in reduced glycemia, glucosuria, and ketonuria as well as a rise in the respiratory quotient indicating carbohydrate utilization [16, 17]. One year later, the Nobel Prize in Physiology or Medicine was awarded for the discovery of insulin. The University of Toronto immediately gave pharmaceutical companies license to manufacture insulin and Eli Lilly was the first to produce insulin on a large scale [16]. Since then insulin therapy has improved as newer technology allows the production of synthetic insulin analogues with varying onset, peak, and duration of activity, as well as the development of continuous glucose monitoring and pump delivery of insulin.

Despite the life-saving quality of insulin for type 1 diabetes, and its usefulness in late-stage type 2 diabetes, insulin is not a cure. The guesswork involved in insulin dosing based on dietary intake and exercise is difficult and often results in periods of hyperglycemia, or worse, incidents of hypoglycemia, which can lead to coma and death. Due to the inability to achieve constant euglycemia, long-term secondary complications including heart disease, retinopathy, neuropathy and nephropathy can occur, and on average, life expectancy is decreased by ~ 15 years [2-5]. This highlights the need for better therapies for diabetes and researchers have focused on a plethora of avenues to achieve this including cellular therapies that allow for more physiological release of insulin in response to glucose or drugs as adjuvant therapies to insulin to help reduce glucose fluctuations.

Pancreatic β-cells synthesize and secrete the hormone insulin in response to glucose and other stimuli. Humans have a single insulin gene while rodents have 2 insulin genes at separate loci (*Ins1* and *Ins2*). Insulin is generated as preproinsulin in the rough endoplasmic reticulum

where it is converted to proinsulin through cleavage of the signal peptide [18]. Upon transport into the trans-Golgi network, proinsulin undergoes post-translational modification by prohormone convertase (PC)1/3, PC2, and carboxypeptidase E [18]. This yields the final products of the 51 amino acid mature insulin (composed of the A-chain and B-chain connected by disulfide bonds) and C-peptide, which are stored in secretory granules [18]. Mature insulin is highly conserved among vertebrates with 92% sequence similarity between humans and rodents, whereas C-peptide differs more considerably [18]. Transcription of the insulin gene, preRNA splicing, and mRNA stability are largely regulated by glucose, which controls the recruitment of the transcription factors Pdx-1, MafA, and NeuroD1 [19].

While basal insulin levels are maintained through a constitutive secretory pathway, the majority of vesicles are stored for regulated exocytosis stimulated by glucose, other nutrients, or hormones [18]. Glucose from the bloodstream is transported into the β-cell through glucose transporter (Glut) 1 or 2 (in humans and rodents respectively) and becomes phosphorylated by the rate limiting enzyme glucokinase for entry into glycolysis and cellular respiration [18]. This results in an increase in the ATP/ADP ratio, which is sensed by K_{ATP} channels causing their closure resulting in membrane depolarization [18]. Voltage-gated calcium channels sense this depolarization causing an influx of calcium, which interacts with primed insulin vesicles causing exocytosis [18]. Amino acids and fatty acids can further potentiate glucose-stimulated insulin release [18]. GLP-1 and GIP, incretin hormones produced in the L- and K-cells of the small intestine, are secreted following a nutrient load and augment insulin secretion, while the hormones leptin and somatostatin can suppress insulin secretion [18]. Interestingly, rare monogenic forms of diabetes (MODY, or maturity onset diabetes of the young) can result from heterozygous autosomal dominant mutations in genes encoding glucokinase (MODY 2), Pdx-1 (MODY 4) and NeuroD1 (MODY 6) resulting in disrupted insulin production. Thus, maintaining proper insulin biosynthesis is critical for normal health.

Insulin and metabolism

Insulin is the major anabolic hormone essential for post-prandial nutrient uptake and storage in peripheral tissues. In response to nutrients, insulin is secreted from pancreatic β-

cells and works to maintain blood glucose within a tight range. Following a meal, insulin stimulates glucose uptake in skeletal muscle and adipose tissue by causing translocation of Glut4 to the cell surface [20, 21]. Furthermore, insulin reduces hepatic glucose output by suppressing gluconeogenesis and glycogenolysis and stimulating glycogen synthesis through modulation of the activity of key enzymes in these pathways [20, 21]. Glucose is then stored for use during times of fasting as glycogen in skeletal muscle and liver, and triglycerides in adipose tissue. In addition to glucose metabolism, insulin can also potently affect protein and lipid metabolism. Insulin stimulates the uptake of amino acids into cells, promotes protein synthesis in muscle, adipose tissue, and liver, and inhibits degradation of protein in muscle [20, 21]. As for lipid metabolism, insulin decreases the rate of lipolysis in adipose tissue decreasing plasma fatty acids levels, stimulates fatty acid and triglyceride synthesis in liver and adipose tissue, and represses fatty acid oxidation in muscle and liver [20, 21]. Insulin can also act on the brain to suppress food intake and hepatic glucose output [22]. Therefore, in the healthy state, insulin prevents blood glucose from being elevated, and stores nutrients in appropriate tissues for times of need.

Insulin exerts its metabolic effects through the insulin receptor, which belongs to a class of tyrosine kinase receptors and is present in many tissues including the liver, muscle, white adipose tissue (WAT), brown adipose tissue (BAT), brain, α-cells and β-cells [20]. The receptor consists of two extracellular α subunits and two transmembrane β subunits linked by disulphide bonds. Upon binding of insulin, the receptor undergoes a conformational change, autophosphorylation, and activation of various scaffolding proteins such as insulin receptor substrate (IRS) 1 and IRS2, which can initiate signal transduction through various signalling pathways including the phosphatidylinositol 3-kinase (PI3K) – Akt pathway, which is responsible for most of the metabolic actions of insulin, and the Ras–mitogen-activated protein kinase (MAPK) pathway, which controls cell growth and differentiation [20, 23]. Activated Akt phosphorylates the Akt substrate of 160 kDa (AS160) leading to Glut4 translocation and glucose uptake, glycogen synthase kinase-3 (GSK3) resulting in increased glycogen synthesis, and the transcription factor forkhead box O1 (FOXO1) triggering decreased gluconeogenesis [23].

Various genetic mouse models have been created to investigate the role of insulin signalling throughout the body. Using the Cre-lox system, which employs tissue specific promoters driving Cre recombinase and the floxed insulin receptor gene, organ-specific insulin receptor knockout mouse lines have been generated. Hepatocyte specific insulin receptor knockout mice using the albumin promoter resulted in severe glucose intolerance and hyperinsulinemia, highlighting the importance of direct insulin signalling on the liver in insulin clearance and suppression of hepatic glucose production [24]. Inactivation of insulin receptors specifically in muscle using the muscle creatine kinase promoter resulted in increased adiposity and serum triglycerides and fatty acids but normal blood glucose levels, glucose tolerance, and serum insulin levels [25]. Given that skeletal muscle accounts for up to 70% of insulin-stimulated glucose uptake [26] it was surprising that these mice were not hyperglycemic, thus it has been hypothesized that glucose uptake was increased in WAT and stored as triglyceride [27]. To investigate the direct role of insulin action in adipose tissues, insulin receptors were ablated in WAT and BAT using the adipocyte protein 2 promoter, which led to decreased insulin-stimulated glucose uptake and lipolysis in adipocytes, decreased fat mass and protection from age-related glucose intolerance [28]. Finally, when insulin receptors were knocked out of the brain using the nestin promoter, male and female mice developed diet-related obesity, mild insulin resistance, and elevated serum triglycerides while female mice exhibited increased food intake, evidence for a role of insulin signalling in the brain to control body weight regulation [29].

To investigate the role of full body insulin action, mice were generated which lack both insulin genes or the insulin receptor gene, which were found to develop hyperglycemia and hyperketonemia and die within 2-3 days of birth [30, 31]. Many patients with mutations in the insulin receptor gene have been identified who exhibit symptoms of varying severity (including extreme insulin resistance) depending on the mutation, and often exhibit growth retardation and glucose intolerance or diabetes [32]. Various mutations have also been found in the insulin gene that can disrupt binding to the insulin receptor causing hyperinsulinemia or that can reduce insulin processing leading to hyperproinsulinemia, both of which result in mild

diabetes or glucose intolerance [33]. More severe neonatal diabetes is also associated with autosomal dominant mutations (MODY 10) and recessive mutations in the insulin gene [33].

Given the critical role of insulin in fuel homeostasis, it is not surprising that type 1 and type 2 diabetes are extreme metabolic disorders. In type 1 diabetes characterized by insufficient insulin, hyperglycemia results from impaired glucose uptake and excessive hepatic glucose output, which leads to microvascular complications. In a state of perceived starvation due to the inability to use glucose as fuel, increased fatty acid oxidation leads to the generation of ketone bodies that can be used as the brain for energy. However, excess ketone bodies can cause acidification of the blood resulting in the deadly syndrome diabetic ketoacidosis [34]. Loss of insulin stimulates lipolysis resulting in decreased adipose tissue mass and increased plasma fatty acids and glycerol. A reduction in lipoprotein lipase (LPL) activity decreases lipoprotein catabolism leading to hypertriglyceridemia, a risk factor for atherosclerosis [35]. While type 2 diabetes is characterized by hyperinsulinemia and general insulin resistance, not all tissues are equally insulin resistant, and individual pathways within a certain tissue may exhibit selective insulin resistance [36]. For instance, similar to type 1 diabetes, type 2 diabetes results in elevated blood glucose levels due to impaired glucose uptake and excess hepatic glucose production, however lipid synthesis pathways are stimulated in the liver and adipose tissue [36-38]. In the liver, the master lipid metabolism regulator sterol regulatory binding protein-1c (SREBP-1c) is paradoxically increased stimulating fatty acid synthesis, triglyceride generation, and very low density lipoprotein (VLDL) production thus increasing circulating triglycerides [36, 38]. In addition, the lipogenic effects of insulin remain intact in adipose tissue in the insulin resistant state, which may contribute to continued promotion of obesity in type 2 diabetes [37]. With current treatments for type 1 and type 2 diabetes, it is difficult to normalize all of these metabolic abnormalities.

1.3 The Hormone Glucagon

Glucagon and the glucagon receptor

The hormone glucagon was first described as the pancreatic hyperglycemic factor contaminating crude insulin extracts [39]. Now, glucagon is best known as a counter-regulatory hormone secreted from pancreatic α-cells, which largely opposes the metabolic actions of insulin by promoting hepatic glucose production in response to hypoglycemia. Glucagon is a 29 amino acid peptide hormone with 100% sequence similarity between humans and rodents and is processed from the preproglucagon gene, which is expressed in various tissues including the pancreatic α-cell, intestinal L-cells, and neurons within the nucleus of the solitary tract [40]. In the α-cell, high expression of PC2 predominantly yields glucagon, in addition to glicentin-related pancreatic polypeptide, the intervening peptide 1, and a major proglucagon fragment [40, 41]. A predominance of PC1/3 in the intestinal L-cells and the nucleus of the solitary tract mainly produces GLP-1, GLP-2, glicentin, oxyntomodulin, and intervening peptide 2 [40, 41]. In α-cells binding of the transcription factors Pax6, cMaf, MafB, Foxa1 and Foxa2 to the glucagon promoter region stimulates expression of glucagon gene expression [41].

The mechanism by which glucagon secretion occurs is similar to that of β-cells; however, glucagon release is stimulated by low glucose levels and inhibited by high glucose levels [41]. Similar to β-cells, glucose is taken up through Glut1 or 2, however, the K_{ATP} channels in α-cells require a lower ATP/ADP ratio than β-cells to stimulate their closure [41]. Thus, hypoglycemia triggers inhibition of K_{ATP} channels causing membrane depolarization, opening of calcium and sodium channels, and exocytosis of glucagon granules [41]. In contrast, during hyperglycemia, K_{ATP} channels cause depolarization to a level where calcium and sodium channels are inactive preventing exocytosis [41]. While glucagon secretion is largely controlled directly by glucose, high glucose concentrations stimulate release of insulin, amylin, γ-aminobutyric acid (GABA) and zinc or somatostatin from neighbouring β- and δ-cells, which can indirectly inhibit glucagon release through a paracrine manner [40-42]. In addition to hypoglycemia, stimulation of glucagon secretion is triggered by mixed meal or protein

ingestion (particularly by arginine), and activation of the autonomic nervous system, while GLP-1 and GIP tend to inhibit glucagon secretion [40-42].

Glucagon exerts its metabolic effects through activation of the glucagon receptor, a seven transmembrane G-protein coupled receptor. High affinity glucagon binding sites or *Gcgr* gene expression have been identified in liver and kidney, and to a lesser extent in heart, adipose tissue, the central nervous system (CNS), lymphoblasts, gastrointestinal tract, adrenal gland, spleen, ovary, β-cells, and α-cells [40, 41]. Binding of glucagon to the glucagon receptor can activate the G protein G_q, which leads to activation of the phospholipase C/inositol pathway resulting in Ca^{2+} release from the endoplasmic reticulum stimulating downstream signalling pathways [41]. In addition, the glucagon receptor can activate the G protein $G_{s\alpha}$, which results in stimulation of adenylate cyclase, increases in cyclic adenosine monophosphate (cAMP) production, activation of PKA, and phosphorylation of cAMP response element-binding protein (CREB), which can act as a transcription factor to influence metabolism [41, 43].

Glucagon and metabolism

The main action of glucagon occurs in the liver where it acts to influence glucose and lipid metabolism. During periods of fasting, to prevent blood glucose from dropping too low, insulin is suppressed and glucagon is released to promote synthesis and mobilization of glucose from the liver. Glucagon increases hepatic glucose production by enhancing gluconeogenesis and glycogenolysis, and inhibiting glycolysis and glycogen synthesis. The glucagon to insulin ratio is critical for determining the activity of key enzymes involved in these pathways. Indeed, global and hepatocyte-specific knockout of glucagon receptors in mice results in an identical phenotype of lowered blood glucose levels and improved glucose tolerance highlighting the importance of glucagon signalling on the liver in regulating glucose homeostasis [44, 45]. Glucagon also stimulates uptake of amino acids that can be used as precursors in gluconeogenesis [43]. In the adipocyte, glucagon causes activation of hormone-sensitive lipase (HSL) and stimulate lipolysis promoting the release of glycerol and free fatty acids, which can then be used for gluconeogenesis or ketogenesis, respectively, in the liver [41, 43]. Glucagon also has hypolipidemic effects in the liver as it stimulates fatty acid oxidation,

decreasing substrates for triglyceride synthesis, and thus decreasing VLDL release [46, 47]. Given its hyperglycemic effects, glucagon is injected as a life-saving intervention to treat hypoglycemia [41]. In addition, glucagon has been proven effective when administered intra-nasally [48] and has been tested in a dual-hormone pump in conjunction with insulin [49].

In addition to the liver, glucagon can act on various other tissues in the body to regulate metabolism. Interestingly, central infusion of glucagon into the medial basal hypothalamus (MBH) inhibited hepatic glucose production and improved glucose tolerance in rodents [50]. Various studies have demonstrated that glucagon can control body weight by promoting satiation and energy expenditure [51]. Indeed, glucagon can increase core and BAT temperature, which may play a role in non-shivering thermogenesis during cold exposure [40]. Glucagon also decreases glucose uptake from skeletal muscle, increases insulin production from β-cells, and inhibits gastric motility [41]. Finally, glucagon can increase lipolysis in WAT leading to decreased fat mass [41]. Given these beneficial effects of glucagon on lipolysis and thermogenesis, researchers have tested dual-agonism of glucagon and GLP-1 (to restrain the hyperglycemic effect of glucagon) and triagonism of glucagon/GLP-1/GIP to much success as an anti-obesity agent [41, 52].

Generation of various genetic knockout models have provided insight into the role of glucagon signalling throughout the body. PC2 null mice exhibit severely impaired processing of proglucagon (and many other hormones), fasting hypoglycemia, a reduced rise in blood glucose following injection of glucose, and a marked increase in a-cell hyperplasia [53]. Administration of exogenous glucagon to PC2 null mice corrects blood glucose levels and α-cell hyperplasia highlighting the importance of PC2 in processing of glucagon and the role of glucagon in preventing hypoglycemia [54]. Mice with a knockout of the proglucagon gene have been generated; however, dissecting the specific role of glucagon is difficult as the mice are deficient for all of the glucagon gene-derived products including GLP-1 [55, 56]. However, by administering exogenous glucagon in these mice, it was demonstrated that glucagon is essential for adaptive thermogenesis in BAT [57]. In an attempt to explore the effect of near total ablation of α-cells, a mouse line was generating expressing diphtheria toxin receptor

(DTR) driven by a portion of the glucagon promoter excluding the intestinal-specific cis-regulatory elements of the glucagon gene. Despite 98% ablation of α-cells, circulating glucagon levels were reduced by only 35% 1 week following diphtheria toxin (DT) delivery, were normalized at 6 months, and no alteration in glucose homeostasis was observed [58]. To investigate the effect of complete blockade of glucagon signalling, mice with a global knockout of glucagon receptors were generated and exhibit reduced blood glucose levels, improved glucose tolerance, α-cell hyperplasia and hyperglucagonemia, increased circulating and pancreatic GLP-1, inappropriately high release of triglyceride and fatty acids into the plasma during fasting, a lean phenotype, and protection from diet induced obesity underlining the role of glucagon in glucose and lipid metabolism [44, 47, 59]. Mice with a hepatocyte specific attenuation of glucagon receptors also exhibit lowered blood glucose levels and enhanced glucose tolerance, similar in magnitude to mice with full body knockout of the receptor, emphasizing the role of glucagon action on the liver in regulating glucose homeostasis [45]. In addition, liver specific knockout of glucagon receptors also results in α-cell hyperplasia, thus it was hypothesized that a hepatocyte-derived factor is responsible for proliferation of α-cells [45]. Indeed, it has been recently reported that inhibiting hepatic glucagon receptors suppresses amino acid catabolism, raising circulating levels of amino acids, which act to stimulate α-cell hyperplasia in a mechanistic target of rapamycin (mTOR)-dependent manner [60].

Mutations in the glucagon receptor gene have been identified in humans. Similar to glucagon receptor knockout mice, a patient with a homozygous P86S missense mutation in the glucagon receptor gene also exhibited α-cell hyperplasia and hyperglucagonemia [61]. In addition, in a study of six patients with glucagon cell adenomatosis, three were found to harbor germline mutations in the glucagon receptor gene [62]. The first patient exhibited a homozygous insertion mutation resulting in a premature stop codon which was associated with hyperglucagonemia [62]. The second patient, which showed two heterozygous point mutations leading to premature stop codons, also displayed elevated circulating glucagon levels [62]. The third patient had two homozygous missense mutations, which may disturb the function of the receptor [62]. The patients with glucagon cell adenomatosis that exhitied glucagon gene

mutations had macrotumors and a higher density of hyperplastic islets and microtumors compared to patients without these mutations [62]. Finally, a G40S missense mutation has been found to be associated with non-insulin dependent diabetes mellitus in some populations [63, 64], which may paradoxically result in a receptor with decreased sensitivity to glucagon [65].

Clinical utility of glucagon suppression therapy

In addition to insufficient insulin action, patients with diabetes also exhibit dysregulated glucagon action. In type 1 diabetes and late stage type 2 diabetes, the secretory response of the α-cell to low blood glucose levels is defective, which can lead to hypoglycemic episodes [66]. In addition, severe hyperglucagonemia is present in diabetic ketoacidosis and poorly controlled type 1 diabetes [67-69] and a modest elevation in circulating glucagon is observed in type 2 diabetes [69-71]. Inappropriately high circulating glucagon levels contributes to fasting and post-prandial hyperglycemia through excessive hepatic glucose production [69]. Interestingly, it has been proposed that volatility in blood glucose levels due to insulin injection therapy may be due to insufficient suppression of glucagon [72, 73]. In the non-diabetic state, α-cells are in close proximity to β-cells and thus come in contact with highly concentrated insulin, which causes suppression of glucagon secretion [72, 73]. However, in the diabetic state characterized by a reduction in insulin producing β-cells, when insulin is injected subcutaneously, low levels of insulin reach the islet and hyperglucagonemia can persist [72, 73]. Therefore, it has been proposed that glucagon suppression therapy may be beneficial in further preventing hepatic glucose output in type 1 and type 2 diabetes.

Several preclinical and clinical studies have demonstrated that glucagon suppression therapy may be useful in treating type 1 and 2 diabetes. Glucagon receptor gene deletion, administration of a glucagon receptor antagonist, and immunoneutralization of glucagon using a monoclonal antibody prevent or reverse hyperglycemia in animal models of insulin-deficient diabetes [74-77]. Interestingly, Eli Lilly's drug LY2409021 (clinicaltrials.gov, NCT01640834) lowers insulin requirements in patients with type 1 diabetes under euglycemic clamp conditions [78]. In addition, immunoneutralization of endogenous glucagon, glucagon

receptor antisense oligonucleotides or siRNA, and genetic deletion of glucagon receptors ameliorate glucose metabolism in rodent models of obese, type 2 diabetes [59, 79-81]. These promising results led pharmaceutical companies to produce small molecule glucagon receptor antagonists that have been tested in numerous clinical trials for patients with type 2 diabetes [82]. Various agents from Eli Lilly, Merck, Pfizer and Ligand Pharmaceuticals reduce fasting blood glucose, mean daily blood glucose and HbA1c [83-87]. Interestingly, glucagon receptor knockout mice do not exhibit hypoglycemia until 24 hours of fasting [44], and the number of hypoglycemic events were not increased in patients receiving glucagon receptor antagonists [83, 84, 86, 87]. However, despite these promising benefits, many of these agents have been discontinued, potentially due to side effects such as elevated serum cholesterol, triglycerides and transaminase levels and increased blood pressure and liver fat [83-88]. Additional concerns over the use of glucagon suppression therapy have been raised including glycogen storage alterations and malignant transformation of α-cells [89]. Glucagon receptor null mice also have dramatically increased liver glycogen and reduced muscle glycogen [74], reminiscent of glycogen storage disease, and this should be considered when treating patients with diabetes. Furthermore, global and liver specific glucagon receptor knockout mice are characterized by hyperglucagonemia associated with severe α-cell hyperplasia, and elevated glucagon levels are observed in patients receiving glucagon receptor antagonists [83, 84, 86]. Thus, these risks may limit the clinical utility of glucagon receptor antagonists for the treatment of diabetes.

1.4 The Hormone Leptin

Leptin and the leptin receptor

In addition to insulin and glucagon, the hormone leptin also plays a role in glucose metabolism. The discovery of leptin began over 50 years ago when two mouse lines with inheritable obese and diabetic phenotypes spontaneously developed at the Jackson Laboratory. In 1949, mice characterized by massive obesity, hyperphagia, and mild diabetes were discovered and aptly named *obese* or *ob/ob* mice [90]. Sixteen years later another mutant mouse was discovered and found to have obesity, hyperphagia, and severe life-shortening diabetes and thus dubbed *diabetes* or *db/db* mice [91]. Interestingly these mice had identical

phenotypes when back-crossed onto the same genetic background [92, 93], and since the *ob* and *db* mutations were mapped to chromosome 6 and chromosome 4 respectively, it was postulated that the mutations occurred in genes involved in the same metabolic pathway [94]. To test this hypothesis, Coleman performed elegant parabiosis studies involving surgical joining of two mice to establish cross-circulation [94]. When *ob/ob* mice were parabiosed with a wildtype partner, the *ob/ob* mouse exhibited decreased food intake, body fat, and blood glucose levels suggesting that the *ob/ob* mice were missing a blood-borne satiety factor [95]. In contrast, when *db/db* mice were parabiosed to *ob/ob* or wildtype mice, the *ob/ob* or wildtype partner died of hypoglycemia and starvation suggesting the *db/db* mice overproduced and may be non-responsive to this blood-borne satiety factor, perhaps due to a defective receptor [96]. Indeed, more than 20 years later it was found through positional cloning that the *ob* gene encoded leptin [97] while the *db* gene encoded the leptin receptor [98].

Since the discovery of *ob/ob* and *db/db* mice, rare cases of humans carrying mutations in leptin or the leptin receptor gene have also been found. Similar to *ob/ob* mice, humans with congenital leptin deficiency due to the synthesis of biologically inactive truncated leptin, exhibit diverse metabolic defects including obesity, diabetes, dyslipidemia, hormonal and immune deficiencies, and cognitive impairments, and exogenous leptin therapy in these patients can dramatically improve signs, symptoms and metabolic markers of disease [99-108]. Humans with mutations in the leptin receptor gene also exhibit signs and symptoms similar to patients with mutations in the leptin gene; however, they exhibit high circulating leptin levels [109-115].

The hormone leptin is a 167 amino acid protein with ~80% sequence homology between humans and rodents [97]. Leptin is secreted primarily from WAT into the bloodstream through a constitutive pathway [116, 117] and can be transported across the blood brain barrier [118]. Various factors have been found to modulate leptin levels including overfeeding, insulin, and glucocorticoids, which stimulate leptin expression and fasting, β-adrenergic agonists, and cold exposure, which decrease leptin expression [119]. Leptin is also expressed in BAT [120, 121], mammary gland [122], placenta [123, 124], skeletal muscle [125], stomach [126], and pituitary

gland [127]; however, the relative contribution from these tissues to total circulating leptin levels is negligible [128]. These extra-adipocyte sources of leptin may have an autocrine or paracrine effect on nearby cells [128]. Circulating leptin levels are generally proportional to fat mass [129, 130], relaying information regarding the mass of energy stores to other tissues [131]. In mice and humans, fasting induces a dramatic fall in circulating leptin levels, disproportionally to fat mass, which triggers food-searching behaviour [132, 133]. Starvation leads to neuroendocrine alterations and infertility, which can be reversed with leptin administration [132]. Thus it has been hypothesized that the evolutionary importance of leptin is not to induce satiety when food is abundant, but to protect from the detrimental effects of famine on survival by conserving energy until food becomes available [134].

Leptin exerts its metabolic effects through the leptin receptor, which is a member of the interleukin-6-type cytokine receptor family. Multiple alternative splicing events result in six isoforms of the leptin receptor, LepRa-f. All isoforms have an identical extracellular leptin binding domain, and all isoforms with the exception of LepRe contain the transmembrane region but differ in the length of the intracellular tail [135]. Only isoform LepR-b, the long form of the leptin receptor, contains all the intracellular motifs required for activation of the janus kinase-signal transducers and activators of transcription (Jak-STAT) signal transduction pathway [136-138]. A 106 nucleotide insertion mutation in the leptin receptor gene of *db/db* mice causes a premature stop codon and terminates the intracellular domain resulting in a truncated LepR-b isoform [98, 135]. Given that *db/db* and *ob/ob* mice have identical phenotypes when crossed onto same genetic background [92, 93] suggests that the majority of the metabolic effects of leptin are mediated by the LepR-b isoform. Little is known about the role of the other isoform of the leptin receptor, with the exception of LepR-a, which was originally hypothesized to affect leptin transport and clearance through the blood brain barrier [139]. Specific knockout of LepR-a results in very modest improvements in glucose tolerance on a chow diet and a slight propensity for increased fat mass and a decreased cerebral spinal fluid/plasma ratio of leptin on a high fat diet [140]. Whether the isoforms of the leptin receptor are involved in metabolism are currently unknown.

Lepr-b homo-oligomers exist at the cell membrane and undergo a conformational change upon leptin binding [141]. Following this conformational change, constitutively receptor-associated Jak2 undergoes autophosphorylation, which promotes the phosphorylation of key tyrosine resides in the intracellular domain, Tyr985, Tyr1077, and Tyr1138 [131]. Each of these residues recruits distinct intracellular proteins to induce signalling cascades with different metabolic outcomes. Phosphorylation of Tyr985 promotes the recruitment of the SH2-containing tyrosine phosphatase-2 (SHP2), which activates the extracellular signal-regulated kinase cascade [131]. Phosphorylated Tyr1077 and Tyr1138 recruits and phosphorylates STAT5 and STAT3 respectively [131]. Activated STAT molecules homo-dimerize resulting in nuclear translocation where they act as transcription factors to affect various target genes [142]. Notably, after prolonged leptin stimulation, STAT3 induces suppressor of cytokine signalling 3 (SOCS3) expression, which acts in a negative feedback loop to impair leptin signalling by binding Tyr985 or Jak2 and preventing phosphorylation [143-145]. Protein tyrosine phosphatase 1B (PTP1B) has also been implicated in the inhibition of leptin signalling as it dephosphorylates Jak2 [142]. Interestingly, in certain tissues there may be cross-talk between the leptin and insulin signalling pathways since Jak2 phosphorylation can activate the IRSs/PI3K pathway [146-149].

Leptin receptors are expressed in various locations within the CNS [150] and the periphery [126, 137, 151-170]. There is ample evidence from studies using *ob/ob* mice, lean mice, or insulin-deficient rodents that leptin acts through the CNS to suppress insulin [171-175], glucagon [176, 177], and corticosterone [176] from β-cells, α-cells and the adrenal gland respectively, and increase muscle [178-183] and liver [184-187] insulin sensitivity. Although it is believed that the majority of body weight and blood glucose reducing effects of leptin are mediated through the CNS (further described in upcoming sections), leptin receptors are also present in peripheral organs, which have led various researchers to investigate the direct effect of leptin on these tissues [188]. There is conflicting evidence as to whether leptin signals directly on β-cells to inhibit insulin secretion. While $LepR^{fl/fl}$ RIP-cre and $LepR^{fl/fl}$ Pdx1-cre mice are characterized by hyperinsulinemia [189, 190], when the less promiscuous Ins1-cre is used, hyperinsulinemia does not develop [191]. While investigating the direct role of leptin

action on α-cells, it was found that a partial ablation of LepR from ~43% of α-cells using a $LepR^{fl/fl}$ Glg-cre did not alter glucose or lipid metabolism in mice [192], and similar results were found using the more efficient iGlu-cre [191], suggesting that leptin receptors in α-cells do not play a critical role in glucose homeostasis. In addition, leptin suppressed adrenocorticotropic hormone-stimulated cortisol/corticosterone release from primary cultures of bovine [193], rat, and human [165] adrenocortical cells suggesting that leptin may also have a direct effect on adrenocortical cells within the hypothalamic-pituitary-adrenal axis. Moreover, treatment of isolated rodent soleus with leptin alone or in combination with insulin resulted in increased muscle glycogen synthesis and glucose uptake, glucose oxidization, and glycogen synthesis [194-196]. Interestingly, in contrast to the central effects of leptin on liver, $LepR^{fl/fl}$ Alb-cre mice exhibit enhanced hepatic insulin sensitivity. Thus leptin may act through both indirect and direct mechanisms on tissues throughout the body.

Effect of leptin on body weight regulation

As demonstrated by Coleman's parabiosis experiments, leptin is a potent satiety factor. The absence of leptin in *ob/ob* mice, KiloRats, and humans with congenital leptin deficiency all result in massive obesity, hyperphagia and reduced metabolic rate, all of which are improved with exogenous leptin therapy [102, 105, 175, 197-199]. Even in lean mice, leptin administration can suppress food intake and stimulate energy expenditure [199, 200]. Low doses of intracerebroventricular (ICV) leptin exert beneficial effects on body weight regulation identical to those of systemic leptin, demonstrating that the brain is a key target for the action of leptin on energy homeostasis [200, 201]. Moreover, a complete rescue of obesity is observed following re-expression of leptin receptors in the brain of *db/db* mice [202] and obesity is induced following neuronal deletion of leptin receptors in wildtype mice [203]. Leptin receptors are widely expressed throughout the CNS, particularly in various regions of the hypothalamus including the arcuate nucleus of the hypothalamus (ARC), ventromedial nucleus of the hypothalamus (VMH), lateral hypothalamic area (LHA), and dorsomedial hypothalamic nucleus (DMH), as well as extra-hypothalamic regions such as the nucleus of the solitary tract of the hind brain [150, 151, 204-207]. Within the ARC, leptin responsive neurons include those that express pro-opiomelanocortin (POMC) as well as neurons that co-

express agouti-related peptide (AgRP) and neuropeptide Y (NPY) [208, 209]. Leptin stimulates the release of the anorexigenic peptide α-melanocyte stimulating hormone (α-MSH) from POMC neurons, which can bind to melanocortin-4 receptors (MC4R) on neurons within the paraventricular nucleus of the hypothalamus (PVN) to induce satiety and increase in energy expenditure [208, 209]. Conversely, during times of fasting, a fall in leptin levels reduces inhibition of the orexigenic peptides AgRP and NPY, which act on neurons expressing MC4R or the NPY receptors to stimulate food intake and suppress energy use [208-210].

Despite the well characterized role of the MC4R network on energy homeostasis, other factors may be involved. Mice lacking leptin receptors in either POMC or AgRP expressing neurons exhibit only mild obesity [211, 212] and restoration of leptin receptors in POMC neurons of *db/db* mice partially reduces adiposity [213]. In addition, mice lacking leptin receptors in both of these neuron populations exhibit more severe obesity, yet they do not recapitulate the *db/db* phenotype [212], suggesting other neuron populations may be involved in mediating the body weight reducing effects of leptin. Within the VMH, steroidogenic factor-1 (SF1) expressing neurons have been implicated in body weight regulation [214]. Knockout of leptin receptors in SF1 neurons resulted in modestly increased body weight [215, 216] and reconstitution of leptin receptors in *db/db* mice partially reduces adiposity [217]. Leptin receptors are also expressed in the nucleus of the solitary tract of the hind brain where leptin may act to promote satiety in conjunction with GLP-1 or cholecystokinin [218]. Thus, the neural networks by which leptin regulated body weight regulation are complex.

Body weight independent effect of leptin on metabolism

In addition to defects in body weight regulation, rodents lacking the gene encoding leptin (*ob/ob* mice or KiloRat) [90, 95, 175, 197, 219, 220] or the leptin receptor (*db/db* mice, Zucker diabetic fatty rats, and JCR:LA-cp or SHR/N-cp rats) [91, 95, 96, 221-224] are commonly characterized by insulin resistance, hyperinsulinemia, impaired glucose tolerance, and in some cases, chronic hyperglycemia. In humans lacking leptin or its receptor due to rare mutations, obesity is also evident [99-115], and though impairments to glucose tolerance are not as severe

as in rodents, hyperinsulinemia is often present [102]. Leptin therapy in leptin-deficient rodents and humans improves all of their metabolic abnormalities [102, 105, 175, 197].

The improvement in hyperglycemia following leptin therapy in rodents was initially attributed to the secondary effects of reduced body weight, however numerous observations suggest that leptin can have metabolic effects independent of reductions in body weight. First, in *ob/ob* and *db/db* mice, hyperinsulinemia precedes obesity, suggesting that impairments to glucose regulation occur distinctly from weight gain [220, 225]. Second, pair feeding *ob/ob* mice to consume the same amount of food as leptin treated *ob/ob* mice did not improve blood glucose or plasma insulin to the same extent as leptin treatment [226]. Third, a low dose of leptin that was unable to lower body weight was still capable of normalizing blood glucose and insulin levels in *ob/ob* mice [175]. Fourth, acute disruption of leptin signalling using a leptin antagonist raised blood glucose and plasma insulin levels before altering body weight [187]. Fifth, rodents and humans with lipodystrophy that had loss of fat tissue and had extremely low leptin levels, also exhibited hyperglycemia, hyperinsulinemia and insulin resistance, which were corrected by leptin therapy [227-229]. Lastly, insulin-deficient rodents, which had depleted WAT depots, exhibited hyperglycemia, insulin resistance, and impaired glucose tolerance, all of which were improved by leptin therapy [167, 176, 177, 230-235]. Together, these findings demonstrate that leptin signalling can influence glucose regulation independent of its effects on body weight.

Leptin therapy in insulin-deficient diabetes

Recently, we and others have shown that leptin administration can reverse the hyperglycemia in insulin-deficient rodents. In cases of chemically-induced (streptozotocin (STZ)-rodents [167, 176, 177, 230, 233, 234, 236-239]), virally-induced (Biobreeding rats [240]), and autoimmune-mediated (non-obese diabetic (NOD) mice [232, 233]) β-cell destruction, induction of supraphysiological levels of circulating leptin or ICV leptin delivery can have considerable positive effects on both glucose and lipid metabolism. Remarkably, leptin monotherapy decreased hyperphagia [176, 177, 230, 232, 233, 236-238], stabilized body weight [167, 177, 230, 232, 233, 236-238], reduced hyperglycemia [167, 177, 230, 232, 233,

236-238] and ketoacidosis [167, 177, 232, 233], increased insulin sensitivity [167, 234, 236], abolished glucosuria [176, 177, 232, 233], normalized water intake [176] and urine output [177], diminished plasma lipids [177, 232-234, 236] and overall, extended lifespan [177, 230, 232, 238]. Despite tremendous interest in this topic, the underlying mechanisms behind the diabetes-reversing effects of leptin in insulin-deficient diabetes remain unclear.

Leptin therapy also can elicit a plethora of effects in peripheral tissues to normalize glucose and lipid metabolism in insulin-deficient diabetes. Leptin can potently reduce circulating levels of the counter-regulatory hormones glucagon and corticosterone. Expression of hepatic glucokinase, a marker of glucose utilization, was increased [237] while levels of glucose 6-phosphatase and Glut2, markers of hepatic glucose production and release of glucose into the plasma, were decreased with leptin therapy [177, 232, 237]. Moreover, it has been reported that lower rates of hepatic gluconeogenesis through conversion of pyruvate and glycerol to glucose are associated with leptin treatment [231, 235, 241]. Leptin can also stimulate glucose uptake into BAT [176, 230], soleus muscle [230, 242], red gastrocnemius muscle [176, 242], heart and brain [176]. Uncoupling protein 1 (Ucp1), which mediates non-shivering thermogenesis, was increased in BAT upon leptin administration [237]. Furthermore, markers of fatty acid utilization, LPL, HSL, and carnitine palmitoyltransferase I (CPT1), were normalized in BAT upon leptin induction [237]. Similarly in muscle, expression of fatty acid binding protein and CPT1 were up-regulated and restored with leptin treatment suggesting increased β-oxidation [177, 237]. In addition, leptin potently decreased plasma cholesterol, triglycerides, and fatty acids [177, 232, 233, 237] associated with uncontrolled insulin-deficient diabetes. Even in studies where diabetic controls do not have marked elevations in plasma lipids, leptin decreased lipids beyond that of healthy controls [177, 232, 233, 237]. Overall, leptin plays an integral role in regulating hepatic glucose production, peripheral glucose uptake, and energy expenditure.

The mechanism by which leptin lowers blood glucose in insulin-deficient diabetes is not fully clear but is largely mediated through action on the CNS. ICV injection of leptin that results in negligible peripheral leptin levels restores euglycemia and insulin sensitivity in

insulin-deficient rodents [176, 177, 237, 243] providing compelling evidence that central leptin signalling is sufficient for potent glucose-lowering actions of leptin. Leptin injection into the VMH of STZ-diabetic rats completely normalized blood glucose levels and hepatic glucose production [244]. However, in STZ-injected mice, knockout of leptin receptors in SF-1 neurons, which are found within the VMH, did not block the glucose lowering action of leptin, suggesting a role for other cells within the VMH [230]. Another study in STZ-injected mice found that knockout of GABA in leptin receptor expressing neurons did not prevent the glucose lowering actions of leptin [245], suggesting that GABA itself does not mediate the effect of leptin on glucose lowering. The loss of leptin receptors in POMC neurons within the ARC of STZ-injected mice only partially prevented leptin-mediated reversal of hyperglycemia [230]. In STZ-diabetic MC4R knockout rats, leptin was unable to lower blood glucose levels, suggesting that MC4R, the receptor mediating the downstream effects of α-MSH from POMC neurons, contributes to the glucose lowering effects of central leptin signalling [246]. While leptin signalling in AgRP expressing neurons appears to be critical for the leptin-mediated reversal of diabetes in obese insulin resistant mice [217], their role is unclear in insulin-deficient diabetes. Additional studies are warranted to better understand the neurocircuitry involved in the mechanism of leptin action.

It could be hypothesized that leptin normalizes glucose homeostasis in STZ-diabetic rodents by increasing insulin levels or promoting β-cell regeneration. However, leptin reportedly has no discernable effect on plasma or pancreas insulin levels [177] or β-cell mass [233] and further, when leptin therapy is withdrawn, hyperglycemia returns [177]. In addition, due to the potent inhibitory action of leptin on food intake [247], it may be postulated that leptin normalizes blood glucose in insulin-deficient diabetes by decreasing food consumption. However, during pair feeding studies, despite consuming identical amounts of food as the leptin treated group, pair-fed STZ-rodents did not exhibit normalization of hyperglycemia [177, 233, 236, 237], basal glucose production rate, or plasma cholesterol, triglycerides, or free fatty acid levels, and only experienced a modest improvement in hepatic insulin sensitivity [236]. Interestingly, pair feeding worsened body weight loss in diabetic rats, while leptin maintained a similar body weight loss as in a hyperphagic STZ-vehicle group [233, 236, 237],

suggesting that the anabolic changes may counteract the anorexic effect of leptin. Moreover, it has been speculated that the additional metabolic benefits of leptin treatment in STZ-rodents may be secondary to the normalization of blood glucose. This has been investigated by treating diabetic rats with phloridzin, a drug that normalizes blood glucose independently of insulin by increasing glucose output in the urine. Despite reducing blood glucose levels to that of the leptin treated group, phloridzin treated rats exhibited only moderate improvements in basal glucose production rate and hepatic insulin sensitivity, and did not correct lipid metabolism [236].

Our laboratory has also ruled out the requirement of UCP1 expression and hepatic leptin receptor signalling for the glucose lowering effect of leptin. Since leptin increases glucose uptake and *Ucp1* expression in BAT [176, 230, 237], and UCP1 can mediate non-shivering thermogenesis and energy dissipation as heat, it was hypothesized that UCP1 may be required for the anti-diabetic effect of leptin. To test this, *Ucp1KO* mice rendered diabetic using STZ were treated with exogenous leptin therapy. Leptin was effective in lowering blood glucose to a similar degree in *Ucp1KO* and *Ucp1WT* mice, suggesting that the mechanism is UCP1 independent [248]. In addition, although the glucose lowering effect of leptin appears to be largely mediated by the CNS, leptin receptors are also found in the periphery. Since the liver is a metabolic hub involved in glucose and lipid metabolism it may be hypothesized that hepatic leptin receptor signalling is important for glucose reduction. To test this, mice with a floxed leptin receptor gene were crossed with mice expressing Cre recombinase under the control of the albumin promoter. However, leptin lowered blood glucose levels to the same extent in mice with and without leptin signalling in the liver [167].

Another potential mechanism by which leptin may decrease blood glucose is through decreased glucagon action. Overt hyperglucagonemia is present in uncontrolled insulin-deficient diabetes which contributes to hyperglycemia (as described in section 1.3) [249-251]. We and others have shown that leptin therapy normalizes circulating glucagon levels in STZ-diabetic rodents [167, 230, 233] or NOD mice [233] as well as decreases levels of hepatic phosphorylated CREB, indicative of diminished glucagon action in the liver. However,

glucagon suppression may not be necessary or sufficient for the metabolic actions of leptin. For instance, a low dose of leptin administered to STZ-diabetic mice, enough to restore physiological circulating leptin levels, induced only a slight reduction in blood glucose despite a normalization of plasma glucagon levels [234]. Additionally, in STZ-diabetic rats leptin therapy normalized blood glucose after 6 hours, while glucagon levels were normalized 24 hours after leptin therapy, thereby temporally separating the two effects [235]. Therefore, although glucagon suppression may be beneficial for diabetes, it does not appear to contribute to the glucose lowering action of leptin.

The reduction in corticosterone levels may be more critical for the glucose lowering actions of leptin, which may act to reduce WAT lipolysis thus suppressing the release of gluconeogenic and ketogenic substrates. Adrenalectomy, which reduces corticosterone levels, effectively lowered blood glucose levels in STZ-diabetic rats [241]. In addition, when the leptin-induced drop in corticosterone was prevented by administering exogenous corticosterone the glucose lowering actions of leptin were largely blocked [241]. Addition of an adipose triglyceride lipase inhibitor to rats receiving leptin and exogenous corticosterone restored the glucose lowering effect of leptin suggesting that leptin suppresses lipolysis through reduced corticosterone [241]. Indeed leptin decreases circulating fatty acid and glycerol levels [167, 231, 235, 241, 252], and reduced whole-body glycerol, palmitic acid, and β-hydroxybutyrate turnover indicative of suppressed lipolysis [235, 241].

It has been shown that the leptin-induced suppression of lipolysis drives a reduction in gluconeogenesis. The main substrates for gluconeogenesis are lactate, alanine, and glycerol. Glycerol is released along with fatty acids upon lipolysis of triacylglycerol (TAG), and glycogen can supply glucose through glycogenolysis. Leptin treated mice have severely depleted energy-yielding substrates in the liver such as glucose, acetyl coenzyme A (CoA), TAG, and glycogen [231, 241]. Although hepatic glycogen levels were reduced with leptin therapy, they were depleted prior to blood glucose lowering [231] suggesting that reduced glycogenolysis does not drive glucose lowering in insulin-deficient diabetes. In another study, leptin therapy normalized blood glucose levels but did not affect plasma lactate and alanine

levels suggesting that the glucose lowering actions of leptin are not dependent on reduction of these gluconeogenic substrates [231]. However plasma ketones, glycerol, fatty acids and TAG were gradually reduced in a manner which mimicked the reduction of blood glucose levels [231], consistent with other studies [235, 252]. In addition, in STZ diabetic rats, leptin reduced conversion of glycerol and pyruvate to glucose through hepatic gluconeogenesis [235]. Leptin therapy reduced acetate turnover [235], plasma acetate levels [235] and liver acetyl-CoA concentrations [231, 235, 241] but not free CoA levels [231] suggesting elevated glucose oxidation compared to lipid oxidation in the liver. The reduction in acetyl-CoA may in turn prevent the flux of pyruvate converted to glucose thereby lowering glucose levels. To investigate whether the glucose lowering of leptin can be blocked by supplying these gluconeogenic substrates, an acute injection of glycerol [231], infusion of a lipid emulsion with heparin (to increase glycerol and fatty acids) [235] or infusion of acetate to increase hepatic acetyl-CoA levels [241], were administered to leptin treated diabetic mice or rats. Injection of glycerol increased blood glucose levels, but not to the level of diabetic controls [231]. Similarly, infusion of a lipid emulsion completely reversed the glucose lowering effect of leptin [235], suggesting that depleted glycerol may contribute but depleted fatty acids may play a larger role in the anti-diabetic effect of leptin. Finally, acetate administration, which increases hepatic CoA levels, completely blocked the glucose lowering effect of leptin [241]. Therefore, reductions in glycerol, fatty acids, and acetyl CoA are critical for leptin to lower blood glucose.

The hypothesis that leptin reduces gluconeogenesis through suppression of lipolysis in insulin deficient diabetes is surprising given the well-known fat burning effect of leptin. Under normal circumstances, hyperleptinemia is a signal of sufficient energy stores resulting in the limiting of lipid storage and promoting of a negative energy balance. Indeed, in leptin deficient and lean rodents, leptin therapy causes loss of WAT mass through increased lipolysis [175, 197, 253]. Furthermore, adipocytes from lean or *ob/ob* mice acutely cultured with leptin had increased glycerol release, indicative of increased lipolysis [254, 255]. In addition, lean rodents or *ob/ob* mice that were injected with a single bolus of leptin exhibited an increase of both plasma glycerol and fatty acids *in vivo* [256, 257], as well as increase in glycerol release

in vitro [258, 259]. However, in leptin treated insulin-deficient rodents, plasma glycerol and fatty acids are depleted [231, 235, 241]. This could be consistent with leptin increasing lipolysis until the small WAT stores present in insulin-deficient animals are depleted, thus causing reduced plasma glycerol and fatty acids due to triglyceride depletion rather than suppression of lipolysis. Interestingly, in STZ-injected mice, several days of hyperleptinemia are often required to observe an improvement in hyperglycemia [167, 176, 230, 232, 233], suggesting that leptin may take time to delete energy stores, and thus gluconeogenic substrates, through lipolysis. One exception includes a study using a leptin replacement dose for 6 hours; however, the rats in this study underwent a severe 18 hour fast, which may be required mimic the depletion of energy stores [235]. Thus, the effect of leptin on lipolysis in insulin-deficient diabetes warrants further investigation.

Due to the promising results of leptin therapy in insulin-deficient rodents, researchers initiated a clinical trial testing leptin as an adjunct to insulin for treatment of patients with type 1 diabetes aiming to lower total insulin requirements and suppressing fluctuations in glucose levels (clinicaltrials.gov; NCT01268644). Patients were treated with 0.04-0.08 mg/kg/day recombinant methionyl human leptin via s.c. injection for 20 weeks. Although body weight, percent body fat, and insulin dose were reduced due to leptin therapy, HbA1c, fasting blood glucose and blood glucose, TAG, fatty acids, and glucagon measured over a 24 hour period were unaltered, suggesting that leptin was not efficacious in improving glycemic control in these patients [260]. This trial has been terminated and it is unclear whether leptin therapy will continue to be pursued for type 1 diabetes.

Leptin therapy in type 2 diabetes

Following studies demonstrating the impressive weight loss capabilities of leptin therapy in leptin deficient mice, it was speculated that leptin may be a powerful tool for the treatment of obesity and type 2 diabetes. However, it was soon discovered that individuals with common obesity are hyperleptinemic, suggestive of leptin resistance [261-263]. In clinical trials testing leptin therapy in obese individuals, no changes in body weight or adiposity were observed [264] or the amount of weight lost was considerably variable between patients [265]. This is

consistent with hyperleptinemic diet-induced obese mice that require higher doses of leptin than lean littermates to achieve half-maximal effective doses for weight loss and fat reduction [266]. In addition, although leptin injections in a rat model of obese type 2 diabetes normalized fasting blood glucose [267], leptin treatment in humans with type 2 diabetes did not increase insulin-mediated stimulation of glucose disposal [268] nor meaningfully reduce HbA1c [269]. To combat leptin resistance, recent pre-clinical studies have shifted the focus from exogenous leptin therapy to strategies that enhance endogenous leptin sensitivity [270-274].

Various mechanisms may contribute to leptin resistance including decreased leptin transport through the blood brain barrier and inhibition at the level of the leptin receptor. Obese humans and mice exhibit impaired leptin transport through the blood brain barrier, which may decrease contact with LepR-b expressing neurons critical for regulation of body weight and glucose metabolism [261, 275]. However, additional mechanisms may be involved given that high fat fed mice given ICV leptin have an attenuated body weight reducing effect [276]. Impaired activation of the Jak/STAT pathway as well as elevated expression of the negative regulators SOCS3 and PTP1B have been found in neurons of diet-induced obese rodents [143, 276-278]. SOCS3 dephosphorylates Tyr985 and Jak2 to impair leptin signalling [131] and haploinsufficiency of SOCS3 enhances leptin sensitivity and protects against diet-induced obesity [279] while overexpression of SOCS3 has the opposite effect [280]. PTP1B dephosphorylates Jak2 and knockout of PTP1B in mice results in hypersensitivity to leptin and protection from obesity [281]. Thus, impaired leptin transport through the blood brain barrier or dysregulated SOCS3 or PTP1B may play a role in the development of leptin resistance and inability of leptin to be therapeutic in obese patients with type 2 diabetes.

1.5 Thesis Investigation

Various preclinical studies have demonstrated that leptin can have profound beneficial effects on glucose metabolism in rodent models of insulin-deficient diabetes. Furthermore, the therapeutic potential of glucagon suppression therapy has been demonstrated for type 1 and type 2 diabetes. The overall goal of this dissertation was to investigate the effects of leptin

administration or glucagon suppression therapy in rodent models of diabetes. Multiple studies have suggested that some of the metabolic manifestations of insulin-deficient diabetes are due to hypoleptinemia as opposed to hypoinsulinemia, and insulin therapy normalizes plasma leptin levels. In Chapter 3, we aimed to investigate the contribution of increased leptin action to the benefits of insulin therapy we tested insulin therapy in STZ-diabetic mice on an *ob/ob* background or that were given a leptin antagonist to determine if blocking leptin action would blunt the glucose lowering effects of insulin therapy. It has been shown that leptin increases plasma IGFBP2 in *ob/ob* mice and that massive overexpression of IGFBP2 to beyond levels seen with leptin therapy, was capable of reversing obesity and diabetes in various mouse models of diabetes. In Chapter 4, we aimed to determine whether IGFBP2 is a physiologically relevant mediator of leptin action. To do this we used a low dose of adenovirus to express IGFBP2 to levels seen with leptin therapy in *ob/ob* mice, knocked down IGFBP2 in wildtype mice to examine the physiological role, and prevented the leptin-mediated increase in IGFBP2 using siRNA to determine if this blocks the effect of leptin. Strikingly, leptin can also reverse hyperglycemia in rodent models of insulin-deficient diabetes. This suggested that leptin acts independently of insulin, challenging the dogma that life without insulin is impossible. However, none of the models used were 100% lacking insulin. Therefore, in Chapter 5, we aimed to determine whether insulin is required for the glucose lowering actions of leptin we tested leptin therapy in STZ-diabetic mice given an insulin receptor antagonist or in insulin knockout (*InsKO*) mice. Excess glucagon contributes to hyperglycemia in diabetes, and suppression of glucagon action holds potential as a therapy for type 1 and 2 diabetes. In Chapter 6 we aimed to investigate the effects of Gcgr siRNA encapsulated in LNP on diabetes in STZ diabetic and high fat diet (HFD)/STZ diabetic mice. Finally, given that glucagon suppression has been effective in rodent models of insulin deficiency, it has been hypothesized that glucagon antagonism can overcome the catabolic consequences of reduced insulin. In Chapter 7, we aimed to determine whether inhibiting glucagon can overcome the complete lack of insulin, we have characterized *InsKO* mice with and without *Gcgr* gene inactivation. Collectively the studies help elucidate the mechanism of action of leptin and the therapeutic benefit of glucagon suppression therapy as a treatment for diabetes.

CHAPTER 2 – MATERIALS AND METHODS

2.1 Animals

Housing and facilities

All experiments were approved by The University of British Columbia Animal Care Committee and carried out in accordance with the Canadian Council on Animal Care guidelines. All mice were housed at 21°C in filter-top cages containing red plastic mouse huts and nesting material with EcoFRESH bedding (Absorption Corp, Ferndale, WA, USA) for studies in Chapter 4 or Betachip bedding (Northeastern Products, Warrensburg, NY, USA) for studies in Chapters 3/5/6/7. The majority of mice were housed in groups of 4-5 mice/cage except if mice were separated due to fighting or cage mates reached humane endpoint and were euthanized. All experiments, except those in Chapter 7, were performed at the Centre for Disease Modelling (a specific-pathogen free facility) where mice were housed on a 12-h:12-h light-dark cycle with *ad libitum* access to food (2918, Harlan Laboratories, Indianapolis, IN, USA) and water. Experiments in Chapter 7 were performed in the Modified Barrier Facility (in the conventional zone of the facility) where mice were housed on a 14:10 hour light-dark cycle with *ad libitum* access to food (5053, PicoLab Rodent Diet 20, LabDiet, St. Louis, MO, USA) and water. To allocate animals to groups, basal measurements were inputted into a script that tested up to 10^7 random groupings and performed an analysis of variance (ANOVA) to pick the grouping with the highest p value to ensure minimal differences between groups prior to treatment or a similar procedure was performed manually by testing less groups. Researchers were not blinded to groups when performing experiments and analyzing results.

C57BL/6J and ob/ob mice

Male C57BL/6J mice (stock 000664), *ob/ob* mice (stock 000632 on the C57BL/6J background), and wildtype or heterozygous (*+/ob*) colony controls from the *ob/ob* colony were obtained from the Jackson Laboratory (Bar Harbor, ME, USA) and acclimatized on arrival for at least a week. *Ob/ob* colony controls were genotyped using the primers in Table 1, followed by Dde I digestion.

Table 1. Primer sequences used for genotyping

Genotyping primers for either PCR or probe based qPCR

Target	Forward (5′-3′)	Probe (5′-3′)	Reverse (5′-3′)
Lep[1]	TGTCCAAGATGGACCAGACTC	N/A	ACTGGTCTGAGGCAGGGAGCA
Ins1	TCAGTGCTGCACCAGCATCT	N/A	TCCAGATACTTGAATTATTCCTGGTGTTTTATCAC
Ins1 specific *Neo*[2]	ACAACGTCGAGCACAGCTGC	N/A	CAGGAAGCAGAATTCCAGATACTTGAATTATTCCT
Ins2	GGTCCTTGGTGGTAGTAACTTG	GCAGTGCTCTATGAGGGCCCTAAA	GCCTCTAAAGCCTACTCATCTTC
Ins2 specific *LacZ*	CTGTATGAACGGTCTGGTCTTT	TTGCCCGGATAAACGGAACTGGAA	CGCTATGACGGAACAGGTATT
Gcgr	ACGGTACAGCCAGAAGATTG	CGATGACCTCAGTGTGAGCGTCTG	AGCAAGGTGAGGCATGAG
Gcgr specific *Neo*[2]	CAGCCTCTGTTCCACATACA	ATCAGAAGCTTGGATCTGACTGCTCC	AGCAAGGTGAGGCATGAG

[1]Amplifies a region of the leptin gene containing the *ob* mutation (*WT*, *Het*, and *KO* gene products can be distinguished following Dde I digestion)
[2]One primer anneals in the *Gcgr* or *Ins1* and the other anneals in the *Neo* gene to allow for detection of each gene specific mutant

Generation of InsKO mice

Mice lacking both *Ins1* alleles and one *Ins2* allele, (referred to as *Ins1KO/Ins2Het*) with a mixed genetic background (predominantly C57BL/6 and 129 strains), were supplied by Dr. James D. Johnson (The University of British Columbia, Vancouver, BC, Canada), and were originally described by Duvillie *et al.* [31]. These mice were bred to generate male and female *Ins1KO/Ins2KO* (referred to as *InsKO*) mice and *Ins1KO/Ins2Het* (referred to as *Het*) controls. Mice were genotyped using the primers found in Table 1. Near the expected day of birth, cages containing pregnant mothers were checked in the morning and evening for the presence of litters. Then every morning and evening (as close to 12 hours apart as possible) from post-natal day (P) 0.5 the pups were monitored. The mothers were moved to an empty cage, the cage containing pups was placed on a heating pad and the top of the nest was gently opened to expose the pups. Pups were marked with permanent marker and weighed, and *InsKO*s were suspected based on their reduced body weight. For injection, Lantus insulin glargine (Sanofi-Aventis Inc., Laval, QC, Canada) was diluted to a dose of 5 U/mL in Hams F10 media (Sigma-Aldrich, Oakville, ON, Canada) and remade approximately every 2 days. To ensure survival, suspected *InsKOs* pups were injected subcutaneously with 0.05 U/g insulin from P0.5 - 5, then

each day the dose was reduced by 0.005 U/g until P13 and this dose (0.01 U/g) was continued on P14-15. Exceptions include sometimes injecting 0.075 U irrespective of weight on P0.5, or if a pup did not gain body weight since the last injection, the dose was reduced by half or skipped and then insulin therapy was continued at a lower dose. This injection procedure was developed through trial and error by HC Denroche who found that a constant injection dose of 0.05 or 0.03 U/g was unable to keep the *InsKO* pups alive, while they survived when the dose decreased from 0.05 to 0.01 U/g. On P13-15, *InsKO* mice underwent a transplantation of ~100-150 islets into the anterior chamber of the eye from 10 - 32 week old male or female donors. Mice used in Figures 21/22/24/25 received islets from *Ins1KO/Ins2Het* donors and were split into groups that were matched for sex and body weight prior to starting leptin/vehicle treatment. Mice used in Figure 23 received islets from *Ins1KO/Ins2WT* or *Ins1WT/Ins2KO* donors and all mice were given leptin therapy. At ~10 weeks of age, on day 4 of leptin/vehicle therapy (see below for details of leptin therapy), *InsKO* mice underwent enucleation of the graft-bearing eye and *Het* controls underwent enucleation to control for the effect of the surgery. Details of islet isolation, islet transplantation and enucleation are described below.

Generation of GcgrKO/InsKO mice

Mice with global *Gcgr* knockout that were backcrossed for seven generations onto a C57BL/6J background, originally described by Gelling *et al.* [44], were supplied by Dr. Daniel J. Drucker (The University of Toronto, Toronto, ON, Canada). Male *GcgrKO* mice were bred with female *Ins1KO/Ins2Het* mice and their progeny was subsequently bred according to the breeding scheme in Figure 1, to generate male and female experimental and control mice. If mice with the *GcgrKO* genotype were required for breeding, males were used, as it is known that pups from *GcgrKO* mothers die *in utero* or shortly after birth [282]. Mice were genotyped using primers found Table 1. For pup characterization, *GcgrHet/InsKO* and *GcgrKO/InsKO* pups were not treated with insulin. For young adult characterization, *GcgrHet/InsKO* and *GcgrKO/InsKO* mice were treated twice daily with s.c. injections of insulin as described above. On P13-15, *GcgrHet/InsKO* and *GcgrKO/InsKO* mice underwent a transplantation of ~130 islets into the anterior chamber of the eye from 20 - 21 week old male *Ins1KO* donors with 0-2 alleles of *Gcgr* and 1-2 alleles of *Ins2*. The genotype of the islets transplanted were not

tracked per recipient mouse, which may have been from donor mice of single genotype or a mix of genotypes. At 4 weeks of age, *GcgrHet/InsKO* and *GcgrKO/InsKO* mice underwent enucleation of the graft-bearing eye and *GcgrHet/InsHet* and *GcgrKO/InsHet* controls underwent enucleation to control for the effect of the surgery. Details of islet isolation, islet transplantation and enucleation are described below.

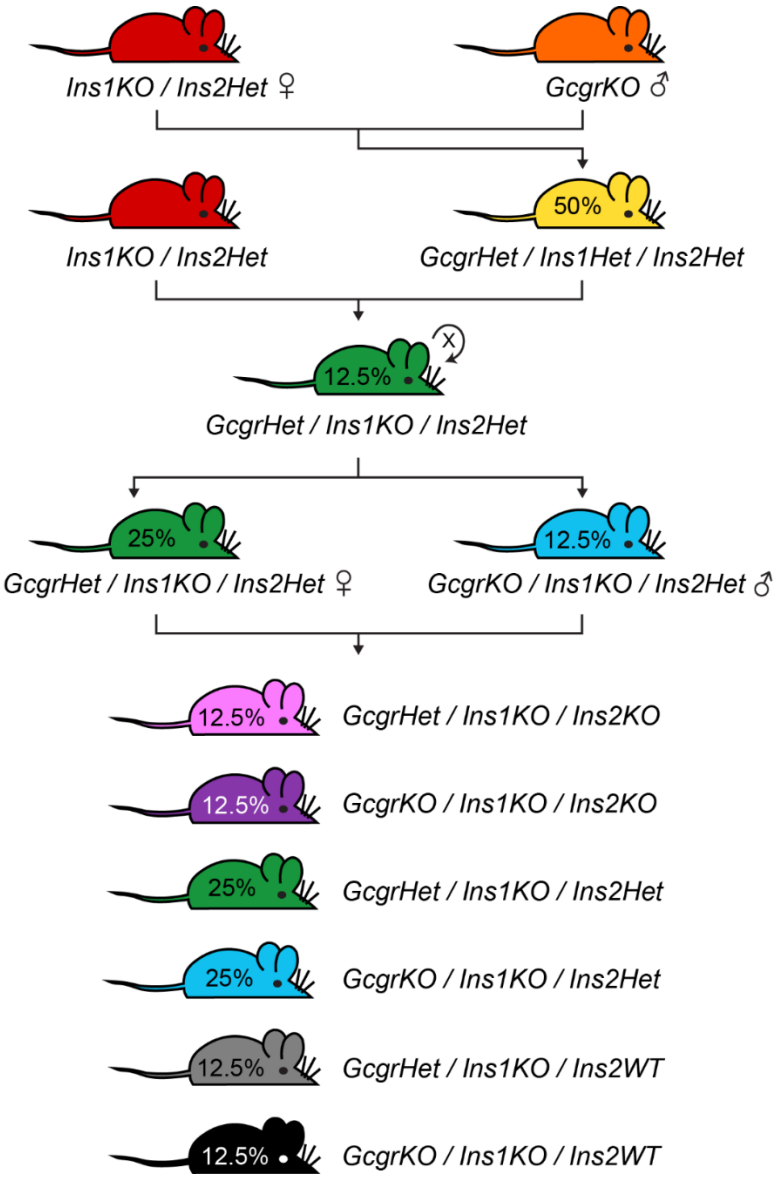

Figure 1. Breeding scheme for the generation of *GcgrKO/InsKO* mice. Schematic depicting the generation of *GcgrKO/InsKO* and littermates used in Chapter 7. If mice with the *GcgrKO* genotype were required, males were used as it is known that pups from *GcgrKO* mothers die *in utero* or shortly after birth. Male and female mice were used for experiments.

Islet isolation, islet transplantation and enucleation in InsKO mice

Islets were isolated using procedures previously described by Lacy *et al.* [283] and Salvalggio *et al.* [284]. Mice used as islet donors were euthanized and the pancreatic duct was injected with ~5 mL of ice cold Type XI collagenase (1000 U/mL; Sigma-Aldrich, Oakville, ON, Canada) dissolved in filter-sterilized Hank's balanced salt solution (HBSS – 137 mM NaCl, 5.4 mM KCl, 4.2 mM NaH_2PO_4, 4.1 mM KH_2PO_4, 10 mM HEPES, 1 mM $MgCl_2$, 5 mM glucose, pH 7.2). The pancreas was then excised, placed in a 37°C water bath for ~12 minutes, and shaken vigorously to dissociate the pancreatic tissue, then 40 mL of ice cold HBSS with 1 mM $CaCl_2$ was added to stop digestion. The pancreas solution was centrifuged for 1 min at 1000 rpm at 4°C and resuspended in HBSS+$CaCl_2$ twice, then resuspended in Hams F10 media (Sigma-Aldrich, Oakville, ON, Canada) supplemented with 7.5% bovine serum albumin (Life Technologies; Burlington, ON, Canada), and 1% penicillin-streptomycin (Life Technologies; Burlington, ON, Canada). Islets were then filtered through a 70 µm cell strainer (BD Biosciences, San Diego, CA, USA) and hand-picked 2 times to purify the islets from contaminating exocrine tissue or lymph nodes. Islets were either transplanted immediately, or incubated for up to 3 days at 37°C (humidified air, with 5% CO_2) in Hams F10 media supplemented with 7.5% bovine serum albumin and 1% penicillin-streptomycin prior to transplantation. Islets that were cultured appeared healthy upon visual inspection as indicated by smooth round surface and lack of dark hypoxic center.

Islet transplantation was performed by M Mojibian. Islets in a minimal volume (10 – 20 µL) were aspirated into a stripper micropipette (MXL3-200, MidAtlantic Diagnostics, Mt Laurel, NJ, USA) that was attached to the modified micro-manipulator. Mice were anesthetized under isoflurane, administered s.c. injections of ketoprofen (5 mg/kg; Merial, Baie d'Urfé, QC, Canada), and positioned under a stereomicroscope and proparacaine ophthalmic topical analgesic (0.5% solution) was applied to the eye to minimize and relieve postoperative pain. Using a 27G needle the cornea was punctured close to the sclera, the micropipette was gently inserted into the anterior chamber of the eye, and the islets were injected using the micro-manipulator.

Enculeation surgery was performed by M Mojibian. In adulthood, *InsKO*, *GcgrHet/InsKO* and *GcgrKO/InsKO* mice with islets in the anterior chamber of the eye, underwent enucleation of the graft bearing eye to render the mice completely insulin-deficient. Control animals also underwent enucleation to control for the effect of the surgery. Mice were anesthetized under isoflurane, administered ketoprofen (5 mg/kg; Merial, Baie d'Urfé, QC, Canada), and positioned under a stereomicroscope. Two non-absorbable sutures were tied behind the eye to stop blood flow, the eye was removed anterior to the sutures using micro-scissors, and Gluture Tissue Adhesive (World Precision Instruments, Sarasota, FL, USA) was applied to prevent unraveling of the sutures and to close the wound.

Generation of STZ-diabetic mice

STZ (Sigma-Aldrich, Oakville, ON, Canada) was freshly prepared in a pH 4.5 acetate buffer (118 mM $C_2H_3NaO_2$, 38.5 mM NaCl) and administered at a dose of 180 mg/kg STZ in male C57BL/6J mice, *ob/ob* mice, or *+/ob* controls from the *ob/ob* colony or at a dose of 140 mg/kg STZ in *ob/ob* mice. C57BL/6J mice received the injection at 9 weeks of age while *ob/ob* and *+/ob* mice received the injection at 6 weeks of age. Diabetes was defined as two consecutive blood glucose measurements >20 mM. Non-diabetic controls received an i.p. injection of acetate buffer alone.

Generation of HFD/STZ-diabetic mice

Male C57BL/6J mice were put on a 60% HFD (D12492i, Research Diets, Inc., New Brunswick, NJ, USA) at 6 weeks of age and remained on the diet until the end of the study. At 10 weeks of age, STZ (Sigma-Aldrich, Oakville, ON, Canada) was prepared in a pH 4.5 acetate buffer (118 mM $C_2H_3NaO_2$, 38.5 mM NaCl) and administered at a dose of 100 mg/kg. Diabetes was defined as one consecutive blood glucose measurement >13 mM (measurements ranged from 13 – 27 mM). Controls were put on a 10% low fat diet (LDF) (D12450i, Research Diets, Inc., New Brunswick, NJ, USA) at 6 weeks of age and did not receive an i.p. injection of acetate buffer. Composition of the diets can be found in Table 2.

Table 2. Composition of 60% and 10% fat diets

	D12492 (60% fat)		D12450B (10% fat)	
Component	**g (%)**	**kcal (%)**	**g (%)**	**kcal (%)**
Protein	26.2	20	19.2	20
Carbohydrate	26.3	20	67.3	70
Fat	34.9	60	4.3	10
kcal/g	5.24		3.85	
Ingredient	**g**	**kcal**	**g**	**kcal**
Casein, 30 Mesh	200	800	200	800
L-Cystine	3	12	3	12
Corn Starch	0	0	315	1260
Maltodextrin 10	125	500	35	140
Sucrose	68.8	275.2	350	1400
Cellulose, BW200	50	0	50	0
Soybean Oil	25	225	25	225
Lard	245	2205	20	180
Mineral Mix S10026	10	0	10	0
DiCalcium Phosphate	13	0	13	0
Calcium Carbonate	5.5	0	5.5	0
Potassium Citrate, 1 H_2O	16.5	0	16.5	0
Vitamin Mix V10001	10	40	10	40
Choline Bitartrate	2	0	2	0
FD&C Blue Dye #1	0.05	0	0	0
FD&C Yellow Dye #5	0	0	0.05	0
Total	**773.85**	**4057**	**1055.05**	**4057**

2.2 Blood and Plasma Analytes

All parameters were measured following a 4 hour fast (between ~11 AM and 3 PM) except for Chapter 7 in which all parameters were measured in the *ad libitum* fed state, unless otherwise specified. All measurements were performed in singlicate. All blood glucose measurements were taken via the saphenous vein, except those from *InsKO, GcgrHet/InsKO* and *GcgrKO/InsKO* mice which were taken via the tail-vein, using a OneTouch Ultra Glucometer (LifeScan, Burnaby, BC, Canada) with a detection limit of 1.1 – 33.3 mM. In some instances when glucometer readings were <1.1 mM they were assigned a value of 1.1, or if they were >33.3 mM they were assigned a value of 33.3 mM and statistical analysis was not performed. In other instances, samples that fell over the detection limit were diluted with

non-diabetic blood of known glucose concentration, re-assayed, and original blood glucose levels calculated. Hemoglobin A1c (HbA1c) levels were measured using a Siemens DCA 200 Vantage Analyzer (Siemens Healthcare Diagnostics, Tarrytown, NY, USA) from whole blood. Blood samples were taken from either the saphenous vein and collected into heparinized capillary tubes or by cardiac puncture into a needle coated with heparin to achieve a final concentration of 4.0 U heparin/mL whole blood. Plasma was separated by centrifugation of samples at 4600 RCF for 9 minutes at 4°C. Insulin (Ultrasensitive Mouse Insulin ELISA, ALPCO, Salem, NH, USA), IGFBP2 (Mouse/Rat IGFBP-2 ELISA, ALPCO, Salem, NH, USA), leptin (Mouse Leptin ELISA, Crystal Chem, Downers Grove, IL), corticosterone (Corticosterone ELISA, Abcam, Toronto, ON, Canada), glucagon (Glucagon ELISA, Mercodia, Salem, NC, USA), β-hydroxybutyrate (β-Hydroxybutyrate LiquiColor Test, Stanbio, Boerne, TX), triglycerides and glycerol (Serum Triglyceride Determination Kit, Sigma-Aldrich, Oakville, ON, Canada), cholesterol (Cholesterol E, Wako Diagnostics, Richmond, VA, USA) and free fatty acids (HR Series NEFA HR[2] Kit, Wako Diagnostics, Richmond, VA, USA) were measured from plasma samples according to manufacturer instructions or by scaling down the assay to a 96-well format. If samples fell above the standard curve of the assay, samples were diluted and reassayed. For samples from polyethylene glycol (PEG)lyated leptin injected mice, plasma leptin levels were analyzed using a PEGylated leptin standard curve as the PEGylated leptin was found to produce a lower absorbance signal than non-PEGylated leptin.

2.3 Experimental Manipulations

Overexpression of IGFBP2 by adenoviral transfer

RK Baker generated the Ad-IGFBP2 construct. Briefly, the mIGFBP2 open reading frame was removed from pCMV SPORT6 mIGFBP2 (Open Biosystems, Huntsville, AL, USA) with EcoRI and NotI, and subcloned into pShuttle (Clontech, Palo Alto, CA, USA) downstream of the cytomegalovirus (CMV) promoter and a heterologous intron (rabbit beta globin intron 2). The transgene was then excised with I-CeuI and PI-SceI and ligated into pAdeno-X (Clontech, Palo Alto, CA, USA). The adenovirus was generated and propagated

by transfecting HEK293 cells with ligated construct and culture supernatant was sent to ViraQuest Inc. (North Liberty, IA, USA) for further expansion and purification by CsCl gradient. The adenovirus expressing β-galactosidase (Ad-β-gal) expressing lac-Z under the CMV promoter was generated as previously described [285]. In Chapter 4, to investigate the effect of IGFBP2 expression *in vivo*, 9 week old male *ob/ob* mice were injected intravenously via the tail vein with either 10^8 plaque forming units (low dose) or 10^9 plaque forming units (high dose) of Ad-IGFBP2 or Ad-β-gal.

Preparation of lipid nanoparticles siRNA systems and in vivo delivery

Lipid nanoparticles siRNA systems were prepared by the Cullis laboratory (The University of British Columbia, Vancouver, BC, Canada). IGFBP2 siRNAs were purchased as Stealth RNAi siRNA (Invitrogen, Burlington, ON, Canada). Gcgr siRNAs and the factor VII (FVII) siRNA were purchased from Integrated DNA Technologies (Coralville, IA, USA). siRNA sequences are outlined in Table 3. The lipids, 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) and cholesterol were obtained from Avanti Polar Lipids (Alabaster, AL, USA). The ionizable lipid 2,2-dilinoleyl-4-(2-dimethylaminoethyl)-[1,3]-dioxolane (DLin-KC2-DMA) and N-[(methoxy polyethylene glycol 2000 carbamyl]-1,2-dimyristyloxlpropyl-3-amine (PEG-c-DMA) were purchased from AlCana Technologies (Vancouver, BC, Canada).

Lipid nanoparticle siRNA systems were synthesized by rapid mixing of lipid components with siRNA using a microfluidic micromixer as described previously [286]. Briefly, a lipid mixture in ethanol composed of DLin-KC2-DMA/DSPC/cholesterol/PEG-c-DMA at a molar ratio of 50/10/39/1 was rapidly combined with 3 volumes of siRNA dissolved in 25 mM acetate, pH 4.0 in a microfluidic micromixer provided by Precision Nanosystems (Vancouver, BC, Canada) with a combined final flow rate of 4 mL/min. siRNA-to-lipid ratio was maintained at 0.06 (wt/wt). Ethanol was removed by 4 hour dialysis of lipid nanoparticle formulations in 50 mM 2-(N-morpholino)ethanesulfonic acid/sodium citrate buffer, pH 6.7 followed by an overnight dialysis in 1X phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8 mM Na_2HPO_4), pH 7.4. All lipid nanoparticles have mean

diameter of ~60 nm in number mode as measured by a Zetasizer NanoZS (Malvern, Worcestershire, UK). siRNA concentration was determined by measuring absorbance at 260 nm and lipid concentration was measured by using the Cholesterol E assay (Wako Diagnostics, Richmond, VA, USA).

For all studies, siRNA was administered via the tail vein to allow for delivery to liver [287]. In Chapter 4, 9 week old male *ob/ob* mice or 13 week old male C57BL/6J mice were injected with 5 mg/kg lipid nanoparticle encapsulating one of three IGFBP2 siRNAs or FVII siRNA. Empty particles and PBS were also used as controls. In Chapter 6, for Figure 26, 5 mg/kg lipid nanoparticle encapsulating one of three Gcgr siRNAs or FVII siRNA were injected. For Figure 27, 5 mg/kg lipid nanoparticle encapsulating Gcgr siRNA 2 or FVII siRNA were injected. For Figures 28/29/32, 10 mg/kg lipid nanoparticle encapsulating Gcgr siRNA 2 or FVII siRNA were injected. For Figure 31, 10 mg/kg lipid nanoparticle encapsulating an equal mix of Gcgr siRNA 1 and 2 or FVII siRNA were injected.

Table 3. siRNA sequences

siRNA	Sense (5′-3′)	Antisense (5′-3′)
IGFBP2 siRNA 1[1]	GCCAUCUCUUCUACAACGAGC AGCA	GCAGUGCAAGAUGUCUCUGA ACGGA
IGFBP2 siRNA 2[1]	GCAGUGCAAGAUGUCUCUGAA CGGA	UCCGUUCAGAGACAUCUUGC ACUGC
IGFBP2 siRNA 3[1]	CCCACAGCAGGUUGCAGACAG UGAU	AUCACUGUCUGCAACCUGCU GUGGG
Gcgr siRNA 1	mGmGAmGAmAGCCAUmGUmU AmUCmUAUGAAmCT	AGmUUCAUAmGAmUAmACAU GGCUUCmUCmCmAmU
Gcgr siRNA 2	mGmUAmACmAGAACCmUUmCG mACmAAGUACmUC	GAmGUACUUmGUmCGmAAGG UUCUGUmUAmCmAmG
Gcgr siRNA 3	mAmCCmCGmAAACUAmCAmUC mCAmUGGGAAmCC	GGmUUCCCAmUGmGAmUGUA GUUUCGmGGmUmGmC
FVII siRNA	GGAucAucucAAGucuuAcT*T	GuAAGAcuuGAGAuGAuccT*T

[1] Invitrogen Stealth siRNA used proprietary chemical modifications to restrict off-target effects
Lowercase ‘m_’ indicates 2’O-methyl modifications
Lowercase bases indicated 2’fluoro modifications
Asterisks indicate phosphothioate linkages

Leptin therapy

In Chapters 4/5/7 leptin was delivered via mini-osmotic pump. *Ob/ob* mice were given a dose of either 0.8 µg/day or 5 µg/day of recombinant mouse leptin (Peprotech, Rocky Hill, NJ, USA) prepared in sterile water and administered via Alzet 1004 mini-osmotic pumps (DURECT Corporation, Cupertino, CA, USA). Male STZ-diabetic mice were given a dose of 20 µg/day of recombinant mouse leptin (Peprotech, Rocky Hill, NJ, USA) prepared in sterile water and administered via Alzet 1007D mini-osmotic pumps (DURECT Corporation, Cupertino, CA, USA). Water (vehicle) filled pumps were used as controls. Pumps were incubated at 37°C in sterile saline according to the manufacturer's instruction prior to s.c. implantation. Mice were anesthetized with isoflurane, given s.c. injections of ketoprofen (5 mg/kg; Merial, Baie d'Urfé, QC, Canada) and bupivicaine (6 mg/kg; Hospira Healthcare Corporation; Montreal, QC, Canada), and implanted with the pump subcutaneously. Non-diabetic controls received sham surgery. Surgical incisions were closed with sutures or wound clips.

In Chapters 3/5/6 PEGylated mouse leptin (Protein Laboratories Rehovot Ltd., Rehovot, Israel) was delivered by daily i.p. or s.c. injection. *Ob/ob* mice were given a dose of 5 µg/day, *InsKO* mice were given a dose of 1 µg/g body weight/day (for Figures 21/22/24/25) or 0.5 µg/g body weight/day (for Figure 23), and HFD/STZ-diabetic mice received 20 µg/day. Control mice received water (vehicle) injections.

Insulin therapy

In Chapters 3 and 5, STZ-diabetic *ob/ob*, *+/ob*, or C57BL/6J mice were subcutaneously implanted with one slow release insulin pellet (LinShin Inc., Toronto, ON Canada). Mice were anesthetized with isoflurane, given a s.c. injection of ketoprofen (5 mg/kg; Merial, Baie d'Urfé, QC, Canada) and bupivicaine (6 mg/kg; Hospira Healthcare Corporation; Montreal, QC, Canada), and implanted with the insulin pellet. Non-diabetic controls received sham surgery. Surgical incisions were closed with sutures or Gluture Tissue Adhesive (World Precision Instruments, Sarasota, FL, USA).

Leptin antagonist plasmid delivery

The plasmids were generated by RK Baker. Briefly, the mouse leptin antagonist open reading frame (mouse leptin containing D23L, L39A, D40A, and F41A mutations) with an XbaI site 5' of the start codon and a SalI site 3' to the stop codon was obtained as a chemically synthesized, double-stranded DNA fragment from Integrated DNA Technologies (Coralville, IA, USA). This fragment was digested with XbaI and SalI then ligated into the NheI and XhoI sites in the pLIVE vector (Mirus Bio; Madison, WI, USA) downstream of a murine albumin promoter. The leptin antagonist plasmid (pLA) was then sequenced, amplified with endotoxin-free gigapreps (QIAgen; Hilden, Germany), and resuspended in sterile water. The empty (pEmpty) pLIVE vector was used as a control. Healthy or STZ-diabetic C57BL/6J mice were administered 50 µg of leptin antagonist plasmid or empty (control) plasmid via hydrodynamic tail-vein injection, which was performed by MM Kwon. This delivery method involved 2.0–2.5 ml of saline solution containing the plasmid DNA being injected into the via tail vein over 5–8 seconds.

Leptin antagonist peptide delivery

PEGylated super active mouse leptin antagonist (PEG-SMLA, Protein Laboratories Rehovot Ltd., Rehovot, Israel) containing D23L, L39A, D40A, and F41A mutations was delivered via daily i.p. injection at a dose of 5 mg/kg/day to healthy or STZ-diabetic C57BL/6J mice.

Insulin antagonist (S961) delivery

Following a 4 hour fast, mice were injected i.p. with 24 nmol/kg S961 (Novo Nordisk, Bagsvaerd, Denmark) or PBS (vehicle) as a control either alone (day 5), or 30 min preceding an oral gavage of 1.5 g/kg 30% dextrose (day 7) (Fisher Scientific, Ottawa, ON, Canada).

2.4 *In vivo* Assays

Oral glucose tolerance test

Mice were fasted for 4 hours and given an oral gavage of either 1.5 g/kg of 30% dextrose or 2 g/kg of 50% dextrose (Fisher Scientific, Ottawa, ON, Canada) and blood glucose was measured via the saphenous vein at the indicated time points post-gavage. In some instances when glucometer readings were >33.3 mM they were assigned a value of 33.3 mM and statistical analysis was not performed. In other instances, samples that fell over the detection limit were diluted with non-diabetic blood of known glucose concentration, re-assayed, and original blood glucose levels calculated. Area under the curve (AUC) was measured from 0.

Insulin tolerance test

Mice were fasted for 4 hours and given an intraperitoneal injection of 1 U/kg insulin (Novolin ge Toronto, Novo Nordisk, Mississauga, ON, Canada) and blood glucose was measured via the saphenous vein at the indicated time points post-injection.

Fasting tolerance test

In Chapter 5 blood glucose and body weight were measured prior to fasting. If mice became hypoglycemic while fasting (<1.1 – 2.3 mM) food was returned, or glucose was injected.

Survival curve

In Chapters 5 and 7, following enucleation, *InsKO, GcgrHet/InsKO* and *GcgrKO/InsKO* mice were monitored every few hours in order to euthanize animals as they reached humane endpoint. For the leptin treated *InsKO* mice, due to the anorexic effect of leptin, significant body weight loss occurred before enucleation. Therefore, regardless of total weight lost, mice requiring euthanasia were hunched, displayed piloerection, and were lethargic or non-responsive.

2.5 Analysis of Mouse Tissues

Hepatic lipid content

Hepatic triglycerides and cholesterol were quantified by a modified protocol from Briaud *et al.* [288]. Liver (~150 µg for adult mice and ~20 µg for pup mice) was collected following a 4 hour fast (adult mice) or in the ad libitum fed state (pup mice) and immediately flash frozen in liquid N_2. Liver was homogenized with an Ultra-Turrax in 3 mL of chloroform:methanol (2:1). Lipids were extracted first with 1.5 mL of ice cold water and second with 750 µL of ice cold water. A portion (500 µL for adult mice and 1400 µL for pup mice) of the organic layer was dried under N_2 gas and 30 µL of Thesit (Sigma-Aldrich, Oakville, ON, Canada) was added and mixed under N_2 gas. Standards containing cholesterol (Wako Diagnostics, Richmond, VA, USA) and triolein (Sigma-Aldrich, Oakville, ON, Canada) were also dried down under N_2 gas and mixed with Thesit. Water (300 µL) was added and samples were incubated at 37°C for 30 minutes with intermittent vortexing. Triglycerides were assayed using the Serum Triglyceride Determination kit (Sigma-Aldrich, Oakville, ON, Canada) and total cholesterol was quantified using the Cholesterol E kit (Wako Diagnostics, Richmond, VA, USA) according to the manufacturer instructions.

Quantification of hepatic transcripts using RT-qPCR

Liver was collected following a 4 hour fast and stored in RNA*later* (Qiagen, Mississauga, ON, Canada) overnight at 4°C. Then the RNA*later* was removed and tissues were stored at -80°C. To extract RNA, liver was homogenized using a Tissue Tearer in TRI Reagent according to manufacturer instructions (Ambion, Streetsville, ON, Canada). Next, RNA was DNase treated (New England Biolabs, Whitby, ON, Canada) and cDNA was generated using the iScript cDNA synthesis kit (Bio-Rad, Mississauga, ON, Canada). Beta-2-microglobulin (*B2m*) was used as a reference gene, which was selected from 6 potential reference genes (beta-actin (*Actb*), *B2m*, glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*), phosphoglycerate kinase (*Pgk1*), and peptidylprolyl isomerase A (*Ppia*)) based on stability between experimental groups assessed by geNorm software [289] and PCR efficiency. The Sybr Green method was employed using SsoFast EvaGreen Supermix with Low ROX (Bio-Rad, Mississauga, ON,

Canada) and relative transcript levels were calculated using the Pfaffl method [290]. Primer sequences can be found in Table 4.

Table 4. Primer sequences for RT-qPCR

Target	Forward Primer (5'-3')	Reverse Primer (5'-3')
Actb	AAGAGCTATGAGCTGCCTGA	TACGGATGTCAACGTCACAC
B2m	GGCCTGTATGCTATCCAGAA	GAAAGACCAGTCCTTGCTGA
Gapdh	CTGGAGAAACCTGCCAAGTA	TGTTGCTGTAGCCGTATTCA
Hprt1	GCTGACCTGCTGGATTACAT	TTGGGGCTGTACTGCTTAAC
Igfbp1	GATCGCCGACCTCAAGAAATG	CCTCTAGTCTCCAGAGACCCAG
Igfbp2	ACCCCTTGCCAGCAGGAGTTGGA	TCCCTGGATGGGCTTCCCGGT
Igfbp3	GACAGAATACGGTCCCTGCC	GGAGCATCTACTGGCTCTGC
Igfbp4	CCACCCCAAACAGTGTCACC	CTCAGACTCCAAGCCAGGTC
Igfbp5	CGACTGTTGTCATTTGCCAGC	CTTTGTGTTGCTCCATGTTCCG
Igfbp6	GGGATGCAGACTGGTTGTCG	CCTCCTTGGGGTTTGCTCTC
Pgk1	GCAGATTGTTTGGAATGGTC	TGCTCACATGGCTGACTTTA
Ppia	AGCTCTGAGCACTGGAGAGA	GCCAGGACCTGTATGCTTTA

2.6 Histology

Tissue processing and hematoxylin and eosin staining

Pancreata and liver were harvested from mice following a 4 hour fast, fixed overnight in 4% paraformaldehyde (pH 7.2) at 4°C, then rinsed in 70% ethanol. Tissues were then embedded in paraffin and sectioned by Wax-it Histology Services Inc (5 µm thickness; Vancouver, BC, Canada). Liver sections were stained with hematoxylin and eosin by Wax-it Histology Services Inc (Vancouver, BC, Canada) and visualized under light microscopy.

Quantification of alpha cell area and immunofluorescent staining

Antibodies used are detailed in Table 5. Sections were immunostained with primary antibodies overnight at 4°C then incubated with secondary antibodies for 1 hour at room temperature. Cell nuclei were then counterstained with VECTASHIELD HardSet Mounting Medium with DAPI (Vector Laboratories, Burlingame, CA, USA). Whole slide fluorescence scanning was performed using an ImageXpress Micro Imaging System (Molecular Devices

Corporation, Sunnyvale, CA, USA). Individual images were stitched together to recreate the pancreas area and then quantified using MetaXpress software. For α-cell area, glucagon positive area was expressed relative to the pancreas area; 3 sections were quantified per animal, separated by 200 µm. For Glut2/insulin staining, 1 section per animal was stained.

Table 5. Antibodies used for immunofluorescence

α-Cell Area Staining									
Primary Antibody					**Secondary Antibody**				
Antigen	**Host**	**Dilution**	**Manufacturer**	**Cat. #**	**2° Antibody**		**Dilution**	**Manufacturer**	**Cat. #**
Glucagon	Mouse	1:1000	Sigma-Aldrich	G2654	Alexa-Fluor-	Goat-anti-mouse	1:1000	Invitrogen	A11032
Glut2/Insulin Staining									
Primary Antibody					**Secondary Antibody**				
Antigen	**Host**	**Dilution**	**Manufacturer**	**Cat. #**	**2° Antibody**		**Dilution**	**Manufacturer**	**Cat. #**
Glut2	Rabbit	1:500	Millipore	07-1402	Alexa-Fluor-	Goat-anti-rabbit	1:1000	Invitrogen	A11034
Insulin	Mouse	1:250	Cell Signaling	8138BF	Alexa-Fluor-	Goat-anti-mouse	1:1000	Invitrogen	A21424

2.7 Data Analysis

Data are presented as mean±SEM or individual mice. Data were analyzed using an unpaired student's t-test for bar graphs with two groups, an ordinary 1-way ANOVA with Bonferroni, Dunnett, Sidak or Tukey post-hoc testing for bar graphs with three or more groups, a repeated measures 2-way ANOVA with Bonferroni, Dunnett, Sidak or Tukey post-hoc testing for line graphs or the Kaplan-Meier method with the log rank test for survival curves. Statistical analysis was performed using GraphPad Prism 6.05 (La Jolla, CA) where the experimental unit is a single mouse and significance was set at $P<0.05$.

CHAPTER 3 – CONTRIBUTION OF LEPTIN TO THE BENEFICIAL EFFECTS OF INSULIN THERAPY

3.1 Introduction

With the discovery of insulin in 1922 [17], type 1 diabetes has changed from a uniformly fatal condition to a lifelong, but treatable disease. It is often thought that the diabetic manifestations in uncontrolled type 1 diabetes are directly due to insulin loss. However, humans and rodents with insulin-deficient diabetes also exhibit dramatically decreased plasma leptin levels due to the inability of insulin to promote lipogenesis and leptin synthesis [291, 292]. Indeed there is a rapid decrease in leptin in STZ-injected rats that is restored upon insulin treatment [293]. In addition, leptin levels are low in children with new-onset type 1 diabetes compared to controls and are increased within 24 hours of insulin therapy and normalized within 3 months of therapy [294, 295]. Therefore, we have questioned if leptin deficiency is responsible for some of the metabolic derangements in uncontrolled insulin-deficient diabetes and whether some of the beneficial actions of insulin therapy may be due to increased leptin levels. In an attempt to dissect the role of leptin deficiency in insulin-deficient states, German *et al.* administered a dose of leptin high enough to restore normal leptin levels to STZ-diabetic rats [234]. Although leptin replacement therapy only caused a minute decrease in blood glucose levels, insulin resistance, a common symptom of insulin-deficiency, was completely normalized [234]. In addition, Denroche *et al.* demonstrated that a dose of leptin resulting in restoration of normal leptin levels was also capable of normalizing plasma triglycerides, fatty acids, and β-hydroxybutyrate levels in STZ-diabetic mice [296]. There is also evidence that hypoleptinemia, and not hypoinsulinemia, induces hyperphagia and suppresses the hypothalamic-pituitary-adrenal axis in STZ diabetes [297, 298]. Therefore, while it is clear that attenuation of leptin action does contribute to the metabolic defects seen in insulin-deficient diabetes, it is unknown to what extent it is involved in the multitudinous symptoms.

In this Chapter we hypothesized that a proportion of the therapeutic effects of insulin on insulin-deficient diabetes is due to increased leptin action. To investigate this we first

attempted to develop a model of insulin-depleted leptin-deficient mice by STZ-treating *ob/ob* mice to determine if insulin therapy is impaired in the absence of a rise in circulating leptin levels. Complementary to this we blocked an increase in leptin action via a leptin antagonist in insulin treated STZ-diabetic mice to determine if this would prevent normalization of diabetic symptoms. Our data suggest that leptin action may contribute to the improvement in hyperglycemia and glucose tolerance following insulin therapy. All of the data in this Chapter are unpublished.

3.2 Results

STZ injected ob/ob mice experience severe weight loss and mortality

To determine if insulin therapy is effective in insulin-deficient diabetes when leptin levels cannot be raised, we first attempted to generate a leptin-deficient model of insulin-deficient diabetes by injecting *ob/ob* mice with STZ. Obese leptin-deficient *ob/ob* mice exhibit β-cell expansion and hyperinsulinemia, and are typically used as a model of type 2 diabetes [94, 225, 299]; however, we required an insulin-deficient state to test our hypothesis. Previous reports have shown that STZ injection in *ob/ob* mice [300, 301] or *db/db* mice [302] is not as effective at destroying β-cells as it is in wildtype littermates. We hypothesize that the ineffectiveness of STZ may be due to decreased Glut2 expression in *ob/ob* and *db/db* islets due to the diabetic environment [303, 304], and Glut2 is required for STZ to enter and destroy β-cells [305]. Therefore, to allow *ob/ob* mice to be rendered diabetic by STZ-injection, we treated 5 week old *ob/ob* mice with daily i.p. injections of PEGylated leptin at a dose of 5 μg/day until their body weight reached that of *+/ob* controls (Figure 2A). This dose of leptin corrects derangements characteristic of *ob/ob* mice as glucose metabolism is dramatically and rapidly normalized [306, 307] and fertility is restored. On day 9, leptinized-*ob/ob* mice and *+/ob* controls were administered STZ at a dose of 180 mg/kg then leptin treatment was ceased. Following STZ injection, *ob/ob*-STZ mice continued to rapidly decrease in body weight losing almost 20% by day 13, and all mice had reached humane endpoint by day 16, 1 week after STZ injection (Figure 2A-D). Despite being given the same dose of STZ, *+/ob* mice did not experience as severe body weight loss following STZ injection, losing ~2% body weight by

day 13 (Figure 2A-D). On day 13 *ob/ob*-STZ mice trended towards increased blood glucose levels compared to untreated *ob/ob* controls while *+/ob*-STZ mice were significantly more hyperglycemic than untreated *+/ob* mice (11.6±1.6 mM *ob/ob*, 18.5±1.8 mM *ob/ob*-STZ, 7.4±0.7 mM *+/ob*, 20.9±2.3 mM *+/ob*-STZ, Figure 2E&F).

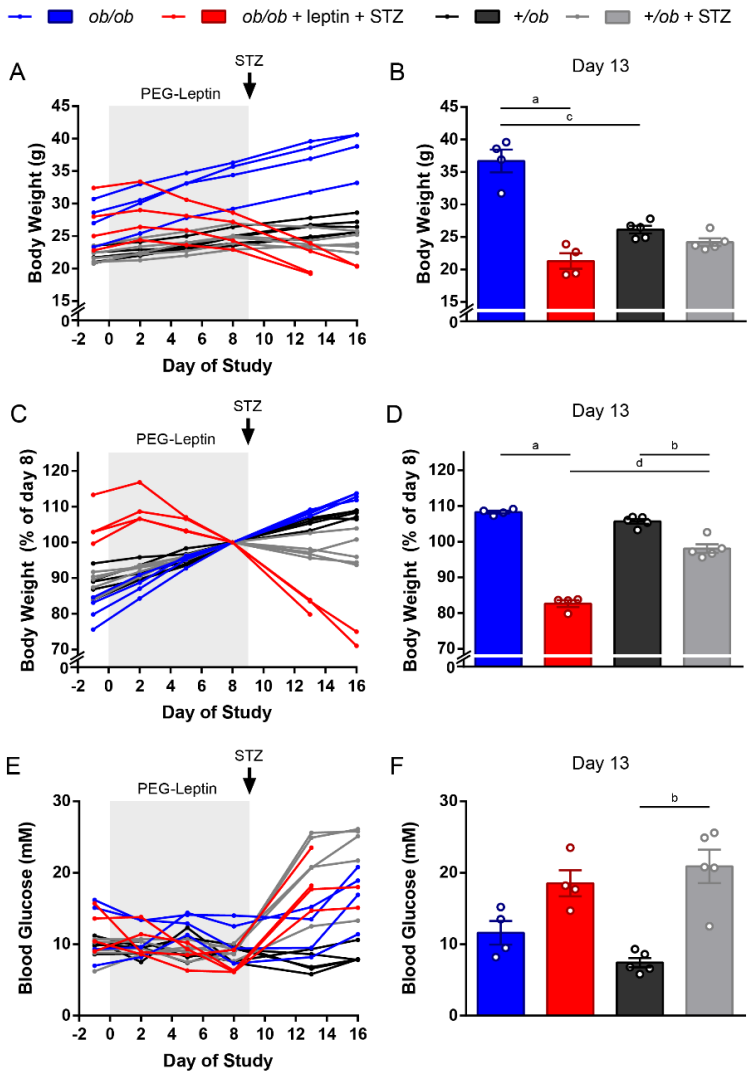

Figure 2. STZ injected *ob/ob* mice experience severe weight loss and mortality. From day 0 to 9 *ob/ob* received daily i.p. injections of PEGylated leptin. On day 9, leptinized-*ob/ob* mice and heterozygous (*+/ob*) mice received 180 mg/kg of STZ. Groups of *ob/ob* and *+/ob* mice that received an injection of buffer alone served as controls. Raw body weight (A), body weight normalized to day 8 to observe the effect of STZ injection (C), and blood glucose levels (E) were measured throughout the study following a 4 hour fast or at humane endpoint. Plots are shown for individual mice since some mice reached humane endpoint before the end of the study. Raw body weight (B), body weight normalized to day 8 (D) and blood glucose levels (F) on day 13 are shown as mean ± SEM. Groups were compared using a 1-way ANOVA with Tukey post-hoc testing; a, P<0.05 *ob/ob* vs *ob/ob* + leptin + STZ; b, P<0.05 *+/ob* vs *+/ob* + STZ; c, P<0.05 *ob/ob* vs *+/ob*; d, P<0.05 *ob/ob* + leptin + STZ vs *+/ob* + STZ, n=4-5.

Since blood glucose levels were not significantly different between *ob/ob* and *ob/ob*-STZ mice, it was unclear whether the STZ was successful in causing insulinopenia in these mice. Therefore, we measured plasma insulin levels and found that despite hyperinsulinemia in *ob/ob* mice, STZ injection reduced insulin levels close to the limit of detection of the assay similar to *+/ob*-STZ mice (Figure 3A). In addition, β-cell area in *ob/ob*-STZ mice was significantly reduced by 94% to levels similar to that of *+/ob* mice treated with STZ which had been reduced by 78% compared to non-diabetic *+/ob* controls (Figure 3B). The success of β-cell destruction may be due to increased Glut2 expression in β-cells of leptinized-*ob/ob* mice compared to untreated *ob/ob* controls (Figure 3C), however, as these images are from one mouse per group additional biological replicates will be required to be more confident in this finding.

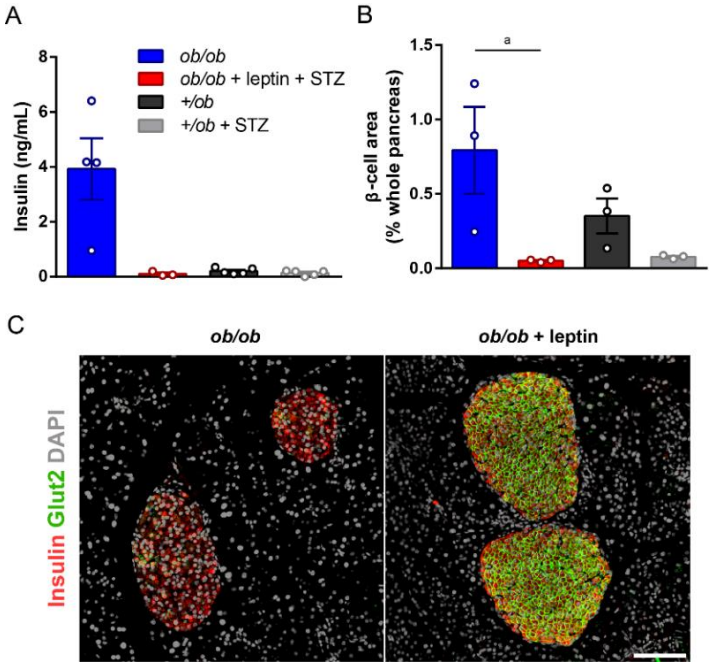

Figure 3. Increased Glut2 in leptinized-*ob/ob* mice facilitates entry of STZ into β-cells causing toxicity and reduced β-cell area. For 9 days *ob/ob* received daily i.p. injections of PEGylated leptin then leptinized-*ob/ob* mice and *+/ob* mice received an injection of STZ at a dose of 180 mg/kg. Groups of *ob/ob* and *+/ob* mice received an injection of buffer alone. As STZ-*ob/ob* mice reached humane endpoint (day 13 or 16) or the rest of the groups reached the end of the study and were fasted for 4 hours (day 16) plasma insulin was measured (A) and β-cell area was analyzed (B). Representative images of pancreata from 1 *ob/ob* and 1 *ob/ob* + leptin mouse prior to STZ injection co-stained for insulin (red), Glut2 (green), and DAPI (grey); scale bar represents 100 µm (C). Many samples in (A) fell close to or below the limit of detection of the assay (0.019 ng/mL) and therefore statistical analysis were not performed. In (B), groups were compared using a 1-way ANOVA with Tukey post-hoc testing; a, $P<0.05$ *ob/ob* vs *ob/ob* + leptin + STZ. The following groups were compared but no statistical differences were detected: *+/ob* vs *+/ob* + STZ; *ob/ob* vs *+/ob*; *ob/ob* + leptin + STZ vs *+/ob* + STZ. Data are mean ± SEM, n=3-4 for (A, B), n=1 for (C).

It is surprising that despite severe β-cell destruction, *ob/ob*-STZ mice were not overtly hyperglycemic. In addition to β-cells, STZ can enter the liver, kidney and neurons via Glut2 causing DNA damage [305], which can result in toxicity and death in mice at high doses. We postulated that leptin therapy in *ob/ob* mice may have increased their sensitivity to STZ toxicity resulting in sickness causing severe body weight loss, lowering of food intake and thus lowering of blood glucose levels, and ultimately death, therefore we repeated the study with a lower dose of STZ. Again, daily leptin injections were delivered to *ob/ob* mice until their body weight matched that of *+/ob* controls (Figure 4A), then on day 7 they received 140 mg/kg STZ while *+/ob* mice received 180 mg/kg. Similar to the previous study *ob/ob*-STZ mice rapidly lost weight following STZ injection (Figure 4A). However, at 1 week following STZ injection none of the mice had reached humane endpoint, and the mice became more severely hyperglycemic, which may suggest less STZ toxicity in non-β-cell tissues than the previous study (Figure 4B). The degree of β-cell destruction should be characterized at the 140 mg/kg STZ dose in *ob/ob* mice as it was at the 180 mg/kg dose.

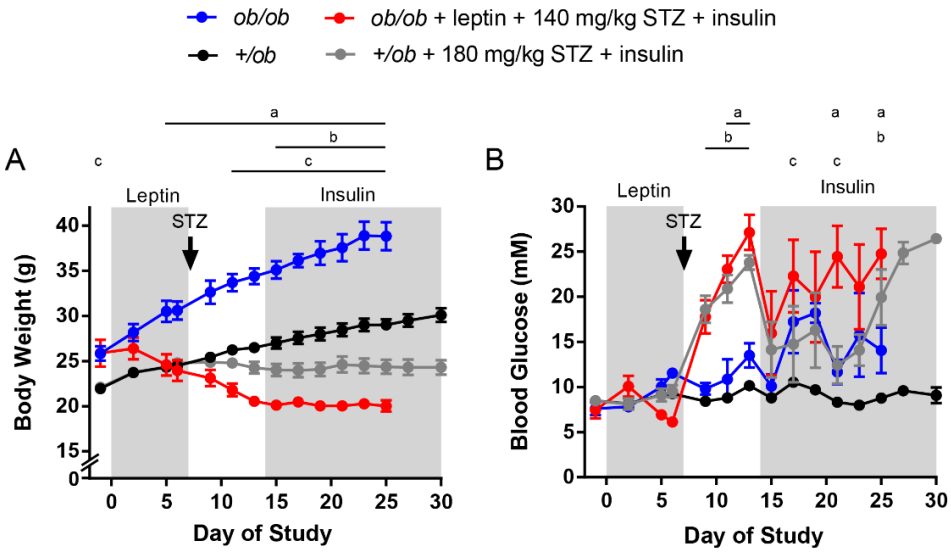

Figure 4. STZ diabetic *ob/ob* mice respond less robustly to insulin therapy than STZ diabetic controls. From day 0 to 7 *ob/ob* received daily i.p. injections of PEGylated leptin. On day 7, leptinized-*ob/ob* mice received 140 mg/kg STZ and *+/ob* mice received 180 mg/kg STZ. Groups of *ob/ob* and *+/ob* mice that received an injection of buffer alone served as controls. On day 14 STZ diabetic mice were implanted with 1 slow-release insulin pellet. Four hour fasted body weight (A) and blood glucose (B) were measured throughout the study. Groups were compared using a repeated measures 2-way ANOVA with Tukey post-hoc testing from day -1 to 25; a, $P<0.05$ *ob/ob* vs *ob/ob* + leptin + STZ; b, $P<0.05$ *+/ob* vs *+/ob* + STZ; c, $P<0.05$ *ob/ob* vs *+/ob*; d, $P<0.05$ *ob/ob* + leptin + STZ vs *+/ob* + STZ. Data are mean ± SEM, n=3-6.

STZ diabetic ob/ob mice respond less robustly to insulin therapy than STZ diabetic controls

If an increase in leptin action contributes to the beneficial effects of insulin therapy, we would expect that *ob/ob*-STZ mice would not respond to insulin therapy as well as *+/ob*-STZ mice with intact leptin expression. To test this, we implanted one slow release insulin pellet into the STZ diabetic *ob/ob* and *+/ob* mice. While both groups experienced a decrease in blood glucose levels, on day 17 and 21 blood glucose levels were significantly higher in the *ob/ob*-STZ mice compared to the *+/ob*-STZ mice (24.5±3.4 mM *ob/ob*-STZ, 12.4±2.0 mM *+/ob*-STZ, $p<0.05$ on day 21, Figure 4B) suggesting that leptin may partially mediate blood glucose lowering during insulin therapy. However, due to variability in blood glucose levels in response to insulin pellet therapy, additional biological replicates could be used to gain confidence in this conclusion.

Wildtype mice expressing a leptin antagonist exhibit increased body weight, impaired oral glucose tolerance and elevated plasma insulin levels

As a complementary study to testing insulin therapy in STZ-diabetic *ob/ob* mice, we investigated the effect of leptin antagonism on insulin therapy in STZ-diabetic wildtype mice. Our first approach was to deliver a plasmid encoding a leptin antagonist via hydrodynamic gene transfer, which can induce sustained, supraphysiological expression [308, 309]. The leptin antagonist contained D23L/L39A/D40A/F41A mutations in the mouse leptin gene that increases binding to the leptin receptor by 64-fold compared to normal leptin, thereby competitively binding to, but not activating, the receptor. The PEGylated version of this antagonist can dramatically increase body weight and abdominal fat when injected into mice [310]. To test the effectiveness of our leptin antagonist plasmid, we injected 50 µg of leptin antagonist plasmid (pLA), or the empty plasmid (pEmpty) as a control, via hydrodynamic tail-vein injection into wildtype mice. The leptin antagonist was capable of significantly increasing body weight by 13% compared to controls on day 19 (29.1±0.6 g pLA, 25.8±0.5 g pEmpty, $P<0.05$, Figure 5A). Although fasting blood glucose and blood glucose levels during an oral glucose tolerance test were not significantly different between treatment groups, the area under the curve (AUC) throughout the oral glucose tolerance test showed slightly impaired glucose tolerance due to blocking leptin action (Figure 5B-D). Finally, plasma insulin levels measured

on day 15 were significantly elevated in response to leptin antagonist plasmid injection compared to controls (1.6±0.3 pLA, 0.6±0.1 pEmpty, $p<0.05$, Figure 5E). Therefore, given the body weight gain, impairment in glucose tolerance, and increase in insulin levels, similar to what is observed in *ob/ob* mice [175, 307] and other studies using leptin antagonists [187, 310], we believe this method of leptin antagonist delivery was successful.

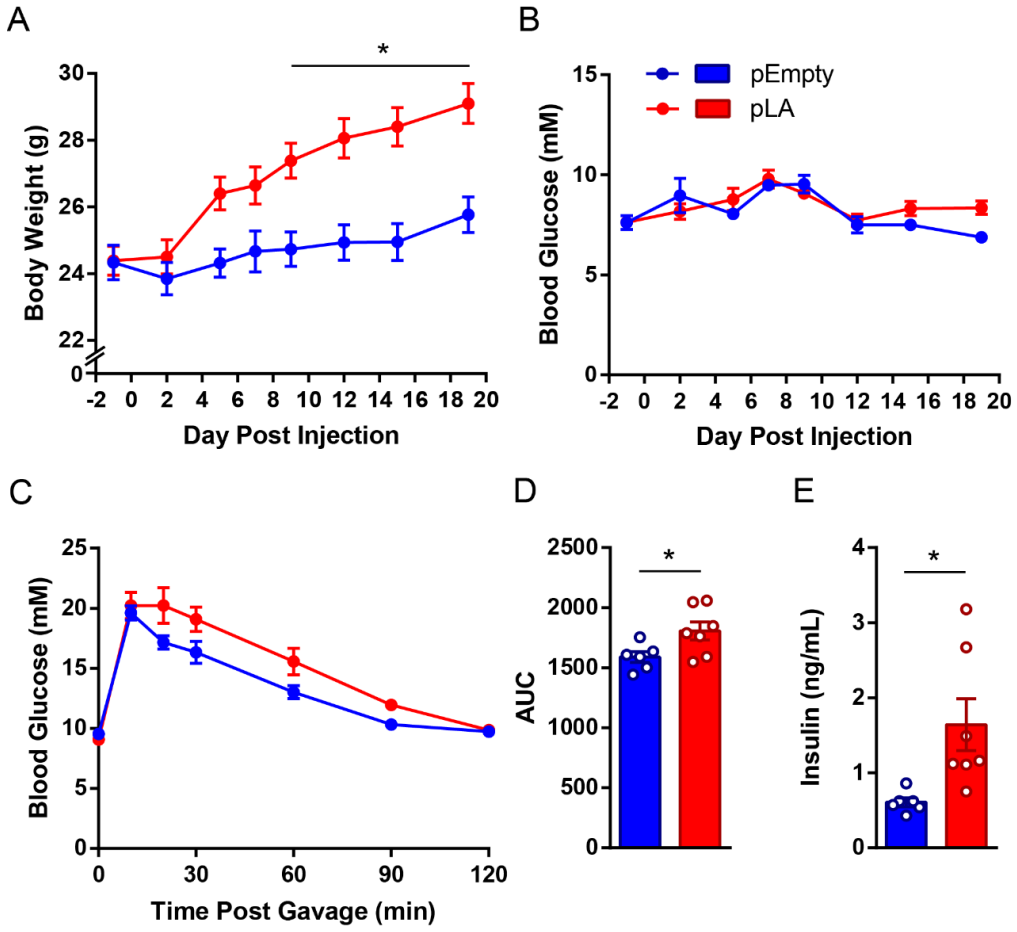

Figure 5. Wildtype mice expressing leptin antagonist exhibit increased body weight, impaired oral glucose tolerance and elevated plasma insulin levels. On day 0 wildtype mice were injected with a plasmid encoding a leptin antagonist (pLA) or the empty plasmid (pEmpty) as a control via hydrodynamic tail-vein injection. Four hour fasted body weight (A) and blood glucose (B) were measured throughout the study. On day 9, mice were gavaged with 2 g/kg glucose following a 4 hour fast, blood glucose levels were monitored (C) and area under the curve was calculated (D). On day 15, 4 hour fasted plasma insulin levels were analyzed (E). Groups were compared using a repeated measures 2-way ANOVA with Sidak post-hoc testing or an unpaired student's t-test; *, $P<0.05$. Data are mean ± SEM, n=6-7.

STZ diabetic mice experience an attenuated insulin-mediated lowering of blood glucose when expressing a leptin antagonist

If increased leptin action due to insulin treatment has direct benefits on diabetic symptoms, we anticipate that leptin antagonism in insulin treated STZ-diabetic mice will reduce the effectiveness of insulin therapy by elevating fasting blood glucose and impairing glucose tolerance. To test this we injected the leptin antagonist plasmid or the empty plasmid as a control into STZ diabetic mice on day -3. The injection itself caused no effect on body weight but decreased blood glucose by approximately 3 mM in both groups compared to STZ mice that were not injected with plasmid as measured on day -1 (Figure 6A&B). On day 0 both plasmid injected groups were implanted with one slow release insulin pellet while non-injected STZ-diabetic mice were given sham surgery. STZ diabetic mice lost weight compared to non-diabetic controls, insulin therapy prevented further weight loss, and there was no difference between mice receiving the leptin antagonist plasmid versus the empty plasmid (Figure 6A). STZ injection resulted in hyperglycemia, which was improved by insulin therapy (Figure 6B), although blood glucose levels were extremely variable in individual mice. Despite this variability, on day 8 and 11 blood glucose levels were significantly higher in the mice receiving leptin antagonist versus control plasmid (7.9±0.6 non-diabetic controls, 25.2±0.7 STZ, 8.9±1.3 STZ-insulin+pEmpty, 17.1±2.5 STZ-insulin+pLA, $p<0.05$, Figure 6B). We performed oral glucose tolerance tests on day 6 and 14 and found that glucose tolerance was worsened by STZ diabetes, and improved by insulin therapy, however there were no difference between the plasmid injected groups (Figure 6C-F). Therefore, increased leptin action during insulin therapy may play a role in regulating fasting blood glucose levels.

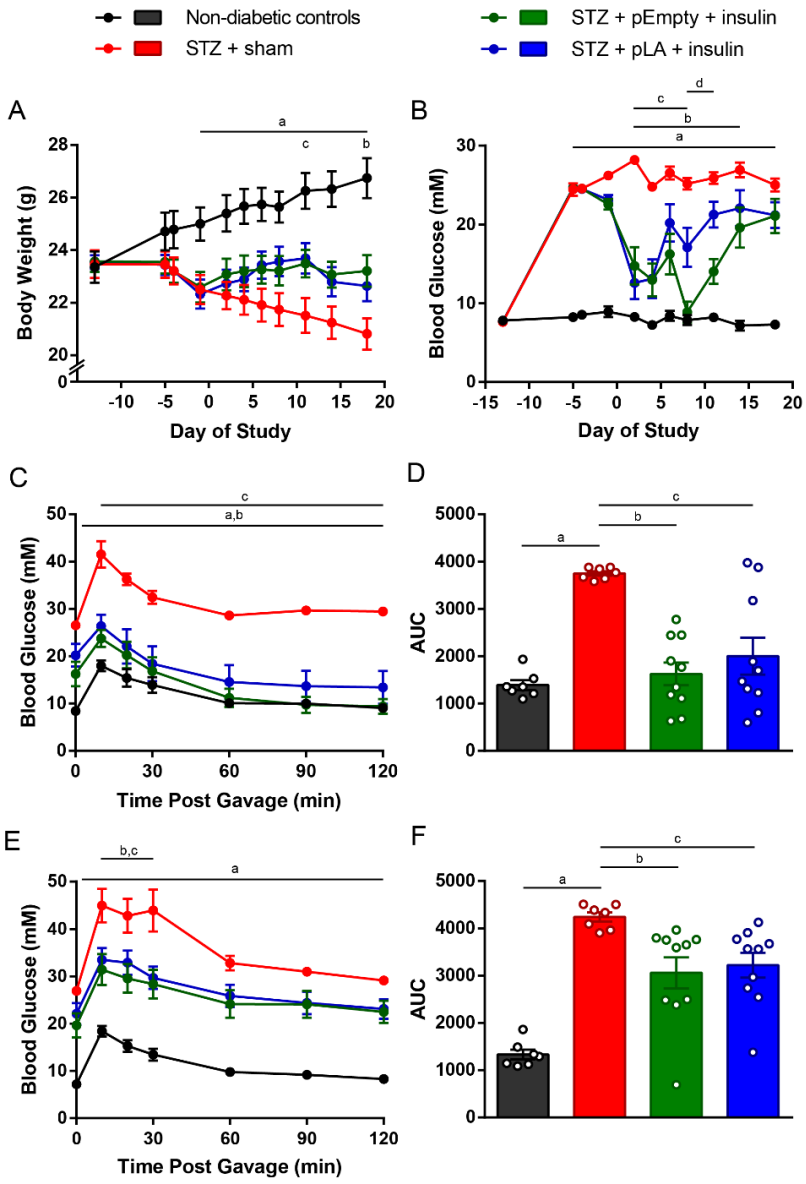

Figure 6. STZ diabetic mice experienced an attenuated insulin-mediated lowering of blood glucose when expressing a leptin antagonist. Insulin-deficient diabetes was induced in C57BL/6J mice by injecting 180 mg/kg STZ on day -13; vehicle was administered to non-diabetic controls. On day -3 diabetic mice were injected with a plasmid encoding a leptin antagonist (pLA) or the empty plasmid (pEmpty) via hydrodynamic tail-vein injection. On day 0, diabetic mice that had been injected with the plasmid were implanted with 1 slow-release insulin pellet while mice that did not receive a plasmid were given sham surgery. Four hour fasted body weight (A) and blood glucose (B) were measured throughout the study. On day 6 (C&D) and day 14 (E&F) an oral glucose tolerance test was performed using 1.5 g/kg glucose and area under the curve (AUC) was calculated. Groups were compared using a 1-way ANOVA or a repeated measures 2-way ANOVA with Tukey post-hoc testing; a, $P<0.05$ non-diabetic controls vs STZ; b, $P<0.05$ STZ vs STZ+insulin+pEmpty; c, $P<0.05$ STZ vs STZ+insulin+pLA; d, $P<0.05$ STZ+insulin+pEmpty vs STZ+insulin+pLA. Data are mean ± SEM, n=7-10.

STZ diabetic mice respond less robustly to insulin therapy when leptin action is blocked

While the plasmid expressing the leptin antagonist did induce a phenotype consistent with inhibited leptin action, it was not as potent as that induced by the PEGylated version of the leptin antagonist. While in our study body weight was increased by 13% by day 19 (Figure 5A) the PEGylated leptin antagonist is capable of increasing body weight by 40% over the same time period [310]. Therefore we opted to repeat the study in insulin treated STZ-diabetic mice using the PEGylated peptide to determine if this would elicit larger differences in blood glucose and glucose tolerance. Non-diabetic controls or STZ diabetic mice implanted with one slow release insulin pellet received either daily i.p. injections of PEGylated leptin antagonist or vehicle as a control. STZ diabetic mice given sham surgery were also used as controls. The PEGylated version of the leptin antagonist delivered via daily injection was indeed more effective than the non-PEGylated antagonist expressed via a plasmid since it caused a 20% increase in body weight by day 7 (26.9±0.7 g Buffer + vehicle, 32.2±0.8 g Buffer + antagonist, $p<0.05$, Figure 7A) although there were no differences in blood glucose or oral glucose tolerance due to the leptin antagonist in non-diabetic controls (Figure 7B-D). STZ injection caused weight loss compared to buffer treated controls, insulin therapy trended to prevent further weight loss, and there were no significant differences between mice receiving the antagonist versus vehicle injections (Figure 7A). Hyperglycemia developed following STZ injection and strikingly the insulin-mediated lowering of blood glucose was blunted due to the leptin antagonist, reaching maximum separation on day 7 (7.8±0.4 Buffer + vehicle, 10.4±0.3 Buffer + antagonist, 29.6±1.2 STZ-sham + vehicle, 12.8±2.8 STZ-insulin + vehicle, 25.4±1.7 STZ-insulin + antagonist, $p<0.05$, Figure 7B). During an oral glucose tolerance test, blood glucose was higher throughout the curve in STZ-diabetic mice that received the antagonist versus vehicle injection (Figure 7C) resulting in a modest impairment in AUC (Figure 7D). However, due to differences in basal blood glucose levels, when the AUC is calculated from baseline, the response to oral glucose was similar between all groups (data not shown). Therefore this provides further evidence that increased leptin action during insulin therapy may play a role in regulating glucose homeostasis.

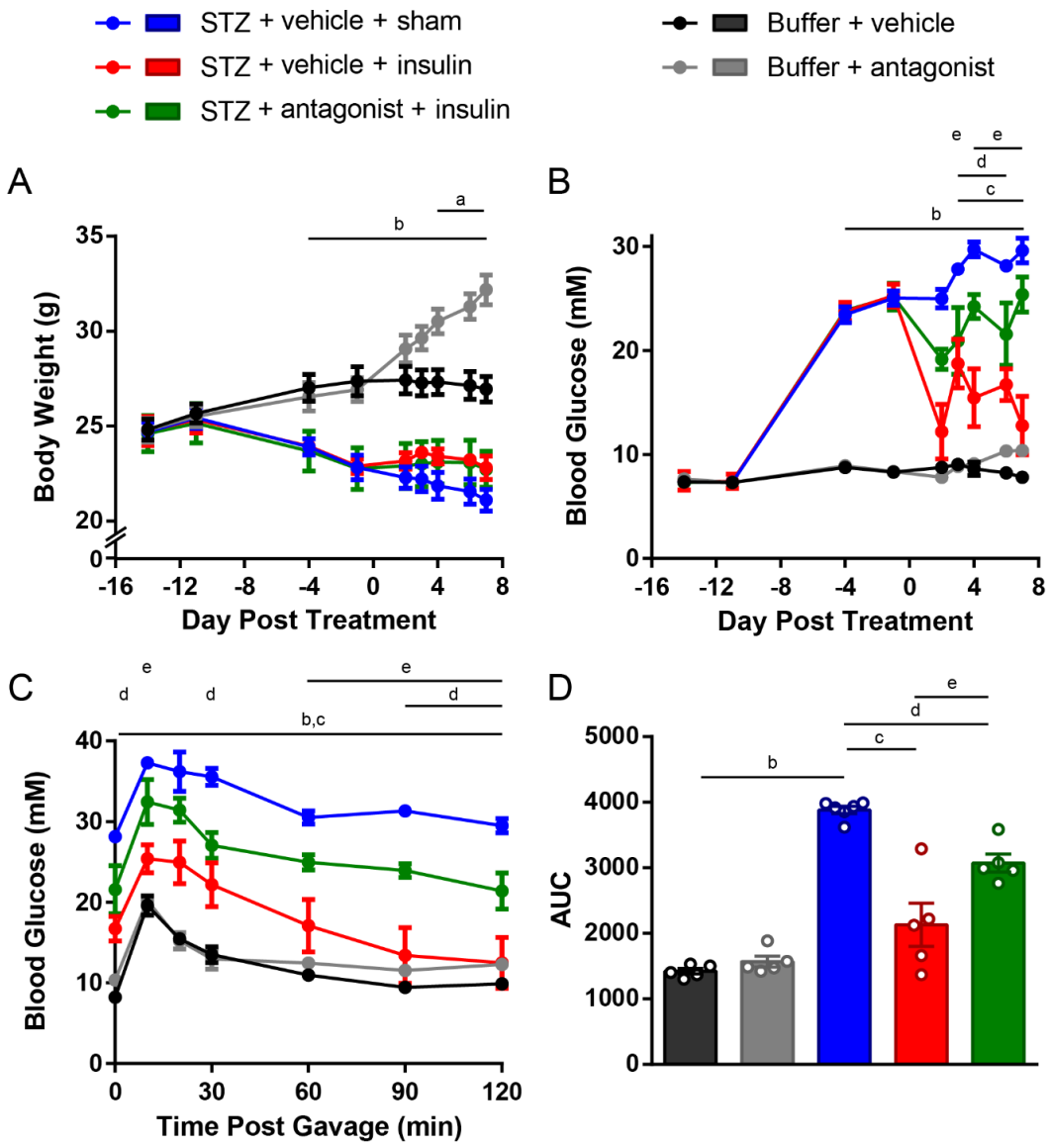

Figure 7. STZ diabetic mice respond less robustly to insulin therapy when leptin action is blocked. Insulin-deficient diabetes was induced in C57BL/6J mice by injecting 180 mg/kg STZ on day -9; buffer was administered to non-diabetic controls. On day 0, diabetic mice were either implanted with 1 slow-release insulin pellet or given sham surgery. Starting on day 0 mice were given daily i.p. injections of either 5 mg/kg PEGylated leptin antagonist or vehicle. Four hour fasted body weight (A) and blood glucose (B) were measured throughout the study. On day 6 an oral glucose tolerance test was performed using 1.5 g/kg glucose (C) and area under the curve (AUC) was calculated (D). Groups were compared using a 1-way ANOVA or a repeated measures 2-way ANOVA with Tukey post-hoc testing; a, $P<0.05$ buffer+vehicle vs buffer+antagonist; b, $P<0.05$ buffer+vehicle vs STZ-sham+vehicle; c, $P<0.05$ STZ-sham+vehicle vs STZ-insulin+vehicle; d, $P<0.05$ STZ-sham+vehicle vs STZ-insulin+antagonist; e, $P<0.05$ STZ-insulin+vehicle vs STZ-insulin+antagonist. Data are mean ± SEM, n=5-6.

Next we measured epididymal fat pad weight and as expected found that the leptin antagonist in non-diabetic controls caused an increase in fat pad weight while STZ injection caused depletion of WAT (Figure 8A). Although there was only a trend for increased fat pad weight due to the insulin pellet (Figure 8A), there was a restoration in plasma leptin levels from below the limit of detection to levels seen in non-diabetic controls (Figure 8B). Finally we measured various plasma analytes to determine if any were affected by leptin antagonism. No differences in plasma glucagon, fatty acid, or glycerol levels were found between any of the groups compared (Figure 9A-C). Interestingly plasma cholesterol levels were increased in non-diabetic controls given the leptin antagonist, while no changes were observed due to STZ, insulin, or leptin antagonist in diabetic mice (Figure 9D). Uncontrolled diabetes resulted in elevated plasma β-hydroxybutyrate and triglyceride levels, which were normalized in both insulin treated groups despite leptin antagonist treatment suggesting that insulin can normalize these parameters independently of increased leptin action (Figure 9E&F).

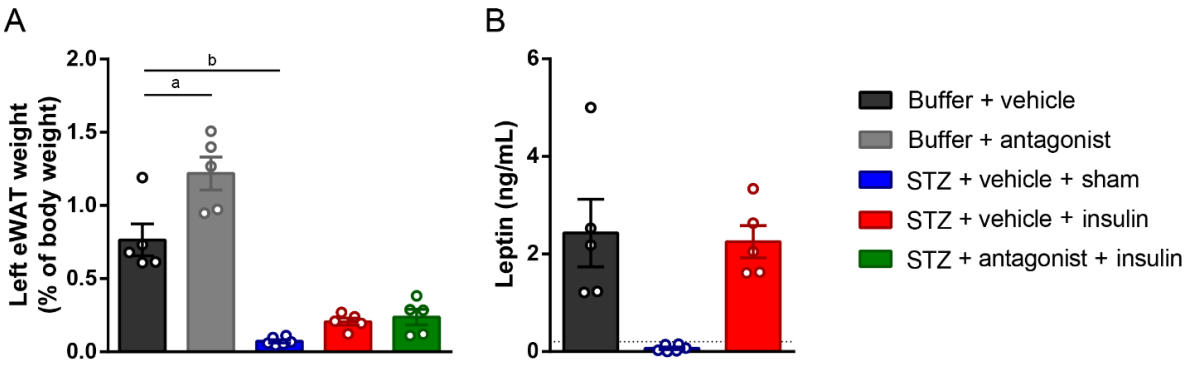

Figure 8. Leptin antagonist peptide increased epididymal WAT weight in non-diabetic mice and insulin therapy restores leptin levels in STZ-diabetic mice. Insulin-deficient diabetes was induced in C57BL/6J mice by injecting 180 mg/kg STZ on day -9; buffer was administered to non-diabetic controls. On day 0, diabetic mice were either implanted with 1 slow-release insulin pellet or given sham surgery. Starting on day 0 mice were given daily i.p. injections of either 5 mg/kg PEGylated leptin antagonist or vehicle. On day 7, following a 4 hour fast, epididymal WAT (eWAT) weight was measured (A) and plasma leptin levels were measured (B). The dotted line in (B) represents the lower limit of the standard curve (0.2 ng/mL). Groups were compared using a 1-way ANOVA with Tukey post-hoc testing; a, $P<0.05$ buffer+vehicle vs buffer+antagonist; b, $P<0.05$ buffer+vehicle vs STZ-sham+vehicle; c, $P<0.05$ STZ-sham+vehicle vs STZ-insulin+vehicle; d, $P<0.05$ STZ-sham+vehicle vs STZ-insulin+antagonist; e, $P<0.05$ STZ-insulin+vehicle vs STZ-insulin+antagonist. Data are mean ± SEM, n=5-6.

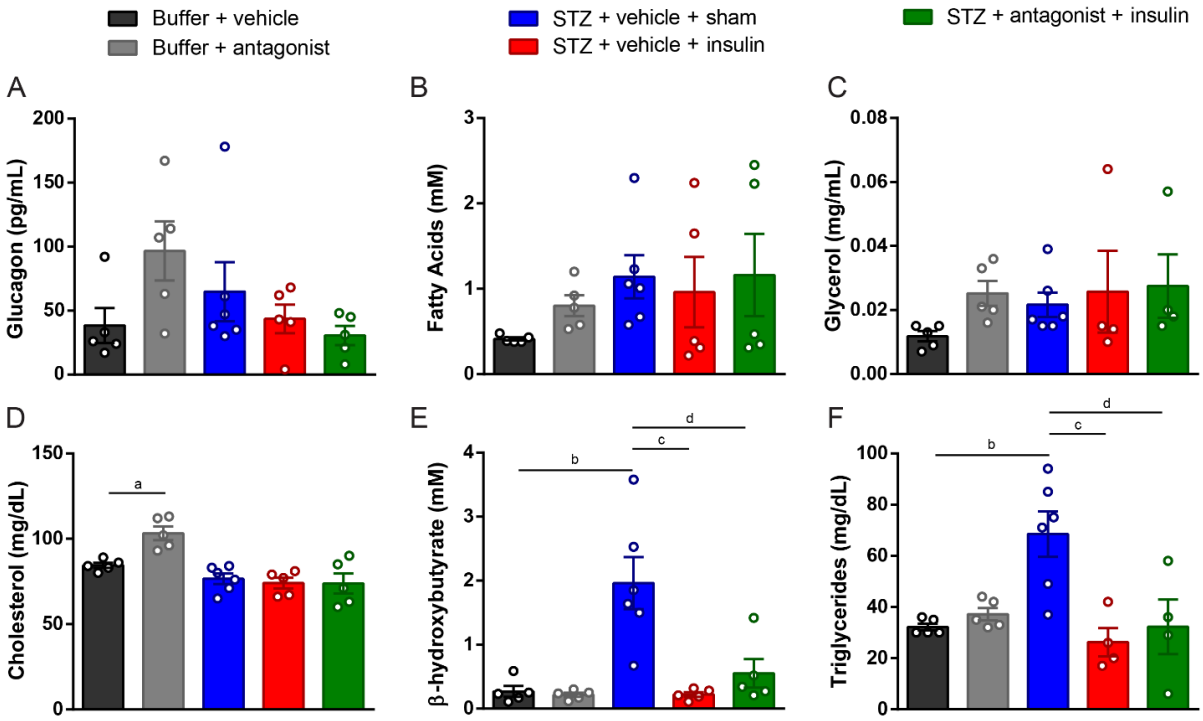

Figure 9. Leptin antagonist peptide increases plasma cholesterol in non-diabetic mice but does not affect ability of leptin to reduce ketones or triglycerides in STZ-diabetic mice. Insulin-deficient diabetes was induced in C57BL/6J mice by injecting 180 mg/kg STZ on day -9; buffer was administered to non-diabetic controls. On day 0, diabetic mice were either implanted with 1 slow-release insulin pellet or given sham surgery. Starting on day 0 mice were given daily i.p. injections of either 5 mg/kg PEGylated leptin antagonist or vehicle. On day 7, following a 4 hour fast, plasma glucagon (A), fatty acids (B), glycerol (C), total cholesterol (D), β-hydroxbutyrate (E) and triglycerides (F) were measured. Groups were compared using a 1-way ANOVA with Tukey post-hoc testing; a, $P<0.05$ buffer+vehicle vs buffer+antagonist; b, $P<0.05$ buffer+vehicle vs STZ-sham+vehicle; c, $P<0.05$ STZ-sham+vehicle vs STZ-insulin+vehicle; d, $P<0.05$ STZ-sham+vehicle vs STZ-insulin+antagonist; e, $P<0.05$ STZ-insulin+vehicle vs STZ-insulin+antagonist. Data are mean ± SEM, n=5-6.

3.3 Discussion

In this Chapter, we have attempted to generate a leptin deficient type 1 diabetic mouse model by injecting STZ into leptinized-*ob/ob* mice in order to investigate the contribution of increased leptin action to the beneficial effects of insulin therapy. In our first study, using the higher dose of STZ (180 mg/kg) *ob/ob* mice rapidly lost weight and reached humane endpoint despite having only moderately elevated blood glucose levels. We suspected that weight loss and death may have been due to increased sensitivity to STZ toxicity in extra-pancreatic tissue such as the liver, kidney, and neurons [305], however we did not investigate this directly. As

the mice approached humane endpoint, they may have ceased feeding thus resulting in reduced blood glucose levels. We hypothesized that if this were the case, administrating a lower dose of STZ may increase survival and cause more severe hyperglycemia. Indeed this is what occurred when we injected 140 mg/kg STZ into leptinized-*ob/ob* mice. Substantial body weight loss following STZ in the *ob/ob* mice in both studies could suggest that the small amount of leptin normally present following STZ injection in *+/ob* mice is important for maintaining body weight potentially due to the insulin sensitizing actions of leptin [311]. In addition, since leptin deficient *ob/ob* mice are insulin resistant and develop hyperinsulinemia in order to compensate, reducing insulin levels may be more damaging on glucose homeostasis and survival. This idea is consistent with another model we have generated in our laboratory where male *ob/ob* mice lacking three insulin alleles resulting in reduced insulin levels exhibit a phenotype reminiscent of type 1 diabetes in humans with wasting of body weight to below that of healthy controls, severe hyperglycemia and glucose intolerance, and premature death [312].

Following the generation of *ob/ob*-STZ mice, we tested whether the inability of insulin to increase leptin action would hinder the beneficial effects of insulin therapy. The method of insulin delivery in this study was implantation of a single slow-release, standard size insulin pellet per mouse. The insulin pellets contain bovine, porcine, and recombinant human insulin, which produce anti-insulin antibodies in mice, thus we were unable to measure plasma insulin levels via an antibody-based assay. However, given that the *ob/ob*-STZ mice are smaller than the *+/ob*-STZ controls, the insulin pellet would deliver a higher dose of insulin per g of body weight in the *ob/ob*-STZ mice. Despite the higher dose of insulin, insulin treatment in *ob/ob*-STZ mice failed to lower blood glucose to the same extent as in insulin treated *+/ob*-STZ mice, which may suggest that increased leptin levels due to insulin therapy may play a role in reducing blood glucose levels. However, there are various potential confounders related to this study, which make interpretation of the results difficult. It is still unclear whether the 140 mg/kg dose of STZ caused liver, kidney, or neuron toxicity, especially considering a dose of 160 mg/kg also resulted in 100% death (data not shown). In addition, *ob/ob*-STZ mice were slightly more hyperglycemic prior to insulin therapy than *+/ob*-STZ mice making the data

harder to interpret. Finally, due to a low sample size combined with dramatic variability in blood glucose levels following insulin pellet therapy the significant differences between the groups are neither large nor sustained. These complications could be rectified given further troubleshooting of the experiment, including testing various doses of leptin and STZ, experimenting with different insulin delivery methods such as mini-osmotic pump or injection, testing different insulin doses and fasting lengths, as well as increasing the sample size to gain more power.

We next took a complementary approach of test the effect of leptin antagonism on insulin therapy in STZ-diabetic wildtype mice using two different delivery methods of the antagonist. The non-PEGylated version of the antagonist expressed from a plasmid was less potent causing a ~7% increase in body weight by day 7 while the PEGylated version of the antagonist delivered at 5 mg/kg/day via i.p. injection was significantly more effective - raising body weight by ~20% by day 7. The effect of the PEGylated antagonist is similar to published data demonstrating a ~23% increase in body weight by day 7 following injection of 6.25 mg/kg/day [310]. Leptin antagonism also resulted in increased adiposity as measured by epididymal WAT pad weight, a 2.7 fold elevation in plasma insulin levels, and a modest impairment in oral glucose tolerance as measured by AUC, but no differences in fasting blood glucose levels. These results are consistent with a study using the earlier version of the PEGylated leptin antagonist (L39A/D40A/F41A without the D23L mutation), which resulted in larger weight gain, elevated fasting and glucose-stimulated plasma insulin levels and impaired whole-body insulin sensitivity, while blood glucose and oral glucose tolerance remained similar to that of controls [187].

We have assessed the effectiveness of the pharmacological antagonism based on phenotypic data; however, we have not measured the levels of circulating leptin antagonist, because the antagonist cross-reacts with native leptin in the Crystal Chem Leptin ELISA. Thus, we cannot discriminate between the antagonist and exogenous leptin using this assay. This could be investigated by injecting the leptin antagonist plasmid or peptide into *ob/ob* mice, which have no endogenous leptin. The leptin ELISA could be performed on plasma samples

from these mice, using a standard curve generated from the commercially available antagonist, to determine the levels of circulating antagonist. In addition, obtaining a measure of leptin activity such as levels of p-STAT3/STAT3 in target cells could help quantify the level of leptin antagonism in our studies.

Similar to the results of the insulin treatment in *ob/ob*-STZ mice, our data using leptin antagonism suggest that increased leptin action due to insulin therapy may play an important role in improving glucose homeostasis. In insulin treated STZ diabetic mice given either the leptin antagonist plasmid or PEGylated peptide, the glucose lowering effect of insulin was partially blocked. Moreover, in the study involving the PEGylated peptide, oral glucose tolerance was also significantly impaired compared to controls. Taken together our results reveal that not only does hypoleptinemia contribute to the metabolic defects in insulin-deficient diabetes, but that increased leptin levels in response to insulin contribute to some of the beneficial effects of the therapy.

CHAPTER 4 – REQUIREMENT OF IGFBP2 FOR THE METABOLIC ACTIONS OF LEPTIN IN *ob/ob* MICE

4.1 Introduction

The adipocyte-derived hormone leptin has well known effects on body weight regulation, but also has profound effects on glucose homeostasis [188]. Indeed, leptin-deficient *ob/ob* and leptin receptor-deficient *db/db* mice are not only obese, but also exhibit hepatic steatosis, hyperinsulinemia, insulin resistance, glucose intolerance and often fasting hyperglycemia [94, 175, 239, 313]. The metabolic manifestation of a loss of leptin action in mice is highly similar to type 2 diabetes. Humans that lack leptin action due to leptin deficiency or lipodystrophy also exhibit these metabolic abnormalities [101, 105, 229]. Leptin replacement treatment can ameliorate these metabolic aberrations in both mice and humans [101, 105, 175, 229]. Interestingly, many of the metabolic actions of leptin are not secondary to its well-established effects on body weight [175, 313, 314]. Rather leptin appears to directly regulate metabolism and glucose homeostasis, although the mechanism by which leptin contributes to glucose regulation has not been fully elucidated.

There has been substantial interest in IGFBP2 as a mediator of leptin action on metabolism. IGFBPs were first characterized by their ability to bind insulin-like growth factors (IGFs). These binding proteins may work in an IGF dependent manner either by increasing IGF half-life or inhibiting IGF action [315], or in an IGF independent manner by acting through integrin receptors [316, 317]. In *ob/ob* mice, which have abnormally low IGFBP2 levels, leptin treatment was found to restore *Igfbp2* expression in the liver, a major site of *Igfbp2* expression in the adult mouse [318], as well as circulating IGFBP2 levels [313]. Overexpression of IGFBP2 by adenoviral transfer normalized fasting blood glucose and insulin levels, glucose tolerance, hepatic insulin sensitivity, and hepatic steatosis in *ob/ob* mice [313]. In addition, overexpression of IGFBP2 in other models of type 1 and 2 diabetes, including diet-induced obese, A^{Y}/a (agouti) and STZ injected mice, corrected their hyperglycemia and glucose intolerance [313]. Therefore, induction of IGFBP2 can improve aberrant glucose homeostasis

in many models of diabetes in a manner similar to leptin, suggesting that IGFBP2 may be a critical mediator of leptin action on glucose metabolism. However, in these overexpression studies IGFBP2 levels were induced to levels of 6000 ng/mL or more, while *ob/ob* levels are ~35 ng/mL and wildtype levels are ~350 ng/mL [313]. Therefore, thus far the glucoregulatory effects of only supraphysiological levels of IGFBP2 have been reported, and the metabolism normalizing function of IGFBP2 may not reflect the physiological role of IGFBP2 in response to leptin.

While pharmacological levels of IGFBP2 evidently have therapeutic value it is currently unclear what role IGFBP2 plays in maintaining normal metabolic physiology. Characterization of *Igfbp2KO* mice revealed only a subtle metabolic phenotype [319-321]. *Igfbp2KO* mice are neither glucose intolerant nor insulin resistant as measured by glucose tolerance and insulin tolerance tests [319]. *Igfbp2KO* mice also display no difference in the rate of body weight gain or absolute body weight at 8 weeks of age compared to wildtype littermates [319]. However, at 16 weeks of age male *Igfbp2KO* mice were mildly heavier than wildtypes due to greater percent body fat [319]. The lack of profound phenotype observed in *Igfbp2KO* mice may be due to compensation by other IGFBPs, which are upregulated in *Igfbp2KO* mice [319, 320]. In contrast, some studies investigating the effect of modest overexpression of IGFBP2 support a protective role of IGFBP2 in glucose metabolism. For example, female transgenic mice overexpressing human IGFBP2 under its native promoter have 2.2 fold higher circulating IGFBP2 and do not have reduced fasting glucose or insulin levels, but are protected from age- and diet-induced obesity and insulin resistance [322]. Together, these studies suggest that lower than normal IGFBP2 levels may be permissive for obesity development, and that an increase in IGFBP2 levels may prevent obesity, but these effects are modest.

In this Chapter we hypothesized that IGFBP2 is a mediator of leptin function on glucose metabolism. To investigate this we first assessed whether IGFBP2 alone is sufficient to mimic the actions of leptin when expressed at levels comparable to those achieved in response to leptin treatment. We then sought to observe whether IGFBP2 is necessary for normal

metabolism via acute siRNA knockdown of IGFBP2 in wildtype mice. Finally, we assessed whether IGFBP2 is required for leptin-mediated normalization of metabolism in *ob/ob* mice. Our results support the notion that physiological levels of IGFBP2 are neither sufficient nor required for the action of leptin on glucose homeostasis. All data in this Chapter except for Figure 12 are published in *Endocrinology* [307].

4.2 Results

Induction of IGFBP2 to levels similar to those following leptin treatment does not mimic the actions of leptin in ob/ob mice

We first sought to over-express IGFBP2 in *ob/ob* mice without leptin treatment, aiming to achieve plasma IGFBP2 levels similar those induced by low dose, metabolically significant leptin treatment in these mice. To serve as a reference point, *ob/ob* mice were treated with a low dose (0.8 μg/day) of leptin via a mini-osmotic pump. We and others have demonstrated that because *ob/ob* mice are very sensitive to leptin they do not require levels of leptin found in wildtype mice to reduce body weight and plasma insulin levels [175, 306, 313, 314, 323]. This dose of leptin increased plasma leptin levels from undetectable (<0.2 ng/mL) in the vehicle group to 0.8±0.2 ng/mL in the leptin treated group, which was subphysiological compared to wildtype controls that had levels of 5.7±1.0 ng/mL (Figure 10A). Separate groups of mice received IGFBP2 treatment (without leptin) through administration of either a low dose (10^8 plaque forming units; pfu) or high dose (10^9 pfu) of an adenovirus expressing IGFBP2 (Ad-IGFBP2). A control adenovirus expressing β-galactosidase (Ad-β-gal) was administered to a separate group of *ob/ob* mice. As expected, prior to leptin treatment, IGFBP2 levels were lower in *ob/ob* mice averaging ~35 ng/mL compared to ~325 ng/mL in wildtype mice (Figure 10B). However, plasma IGFBP2 levels in the leptin treated group were significantly higher than the vehicle group from day 5 post-treatment onward ($P<0.05$), and by 15 days post leptin treatment had increased to 173±8 ng/mL (Figure 10B). Importantly, the low dose Ad-IGFBP2 treatment induced plasma IGFBP2 levels at a similar rate and extent to leptin treatment (Figure 10B). IGFBP2 levels were significantly higher in the low dose Ad-IGFBP2 group than in the low dose Ad-β-gal control group from day 5 onward, and by day 15

post treatment had increased to 202±60 ng/mL ($P<0.05$). In contrast, a high dose of Ad-IGFBP2 increased plasma IGFBP2 levels even further, to 33046±5082 ng/mL by day 2 ($P<0.05$ vs high dose Ad-β-gal) and remained at supraphysiological levels throughout the experiment (4080±294 ng/mL on day 15) (Figure 10B). These plasma IGFBP2 levels are similar to those achieved by Hedbacker *et al.*, who reported IGFBP2 levels >6000 ng/mL one week after administration of IGFBP2 adenovirus [313]. Given the induction of similar IGFBP2 levels in the leptin treated and the low dose Ad-IGFBP2 groups, we used these two groups of animals to further investigate the physiological contribution of IGFBP2 to the glucoregulatory actions of leptin.

We next sought to determine whether IGFBP2 alone could mimic the body weight reducing effects of leptin treatment in *ob/ob* mice. Prior to treatment, obese *ob/ob* mice weighed ~44 g while wildtype controls weighed ~24 g. Leptin treatment attenuated weight gain, such that body weight was significantly lower than that of vehicle treated controls by 4% on day 2 and 13% by day 15 ($P<0.05$, Figure 10C). Although the low Ad-IGFBP2 treated mice had plasma IGFBP2 levels similar to those of leptin treated mice, their body weights did not differ from the low Ad-β-gal group (Fig 10C). High dose Ad-IGFBP2 treatment did reduce body weight compared to the high dose Ad-β-gal control (by 6% at day 15; $P<0.05$, Figure 10C), although weight loss occurred more slowly and to a lesser degree than in the leptin treated group. These data suggest that while high levels of IGFBP2 can induce weight loss in *ob/ob* mice, IGFBP2 at physiological levels does not induce weight loss in *ob/ob* mice.

To ascertain the effect of IGFBP2 induction on glucose homeostasis, we measured 4 hour fasted blood glucose and plasma insulin. At baseline, *ob/ob* mice were not overtly hyperglycemic, and blood glucose levels did not change in response to leptin therapy or treatment with Ad-IGFBP2 or control adenovirus (Figure 10D). However, despite fasting euglycemia, *ob/ob* mice were hyperinsulinemic compared to wildtype controls (~10 ng/mL vs ~1 ng/mL insulin prior to treatment; Figure 10E). As expected, by day 2 leptin treated mice had lower insulin levels than vehicle treated controls (3.2±0.4 ng/mL vs 11.9±1.8 ng/mL, $P<0.05$), which was maintained until day 12 (3.6±0.6 ng/mL vs 11.3±2.2 ng/mL, $P<0.05$)

(Figure 10E). No difference in insulin levels were observed between the low Ad-IGFBP2 and low Ad-β-gal groups or between the high Ad-IGFBP2 and high Ad-β-gal groups (Figure 10E). We did observe a decrease in insulin levels in the high Ad-IGFBP2 group between day 2 and day 5 (from 14.2±1.9 ng/mL to 4.5±1.5 ng/mL), but we attribute this to non-specific virus effects since the high Ad-β-gal control group displayed the same decrease (from 12.5±3.2 ng/mL at day 2 to 4.1±1.0 ng/mL at day 5) (Figure 10E). Therefore, although exogenous leptin treatment and Ad-IGFBP2 treatment induced similar plasma IGFBP2 levels, treatment with leptin but not Ad-IGFBP2 reduced plasma insulin. These results indicate that IGFBP2 alone is not sufficient to mimic the insulin lowering effects of leptin in *ob/ob* mice.

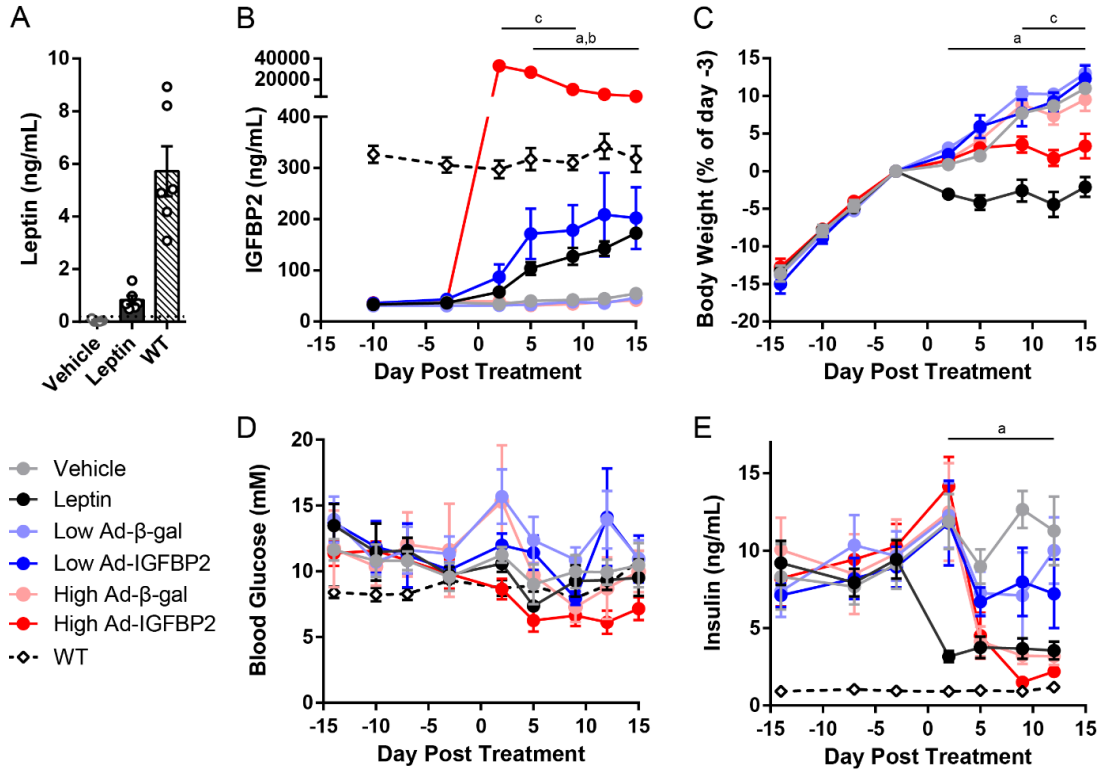

Figure 10. Low dose Ad-IGFBP2 treatment does not mimic the body weight, and plasma insulin reducing effects of leptin. Nine week old *ob/ob* mice were treated (day 0) with either 0.8 µg/day leptin or vehicle as a control via mini-osmotic pumps, or either low or high dose Ad-IGFBP2 or Ad-β-gal as a control. Wildtype mice not receiving any treatment served as a reference point. Plasma leptin levels were measured on day 12 following a 4 hour fast (A). Plasma IGFBP2 (B), body weight (C; expressed as % change over baseline), blood glucose (D) and plasma insulin (E) were measured after a 4 hour fast. Statistical analysis was not performed on (A) since some samples fell below the limit of detection denoted by the dotted line. Statistical analyses were performed using a repeated measures 2-way ANOVA with Bonferroni post-hoc testing. The following groups were compared: a, $P<0.05$ leptin vs vehicle; b, $P<0.05$ low Ad-IGFBP2 vs low Ad-β-gal; c, $P<0.05$ high Ad-IGFBP2 vs high Ad-β-gal. Data are mean±SEM, n=4-6.

We next assessed the effect of IGFBP2 expression on glucose tolerance (Figure 11A&B). *Ob/ob* mice treated with low dose leptin had lower and earlier peak blood glucose levels than vehicle treated mice, all of whom had glucose levels exceeding the maximum detection limit (33.3 mM) of the glucometer (26.5 mM at 10 min for leptin treated group vs 33.3 mM at 20 min for vehicle treated animals, Figure 11A). The AUC was similar between the groups (Figure 11B), although this may in part be due to limitations in our ability to accurately quantify high glucose levels with the glucometer. Mice treated with low dose Ad-IGFBP2 or Ad-β-gal had comparable glucose levels and glucose excursion to the vehicle treated group, while mice treated with high dose Ad-IGFBP2 had markedly improved glucose tolerance compared to control virus treated mice (Figure 11B).

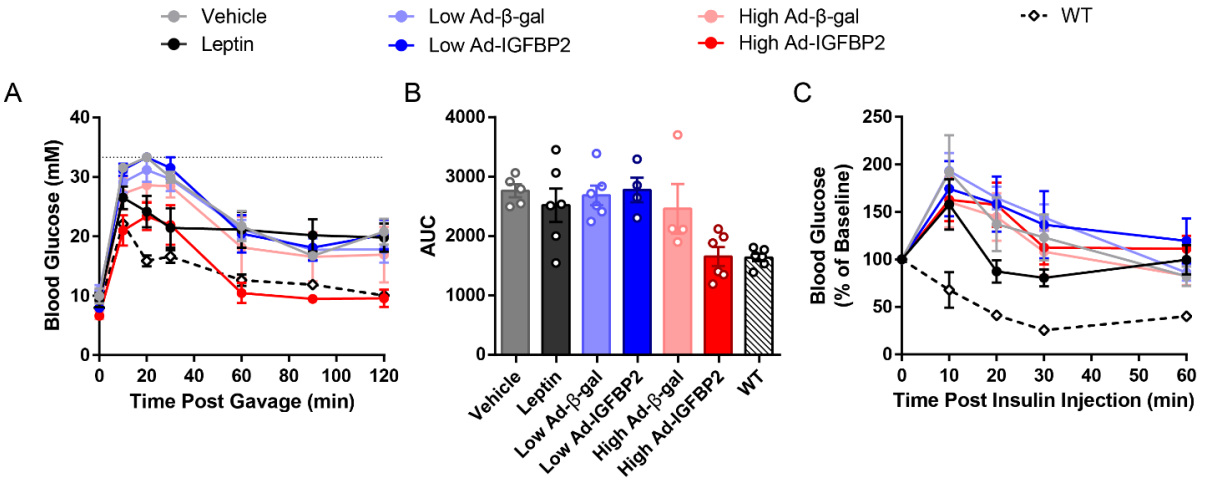

Figure 11. Oral glucose tolerance and insulin tolerance in *ob/ob* mice receiving leptin or Ad-IGFBP2 treatment. Oral glucose tolerance and insulin tolerance tests were performed on *ob/ob* mice receiving either 0.8 µg/day leptin or vehicle, or low dose (10^8 pfu) or high dose (10^9 pfu) Ad-IGFBP2 or Ad-β-gal. On day 9 following a 4 hour fast, mice were gavaged with 1.5 g/kg glucose, blood glucose responses were measured (A) and area under the curve was calculated (B). On day 15 following a 4 hour fast, mice were administered 1 U/kg insulin by intraperitoneal injection and blood glucose was measured (C). During the glucose tolerance test, at least one sample in every *ob/ob* group fell over the limit of detection (denoted by the dotted line) and was assigned a value of 33.3 mM; therefore, statistical analysis was not performed in (A) or (B). For the ITT, statistical analyses were performed using a repeated measures 2-way ANOVA with Bonferroni post-hoc testing. The following groups were compared: leptin vs vehicle; low Ad-IGFBP2 vs low Ad-β-gal; high Ad-IGFBP2 vs high Ad-β-gal; however, no statistical differences were detected. Data are mean±SEM, n=4-6.

In addition to impaired glucose tolerance, all *ob/ob* groups had a notably impaired glucose-lowering response to insulin compared to wildtype controls (Figure 11C). In response to insulin treatment, blood glucose levels in wildtype mice dropped to 26% of baseline by 30 min after injection, while none of the groups of *ob/ob* mice exhibited this response. We speculate that the increase in blood glucose levels following provision of insulin in *ob/ob* mice was caused by a stress response due to the injection combined with severe insulin resistance, and this phenomenon has been observed in other studies [324, 325]. Leptin treatment did not restore normal insulin sensitivity in *ob/ob* mice, although leptin treated mice did display a trend towards having lower blood glucose levels at 20 and 30 minutes post injection compared to all other *ob/ob* groups; however, this trend did not reach significance. Insulin tolerance profiles were superimposable between the low and high Ad-IGFBP2 groups compared to their respective controls. Therefore, induction of low levels of IGFBP2 did not improve glucose or insulin tolerance in a similar manner as low dose leptin treatment.

Finally, we assessed the effect of leptin and IGFBP2 on hepatic lipid accumulation (Figure 12). *Ob/ob* mice receiving vehicle had substantial ectopic fat deposition in the liver as denoted by large white lipid droplets that were absent in wildtype mice as visualized by hematoxylin and eosin staining (Figure 12A). Leptin treatment appeared to reduce the size of the lipid droplets compared to vehicle treated *ob/ob* mice (Figure 12A), however reduced hepatic triglycerides and cholesterol were not observed due to leptin therapy (Figure 12B&C). Leptin has well known effects on reducing hepatic triglyceride levels [326, 327]; however, the dose of leptin used in this study may have been too low to elicit this effect. It is unclear why there appears to be a discrepancy between the qualitative H&E images and the quantitative triglyceride assessment. Digital image analysis of images of a larger section of liver may have given a less biased quantitative measurement than visual inspection; however, this was not performed. No differences in hepatic steatosis were observed between the low Ad-IGFBP2 and low Ad-β-gal groups or between the high Ad-IGFBP2 and high Ad-β-gal groups, all which looked similar to the vehicle treated *ob/ob* mice (Figure 12A-C).

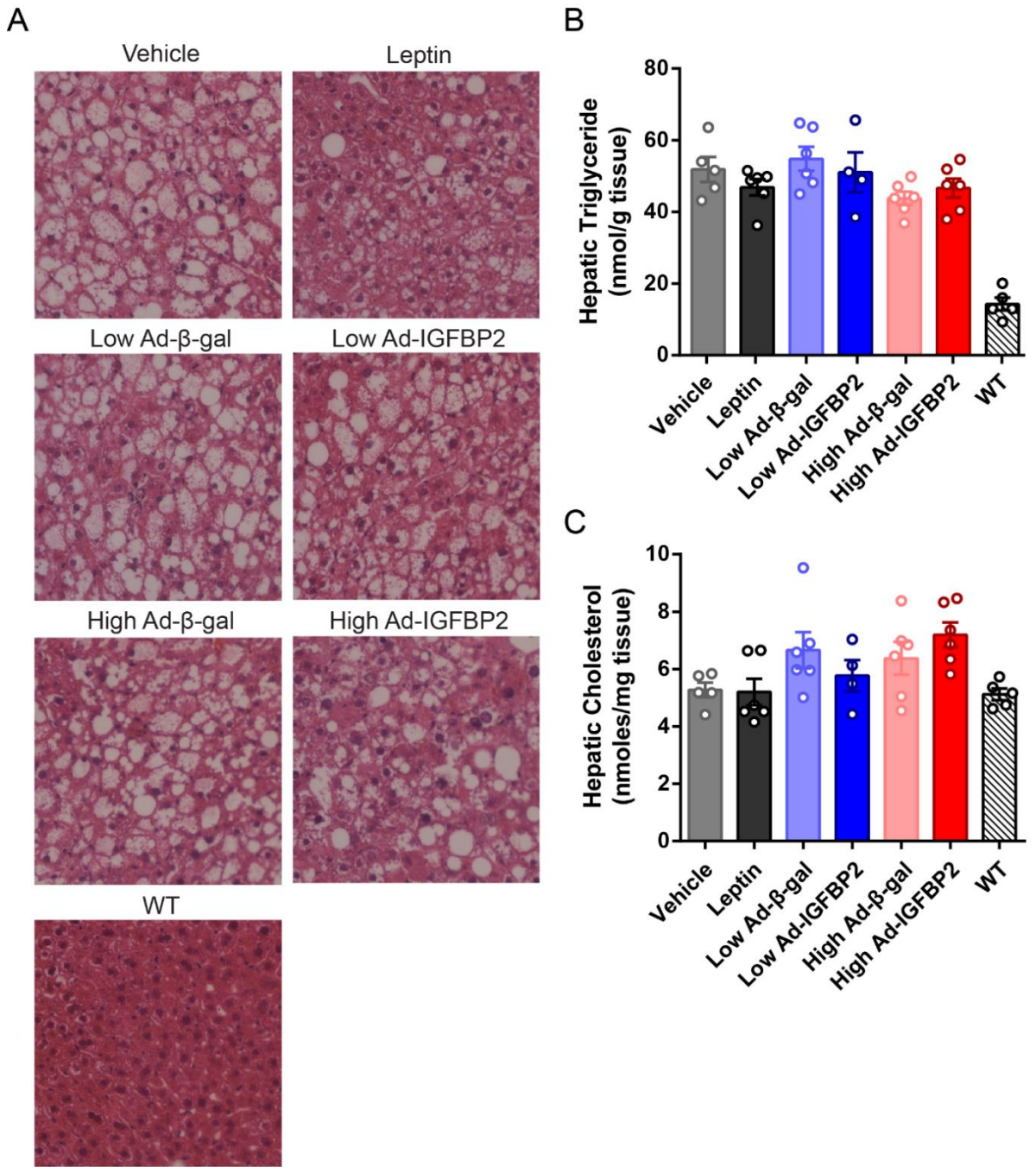

Figure 12. Hepatic lipid content in *ob/ob* mice receiving leptin or Ad-IGFBP2 treatment. Liver sections from *ob/ob* mice receiving either 0.8 µg/day leptin or vehicle, or low dose (10^8 pfu) or high dose (10^9 pfu) Ad-IGFBP2 or Ad-β-gal or from wildtype mice were stained with hematoxylin and eosin. Representative images are shown in (A). Four hour fasted hepatic triglyceride (B) and total cholesterol (C) content were measured on day 15. Statistical analyses were performed using 1-way ANOVA with Bonferroni post-hoc testing. The following groups were compared: leptin vs vehicle; low Ad-IGFBP2 vs low Ad-β-gal; high Ad-IGFBP2 vs high Ad-β-gal; however, no statistical differences were detected. Data are mean ± SEM, n=4-6.

Acute knockdown of IGFBP2 in C57BL/6J mice does not alter glucose homeostasis

We next investigated whether acute knockdown of IGFBP2 in wildtype mice would recapitulate any aspects of the *ob/ob* phenotype. In order to achieve knockdown of plasma IGFBP2 levels we assessed the knockdown efficiency of 3 different IGFBP2 siRNAs relative to a FVII siRNA control. We selected lipid nanoparticles as a vehicle for IGFBP2 siRNA delivery, since lipid nanoparticles efficiently target siRNA to the liver *in vivo* [287, 328], and the liver is suspected to be a major source of circulating IGFBP2 [313, 318]. siRNAs were introduced into wildtype mice via tail-vein injection at a dose of 5 mg/kg. IGFBP2 siRNA caused a rapid and dramatic reduction in circulating IGFBP2 levels (Figure 13A). By day 1 post injection IGFBP2 levels were decreased by at least 92% from pre-treatment levels (~350 ng/mL) in all IGFBP2 siRNA groups. We also observed an acute decrease in IGFBP2 levels in the FVII control group on day 1, although IGFBP2 returned to pre-treatment levels by day 10. We attribute this observation to the high dosage of lipid nanoparticles since empty particles produced the same effect (Figure 14). Significant knockdown of IGFBP2 levels was thus achieved from day 3 to days 20, 24, or 29 in mice treated with IGFBP2 siRNA #1, 2, or 3 respectively, compared to the FVII control group ($P<0.05$, Figure 13A). Thus we concluded that the IGFBP2 siRNA approach was successful in reducing plasma IGFBP2.

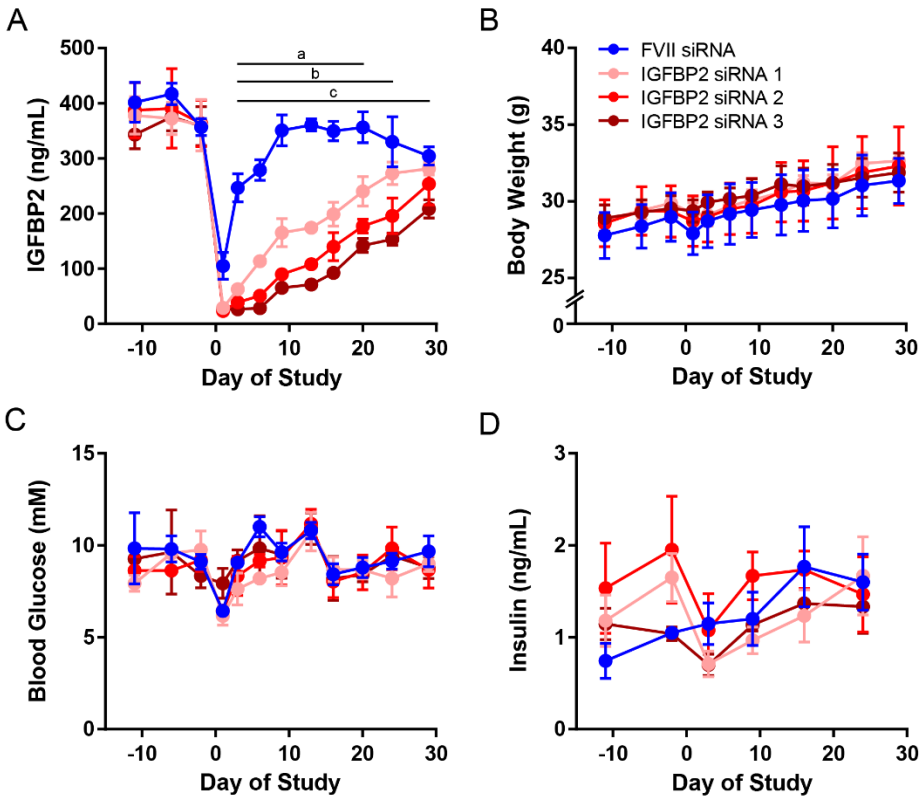

Figure 13. Acute knockdown of IGFBP2 does not affect body weight, blood glucose or plasma insulin. Thirteen week old C57BL/6J mice were treated with 5 mg/kg lipid nanoparticles encapsulating one of three IGFBP2 siRNAs or FVII siRNA by tail-vein injection on day 0. Plasma IGFBP2 (A), body weight (B), blood glucose (C) and plasma insulin (D) were measured following a 4 hour fast. Statistical analyses were performed using a repeated measures 2-way ANOVA with Bonferroni post-hoc testing. The following groups were compared: a, P<0.05 FVII vs IGFBP2 siRNA 1; b, P<0.05 FVII vs IGFBP2 siRNA 2; c, P<0.05 FVII vs IGFBP2 siRNA 3. Data are mean±SEM, n=3.

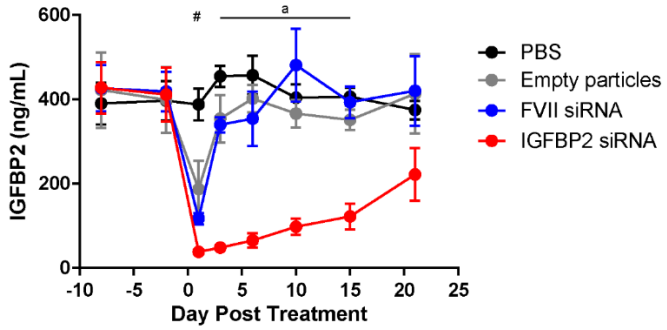

Figure 14. Effect of lipid nanoparticles on circulating IGFBP2 levels. Nine week old C57BL/6J mice were treated with 5 mg/kg lipid nanoparticles encapsulating IGFBP2 siRNA #3, FVII siRNA, empty nanoparticles, or PBS by tail-vein injection on day 0. Plasma IGFBP2 was measured throughout the study following a 4 hour fast. Statistical analyses were performed using two-way ANOVA with Bonferroni post-hoc testing. All groups were compared to PBS: #, P<0.05 for all groups vs PBS; a, P<0.05 IGFBP2 siRNA vs PBS. Data are mean±SEM, n=3.

Our untreated *ob/ob* mice had a range of plasma IGFBP2 levels from 19-76 ng/mL, compared to ~350 ng/mL in wildtype mice. Using the *ob/ob* mouse as a model, we define an IGFBP2 level of <75 ng/mL a state of insufficiency. Therefore, IGFBP2 siRNA 1, 2 and 3 produced IGFBP2 insufficiency for 3, 6 and 13 days respectively. Despite substantially reducing plasma IGFBP2 levels over this time, body weight and glucose homeostasis were not altered by IGFBP2 knockdown in any of the 9 mice treated with IGFBP2 siRNA. The weight of mice treated with IGFBP2 siRNA was indistinguishable from control mice over the course of the experiment (Figure 13B). Likewise, 4 hour fasted blood glucose values were unchanged between the groups regardless of plasma IGFBP2 levels (Figure 13C). Maintenance of glucose homeostasis was not a result of elevated plasma insulin levels, since no statistically significant differences in 4 hour fasted plasma insulin levels were observed amongst any of the IGFBP2 knockdown mice compared to controls (Figure 13D). These results suggest that physiological levels of plasma IGFBP2 are not essential for maintenance of normal fasted body weight, blood glucose or insulin levels.

IGFBP2 does not contribute to leptin-mediated improvements in body weight, blood glucose, and plasma insulin of ob/ob mice

We next investigated whether plasma IGFBP2 is necessary for the metabolic actions of leptin by treating *ob/ob* mice with leptin while knocking down IGFBP2. Mice were injected with IGFBP2 siRNA #3 or FVII siRNA one day before implantation of mini-osmotic pumps delivering either 5 μg/day leptin or vehicle. Both leptin treated groups had elevations in plasma leptin levels from undetectable amounts to levels similar to wildtype mice on day 11 (Figure 15A). Upon FVII siRNA injection and leptin treatment, plasma IGFBP2 levels increased linearly and eventually surpassed wildtype levels reaching 691±62 ng/mL by day 11 (Figure 15C). In contrast, *ob/ob* mice treated with IGFBP2 siRNA were unable to mount this response to leptin treatment, and maintained lower IGFBP2 levels compared to controls throughout the study ($P<0.05$ vs FVII siRNA from day 5 onward) (Figure 15C). On day 11 IGFBP2 levels in leptin treated, IGFBP2 knockdown mice were only slightly elevated compared to vehicle treated controls (142±23 ng/mL compared to 60±3 ng/mL; $P<0.05$, Figure 15C). This was consistent with hepatic transcript levels as the FVII siRNA group displayed a 17±3 fold

increase in *Igfbp2* mRNA levels over vehicle, and this increase was blunted in the IGFBP2 siRNA group, which experienced only a 3±1 fold increase over vehicle (<0.05 Figure 15B). Therefore, we successfully reduced the leptin-mediated increase in IGFBP2.

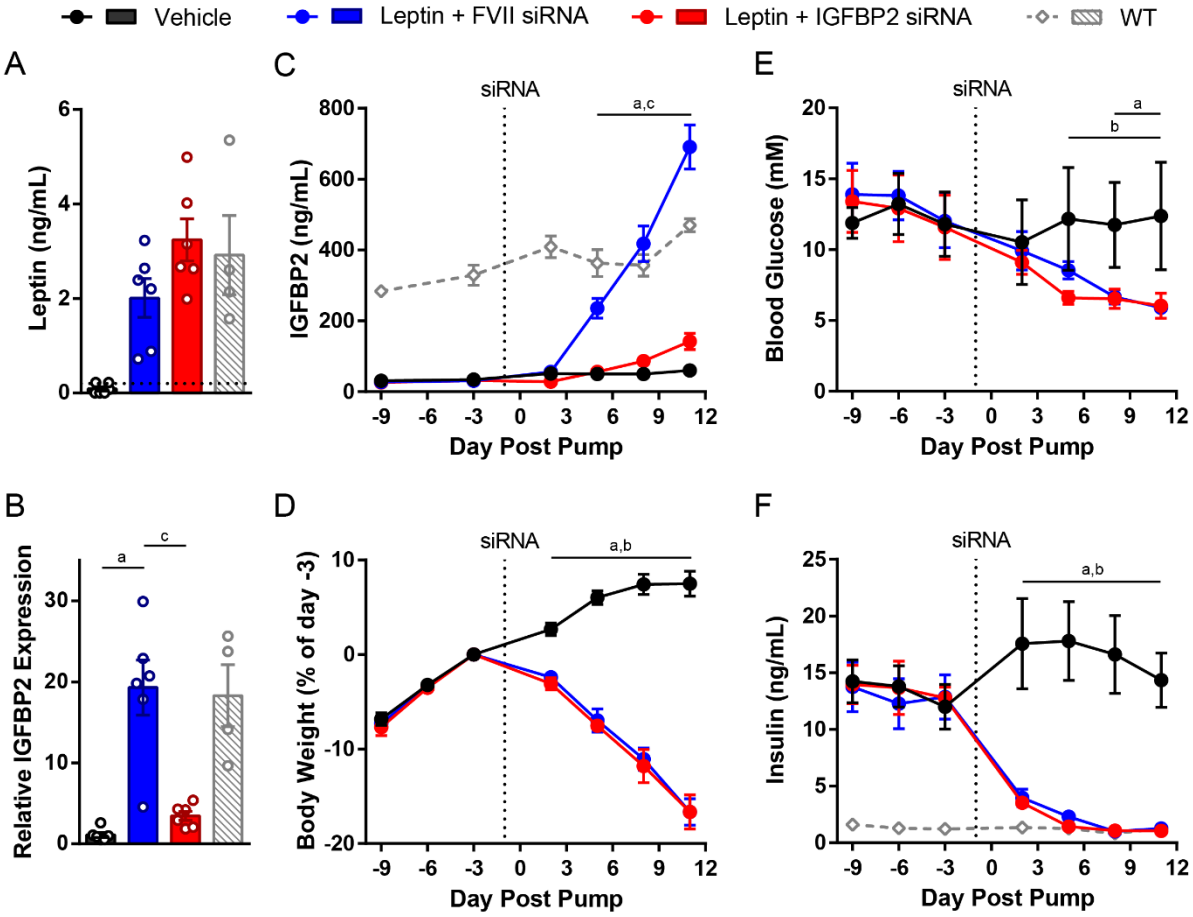

Figure 15. Prevention of the IGFBP2 response to leptin does not block leptin-mediated effects on body weight, blood glucose or plasma insulin. On day -1, nine week old *ob/ob* mice were treated with 5 mg/kg lipid nanoparticles encapsulating either FVII siRNA or IGFBP2 siRNA #3 by tail-vein injection (the vehicle group did not receive siRNA). On day 0, mice were implanted with mini-osmotic pumps delivering 5 µg/day leptin or vehicle. Wildtype mice did not receive either treatment. Plasma leptin levels (A) and IGFBP2 transcript levels (B) were measured on day 11 following a 4 hour fast. Plasma IGFBP2 (C), body weight (D, expressed as % change over baseline), blood glucose (E) and plasma insulin (F) were measured following a 4 hour fast. Statistical analyses were performed using a 1-way ANOVA or a repeated measures 2-way ANOVA with Bonferroni post-hoc testing. Statistical analysis was not performed on leptin measurements (A) as some samples were below the limit of detection denoted by the dotted line. The following groups were compared: a, P<0.05 vehicle vs FVII siRNA; b, P<0.05 vehicle vs IGFBP2 siRNA; c, P<0.05 FVII siRNA vs IGFBP2 siRNA. Data are mean±SEM, n=4-6.

Despite blocking an increase in plasma IGFBP2, leptin was able to reduce body weight and improve glucose metabolism. By day 11 of leptin treatment, body weight had decreased by ~17% in both the IGFBP2 and FVII siRNA groups (Figure 15D). Upon leptin treatment, both IGFBP2 and control knockdown groups experienced a similarly modest decrease in fasting blood glucose, which were statistically lower compared to vehicle treated controls on day 11 (Figure 15E; 12±3.8 mM, 6.0±0.9 mM, 5.9±0.3 mM for vehicle, IGFBP2 siRNA and FVII siRNA respectively; $P<0.05$). In a similar manner, leptin treatment normalized fasting plasma insulin levels in *ob/ob* mice irrespective of whether IGFBP2 was knocked down. Within 2 days of leptin administration, plasma insulin levels in both IGFBP2 and FVII siRNA groups were significantly lower than the vehicle treated *ob/ob* mice and had reached near-wildtype levels ($P<0.05$, Figure 15F), well before plasma IGFBP2 levels had been restored to wildtype levels (Figure 15C). Therefore, leptin treatment of *ob/ob* mice decreased body weight, fasting blood glucose and fasting plasma insulin to the same extent irrespective of whether IGFBP2 levels had been knocked down.

Finally, we assessed glucose tolerance in *ob/ob* mice in response to leptin treatment with or without IGFBP2 knockdown (Figure 16). As expected, leptin treatment improved glucose tolerance. While vehicle treated *ob/ob* mice had impaired glucose tolerance compared to wildtypes, leptin treated-FVII knockdown *ob/ob* mice exhibited a glucose excursion that was more similar to wildtype controls than vehicle treated *ob/ob* mice, with significantly lower glucose levels compared to vehicle from 60 min onward (Figure 16A), although the trend towards a lower integrated AUC did not reach statistical significance (Figure 16B; $P=0.11$ for FVII siRNA vs vehicle). Similarly, the mice given leptin in conjunction with IGFBP2 siRNA had a trend towards improved glucose tolerance over the vehicle treated *ob/ob* mice, although neither individual glucose measurements nor integrated AUC reached statistical significance (Figure 16).

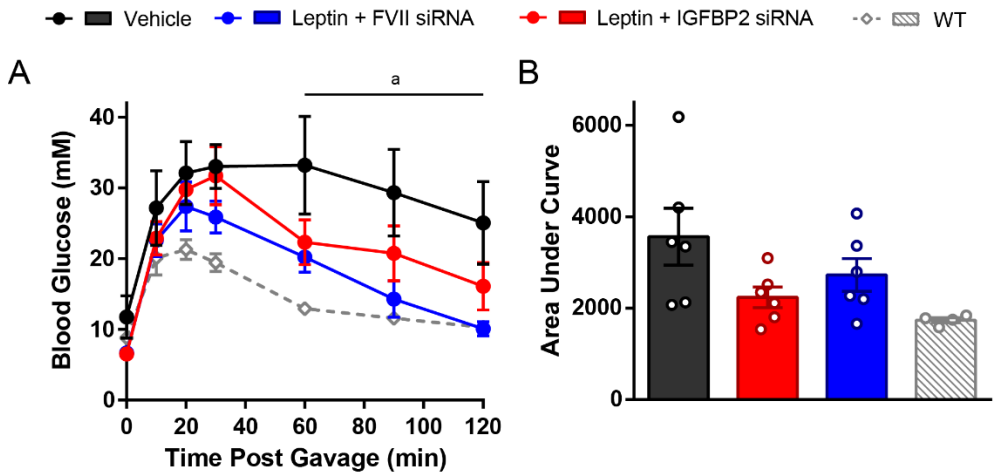

Figure 16. Oral glucose tolerance in *ob/ob* mice receiving leptin and either IGFBP2 or FVII siRNA. Nine week old *ob/ob* mice received 5 mg/kg lipid nanoparticles encapsulating containing either FVII or IGFBP2 siRNA on day -1 followed by 5 µg/day leptin (or vehicle as a control in mice that did not receive siRNA) on day 0. On day 8 post-treatment, mice were gavaged with 1.5 g/kg glucose following a 4 hour fast, blood glucose levels were monitored (A) and area under the curve was calculated (B). Statistical analyses were performed using a 1-way ANOVA or a repeated measures 2-way ANOVA with Bonferroni post-hoc testing. For (A) a, $P<0.05$ vehicle vs FVII siRNA; no significant differences were observed between vehicle vs IGFBP2 siRNA or FVII siRNA vs IGFBP2 siRNA. No statistical differences were observed in (B). Data are mean±SEM, n=4-6.

Mice with a lifelong ablation of IGFBP2 display 2-4 fold increases in hepatic *Igfbp-1*, *-3*, *-4*, *-5* and *-6* mRNA levels and it has been suggested that the lack of metabolic phenotype in these mice may be due to this compensation by other IGFBPs [319]. Therefore, we measured other IGFBP transcript levels in the liver of our acute IGFBP2 knockdown model (Figure 17). We did not find any statistical differences between the *ob/ob* groups, indicating that upregulation of other IGFBPs does not occur in this model (Figure 17).

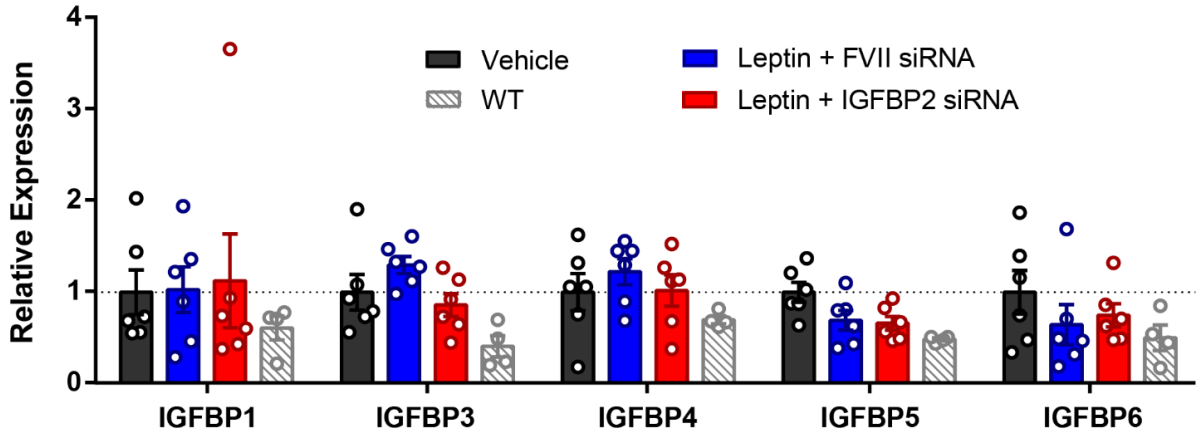

Figure 17. Hepatic IGFBP-1, -3, -4, -5 and -6 mRNA levels do not change in *ob/ob* mice receiving leptin and either IGFBP2 or FVII siRNA. Nine week old *ob/ob* mice received 5 mg/kg lipid nanoparticles encapsulating either FVII or IGFBP2 siRNA on day -1 followed by 5 µg/day leptin (or vehicle as a control in mice that did not receive siRNA) on day 0. Liver samples were collected on day 11 following a 4 hour fast and IGFBP-1, -3, -4, -5 and -6 mRNA levels were measured. Statistical analyses were performed using 1-way ANOVA with Bonferroni post-hoc testing. The following groups were compared: vehicle vs FVII siRNA; vehicle vs IGFBP2 siRNA; FVII siRNA vs IGFBP2 siRNA; however, no statistical differences were detected. Data are mean±SEM, n=4-6.

4.3 Discussion

The ability of leptin to improve various metabolic abnormalities in models of leptin deficiency [105, 175], lipodystrophy [229], and insulin-deficient diabetes [232, 233] has generated interest in understanding the mechanism of these effects. Hedbacker *et al.* uncovered IGFBP2 as a leptin-regulated gene, and determined that vastly overexpressing IGFBP2 alone using adenoviral transfer can induce effects similar to those of leptin [313]. However, the supraphysiological plasma levels of IGFBP2 induced in these studies may not reflect physiological actions of IGFBP2 and the reported effects could have been a result of pharmacological doses. Therefore, we sought to delineate the physiological role of IGFBP2 in glucose metabolism using three complementary approaches. Firstly, we assessed whether physiological levels of IGFBP2 were sufficient to recapitulate the glucose normalizing effects of leptin in *ob/ob* mice. Using an adenoviral vector, we induced IGFBP2 expression in *ob/ob* mice to obtain plasma levels of IGFBP2 similar to those induced by leptin treatment. Secondly, we assessed whether IGFBP2 is essential for glucose homeostasis in wild type mice by siRNA-mediated IGFBP2 knockdown. Finally, we investigated whether IGFBP2 is required for the

therapeutic effects of leptin treatment in *ob/ob* mice by blocking leptin-mediated increases in IGFBP2 using siRNA. Collectively these studies reveal that physiological levels of IGFBP2 are neither necessary nor sufficient for normal glucose homeostasis in the fasting state, or for the therapeutic actions of leptin replacement therapy.

In contrast to physiological levels of IGFBP2, treatment with supraphysiological levels of IGFBP2 affects some aspects of glucose metabolism. The results from our high dose Ad-IGFBP2 study are largely in agreement with those of Hedbacker *et al.* [313]: in our study high dose Ad-IGFBP2 treatment induced a reduction in body weight compared to controls by day 9 of overexpression, similar to the finding by Hedbacker *et al.* where significant weight loss was not achieved until a week following IGFBP2 overexpression [313]. It is noteworthy that weight loss was delayed and diminished in magnitude in Ad-IGFBP2 treated mice compared to leptin treated mice, which had lost significant weight by day 2. This suggests that the mechanism of weight loss induced by leptin and IGFBP2 in *ob/ob* mice may be at least partially distinct. The high dose Ad-IGFBP2 treatment of *ob/ob* mice also decreased fasted plasma insulin levels; however, an identical decrease was observed in the high dose Ad-β-gal control group, suggesting a non-specific adenovirus effect on glucose homeostasis as has been previously reported [329], or related to protein overexpression in the liver. Hedbacker *et al.* did not report virus-induced hypoinsulinemia; this difference compared to the current results may be related to our use of a control virus employing a construct expressing lac-Z under the CMV promoter instead of an empty virus containing only the CMV promoter [313]. Interestingly the high Ad-IGFBP2 treatment improved glucose tolerance compared to control animals, similar to the Hedbacker *et al.* report [313], although no differences in whole body insulin tolerance were observed. This suggests that in the fed state pharmacological levels of IGFBP2 may modulate glucose handling. This is consistent with a previous report that demonstrated that transgenic mice expressing human IGFBP2 at levels that increased total circulating IGFBP2 levels by 2.2 fold, were protected from age- and diet-induced obesity and glucose intolerance [322]. Therefore, our data add additional support to the notion that pharmacological doses of IGFBP2 may influence glucose metabolism.

The phenotype we observed in mice with siRNA-mediated knockdown of IGFBP2 is consistent with that observed following chronic ablation of IGFBP2 in wildtype mice. Male *Igfbp2KO* mice are neither glucose intolerant nor insulin resistant, although they display a mild increase in body fat by 16 weeks of age [319]. This lack of a substantial metabolic phenotype may be due to compensation through upregulation of other IGFBPs [319, 320]. Therefore, we aimed to acutely knockdown IGFBP2 in wildtype mice to minimize the opportunity for compensation due to lifelong gene ablation. We observed that body weight, fasting blood glucose, and fasting plasma insulin were unchanged following rapid and efficient knockdown of plasma IGFBP2. We anticipated that if IGFBP2 were vital for physiological leptin action in wildtype mice, we would have observed changes in metabolic control with acute knock down of IGFBP2. Indeed, acute inhibition of leptin action in wildtype mice via PEGylated mouse leptin antagonist increased body weight [330], plasma insulin, and glucose stimulated insulin secretion, and impaired whole-body insulin sensitivity [187] only after a few days of treatment. Therefore, our study suggests that IGFBP2 is not required for maintenance of glucose homeostasis. In addition to investigating the requirement of IGFBP2 in normal mice, we found that IGFPB2 is not necessary for normalization of metabolism by leptin therapy in *ob/ob* mice. Therefore, we conclude that IGFBP2 is not required either for proper metabolic control or the glucoregulatory action of leptin.

Our studies and other reports do not support a substantial role for IGFBP2 in maintaining fasting glucose homeostasis, and the function of IGFBP2 remains largely unclear. *Igfbp2KO* mice do not have a major metabolic phenotype, although they have a ~25% decrease in spleen weight and a ~15% increase in liver weight [321], as well alterations in bone turnover and architecture [319]. Moreover, transgenic mice overexpressing mouse IGFBP2 under the CMV promoter, driving a 3-fold increase in circulating IGFBP2 levels, had only a mild reduction in postnatal body weight gain, which was attributed to changes in carcass and organ weights rather than fat mass reductions [331]. Among the most compelling studies connecting IGFBP2 to glucose regulation is a report that mice expressing human IGFBP2 under its native promoter are protected from age- and diet-related increases in adiposity and glucose intolerance [322]. It has been speculated that these effects of IGFBP2 might arise from its inhibition of IGF-1,

which affects organ growth and adipocyte differentiation. Therefore, these studies illustrate that many other perturbations are observed when manipulating IGFBP2 levels that may be entirely independent of leptin action. While leptin clearly regulates IGFBP2 levels, IGFBP2 does not appear to be a critical mediator of leptin action on glucose and body weight regulation. Nevertheless, IGFBP2 may be physiologically important for axes of leptin action beyond glucose regulation, a possibility that will require further investigation.

CHAPTER 5 – EFFECT OF LEPTIN THERAPY IN INSULIN KNOCKOUT MICE

5.1 Introduction

It is widely believed that insulin is the only hormone capable of normalizing the catabolic consequences of type 1 diabetes. However, leptin can normalize many metabolic processes in rodents with insulin-deficient diabetes [167, 176, 230-235], prompting testing in patients. Previous reports claimed that the glucose-lowering actions of leptin are insulin-independent, challenging the dogma that life without insulin is not possible. However, those studies used chemical or immune destruction of β-cells, which are <100% efficient. Mice with insulin-deficient diabetes induced by autoimmune destruction of β-cells [233], STZ, or DT administration to *RIP-DTR* mice [230] exhibit islet insulin immunoreactivity and untreated mice survive for multiple weeks. As mice in which both insulin genes are knocked out (*Ins1KO/Ins2KO*, herein referred to as *InsKO* mice), survive for only ~2 days after birth [31] without insulin treatment it is suggesting that these other models are not insulin-free. Given that leptin treated STZ-diabetic mice exhibit vastly improved insulin sensitivity, even beyond that of healthy controls [167, 234], it is possible that residual insulin contributed to the reversal of diabetes with leptin therapy in prior studies. In this Chapter we hypothesized that leptin can reverse diabetes independent of insulin. To investigate this we tested leptin therapy in two complementary models: mice with combined STZ injection and insulin receptor antagonism to maximize abrogation of insulin action, and *InsKO* mice devoid of insulin. All data in this Chapter are published in *Endocrinology* [252].

5.2 Results

Insulin receptor antagonism does not reverse the blood glucose lowering effect of leptin in STZ-diabetic mice

We rationalized that if the actions of leptin on reducing hyperglycemia were insulin dependent then antagonism of insulin signalling would decrease the effectiveness of leptin therapy. STZ-injected mice were implanted with an insulin pellet or a pump delivering either vehicle or 20 μg/day leptin. All STZ-injected groups lost body weight compared to baseline and neither leptin nor insulin treatment altered body weight compared to vehicle treated controls (Figure 18A). Both leptin and insulin treatments reduced 4 hour fasting blood glucose by day 2 compared to STZ-vehicle controls (10.8±2.0 mM STZ-leptin, 12.5±2.0 mM STZ-insulin, 22.6±0.7 mM STZ-vehicle, $P<0.05$, Figure 18B). STZ injection depleted leptin levels, insulin treatment restored leptin levels, and leptin therapy induced supraphysiological leptin levels (2.0±0.3 ng/mL non-diabetic controls, 0.3±0.0 ng/mL STZ-vehicle, 5.2±0.5 ng/mL STZ-insulin, 36.0±6.1 ng/mL STZ-leptin, Figure 18C). To determine if blocking residual insulin action would affect leptin-normalized blood glucose levels, fasted mice were administered the insulin receptor antagonist S961 or vehicle on day 5. Injection of S961 increased blood glucose in the insulin treated mice after 2 hours (4.8±0.5 mM STZ-insulin + vehicle, 21.1±4.1 mM STZ-insulin + S961, $P<0.05$), demonstrating the effectiveness of this antagonist, even in the presence of exogenous insulin. However, this dramatic elevation of blood glucose was not observed in the leptin treated group (4.7±1.1 mM STZ-leptin+vehicle, 7.7±2.7 mM STZ-leptin+S961, Figure 18D&E). Similar to the fasted state, 30 min after an oral glucose load, blood glucose levels were significantly elevated in the insulin treated group receiving S961 compared to vehicle (7.1±1.4 mM STZ-insulin vehicle, 36.6±3.8 mM STZ-insulin + S961, $P<0.05$, Figure 18F). In contrast there was no significant difference due to the insulin receptor antagonist in the leptin treated group (23.1±3.3 mM STZ-leptin + vehicle, 26.6±2.4 mM STZ-leptin + S961, Figure 18F&G). Therefore, the ability of leptin to improve hyperglycemia in STZ-diabetic mice is unaffected by insulin receptor antagonism suggesting that leptin improves glycemia independently of insulin in this model.

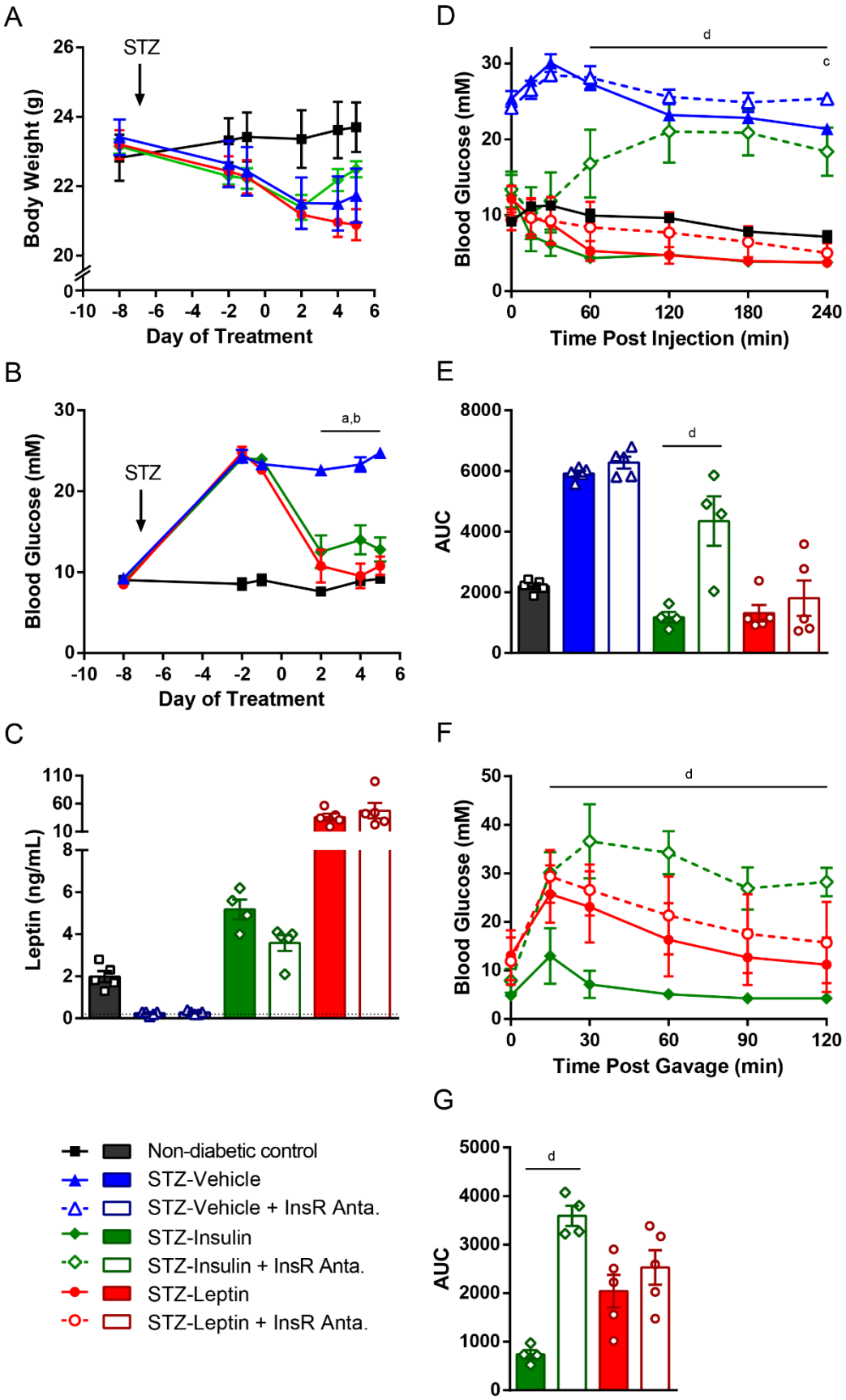
A
STZ
Body Weight (g)
Day of Treatment
B
STZ
a,b
Blood Glucose (mM)
Day of Treatment
C
Leptin (ng/mL)
D
d
c
Blood Glucose (mM)
Time Post Injection (min)
E
d
AUC
F
d
Blood Glucose (mM)
Time Post Gavage (min)
G
d
AUC
Non-diabetic control
STZ-Vehicle
STZ-Vehicle + InsR Anta.
STZ-Insulin
STZ-Insulin + InsR Anta.
STZ-Leptin
STZ-Leptin + InsR Anta.

Figure 18. Insulin receptor antagonism does not reverse the blood glucose lowering effect of leptin in STZ-diabetic mice. Insulin-deficient diabetes was induced in 9-week-old C57BL/6J mice by injecting 180 mg/kg STZ on day -7; vehicle was administered to non-diabetic controls. On day 0, STZ-diabetic mice were implanted with either 1 insulin pellet or a pump delivering 20 µg/day leptin or vehicle; sham surgery was performed on non-diabetic controls. Body weight (A) and blood glucose (B) were measured throughout the study following a 4 hour fast. Plasma leptin levels were measured on day 8 (C) and the limit of detection is denoted by the dotted line. On day 5, mice fasted 4 hours were given an i.p. injection of either 24 nmol/kg S961 InsR antagonist (InsR Ant.) or vehicle, after which blood glucose was measured (D) and the AUC was calculated (E). On day 7 mice fasted for 4 hours were given an i.p. injection of either 24 nmol/kg S961 InsR Ant. or vehicle, 30 min prior to an oral gavage of 1.5 g/kg glucose, and blood glucose was measured (F) and AUC was calculated (G). For statistical analysis, the following groups were compared using 1- or 2- way ANOVA with Tukey post-hoc testing: a, $P<0.05$ STZ-vehicle vs STZ-insulin; b, $P<0.05$ STZ-vehicle vs STZ-leptin; c, $P<0.05$ STZ-vehicle vs STZ-vehicle + InsR Ant.; d, $P<0.05$ STZ-insulin vs STZ-insulin + InsR Ant.; e, $P<0.05$ STZ-leptin vs STZ-leptin + InsR Ant. Data are mean±SEM, n=4-5.

Leptin prolongs survival and induces fasting hypoglycemia in InsKO mice

As a complementary study we tested whether leptin therapy could extend lifespan and normalize glucose metabolism in *InsKO* mice. If left untreated *InsKO* mice die within 48 hours of birth [31]. Therefore, to maintain *InsKO* mice into adulthood, they were injected twice daily with insulin until 14 days of age when they underwent an islet transplantation into the eye. We predicted that a pump surgery would be too severe on the *InsKO* mice, which may have compromised survival results; therefore, we opted to use daily PEGylated leptin injections. Daily injections of PEGylated leptin produces similar effects to non-PEGylated leptin delivered via pump in STZ-diabetic mice (Figures 19&20). At ~10 weeks old, starting on day 0 of the study, *InsKO* mice were treated daily with PEGylated leptin or vehicle and *Ins1KO/Ins2Het* (*Het*) controls received vehicle. Four days of leptin and insulin co-therapy was performed as leptin requires several days to normalize fasting glycemia [167, 230-233]. On day 4 the eye containing islets was enucleated to render the mice entirely insulin-deficient. As expected, 100% (5/5) of vehicle treated *InsKO* mice rapidly lost weight, were hunched, displayed piloerection, were lethargic or non-responsive and reached humane endpoint within 24 hours of enucleation (Figure 21A&B). Although 36% (5/14) of leptin treated *InsKO* mice also reached humane endpoint, 64% (9/14) survived until the end of the study (day 11; Figure 21A). The leptin treated *InsKO* mice lost substantial mass prior to enucleation, consistent with the well-established body weight reducing effect of leptin. Despite losing more mass following enucleation, the leptin treated mice did not exhibit signs of deteriorating body condition

(Figure 21B). Thus astonishingly, leptin is capable of promoting survival in the complete absence of insulin.

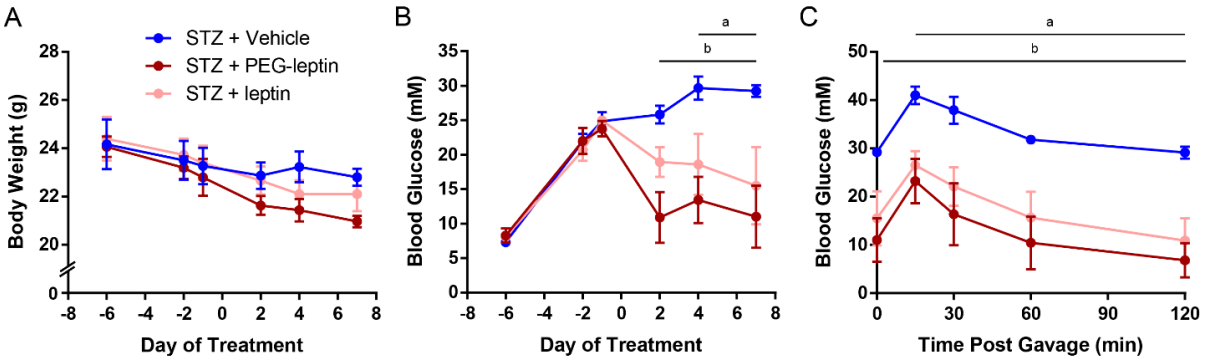

Figure 19. Leptin and PEGylated leptin similarly lower fasting blood glucose in STZ-diabetic mice. On day -6 C57BL/6J mice were treated with 180 mg/kg STZ to induce insulin-deficient diabetes. On day 0, mice received either a pump delivering vehicle and daily intraperitoneal vehicle injections (STZ+Vehicle), a pump delivering 10 µg/day leptin and daily intraperitoneal vehicle injections (STZ+Leptin), or a pump delivering vehicle and daily intraperitoneal injections of 10 µg/day PEGylated leptin (STZ+PEGylated leptin). Body weight (A) and blood glucose (B) were measured throughout the study following a 4 hour fast. On day 7, an oral glucose tolerance test using 1.5 g/kg glucose was performed (C). The following groups were compared using a repeated measures 2-way ANOVA with Tukey post-hoc testing: a, STZ+Vehicle vs STZ+Leptin; b, STZ+Vehicle vs STZ+PEG-leptin; $P<0.05$. No differences were detected between the STZ+Leptin and STZ+PEG-leptin groups. Data are mean ± SEM, n=3.

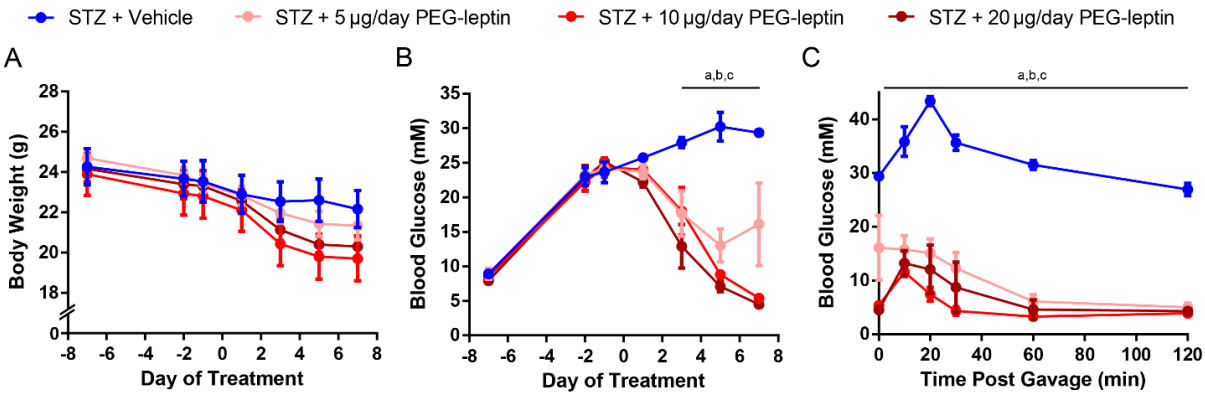

Figure 20. PEGylated leptin at 5, 10, and 20 µg/day lowers fasting blood glucose in STZ-diabetic mice. On day -7, C57BL/6J mice were treated with 180 mg/kg STZ to induce insulin-deficient diabetes. On day 0, mice received daily intraperitoneal injections of either vehicle, or 5, 10, or 20 µg/day of PEGylated leptin. Body weight (A) and blood glucose (B) were measured throughout the study following a 4 hour fast. On day 7, an oral glucose tolerance test using 1.5 g/kg glucose was performed (C). The following groups were compared using a repeated measures 2-way ANOVA with Tukey post-hoc testing: a, STZ+Vehicle vs STZ+5 µg/day of PEG-leptin; b, STZ+Vehicle vs STZ+10 µg/day of PEG-leptin; c, STZ+Vehicle vs STZ+20 µg/day of PEG-leptin. Data are mean ± SEM, n=3.

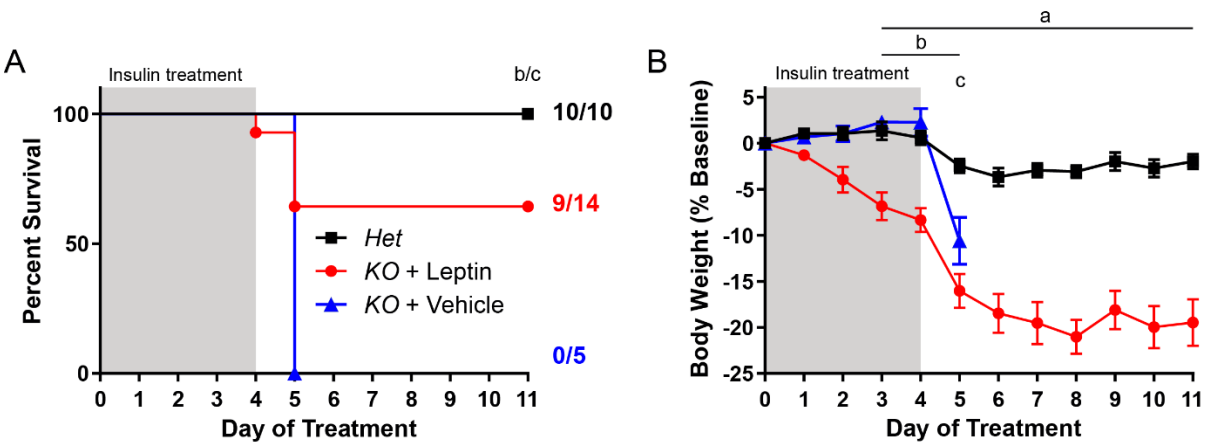

Figure 21. Leptin prolongs survival in *InsKO* mice. *InsKO* mice with islet transplants in the eye were given daily injections of leptin or vehicle starting on day 0. On day 4 mice underwent enucleation to become completely devoid of insulin. *Het* controls also underwent enucleation and received vehicle injections. Survival (A) and body weight (B) were measured throughout the study. The following groups were compared using the Kaplan-Meier method with the log rank test from day 4 onward for (A) or a repeated measures 2-way ANOVA with Tukey post-hoc testing for (B): a, P<0.05 *Het* vs *KO* + Leptin; b, P<0.05 *KO* + Leptin vs *KO* + Vehicle; c, P<0.05 *Het* vs *KO* + Vehicle; d, P<0.05 *Het* (Fast) vs *KO* + Leptin (Fast). Data are % survival or mean±SEM, n=4-10.

Given that some leptin treated *InsKO* mice had extended survival, we speculated that they may exhibit improved glucose metabolism. However, in the leptin treated *InsKO* mice that survived, blood glucose was volatile, ranging from hyperglycemia in the fed state to hypoglycemia within only 2-6 hours fasting. On day 7, *Het* controls had a fed blood glucose ranging from 7.1 - 9.3 mM and fasting blood glucose of 4.1 - 7.5 mM (Figure 22A&B). In contrast, the leptin treated *InsKO* mice exhibited overt fed hyperglycemia of 23.8 - >33.3 mM that was reduced to <1.1 - 10 mM within 4 hours of fasting (Figure 22A&B). To investigate the timeframe in which the mice became hypoglycemic, blood glucose was measured hourly following removal of food. During the fast leptin treated *InsKO* mice lost substantial weight relative to *Hets* (-9.3±0.6% vs -4.2±0.5% at 4 hours fasting, Figure 22C). Individual blood glucose plots are graphed from day 9 in which leptin treated *InsKO* mice reached dangerously low blood glucose levels (1.3 - 2.3 mM) in 3-5 hours of fasting and were rescued by returning food or injecting glucose (Figure 22D). This rescue led to rapid hypergylcemia relative to the healthy *Hets*, which maintained steady glucose levels after returning food (Figure 22D). Therefore, despite having extended survival, leptin treated *InsKO* mice did not have normal glucose homeostasis.

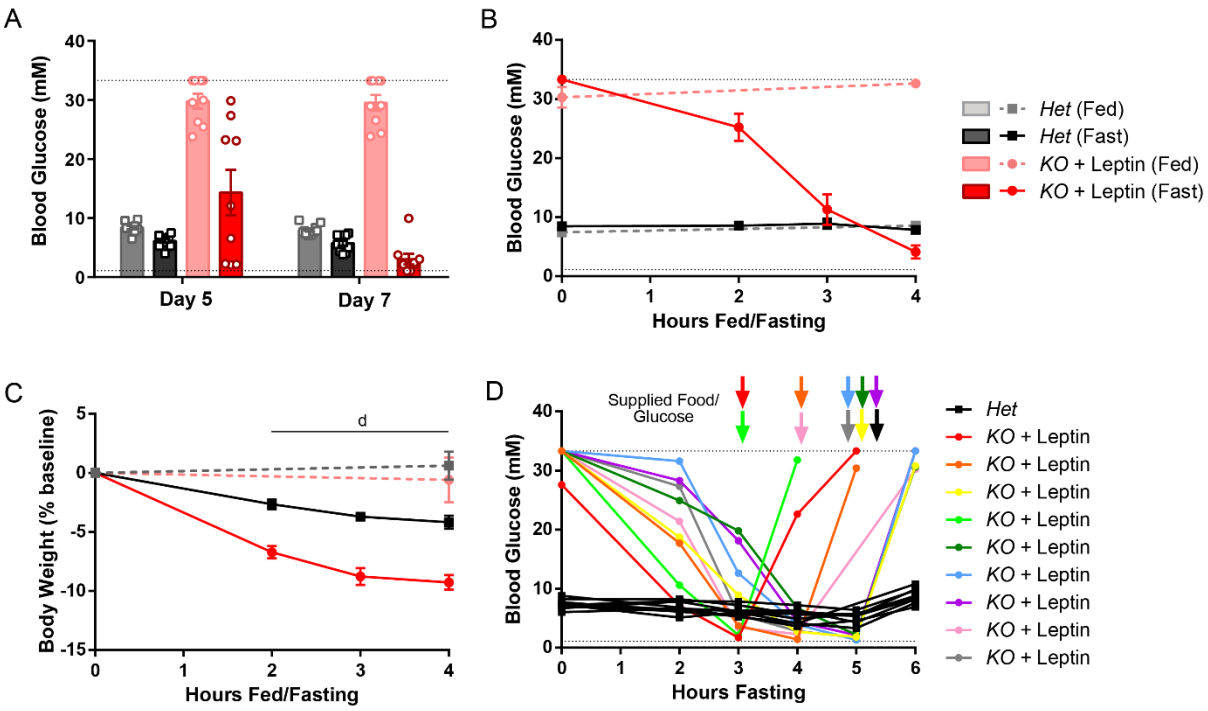

Figure 22. Leptin induces fasting hypoglycemia in *InsKO* mice. *InsKO* mice with islet transplants in the eye were given daily injections of leptin or vehicle starting on day 0. On day 4 mice underwent enucleation to become completely devoid of insulin. *Het* controls also underwent enucleation and received vehicle injections. Day 5 and 7 blood glucose levels were measured in the fed state and after fasting up to 4 hours (A). Hourly tracking of blood glucose (B) and body weight (C) during a 4 hour fast on day 11. Hourly tracking of blood glucose of individual mice during a fast on day 9 (D). Coloured arrows denote when *InsKO* mice became hypoglycemic and were rescued with food or glucose; at 5 hours the *Het*s received food. Statistical analysis was not performed on blood glucose values as every *InsKO* group had at least 1 sample above (>33.3 mM) or below (<1.1 mM) the detection limit of the glucometer, which are indicated by the dotted lines. The following groups were compared by a repeated measures 2-way ANOVA with Tukey post-hoc testing: a, P<0.05 *Het* vs KO + Leptin; b, P<0.05 *KO* + Leptin vs *KO* + Vehicle; c, P<0.05 *Het* vs *KO* + Vehicle; d, P<0.05 *Het* (Fast) vs *KO* + Leptin (Fast). Data are mean±SEM (A-C) or individual mice (D), n=4-10.

Since leptin therapy rescued *InsKO* mice from death, we aimed to determine how long leptin treatment could prolong survival in a second cohort of *InsKO* mice. Similar to the first cohort, on day 11, 71% (5/7) of leptin treated *InsKO* remained; however, by day 26 all had succumbed to their diabetic symptoms (Figure 23A). Again we observed significant weight loss and a rapid decline in blood glucose from fed hyperglycemia to near fatal hypoglycemia by 6 hours of fasting in the leptin treated *InsKO* mice (Figure 23B&C). Thus leptin effectively lowered fasting glycemia but did not normalize glycemic control in the absence of insulin.

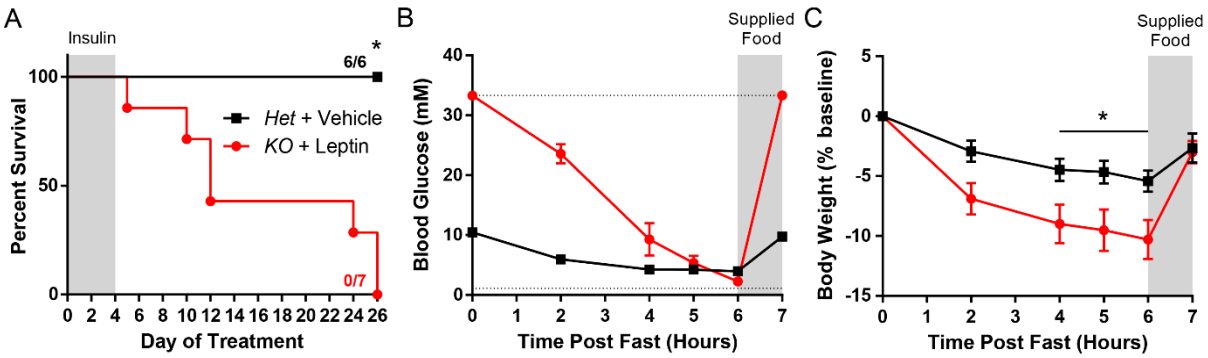

Figure 23. Leptin prolongs survival and induces fasting hypoglycemia in *InsKO* mice. *InsKO* mice with islet transplants in the eye were given daily injections of leptin or vehicle starting on day 0. On day 4 mice underwent enucleation to become completely devoid of insulin. *Het* controls also underwent enucleation and received vehicle injections. Survival was measured until all *InsKO* mice reached humane endpoint (A). Hourly fasting blood glucose (B) and body weight (C) on day 11. Statistical analysis was not performed on blood glucose values as every *InsKO* group had at least 1 sample above (>33.3 mM) or below (<1.1 mM) the detection limit of the glucometer. Groups were compared using the Kaplan-Meier method with the log rank test from day 4 onward (A) or a repeated measures 2-way ANOVA with Bonferroni post-hoc testing (C); *, P<0.05 *Het* + vehicle vs *KO* + Leptin. Data are % survival (A) or mean±SEM (B, C), n=5-6.

Supraphysiological levels of leptin reduce plasma corticosterone, glucagon, β-hydroxybutyrate, triglycerides, cholesterol, free fatty acids and glycerol in InsKO mice

Since leptin has a remarkable ability to prolong survival of *InsKO* mice we assessed several metabolic parameters in the leptin treated mice, which were improved with leptin treatment in other models of insulin-deficient diabetes. On day 4-5, as the vehicle- and leptin treated *InsKO* mice reached humane endpoint, blood samples were collected prior to euthanasia. At the same time, some samples were collected from healthy leptin treated *InsKO* mice and *Hets* in the *ad libitum* fed state for comparison. Results were highly variable, likely a reflection of variations in health, when the animals last ate, their activity level, and time of day they were euthanized. On day 11 the remaining healthy mice were either fed or fasted for 4 hours and euthanized (corresponds to Figure 22B&E). Although comparing control and experimental groups on different days is confounded in that we cannot delineate primary effects of leptin from secondary effects of the improved metabolic state, it was necessary as the control mice rapidly deteriorated. Despite being at humane endpoint, *InsKO* mice exhibited plasma analyte profiles similar to other models of insulin-deficient diabetes. As expected on day 11 the leptin treated *InsKO* mice had supraphysiological levels of leptin compared to

controls (1471.8±364.5 ng/mL *InsKO*+leptin fed, 1.5±0.9 ng/mL *InsKO*+vehicle fed, 2.3±0.6 ng/L *Het* fed, Figure 24A). Interestingly although the leptin treated *InsKO* mice that reached humane endpoint were hyperleptinemic, they had the lowest plasma leptin of all the leptin treated mice and were more similar to the vehicle treated mice than the healthy leptin treated *InsKO* mice for all other parameters (Figure 24&25).

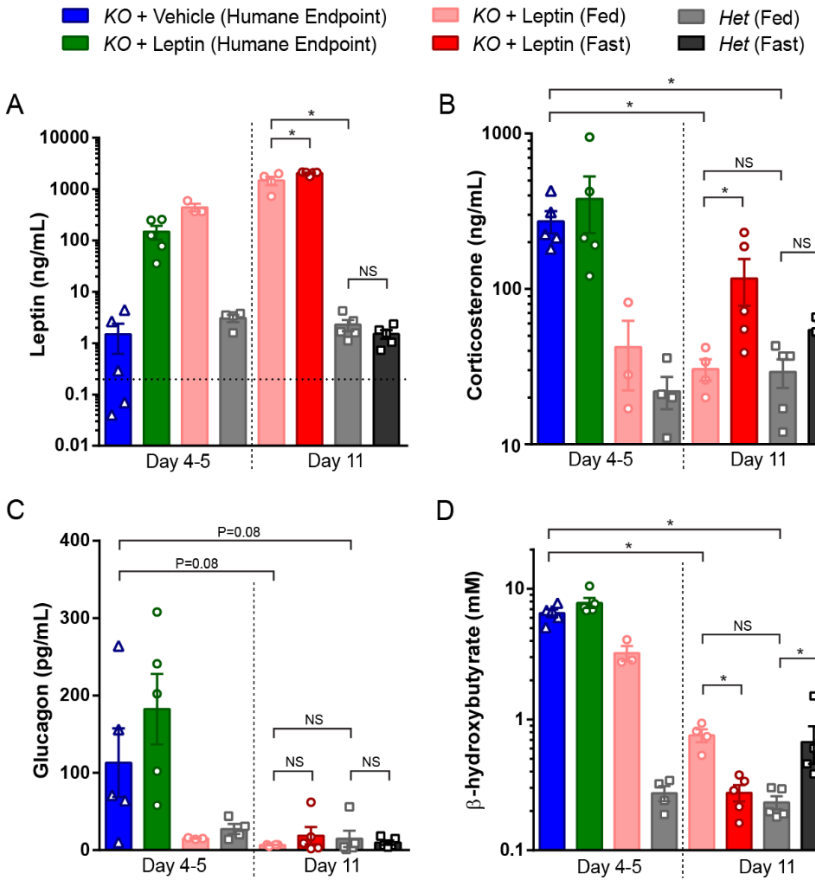

Figure 24. Supraphysiological levels of leptin reduce plasma corticosterone, glucagon, and β-hydroxybutyrate in *InsKO* mice. *InsKO* mice with islet transplants in the eye were given daily injections of leptin or vehicle starting on day 0. On day 4 mice underwent enucleation to become completely devoid of insulin. *Het* controls also underwent enucleation and received vehicle injections. On day 4-5 of treatment, all vehicle treated *InsKO* mice and some leptin treated *InsKO* mice reached humane endpoint and plasma samples were collected (Refer to Figure 21A). At the same time plasma samples were also collected from 3 healthy leptin treated *InsKO* and 4 *Het*s. On day 11, half of the leptin treated *InsKO* and *Het*s were left feeding, while the other half were fasted for 4 hours, and plasma samples were collected (corresponds to Figure 22B&C). Plasma leptin (A), corticosterone (B), glucagon (C), and β-hydroxybutyrate (D) were assayed. For statistical analysis, comparisons were made using a 1-way ANOVA with Bonferroni post-hoc testing between *KO* + vehicle day 4-5, *KO* + Leptin (Fed) day 11, and *Het* (Fed) day 11, as well as between *KO* + Leptin fast vs fed and *Het* fast vs fed on day 11. For (A), some samples from the *KO* + vehicle group fell below the detection limit, denoted by the dotted line, and therefore were not compared in the statistical analysis. *, $P<0.05$; NS = not significant. Data are mean±SEM, n=3-5.

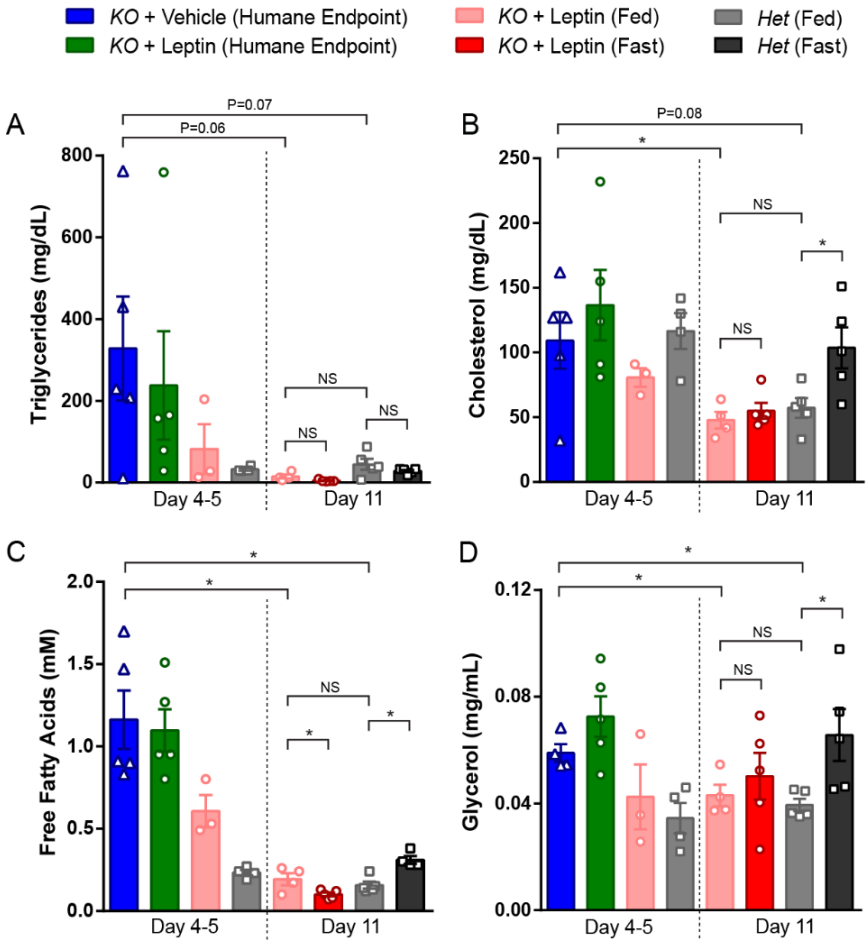

Figure 25. Supraphysiological levels of leptin triglycerides, cholesterol, free fatty acids and glycerol in *InsKO* mice. *InsKO* mice with islet transplants in the eye were given daily injections of leptin or vehicle starting on day 0. On day 4 mice underwent enucleation to become completely devoid of insulin. *Het* controls also underwent enucleation and received vehicle injections. On day 4-5 of leptin treatment, all vehicle treated *InsKO* mice and some leptin treated *InsKO* mice reached humane endpoint and plasma samples were collected (Refer to Figure 21A). At the same time plasma samples were also collected from 3 healthy leptin treated *InsKO* and 4 *Het*s. On day 11, half of the leptin treated *InsKO* and *Het*s were left feeding, while the other half were fasted for 4 hours, and plasma samples were collected (corresponds to Figure 22B&C). Plasma triglycerides (A), total cholesterol (B), fatty acids (C), and glycerol (D) were assayed. For statistical analysis, comparisons were made using a 1-way ANOVA with Bonferroni post-hoc testing between *KO* + vehicle day 4-5, *KO* + Leptin (Fed) day 11, and *Het* (Fed) day 11, as well as between *KO* + Leptin fast vs fed and *Het* fast vs fed on day 11. *, $P<0.05$; NS = not significant. Data are mean±SEM, n=3-5.

Both the counter-regulatory hormones corticosterone and glucagon have been implicated in the glucose-lowering action of leptin. Fed corticosterone was dramatically reduced in *InsKO* mice by leptin treatment vs vehicle (29±6 ng/mL vs 272±45 ng/mL, $P<0.05$, Figure 24B). Although leptin normalized corticosterone in the hyperglycemic fed state, upon fasting, the

mice displayed a robust counter-regulatory response, similar to that of *Hets* (Figure 24B). Therefore, the hypoglycemia in leptin treated *InsKO* mice does not require suppressed corticosterone levels. Hyperglucagonemia is characteristic of STZ induced diabetes and leptin treatment lowers plasma glucagon levels, as well as hepatic p-CREB levels, a downstream mediator of glucagon receptor signalling [167, 230, 232-234]. However, it has also been suggested that leptin therapy can normalize hyperglycemia prior to normalization of glucagon [235] and can reverse hyperglucagonemia without restoring normoglycemia [234]. Leptin treated *InsKO* mice had a trend towards normalized glucagon levels in the hyperglycemic fed state vs vehicle treated mice, (6±1 pg/mL vs 113±44 pg/mL, p=0.08, Figure 24C). Therefore, leptin can reduce glucagon levels independently of insulin, which may contribute to life extension and improved glycemia [75].

We next investigated parameters involved in lipid metabolism. Interestingly, although the leptin treated *InsKO* mice had overt hyperglycemia in the fed state, plasma β-hydroxybutyrate was significantly reduced relative to controls receiving vehicle (0.8±0.1 mM vs 6.5±0.5 mM, P<0.05, Figure 24D). As expected, upon fasting the *Het* controls had an increase in β-hydroxybutyrate; however, the leptin treated *InsKO* mice did not mount this response (Figure 24D). This is consistent with results in the STZ model [231] and suggests that endogenous glucose and ketone production during fasting is largely suppressed by leptin treatment. In addition, in the fed state plasma triglycerides were reduced with leptin treatment vs vehicle (15±9 mg/dL vs 328±127 mg/dL, P=0.06, Figure 25A) as was plasma cholesterol (48±5 mg/dL vs 109±22 mg/dL, P<0.05, Figure 25B), which is consistent with what has been previously observed in the STZ and NOD models [167, 234]. Fatty acids were reduced to normal levels with leptin treatment vs vehicle in the fed state, (0.19±0.03 mM vs 1.16±0.18 mM, P<0.05, Figure 25C). Upon fasting, the healthy *Hets* increased fatty acids (0.15±0.02 mM to 0.31±0.02 mM, P<0.05); however, the opposite was observed in the leptin treated *InsKO* mice (0.19±0.03 mM to 0.10±0.01 mM, P<0.05), in agreement with previous studies [235] (Figure 25C). Glycerol levels were moderately reduced with leptin treatment vs vehicle (0.043±0.003 mg/mL vs 0.059±0.003 mg/mL, p<0.05) and upon fasting were not lowered

relative to the fed state in the leptin treated group (Figure 25D). These data suggest that glycerol depletion is not required for hypoglycemia in the leptin treated *InsKO* mice.

5.3 Discussion

It has been proposed that leptin therapy can enable insulin-deficient rodents to thrive without insulin [233] and touted that unlike insulin injections, leptin does not cause hypoglycemia [230] and is therefore a promising alternative or additive to insulin therapy. We found that while remarkably leptin can extend lifespan in the complete absence of insulin, glucose homeostasis was not normalized and leptin treated mice deteriorated. Interestingly when diabetic mice are treated with leptin either intracerebroventricularly [176, 177, 230], or systemically by adenovirus [233], mini-osmotic pump [167, 231, 232], or daily PEGylated leptin injections, both fasting and fed blood glucose levels are improved [167, 230-233]. Conversely, leptin treated *InsKO* mice exhibit fed hyperglycemia and fasting hypoglycemia. We have previously demonstrated that leptin treated STZ-diabetic mice can become hypoglycemic if fasted for 9-15 hours [231] and therefore believe that leptin-induced blood glucose lowering is sensitive to fasting duration. However, at comparable fasting lengths the hypoglycemia in the *InsKO* mice is far more severe than STZ-diabetic mice. We speculate this is could be due to the complete absence of insulin in the *InsKO* mice compared to STZ-diabetic mice or differences is the dose of leptin used.

Similar to reports in NOD and STZ-diabetic mice, leptin therapy in *InsKO* mice reduced corticosterone, glucagon, β-hydroxybutryate, triglycerides, total cholesterol, fatty acids and glycerol suggesting these actions of leptin are independent of insulin [167, 231, 234, 235]. Interestingly, all of these parameters were reduced in the fed hyperglycemic state and only fatty acids and β-hydroxybutryate were further depleted upon fasting. This suggests that in the *InsKO* model reductions in corticosterone, glucagon, triglycerides, cholesterol, and glycerol are not sufficient to reduce fasting glycemia but fatty acids and β-hydroxybutryate may play an important role.

There are conflicting views over the importance of suppressing counter-regulatory hormones to the glucose-lowering action of leptin. Studies have reported both normalization of [234, 235] and no change in [167] corticosterone in response to leptin therapy. Furthermore, one report claims that leptin reverses diabetes by reduction of corticosterone through suppression of the hypothalamic–pituitary–adrenal axis [235], while a subsequent study challenges this conclusion [332]. Although hyperglucagonemia can be normalized with leptin treatment [167, 230, 232, 233, 235], glucagon lowering has been observed after blood glucose is normalized by leptin treatment [235], and low dose leptin that induced only a minute decrease in blood glucose normalized plasma glucagon [234]. Our data suggest that the fasting hypoglycemia in leptin treated *InsKO* mice may be independent of corticosterone and glucagon action.

Taken together, the precise glucose-lowering mechanisms of leptin remain to be fully elucidated and warrant further investigation, particularly given that leptin has been considered as a potential therapy for type 1 diabetes. Although leptin and insulin co-therapy may reduce insulin requirements, the risk of hypoglycemia with leptin use should promote caution whether used with or without insulin.

CHAPTER 6 – EFFECT OF GLUCAGON RECEPTOR siRNA IN MODELS OF TYPE 1 AND TYPE 2 DIABETES

6.1 Introduction

Hyperglucagonemia is present in type 1 [67, 68] and type 2 [70, 71] diabetes, and contributes to elevated blood glucose by stimulating glycogenolysis, gluconeogenesis, and ketogenesis while suppressing glycogen synthesis. Moreover, suppression of glucagon action by *Gcgr* gene deletion, glucagon immunosuppression, or Gcgr antagonist can reduce hyperglycemia in models of both type 1 and type 2 diabetes. To investigate the effect of suppressing glucagon action in a model of insulinopenic diabetes, *GcgrWT* and *GcgrKO* mice were injected with the beta cell toxin STZ [74, 75]. *GcgrWT* mice become hyperglycemic, hyperketonemic, polyuric, and cachectic, while remarkably *GcgrKO* mice were protected from these diabetic symptoms [74, 75]. In addition, immunoneutralization of glucagon using a monoclonal antibody reduced hyperglycemia in alloxan-diabetic rabbits [76]. Moreover, in STZ-injected mice, weekly treatment with a Gcgr antibody completely normalized blood glucose levels for up to 12 weeks, concomitant with an improvement in HbA1c levels [77]. Similarly, immunoneutralization of endogenous glucagon improved oral glucose tolerance and reduced hepatic glucose output in obese leptin-deficient *ob/ob* mice [79], and Gcgr antisense oligonucleotides or siRNA diminished hyperglycemia and improved oral glucose tolerance in obese leptin receptor null *db/db* mice [59, 80]. Finally, genetic deletion of *Gcgr* in diet-induced obese mice or *db/db* mice prevented obesity, hyperinsulinemia, and hyperglycemia [81]. Therefore, inhibiting glucagon action can improve diabetic signs in various models of diabetes.

The hormone leptin, well known for its role in body weight regulation, has also shown promise as a glucose-lowering therapy. In rodent models of insulin-deficient diabetes, leptin monotherapy can potently reduce diabetic symptoms and normalize hyperglycemia [167, 177, 230, 232, 233, 235, 241, 252]. Interestingly, leptin can reduce circulating glucagon levels and levels of hepatic p-CREB indicative of reduced Gcgr signalling [230, 232, 233], which has

been thought to be important for the glucose-lowering mechanism of leptin. However, in type 2 diabetes, leptin monotherapy appears to be less efficacious as a glucose-lowering agent. Although leptin injections in a rat model of obese type 2 diabetes normalized fasting blood glucose [267], leptin treatment in humans with type 2 diabetes did not increase insulin-mediated stimulation of glucose disposal [268] or meaningfully reduce HbA1c [269]. The failure of leptin to improve type 2 diabetes may be due to leptin resistance as many obese individuals are hyperleptinemic [261].

In this Chapter we hypothesized that Gcgr siRNA delivered using LNP technology or leptin therapy will be an effective treatment in mouse models of type 1 and type 2 diabetes. LNPs are capable of effectively and safely delivering genetic drugs such as siRNA to target tissues, and are the most clinically advanced delivery systems for siRNA, with multiple LNP-siRNAs in clinical trials for the treatment of various diseases [333]. In addition, LNPs effectively target the liver, where glucagon exerts most of its biological functions. We find that Gcgr siRNA can potently improve glucose metabolism in both STZ (a model of type 1 diabetes) and HFD/STZ (a model of type 2 diabetes) diabetic mice. However, while leptin was able to improve both glucose and lipid metabolism in STZ-diabetic mice, no changes were observed in HFD/STZ-diabetic mice given leptin treatment. All data in this Chapter were submitted to *Molecular Metabolism* and we have been invited to perform minor revisions and resubmit our manuscript.

6.2 Results

Gcgr siRNA lowers blood glucose and improves oral glucose tolerance in wildtype mice

To identify an effective Gcgr siRNA, 1 of 3 Gcgr siRNAs, or FVII siRNA used as a control, were encapsulated into LNPs and delivered via the tail-vein to wildtype mice at a dose of 5 mg/kg and glucose homeostasis was assessed. A group of mice receiving PBS served as a control and were no different from mice that received FVII siRNA demonstrating that the particles themselves had no effect on the parameters measured (Figure 26). None of the Gcgr siRNAs affected bodyweight (Figure 26A). Strikingly, despite the mice already being healthy,

Gcgr siRNA 1 and 2 reduced blood glucose (8.8±0.4 mM PBS, 8.1±0.3 mM FVII siRNA, 5.1±0.2 mM Gcgr siRNA 1, 5.6±0.1 mM Gcgr siRNA 2, 8.1±0.4 mM Gcgr siRNA 3, day 7, P<0.05, Figure 26B) and improved oral glucose tolerance (Figure 26C&D). In addition, Gcgr siRNA 1 and 2 caused an elevation in circulating glucagon levels (21±6 pg/mL PBS, 14±3 pg/mL FVII siRNA, 91±2 pg/mL Gcgr siRNA 1, 140±30 pg/mL Gcgr siRNA 2, 36±13 pg/mL Gcgr siRNA 3, P<0.05, Figure 26E) without a corresponding increase in α-cell area (Figure 26F). Notably, our acute knockdown model exhibited a similar phenotype to mice with a lifelong, full-body knockout of glucagon receptors, which also display reduced blood glucose, improved glucose tolerance, and increased glucagon levels [44]. We chose either Gcgr siRNA 2 or a combination of Gcgr siRNA 1 and 2 for continuing our studies in diabetic models.

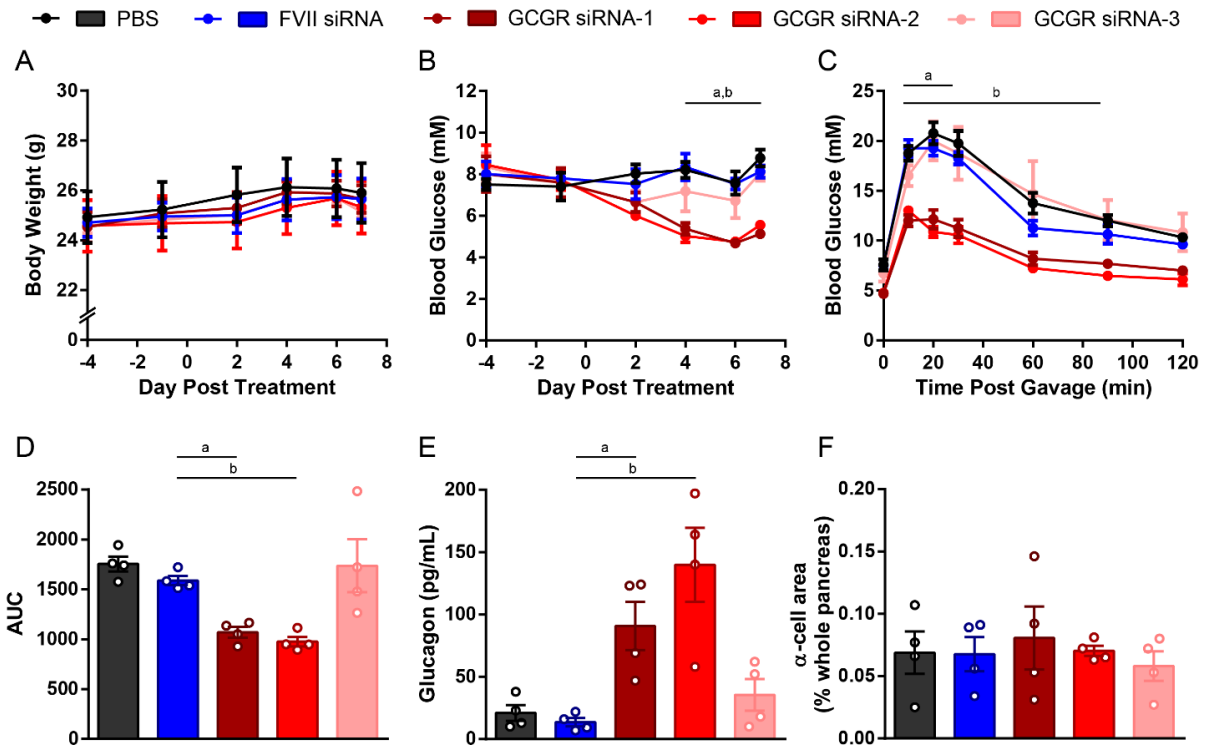

Figure 26. Glucagon receptor siRNA reduces blood glucose, improves oral glucose tolerance, and increases glucagon without affecting α-cell area in wildtype mice. C57BL/6J mice received Gcgr siRNA or FVII siRNA at a dose of 5 mg/kg or PBS on day 0. Four hour fasted body weight (A) and blood glucose (B) were measured throughout the study. On day 6 an oral glucose tolerance test was performed using 2 g/kg glucose (C) and area under the curve (AUC) was calculated (D). Plasma glucagon (E) and α-cell area (F) were analyzed on day 7. Each group was compared to the FVII siRNA treated group by a 1-way ANOVA or a repeated measures 2-way ANOVA with Dunnett post-hoc testing; a, P<0.05 Gcgr siRNA 1 vs FVII siRNA; b, P<0.05 Gcgr siRNA 2 vs FVII siRNA. Data are mean ± SEM, n=4.

5 mg/kg Gcgr siRNA does not improve glucose and lipid metabolism as effectively as leptin therapy in STZ-diabetic mice

Next, we sought to determine whether Gcgr siRNA could improve diabetic symptoms in a model of insulin-deficient diabetes and to compare this treatment to leptin, which is a potent glucagon suppressor and can normalize blood glucose levels in mouse models of insulin-deficient diabetes [167, 177, 230, 232, 233]. Mice were rendered diabetic by STZ injection and treated with Gcgr siRNA (5 mg/kg by tail-vein injection) and/or leptin therapy (20 μg/day by mini-osmotic pump), and FVII siRNA was used as a control resulting in the following groups: non-diabetic controls, STZ-FVII siRNA, STZ-Gcgr siRNA, STZ-leptin + FVII siRNA, STZ-leptin + Gcgr siRNA. The leptin + Gcgr siRNA group was nearly identical to the leptin + FVII treated group for all metabolic parameters measured (Figure 26). As expected, STZ-FVII mice lost weight compared to non-diabetic controls, and neither Gcgr siRNA nor leptin treatment affected body weight (Figure 27A). While blood glucose was completely normalized by leptin therapy, Gcgr siRNA only modestly reduced blood glucose at this dose (7.3±0.3 mM non-diabetic controls, 29.5±0.6 mM FVII siRNA, 9.9±0.7 mM leptin+FVII siRNA 1, 19.4±0.7 mM Gcgr siRNA, 7.8±1.1 mM leptin+Gcgr siRNA, day 7, $P<0.05$, Figure 27B). In addition, leptin normalized oral glucose tolerance while Gcgr siRNA caused only a partial improvement (Figure 27C&D). However, due to differences in basal blood glucose levels, when the AUC is calculated from baseline, the response to oral glucose was similar between all groups (data not shown). As expected based on Figure 26E, groups receiving Gcgr siRNA displayed supraphysiological levels of circulating glucagon (Figure 27E), and groups receiving leptin treatment exhibited supraphysiological levels of plasma leptin (Figure 27F).

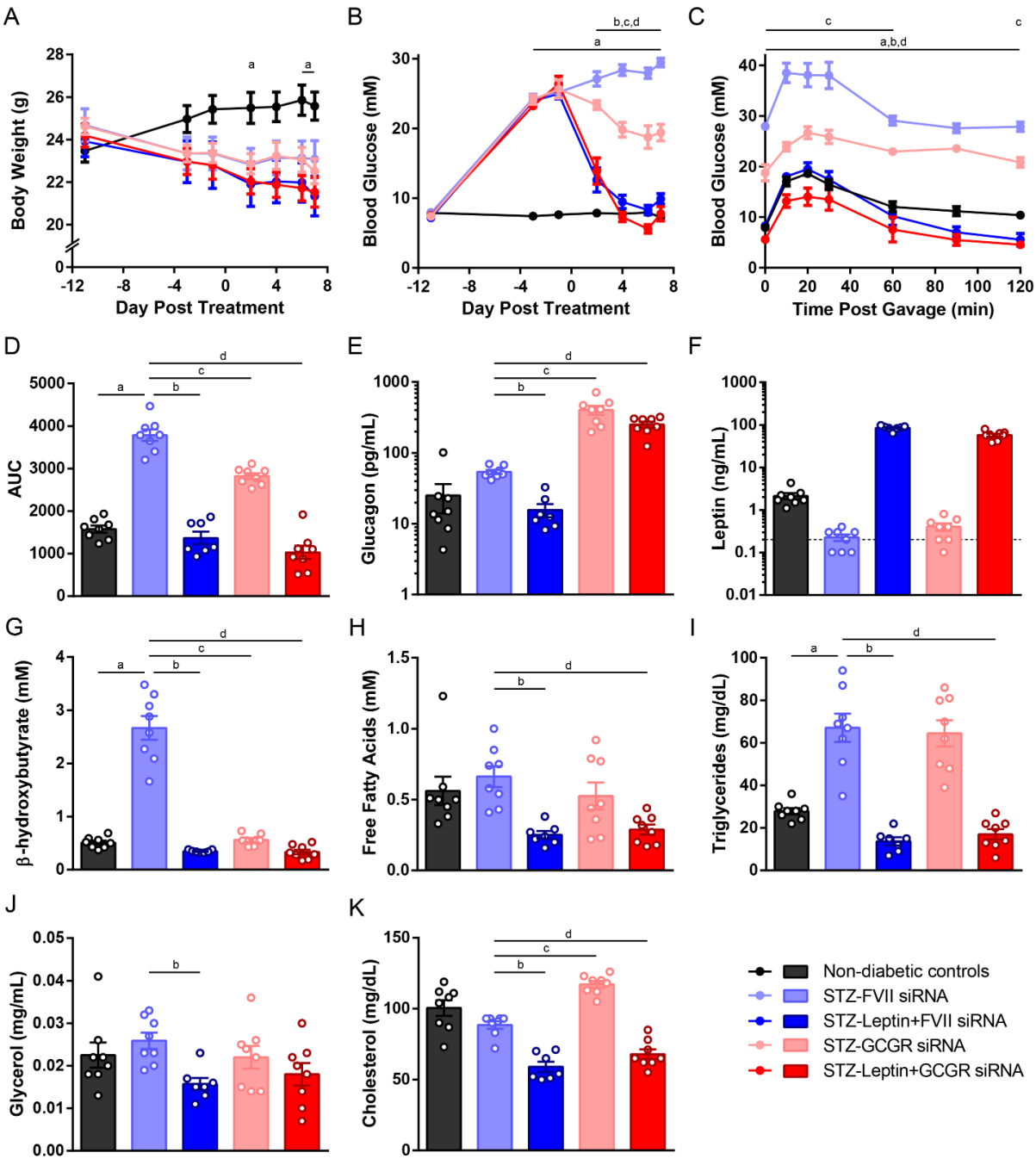
A
Body Weight (g)
Day Post Treatment
B
Blood Glucose (mM)
Day Post Treatment
C
Blood Glucose (mM)
Time Post Gavage (min)
D
AUC
E
Glucagon (pg/mL)
F
Leptin (ng/mL)
G
β-hydroxybutyrate (mM)
H
Free Fatty Acids (mM)
I
Triglycerides (mg/dL)
J
Glycerol (mg/mL)
K
Cholesterol (mg/dL)
Non-diabetic controls
STZ-FVII siRNA
STZ-Leptin+FVII siRNA
STZ-GCGR siRNA
STZ-Leptin+GCGR siRNA

Figure 27. Effect of leptin or 5 mg/kg Gcgr siRNA on glucose and lipid metabolism in STZ diabetic mice. Insulin-deficient diabetes was induced in C57BL/6J mice by injecting 180 mg/kg STZ on day -11; vehicle was administered to non-diabetic controls. STZ diabetic mice were treated with either FVII siRNA, Gcgr siRNA, FVII siRNA + leptin, or Gcgr siRNA + leptin on day 0. siRNA was delivered at a dose of 5 mg/kg via tail-vein injection and leptin was administered at a dose of 20 μg/day via mini-osmotic pump. Four hour fasted body weight (A) and blood glucose (B) were measured throughout the study. On day 6 an oral glucose tolerance test was performed using 1.5 g/kg glucose (C) and area under the curve (AUC) was calculated (D). Four hour fasted plasma glucagon (E), leptin (F), β-hydroxybutyrate (G), fatty acids (H), triglycerides (I), glycerol (J) and total cholesterol (K) were analyzed on day 7. The dotted line in (F) represents the limit of detection of the assay (0.2 ng/mL). Each group was compared to the FVII siRNA treated group by a 1-way ANOVA or a repeated measures 2-way ANOVA with Dunnett post-hoc testing; a, $P<0.05$ non-diabetic controls vs FVII siRNA; b, $P<0.05$ Leptin+FVII siRNA vs FVII siRNA; c, $P<0.05$ Gcgr siRNA vs FVII siRNA; d, $P<0.05$ Leptin+Gcgr siRNA vs FVII siRNA. Statistical analysis was not performed on leptin measurements (F) as some samples were below the limit of detection. Data are mean ± SEM, n=7-8.

To ascertain how both treatments would affect lipid metabolism, we measured circulating β-hydroxybutyrate, fatty acids, triglycerides, glycerol, and total cholesterol (Figure 27G-K). FVII siRNA treated mice with uncontrolled diabetes displayed elevated β-hydroxybutyrate, which was normalized by both leptin and Gcgr siRNA (0.51±0.04 mM non-diabetic controls, 2.67±0.22 mM FVII siRNA, 0.38±0.03 mM leptin+FVII siRNA, 0.56±0.04 mM Gcgr siRNA, 0.33±0.05 mM leptin+Gcgr siRNA, $P<0.05$, Figure 27G). Conversely, triglycerides that were raised in the FVII siRNA group were normalized by leptin but unchanged by Gcgr siRNA (Figure 27I). Interestingly, although plasma fatty acids, glycerol, and cholesterol were not elevated in the FVII siRNA group compared to non-diabetic controls, leptin was capable of reducing all of these parameters, while Gcgr siRNA had no effect on fatty acids or glycerol and modestly raised cholesterol levels (Figures 27H/J/K). Therefore, while leptin is capable of completely normalizing blood glucose, glucose tolerance, and lipid metabolism, this dose of Gcgr siRNA only partially improved blood glucose and glucose tolerance, fully normalized ketones, had no effect on triglycerides and increased cholesterol.

10 mg/kg Gcgr siRNA reduces blood glucose, increases plasma glucagon, normalizes β-hydroxybutyrate and increases cholesterol in STZ-diabetic mice

Since 5 mg/kg Gcgr siRNA only partially improved glucose homeostasis in STZ-diabetic mice, we investigated if increasing the dose may elicit a more potent effect. Similar to the previous study, compared to non-diabetic controls, STZ caused weight loss in FVII siRNA

treated mice, and this was unaffected by Gcgr siRNA (Figure 28A). While 5 mg/kg Gcgr siRNA had reduced blood glucose by 45% by day 7 (Figure 27B), 10 mg/kg reduced glucose by 76% by day 7, but was still unable to normalize blood glucose (Figure 28B). For the remainder of the plasma analytes measured, the results were similar to the 5 mg/kg dose of siRNA. Gcgr siRNA induced supraphysiological levels of circulating glucagon (Figure 28C). STZ injection depleted leptin levels in both FVII and Gcgr siRNA treated mice to near the detection limit of the assay (Figure 28D). β-hydroxybutyrate levels were increased in FVII siRNA controls and normalized by Gcgr siRNA treatment (Figure 28E). Triglycerides were elevated in FVII siRNA mice and unchanged due to Gcgr siRNA (Figure 28G). Fatty acid and glycerol levels were not elevated in FVII siRNA treated controls and remained unchanged due to Gcgr siRNA (Figure 28F&H). Finally, total cholesterol levels were elevated due to Gcgr siRNA compared to FVII siRNA (Figure 28I). Therefore, 10 mg/kg Gcgr siRNA can lower blood glucose and normalize ketones, but does not lower triglycerides and increased cholesterol.

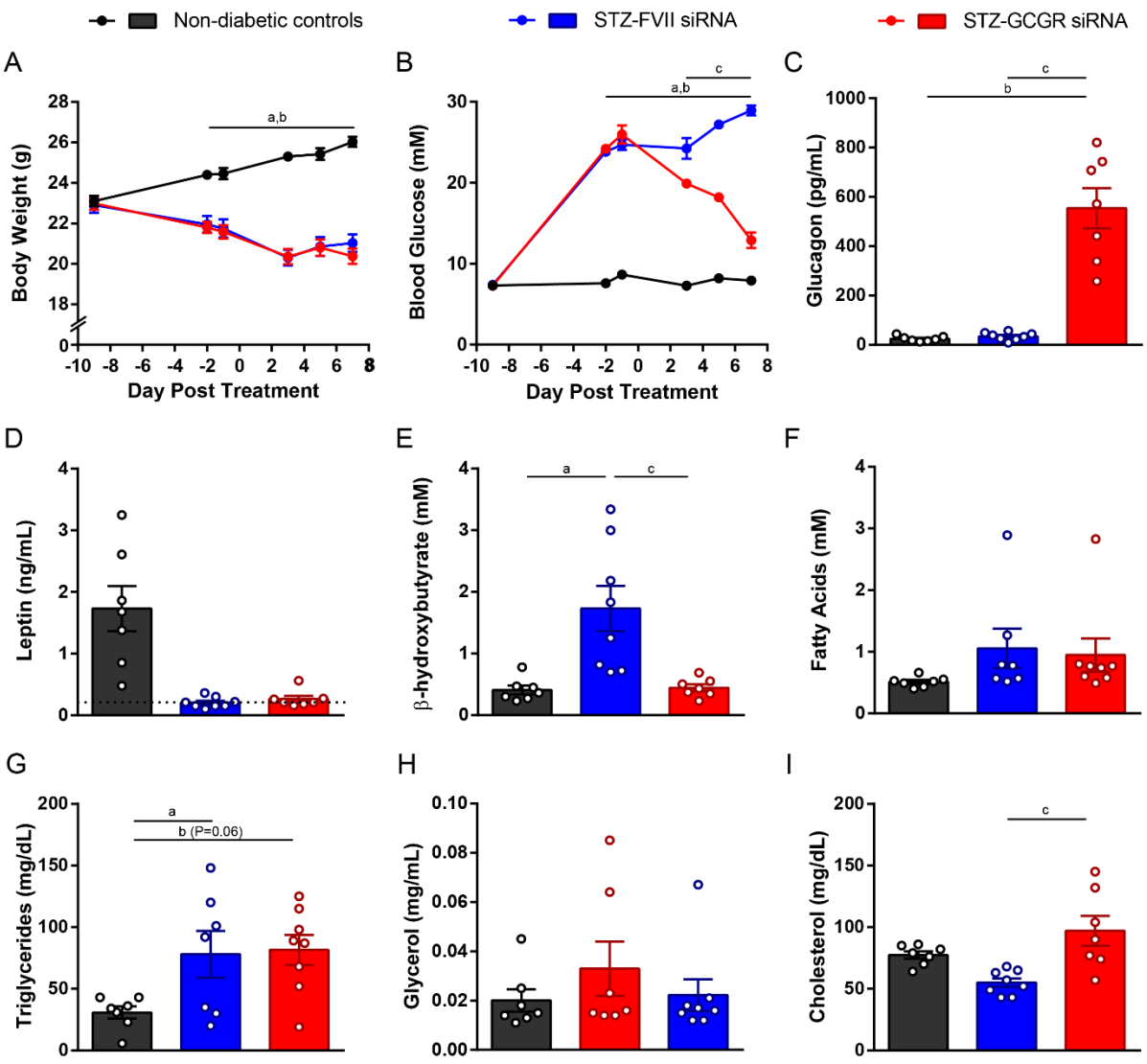

Figure 28. 10 mg/kg Gcgr siRNA reduces blood glucose, increases plasma glucagon, normalizes β-hydroxybutyrate and increases cholesterol in STZ diabetic mice. Insulin-deficient diabetes was induced in C57BL/6J mice by injecting 180 mg/kg STZ on day -8; vehicle was administered to non-diabetic controls. On day 0, STZ diabetic mice were treated with FVII siRNA or Gcgr siRNA at a dose of 10 mg/kg and compared to untreated non-diabetic controls. Four hour fasted body weight (A) and blood glucose (B) were measured throughout the study. Four hour fasted plasma glucagon (C), leptin (D), β-hydroxybutyrate (E), fatty acids (F), triglycerides (G), glycerol (H) and total cholesterol (I) were analyzed on day 7. Groups were compared using a 1-way ANOVA or a repeated measures 2-way ANOVA with Tukey post-hoc testing; a, $P<0.05$ non-diabetic controls vs FVII siRNA; b, $P<0.05$ non-diabetic controls vs Gcgr siRNA; c, $P<0.05$ FVII siRNA vs Gcgr siRNA. Statistical analysis was not performed on leptin measurements (D) as some samples were below the limit of detection, which is denoted by the dotted line. Data are mean ± SEM, n=7-8.

A single injection of 10 mg/kg Gcgr siRNA reduces blood glucose for 3 weeks and improves oral glucose tolerance in STZ-diabetic mice

Next, we assessed how long a single injection of Gcgr siRNA could improve diabetic symptoms in STZ-diabetic mice. Both STZ-injected groups lost weight compared to non-diabetic controls and Gcgr siRNA treated mice did not differ from FVII siRNA treated mice (Figure 29A). Gcgr siRNA significantly reduced blood glucose from day 4 - 29, reaching the lowest value by day 10 (8.2±0.4 mM non-diabetic controls, 23.2±1.6 mM STZ+FVII-siRNA, 10.6±0.4 mM STZ+Gcgr-siRNA, $P<0.05$, Figure 29B). Gcgr siRNA also improved oral glucose tolerance as measured by AUC (1340±60 non-diabetic controls, 3700±150 STZ+FVII-siRNA, 2150±170 STZ+Gcgr-siRNA, day 10, $P<0.05$, Figure 29C&D). Therefore a single injection of Gcgr siRNA was capable of ameliorating blood glucose for over 3 weeks in STZ-diabetic mice.

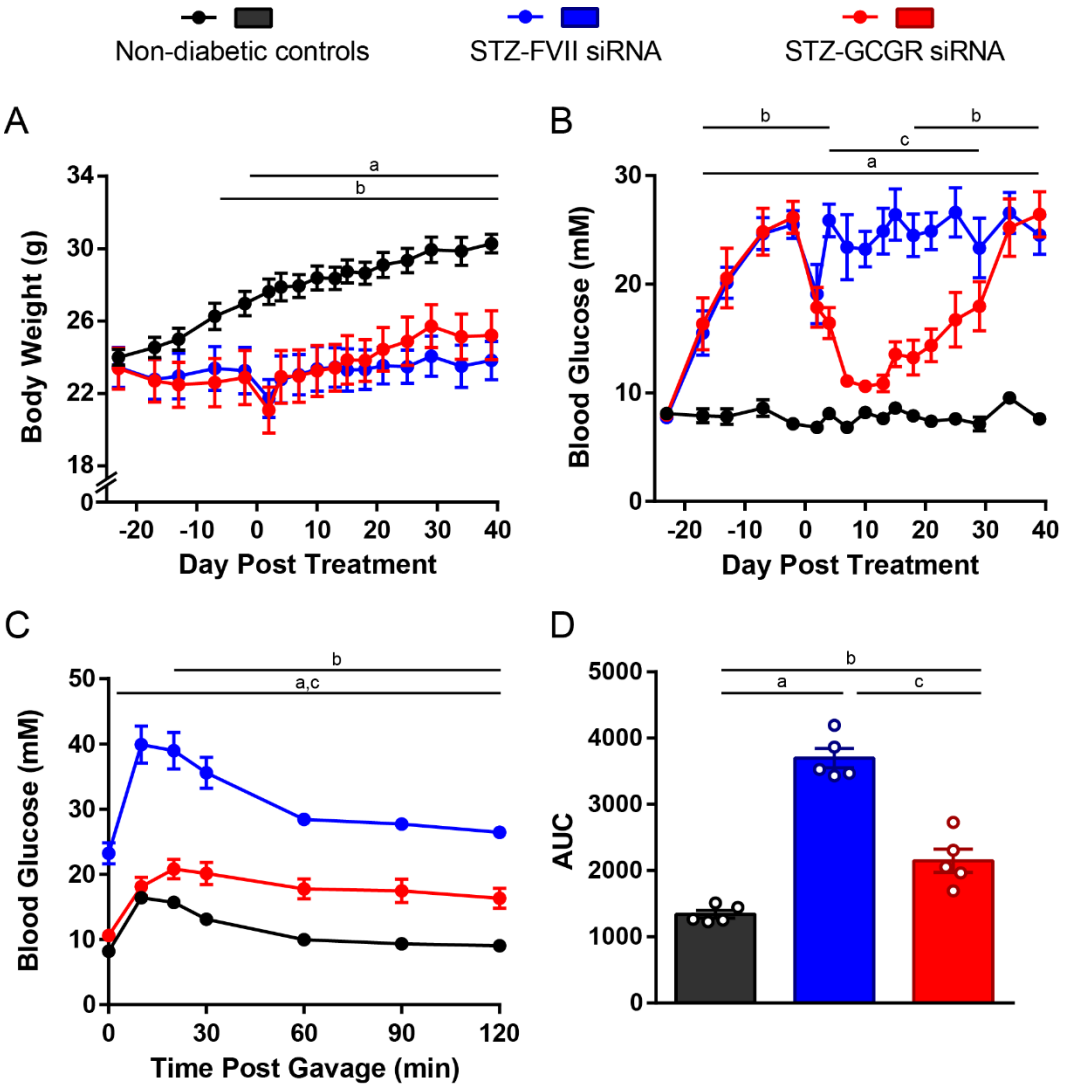

Figure 29. A single injection of 10 mg/kg Gcgr siRNA reduces blood glucose for 3 weeks and improves oral glucose tolerance in STZ diabetic mice. Insulin-deficient diabetes was induced in C57BL/6J mice by injecting 180 mg/kg STZ on day -22; vehicle was administered to non-diabetic controls. STZ diabetic mice were treated with FVII siRNA or Gcgr siRNA at a dose of 10 mg/kg on day 0 and compared to untreated non-diabetic controls. Four hour fasted body weight (A) and blood glucose (B) were measured throughout the study. On day 10 an oral glucose tolerance test was performed using 1.5 g/kg glucose (C) and area under the curve (AUC) was calculated (D). Groups were compared using a 1-way ANOVA or a repeated measures 2-way ANOVA with Tukey post-hoc testing; a, P<0.05 non-diabetic controls vs FVII siRNA; b, P<0.05 non-diabetic controls vs Gcgr siRNA; c, P<0.05 FVII siRNA vs Gcgr siRNA. Data are mean ± SEM, n=5.

Leptin therapy does not affect body weight, blood glucose, or oral glucose tolerance in HFD/STZ-diabetic mice

Since both Gcgr siRNA and leptin therapy were efficacious in a model of type 1 diabetes, we sought to test these treatments in HFD/STZ mice, a model of type 2 diabetes. HFD/STZ-diabetic mice were put on a 60% HFD at 6 weeks of age and given a moderate dose of 100 mg/kg STZ at 10 weeks of age. At 12 weeks of age, we tested leptin therapy by administering daily i.p. injections of PEGylated leptin or vehicle to HFD/STZ mice and compared results to 10% LFD controls. HFD mice were initially heavier than LFD controls, however following STZ injection, the body weights converged, and there was no effect of leptin vs vehicle (Figure 30A). Although blood glucose insulin-deficient tended to be lower in the leptin treated group, there was no significant difference between leptin and vehicle treatment (6.9±0.3 mM LFD, 19.3±1.9 mM HFD/STZ+vehicle, 17.1±2.5 mM HFD/STZ+leptin, day 14, $P>0.05$, Figure 30B). Similarly, although blood glucose values were lower in the leptin treated group than the vehicle treated group at all time points during the oral glucose tolerance test, there were no significant differences between the groups in the glucose response curves or the AUC (Figure 30C&D). This leptin injection regimen resulted in extremely high circulating leptin levels (2.9±0.7 ng/mL LFD, 7.5±1.5 ng/mL HFD/STZ+vehicle, 2809.6±259.6 ng/mL HFD/STZ+leptin, day 14, $P>0.05$, Figure 30E). Therefore, despite supraphysiological leptin levels, leptin did not significantly lower blood glucose levels or improve oral glucose tolerance in HFD/STZ-diabetic mice, contrary to the potent glucose-lowering effect of leptin in STZ diabetic mice (Figure 27).

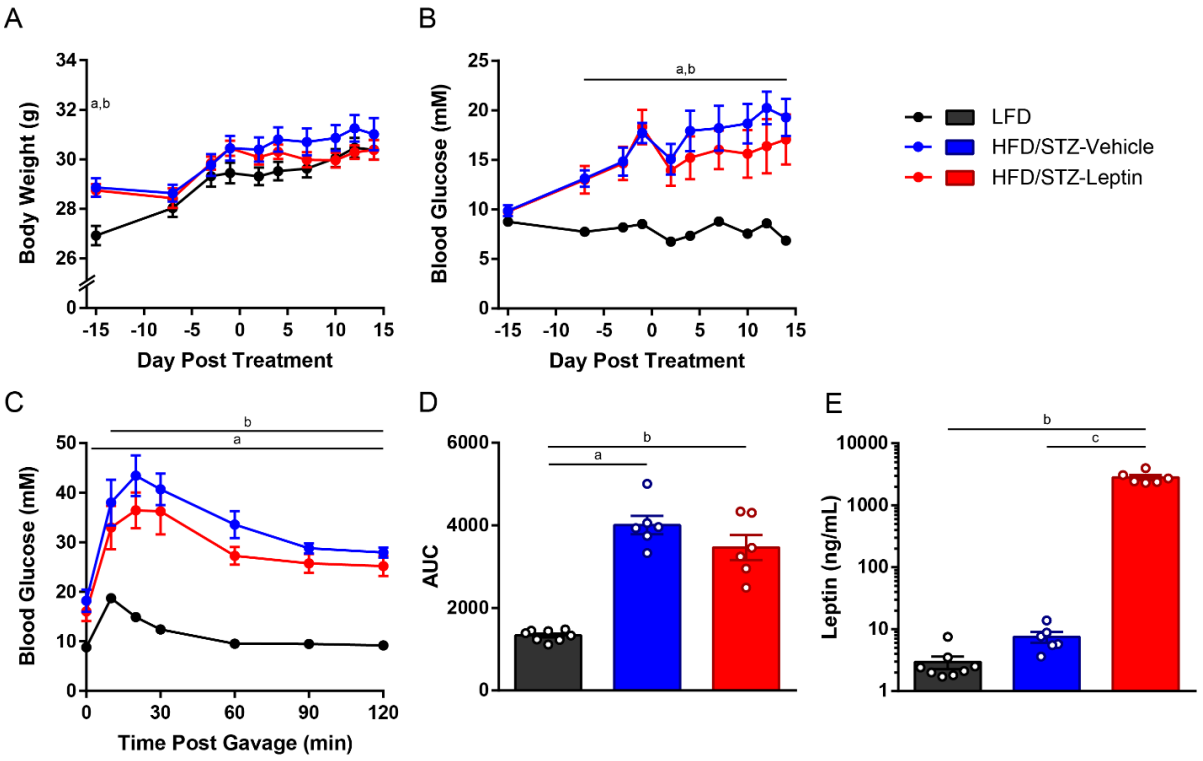

Figure 30. Leptin therapy does not affect body weight, blood glucose, or oral glucose tolerance in HFD/STZ diabetic mice. C57BL/6J mice on a HFD were injected with 100 mg/kg STZ on day -14. Starting on day 0, HFD/STZ diabetic mice were intraperitoneally injected daily with PEG-leptin at a dose of 20 µg/day or vehicle and compared to untreated LFD fed mice. Four hour fasted body weight (A) and blood glucose (B) were measured throughout the study. On day 7 an oral glucose tolerance test was performed (C) and area under the curve (AUC) was calculated (D). Plasma leptin was measured on day 14 following a 4 hour fast (E). Groups were compared using a 1-way ANOVA or a repeated measures 2-way ANOVA with Tukey post-hoc testing; a, P<0.05 LFD vs vehicle; b, P<0.05 LFD vs leptin; c, P<0.05 vehicle vs leptin. Data are mean ± SEM, n=6-8.

10 mg/kg Gcgr siRNA reduces blood glucose and improves oral glucose tolerance in HFD/STZ-diabetic mice

Next we injected Gcgr siRNA or FVII siRNA at a dose of 10 mg/kg into HFD/STZ to determine if the glucose reducing effect would be as potent as it was in STZ-diabetic mice. The body weights of HFD/STZ-diabetic mice that received Gcgr siRNA were no different than those that received FVII siRNA (Figure 31A). Remarkably, blood glucose was normalized to that of LFD controls due to Gcgr siRNA treatment (8.7±0.4 mM LFD, 19.5±1.8 mM HFD/STZ+FVII siRNA, 7.4±0.4 mM HFD/STZ+Gcgr siRNA, day 14, P<0.05, Figure 31B). Moreover, oral glucose tolerance was greatly improved due to Gcgr siRNA treatment as

revealed by blood glucose values throughout the test and AUC measurements (1600±100 LFD, 4200±100 HFD/STZ+FVII siRNA, 2400±200 HFD/STZ+Gcgr siRNA, day 14, $P<0.05$, Figure 31C&D). Next, we measured various plasma analytes to better understand how Gcgr siRNA affected metabolic processes in this model. Similar to our previous studies, Gcgr siRNA induced supraphysiological levels of circulating glucagon (Figure 31E). Plasma leptin, β-hydroxybutyrate, fatty acids, glycerol, and cholesterol were unchanged due to HFD/STZ induced diabetes and there were no differences between FVII and Gcgr siRNA treated groups (Figure 31F/G/H/J/K). Lastly, plasma triglycerides were increased in the FVII siRNA group and remained unchanged due to Gcgr siRNA treatment (Figure 31I). Therefore, not only can Gcgr siRNA improve glucose metabolism in the STZ model of severe insulin deficiency, it can also potently lower blood glucose and improve oral glucose tolerance in the HFD/STZ model of type 2 diabetes, but does not improve plasma triglycerides.

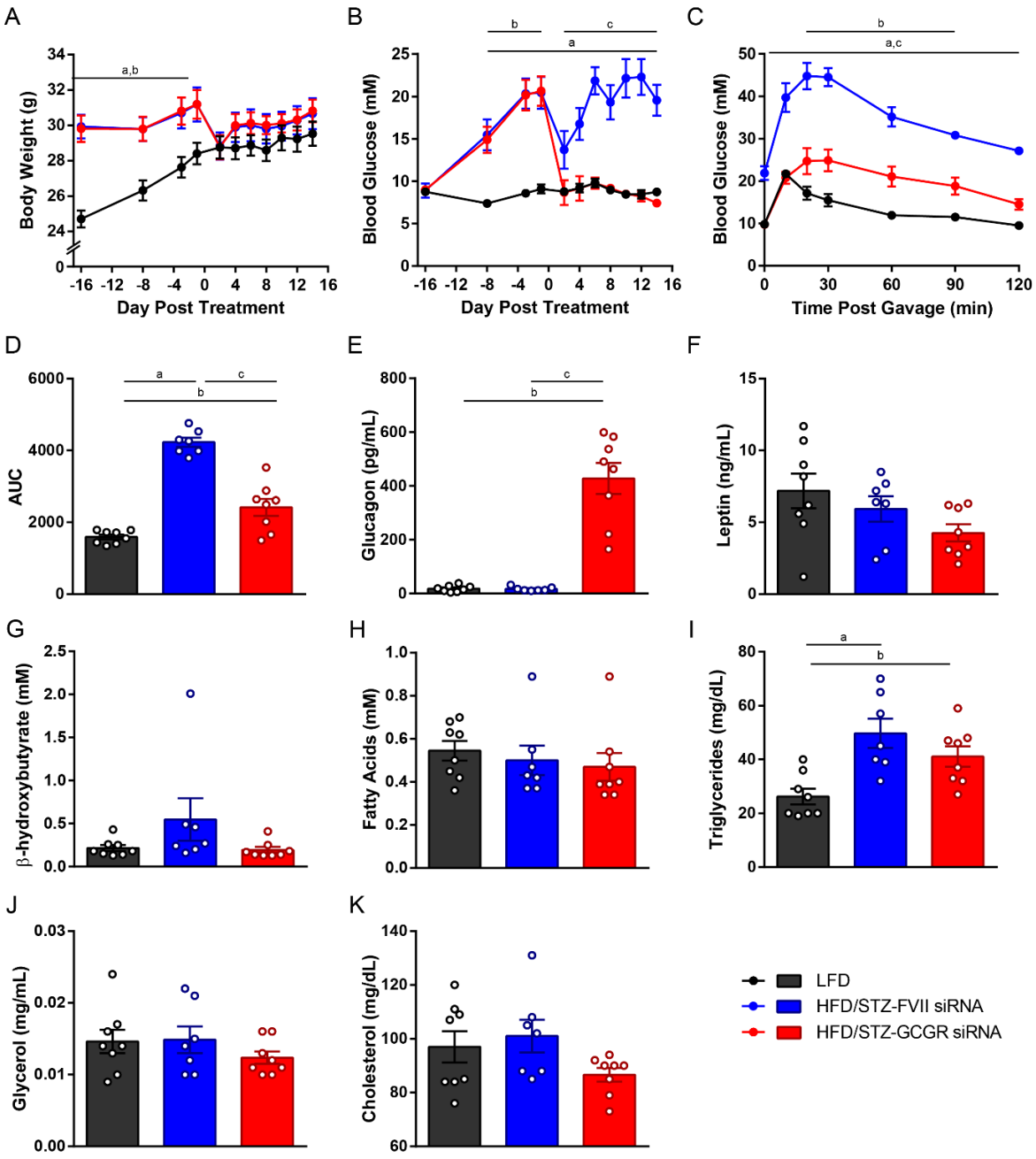

Figure 31. 10 mg/kg Gcgr siRNA reduces blood glucose and improves oral glucose tolerance in HFD/STZ diabetic mice. C57BL/6J mice on a HFD were injected with 100 mg/kg STZ on day -15. HFD/STZ diabetic mice were treated with FVII siRNA or Gcgr siRNA at a dose of 10 mg/kg on day 0 and compared to untreated LFD fed mice. Four hour fasted body weight (A) and blood glucose (B) were measured throughout the study. On day 6 an oral glucose tolerance test was performed using 1.5 g/kg glucose (C) and area under the curve (AUC) was calculated (D). Four hour fasted plasma glucagon (E), leptin (F), β-hydroxybutyrate (G), fatty acids (H), triglycerides (I), glycerol (J) and total cholesterol (K) were analyzed on day 14. Groups were compared using a 1-way ANOVA or a repeated measures 2-way ANOVA with Tukey post-hoc testing; a, $P<0.05$ LFD vs FVII siRNA; b, $P<0.05$ LFD vs Gcgr siRNA; c, $P<0.05$ FVII siRNA vs Gcgr siRNA. Data are mean ± SEM, n=7-8.

A single injection of 10 mg/kg Gcgr siRNA reduces blood glucose for 2 months and improves oral glucose tolerance in HFD/STZ-diabetic mice

Because Gcgr siRNA can robustly normalize diabetic symptoms in HFD/STZ mice we aimed to determine how long a single injection could lower blood glucose in this model. Similar to our previous studies there were no differences in body weight between Gcgr siRNA and FVII siRNA treated mice (Figure 32A). Strikingly, blood glucose in the Gcgr siRNA treated group were indistinguishable from LFD controls for approximately 1 month (8.6±0.5 mM LFD, 16.8±2.4 mM HFD/STZ+FVII siRNA, 8.2±0.9 mM HFD/STZ+Gcgr siRNA, day 30, $P<0.05$) and remained significantly reduced compared to FVII siRNA controls until day 71 (Figure 32B). Due to the long-term amelioration in blood glucose, we measured HbA1c on day 24 and 48 and found that levels were reduced in mice that received Gcgr siRNA (Figure 32C&D). On day 6 mice receiving Gcgr siRNA had normalized oral glucose tolerance (Figure 32E&F), and this effect waned by day 43 (Figure 32G&H). Therefore, a single injection of Gcgr siRNA can reduce blood glucose for 2 months, improve oral glucose tolerance, and reduce HbA1c levels in HFD/STZ mice.

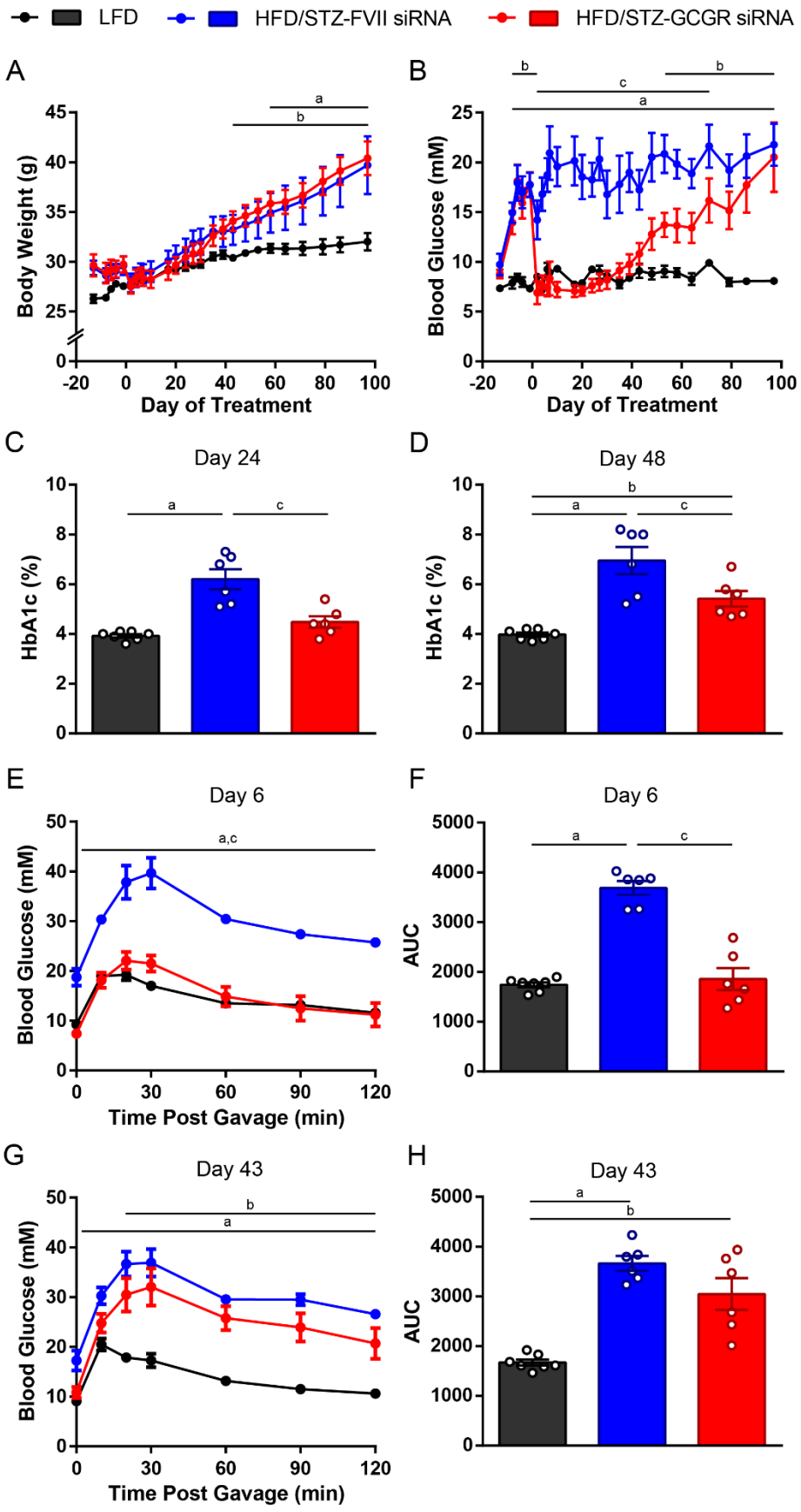
LFD
HFD/STZ-FVII siRNA
HFD/STZ-GCGR siRNA
A
Body Weight (g)
Day of Treatment
B
Blood Glucose (mM)
Day of Treatment
C
Day 24
HbA1c (%)
D
Day 48
HbA1c (%)
E
Day 6
Blood Glucose (mM)
Time Post Gavage (min)
F
Day 6
AUC
G
Day 43
Blood Glucose (mM)
Time Post Gavage (min)
H
Day 43
AUC

Figure 32. A single injection of 10 mg/kg Gcgr siRNA reduces blood glucose for 2 months and improves oral glucose tolerance in HFD/STZ diabetic mice. C57BL/6J mice on a HFD were injected with 100 mg/kg STZ on day -13. HFD/STZ diabetic mice were treated with FVII siRNA or Gcgr siRNA at a dose of 10 mg/kg on day 0 and compared to untreated LFD fed mice. Four hour fasted body weight (A) and blood glucose (B) were measured throughout the study. HbA1c was analyzed on day 24 (C) and 48 (D). On day 6 an oral glucose tolerance test was performed using 1.5 g/kg glucose (E) and area under the curve (AUC) was calculated (F). On day 43 an oral glucose tolerance test was performed using 1.5 g/kg glucose (G) and area under the curve (AUC) was calculated (H). Groups were compared using a 1-way ANOVA or a repeated measures 2-way ANOVA with Tukey post-hoc testing; a, $P<0.05$ LFD vs FVII siRNA; b, $P<0.05$ LFD vs Gcgr siRNA; c, $P<0.05$ FVII siRNA vs Gcgr siRNA. Data are mean ± SEM, n=6-7.

6.3 Discussion

We report that administration of Gcgr siRNA encapsulated in LNPs can dramatically improve glucose metabolism in wildtype mice, as well as STZ and HFD/STZ-diabetic mice, models of type 1 and type 2 diabetes [334, 335], respectively. The phenotype in our acute knockdown model closely resembles that of *GcgrKO* mice suggesting highly efficient knockdown of the Gcgr. *GcgrKO* mice exhibited a ~30% reduction in blood glucose levels and AUC of an oral glucose tolerance test compared to *GcgrWT* mice [44], while mice receiving Gcgr siRNA exhibited a reduction of ~40% of both of these parameters compared to FVII siRNA controls. While *GcgrKO* mice had an excess of 250 fold higher circulating glucagon levels concomitant with extreme α-cell hyperplasia [44], a ~5 fold increase in plasma glucagon with no alteration in α-cell area was observed on day 7 of our study. This may reflect the acute nature of our knockdown model, and suggest that the rise in circulating glucagon in our study may be due to increased glucagon transcription, translation, and/or secretion whereas increased α-cell mass likely contributes to hyperglucagonemia in *GcgrKO* mice. To directly assess efficiency of knockdown hepatic *Gcgr* mRNA, Gcgr protein, or p-CREB/CREB (a measure of glucagon receptor signalling) levels could be quantified.

Following characterization of our knockdown model in wildtype mice, we found that Gcgr siRNA diminished blood glucose and improved oral glucose tolerance in insulin-deficient STZ-diabetic mice. Increasing the dose of Gcgr siRNA from 5 to 10 mg/kg further improved glucose homeostasis, and a single injection reduced blood glucose for 3 weeks. These results,

along with other reports of hyperglycemia normalization or prevention using *GcgrKO* or glucagon/Gcgr antagonizing antibodies in rodent models of insulin-deficient diabetes, have challenged the insulinocentric dogma of diabetes by suggesting that glucagon excess rather than insulin deficiency is critical for the development of diabetes. However, recent reports demonstrate that as insulin deficiency becomes more severe, glucagon blockage is less efficacious. Steenberg *et al.* suppressed glucagon action through α-cell ablation by giving DT to mice expressing DTR driven by the glucagon promoter, glucagon immunoneutralization, or Gcgr antagonism, and found that STZ injection was still capable of inducing hyperglycemia and impaired glucose tolerance [336]. In addition, Damond *et al.* generated mouse models with more severe insulin deficiency by using a combining STZ injection with insulin receptor antagonism, as well as β-cell ablation by giving DT to mice expressing DTR driven by the insulin promoter [337]. When these mice were put on a *GcgrKO* background or treated with a Gcgr monoclonal antibody, hyperglycemia still developed [337]. Finally, in *InsKO* mice completely devoid of insulin, *Gcgr* gene deletion did not prevent the development of diabetes (data from Chapter 7). Taken together, these studies suggest that sufficient levels of basal insulin may be required for glucagon suppression to be therapeutic for insulin-deficient diabetes.

In addition to lowering blood glucose, Gcgr siRNA also potently suppressed ketones, but did not affect lipid metabolism in STZ-diabetic mice. Strikingly, the effect on ketosis may be even more potent than the effect on blood glucose, since the lower dose of siRNA, which only modestly reduced blood glucose, was capable of restoring ketones to control levels. This phenomenon has been previously observed when STZ-diabetic mice were given a GLP-1 receptor agonist or glucagon-neutralizing antibody that reversed hyperglucagonemia and blood glucose levels were unchanged while ketones were lowered [338]. However, at both doses of Gcgr siRNA, plasma triglycerides, plasma fatty acids and glycerol were unchanged, and plasma cholesterol was increased. Interestingly, clinical trials in patients with type 2 diabetes receiving Gcgr antagonists [83, 85] as well as a study involving *db/db* mice receiving Gcgr siRNA [80] have reported increased low-density lipoprotein cholesterol (LDL-C). We only observed an increase in total plasma cholesterol due to Gcgr siRNA in STZ-diabetic mice and

not HFD/STZ-diabetic mice, but did not measure the individual lipoprotein components. In contrast to Gcgr siRNA treatment, leptin therapy suppressed plasma ketones, fatty acids, triglycerides, glycerol, and cholesterol, consistent with previous studies [167, 231-233, 235, 241]. Therefore, this suggests that glucagon reduction by leptin may contribute to improving hyperglycemia and ketosis but not the suppression of lipid metabolism.

After observing the glucose-lowering effect of Gcgr siRNA in STZ diabetes, we sought to determine whether this effect could be extended to a model of type 2 diabetes, the HFD/STZ mouse. Strikingly, a single injection of Gcgr siRNA reduced blood glucose levels for approximately 2 months, and improved HbA1c levels in this model. In addition, in contrast to the beneficial effects of leptin monotherapy in STZ-diabetic mice, leptin treatment in HFD/STZ mice did not improve diabetic symptoms. Despite immensely high leptin levels, there was no improvement in blood glucose levels and oral glucose tolerance. Other studies have reported that leptin therapy in HFD/STZ mice caused a partial improvement in blood glucose and glucose tolerance [339, 340]. Discrepancies between these studies and ours may be due to differences in the timing of STZ relative to HFD treatment, the dose of STZ, the % of fat in the diet, or method of leptin delivery [339, 340]. Nonetheless, a dose of leptin that would have normalized hyperglycemia in STZ diabetic mice does not normalize diabetic symptoms in HFD/STZ diabetic mice, possibly due to leptin resistance. Similar to obese humans with type 2 diabetes, HFD/STZ mice exhibited 2.5 fold elevated leptin levels compared to LFD controls, which may suggest leptin resistance. Several pre-clinical studies have identified strategies to enhance endogenous leptin sensitivity [270-274, 339], which may help overcome leptin resistance and unleash the body weight and blood glucose lowering effects of leptin.

Liver specific suppression of glucagon action may be more beneficial than whole body inhibition in treating metabolic disease. Liver specific *GcgrKO* mice exhibit improvements in fasting blood glucose and glucose tolerance to the same degree as that which is seen in global *GcgrKO* mice [44, 45] highlighting the importance of glucagon action on the liver and glucose homeostasis. Interestingly, outside the liver, glucagon can exert beneficial effects in the brain

by inhibiting glucose production, improving glucose tolerance [50], promoting satiety and increasing energy expenditure [51], in the muscle by promoting glucose uptake, and in WAT by increasing lipolysis [41]. Therefore, administration of Gcgr siRNA, which largely targets the liver and increases circulating glucagon levels, could elicit added benefits of extra-hepatic glucagon signalling compared to a Gcgr antagonist targeting glucagon receptors throughout the body.

Together our results indicate that Gcgr siRNA encapsulated in LNPs is an effective therapy in mouse models of type 1 and type 2 diabetes. Utilization of small molecule Gcgr antagonists for the treatment of glycemic control has been of interest and is currently in phase 2 clinical trials for patients with type 2 diabetes (clinicaltrials.gov, NCT02851849). In addition, LNP delivery of siRNA is currently in clinical trials for the treatment of various liver-related diseases. Therefore, Gcgr siRNA delivery via LNPs may hold therapeutic potential for the treatment of diabetes in humans.

CHAPTER 7 – EFFECT OF GLUCAGON RECEPTOR GENE DELETION IN INSULIN KNOCKOUT MICE

7.1 Introduction

Hyperglucagonemia is present in many forms of diabetes [71, 341], and contributes to elevated blood glucose by promoting glycogenolysis, gluconeogenesis, and ketogenesis while inhibiting glycogen synthesis. Remarkably, mice with whole body *Gcgr* gene deletion (*GcgrKO*) are protected from STZ-induced diabetes [74, 75]. While *GcgrWT* mice developed severe hyperglycemia, hyperketonemia, and cachexia and had to be euthanized 6 weeks post-STZ injection, *GcgrKO* mice remained healthy [74, 75]. Moreover, as detailed in section 6.1, other studies have shown that glucagon suppression can improve glucose homeostasis in healthy and diabetic models [44, 76, 77, 342]. These studies support the idea that elevated glucagon action is required for hyperglycemia in insulin-deficient diabetes. However, similar to the rationale described in section 5.1, we believe that the models that have been used to test glucagon suppression therapy in insulin-deficient rodents are not 100% devoid of insulin. Therefore, in this Chapter we aimed to determine whether loss of glucagon action improves glucose metabolism and promotes survival in *InsKO* mice and hypothesized that glucagon receptor gene deletion will not be able to overcome the complete absence of insulin. To investigate this we characterized mice with *Gcgr*, *Ins1* and *Ins2* gene deletions. All data in this Chapter were published in *Molecular Metabolism* [343].

7.2 Results

Gcgr gene deletion does not promote survival in InsKO pups

First, we aimed to determine whether *Gcgr* gene deletion would extend survival of *InsKO* pups. All male and female pups compared had 0 *Ins1* alleles, 0-1 *Gcgr* alleles and 0-2 *Ins2* alleles. *GcgrHet/InsWT*, *GcgrKO/InsWT*, *GcgrHet/InsHet*, and *GcgrKO/InsHet* pups were used as healthy controls, while *GcgrHet/InsKO* and *GcgrKO/InsKO* pups tested the effect of

Gcgr gene deletion on *InsKO* pups. All controls survived and gained weight throughout the first week of life (demonstrating that *Gcgr* gene deletion with the *InsWT* or *Het* genotype did not affect these parameters), while both *GcgrHet/InsKO* and *GcgrKO/InsKO* failed to gain mass and died at a similar rate, surviving no longer than 6 days (Figure 33A&B). Therefore, *Gcgr* gene deletion does not promote survival of *InsKO* pups.

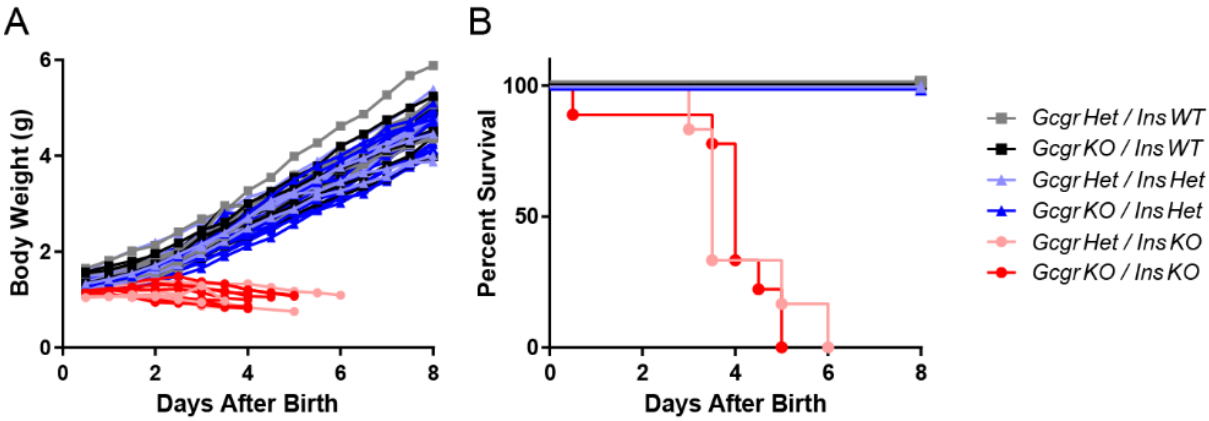

Figure 33. *Gcgr* gene deletion does not promote survival in *InsKO* pups. *Ins1KO* pups with 0-1 alleles of *Gcgr* and 0-2 alleles of *Ins2* were tracked twice daily for body weight (A) and survival (B) for 8 days after birth. For A statistical analysis was not performed, for B no statistical differences were observed between *GcgrHet/InsKO* and *GcgrKO/InsKO* when compared using the Kaplan-Meier method with the log rank test. Data are graphed as individual pups (A) and % survival (B), n=6-17.

Gcgr gene deletion modestly improves body weight, blood glucose and plasma ketones but does not normalize plasma leptin, triglycerides, fatty acids or hepatic cholesterol accumulation in InsKO pups

We assessed if loss of glucagon action in *InsKO* pups improved metabolism by analyzing metabolic parameters associated with glucose metabolism in P1 pups. Results were variable, likely a reflection of litter size, when the animals last fed, and the time of day the samples were collected. *Gcgr* gene deletion with the *InsWT* or *Het* genotype did not affect any of the parameters measured. At P1 *GcgrHet/InsKO* pups weighed 30% less than controls, which was marginally increased in *GcgrKO/InsKO* pups but still 20% less than the control groups (Figure 34A). As expected, *GcgrHet/InsKO* pups were hyperglycemic compared to controls. While still hyperglycemic, this was modestly decreased by 24% in *GcgrKO/InsKO* pups (Figure 34B) relative to *GcgrHet/InsKO* pups. Similarly, plasma β-hydroxybutyrate levels were 9-fold

higher in *GcgrHet/InsKO* pups compared to controls and reduced in *GcgrKO/InsKO* pups to 4-fold that of controls (Figure 34C). It has been published that adult *GcgrKO* mice exhibit elevated glucagon levels [44, 75]. At P1, glucagon levels tended to be elevated in the *GcgrKO/InsWT* pups and were significantly higher than controls in *GcgrKO/InsKO* pups (Figure 34D). Therefore, *Gcgr* gene deletion modestly improved hyperglycemia and hyperketonemia in *InsKO* pups.

Since insulin-deficient diabetes is associated with aberrant lipid metabolism, we characterized lipid homeostasis in P1 pups. As expected, due to the positive effect of insulin on adipogenesis and leptin production [344], *GcgrHet/InsKO* pups had undetectable plasma leptin levels that were unchanged due to *Gcgr* gene deletion (Figure 34E). Plasma triglycerides and free fatty acids were increased in *GcgrHet/InsKO* pups relative to controls and *Gcgr* gene deletion did not alter this (Figure 34F&G). Plasma glycerol and cholesterol in *GcgrHet/InsKO* pups were similar to controls and unchanged due to the loss of the *Gcgr* (Figure 34H&I). Finally, hepatic cholesterol accumulation was unaffected by *Gcgr* gene deletion, and hepatic triglyceride levels in *InsKO* pups were comparable to that of controls (Figure 34J&K). Therefore, *Gcgr* gene deletion does not normalize lipid metabolism in *InsKO* pups.

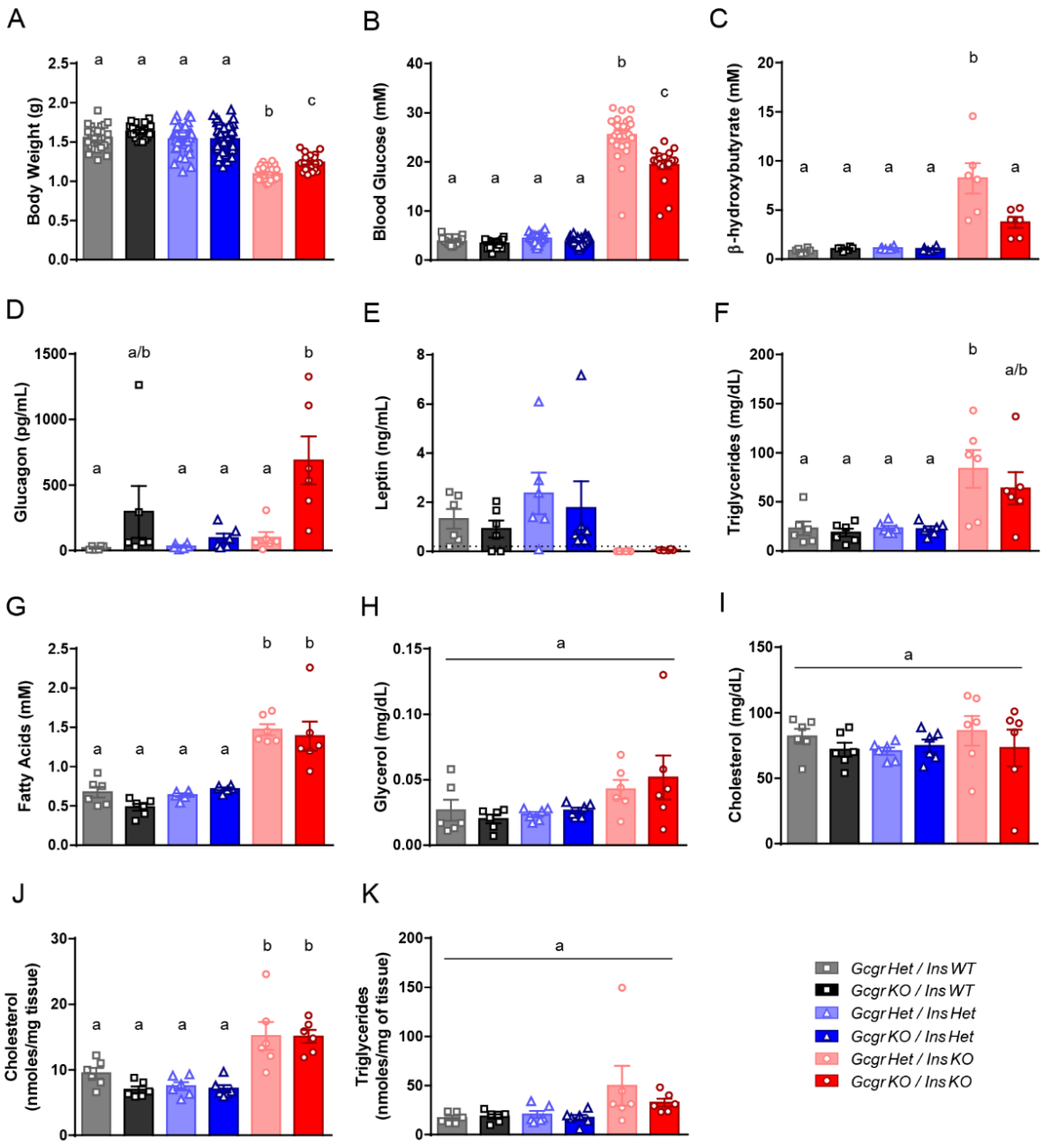

Figure 34. *Gcgr* gene deletion modestly improves body weight, blood glucose and plasma ketones but does not normalize plasma leptin, triglycerides, fatty acids or hepatic cholesterol accumulation in *InsKO* pups. On P1in the random fed state, *Ins1KO* pups with 0-1 alleles of *Gcgr* and 0-2 alleles of *Ins2* were harvested to measure body weight (A), trunk blood was collected to measure blood glucose (B) and plasma β-hydroxybutyrate (C), glucagon (D), leptin (E), triglycerides (F), fatty acids (G), glycerol (H), and total cholesterol (I), and liver was collected to quantify hepatic total cholesterol (J) and triglycerides (K). All groups were compared by 1-way ANOVA with Tukey post-hoc testing and different superscripts (a, b, c) are significantly different from each other within each graph. Statistical analysis was not performed on leptin measurements (D) as some samples were below the limit of detection, which is denoted by the dotted line. Data are mean±SEM, n=19-59 for A&B, n=6 for C-K.

Gcgr gene deletion modestly lowers blood glucose but does not promote survival in adult InsKO mice

We have determined that *GcgrKO/InsKO* pups do not have extended survival; however because insulin is involved in growth and development [345], we sought to determine the effect of glucagon suppression in adult *InsKO* mice. As previously published and described in Chapter 5, male and female *InsKO* mice were maintained to adulthood using insulin injections and islet transplantation into the eye [252]. *GcgrKO/InsKO* mice failed to grow rapidly following weaning despite post-transplant euglycemia (Figure 35A&B). Despite receiving the same number of islets, *GcgrKO/InsKO* mice had normal blood glucose levels compared to hyperglycemic *GcgrHet/InsKO* mice suggesting that the loss of *Gcgr* aids in improving hyperglycemia when insulin is present. On day 0 of the study the eye containing the exogenous islets was enucleated to render the *InsKO* mice insulin-deficient; *InsHet* mice underwent enucleation to control for the effect of surgery. Following enucleation all *InsKO* mice rapidly lost weight, were hunched, displayed piloerection, were lethargic or non-responsive and reached humane endpoint at a similar rate, by day 6 of the study (Figure 35C). As graphed in Figure 35D-F, the kinetics of this weight loss were similar between *GcgrHet/InsKO* and *GcgrKO/InsKO* mice. Therefore, in young adult mice, *Gcgr* gene deletion was unable to prevent body weight loss and death associated with a complete loss of insulin.

We next sought to determine whether *Gcgr* gene deletion improved blood glucose levels in young adult *InsKO* mice. *InsKO* mice rapidly became hyperglycemic upon removal of the graft. At day 0.75, 1.25 and 1.75 blood glucose of *GcgrKO/InsKO* mice was modestly lower than that of *GcgrHet/InsKO* mice (Figure 35G-K). Interestingly, at humane endpoint, 3 of the *GcgrKO/InsKO* mice were hypoglycemic (<1.3 mM), which was not observed in the *GcgrHet/InsKO* mice (Figure 35K and data not shown). In conclusion, *Gcgr* gene deletion modestly reduced blood glucose throughout the study and even resulted in hypoglycemia in some mice, but did not permit survival in young adult *InsKO* mice.

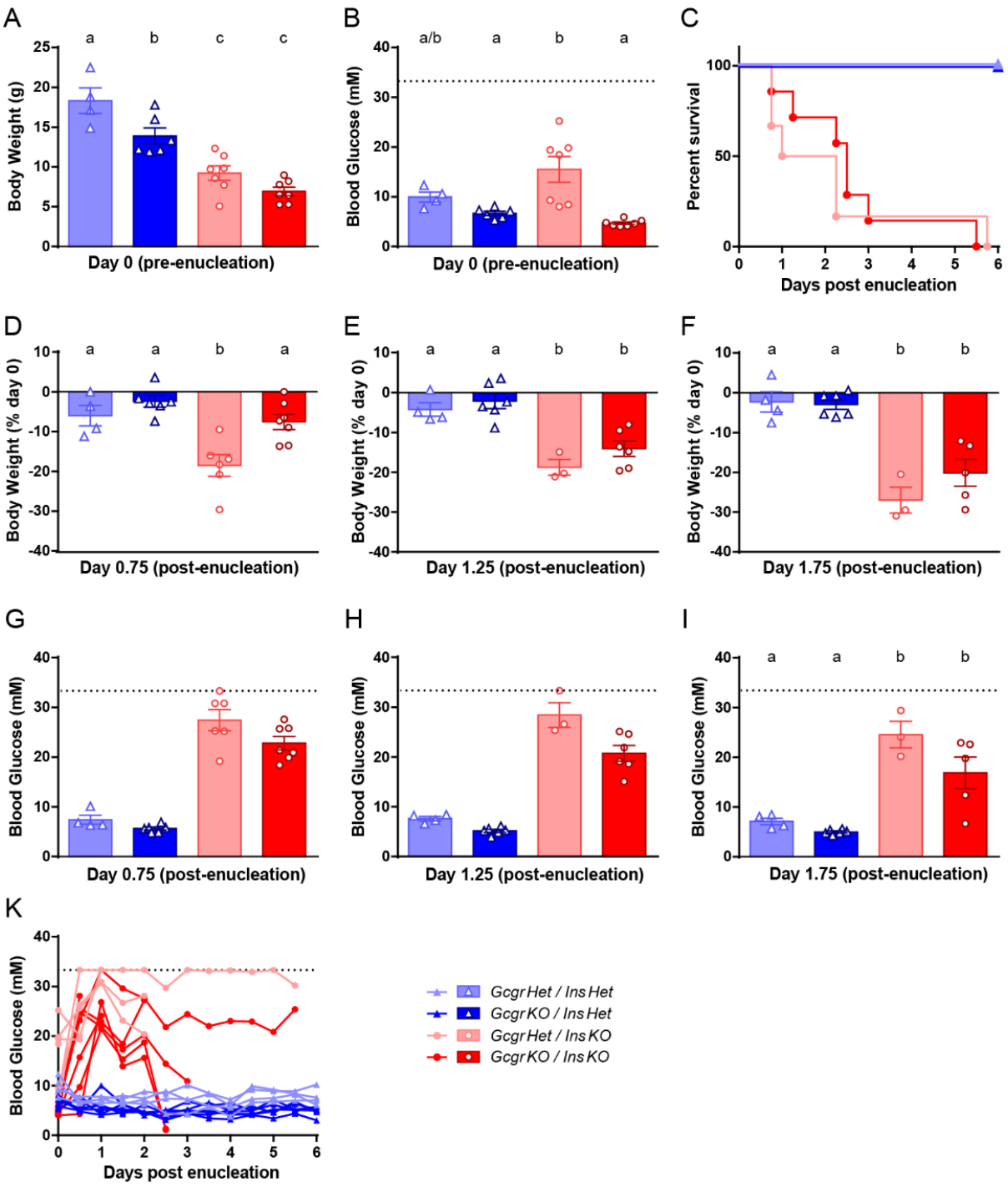
A
a
b
c
c
Body Weight (g)
Day 0 (pre-enucleation)
B
a/b
a
b
a
Blood Glucose (mM)
Day 0 (pre-enucleation)
C
Percent survival
Days post enucleation
D
Body Weight (% day 0)
Day 0.75 (post-enucleation)
E
Body Weight (% day 0)
Day 1.25 (post-enucleation)
F
Body Weight (% day 0)
Day 1.75 (post-enucleation)
G
Blood Glucose (mM)
Day 0.75 (post-enucleation)
H
Blood Glucose (mM)
Day 1.25 (post-enucleation)
I
Blood Glucose (mM)
Day 1.75 (post-enucleation)
K
Blood Glucose (mM)
Days post enucleation
GcgrHet / InsHet
GcgrKO / InsHet
GcgrHet / InsKO
GcgrKO / InsKO

Figure 35. *Gcgr* gene deletion modestly lowers blood glucose but does not promote survival in adult *InsKO* mice. *InsKO* mice with and without *Gcgr* gene deletion were kept alive to 4 weeks of age using insulin injections and an islet transplant into the eye. On day 0 of the study, the eye containing islets was enucleated rendering the mice completely insulin-deficient. Day 0 absolute body weight (A) and blood glucose levels (B) prior to enucleation in the random fed state. Following enucleation, survival (C) and day 0.75 (D), 1.25 (E) and 1.75 (F) body weight loss were measured. Random fed blood glucose at day 0.75 (G), 1.25 (H), 1.75 (I) and throughout the study (H) are graphed. The dotted line in (B, G-K) is the upper detection limit of the glucometer (33.3 mM). For A, B, D-F, and I, all groups were compared by 1-way ANOVA with Tukey post-hoc testing and different superscripts (a, b, c) are significantly different from each other within each graph. For B, no statistical differences were observed between *GcgrHet/InsKO* and *GcgrKO/InsKO* when compared using the Kaplan-Meier method with the log rank test. Statistical analysis was not performed on G and H as one blood glucose value fell above the limit of detection and was assigned a value of 33.3 mM, or in K because the n value decreased over time. Data are graphed as mean±SEM (A, B, D-I), % survival (C), or individual mice (K), n=4-7, throughout study *InsKO* mice reach humane endpoint therefore n is reduced in E, F, H, and I.

Gcgr gene deletion increases plasma glucagon, normalizes plasma ketones, and does not affect plasma cholesterol or fatty acids in adult InsKO mice

Finally, we measured various metabolic parameters in young adult *InsKO* mice with and without *Gcgr* gene deletion. Similar to P1 pups, *GcgrKO/InsKO* mice had substantially elevated plasma glucagon levels compared to all other groups, while the *GcgrKO/InsHet* group trended towards increased levels of glucagon compared to *GcgrHet* groups (Figure 36A). As expected, β-hydroxybutyrate levels were substantially elevated in *GcgrHet/InsKO* mice. The *GcgrKO/InsKO* mice trended (P=0.09) to have lower β-hydroxybutyrate levels, which were similar to that of controls; however, this did not reach statistical significance (Figure 36B). Plasma cholesterol levels were similar between controls and *InsKO* mice, and fatty acids levels were increased in *GcgrKO/InsKO* compared to controls (Figure 36C&D).

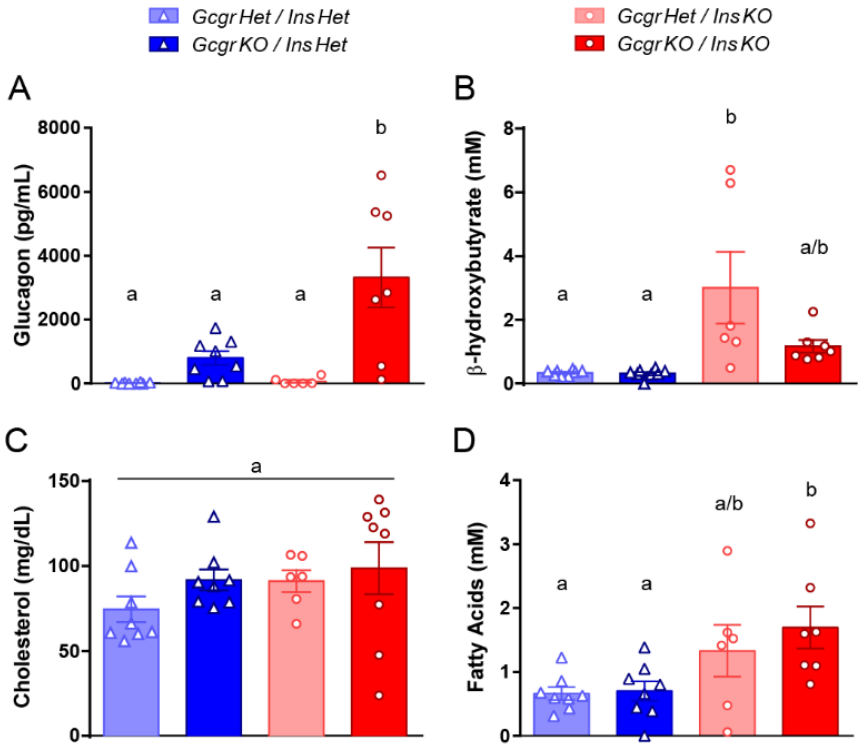

Figure 36. *Gcgr* gene deletion increases plasma glucagon, normalizes plasma ketones, and does not affect plasma cholesterol or fatty acids in adult *InsKO* mice. At humane endpoint (*InsKO* mice) or the end of the study (controls) cardiac blood was collected to measure plasma glucagon (A), β-hydroxybutyrate (B), total cholesterol (C), and fatty acids (D) in the random fed state. All groups were compared by 1-way ANOVA with Tukey post-hoc testing and different superscripts (a, b, c) are significantly different from each other within each graph. Data are mean±SEM, n=6-8.

7.3 Discussion

Recent evidence suggests that glucagon itself causes the lethal catabolic consequences of insulin deficiency [73]. Remarkably, despite a 90% reduction in insulin, *GcgrKO* mice remained euglycemic and healthy compared to controls that required euthanization 6 weeks post-STZ [75, 342]. However, we found that 100% elimination of insulin alongside *Gcgr* gene deletion in mice does not extend survival. At both P1 and in early adulthood, *GcgrKO/InsKO* mice failed to gain mass or lost a substantial amount of weight and survived no longer than that of *GcgrHet/InsKO* controls. In a previous study, we generated *InsKO* mice on a *GcgrWT* background and found that these mice survived for ~1 day following islet graft removal, but survived for fewer than 6 days in the current study. While the reduction of *Gcgr* may have had

a slight protective effect on *InsKO* mice, other differences between the two studies include the background strain, age, diet, and facility. Nonetheless, it is clear that *Gcgr* gene deletion cannot prolong survival in mice completely lacking insulin.

The loss of glucagon action in the complete absence of insulin does moderately improve aspects of metabolism. Glucagon not only plays a role in the fasting state but also in the fed state, which is evident as *Gcgr* gene deletion can normalize fed blood glucose levels and glucose tolerance [74, 75, 342]. In our study, *GcgrKO/InsKO* mice only experienced a partial improvement in the fed blood glucose levels in both P1 pups and young adults. Furthermore, although we saw modest blood glucose reduction throughout the study in the fed state, at euthanasia, some of the *GcgrKO/InsKO mice* were hypoglycemic, a state not observed in the *GcgrHet/InsKO* mice. The feeding state of the mice at the time of euthanization is unclear. Therefore, we speculate that if we had fasted the *GcgrKO/InsKO* mice, they may have experienced a larger decrease in blood glucose due to insufficient hepatic glucose production due to inability to mobilize glycogen stores. Furthermore, it has been shown that *Gcgr* gene deletion normalizes ketones [75]. Since *GcgrHet/InsKO* mice had highly variable ketones, levels only trended towards improvement in *GcgrKO/InsKO* adult mice and although we observed a significant improvement in ketones in P1 pups, levels in *GcgrKO/InsKO* mice still remained 4 fold higher than healthy controls. Interestingly in both pups and adults, loss of the *Gcgr* gene did not improve aberrant lipid metabolism in the *InsKO* mice. Thus while loss of glucagon action can improve some aspects of metabolism, metabolism is not normalized in the absence of both insulin and glucagon action.

While several reports indicate that loss of glucagon action can protect rodents from diabetes [74-77, 342], some studies suggest a less substantial role for glucagon in diabetes pathogenesis [336, 338]. Meek *et al.* suppressed glucagon action in STZ-diabetic rats using a Glp-1 receptor agonist or a glucagon-neutralizing antibody, both of which did not change blood glucose levels, but lowered ketone levels, suggesting that hyperglucagonemia is required for ketosis but not hyperglycemia [338]. Their report only partially agrees with our data as we observed minor improvements in both blood glucose and ketones. Furthermore, Steenberg *et*

al. report that ablation of glucagon expressing cells using Gcg-DTR mice does not improve glucose tolerance but modestly prevents further deterioration in STZ-diabetic mice [336]. Collectively, we demonstrate that the absence of glucagon signalling can modestly improve diabetic symptoms in *InsKO* mice, but this improvement was insufficient in promoting survival. Therefore, we have provided unequivocal evidence that it is not possible to protect from the catabolic consequences due to the complete loss of insulin by eliminating glucagon action.

CHAPTER 8 – CONCLUSIONS AND FUTURE DIRECTIONS

It has been assumed that the only hormone able to reverse the catabolic repercussions of diabetes is insulin. However, various studies have shown that the adipocyte-derived hormone leptin plays a role in regulating glucose homeostasis and can normalize blood glucose in obese leptin deficient *ob/ob* mice as well as insulin-deficient mice. In addition, hyperglucagonemia is present in many patients with type 1 and type 2 diabetes [67-71], and glucagon receptor antagonism has shown promise as a glucose-reducing therapy. The overarching goal of this thesis was to investigate the mechanism of the glucose lowering actions of leptin and the effects of glucagon suppression therapy in mouse models of diabetes. To achieve this we probed the role of increased leptin action during insulin therapy, determined the role IGFBP2 in the glucose lowering actions of leptin, investigated the necessity of insulin for leptin and glucagon suppression therapy, and explored the potential of glucagon suppression therapy via Gcgr siRNA delivered by lipid nanoparticles.

The first part of this thesis focuses on the benefit of leptin action indirectly as a result of insulin therapy. By testing insulin therapy in STZ-diabetic *ob/ob* mice not capable of increasing plasma leptin levels, as well as in STZ diabetic wildtype mice given a leptin antagonist, we have demonstrated that the increased leptin action due to insulin therapy contributes to lowering of blood glucose levels. If the *ob/ob*-STZ mice had not lost weight so dramatically and reached humane endpoint, additional experiments could have elucidated the role of insulin-stimulated leptin release from adipose tissue in insulin therapy by comparing insulin treatment in *ob/ob*-STZ mice with and without leptin expressing adipose tissue transplants. Nonetheless, these data are consistent with the current literature, which suggests that some metabolic derangements in insulin-deficient diabetes are directly due to hypoleptinemia as opposed to hypoinsulinemia. Various studies report that administration of a leptin at a dose that normalizes plasma leptin levels in STZ-diabetic rodents only moderately affects blood glucose but completely restores hyperphagia, insulin resistance, and hyperlipidemia [234, 296-298]. Taken together, these data highlight the consequences of leptin deficiency due to insulin depletion.

One avenue of leptin research we are interested in is elucidating the downstream metabolic pathways by which leptin reverses hyperglycemia. In 2010, an article was published that suggested IGFBP2 may be a mediator of leptin action on glucose homeostasis. By performing a microarray, *Igfbp2* mRNA was identified as being significantly upregulated in the liver of *ob/ob* mice following leptin treatment [313]. Further, when IGFBP2 levels were raised to supraphysiological levels using an adenovirus fasting blood glucose levels, insulin levels, glucose tolerance, hepatic insulin sensitivity, and hepatic steatosis were normalized in *ob/ob* mice [313]. Based on these preclinical data the authors filed a patent for treating type 1 diabetes, type 2 diabetes, insulin resistance, and hepatic steatosis by administering IGFBP2 (patent #8404639). Interestingly, their article reports that beneficial effects were observed when IGFBP2 levels were raised to at least 6000 ng/mL while the patent reveals that levels were raised to 6884 – 107200 ng/mL, which is 170 – 2680 fold higher than levels in *ob/ob* mice, while the patent only covers use of 2.5 – 5 fold higher levels in humans. This thesis now shows that a lower dose of IGFBP2 similar to what is induced by leptin therapy (5 fold higher than *ob/ob* levels and close to wildtype levels) does not have any beneficial effects on the parameters we measured. If this phenomenon in mice holds true to humans, this information may nullify the usefulness of the patent and usefulness of IGFBP2 as a therapy. Given that we found that IGFBP2 does not likely mediate the effects of leptin, we did not continue pursuing IGFBP2 as a topic of interest. Since the publication of our data, an article by Yau *et al.* has demonstrated that leptin-mediated increases in IGFBP2 may be important for increasing insulin sensitivity in human muscle [346], which suggests that testing in humans or human tissue may be required to investigate the role of IGFBP2 in glucose homeostasis in humans.

Another mechanism tested in this thesis was whether leptin mediates glucose lowering in insulin-deficient diabetes through an insulin-sensitizing effect. Throughout the past decade, several studies have demonstrated that leptin therapy can reverse diabetic symptoms in various rodent models of insulin-deficient diabetes with varying levels of insulinopenia leading authors to claim that leptin can reverse diabetes in the absence of insulin. Yet, since insulin depletion is not complete in these models and leptin can potently increase insulin sensitivity [167, 234], leptin may reverse diabetes by enhancing insulin action to a degree where residual insulin may

be useful. By testing leptin therapy in *InsKO* mice, the data in this thesis reveal that while leptin can reverse some of the diabetic symptoms the treatment is not perfect. Although leptin increased survival in *InsKO* mice, they lasted less than 3 weeks before succumbing. In addition, while leptin therapy in other models of insulin-deficient diabetes improves fed or glucose-induced glycemia, leptin treated *InsKO* mice had overt hyperglycemia in the fed state. Despite strikingly high blood glucose levels, fasting induced dramatic fasting hypoglycemia. These data were published as a Brief Report where original research of important interest can be concisely presented. However, it would have been interesting to do a more thorough characterization of these mice to determine whether leptin therapy has the same effects on other parameters as has been demonstrated in STZ diabetic mice. For instance it would be interesting to perform metabolic cage experiments to investigate food intake and energy expenditure, perform an analysis of insulin sensitivity such as an insulin tolerance test or hyperinsulinemic euglycemic clamps, and to perform a thorough investigation of hepatic glucose production such as those performed by Perry *et al.* [235, 241]. In addition, by delivering ICV leptin, it could be determined whether the effects we observed in *InsKO* mice are mediated by the CNS, as has been reported in other models of insulin-depleted diabetes. However, these studies would be difficult given the challenges in keeping the mice alive.

This thesis highlights the risk of fasting hypoglycemia as well as the importance of the fed to fasting transition on lowering of blood glucose by leptin. While it has been touted that unlike insulin injections, leptin does not cause hypoglycemia [230] our laboratory has demonstrated that prolonged fasting for 9-15 hours can result in hypoglycemia in leptin treated STZ mice [231]. This timeframe was accelerated to 3-6 hours in the current study potentially due to the complete absence of insulin in the *InsKO* model or the higher dose of leptin given to *InsKO* mice. Given that leptin has been tested in patients with type 1 diabetes as an additive to insulin therapy to reduce insulin requirements and reduce fluctuations in blood glucose levels, it is critical that the potential for hypoglycemia be considered. In addition, studies often only report *ad libitum* fed, or 2 hour fasting measurements in leptin treated mice versus controls. By measuring various parameters in the fed and fasted state as blood glucose levels descend from hyper- to hypo-glycemia, we reveal additional information about what

parameters were already normalized in the hyperglycemic fed state and are unlikely involved in the glucose lowering effect of leptin such as corticosterone, as well as parameters that have unexpected drops compared to healthy controls, which may explain the glucose lowering effect of leptin such as fatty acids and β-hydroxybutyrate. Given the decreasing costs of omics experiments, it would be interesting to perform transcriptomics, proteomics, and metabolomics profiling in the liver of mice with insulin-deficient diabetes given leptin in the fed and fasted state compared to controls to obtain a bigger picture of the metabolic changes driving a reduction in blood glucose.

Recent work by Perry *et al*. strongly underlines the suppression of lipolysis and hepatic glucose production in the mechanism of leptin action. In fasted STZ diabetic rats, leptin reduced whole-body acetate, glycerol, fatty acid and β-hydroxybutyrate turnover [235, 241]. This was associated with decreased rates of adipocyte lipolysis and reduced conversion of glycerol and pyruvate to glucose through hepatic gluconeogenesis [235, 241]. In addition, these effects were reversed by administration of a lipid emulsion increasing fatty acids and glycerol, corticosterone or acetate [235, 241]. The results in this thesis are largely in agreement with these studies in that leptin reduced glycerol, fatty acid, and β-hydroxybutyrate levels in the fed state in *InsKO* mice. In addition, upon fasting, *InsKO* mice experienced a further reduction in fatty acids and β-hydroxybutyrate, which corresponded to a reduction in glycemia suggesting these parameters may be critical for glucose reduction. However, while we observed a decrease in circulating corticosterone in the hyperglycemic fed state, the *InsKO* mice experienced a robust increase in corticosterone levels upon fasting-induced hypoglycemia. This suggests that corticosterone is indispensable for leptin-mediated glucose reduction. Furthermore, Morton *et al.* also investigated the role of reduced corticosterone in mediating the glucose lowering effects of leptin. They report that neither adrenalectomy-induced glucocorticoid deficiency nor glucocordicoid receptor inhibition blockade reduced hyperglycemia in STZ-diabetic rats [332]. In addition, they found that exogenous corticosterone administration did not block the glucose lowering by leptin [332]. It has been postulated that the contradiction between these studies may be due to sucrose in the drinking water of adrenalectomized mice and higher insulin levels in STZ-diabetic rats in the Morton

article [241]. Discrepancies between the Perry report and our data may be due to differences in models used (STZ vs *InsKO*) or the degree of glucose reversal at the time of sample collection (euglycemic vs hypoglycemic). Thus, the importance of the leptin-mediated reduction in corticosterone in improving glucose metabolism is unclear.

Since leptin is a potent glucagon suppressor, it has been postulated that reduced glucagon action contributes to the glucose-lowering effect of leptin therapy in insulin-deficient diabetes [232, 233]. Initially our Gcgr siRNA vs leptin therapy study was focused on investigating the contribution of glucagon suppression to the glucose lowering actions of leptin, however our focus changed to characterizing the Gcgr siRNA therapy alone. Nonetheless, data from our study as well as data from other groups provide evidence suggesting that glucagon may not be critical for leptin-mediated improvement in glycemia. First, a low dose of leptin in rats normalized plasma glucagon levels, but lowered blood glucose levels only slightly [234]. In addition, while leptin treatment in rats normalized blood glucose within 6 hours, glucagon was only suppressed by 24 hours of leptin therapy, thereby temporally disconnecting the two effects [235]. Finally, the importance of inhibited lipolysis, reduced plasma fatty acids and glycerol, and thus reduced gluconeogenesis underlying the glucose-lowering mechanism of leptin have been demonstrated in mice and rats [231, 235, 241]. The data in this thesis reveal that fatty acid and glycerol levels are reduced with leptin therapy, but this effect was not seen following injection of Gcgr siRNA. Therefore, while inhibiting glucagon action alone may be beneficial in insulin-deficient diabetes, leptin action exerts additional effects that can reduce hyperglycemia. To complement these findings a study where the leptin-mediated suppression of glucagon is prevented by exogenous glucagon delivery could reveal whether leptin can reverse diabetes independently of a lowering of glucagon action.

We next shifted our focus to investigating glucagon suppression therapy by testing Gcgr siRNA in models of insulin-deficient diabetes, as well as glucagon receptor gene deletion in *InsKO* mice. In various preclinical studies, suppression of glucagon action by *Gcgr* gene deletion, glucagon immunosuppression, or Gcgr antagonist can ameliorate diabetic symptoms in models of both type 1 and type 2 diabetes [59, 74-77, 79-81]. In addition, it has been claimed

that glucagon excess rather than insulin deficiency is required for development of the metabolic manifestations of diabetes [72]. The data in this thesis reveal that Gcgr siRNA delivered via LNP is very effective at improving diabetic symptoms in STZ and HFD/STZ diabetic mice. LNP are the most advanced systems for delivering siRNA *in vivo* and very effective knockdown has been observed at doses as low as 0.01 mg/kg in rodents and 0.1 mg/kg in nonhuman primates [328]. Therefore, the dose of 10 mg/kg siRNA used in these studies is quite high as toxic effects begin to be observed at doses of 20 mg/kg (Dr. Yuen Yi Tam, The University of British Columbia, Vancouver, BC, Canada, personal communication). It would be interesting to test repeat administration of Gcgr siRNA-LNP to determine if even longer term normalization of blood glucose levels can be achieved, and to test for any signs of liver toxicity such as the presence of liver enzymes in the plasma. In addition, to make these results more clinically relevant we could test Gcgr siRNA-LNP in conjunction with insulin therapy in models of insulin-deficient diabetes to determine the risk of hypoglycemia. Moreover, troubleshooting the use of different cationic lipids in the LNP, siRNA sequences, and modifications on the siRNA could allow for more effective Gcgr siRNA knockdown, a stronger glucose lowering phenotype, and longer term reversal of diabetic symptoms.

Given various reports, as well as data in Chapter 6, of glucagon suppression therapy being efficacious in insulin-deficient diabetes, it was hypothesized that glucagon excess rather than insulin deficiency was necessary for the development of diabetes [74, 77]. Therefore, we tested the effect of glucagon gene deletion in *InsKO* mice. Although the *InsKO* model may not be directly clinically relevant given that typically patients with type 1 diabetes do not completely lack insulin, insulin deficiency can be severe in patients with type 1 diabetes [347]. This model is unique in that it can provide a mechanistic understanding of the role of insulin levels in glucagon suppression therapy. The data from these studies reveal that in the context of complete insulin deficiency, blocking glucagon action can only have minor beneficial effects on blood glucose and plasma ketone levels with no lengthening of survival. These data fit nicely into the growing body of literature on this topic suggesting that as insulin deficiency gets more severe, glucagon suppression is less effective.

The mechanism by which glucagon suppression therapy lowers blood glucose has been proposed to be through increased GLP-1 levels. *GcgrKO* mice, which are protected from STZ-induced diabetes, exhibit increased proglucagon expression and processing that leads not only to higher glucagon levels, but elevated circulating and pancreatic GLP-1 levels (total GLP-1 and GLP-1 amide) [44]. In addition, levels of active GLP-1 are elevated in mice given a Gcgr monoclonal antibody [348]. Total GLP-1 levels were raised in patients with type 2 diabetes given glucagon receptor antagonists [83, 84, 86], however active GLP-1 levels were inconsistently elevated [86]. Intriguingly, one study demonstrated that loss of GLP-1 action (through GLP-1 receptor gene deletion or GLP-1 antagonism) worsened non-fasting blood glucose levels and glucose tolerance in STZ-diabetic *GcgrKO* mice or mice given a Gcgr antagonist [342]. Another study reported that although GLP-1 receptor or FGF21 antagonism alone did not cause hyperglycemia in STZ-injected *GcgrKO* mice, co-antagonism resulted in substantially impaired glucose tolerance [349]. These studies suggest that non-insulinotropic effects of GLP-1 may contribute to the metabolic benefit observed in mice with blockade of glucagon receptor signalling. It would be interesting to determine if this effect was observed in healthy or diabetic mice given Gcgr siRNA or *InsKO* mice with *Gcgr* gene deletion.

Recently concern has grown over rigor, reporting, and reproducibly of research [350-352] and it has been reported that more than 70% of researchers have tried and failed to reproduce another researchers' science and more than 50% fail to reproduce their own findings [353]. In addition, there has been a push to increase the quality and reporting on animal studies such as using the Animals in Research: Reporting *In vivo* Experiments (ARRIVE) guidelines [354, 355]. Most of the effect sizes in our data are very large and for certain experiments we have repeated our studies in various cohorts, which increases the power and confidence in our data. However, our sample sizes can also be considered small thus thoughtful consideration should be given to the robustness of our conclusions. The data in Chapter 3 are the most preliminary data in this thesis, therefore when describing these data we have attempted to point out caveats of the experiments, use cautious wording, and not to over-interpret the results. The use of N=1 in Figure 3 was included as increased Glut2 expression by leptin was expected, striking, and may be of interest to readers of this thesis but must be repeated to ensure the result is reproducible. In Chapter 4 Figures 10-12, a sample size of 4-6 may be considered low.

Indeed if we were attempting to prove a null hypothesis that low dose IGFBP2 has no effect on glucose metabolism we may require significantly more mice. However, our goal was not to detect small effects of IGFBP2 treatment but rather to determine if IGFBP2 is entirely responsible for the metabolic actions of leptin as was reported. Since the low Ad-IGFBP2 group achieved similar levels of IGFBP2 to leptin treated animals, but there is a significantly differential effect between leptin treatment and IGFBP2 treatment, we feel it is reasonable to conclude that IGFBP2 does not mediate the robust effects of leptin. In addition in Figure 13 although the IGFBP2 siRNA groups have an N=3 each, we have tested 3 different IGFBP2 siRNAs and all were similarly effective with no evidence of altered body weight, blood glucose, or plasma insulin giving us more confidence that IGFBP2 does not regulate glucose metabolism in mice. In Chapter 5, although some of the groups had a low sample size value (N=3-5) the experiment was repeated in 2 cohorts of mice evoking similar results, and the effects are so dramatic (death vs life, extreme changes in blood glucose in *InsKO* vs tight regulation in *Het* controls) that we are assured of our results. For data in Chapter 6, Gcgr siRNA delivery was repeated in 3 cohorts of STZ diabetic mice and 2 cohorts of HFD/STZ producing similar results. Moreover, although the LNP delivery method is novel, as well as testing glucagon suppression in HFD/STZ mice, the idea that glucagon suppression can be beneficial for diabetes has been previously shown and thus our results and not unexpected. In Chapter 7, variability in the blood glucose and ketone data in adult mice (Figures 35&36) due to variable health status in mice and low sample size could potentially be rectified by increasing the number of biological replicates in this study. However, the results from pups and survival data in both age groups is clear. Lastly, given the fact that the same experiment can yield different results when performed in various facilities (potentially due to differences in chow diets, makeup of microbiota, etc.) all of our studies could benefit from being repeated at another facility.

The statistical analyses performed throughout this thesis were either Student's t-test, 1-way ANOVA, or repeated measures 2-way ANOVAs, with the exception of the survival curves, and the data is presented as mean±SEM. The analyses were presented as they had been published and had been performed this way given the convention in the literature. However,

reporting the median may be more useful since it is less sensitive to outliers than the mean. In addition, presenting the error as standard deviation or 95% confidence intervals can allow the differences between groups to be more easily understood. Moreover, it should be noted that when testing with small sample sizes, as has been performed in some studies through this thesis, non-parametric tests may be more statistically robust. Given the large effect sizes seen in our results choosing a different statistical test is unlikely to change our main conclusions.

In addition to specific caveats pertaining to individual experiments, which have been discussed throughout this thesis, general limitations include the use of only male mice as well as difficulty in translating results from animal models to human physiology. For the majority of studies performed in this thesis only male mice were tested. For the studies involving the *InsKO* model, male and female mice were used for experiments since breeding was done at our facility; however, due to low sample size the data from both sexes were pooled such that no sex differences could be detected. Typically, male mice are selectively used in scientific testing to cut costs and reduce variability in results due to the 4-5 day estrous cycle in female mice. However, various studies illustrate important sex differences in rodents and humans relating to glucose and lipid metabolism [356-358]. Therefore, it would be prudent to perform these studies using female mice to investigate any potential interactions with sex steroid hormones. In addition to using only male mice, animal experiments in general often provide limited translational potential to humans, for various reasons including differences between animal and human physiology, increased genetic variability in humans, and use of rodent models that do not accurately replicate human diseases [359]. Although the research in this thesis is largely investigating the mechanism behind the glucose lowering actions of leptin and inhibition of glucagon action, it may be valuable to test these therapies in a rodent model that has a pathophysiology more similar to human diabetes. For instance STZ destruction of β-cells to induce insulin deficiency is chemically-mediated, rapid and severe while diabetes in NOD mice is immune-based and more closely resembles human type 1 diabetes. These caveats should be kept in mind when considering the results from these experiments.

The preclinical studies executed in this thesis are clinically relevant, as leptin therapy has been tested clinically in patients with diabetes. Throughout the duration of these studies in this PhD thesis, we eagerly awaited the results of the clinical trial testing the addition of leptin therapy to insulin therapy in patients with type 1 diabetes to determine if blood glucose control would be tightened, whether insulin requirements would decline, and whether the incidence of hypoglycemia would increase. Finally, the results revealed that although body weight, percent body fat, and insulin dose were reduced due to leptin therapy, HbA1c, blood glucose and lipids were unaltered, suggesting that leptin was not efficacious in improving glycemic control in these patients [260]. Interestingly, although on average HbA1c and insulin dose were unchanged, individual responses were variable with 1 patient (out of 8) experiencing a meaningful reduction of HbA1c of -0.7% [260]. In contrast to the majority of studies that test leptin monotherapy in severely insulin-deficient, hypoleptinemic, and hyperglycemic rodents [167, 231-233, 235, 241], the patients enrolled in this study were insulin treated, had normal baseline leptin levels, and had better glycemic control, therefore there is less potential for improvement in these patients [260]. We have reported that a dose of leptin that raises circulating leptin by ~6 - 12 fold higher than non-diabetic controls can normalize 4 hour fasting blood glucose levels in STZ-diabetic mice [167, 231, 248]. The dose used in the clinical trial only raised leptin levels by approximately 2 fold, thus a higher dose of leptin may elicit a more substantial glycemic response [260]. Interestingly, while leptin has a well-known lipolytic/fat-burning effect in *ob/ob* and lean mice [175, 197, 253-259], studies in insulin-deficient rodents have highlighted the importance of leptin-mediated suppression of lipolysis in reversing hyperglycemia [231, 235, 241]. However, leptin treated patients had significantly reduced percent body fat indicative of increased lipolysis [260], suggesting that leptin may be acting via a different mechanism than what has been demonstrated in STZ-diabetic rodents. This clinical trial has been terminated and it is unclear whether leptin therapy will continue to be pursued for type 1 diabetes.

Over the course of the past few years a series of glucagon receptor antagonists have entered clinical trials for patients with type 2 diabetes [82]. Despite demonstrating safety of the drug and success in lowering blood glucose and HbA1c, many agents have been removed from the

pipeline. The agents were likely discontinued due to an insufficient benefit to risk ratio as many glucagon receptor antagonists increased blood pressure, liver fat, and serum cholesterol, triglycerides and transaminase levels [83-88]. Currently, Ligand Pharmaceuticals has a drug in phase 2 clinical trials (clinicaltrials.gov, NCT02851849), although it is unclear whether this drug will suffer the same fate as others. It would be interesting to determine the benefits and risks of Gcgr siRNA delivered by LNP in humans.

For both leptin and glucagon suppression therapy, personalized medicine may allow for these treatments to truly benefit select populations of patients as their genetic makeup may cause the patient to be more sensitive to the benefits and less susceptible to any side effects. However, this will not likely happen in the near future given that the field of personalized medicine is in its infancy and the amount of effort to determine which patients may benefit from the treatment may not be cost effective. Whether or not these therapies are ever approved, it is hoped that the data in this thesis provide a better understanding of the metabolic pathways involved in leptin therapy and glucagon suppression therapy, which may allow for the development of alternative strategies and novel therapeutic targets for the treatment of diabetes.

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