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Mechanisms of beta-cell deficiency in gestational diabetes and strategies to reverse hyperglycemia

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Abstract

Gestational diabetes mellitus (GDM) is an increasingly prevalent form of diabetes that first appears during pregnancy, and reverses after parturition in most cases. Nonetheless, GDM is associated with adverse maternal and fetal health outcomes. There is currently no reliable method of intervention for GDM and a limited understanding of the mechanisms of impaired endocrine adaptability in GDM. In this thesis, I aimed to address these knowledge gaps by establishing a mouse model for the study of suboptimal endocrine adaptations during pregnancy. This was accomplished using a dietary low protein (LP) insult during fetal and neonatal development, which programs for suboptimal pancreas development in the offspring, and performing histomorphometric analyses on fixed pancreas tissues. Female offspring displayed glucose intolerance during their own pregnancy that was apparent by gestational day (GD) 18.5 and characterized by reduced β-cell mass (BCM) and α-cell mass (ACM) relative to control-fed animals. Using this model, I provided evidence that pancreatic maladaptations at GD18.5 persisted at postpartum day 7.5, contributing to glucose intolerance until 1 month after parturition. To provide mechanistic insights of reduced BCM expansion in GDM, I investigated the contribution of α- to β-cell transdifferentiation *via* immunofluorescence cell counting analysis of fixed pancreas tissues. I identified maladaptations of α-cell plasticity in glucose-intolerant mice, as demonstrated by reduced α-cell proliferation, leading to reduced ACM expansion relative to controls. Additionally, these animals presented with hyperglucagonemia. These findings demonstrated that, in addition to β-cells, insufficient pancreatic α-cell adaptations can also contribute to GDM pathogenesis. Although there were differences in the percentages of bihormonal (Insulin^{+}Glucagon^{+}) cells in LP *vs.* control pregnancy, genetic lineage tracing in control pregnancy using Glucagon-Cre/Rosa26-eYFP mice revealed a negligible amount of α- to β-cell transdifferentiation contributing to BCM expansion. Finally, I used the animal model to test a therapeutic intervention for GDM through the attempted manipulation of BCM using the artemisinin, artesunate. Artesunate-treated animals had improved glucose tolerance, although the glucose-lowering effect was attributed to the acetone vehicle. Collectively, this thesis has

identified mechanisms of impaired endocrine pancreas adaptability in GDM and has established a mouse model that can be used to explore novel therapeutics.

Keywords

Alpha-cell, Beta-cell, Gestational Diabetes Mellitus, Glucose Tolerance, Inflammation, Insulin Secretion, Low Protein Diet, Mouse, Pancreas, Postpartum, Pregnancy

Summary for Lay Audience

Diabetes occurs when there is a loss or dysfunction of insulin-producing β-cells in the pancreas, leading to elevated blood sugar levels. Diabetes is often classified as either being type 1 or type 2. However, gestational diabetes mellitus (GDM) is another type of diabetes that first presents during pregnancy and is becoming increasingly prevalent. Although human pancreas samples during GDM are scarce, it is believed that β-cell dysfunction is a major driver of GDM pathogenesis. In this thesis, I sought to develop a mouse model that can be used to better understand the reasons for suboptimal pancreas adaptations in GDM. First, I established the mouse model using a dietary insult (low protein) during early life, which results in suboptimal pancreas development in the offspring. As is diagnosed in humans, these animals presented with GDM identified during late gestation (which in mice is around gestational day 18.5) due to impairments in β-cell number and the capacity for insulin release. Since many women go on to develop type 2 diabetes mellitus after delivery, I also presented evidence that these impairments in the pancreas are still present following birth and contribute to high blood sugar levels until at least one month postpartum. Using our mouse model, I demonstrated that diabetes develops not only due to impairments in β-cells, but also due to abnormalities in pancreatic α-cells, which work antagonistically with β-cells to secrete glucagon. Finally, I identified a therapeutic effect in GDM where there was a reversal of diabetes in animals treated with a chemical that likely damages the gut equivalent to transient fasting. This thesis characterized a novel mouse model of GDM and provides new information about mechanisms of suboptimal pancreas adaptations that can be used to explore methods of treatment.

Co-Authorship Statement

All studies presented in this thesis were performed primarily by Sandra K. Szlapinski in the laboratory of Dr. David Hill with the assistance of the co-authors listed below. For all experimentation, the contribution from Dr. David Hill was of an intellectual nature with respect to the experimental design, data analysis/interpretation and manuscript preparation.

Chapter 2: Renee King and Gabrielle Retta assisted with some immunohistochemical staining and analysis for the alpha-cell mass experiments. Erica Yeo assisted with some pancreas surface area measurements for beta-cell mass experiments. Brenda Strutt performed the injections for glucose tolerance tests and performed collagenase injections into the common bile duct for islet isolation experiments.

Chapter 3: Anthony Botros assisted with immunohistochemical staining and analysis for the 1-month postpartum timepoint. Renee King and Gabrielle Retta assisted with some immunohistochemical staining and analysis for the alpha-cell area measurements. Sarah Donegan performed the PD-L1 ELISA assay. Brenda Strutt performed the injections for glucose tolerance tests. Shannon Seney provided technical assistance with the Luminex Multiplex experiment.

Chapter 4: Jamie Bennett assisted with immunohistochemical staining and cell counting analysis for the transitional cell data (Insulin^{+}YFP^{+}Glucagon^{+}). Brenda Strutt produced the double transgenic Gcg-Cre/YFP mice and maintained animal colonies.

Chapter 5: Jamie Bennett assisted with immunohistochemical staining and cell counting analysis for the bihormonal (Insulin^{+}Glucagon^{+}) cells. Brenda Strutt performed the injections for glucose tolerance tests.

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List of Abbreviations, Symbols, and Nomenclature

α	alpha
β	beta
γ	gamma
δ	delta
ε	epsilon
ACM	alpha cell mass
ADP	adenosine di-phosphate
ANOVA	analysis of variance
APJ	apelin receptor
ATP	adenosine tri-phosphate
AUC	area under the glucose tolerance curve
BCM	beta cell mass
BMI	body mass index
C	control
CCND2	cell-cycle protein cyclin-D2
CNP	control non-pregnant
CP	control pregnant
Cre	cyclization recombinase enzyme
Ct	cycle threshold
DAPI	4’,6–diamidino–2–phenlindole
DM	diabetes mellitus
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
DOX	doxycycline
DYRK1A	dual-specificity tyrosine-regulated kinase 1A
E	emybronic day
g	gram
Gck	glucokinase
GD	gestational day

GDM	gestational diabetes mellitus
GLP-1	glucagon-like peptide 1
Glut	glucose transporter
GSIS	glucose-stimulated insulin secretion
GSL	glycosphingolipid
HBSS	Hank's buffered salt solution
HCl	hydrochloric acid
HFD	high fat diet
HNF	hepatocyte nuclear factor
HOMA-IR	homeostatic model assessment of insulin resistance
IL	interleukin
Ins	insulin
IPGTT	intraperitoneal glucose tolerance test
IRS	insulin receptor substrate
IUGR	intra-uterine growth restriction
Ki67	antigen identified by monoclonal antibody Ki67
LP	low protein
LPNP	low protein non-pregnant
LPP	low protein pregnant
MafA	V-maf musculoaponeurotic fibrosarcoma oncogene homolog A
MafB	V-maf musculoaponeurotic fibrosarcoma oncogene homolog B
MAPK	mitogen-activated protein kinase
MODY1	maturity onset diabetes of the young type 1
MPC	multipotent progenitor cells
MRI	magnetic resonance imaging
mRNA	messenger ribonucleic acid
n	sample size
NaCl	sodium chloride
NeuroD	neurogenic differentiation-1
NF-kB	nuclear factor kappa-light-chain-enhancer of activated B cells
Ngn3	neurogenin 3

Nkx2.2	NK2 transcription factor related, locus 2
Nkx6.1	NK6 homeobox 1
OCT	optimal cutting temperature medium
p	p-value, probability
Pax4	paired box gene 4
Pax6	paired box gene 6
PBS	phosphate-buffered saline
PCR	polymerase chain reaction
PD-L1	programmed death-ligand
PDL	partial duct ligation
Pdx1	pancreatic duodenal homeobox-1
PET	positron emission tomograph
PFA	paraformaldehyde
PI3k	phosphatidylinositol 3-kinase
PND	postnatal day
PPD	postpartum day
PRLR	prolactin receptor
Ptf1	pancreas associated transcription factor 1a
qPCR	quantitative (real-time) RT-PCR
RNA	ribonucleic acid
ROS	reactive oxygen species
SEM	standard error of the mean
SGLT2	sodium-glucose cotransporter 2
SPECT	single photo emission computed tomography
STZ	streptozotocin
T1DM	type 1 diabetes mellitus
T2DM	type 2 diabetes mellitus
TAM	tamoxifen
Th1	helper T-cell-1
TNF-α	tumor necrosis fator alpha
Tph1	tryptophan hydroxylase

TUNEL	terminal deoxynycleotidyl transferase dUTP nick end labeling
VEGFA	vascular endothelial growth factor A

Chapter 1

Sections of this chapter have been published:

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1 Introduction

The disease report conducted by the World Health Organization from 2000 to 2015 lists diabetes mellitus (DM), which was not on the report previously, as the top 6th leading cause of death in 2015 killing 1.6 million people worldwide [1]. DM is a metabolic disorder characterized by increased levels of glucose in the blood (hyperglycemia). When left uncontrolled, DM can result in multiple adverse health complications such as damage to the nerves and blood vessels increasing the risk of cardiovascular disease [2], nephropathy [3] and retinopathy [4].

1.1 Diabetes

There are two main types of diabetes, Type I diabetes mellitus (T1DM) and Type II diabetes mellitus (T2DM). T1DM is characterized by insufficient insulin production while T2DM occurs when the body is unable to effectively use insulin. Insulin is a hormone produced by pancreatic beta (β)-cells, which are cells located in the endocrine portion of the pancreas called the islets of Langerhans. Insulin binds the insulin receptor, a tyrosine kinase, on target tissues (liver, adipose, muscle) resulting in autophosphorylation and subsequent phosphorylation of downstream targets [5]. This ultimately leads to translocation of vesicles containing glucose transporters (i.e. Glut1, Glut4) to the cell membrane and regulates glucose uptake in target tissues [5].
Insulin was first isolated by Sir Frederick Banting and colleagues Charles Best, James Collip and John Macleod in 1922 which revolutionized treatment for individuals with T1DM [6]. This life-saving treatment is now given *via* exogenous insulin therapy. The protein, insulin, was isolated from pancreas extract samples and injected into dogs with pancreatectomy-induced diabetes, which resulted in lowering of blood glucose levels.

In individuals with T1DM, insulin deficiency occurs due to autoimmune destruction of insulin producing β-cells [5]. Autoimmune destruction of β-cells is mediated by T-cell activation through direct cell toxicity and β-cell specific autoantibodies [7]. T1DM is the less common form of diabetes as it accounts for only 5-10% of all cases of diabetes.

Symptoms include fatigue, weight loss, polyuria and dehydration. Individuals with T1DM require exogenous insulin therapy to maintain euglycemia. Transplantation of cadaveric human islets or whole pancreas as a strategy for diabetes reversal has also been undertaken but is limited by the shortage of organ supply from deceased donors which does not meet the demand for islet transplantations [8]. A combination of predispositions for T1DM have been suggested including viral exposure and genetic susceptibility [9].

T2DM is the more common form of diabetes (>90% of patients with diabetes) and occurs when insulin secretion is suboptimal [10]. T2DM often includes peripheral insulin resistance, meaning that target tissues are unable to respond to insulin resulting in hyperglycemia. However, the major driver of T2DM is suggested to be β-cell dysfunction with particularly a marked reduction of first-phase insulin secretion [11]. Individuals with T2DM can manage blood glucose levels with lifestyle changes including diet and exercise. Antihyperglycemic therapeutics can also be used, such as glucagon-like peptide 1 (GLP-1) receptor agonists, metformin and sodium-glucose cotransporter 2 (SGLT2) inhibitors. Each therapeutic works to either decrease blood glucose levels or increase insulin secretion in effort to attain euglycemia. Metformin reduces gluconeogenesis in the liver, GLP-1 receptor agonists enhance glucose-stimulated insulin secretion (GSIS), and SGLT2 inhibitors block the reabsorption of filtered glucose in the kidney [12]. The prevalence of T2DM is drastically increasing as the susceptibility is influenced by lifestyle factors such as obesity, age and a sedentary lifestyle [13]. This is concerning due to the various health risks associated with T2DM including cardiovascular disease, neuropathy and nephropathy [12].

Both T1DM and T2DM show the vital role that pancreatic β-cells play in maintaining euglycemia which demonstrates the important role of the pancreas in the physiology of regulating glucose homeostasis.

1.2 Pancreas Anatomy and Development

The pancreas is an organ located in the abdominal cavity. In humans, the head of the pancreas is attached at the initial curve of the duodenum of the small intestine, while the tail is attached to the spleen (Fig. 1.1). The body of the pancreas is found between the tail and head. This organization differs from that observed in mice, where 3 less defined lobes (duodenal, gastric and splenic) are present. The pancreas has both endocrine and exocrine functions, with only ~1-2% of the pancreas being endocrine despite its critical role in glucose homeostasis. Endocrine cells of the pancreas are congregated in the islets of Langerhans and secrete various hormones. Exocrine cells comprise the remaining ~98% of the pancreas including acinar and duct cells which secrete pancreatic fluids containing digestive enzymes into the small intestine.

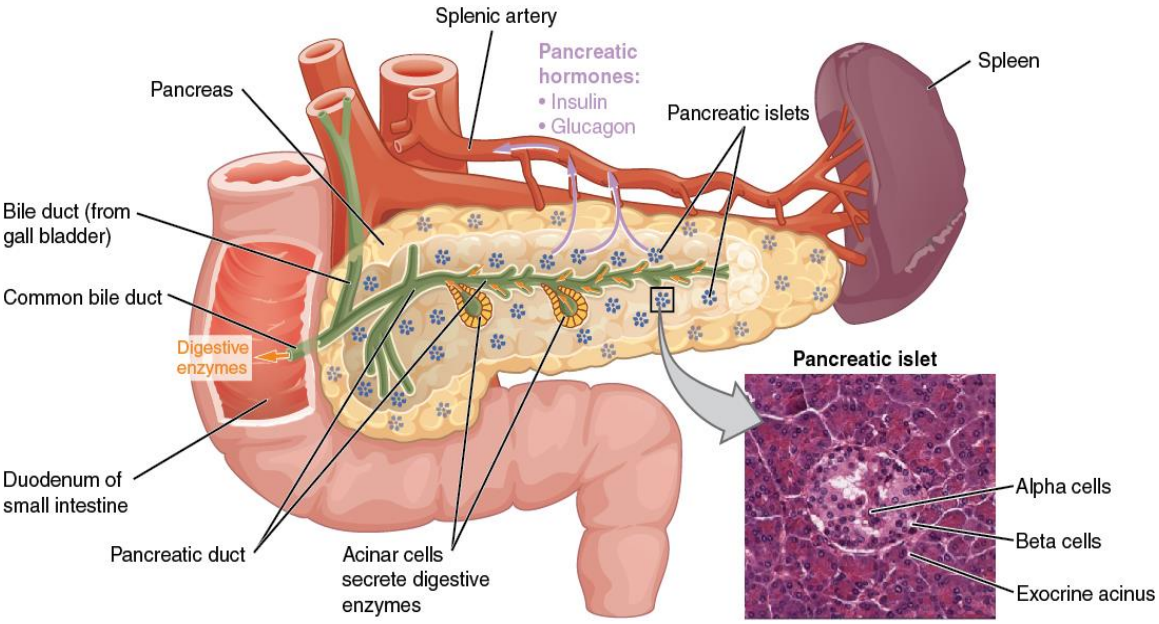

Figure 1.1. Anatomical location of the pancreas

The pancreas is located behind the stomach in the upper left abdomen. The endocrine portion, the islets of Langerhans, are found dispersed throughout the head and tail of the pancreas and function to regulate glucose metabolism. The exocrine portion is characterized as a highly branched ductal system, which secretes digestive enzymes into the small intestine through the pancreatic duct. *Reproduced from Human Anatomy and Physiology, an OpenStax College resource [14].*

All pancreatic progenitor cells express the transcription factor pancreatic duodenal homeobox-1 (*Pdx1)* [15], which is a key regulator of pancreas development, β-cell differentiation and maintenance of β-cell function in mature β-cells [16]. In mice, development of pancreatic tissue begins at embryonic day (E) 8.5 when expression of *Pdx1* is induced in the endodermal epithelium of the foregut [17]. This is followed by formation of the dorsal and ventral pancreatic buds from the foregut endoderm at E9.5. Expression of pancreas associated transcription factor 1a (*Ptf1),* the key transcriptional regulator promoting exocrine cell specification [18], is initiated at E9.5, with *Pdx1* co-expression from E9.5-12.5 [19]. Both *Pdx1* and *Ptf1a* are essential for appropriate endocrine and exocrine pancreas lineage specification, as animal models lacking these transcription factors demonstrate pancreatic defects such as incomplete branching, expansion and differentiation [18,20]. Each pancreatic bud develops into a highly branched ductal-tree structure of undifferentiated ductal epithelium [21]. By E14.5, both pancreatic buds rotate and fuse into a single organ [21]. Contained within the dorsal and ventral pancreatic buds are multipotent progenitor cells (MPC) forming a multilayered epithelium. The MPCs are able to give rise to endocrine, acinar and duct cells. From E12.5-15.5, the MPCs in the dorsal bud proliferate causing pancreatic bud expansion. Endocrine cells are present from the beginning of pancreatic development arising from MPCs in the gut endoderm by E9.5, while acinar cell clusters differentiate from ductal epithelium and are visible by E14.5 [22,23]. The differentiation of MPCs into either endocrine/ductal or acinar exocrine lineages occurs as MPCs are segregated into either the trunk or tip domains, respectively. The allocation of MPCs to either domain is regulated by the balance of the transcription factors *Ptf1a,* which favors tip formation, and homeobox protein *Nkx6.1,* which induces trunk formation [23,24]. Thus, both endocrine and exocrine compartments in the mouse and human fetus arise from endodermal pancreatic epithelium during development. However, the expression of the transcription factor neurogenin3 (*Ngn3*) plays a major role in the lineage switch that is required for development of all endocrine cell types [25,26].

Expression of the pancreatic hormone, glucagon, occurs as early as E9.5 and is followed by insulin co-expression by E10.5 [21]. Studies have demonstrated an increase in messenger ribonucleic acid (mRNA) encoding endocrine hormones between E14.5-

E20.5, indicating endocrine cell morphological development [27]. On E16.5 the endocrine cells that were previously organized as single cells within the ductal epithelium, become organized as clusters [21]. By E19.5, the endocrine clusters, termed the islets of Langerhans, become regulated by specific transcription factors to produce either alpha (α), β, epsilon (ε), delta (δ) or pancreatic polypeptide/gamma (γ) cells. In both mice and humans, the majority of the islet is composed of α- and β-cells, while the remaining minority of composition of the islet contain δ, γ and ε-cells. Nonetheless, islet composition and architecture vary between these species. In mice, β-cells are localized to the core of the islet, encompassing 60-80% of the islet, while α-cells are contained within the mantle and compose only 10-20%. In contrast, human islets do not display this core-mantle arrangement, with most islet cell types being dispersed throughout the islet. Additionally, in humans, 50-70% of the islet is composed of β-cells while α-cells account for 20-40% [28]. These two endocrine cell types play a critical role in maintaining glucose homeostasis by functioning in an antagonistic manner, whereby the intra-islet hypothesis states that insulin inhibits glucagon secretion [29]. β-cells secrete insulin in response to high blood glucose levels (i.e. fed state), resulting in glycolysis or glucose uptake in peripheral organs and initiating a decrease in blood glucose levels. On the other hand, α-cells secrete glucagon in response to fasting conditions to increase blood glucose levels *via* glycogenolysis in the liver and muscle. In order to maintain glucose homeostasis, islet cells receive information about neighbouring cells through paracrine interactions [29,30]. For example, studies have shown that paracrine intra-islet glucagon signaling is essential for maintaining appropriate secretion of insulin from β-cells [29]. An additional study showed that the pancreatic islet establishes the 'glycemic set-point' in the body [30]. This process relies on paracrine input of neighbouring α-cells in the islet to regulate insulin secretion from β-cells. The transcriptional balance of α- and β-cells is regulated by changes in the expression of V-maf musculoaponeurotic fibrosarcoma oncogene homolog A (*MafA*) and *MafB* transcription factors. *MafA* levels increase in mature β-cells while *MafB* expression becomes restricted to α-cells [31]. In addition to *MafA*, mature β-cells express multiple transcription factors including neurogenic differentiation 1 (*NeuroD*), paired box gene4 (*Pax4*), Homeobox protein Nkx2.2 (*Nkx2.2*), and *Nkx6.1* [19,32]. A transcriptional network listing some of the key

transcription factors involved in endocrine cell lineage development is shown in Figure 1.2. Together with α- and β-cells, islets also contain hormone-producing δ-, γ- and ε-cells. δ-cells produce somatostatin which acts as an important regulator of paracrine inhibition of insulin and glucagon secretion while also controlling gastric emptying [33]. Novel studies have started to explore the mechanisms involved in δ-cell secretion in regulating blood glucose levels in more detail [34–36]. δ-cells have filopodia that enable interaction with many islet cell types despite their low prevalence (~5% of islet cell types) [36]. Somatostatin is released *via* adenosine triphosphate (ATP)-sensitive potassium channels (K_{+ATP} channels) but can also be released in response to glucose stimulation *via* K_{+ATP} channel-independent mechanisms [35]. It is postulated that defective somatostatin secretion can occur in diabetes, warranting continued research efforts to understanding the integrative communication between the multiple islet cell types [35]. Lastly, ε-cells release ghrelin, stimulating appetite and γ-cells produce pancreatic polypeptide in response to food intake proportional to calorie intake, inhibiting pancreas secretions [37,38].

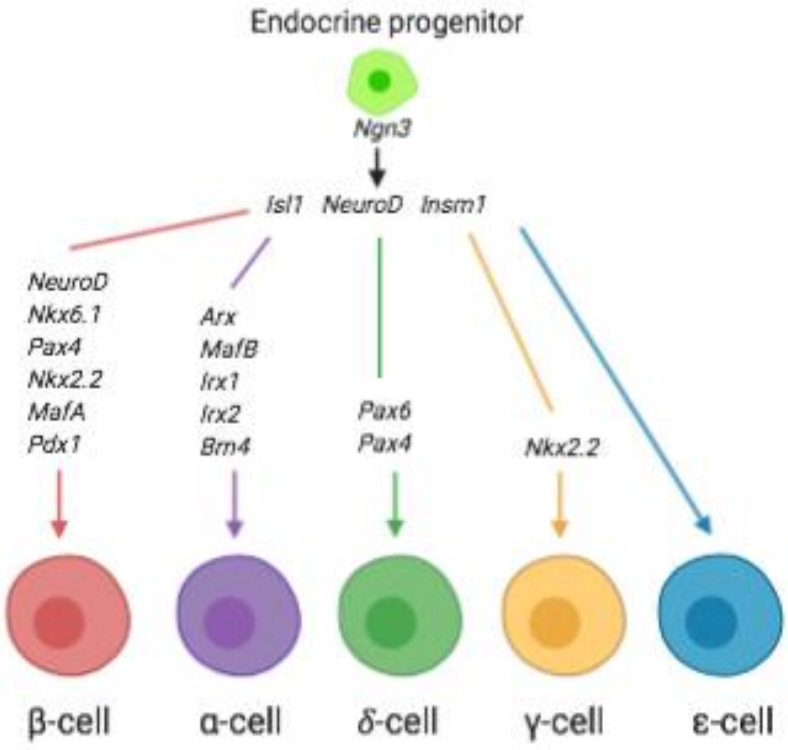

Figure 1.2. Transcriptional pathways involved in endocrine cell specification

Progenitor cells expressing *Ngn3* give rise to all islet cell types. A simplified list of some of the key transcription factors involved in endocrine islet subtype specification are depicted. The expression of different transcription factors ultimately delineates the differentiation of the distinct endocrine cell types. *Figure was created in Biorender*.

In addition to differences in islet architecture between humans and mice, differences also exist in islet development. While in humans islet development is complete at birth, this process continues in mice from E15.5 to postnatally at the end of lactation [39,40]. Islet maturation occurs *via* increased β-cell replication and neogenesis, both of which slow extensively by adulthood [41]. Pancreatic β-cells are considered to be a slowly renewed cell with low turnover in healthy adults. The steady state β-cell replication rate in an adult rat is just over 2% per day [42–44] and this rate is even lower in humans [45]. However, rates of β-cell apoptosis are also low in adulthood, around 0.5% in the rat, allowing for gradual replacement of β-cells and maintenance of β-cell mass (BCM) in adulthood [46]. Thus, BCM is considered to be fairly stable after birth. Nevertheless, the pancreas undergoes extensive remodeling postnatally, which in the rat is characterized by a wave of β-cell apoptosis peaking around postnatal day 14 [41]. This is immediately followed by an additional wave of neogenesis which allows for replacement of β-cells and maintenance of BCM (Fig. 1.3).

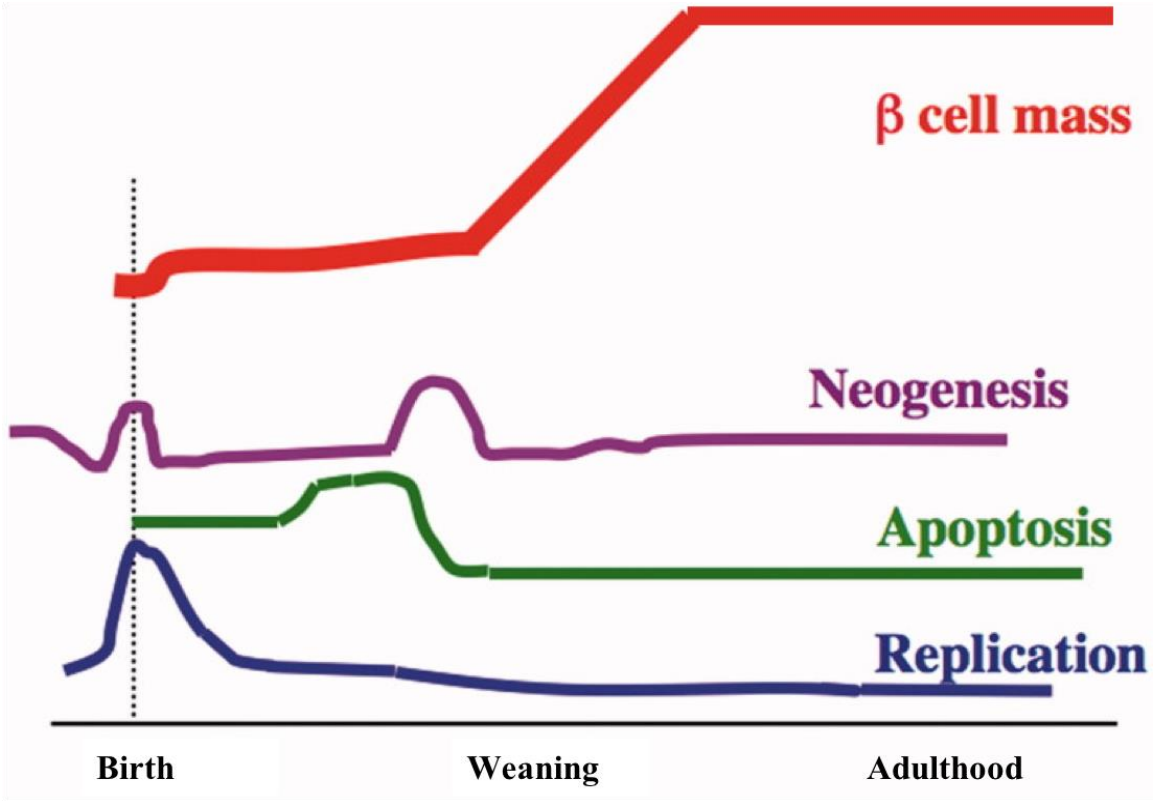

Figure 1.3. Schematic diagram of the dynamic changes in BCM with age

The various determinants of β-cell growth in mice are shown as they change with age. *Reproduced from Bonner-Weir et al. Ups. J. Med. Sci. 2016;121:155-158, with minor revisions.*

1.3 Mechanisms and Dynamics of Insulin Secretion

The first step in the cascade that initiates insulin secretion from pancreatic β-cells involves a glucose molecule entering the β-cell. This occurs *via* glucose-transporter-2, Glut2, which is a transmembrane protein on the β-cell that permits for uptake of glucose across the β-cell membrane, amongst other tissues, in response to high glucose concentrations [47]. An important difference between glucose uptake mechanisms between mice and humans is that human β-cells are able to use both Glut1 and Glut2 to uptake glucose [48]. Nonetheless, Glut1 is considered to be the primary source of glucose uptake in the β-cell in humans [49]. This is in contrast to mice where only Glut2 is used [48]. Once glucose enters the cell, it is phosphorylated by glucokinase (Gck) and converted into ATP *via* multiple steps in glucose metabolism (Fig. 1.4) [50]. The rising ATP:adenosine diphosphate (ADP) ratio triggers the closure of ATP-sensitive K_+ channels, resulting in β-cell membrane depolarization [51]. Depolarization of the β-cell membrane triggers opening of voltage-dependent Ca^{2+} channels and a rapid influx of Ca^{2+} into the cell, triggering exocytosis of insulin-containing granules [11]. To be more effective at reducing blood glucose levels, insulin is secreted in pulses which is postulated to be modulated by oscillations in $[Ca^{2+}]$ [52,53]. Insulin secretion occurs in a biphasic pattern with first phase insulin secretion occurring rapidly within minutes of stimulation and lasting only approximately 2 min [11]. This is followed by second phase insulin secretion, which is considered to be a slow release, but sustained, phase. It is well-established that a loss of first phase insulin secretion and a blunted second phase is characteristic of T2DM [54]. Once insulin is released into the circulation, it can interact with the insulin receptor on peripheral tissues to stimulate glucose uptake *via* the insulin receptor signaling pathway that results in trafficking of Glut4 transport vesicles to the plasma membrane (Fig. 1.4) [55].

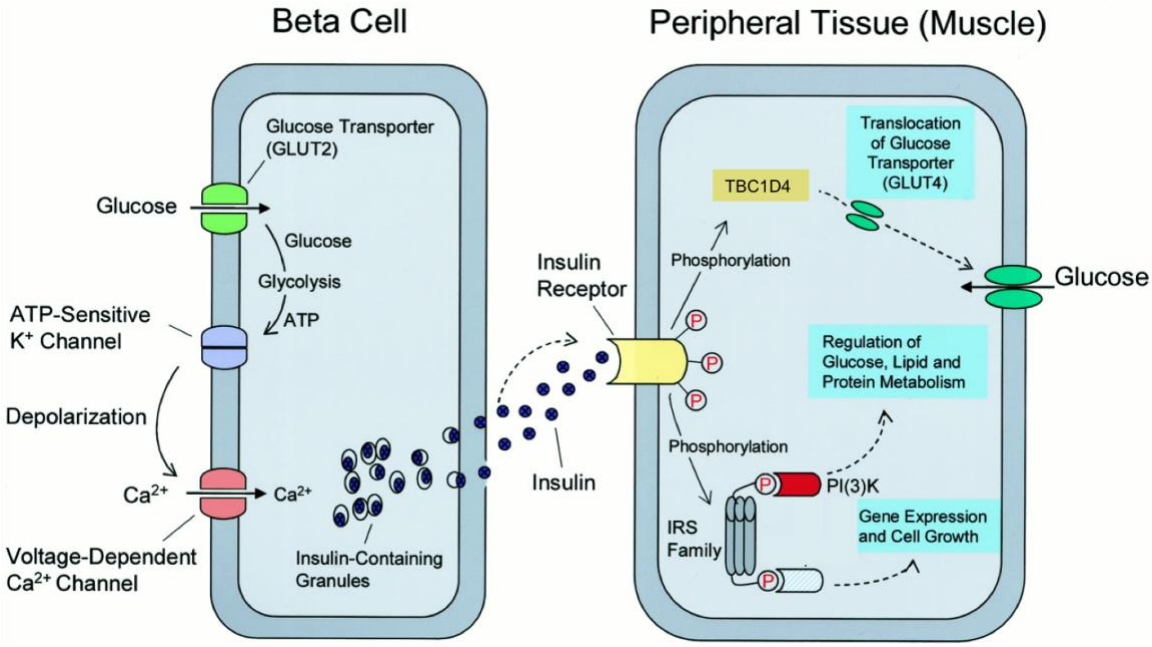

Figure 1.4. Overview of insulin release and action

In mice, glucose enters the β-cell *via* Glut2 and is metabolized *via* glycolysis, generating ATP. The accumulation of ATP in the cytoplasm leads to closure of ATP-sensitive K_+ channels, and depolarization of the plasma membrane. Depolarization of the plasma membrane results in opening of voltage-dependent Ca_{2+} channels. The influx of Ca_{2+} into the cell leads to release of insulin granules, which are carried in the bloodstream to cells throughout the body (i.e. liver, skeletal muscle, adipose) to bind the insulin receptor. Upon binding, autophosphorylation of tyrosine residues occurs in addition to phosphorylation of other cellular proteins, including recruitment of insulin receptor substrates (INSR1/2). This results in recruitment of other proteins, activating various signaling pathways (dashed lines). Ultimately, translocation of Glut4 vesicles to the plasma membrane occurs permitting uptake of glucose into the cell, in addition to activation of pathways that regulate metabolism, transcriptional changes and cell growth. *Reproduced from Abner Louis Notkins J. Biol. Chem. 2002;277:43545-43548, with minor revisions.*

1.4 β-cell Plasticity

As mentioned in section 1.2, β-cell number is considered to remain stable after birth and variations in BCM are minimal in adulthood [56]. Nonetheless, there are many studies that have reported that β-cells display plasticity in injury models and certain physiological situations (i.e. obesity and pregnancy). These findings have drawn interest to understanding the mechanisms of β-cell plasticity and the stimuli for β-cell regeneration in these models. Once elucidated, these mechanisms could be appealing strategies for endogenous pancreatic β-cell replacement for diabetes reversal.

1.4.1 Models to Study β-cell Regeneration

Numerous animal models have been developed where an insult initiates β-cell regeneration and have been pivotal for β-cell plasticity research. Some of these β-cell stresses can be induced by surgical damage (partial pancreatectomy, pancreatic duct ligation), and β-cell destruction (genetic or pharmacological) which will be briefly described below.

Partial pancreatectomy

Partial pancreatectomy involves the removal of 90% of the pancreas resulting in diabetes [57]. In this model, regeneration of both endocrine and exocrine pancreas, in addition to generation of new lobes, was demonstrated in rats [57–59]. These studies were followed by mechanistic lineage tracing experiments that showed that β-cell regeneration occurred mainly through proliferation of pre-existing cells [60], although other studies report that α- to β-cell transdifferentiation could also be occurring [61]. Similar mechanisms might be operating in humans as there is evidence of pancreas regeneration in patients with pancreatectomy [62].

Pancreatic duct ligation

Pancreatic duct ligation (PDL) involves the surgical ligation of the pancreatic duct at the pylorus, resulting in accumulation of exocrine secretions in the body and tail of the pancreas. Studies of pancreas remodelling following PDL in rat have shown the formation of new β-cells from progenitors in ductal epithelium [63] by activation of *Ngn3* which gives rise to all islet cell types [64]. That being said, the origin of these progenitor cells remains controversial (discussed below in section 1.4.3.2) as some experiments suggest that pancreatic ductal cells are not pancreatic progenitors [65–67].

β-cell damage: genetic ablation

Islet cell plasticity has also been demonstrated in transgenic mouse models using *in vivo* cell lineage tracing tools that allow for inducible (doxycycline (DOX) or tamoxifen (TAM) administration) β-cell ablation and tracing of islet cells. In one model, upon DOX administration to transgenic mice (Insulin-rtTA;TET-DTA), targeted ablation of pancreatic β-cells occurred based on driving of insulin promoter and conditional ablation of β-cells in specific transgenic strains. Upon DOX administration in this model, a targeted β-cell loss of 70-80% was observed [68]. In this model, experiments concluded that β-cell regeneration occurred due to replication of pre-existing β-cells. An additional study using TAM administration to investigate β-cell regeneration showed that β-cell proliferation was the driver of regeneration in this model [69].

β-cell damage: pharmacological

The two most commonly used pharmacological agents to induce β-cell ablation for study of β-cell plasticity and regeneration are streptozotocin (STZ) and alloxan. Both drugs enter the β-cell through Glut2 and trigger β-cell death [70]. STZ is a cytotoxic product produced by *Streptomycetes achromogenes* that causes damage to β-cells by entering the β-cell [71]. STZ causes deoxyribonucleic acid (DNA) alkylation and damage ultimately resulting in β-cell death. Alloxan triggers β-cell death by inducing production of reactive oxygen species (ROS). Both models have been widely used to study β-cell regeneration in various animal models [72–77].

1.4.2 β-cell Plasticity with Age

Although there is clear evidence of β-cell regeneration in the many discussed studies above, it is important to note that the capacity for β-cell regeneration varies with age. It has been well-established that β-cell replication declines drastically after birth in both humans and rodents implicating a long lifespan and low turnover rate for β-cells [42,43,56,78–80]. The impairment of this replicative process correlates with the induction of processes preventing the β-cell from re-entering the cell cycle [81,82]. Furthermore, aged islets have been shown to exhibit inflammatory markers, including nuclear factor kappa-light-chain-enhancer of activated B cells (NF-kB), which has been shown to upregulate *socs2*, a gene that inhibits β-cell proliferation [83]. In addition to reduced replicative capacity, some studies in humans show that β-cell function is also impaired with age [84]. The reduced capacity for β-cell regeneration in the adult could also be due to the reduction of multipotent precursor cells, expressing insulin but low levels of glucose-transporter 2, Glut2 ($Ins^{+}Glut2^{LO}$), which have been previously shown to decrease with age in both humans and mice [85].

Regeneration of β-cells following β-cell loss has been shown to occur in young mice [68,86] although studies with aged mice have shown a limited capacity for regeneration [87,88]. Similarly, regeneration might be restricted in non-human primates as a study using STZ-mediated β-cell ablation in middle-aged velvet monkeys did not find a compensatory increase in β-cell replication [89]. Another example in humans showed that young children (between 2 and 9 years old) had complete pancreas regeneration following a pancreatectomy [62]. However, pancreatectomy in adults (39-72 years) did not yield an increase in pancreatic volume [90]. Studies in mice treated with STZ demonstrated that neonates treated with STZ were able to partially regenerate their pancreas. In contrast, mice treated with STZ in adulthood showed reduced regeneration [87]. Elegant studies by Herrera's group investigated the influence of age on islet cell plasticity in β-cell ablated mice and demonstrated β-cell regeneration *via* reprogramming of δ-cells in juvenile mice. Interestingly, this process involved the dedifferentiation of δ-cells, subsequent proliferation and redifferentiation into β-cells which differed from mechanisms that occurred in adults [91]. Taken together, these studies suggest that β-cell

regeneration declines with age in mammals. The reduced replicative capacity of β-cells and reduced β-cell function in aged islets are important considerations to address for regenerative therapies or islet replacement protocols.

1.4.3 Sources of New β-cells From Within the Pancreas

It is important to elucidate the sources of new β-cells in order to be able to better manipulate these populations to increase BCM as a strategy for diabetes reversal. In this section, 5 topics will be discussed including replication from pre-existing β-cells, conversion from ductal progenitors, pancreatic progenitors within the endocrine pancreas, α- to β-cell transdifferentiation, and acinar to β-cell transdifferentiation (Fig. 1.5).

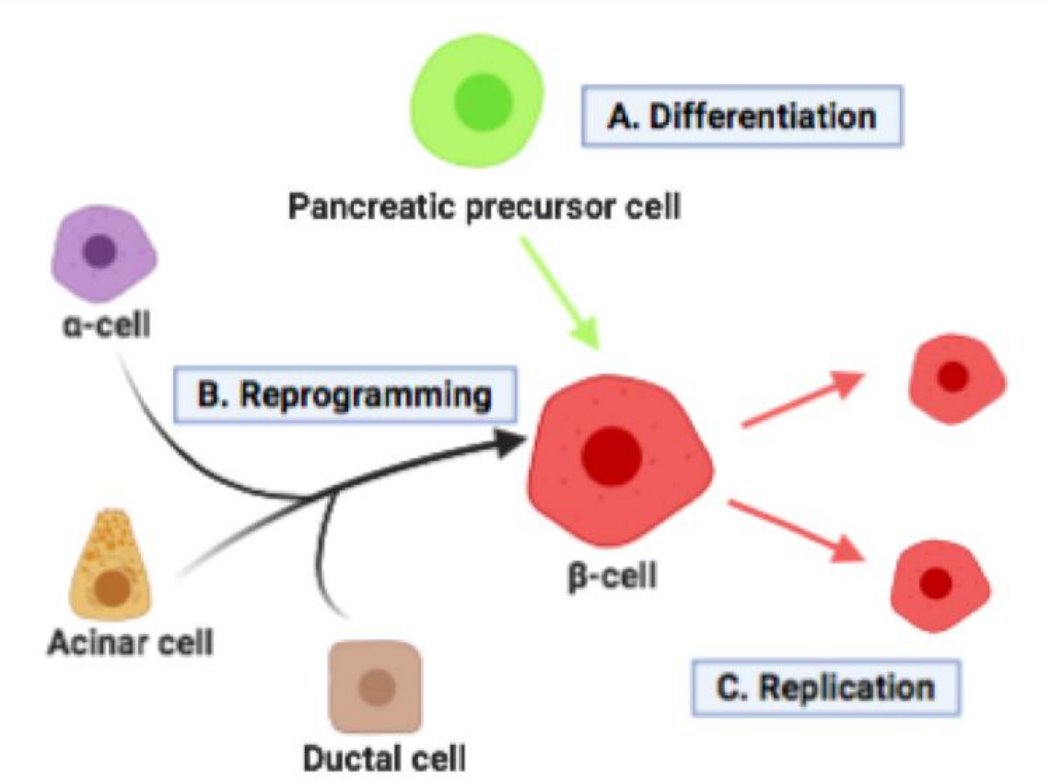

Figure 1.5. Schematic diagram of sources of new β-cells from within the pancreas
Overview of some of the sources of new β-cells from within the pancreas discussed in this thesis. Sources include the differentiation of pancreatic progenitor cells (A), reprogramming of cells from within the exocrine and endocrine pancreas (B), and self-replication of pre-existing β-cells (C). *Figure was created in Biorender.*

1.4.3.1 Replication from Pre-Existing β-cells

As previously mentioned β-cells have a slow cellular turnover. Nonetheless, in stressful situations, such as the injury models described in section 1.4.1, evidence of increased replication from pre-existing β-cells has been documented [60,68]. Additionally, in some physiological situations, such as in pregnancy, increased β-cell replication has also been demonstrated [92]. A milestone study that lineage traced β-cells in young adult mice demonstrated that most β-cells arise by self-replication rather than from a progenitor source [60]. In this study, a pulse-chase experiment was performed using transgenic mice that tagged existing mature insulin-expressing β-cells with human placental alkaline phosphatase following tamoxifen injection. After following the mice for up to a year the authors concluded that the new β-cells were products of self-renewal. Important limitations of this study however arise due to only 30% of β-cells undergoing tamoxifen-induced recombination [93]. Furthermore, tamoxifen-independent recombinase activity poses a technical limitation for mice in these experiments [93,94]. However, additional studies re-affirmed these findings by also detecting no evidence of β-cell neogenesis arising from progenitors to contribute to β-cell regeneration using an innovative DNA double-labelling experiment [95]. In this study the authors also concluded that replication occurred from pre-existing β-cells. In humans, fewer studies have investigated the contribution of β-cell replication, therefore less is known regarding the contribution of this mechanism to β-cell renewal [80,96]. However, there is evidence from pancreas samples that suggests mature human β-cells can proliferate *in vivo* [97]. Thus, manipulation of existing β-cells presents as an attractive strategy to β-cell deficiency reversal.

β-cells possess cell cycle regulators although they are sequestered in the cytoplasm of mature β-cells [98,99]. Transfection of cell cycle regulators into β-cell lines *in vitro* have successfully led to an increase in replicative rate [100]. However, overexpression of oncogenes could increase the risk of carcinogenesis hindering the safety of such therapeutics. Application of growth factors and mitogens, such as growth hormone and placental lactogen, has been shown to increase β-cell replication in mice *in vivo* and *in vitro* [101,102]. Nonetheless, some of these agents have failed to produce a replicative

response in human β-cells. Furthermore, this was accomplished by inducing targeted expression *via* transgene which could result in off-target consequences if administered systemically. Recently, dual-specificity tyrosine-regulated kinase 1A (DYRK1A) was identified and shown to stimulate proliferation of human β-cells *in vitro* and transplanted human β-cells *in vivo* [103–105]. Combined pharmacologic inhibition of DYRK1A and transforming growth factor β superfamily signaling resulted in a synergistic increase in human β-cell proliferation [105]. This occurred due to activation of cyclins and reductions of cell cycle inhibitors. In conclusion, the identification of molecules that stimulate β-cell replication which have reversible effects and are β-cell-specific are needed to optimize strategies of stimulating endogenous β-cell replication as a therapeutic for diabetes reversal.

1.4.3.2 Exocrine Conversion: Differentiation from Ductal Progenitors

An additional endogenous source of β-cell reversal for diabetes would be an existing progenitor population in the pancreas. There are many studies that have implicated pancreatic ductal cells as the source of progenitor cells in the pancreas, dating back to 1911 when it was observed that small endocrine cell clusters were budding from ducts [106]. This was a convincing hypothesis considering endocrine cells and pancreatic ductal cells stem from a common developmental ductal lineage, prior to endocrine lineage delineation by *Ngn3*. An initial study by Xu *et al.* reported evidence of β-cell neogenesis in a PDL model *via* formation of β-cells from *Ngn3*$^{+}$ cells resulting in an increased BCM [64]. Proliferation increased in ductal cells and importantly the *Ngn3*$^{+}$ cells were shown to migrate away from the duct into islet structures. Further evidence of this contribution stems from a study that tagged ductal cells with the Cre-Lox system using carbonic anhydrase II promoter. In this study, new islets were traced back to carbonic anhydrase II-expressing cells as progenitors after PDL [63,107]. These findings were supported by additional pancreas-injury models where β-cell regeneration occurred from pancreatic ducts [23,108,109]. A more recent study importantly showed *via* lineage

tracing that under certain conditions (mild hyperglycemia, gastrin or epidermal growth factor treatment) pancreatic ductal cells can be induced to differentiate into β-cells and reverse diabetes [110]. On the contrary, there are studies that have failed to observe the contribution of ductal cells to β-cell regeneration [65–67,111], which is postulated to be due to potential differences in lineage tracing tools, markers for ductal cells or injury models used. An additional argument is that embryonic-specific transcription factors (*Ngn3/Pax4*) are not expressed during postnatal life, suggesting that postnatal β-cells should arise from an additional source. Nonetheless, convincing data suggesting the therapeutic potential of a ductal progenitor pool has been demonstrated in STZ-treated mice where β-cell regeneration was successful using isolated ductal cells [112,113]. This has also been extrapolated to non-rodents as a recent study showed that β-cell differentiation can occur from ductal progenitor cells in zebrafish [114]. Importantly, human pancreatic ductal cells have been grown *in vitro* and induced to differentiate into glucose-responsive, insulin-producing cells [115]. The authors concluded that the pancreatic ductal epithelium thus serves as a pool of pancreatic progenitor cells [116].

1.4.3.3 Pancreatic Progenitors Within the Endocrine Pancreas

The existence of β-cell progenitors remains one of the most controversial concepts in β-cell biology. Dor's initial landmark study suggested that new β-cells are predominantly generated by self-replication of pre-existing β-cells rather than from new islets arising from a progenitor [60]. Nonetheless, this conclusion remains open-ended as the study does not consider mechanisms of regeneration that can occur in injury models such as the convincing evidence discussed in section 1.4.3.2 and below. In this section, evidence for pancreatic progenitors will be discussed.

A study by Liu *et al.* opposed the work done by Dor, where the same transgenic mouse model was used to track β-cells with age, in addition to STZ-mediated β-cell ablation [77]. In this study, β-cell progenitors were identified that had an immature β-cell

phenotype (lack of *Nkx6.1* and *Glut2*). Importantly, these cells proliferated in STZ-ablated pancreas and were concluded to be a group of progenitor cells with substantial endocrine lineage plasticity. Additional studies have identified putative adult pancreatic stem/progenitor cells in mouse pancreas [117,118]. Suzuki *et al.* isolated progenitor cells with flow cytometry from neonatal pancreas, while Seaberg's study also identified rare single clonal cells from adult mouse pancreas [117,118]. Importantly, these cells were shown to have the capacity to differentiate into functional β-cells and were thus concluded to be a source of multipotent precursors cells in mouse pancreas [118]. Follow-up studies from this group validated Liu's findings that these progenitor cells represent "immature" β-cells, characterized by decreased levels of *Nkx6.1* and *Pdx1*, and lacked *Glut2* [119]. These cells were also found to be capable of proliferation, renewal, and differentiation into multiple endocrine lineages in both isolated mouse and human islet tissues [119]. Importantly, after transplantation into mice with diabetes, both mouse and human pancreatic progenitor cells decreased hyperglycemia in the rodents, demonstrating the therapeutic potential of these progenitors. As discussed in section 1.4.3.2, additional proof for the existence of pancreatic progenitors was shown by lineage tracing studies where one of the origins of the progenitors was suggested to be in ductal cells, as shown by reactivated *Ngn3* expression in endocrine cells [63,64].

Since these landmark studies, additional studies have supported the hypothesis of multipotent pancreatic precursor cells including a progenitor pool expressing insulin but low levels of glucose-transporter 2, Glut2 ($Ins^{+}Glut2^{LO}$). These progenitors have been identified in mouse in addition to human pancreas and have been shown to have the ability to differentiate into mature β-cells under metabolic stress [118,119]. $Ins^{+}Glut2^{LO}$ cells have been shown to decrease with advancing age in both human and mouse pancreas, however they retain a progenitor-type plasticity as they have a higher proliferation rate compared to mature $Ins^{+}Glut2^{HI}$ cells [120]. Thus, these cells may represent a progenitor pool capable of differentiating into new β-cells. An additional recent publication used a marker to identify immature β-cells in a "neogenic niche" at the islet periphery, that are importantly present throughout life [121]. These immature β-cells express insulin, but represent an immature β-cell as they lack key markers (i.e. *Glut2*) including the maturation marker, Urocortin3 [122]. The authors suggested that the

Urocortin-Insulin+ cells represent an intermediate stage in transdifferentiation of α-cells into β-cells (discussed below in section 1.4.3.4). Importantly, Urocortin-Insulin+cells were also identified in human pancreas of varying ages, including donors with T1DM. Although there are inherently going to be challenges in using these populations as a therapeutic, such as the findings that many of these progenitor populations are extremely small, the therapeutic potential of the research has been convincing thus far and could be important for diabetes reversal should the methods be optimized.

1.4.3.4 Endocrine Conversion: α- to β-cell Transdifferentiation

An alternative source for β-cell regeneration for diabetes reversal could be from re-programming of the closely-related glucagon-producing α-cells. After β-cells, α-cells are the most abundant cell type in islets. Importantly, α-cells remain viable in diabetes, and both mice and humans are able to survive without α-cells should existing α-cells be used as a therapeutic [123,124]. As was discussed in section 1.2, the pancreas arises from a common *Pdx1*-expressing progenitor. Upon expression of *Ngn3* in ductal cells, islet lineages develop. Perhaps most interesting in the transcriptional changes in development of specific endocrine cell lineages is the overlap of transcription factors common to both mature α- and β-cells. It was once thought that the development of mature α- and β-cells was static and a definitive lineage. However, studies have discovered that both α- and β-cells are able to interconvert between one another. Remarkable pioneer studies demonstrated that the misexpression of α-cell specific transcription factors, such as *Arx*, in β-cells can result in conversion to α-cells [125]. Conversely, expression of β-cell specific transcription factors, such as *Pax4*, can cause conversion of α-cells into β-cells [126]. Moreover, these new β-cells displayed most characteristics of mature β-cells. On the contrary, β-cells have also been shown to undergo de-differentiation into α-cells, which contributed to loss of BCM in T2DM [127]. The epigenetic chromatin signature of α-cells, resembling stem cells, likely attributes to this remarkable plasticity [128]. Nonetheless, this process has also been shown without genetic manipulation of

transcription factors. One of the first studies demonstrating interconversion between α- and β-cells was reported in 2007 in a β-cell ablated model of diabetes using zebrafish [129]. In this study, the authors lineage traced the regenerated β-cells and found that they arose from a non-β-cell origin located at the periphery of the islet. These studies were followed by lineage tracing experiments to show that α- to β-cell conversion was the main contributor of β-cell regeneration. Interestingly the location of these cells at the periphery of the islet supports the more novel findings of the Urocortin3 study that proposes a neogenic niche where α-cells can convert to β-cells in order to facilitate β-cell regeneration [121]. Additional compelling evidence for this process exists in many models of β-cell regeneration. Studies using a model of extreme β-cell ablation demonstrated β-cell regeneration *via* conversion from α-cells, or δ-cells, depending on the age of the mice [86,91]. The regeneration has been shown to occur even after multiple insults of β-cell ablation and is postulated to arise from a pancreatic ductal cell origin [108]. To compensate for the shortage of α-cells, α-cell neogenesis was stimulated *via* re-activation of *Ngn3* in ductal cells, which enabled subsequent conversion into β-cells upon *Pax4* expression or *Arx* inhibition resulting in a continuous cycle of neogenesis and conversion [108,126,130].

As exciting as these studies are they do have practical limitations as these processes are only observed in extreme and acute models of β-cell ablation, which do not have clinical equivalents. The amount of β-cell loss and injury model used will also determine whether α- to β-cell transdifferentiation occurs and this will further influence the degree of re-programming. Thorel and colleagues showed that β-cell loss must be near total for triggering the re-programming process as in situations of milder β-cell ablation (less than 95%), less α-cell reprogramming occurred and the mechanism of β-cell regeneration was self-replication of existing β-cells [86]. Importantly, an even milder form of β-cell ablation showed no evidence of reprogramming at all. Furthermore, although some studies suggest that this process can occur in humans [131,132], without lineage tracing studies direct evidence is lacking.

Despite these limitations, an endogenous source of β-cell replacement for diabetes, such as the closely related, and increasingly proven to be plastic, α-cell, is appealing if shown

to be feasible. Some molecules have been suggested to promote α- to β-cell conversion including γ-aminobutyric acid (GABA) [132], GLP-1 [133] and artemisinins [134]. Nonetheless, some of these findings remain controversial as a study rebutted the suggestion that artemisinins cause α- to β-cell conversion [135]. Thus, the detailed mechanisms and reasons for differences in these studies should be further addressed. However, continued research efforts to identify stimulators for α- to β-cell conversion are warranted.

1.4.3.5 Exocrine Conversion: Acinar to β-cell Transdifferentiation

As was discussed in section 1.2, the pancreas has both an endocrine and an exocrine portion. In addition to the findings of exocrine ductal cells as a source of progenitor cells discussed in section 1.4.3.2, there is data to support the reprogramming of exocrine acinar cells into insulin-producing β-cells as well [136–139]. Similar to the studies involved in α- to β-cell conversion involving transcriptional manipulation, studies using mouse acinar cells have shown that by expressing β-cell transcription factors (*Pdx1*, *Ngn3* and *MafA*) *via* adenoviral vectors injected into pancreatic parenchyma, new β-cells arose *in vivo*. Furthermore, when these cells were transplanted into rodents with diabetes, hyperglycemia was reduced and importantly recurred upon removal of the graft [138,139]. The new β-cells also resembled a mature β-cell phenotype and have been confirmed to persist for up to 1 year *in vivo* [140]. These results were confirmed *in vitro* using primary human pancreatic exocrine cells cultured in specific conditions (transforming growth factor-B1, Rho-associated kinase inhibitors) that generated cells amongst which 18% were mature, glucose responsive β-cells *in vitro* and *in vivo* [141]. Upon transplantation, these cells were able to prevent diabetes in STZ-β-cell ablated mice. More recently, the same group demonstrated that by suppressing the α-cell specific transcription factor, *Arx*, while simultaneously overexpressing the β-cell specific transcription factor, *Pax4*, there was an enhanced production of functional insulin-producing β-cells from exocrine tissue [142]. When transplanted into mice with diabetes,

there was an immediate and prolonged effect of reduced blood glucose levels. As with previous transdifferentiation studies, some of these studies are nonetheless limited in terms of clinical applications due to the use of viral vectors. Furthermore, the results are still controversial as one publication used *in vivo* lineage tracing after partial pancreatectomy and demonstrated no evidence of acinar to β-cell conversion; rather the authors concluded that new exocrine cells arose from replication of pre-existing acinar cells [143].

In summary, although the topic remains controversial, many studies do provide evidence for multiple alternate endogenous sources of β-cell replacement for diabetes reversal. Most data suggest that β-cells replicate from pre-existing β-cells, although some more severe injury models demonstrate convincing evidence for alternate sources of β-cell re-programming from the other pancreatic cell types discussed above. Importantly, these alternate mechanisms could still be contributing to β-cell replacement, even if the contribution is minor. These mechanisms should be studied further in order to allow for the implementation of targeted therapeutics which increase BCM during pathological conditions characterized by β-cell insufficiency. Translational applications will be limited by the need to produce stable, and functional β-cells. This will be complicated by the need to evaluate the safety of molecules to induce *in vivo* programming of β-cells. Thus, there are many remaining questions, nevertheless the results have tremendous potential to have an influential impact on diabetes research and treatment.

1.5 Metabolic Situations of β-cell Adaptability

In contrast to the models of regeneration discussed in section 1.4.1 that are used to study the sources of new β-cells, in this section, real physiological situations of β-cell adaptability in response to metabolic stress will be discussed. As mentioned in section 1.2, β-cells are considered to be a slowly renewing cell type with low levels of apoptosis and replication enabling for gradual replacement of β-cells to maintain BCM. In contrast, there are compensatory mechanisms that occur in certain physiological situations to rapidly increase BCM. Two such situations where β-cell compensation must occur in

order to maintain euglycemia are the insulin-resistant states of obesity and pregnancy [144].

1.5.1 Obesity

Obesity is described as a pathological condition that involves excess deposition of adipose tissue. It is diagnosed by body mass index (BMI) and fat distribution through the waist-hip ratio. In the context of β-cell biology, obese patients show increased BCM expansion compared to lean individuals [80,145,146] with the increase being from 50-90% [147,148]. Interestingly, one study found a lack of β-cell replication in human samples and the authors suggested that BCM increased *via* neogenesis through differentiation of ductal cells [80]. Two subsequent studies supported this hypothesis, demonstrating a lack of β-cell replication in obese human patients, rather the authors found an increased number of cells coexpressing insulin and a ductal marker, cytokeratin 19, in patients with insulin resistance [80,149]. Interestingly, one study found an increase in the number of bihormonal ($Insulin^{+}Glucagon^{+}$) cells in insulin resistant patients, which could implicate α- to β-cell conversion as a compensatory mechanism for increased insulin demand [149]. Although most reports implicate neogenesis rather than β-cell proliferation to increased BCM expansion in obesity, most authors warrant that the conclusion should be taken with caution. One cannot exclude the possibility that β-cell proliferation was simply too small to be detected, or importantly could occur prior to the insulin resistance manifestation in obesity. Other studies have indeed found evidence of β-cell proliferation contributing to BCM expansion in obesity [150,151]. Thus, it is clear that the exact mechanisms of β-cell expansion in obesity have yet to be delineated. Several downstream effectors of the insulin signaling pathway have been implicated in BCM expansion in animal models of insulin resistance. For example, *FoxM1* activation in islets was shown to increase compensatory β-cell proliferation in obese mice *via* neuronal input [152]. Nevertheless, further elucidation of these mechanisms is warranted.

1.5.2 Pregnancy

An additional metabolic situation requiring successful, and a remarkably reversible, adaptation of pancreatic β-cells is during the insulin-resistant state of pregnancy. There are numerous physiological changes that occur during pregnancy. In humans, one of these changes is driven by the release of placental growth hormone from the placental syncytiotrophoblast, which contributes to a state of peripheral maternal insulin resistance [153]. These changes are also modulated by release of placental lactogen, estrogen, progesterone and other pregnancy hormones [153]. The state of maternal insulin resistance occurs in order to maintain trans-placental transport of glucose to the fetus to ensure optimal fetal development. Nonetheless, in order to compensate for the state of insulin resistance, the maternal pancreas must respond by increasing BCM to maintain euglycemia. As previously mentioned, the steady state β-cell replication rate in adult mammals is low. Remarkably, the rise in levels of the hormones placental lactogen and prolactin during mouse pregnancy have been shown to initiate proliferation of insulin producing β-cells in early pregnancy in order to prepare for adaptation of BCM [101,154]. The insulin resistance is thus countered by an increase in BCM and enhanced insulin secretion which maintains euglycemia in a healthy pregnancy [155,156]. Similar changes in BCM are believed to occur in humans however fewer studies have been performed.

1.5.2.1 Pancreatic Compensation in Mouse *vs.* Human Pregnancy

Pancreatic adaptation in mouse pregnancy has been extensively studied. It is well understood that there is a substantial increase in BCM during gestation in order to compensate for the increased metabolic demand [155,157,158]. In mice, successful adaptation of BCM during pregnancy occurs, in part, due to increased β-cell hypertrophy and proliferation which peak at mid-gestation and are mediated by increased levels of lactogenic hormones [92,155,159]. In mice, placental lactogen-1 is synthesized at early

gestation and peaks on gestational day (GD) 10.5. Mouse placental lactogen-1 is then replaced by mouse placental lactogen 2 which peaks at GD14.5 and remains high throughout the remainder of pregnancy [160]. This is in contrast to humans which only have one placental lactogen (human placental lactogen or human chorionic somatomammotropin) which gradually increases throughout pregnancy. Furthermore, estrogen levels increase during pregnancy which are associated with decreased β-cell apoptosis, suggesting a protective role for β-cells [161]. Collectively, these changes enable for expansion of BCM which peaks towards the latter portion of gestation (GD18.5, in mouse comparable to late gestation in human) [159,162]. Increased GSIS, in part due to a decrease in threshold for glucose stimulation, from β-cells further contributes to the maintenance of euglycemia during the insulin resistant state of pregnancy [155,163,164]. The adaptive increase in BCM is reversible and returns to pre-pregnancy levels after birth through progesterone-mediated increases in β-cell apoptosis [165], concomitant with decreased levels of placental lactogen reducing β-cell proliferation. The mechanisms and timing of these changes in mouse pregnancy are well-established. In contrast, due to a scarcity of pancreas samples from pregnant humans, these adaptive mechanisms in human pregnancy remain unclear.

There have only been two studies exploring changes in endocrine pancreas in human pregnancy. Importantly, both studies found an increase in endocrine pancreas mass in pregnancy thus implicating endocrine adaptation to the metabolic changes of pregnancy in both humans and mice. The first study conducted by Van Assche et al. [166] reported a 2.4-fold increase in β-cell fractional area in pregnant women compared to non-pregnant controls. More recently, Butler et al. [96] found a 1.4-fold increase in β-cell fractional area during pregnancy. Differences in the extent of endocrine pancreas adaptation have been postulated to occur due to varying factors between the two studies (such as women who died in car accidents, women with inflammatory diseases, varying pre-pregnancy BMI, wide ranges of gestational ages). Nonetheless, the studies collectively confirm that β-cell expansion occurs in human pregnancy.

The most controversial studied difference between human and mouse compensatory β-cell mechanisms in pregnancy is in regard to β-cell proliferation and neogenesis. In

addition to differences in distribution and composition of islets between mice and humans [28], adult human β-cells are thought to be very stable and rarely divide [167]. The Butler study found that the increased β-cell fractional area was not due to β-cell proliferation, rather there was an increased number of small islets implicating islet neogenesis as the driver of endocrine pancreas adaptation. In contrast, β-cell proliferation has been shown to peak at mid-gestation in mice driving the compensatory adaptations in endocrine pancreas. Nonetheless, prior to concluding islet neogenesis as the sole contributor to BCM expansion in human pregnancy based on the findings of the Butler study it is important to consider that samples were pooled across all gestational ages. Thus, it is plausible that pooling the samples could have diluted an increase in β-cell proliferation if proliferation occurs in a timing-specific manner such as in mice. Furthermore, it is possible that a much lower rate of β-cell proliferation is sufficient to achieve BCM expansion in humans over 9 months of pregnancy *vs.* 3 weeks in mice which requires a higher rate of proliferation to achieve maximal BCM expansion in a shorter time [154].

Further contributing to the potential difference of β-cell replication as a driver of endocrine pancreas adaptation between humans and mice is the role of lactogenic hormones. In mice, placental lactogen has been shown to drive β-cell replication *via* signaling through the prolactin receptor (PRLR) in pancreatic β-cells [168]. Signaling via PRLR increases serotonin receptor expression, which upon ligand binding further regulates β-cell proliferation and insulin secretion [169]. Studies of lactogen treatment in human β-cells have reported conflicting results, with some studies suggesting that treatment with lactogens increases GSIS and β-cell proliferation [170] in contrast to others which showed a lack of a mitogenic response to lactogens [171]. Differences in humans could be due to lower expression of PRLR on human β-cells than in mice [172]. Evidently there are differences between the behaviour of mouse and human β-cells during pregnancy which require careful consideration when translating animal data to humans. Nonetheless, the scarcity of human pancreas samples in pregnancy poses a challenge to studies in this field.

Although there is evidence to suggest adaptive increases in BCM in pregnancy in both humans and mice, based on current evidence it is likely that the mechanisms leading to

this adaptation differ between mice and humans. Nonetheless, current studies provide clear evidence that both mice and humans rely on compensatory adaptation of β-cells to successfully counter insulin resistance in pregnancy.

1.5.2.2 Mechanisms of Endocrine Compensation During Pregnancy

Since human pancreas samples during pregnancy are sparse, the cellular mechanisms for maternal β-cell expansion during pregnancy have only been possible to decipher in mice. Expansion of maternal BCM in mouse pregnancy is predominantly due to β-cell replication. Adaptation to metabolic demands of pregnancy also involves lowering the threshold for GSIS, β-cell hypertrophy and increased insulin biosynthesis [92,158]. Whether increased GSIS and β-cell proliferation contribute to expansion in humans remains controversial. In this section, the contribution of β-cell progenitors to BCM expansion in pregnancy will be discussed, in addition to unveiling a potential contribution of other islet cell types (Fig. 1.6).

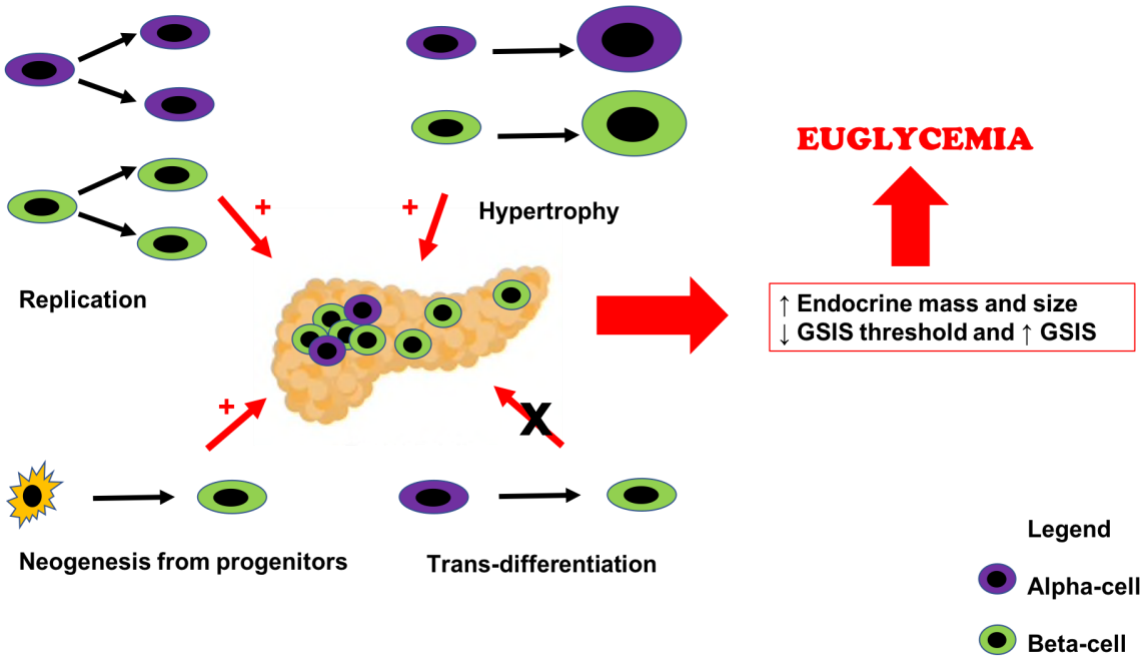

Figure 1.6. Endocrine pancreas adaptations in pregnancy

Beta- and α-cell mass expansion occur in response to increased insulin demand during the insulin resistant state of pregnancy. Endocrine mass expansion occurs due to increased replication, increased hypertrophy of individual cells, and neogenesis from resident progenitor cells. Transdifferentiation of α- to β-cells is also possible. These adaptations maintain euglycemia together with enhanced glucose-stimulated insulin secretion. *Reproduced from Szlapinski et al. Curr. Vasc. Pharmacol. 2020;18.*

β-cell neogenesis

Expansion of maternal BCM in mouse pregnancy is predominantly due to β-cell replication mediated through PRLR signaling, although the source of these cells remains to be determined. Some studies suggest that the majority of BCM expansion occurs through replication of pre-existing β-cells [173–176]. However, there is also evidence *via* lineage tracing that up to 25% of β-cells could arise from non-β-cell progenitors [173,175]. We and others have found an increase in the number of islets during mouse pregnancy which further contributes evidence to this hypothesis [159,177]. Additional studies from our laboratory have shown that the proportion of proliferating multipotent precursor cells ($Ins^{+}Glut2^{LO}$) significantly increased at GD9, which preceded β-cell proliferation at GD12 and facilitated BCM expansion at GD18. The increase in proliferating progenitor cells at GD9 occurred at the same time as an increase in *Pdx1* mRNA expression which is a transcriptional marker for endocrine progenitor and mature β-cells. Thus, these cells may represent a progenitor pool capable of differentiating into new β-cells and in the context of pregnancy are present to facilitate BCM expansion.

Islet cell transdifferentiation

An alternative source of β-cells during pregnancy could be from re-programming of pancreatic glucagon-producing α-cells. The majority of the endocrine islet of Langerhans is composed of α- and β-cells, with the balance being regulated by changes in expression of the *MafA* and *MafB* transcription factors. *MafA* levels increase in mature β-cells while *MafB* expression becomes restricted to α-cells [31]. As discussed in section 1.4.3.5, studies have discovered that both α- and β-cells are able to convert between one another. Thus, an appealing method for regenerating β-cells in situations of β-cell deficiency would be through transdifferentiation of the closely related α-cells.

The lineage-tracing methods used in the studies that failed to detect neogenesis in pregnant mouse models cannot exclude transdifferentiation from other islet types. Thus, it is possible that this process could contribute to a portion of the 25% increase in β-cells from non-β-cell progenitors during pregnancy. Although there was a lack of literature about other islet cell types in gestation in mice, a study was published in 2019 that

investigated the pancreatic α-cell in pregnancy. The study reported that, similar to BCM expansion, ACM expansion occurred in a healthy pregnancy and was maximal at GD18.5 [178]. The authors further concluded that this was due to increased α-cell proliferation which followed a similar mechanism to pancreatic β-cells which proliferate during pregnancy to facilitate BCM expansion. Hypertrophy of α-cells was also observed at GD18.5. Additionally, the study investigated the role of gestational hormones in α-cell adaptations during pregnancy. It was found that in α-tc1.9 cells, placental lactogen and prolactin stimulated α-cell proliferation *in vitro*. This study also claimed that there was a negligible amount of α- to β-cell transdifferentiation occurring in mouse pregnancy, as was postulated to occur in an additional study in 2010 investigating this phenomenon in a healthy pregnancy [173]. However, one must acknowledge that the amount of β-cell loss will determine whether α- to β-cell transdifferentiation occurs and this will further influence the degree of re-programming. Thorel and colleagues showed that β-cell loss must be near total for triggering the re-programming process as in situations of milder β-cell ablation, less α-cell reprogramming occurred [86]. Importantly, an even milder form of β-cell ablation showed no evidence of reprogramming at all. In the case of the healthy animals, there is no loss of β-cells rather an adaptive increase in BCM expansion occurs successfully in pregnancy. Therefore, it is likely that the metabolic stress of pregnancy is insufficient to trigger reprogramming of α-cells and without the stressor of β-cell loss, α- to β-cell transdifferentiation will not occur. In contrast, it is plausible that in a situation of higher metabolic stress in pregnancy, such as in gestational diabetes mellitus (GDM) or in obese pregnancies, α- to β-cell transdifferentiation could occur. In conclusion, this study implicated the importance of other islet cell types in pregnancy that were previously overlooked.

In summary, evidence suggests that the majority of BCM expansion during pregnancy likely occurs due to replication of pre-existing β-cells which is mediated by PRLR signaling of placental hormones. Nonetheless, there is evidence for alternate sources of β-cells. These mechanisms should be studied further in order to allow for the implementation of targeted therapeutics which increase BCM during pathological pregnancies which are characterized by β-cell insufficiency (such as GDM, discussed below in section 1.6).

1.5.2.3 PRLR Signaling-Mediated JAK2/STAT5 Cascade

It is important to understand and identify the mediators influencing adaptive β-cell expansion during pregnancy in order to permit for implementation of targeted therapeutics to reverse this deficiency in pathological pregnancies such as GDM. In this section, some intracellular mechanisms involved in adaptive BCM expansion in pregnancy will be discussed, with many genes being primarily downstream of the lactogens.

PRLR signaling

As previously mentioned, β-cell replication from pre-existing β-cells is the predominant source of BCM expansion in mouse pregnancy. β-cell proliferation is mediated by lactogenic (prolactin and placental lactogen) signaling through the PRLR receptor which is expressed specifically in β-cells in mice [179]. Upon binding of ligand, JAK2 phosphorylates the receptor and allows recruitment and phosphorylation of STAT5 to the nucleus where regulated expression of target genes occurs. The requirement of PRLR signaling in gestational BCM expansion and maintenance of euglycemia was demonstrated in pregnant female mice heterozygous for the PRLR null mutation. These animals were glucose intolerant and had a reduced BCM during pregnancy due to reduced β-cell proliferation [168]. Conversely, overexpression of placental lactogen caused increased β-cell proliferation, and increased BCM leading to hypoglycemia [180].

Signals for adaptive maternal β-cell expansion

The influence of lactogens on adaptive maternal BCM expansion has been well studied and additional studies are discovering various important intracellular signals that mediate these effects. PRLR signaling has been shown to activate multiple signaling pathways in addition to the canonical JAK2/STAT5 pathway, including: mitogen-activated protein kinase (MAPK), phosphatidylinositol 3-kinase (PI3k) and insulin receptor substrate (IRS) 1/2 pathways in order to initiate adaptive BCM expansion [181–185]. Some of these signaling pathways activate cell-cycle proteins and ultimately increase β-cell proliferation. For example, one pathway involves increased expression of tryptophan

hydroxylase (*Tph1*) which is involved in the rate limiting step of serotonin synthesis. Initial studies on the effects of serotonin in islets postulated an inhibitory effect on GSIS. However, recent studies show that serotonin was upregulated in pregnant rat islets and upon inhibition of serotonin synthesis there was decreased gestational β-cell proliferation and BCM expansion resulting in glucose intolerance [169]. Serotonin was also shown to play a role in GSIS [186]. The authors of this study further concluded that serotonin acts downstream of PRLR signaling to drive β-cell proliferation [169]. Another mechanistic pathway involved in mediating an adaptive response in β-cells is *via* suppression of menin by JAK2/STAT5 signaling. In a non-pregnant state menin, a tumor suppressor protein, regulates expression of cyclin dependent kinase inhibitors *p27* and *p18* which inhibit β-cell proliferation by blocking the cell-cycle protein cyclin-D2 (*CCND2*). However, signaling through PRLR during pregnancy decreases levels of menin, which subsequently decreases *p27* and *p18*, enabling *CCND2* to increase β-cell replication and facilitate an adaptive increase in BCM [187,188]. The regulation of menin has been shown to occur due to increased expression of *Bcl6,* a transcriptional repressor of the *Men1* gene [188]. Proof of principle studies showed that when expression of *Men1* was increased in pregnant mice, BCM expansion was impaired due to blocked β-cell proliferation resulting in impaired glucose tolerance during pregnancy.

A second intracellular pathway that signals downstream of JAK2/STAT5 in the PRLR pathway is the PI3k/Akt1 pathway. PRLR signaling acts through this pathway to increase *mTOR* (mechanistic target of rapamycin) signaling which increases β-cell proliferation [185]. The mTOR signaling pathway regulates β-cell proliferation and BCM [189]. Thus, unsurprisingly when this pathway was inhibited by rapamycin in pregnant mice, there were impairments in β-cell proliferation and BCM [190].

1.5.2.4 Transcriptional Regulation of Endocrine Adaptations

The mitogenic response of β-cells in response to pregnancy in mice has been shown to be mediated by changes in expression of transcription factors in the islet. These transcription

factors initiate the processes leading to adaptive β-cell proliferation and BCM expansion in pregnancy. Some of these transcription factors include the orphan nuclear receptor hepatocyte nuclear factor-4 (*HNF-4*)α [191], *Foxm1* [192] and *MafB* [179].

HNF-4α

Mutations in the human ortholog of *HNF-4*α have been shown to cause maturity onset diabetes of the young type 1 (MODY1) [193]. As could be expected, studies in non-pregnant mice lacking *HNF-4*α demonstrated β-cell impairments [194]. It was then shown in pregnant mice that HNF-4α is required for expansion of BCM since upon elimination of HNF-4α from β-cells, proliferation and BCM were reduced leading to glucose intolerance [191].

Foxm1

Mice lacking *Foxm1* have been shown to have a reduced BCM since the transcription factor plays a role in cell proliferation [195]. Unsurprisingly, pregnant mice with pancreatic deletion of *FoxM1* had decreased β-cell replication and BCM contributing to glucose intolerance at late gestation [192]. Inactivation of *FoxM1* prevented lactogen-mediated β-cell proliferation and thus was implicated to be a downstream regulator of lactogens.

MafB

The transcription factor *MafB* is normally restricted to α-cells. Interestingly, during pregnancy in mice, *MafB* expression was induced in a subset of β-cells [196]. Subsequent studies showed that the loss of *MafB* in β-cells decreased gestational proliferation, implicating the transcription factor in gestational β-cell proliferation [179].

Collectively, multiple components discussed in this section are part of the same signaling pathways and are mediated by signaling *via* PRLR. The increase in β-cell proliferation leading to increased BCM and GSIS permit successful endocrine adaptation to counter hormone-mediated insulin resistance that progressively increases during pregnancy. The

importance of mediating an adaptive increase in BCM can be seen in situations of pathology where BCM expansion is suboptimal and can precipitate GDM.

1.6 Gestational Diabetes as a Consequence of Inadequate β-cell Compensation

Although there have been advances in understanding the mechanisms leading to β-cell adaptation during pregnancy there is still much that is unknown about β-cell dysfunction in GDM. However, it appears that a suboptimal increase in BCM is equally as important as a failure to adaptively increase GSIS.

GDM is described as diabetes that first appears during pregnancy with diagnosis occurring around 24-28 weeks of gestation by oral-glucose tolerance test [197]. GDM can develop as a result of severe insulin resistance, insufficient compensation of β-cells and insufficient insulin secretion, leading to maternal hyperglycemia [198]. Thus, GDM occurs due to insufficient β-cell adaptation to compensate for insulin resistance in pregnancy. The incidence of GDM worldwide is around 17% of all pregnancies [197] although a true estimate is difficult to conclude as the incidence will vary depending on the population characteristics and diagnostic criteria. The incidence will only continue to rise as more women enter pregnancy obese or at an older age, both of which are risk factors for GDM [199]. Although obesity increases the risk of developing GDM, many of the women who develop GDM are not obese implicating dysfunction at the level of the β-cell to GDM pathophysiology [199–201]. While GDM reverts after pregnancy in most situations, growing evidence unfortunately associates GDM with adverse maternal and fetal outcomes. In terms of maternal health, GDM can result in pregnancy complications during labour and delivery, and increase the risk of T2DM postpartum [202]. The rates of these manifestations vary ranging between 3% and 90%, nonetheless there is up to a 7-fold increase in risk compared to normoglycemic pregnancies [203]. In terms of the health of the offspring, exposure to GDM *in utero* has been linked to an increased risk of pre-term birth, respiratory distress syndrome, obesity and developing T2DM [204–206]. The increase in incidence of obesity and T2DM observed in children today may be partly

due to the increased cases of GDM. These pathologies in the current generation of youth may lead to further increases in GDM occurrences as they mature, continuing the cycle and implicating the urgent need for a therapeutic to prevent GDM. Currently in Canada, management strategies for GDM involve strict lifestyle management (dietary regulation and exercise) to manage blood glucose levels [197]. If blood glucose targets are not met within 2 weeks, the patient is given insulin or metformin as a treatment to accommodate pancreatic β-cell insufficiency. Thus, dysfunction at the level of the pancreatic β-cell is hypothesized to be the key determinant of GDM pathogenesis.

β-cell defects in GDM

GDM, like most human diseases, is multi-factorial which makes it difficult to determine a specific mechanistic origin. Nonetheless, clinical studies have implicated β-cell failure as a major driver to development of GDM [207] which has been confirmed in animal models of GDM where diabetes occurred when β-cell expansion and β-cell dysfunction failed to compensate for insulin resistance during pregnancy [92,157,168,188,192]. Some factors that contribute to inadequate β-cell compensation and β-cell dysfunction include signaling *via* PRLR, adipokines, inflammation, and oxidative stress. Studies found that the targeted loss of signaling through the PRLR in β-cells of mice resulted in reduced β-cell proliferation and BCM expansion, leading to GDM (Fig. 1.7) [179].

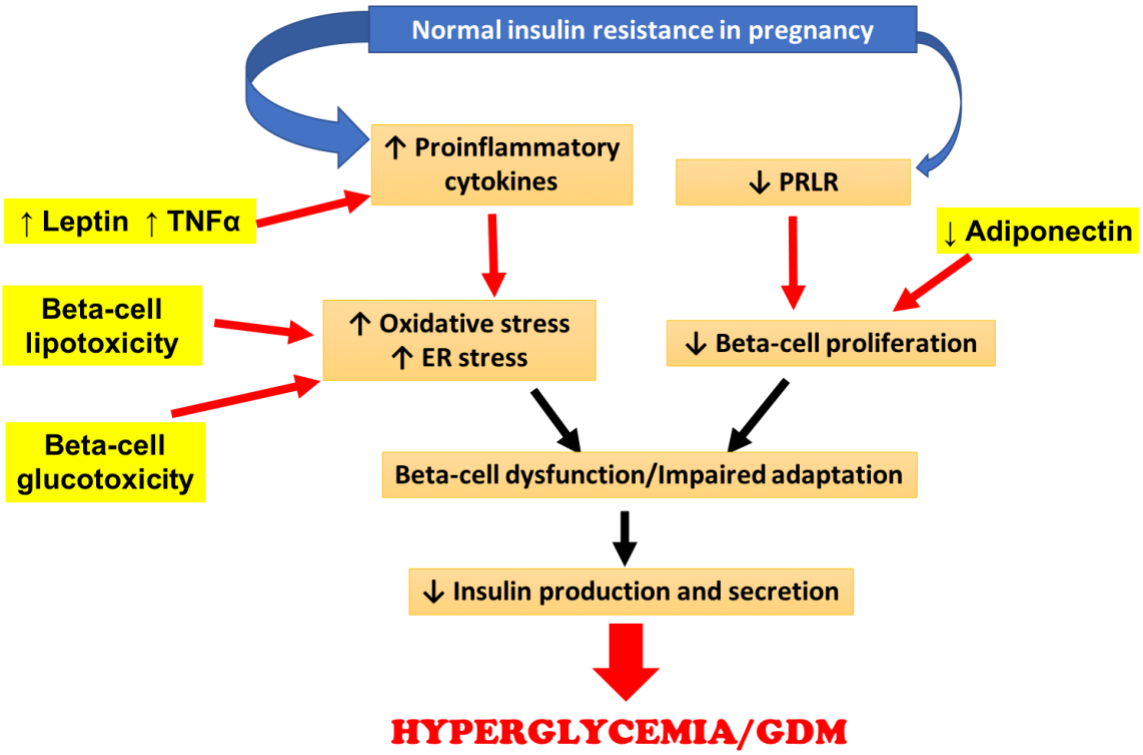

Figure 1.7. Adverse pancreatic β-cell stress during pregnancy can impair adaptation to pregnancy

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Role of inflammation and oxidative stress in β-cell dysfunction in GDM

Cytokines released from adipose tissue influence metabolism during pregnancy; leptin and adiponectin representing two main adipokines that have been shown to be dysregulated in GDM. Changes in adipose-derived adipokines and pro-inflammatory cytokines associated with maternal obesity are likely to exacerbate the risk of β-cell dysfunction during pregnancy, leading to GDM. Circulating leptin, which increases insulin sensitivity, is 2-3 fold higher in pregnancy due to placental as well as adipose expression [208]. Compared to healthy pregnant women, placental leptin expression was increased in patients with GDM and women with high levels of leptin preconception had a 20 times higher incidence of developing GDM [209]. In the context of pancreatic β-cell biology, it has been well-documented that inflammation can contribute to β-cell dysfunction [210]. Inflammation leads to endoplasmic reticulum stress which directly influences β-cell dysfunction in addition to causing decreased insulin sensitivity, as has been documented in GDM patients [211]. Women with GDM have been shown to have increased circulating levels of the proinflammatory cytokines tumor necrosis factor (TNF-α), Interleukin (IL) -1β, and IL-6 which are also associated with β-cell dysfunction [199,212,213]. In one study, human and mouse islets treated *in vitro* with IL-1β showed a reduction in GSIS in addition to β-cell de-differentiation, implicating inflammation to β-cell dysfunction [214]. TNF-α has been shown to contribute to insulin resistance by impairing insulin receptor signaling [104] and observations in women with GDM support the role of TNF-α in the development of insulin resistance [211]. Since leptin increases production of TNF-α, increased levels of leptin in GDM are additive to the pre-existing inflammatory state in late pregnancy that normally contributes to insulin resistance. This increases the metabolic pressure on β-cells to adapt during pregnancy and thus can contribute to the pathophysiology of β-cell dysfunction in GDM. This has also been shown in obesity where both hyper-leptinemia and leptin resistance can impair GSIS and β-cell proliferation [215,216]. In contrast to leptin, adiponectin has been shown to increase β-cell proliferation in mouse islets [217] and was thus, unsurprisingly, shown to influence adaptive BCM expansion in pregnancy. Qiao *et al.* found that pregnant mice with an adiponectin gene knockout had reduced BCM and developed glucose intolerance in pregnancy [218]. Interestingly, the deficiencies were reversed with adiponectin

reconstitution and may be mediated by protection of β-cells against lipotoxic damage [219]. Importantly, hypoadiponectinemia was found and associated with β-cell dysfunction in women with GDM [220]. Collectively these studies implicate inflammation to play a role in dysfunctional β-cell properties in GDM.

Pancreatic β-cell dysfunction can also occur due to oxidative stress, which can be induced in part by chronic hyperglycemia [221]. GDM is also characterized by hyperlipidemia [222] and in the context of β-cell biology, pancreatic β-cells are susceptible to lipotoxicity-induced β-cell dysfunction. Thus, both lipotoxicity and glucotoxicity are contributors to β-cell dysfunction in GDM by causing a buildup of oxidative stress which impairs insulin production and can result in β-cell apoptosis [223].

In conclusion, β-cell dysfunction is one of the key determinants of GDM pathogenesis. Although two studies have looked at β-cells in pregnant humans [96,166], no studies have examined pancreas histology in GDM pregnancies due to lack of human samples and imaging modalities available for *in vivo* examination [224]. Thus, we highly rely on animal models of diabetes in pregnancy to advance our understanding of mechanisms of reduced β-cell adaptability.

1.7 Animal Models of Diabetes in Pregnancy

There are many risk factors for the development of GDM such as being 35 years of age or older or from a high-risk group (Asian, Indigenous, African, Hispanic), in addition to having obesity and GDM in a previous pregnancy [197]. These many factors make it difficult to accurately reproduce the heterogenous pathogenesis of GDM. Nonetheless, many attempts have been made using various approaches that have been reviewed elsewhere [224,225] but will be briefly introduced here. These include: pharmacological, surgical, genetic, and nutritional manipulations.

Pharmacological

One example of a pharmacologically-induced approach for modeling diabetes in pregnancy is *via* STZ-mediated β-cell ablation which can be utilized to portray mild or

severe hyperglycemia depending on the dosage/timing of administration [225]. An advantage of this model is that β-cell destruction can occur rapidly. However, many of these models show severe hyperglycemia which is rarely seen in humans as GDM usually presents as a mild glucose intolerance [224]. Furthermore, the rapid insult does not present true GDM pathogenesis which occurs gradually. An additional limitation is that permanent β-cell destruction and diabetes often remain after pregnancy, which usually does not occur in GDM as symptoms tend to reverse in most women after delivery. Importantly, the β-cell destruction in this model better resembles insulin deficiency and can be described as being more similar to T1DM rather than the progressive insulin resistance and β-cell deficiency that develops during GDM.

Surgical

As discussed in section 1.4.1, one type of surgical manipulation that results in removal of β-cells and has been used to model GDM is partial pancreatectomy. Nonetheless, results in these studies have produced inconsistent findings [224]. Furthermore, this complex technique can result in diabetes onset that can take long to manifest. Importantly, GDM does not occur due to a sudden insult and similarly to the pharmacological manipulation, the GDM phenotype described here better resembles T1DM.

Genetic

The db/db mouse is characterized by a mutation in the leptin receptor gene and is used for studying obesity [226]. Although homozygous females (db/db) are sterile, heterozygotes are fertile and importantly non-pregnant females do not show glucose intolerance [227,228]. During pregnancy, females display increased adiposity contributing to insulin resistance and mild glucose intolerance [228,229]. Thus, this model accurately mimics many features of human GDM. Although obesity is a major driver of GDM, some features of this model better resemble the increased adiposity that is observed in some cases with T2DM. It is important to consider that non-obese individuals develop GDM as well, thus implicating dysfunction at the level of the β-cell rather than due to obesity alone. Furthermore, the use of genetic models is limited for translational use in larger

animal models and simplifies the human condition as GDM is influenced by many genes and environmental factors [224].

Nutritional

Animal models using high-fat diet (HFD) feeding have been used to mimic GDM symptoms where HFD feeding before and during pregnancy results in a GDM phenotype by late gestation in rats [230]. Experiments in mice have reproduced these findings showing that HFD feeding prior to and throughout gestation results in elevated blood glucose and insulin levels during pregnancy [231]. However, the phenotype was present prior to pregnancy in these experiments and thus does not accurately represent the human phenotype. This method has several advantages to modeling the disease, since obesity is a major driver of GDM in humans, and permits for study in larger animals where genetic manipulation is unfeasible [224]. Nonetheless, similarly to the db/db mice, this phenotype better resembles the T2DM condition as the animals show increased adiposity and insulin resistance. Therefore, existing pre-gestational diabetes/obesity is more likely driving the disease in this model which does not take into consideration that lean women also develop GDM.

Although each model presents both advantages and limitations, an important concept of GDM pathogenesis that each model lacks is the progressive development of transient hyperglycemia during pregnancy that is restricted to late gestation and reverts after pregnancy. Thus, continued efforts to produce an accurate model of GDM characterized by defects in β-cell adaptability with restricted hyperglycemia to late pregnancy are needed in order to be able to implement novel methods of intervention. One animal model that has been well-characterized and shown to impact β-cell plasticity is the low protein model of fetal programming.

1.8 Low Protein Model

The hypothesis of fetal programming of adult diseases first formulated by Professor Sir David Barker proposes that the intrauterine environment during development can influence the risk of metabolic diseases later in life in the offspring [232]. More specifically, nutrient availability during fetal and early postnatal life plays an important role in determining adult health. Metabolic disturbances during these critical developmental timepoints, such as dietary restriction, contribute to the development of adult chronic diseases such as T2DM, obesity, and cardiovascular disease [233]. After the hypothesis was formulated, Snoeck and colleagues demonstrated that a low protein (LP) diet during gestation in rats (in comparison to a control, C, diet) resulted in reduced birth weight (intra-uterine growth restricted, IUGR) offspring [234]. The LP diet was made isocaloric to the C diet *via* increased carbohydrate. IUGR is described as the failure of a fetus to achieve its genetic potential for size which in clinical terms would be below the 10_{th} percentile for gestational age [235]. When the dams were maintained on a LP diet during lactation, body weight of offspring was reduced until weaning [236]. IUGR affects the development of multiple organs, including the pancreas [236,237]. Using the established model of dietary protein restriction during pregnancy and lactation, it has been extensively published that dietary insufficiency in early life alters normal pancreatic development in the offspring, which ultimately contributes to impaired glucose homeostasis in adulthood. We found that maternal protein restriction altered cell-cycle kinetics in offspring by increasing the incidence of β-cell apoptosis and decreasing the proliferative rate of β-cells, ultimately resulting in a reduced BCM [236,238,239]. Although offspring of LP-fed dams displayed impaired GSIS, glucose intolerance did not manifest until 130 days of age in female rats [240,241]. In females, glucose intolerance was attributed to decreased BCM. Interestingly, the males in this study displayed insulin resistance in adipose and skeletal muscle in contrast to the reduced BCM observed in females. These findings have also been supported in additional larger animal IUGR models, as in sheep decreased β-cell replication was also shown to result in reduced BCM, in addition to β-cell dysfunction, leading to decreased GSIS [242–244]. Furthermore, IUGR human fetuses have also been shown to have decreased BCM [245].

More recently, β-cell plasticity was examined in mice treated with LP and STZ [246]. The results showed that control fed offspring had largely regenerated their β-cells and replaced BCM after STZ, since young mice normally show a regenerative capability following β-cell loss. Nonetheless, LP exposure limited the capacity for recovery of BCM in both males and females after STZ treatment. In the same study, there was a delayed ability to increase α-cell mass (ACM) implying that mechanisms involved might be common to multiple endocrine cell types. In addition to histological and functional differences in endocrine cells, pancreatic vascularity and signaling between β-cells and endothelial cells has been shown to play a role in β-cell dysfunction of IUGR fetuses. Lower pancreatic islet vascularity has been observed in IUGR humans [245] and animal models of IUGR [247,248]. Importantly, islet size and GSIS was limited by vascular supply [248,249]. Furthermore, expression of angiogenic factor vascular endothelial growth factor A (VEGFA) was decreased in LP rats [234,236]. VEGFA is important in islet development and β-cell function. Proof of concept studies showed that gestational taurine supplementation in the LP rat prevented the decrease in fetal islet vascularity and VEGFA expression [237]. β-cell apoptosis was also attenuated in taurine-supplemented LP rats compared to non-supplemented, resulting in higher BCM [250].

Collectively these studies demonstrate strong evidence for impaired β-cell development and plasticity after exposure to the LP diet during development, which is potentially reversible.

1.8.1. Relevance to Humans

One model that represents the effects of famine on fetal development is the calorie restriction model. However, the effects of famine on fetal development are of lesser concern in North America. In contrast, the LP model shares common features to human placental insufficiency. Placental insufficiency is a major cause of IUGR in North America and complicates 4-8% of pregnancies [251]. Similar to the LP diet model, placental insufficiency in humans can produce a protein deficiency in the fetus [252].

Since placental insufficiency results in both decreased oxygen and nutrient delivery, the LP model permits for differentiation of the specific effects of amino acid deficiency [252]. Importantly, the LP diet has no major effects on maternal physiology (including maternal food intake and weight gain) and no effect on offspring food intake [253]. These findings are important as altered food intake in the mother could add confounding variables to the model and is in contrast to the caloric restriction model which adversely affects both maternal and fetal physiology.

1.9 Rationale, Objectives, Hypothesis

Rationale

GDM seriously impacts the short and long-term health of both the mother and her child. Unfortunately, there is a lack of effective methods for prevention/treatment to reverse β-cell insufficiency in GDM. Given that rates of GDM are increasing, and the *in utero* environment is an important determinant of adult health, it is important to investigate the underlying mechanisms of GDM. The mechanisms leading to GDM are poorly understood and β-cells of pregnant humans with GDM have yet to be analysed. Furthermore, changes in endocrine pancreas of humans with GDM cannot be viewed *in vivo* due to lack of imaging modalities available at present for pregnant humans. However, the reliance on animal models is hindered as models that accurately represent symptoms of GDM are currently lacking. These models present multiple limitations such as demonstrating pre-gestational glucose intolerance and obesity which is not a true diagnosis of clinical GDM, while others better resemble T1DM or T2DM pathogenesis as opposed to the characteristics of insulin resistance seen in conditions such as GDM. Furthermore, these models importantly lack the transient hyperglycemia that is diagnosed at late gestation. Given that β-cell dysfunction is a key determinant to GDM pathogenesis, a better model characterized by impairments of reduced β-cell adaptability is needed so that targeted methods of intervention can be implemented. The LP model

has been well-characterized, and much evidence supports altered pancreatic β-cell development. However, no studies have investigated the plasticity of β-cells during a subsequent time of increased metabolic stress, such as pregnancy. Thus, we sought to use this model in an attempt to produce an animal model of reduced β-cell adaptability in pregnancy that can be used to better understand β-cell insufficiency in GDM.

Hypothesis

It is hypothesized that exposure to LP diet during fetal and neonatal development will impair β-cell adaptability in pregnant F1 females. This will result in glucose intolerance during pregnancy, which can be reversed with treatment.

Objectives

1. Establish a mouse model of impaired glucose tolerance during pregnancy

2. Determine the long-term effects of GDM on glucose tolerance and pancreas histology after pregnancy

3. Investigate the contribution of α- to β-cell transdifferentiation to gestational BCM expansion

4. Test strategies to improve glucose tolerance in pregnancy through the manipulation of BCM

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Chapter 2

2 A Mouse Model of Gestational Glucose Intolerance Through Exposure to a Low Protein Diet During Fetal and Neonatal Development

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2.1 Introduction

Gestational diabetes mellitus (GDM) is a form of diabetes that develops during pregnancy and regresses postpartum. Between 3%-20% of women develop GDM, depending on their risk factors [1]. GDM increases the risk of the mother developing subsequent type 2 diabetes mellitus (T2DM) by up to 7-fold compared to euglycaemic pregnancies [2]. For the offspring, exposure to GDM *in utero* has been linked to an increased risk of childhood obesity and development of T2DM [3].

GDM develops due to insufficient insulin secretion during the relatively insulin-resistant state in pregnancy [4]. The state of peripheral maternal insulin resistance is most prominent during the third trimester when placental growth hormone and placental lactogen levels are highest [5,6]. This ensures normal fetal development by maintaining trans-placental flux of glucose to the fetus. Consequently, maternal euglycaemia is normally maintained through adaptations of β-cell mass (BCM) in maternal pancreas.

Both mouse and human β-cells replicate at a low rate in adulthood (~2% per day) [7,8]. However, the rise in circulating placental lactogen and prolactin during mouse pregnancy has been shown to trigger proliferation of β-cells around gestational day (GD) 12, which increases BCM and enhances insulin secretion [9,10]. In mice, BCM increases *via* β-cell replication and hypertrophy, reaching maximal levels towards late gestation [10,11]. Elevated maternal estrogen levels during pregnancy protect β-cells against apoptosis [12]. As estrogen levels drop after parturition, β-cell apoptosis increases [13] while β-cell proliferation decreases [11,14] returning BCM to pre-pregnancy levels. The compensatory changes in human BCM remain controversial as the dynamics of BCM expansion are hypothesized to be slightly different than in mouse [15]. Nonetheless, the only two human studies conducted to date have both reported an increase in β-cell area in pancreata of pregnant women at post-mortem [16,17]. These data suggest that the pancreas of humans, like mice, should be able to increase BCM and enhance insulin secretion during pregnancy. Consequently, in situations where BCM expansion is suboptimal, GDM can arise [18]. Thus, murine GDM models characterized by alterations to BCM may relate to the pathology in humans as both animal models and genome-wide

association studies in humans implicate β-cell dysfunction as the largest determinant to GDM pathogenesis [19].

There is currently no reproducibly effective prevention or reversal intervention for GDM. As rates of GDM are on the rise, this poses a threat to both the long- and short-term health of the mother and her offspring. Non-invasive imaging to analyse expansion of BCM in human pregnancy has ethical and technical issues making animal models a desirable alternative for studying the mechanisms leading to GDM. Although there are inevitable differences between mouse and human gestation, these differences are well characterized; consequently, mice are considered to be valid models for studies of pregnancy pathophysiology. One important similarity between mouse and human gestation is the fact that both the extent of β-cell dysfunction and insulin resistance play an important role in determining metabolic dysfunction in human and animal models [19]. A useful animal model of GDM would not demonstrate pre-gestational diabetes but show abnormal glucose tolerance as pregnancy progressed. As there are currently few clinically applicable animal models of GDM [20] that meet these criteria, we sought to develop a mouse model relevant to the clinical characteristics of GDM through dietary insult.

It has been established that maternal (F0) dietary protein restriction (low protein (LP) diet) during early life has long-term effects on the endocrine pancreas of the offspring (F1), which contributes to glucose intolerance in adulthood [21]. Offspring born to dams fed a LP diet have reduced BCM as neonates resulting from decreased rates of β-cell proliferation and increased apoptosis [22]. LP-exposed offspring also have impaired β-cell insulin release which further contributes to glucose intolerance in adulthood [23]. Using this well-characterized model, we examined whether female offspring (F1) of LP diet-fed dams (F0) would develop glucose intolerance during pregnancy, and whether this was associated with an altered adaptation of BCM in maternal endocrine pancreas and/or insulin secretion in isolated islets of Langerhans. We hypothesized that female offspring (F1) of LP diet-fed dams would be glucose intolerant during pregnancy as a result of impaired β-cell plasticity and reduced insulin secretion.

2.2 Methods

2.2.1. Ethical Approval

All animal procedures were approved by the Animal Care Committee of Western University in accordance with the guidelines of the Canadian Council for Animal Care (Approval #2018-027).

2.2.2. Animals

Adult (6-week-old) C57BL/6 male and female mice were obtained from Charles River Laboratories (Wilmington, MA, USA) and housed in a temperature-controlled room with 12-h light/dark cycle at Lawson Health Research Institute, London, ON, Canada. Water and food were given *ad libitum*. Timed pregnancies were accomplished by establishing mouse estrous cycling [24]. Individual female and male mice were housed together for mating and separated the following morning. Day zero of pregnancy was determined by identification of a vaginal plug. Females were housed individually for the remainder of pregnancy. F0-females were randomly allocated to either a control (C, 20% protein, Bio-Serv, Frenchtown, NJ, USA) or low protein diet (LP, 8%) group (Fig. 2.1A), where an increase in carbohydrate in LP diet (Table 2.1) yields an isocaloric diet compared to control chow [25]. F0-dams were fed either the LP or C diet throughout gestation and lactation. A total of 24 control and 21 LP litters were used for the study. Since the primary objective of our study was to produce a novel mouse model of GDM we worked only with female offspring. On postnatal day (PND) 21, all female offspring (F1) were weaned onto C diet for the remainder of the study while males were euthanized (Fig. 2.1B). At maturity (PND42), female offspring (F1) born to dams fed either a C or LP diet were randomly allocated into 2 subsequent study groups: pregnant (CP, LPP) or non-pregnant (CNP, LPNP, Fig. 2.1A). All pregnant grouped females were time-mated with C diet-fed males, separated the following morning and housed individually for the remainder of the experiment. Dams were euthanized by CO_2 asphyxia following an intra-peritoneal glucose tolerance test (IPGTT) on a randomly assigned day of gestation (GD9, 12, or 18) for comparison to non-pregnant age-matched F1 females. Maternal (F1) blood

was collected *via* cardiac puncture following the IPGTT and serum insulin and glucagon quantified using an Ultra Sensitive Mouse Insulin ELISA kit (Crystal Chem, Downers Grove, IL, USA) or Mouse Glucagon ELISA kit (Crystal Chem, Downers Grove, IL, USA). Pancreata were removed for fixation in 4% paraformaldehyde and sectioned for histology as previously described [26]. At least three 7 μm-thick cryosections (replicates) were cut from each pancreas with an interval between each section >100 μm.

Table 2.1. Composition (g/100g of diet) of control vs. low protein rodent chow (Bio-Serv, Frenchtown, NJ, USA)

Component	Control	Low Protein
Cornstarch	40.0	40.0
Casein (88% protein)	22.3	8.6
Maltodextrin	13.2	13.2
Sucrose	10.0	23.6
Soybean oil	4.5	4.5
Cellulose	5.0	5.0
Mineral mix	3.5	3.5
Vitamin mix	1.0	1.0
L-Cystine	0.3	0.3
Choline Bitartrate	0.25	0.25
Tert-butyl hydroquinone	0.0014	0.0014

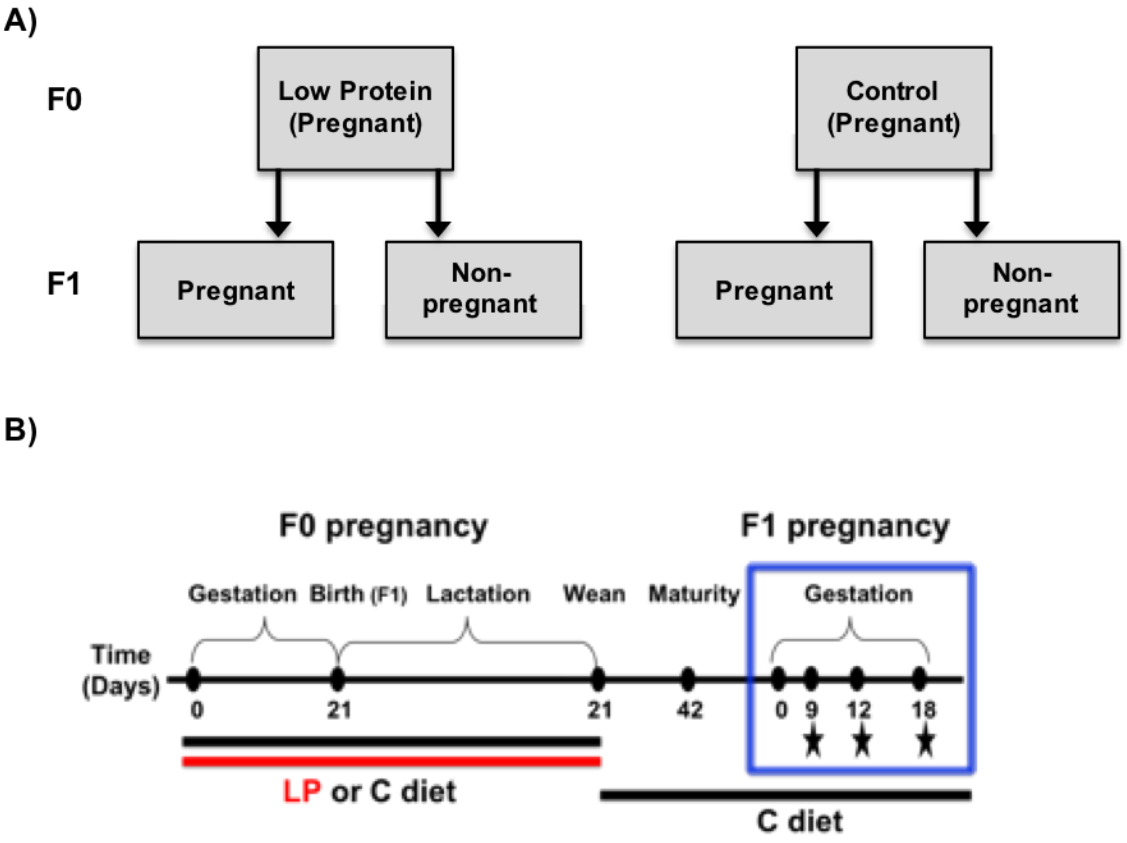


Figure 2.1. Murine model of gestational glucose intolerance

A) Schematic flow chart of experimental groups. F0 females were allocated to LP and C diet groups. F1 female offspring were separated into pregnant, CP and LPP (gestational day 9, 12, 18) and non-pregnant groups (CNP and LPNP). **B)** Timeline for treatment and sample collection. The F1 offspring were exposed to the low protein (LP) or control (C) diet during gestation and lactation and weaned onto control diet. At maturity, F1 females were time-mated with control-fed males. Stars demonstrate timepoints where an intra-peritoneal glucose tolerance test was performed prior to euthanasia and removal of the pancreas for histology (n = 4−7 animals for each group). At each timepoint, serum was also collected.

2.2.3. Glucose Tolerance Test

An intra-peritoneal glucose tolerance test (IPGTT) was performed on all animals in their home cage prior to euthanasia as previously described [27]. For the F0 mice, this was one month after parturition while the IPGTT's for the F1 mice were performed at the assigned gestational day or age for the age-matched group. Mice were fasted for 4-h with free access to water, injected intraperitoneally with 5µl·g^{-1} body weight of 40% glucose solution (2g·kg^{-1} body weight glucose, Sigma Aldrich, St. Louis, Missouri, USA), and blood glucose measured from the tail at 0, 5, 15, 30, 60, 90, and 120 min using a One Touch Ultra2 glucometer. Area under the glucose tolerance curve was analysed using GraphPad Prism software (Version 5.0, La Jolla, CA, USA).

2.2.4. Immunohistochemistry

Immunofluorescence immunohistochemistry was performed to co-localize insulin and Ki-67 as a marker for insulin-immunopositive cells undergoing proliferation as previously described [10]. Slides were viewed by a blinded technician using a Zeiss fluorescence Axioskop microscope and cell counting analysis was performed using Image J [28]. Every insulin-expressing cell was imaged at 20X and counted manually. In this study, an "islet" was considered to contain >5 β-cells, and an extra-islet "cluster" as containing 1-5 β-cells.

Immunofluorescence immunohistochemistry was also performed to localize insulin (β-cells) and glucagon (α-cells) for morphometric analysis. Antibodies against insulin (1:2000, anti-mouse, Sigma-Aldrich, St. Louis, MO, USA, Cat. No:I2018, RRID:AB_260137) and glucagon (1:200, anti-rabbit, Santa Cruz, Santa Cruz, TX, USA, Cat. No:NB110-41547, RRID:AB_805593) were applied to cryosections and incubated overnight at 4°C. The following day, secondary antibodies (1:500 ThermoFisher, Waltham, MA, USA) were applied against the primary antibody using 555 (Cat. No:A-31570, RRID:AB_2536180) and 488 fluorophores (Cat. No:A-21206, RRID:AB_141708), respectively, along with DAPI (4, 6-diamidino-2 phenylindole, dihydrochloride, 1:500, ThermoFisher, Waltham, MA, USA, Cat. No:D3571,

RRID:AB_2307445) to counterstain nuclei. Alpha-cell mass (ACM) and BCM were calculated from at least 2 sections (replicates) per pancreas (n = 4−7 C and LP animals per timepoint) as previously described [10]. β-cell size was calculated by taking the sum of the traced insulin-expressing area and dividing by the total number of β-cells counted for that section. Islets were counted per tissue section and further separated by size into small (less than 5000 μm^2), medium (between 5000 and 10,000μm^2) and large islets (more than 10,000 μm^2). Tissue represented both the head and tail of the pancreas [29].

2.2.5. Islet Isolation and Static Insulin Secretion

Pancreatic islets were isolated from CP (n = 7−9 animals) and LPP (n = 6−8 animals) pancreata on GD18 by collagenase V (Sigma-Aldrich, St. Louis, MO, USA) digestion using a modified sequential Dextran gradient protocol [30,31]. Islets were incubated (37°C) overnight in RPMI media containing 6.5 $mmol \cdot L^{-1}$ D-glucose, 10% fetal calf serum, and 1% Penicillin Streptomycin. The following day, islets were pre-incubated in Krebs buffer solution (KRB's, 119 $mmol \cdot L^{-1}$ NaCl, 4.7 $mmol \cdot L^{-1}$ KCl, 25 $mmol \cdot L^{-1}$ $NaHCO_3$, 2.5 $mmol \cdot L^{-1}$ $CaCl_2 \bullet 2H_2O$, 1.2 $mmol \cdot L^{-1}$ $MgSO_4 \bullet 7H_2O$, 1.2 $mmol \cdot L^{-1}$ KH_2PO_4, 1% bovine serum albumin, 10 $mmol \cdot L^{-1}$ Hepes) containing 2.8 $mmol \cdot L^{-1}$ glucose for 1-h at 37°C. Groups of 10 islets of similar size were collected into 1 mL of KRB's containing either 2.8 $mmol \cdot L^{-1}$ or 16.7 $mmol \cdot L^{-1}$ glucose (1−3 replicate tubes per animal). Insulin release was determined in the supernatant after 90 min at 37°C using an Ultra Sensitive Mouse Insulin ELISA kit (Crystal Chem, Downers Grove, IL, USA).

2.2.6. Statistical Analysis

The sample size of 4−7 animals per variable in either the LP or control groups was calculated based on achieving a statistically significant difference with an expected standard deviation around mean values for BCM and glucose tolerance of 15% or less based on our previous studies [10]. Each animal presented as a single unit of analysis (n) for the experiments with the F0 dams and F1 offspring. For comparisons of litters, each n represented an average value for each litter. Data are presented as mean±SEM, with statistics analysed using GraphPad Prism software (Version 5.0, La Jolla, CA, USA). An

unpaired two-tailed Student's t-test was used to compare treatment groups (LP *versus* C). A two-way ANOVA followed by Bonferroni's post-hoc test was used for comparison between treatment groups (LP *versus* C) at each timepoint during gestation. A repeated measures two-way ANOVA followed by Bonferroni's post-hoc test was used for comparison of IPGTT curves between treatment groups (LP *versus* C) at each timepoint during gestation. Animals with fewer than 2 fetuses or more than 8 were excluded from statistical analyses. Statistical significance was determined as $P < 0.05$.

2.3 Results

2.3.1. F0 Animals

No differences were found between consumption of control or LP diet throughout gestation. Maternal weight gain increased steadily in both control and LP diet-fed dams throughout gestation (Fig. 2.2A). Furthermore, LP diet consumption had no effect on litter size (Fig. 2.2B). There were no differences in the number (Fig. 2.2C) or ratio (Fig. 2.2D) of males to females born to LP *versus* C diet-fed dams. To test for a possible impact of diet on glucose homeostasis, an IPGTT was performed at 1-month postpartum. No differences were found between the IPGTT curves (Fig. 2.2E) or area under the glucose tolerance curves (AUC, Fig. 2.2F) of LP diet-fed females compared to control diet-fed females.

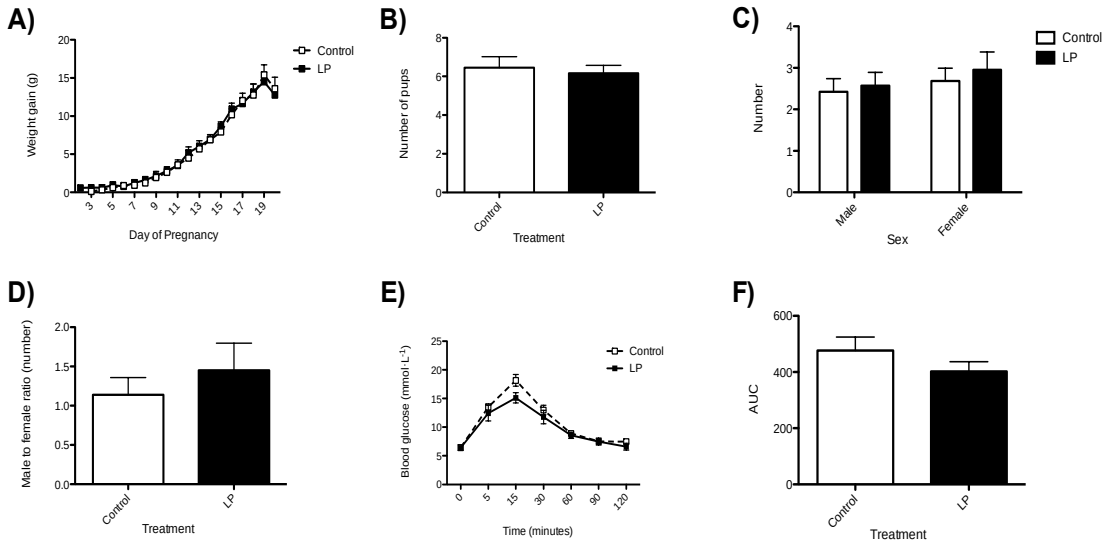


Figure 2.2. Low protein diet during pregnancy does not alter pregnancy characteristics of F0 dams

A–C, low protein (LP) and control (C) diet-fed F0 dams did not statistically differ in mean values for maternal weight gain (A) (n = 13−14 animals for each group), litter size (B) (n = 24 C litters, 21 LP litters), the number of male and female offspring (C) (n = 19 C litters, 20 LP litters), or the ratio of male to female offspring (D) (n = 19 C litters, 20 LP litters). E and F, similarly, glucose tolerance (E) and area under the glucose tolerance curve (F) did not differ (n = 8 C and 7 LP animals for each group).

2.3.2. F1 Animals

Offspring born to dams fed a LP diet weighed less at birth (1.25±0.02g *vs.* 1.34±0.03g, $p<0.05$, unpaired two-tailed Student's t-test, Fig. 3A) and at PND7 (3.56±0.11g *vs.* 4.03±0.15g, $p<0.05$, unpaired two-tailed Student's t-test, Fig. 2.3A). Offspring born to dams fed a LP diet continued to weigh less with age, demonstrating significantly reduced body weights compared to controls at weaning, PND21, (8.20±0.34g *vs.* 9.59±0.36g, $p<0.001$, two-way ANOVA followed by Bonferroni's post-hoc test, Fig. 2.3A), which persisted until maturity, PND42, (14.81±0.27g *vs.* 16.25±0.19g, $p<0.001$, two-way ANOVA followed by Bonferroni's post-hoc test, Fig. 2.3A). The LPP females continued to weigh less than CP females ($p<0.001$) throughout their own pregnancy (Fig. 2.3B). This was especially apparent during late gestation where the LPP females gained significantly less weight compared to CP females (12.78±1.22g *vs.* 15.24±1.44g, $p<0.001$, two-way ANOVA followed by Bonferroni's post-hoc test, Fig. 2.3C). No differences in fetal resorptions were found in LPP females compared to CP females (Fig. 2.3D).

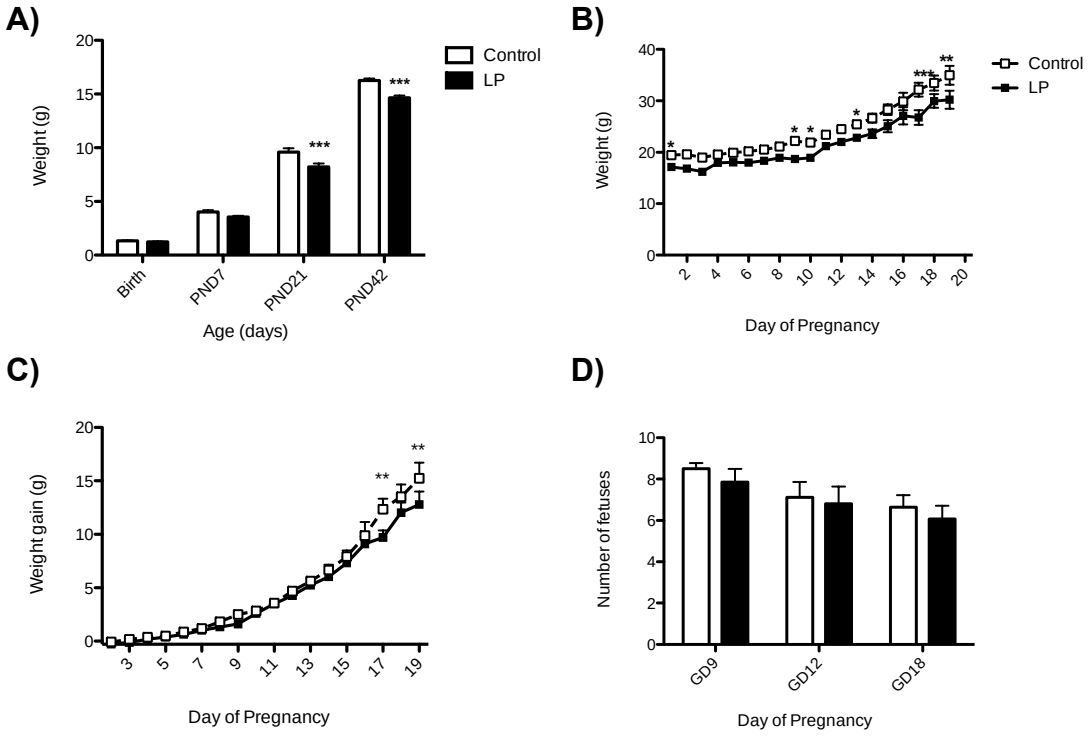


Figure 2.3. Offspring of LP-fed dams show altered pregnancy characteristics

A, offspring of LP-fed mothers weighed less than controls by weaning (postnatal day (PND) 21, n = 13−22 litters for each group). B and C, LPP females weighed less than CP females throughout gestation (B) and put on less weight at late gestation (C) compared to CP females (n = 25 CP and 24 LPP animals). D, the number of fetuses did not differ throughout gestation between CP and LPP females (n = 7−22 animals for each group). ***P < 0.001, **P < 0.01, *P < 0.05, LP vs. C.

2.3.2.1. Offspring of low protein-fed mothers are glucose-intolerant during their own gestation

There were no significant differences in fasting blood glucose levels between LPP and CP females at any timepoint during gestation or between non-pregnant females (CNP, LPNP). Furthermore, no differences in blood glucose levels or AUC were found for non-pregnant (Fig. 2.4A), GD9 (Fig. 2.4B) or GD12 (Fig. 2.4C) offspring born to dams fed a LP or control diet when subjected to an IPGTT. By GD18, LPP females had significantly higher blood glucose levels compared to CP at 5 min (18.88±2.22mmol·L^{-1} *vs.* 10.73±0.97 mmol·L^{-1}, $p<0.001$, repeated measures two-way ANOVA followed by Bonferroni's post-hoc test) but no significant difference was found in AUC (Fig. 2.4D).

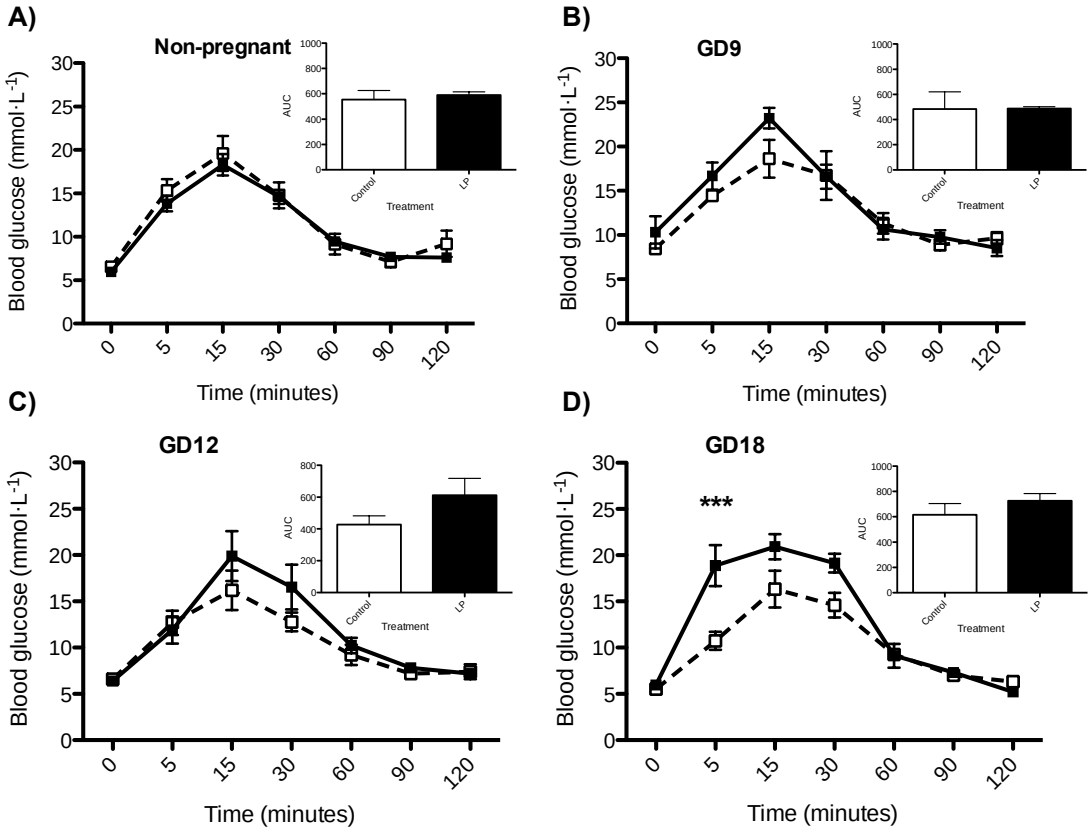


Figure 2.4. Offspring of low protein-fed mothers are glucose intolerant during their own gestation

Blood glucose (mmol/L) and area under the glucose tolerance curve (AUC) data from intraperitoneal glucose tolerance tests performed on offspring from low protein (LP, continuous lines, closed squares) and control-fed (C, dashed lines, open squares) mothers. A–C, there were no differences between blood glucose and AUC levels of non-pregnant (A) (n = 6 CNP and 5 LPNP animals), gestational day (GD) 9 (B) (n = 4 animals for each group), or GD12 (C) (n = 6 animals for each group) LPP and CP females. D, LPP females displayed higher blood glucose levels on GD18 when compared to CP females. However, no differences were found in AUC values (n = 7 CP and 4 LPP females). ***P < 0.001, LP vs. C.

2.3.2.2. Offspring of low protein-fed mothers have altered pancreatic morphology during pregnancy compared to controls

We next evaluated whether impairments in endocrine pancreas could be contributing to the glucose intolerance that was seen in late gestation of LPP females. Expansion of BCM was maximal on GD18 in CP females (Fig. 2.5A). However, BCM was significantly lower in LPP females compared to CP females on GD18 (0.93±0.16g *vs.* 1.96±0.41g, $p<0.01$, two-way ANOVA followed by Bonferroni's post-hoc test). CP females also showed a maximal peak of ACM on GD18 (Fig. 2.5B). However, LPP females had significantly lower ACM expansion on GD18 compared to CP females (0.17±0.05g *vs.* 0.55±0.17g, $p<0.01$, two-way ANOVA followed by Bonferroni's post-hoc test, Fig. 2.5B).

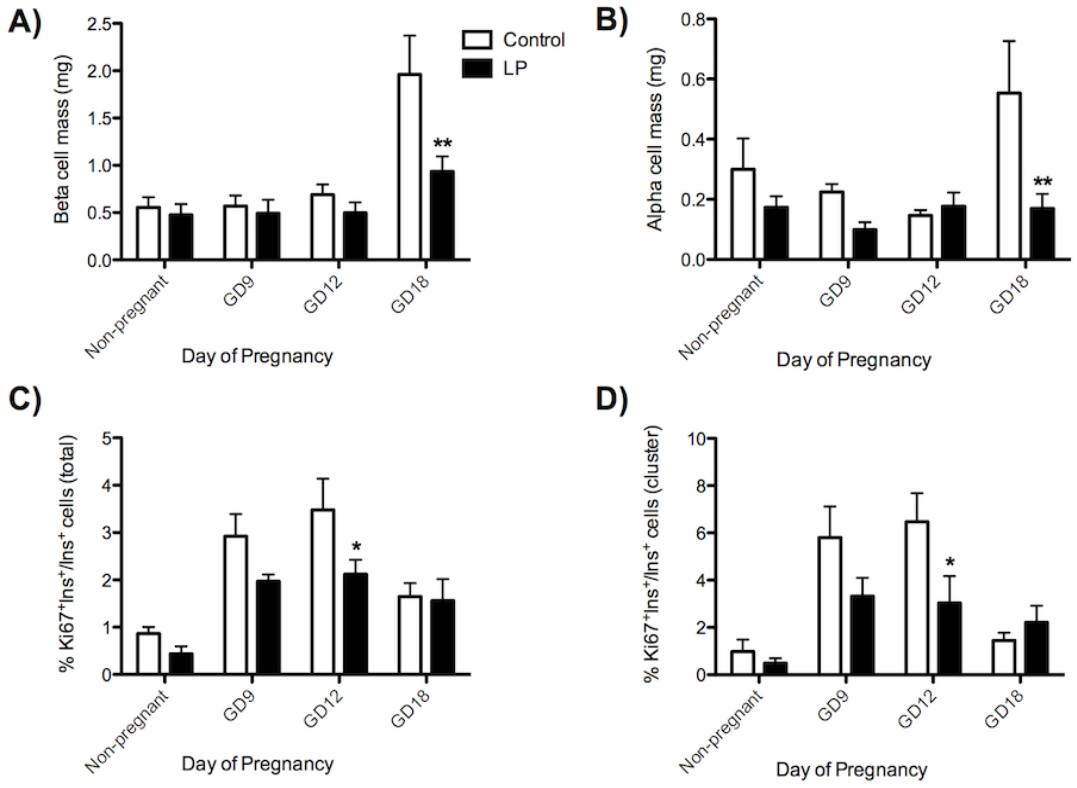


Figure 2.5. Offspring of LP-fed mothers have altered pancreatic morphology during pregnancy compared to controls

β-cell mass expansion (A) and α-cell mass expansion (B) were impaired in LPP females compared to CP females on GD18 (n = 4−6 animals for each group, 2−3 replicates for each animal). C, total β-cell proliferation was reduced during gestation in LPP females on GD12 (n = 4−7 animals for each group, at least 2 replicates for each animal). D, β-cell proliferation was reduced in clusters during gestation in LPP females on GD12 (n = 4−7 animals for each group, at least 2 replicates for each animal). **P < 0.01, *P < 0.05, LP vs. C.

2.3.2.3. Mechanisms of reduced BCM expansion

To determine whether the reduced BCM was due to decreased β-cell proliferation we used immunohistochemistry to identify insulin-containing cells co-localized with the DNA synthesis marker, Ki-67. Proliferating, insulin-expressing cells were identified in both clusters and islets of C and LP animals. Beta-cell proliferation increased during pregnancy in CP females but was significantly reduced in LPP females on GD12 (2.11±0.31%$Ki67^{+}Ins^{+}/Ins^{+}$(total) *vs.* 3.48±0.66%$Ki67^{+}Ins^{+}/Ins^{+}$(total), $p<0.05$, two-way ANOVA followed by Bonferroni's post-hoc test, Fig. 2.5C). The reduced β-cell proliferation at GD12 was specific to small β-cell clusters (3.03±1.14%$Ki67^{+}Ins^{+}/Ins^{+}$(clusters) *vs.* 6.47±1.22%$Ki67^{+}Ins^{+}/Ins^{+}$(clusters), $p<0.05$, two-way ANOVA followed by Bonferroni's post-hoc test, Fig. 2.5D). Representative images of proliferating β-cells (%$Ki67^{+}Ins^{+}$) in non-pregnant, GD12 and GD18 animals are shown in Figure 2.6. We found no evidence of co-localized insulin/TUNEL cells during gestation (GD12 and 18) in either the control or LP diet-exposed groups.

Although there was no effect of treatment on the ratio of α- to β-cells there was a change with day of pregnancy ($p<0.001$, two-way ANOVA, Fig. 2.7A). Mean islet size was reduced at GD18 in LPP females (4323±463μm^2 *vs.* 7967±1542μm^2, $p<0.05$, two-way ANOVA followed by Bonferroni's post-hoc test, Fig. 2.7B), however there were no differences in β-cell size (Fig. 2.7C). No differences in distribution of islet sizes were observed in non-pregnant animals (Fig. 2.7D). Nonetheless, there was a reduction in the number of small islets in LPP compared to CP mice at GD9 (3.80±0.93 *vs.* 12.50±1.43, $p<0.001$, two-way ANOVA followed by Bonferroni's post-hoc test, Fig. 2.7E), GD12 (5.92±1.27 *vs.* 11.33±2.32, $p<0.05$, two-way ANOVA followed by Bonferroni's post-hoc test, Fig. 2.7F), and GD18 (7.90±1.39) *vs.* 17.88±2.98, $p<0.001$, two-way ANOVA followed by Bonferroni's post-hoc test, Fig. 2.7G).

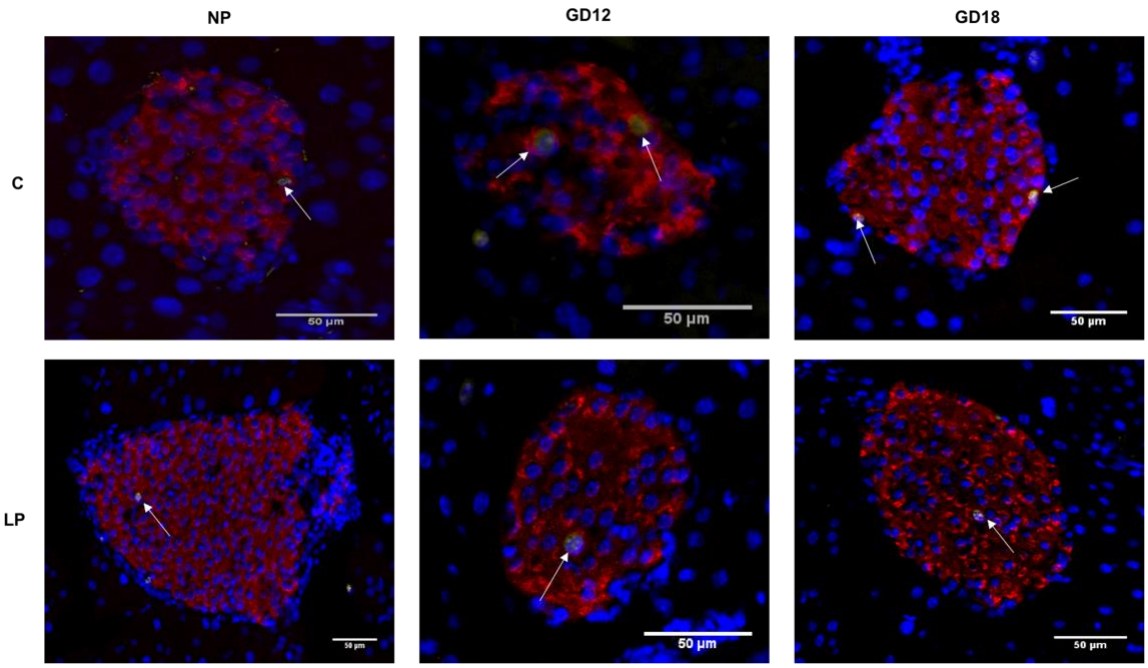


Figure 2.6. LPP females show reduced β-cell replication at GD12 compared to CP females

Representative immunofluorescence images demonstrating insulin (red), Ki-67 (yellow) and nuclei (DAPI, blue) staining of CNP, LPNP, CP and LPP females (at GD12 and GD18). White arrows demonstrate co-localized insulin and Ki-67 cells as an example of proliferating β-cells within an islet. Scale bar represents 50 µm.

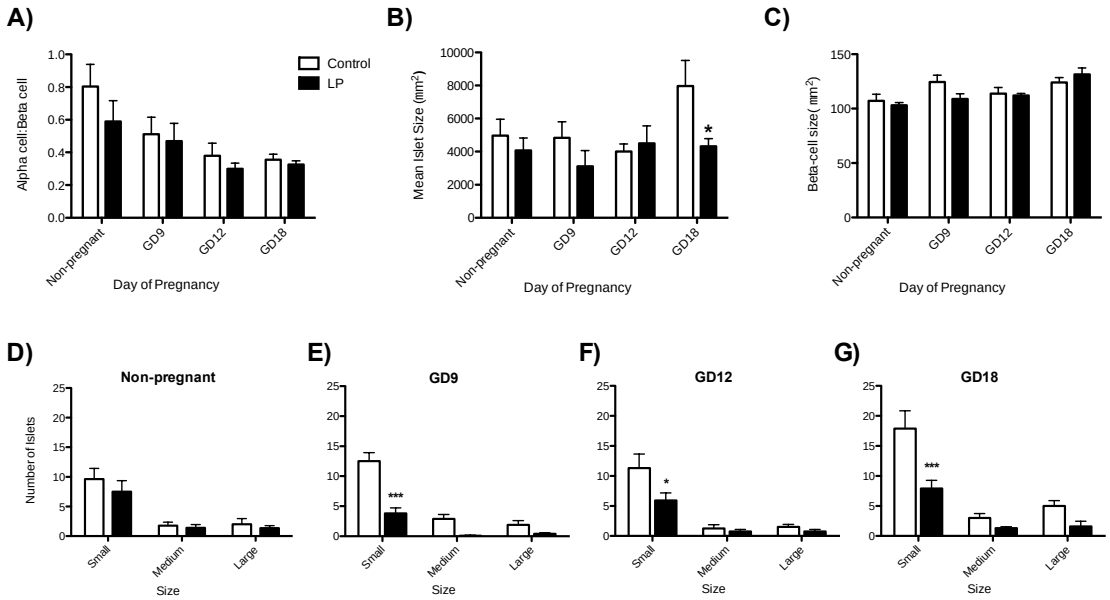


Figure 2.7. LPP females have an altered distribution of islet sizes, contributing to a reduced mean islet size and BCM expansion at late gestation

A, α- to β-cell ratio varies with day of pregnancy but not between dietary groups. B, mean islet size was reduced in LPP females compared to CP females on GD18. C, however, this was not due to a change in β-cell size. D, the number of small islets did not differ in LPNP females. However, the number of small islets was reduced in LPP females on GD9 (E), GD12 (F) and GD18 (G) (n = 4−6 animals for each group, 2−3 replicates for each animal). ***P < 0.001, *P < 0.05, LP vs. C.

2.3.2.4. Offspring of low protein-fed mothers show gestational β-cell dysfunction *in vitro* and *in vivo*

To assess β-cell function, we measured insulin secretion of isolated pancreatic islets from GD18 CP and LPP females. Levels of insulin were similar between LPP and CP females after 90 min in medium containing 2.8 mmol·L^{-1} glucose (Fig. 2.8A). However, islets from LPP females secreted less insulin in the presence of 16.7 mmol·L^{-1} glucose than CP females (0.22±0.04ng·mL^{-1}·$islet^{-1}$ *vs.* 0.49±0.07ng·mL^{-1}·$islet^{-1}$, $p<0.01$, two-way ANOVA followed by Bonferroni's post-hoc test, Fig. 2.8A). To confirm these findings *in vivo*, serum insulin was quantified from blood drawn by cardiac puncture following the IPGTT. Confirming the *in vitro* findings, LPP females had lower serum insulin levels compared to CP females on GD18 (0.57±0.10ng·mL^{-1} *vs.* 1.34±0.25ng·mL^{-1}, $p<0.05$, unpaired two-tailed Student's t-test, Fig. 2.8B). Although serum glucagon levels appeared to be lower in LPP females on GD18, there were no significant differences found when compared to CP females (Fig. 2.8C).

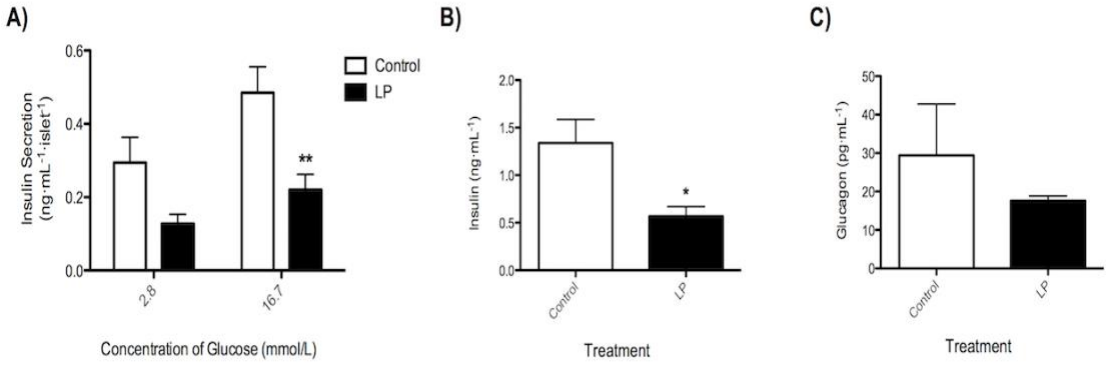


Figure 2.8. Offspring of low protein-fed mothers show gestational β-cell dysfunction *in vitro* and *in vivo*

A, glucose-stimulated insulin secretion was reduced on GD18 from isolated pancreatic islets from LPP females (n = 6−9 animals for each group, 1−3 replicates for each animal). B, serum insulin levels of LPP females were reduced on GD18 (n = 7 animals for each group). C, serum glucagon levels did not differ between CP and LPP females on GD18 (n = 5 animals for each group). **P < 0.01, *P < 0.05, LP vs. C.

2.4 Discussion

This study proposes a novel mouse model of gestational glucose intolerance in which metabolic impairments are restricted to late gestation, as is seen clinically in human GDM. Epidemiological studies in humans have demonstrated strong associations between poor fetal growth, for instance as encountered in times of famine, and susceptibility to metabolic syndrome in adult life [32,33]. These observations have been replicated in maternal malnutrition studies in animals, resulting in permanent changes in tissue composition and cell size in the offspring during adulthood, ultimately contributing to the metabolic syndrome phenotype [34]. In agreement with the concept of developmental origins of health and disease [35], we show in this study that the intrauterine environment influences the risk of metabolic disease in offspring later in life. We previously showed that offspring of LP-fed mothers had reduced BCM and developed glucose intolerance in adulthood [31]. In this study we investigated whether offspring of LP-fed mothers would have a predisposition to glycaemic dysfunction during pregnancy, and the underlying pancreatic physiology that might contribute towards this phenotype. Overall, LP diet did not impact the pregnancy characteristics of F0 dams as no differences in maternal weight gain, litter size/sex of offspring or glucose homeostasis were found. Furthermore, it has previously been shown that isolated islets from pregnant LP-fed rats had a similar response to physiological glucose concentrations compared to controls [36], while another study concluded that a short period of LP-diet consumption did not alter total area under the glucose and insulin curves during a GTT, or basal serum glucose measurements, indicating preservation of pancreatic function [37]. Although this is being extrapolated from rats, we would not anticipate that LP-fed dams in our study would demonstrate gestational glucose intolerance and provide a model for GDM. However, the phenotype was altered in offspring of LP-fed mothers showing a reduced body weight at birth and PND7, which persisted with age and throughout their own pregnancy. Although we do not have data on visceral adipose tissue in our study, a previous study in our laboratory found no differences in visceral adipose tissue between offspring of LP and C-fed rats at 130 days of age [27]. Furthermore, because we are using young, pre-estropausal mice in our study, we anticipate that there would be no differences in visceral adipose tissue present in our model at this age. In humans,

postmenopausal women begin to have increased visceral fat accrual [38]. Therefore, potential differences in visceral adipose tissue in our model might only be seen at estropause which in mice is at 9-12 months of age [39].

In addition to the physical differences found between offspring of LP and C-fed mothers, we found maternal glucose intolerance when LPP females were subjected to an IPGTT at GD18. In comparison to our findings, a similar study using the LP diet model in rats stated that their model did not promote the onset of GDM [40]. However, this claim is made based on AUC data, for which our data are comparable on GD18. Nevertheless, the authors did not include their IPGTT curves, which is where we noted abnormally elevated maternal blood glucose levels in the LPP females. Previous studies found no differences in fasting blood glucose levels in young offspring (PND1, 7, 14 and 30) of LP and C-fed mothers [41]. Furthermore, LPNP female rodents did not demonstrate glucose intolerance until later in adulthood [27,41,42] and the onset in young adults in this study is likely to have been precipitated by the metabolic stress of pregnancy since there were no differences in glucose tolerance between CNP and LPNP. Clinically, since prior GDM increases the risk of the mother developing subsequent T2DM [43], it is plausible that the LPP females could prematurely develop glucose intolerance following pregnancy compared to non-pregnant animals. Future studies investigating metabolic differences and pancreas histology postpartum using our animal model of GDM could prove insightful.

Consistent with previous findings [10,44], we observed that CP females were able to expand BCM to compensate for insulin resistance in pregnancy. However, BCM expansion was impaired in LPP females compared to CP females, as has been postulated to occur in human GDM [11,45,46]. In agreement with our previous work, the expansion of BCM during pregnancy was associated with increased β-cell proliferation [10], which was significantly reduced in LPP females. This was particularly apparent within the small extra-islet endocrine clusters which we have previously shown to be a source of β-cell progenitors [26]. This suggests that the proliferation of progenitors or their differentiation into functional β-cells might be impaired in LPP females. Reduced β-cell proliferation in LPP females contributed to a reduced mean islet size at GD18, consequently contributing

to reduced BCM expansion. Although there were no differences in β-cell size in LPP *versus* CP females, there were fewer small islets in LPP females at GD9, 12 and 18 compared to CP females. Since there was a relative increase in large-sized islets of CP females at GD18 compared to GD9, we postulate that β-cell replication within small islets facilitates islet growth into medium and large sized islets as gestation progresses (GD18). This further contributed to increased BCM expansion in CP females at GD18. However, since LPP females had fewer small islets, there were fewer available to facilitate an adaptive expansion of BCM at GD18. Since there were no differences in islet size distribution in the LPNP *versus* CNP animals, these differences were attributed to the metabolic state of pregnancy. Although increased β-cell apoptosis is seen in offspring of LP-fed mothers [22], here we found no evidence of apoptosis within β-cells of LPP or CP females during a subsequent gestation; thus, excluding the possibility of β-cell apoptosis contributing to the reduced capacity for BCM expansion in LPP females. These results are in agreement with the findings of another animal model of maternal glucose intolerance during gestation in which the authors also reported that β-cell apoptosis did not contribute to the impairment of BCM expansion [47]. These results could be attributed to the contribution of the protein survivin, which normally becomes upregulated during gestation and acts as an inhibitor of apoptosis *via* epidermal growth factor-receptor signalling [48]. In addition to the dynamics of BCM investigated in this study, we are the first to report on α-cell dynamics in the pancreas during mouse pregnancy. Although there was no effect of treatment on the ratio of α- to β-cells there was a change with day of pregnancy. Further to reduced BCM, we found a relative decrease in ACM in CP females throughout gestation when compared to CNP. While CP females replenished ACM by GD18, this was not found in LPP females. There is evidence through lineage tracing of α-cells that they can replenish β-cells following β-cell loss or during β-cell stress *via* transdifferentiation [49,50]. These findings could implicate α- to β-cell transdifferentiation as a contributor to expanded BCM during pregnancy, which might be impaired in LPP females.

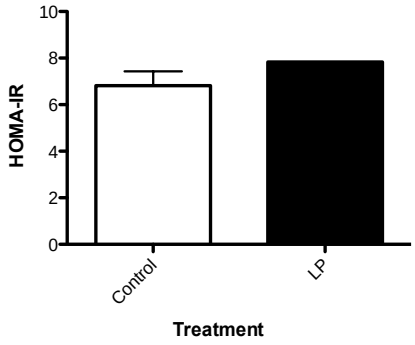
We also examined β-cell functional capacity in our model, since β-cell dysfunction is a key feature of the pathophysiology of GDM [51]. Although insulin release from isolated islets harvested in late pregnancy in response to basal glucose concentration did not differ

between dietary groups, glucose-stimulated insulin secretion (GSIS) was significantly decreased in LPP females. These results are in agreement with other reports in which impaired GSIS was found in islets of offspring of LP-fed mothers as a result of mitochondrial dysfunction in β-cells [52]. Future studies investigating mitochondrial β-cell dysfunction in our model would be insightful as women with GDM demonstrate increased oxidative stress, which has been suggested to contribute to β-cell dysfunction in GDM [53]. We confirmed our *in vitro* findings *in vivo*, showing that LPP females had reduced serum insulin levels at GD18 compared to CP females. These data supported our hypothesis and implicate β-cell dysfunction both *in vivo* and *in vitro* at late gestation in our model of gestational glucose intolerance. Therefore, in our model a combination of reduced BCM and impaired GSIS most likely contributed to the glucose intolerance seen in LPP females. Since our study was limited to changes in pancreas histology we cannot eliminate the possibility that insulin resistance at the level of target tissues contributed to glucose intolerance during pregnancy in offspring of LP-fed mothers. However, this has previously been shown to occur only in late adulthood (130 days) and not within the young adult mice used in these studies [27]. Indeed, pilot studies from our laboratory provide further support for this claim, suggesting no differences in HOMA-IR (a measure of insulin resistance) between CP and LPP females at GD18 (Supplemental Fig. 2.1). Nevertheless, a major strength of our study was the ability to reproduce gestational glucose intolerance during pregnancy complications such as GDM in which glucose intolerance is not diagnosed until late gestation. In our study, glucose intolerance was restricted to GD18 and was not seen in the non-pregnant state as has been shown in other models of gestational glucose intolerance [40]. Furthermore, additional animal models of diabetes in pregnancy that demonstrate pre-gestational obesity and diabetes [54,55] display glucose intolerance prior to conception, which is not a true diagnosis of clinical GDM [56]. Animal models utilizing chemical destruction of β-cells are widely used for modelling pre-gestational and gestational diabetes; however, these models more accurately resemble pre-gestational type-1 diabetes as opposed to the characteristics of insulin resistance seen in conditions such as GDM [57]. Therefore, in comparison to other models, our model of fetal programming of gestational glucose intolerance *via* dietary insult more accurately demonstrates the hyperglycaemic state of GDM, which

occurs only at late gestation. Although our dietary insult involves protein restriction during development, the LP diet is made isocaloric to the control diet through increased carbohydrate, which prompts the question of whether our findings are due to the effects of reduced protein or increased carbohydrate intake. However, the increase in carbohydrate content represents only a 20% increase compared to the more prominent 40% reduction of protein (20% casein *versus* 8%) [58], which suggests that the glycaemic dysfunction and impairments in pancreas histology and function in our model are more likely the result of reduced protein.

Susceptibility to developing GDM arises from a complex combination of both polygenetic and environmental factors. Taking this into account, the developmental programming of adult metabolism utilized in this mouse model of glycaemic imbalance during pregnancy does not reproduce the predominant predisposing causes of human GDM, which include pre-gestational obesity and excessive gestational weight gain [2,59]. Nonetheless, there are a number of anatomic and metabolic similarities including a failure to adequately increase BCM during pregnancy and impaired GSIS in late gestation. Post-mortem studies of human pancreata obtained from pregnant individuals confirmed an increase in endocrine mass during healthy pregnancies compared to the non-pregnant state [16,17], and it has been suggested that failure to adaptively increase BCM might contribute to the risk of GDM in humans [18]. Thus, further research efforts should focus on molecular mechanisms (i.e. signalling *via* prolactin and/or estrogen receptors) leading to reduced BCM expansion during gestation so that targeted interventions could be implemented. Measurements of serum placental lactogen, prolactin and estrogen in our animal model could also prove insightful. In conclusion, the model of hyperglycaemia in pregnancy described in this study could prove useful in evaluating pharmacological interventions aimed at safely increasing BCM or GSIS during pregnancy.

2.5 Supplemental Figures



Supplemental Figure 2.1. HOMA-IR measurements in control compared to LP mice at GD18

Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) values between treatment groups at GD18. *n* = 3 control and 2 LP animals.

2.6 References

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Chapter 3

3 Altered Pancreas Remodeling Following Glucose Intolerance in Pregnancy in Mouse

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3.1 Introduction

Pregnancy presents as a unique situation of endocrine pancreas β-cell adaptation that reverses after parturition. Late pregnancy is characterized by a state of peripheral maternal insulin resistance mediated by placental hormones [1] which is essential for maintaining trans-placental transport of glucose to the fetus for optimal development. To compensate for insulin resistance, the maternal pancreas responds by increasing endocrine pancreatic β-cell mass (BCM) to help maintain euglycemia. An adaptive expansion of BCM has been documented in both mice and humans and is maximal at late gestation (around gestational day (GD) 18 in mice) [2–7]. The adaptive mechanisms of BCM expansion during mouse pregnancy have been studied extensively and occur, in part, due to increased β-cell hypertrophy and proliferation, and the expansion of a β-cell precursor pool, all of which peak at mid-gestation [3,6–8]. These changes are mediated by increased levels of hormones including placental lactogen, prolactin, and estrogen [1,8]. After parturition, progesterone-mediated β-cell apoptosis increases while proliferation decreases concomitant with an absence of placental lactogen, returning BCM to pre-pregnancy levels [8,9]. If BCM expansion is suboptimal, gestational diabetes mellitus (GDM) can arise.

GDM is described as diabetes that first appears during pregnancy and occurs due to insufficient β-cell adaptation to compensate for insulin resistance in pregnancy. The incidence of GDM worldwide is around 7-10% of all pregnancies [10,11] although the incidence will vary depending on the population characteristics and diagnostic criteria. As more women enter pregnancy obese, or at an older age, the incidence of GDM is projected to continue to rise [12]. An increased incidence of GDM is associated with morbidity due to adverse fetal outcomes [13–15] and adverse long-term maternal outcomes including an increased risk of type-2 diabetes mellitus (T2DM) [16] between 3% to 90% [17]. Clinical studies have shown that glucose intolerance after parturition involves β-cell dysfunction [18–21] which can occur, in part, due to inflammation and glucotoxicity-induced oxidative stress in β-cells [22].

Pregnancy can be characterized as a low-grade inflammatory state where the placenta is a major source of cytokines that can alter β-cell function [23]. For example, studies have postulated that 94% of the increased serum levels of tumor necrosis factor (TNF-α) in women during pregnancy results from placental production [24]. The first and early second trimester is a pro-inflammatory environment characterized by helper T-cell-1 (Th1) cytokines, such as TNF-α, interleukin-1β, (IL-1β,), and interferon (IFN)-γ, resulting from implantation and placentation processes [23]. The second trimester is characterized as an anti-inflammatory environment with Th2 cytokines that permit for fetal growth and development [25]. Lastly, the third trimester is characterized by the recurrence of a pro-inflammatory environment in preparation for parturition, as inflammation promotes uterine contractions [26]. Thus, increased cytokine production occurs in healthy pregnancies. However, increased activity of the immune-checkpoint molecule programmed death-ligand 1 (PD-L1), and its receptor, PD-1, play a role in the maintenance of immunological balance between mother and fetus [27] and likely protect maternal β-cells from cytotoxic damage. In healthy pregnancies, PD-L1 is expressed by syncytiotrophoblast cells of the placenta in response to pro-inflammatory stimuli and is increasingly released into the maternal bloodstream as gestation progresses [28]. The interaction between PD-L1 and PD-1 reduces clonal expansion of pathogenic lymphocytes and their associated cytokine release [29]. Therefore, with decreased lymphocyte expansion, cytokine production is decreased, and β-cells are protected against cytokine-induced damage. In the context of GDM and persistent glucose intolerance at postpartum, it is plausible that insufficient levels of PD-L1 cause maternal β-cells to have greater susceptibility to cytotoxic damage, which can contribute to β-cell dysfunction. In GDM pregnancies, the low-grade inflammation that normally takes place in uncomplicated pregnancies is exacerbated [30]. The overexpression of pro-inflammatory cytokines contributes to excessive peripheral insulin resistance in the mother, necessitating enhanced insulin secretion to maintain euglycemia, which is often not met in GDM pregnancies [31]. Thus, it is plausible that a prolonged low-grade inflammatory environment persisting after parturition in the absence of the placenta could result from cytokines released by other organs, such as adipose tissue, which could contribute to β-cell dysfunction postpartum and lead to dysglycemia. Inflammation can

contribute to β-cell dysfunction [32] *via* endoplasmic reticular stress resulting in decreased insulin sensitivity, as has been documented in GDM patients [33].

We investigated the changes that occur in mouse pancreas during pregnancy and found a three-fold increase in BCM on GD18, resulting largely from increased β-cell replication which peaked at GD12 [6,7]. In our previous experiments, female offspring of mice fed a low protein (LP) diet during gestation and lactation were glucose intolerant at GD18 during their own pregnancy when compared to offspring from control diet-fed mothers [6]. Glucose intolerance was associated with reduced β-cell proliferation leading to a lower BCM, in addition to reduced glucose-stimulated insulin secretion (GSIS). Since GDM increases the risk of the mother developing subsequent T2DM, we have used the above mouse model of gestational glucose intolerance to follow animals after parturition and determine whether glucose tolerance normalizes postpartum, and what the long-term effects of glucose intolerance during pregnancy are on maternal pancreas morphometry. The mechanisms contributing to adaptive BCM expansion during pregnancy in mice have been extensively studied. However, few studies exist investigating changes that occur past 7-10 days postpartum and to our knowledge no data exists comparing pancreas morphometry after hyperglycemic and control pregnancy. We aimed to determine: 1) what happens to glucose tolerance and islet morphology postpartum after a normal *vs.* a hyperglycemic pregnancy; and 2) the possible involvement of cytokines and PD-L1 in long-term changes in islet morphology and glucose tolerance postpartum.

3.2 Methods

3.2.1. Animals and Sample Collection

Adult (6-week-old) C57BL/6 male and female mice were obtained from Charles River Laboratories (Wilmington, MA, USA) and housed in a temperature-controlled room with 12-h light/dark cycle at Lawson Health Research Institute, London, ON, Canada. Water and food were given *ad libitum*. Mice showing gestational glucose intolerance were generated using a previously described protocol involving a dietary insult during early

life [6, Chapter 2 of this thesis]. Briefly, timed pregnant F0-females were randomly allocated to either a control (C, 20% protein, Bio-Serv, Frenchtown, NJ, USA) or low protein diet (LP, 8%) group, where an increase in carbohydrate in LP diet yields an isocalorific diet compared to control chow. F0-dams were fed either the LP or C diet throughout gestation and lactation. A total of 12 LP and 12 C litters were used. On postnatal day (PND) 21, female offspring (F1) were weaned onto C diet for the remainder of the study (Fig. 3.1). At maturity (PND42), female offspring (F1) of LP and C diet-fed mothers were randomly allocated into 2 subsequent study groups: pregnant (LPP, CP) or non-pregnant (LPNP, CNP). All pregnant grouped females were mated with C diet-fed males, separated upon confirmation of pregnancy by vaginal plug and housed individually for the remainder of the experiment. Upon birth of pups, CP and LPP females were randomly allocated to one of three timepoints after parturition (postpartum day (PPD), PPD7, PPD30, or PPD90). For consistency, pups from all litters were euthanized at postnatal day 7. Mothers (F1) were euthanized by CO_2 asphyxia following an intra-peritoneal glucose tolerance test (IPGTT) on PPD7, PPD30, or PPD90 for comparison to non-pregnant age-matched F1 females. Data (histology and IPGTT) for non-pregnant and GD18 animals, except for *in vivo* serum quantification, presented here have previously been published and are being used as a comparison to novel postpartum data in this study [6]. Following the IPGTT, maternal (F1) blood was collected *via* cardiac puncture in order to quantify serum insulin, glucagon and PD-L1 quantified using an Ultra Sensitive Mouse Insulin ELISA kit (Crystal Chem, Downers Grove, IL, USA), Mouse Glucagon ELISA kit (Crystal Chem, Downers Grove, IL, USA) and PD-L1 ELISA assay (LifeSpan Biosciences, Seattle, WA, USA), respectively. Data were collected using a BioRad iMark plate reader and analyzed using Microplate Manager Software.

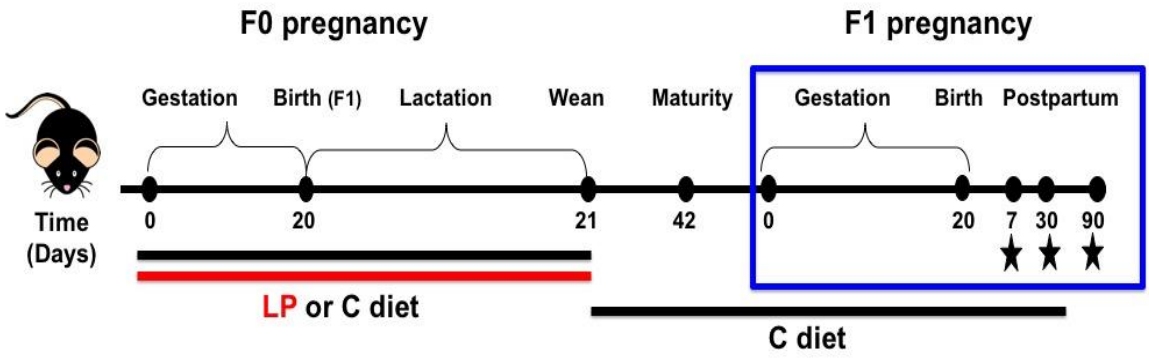


Figure 3.1. Experimental timeline for the generation of mice with gestational glucose intolerance and timepoints for sample collection

Female offspring (F1) were exposed to the low protein (LP) or control (C) diet during gestation and lactation and weaned onto C diet. At maturity, F1 females were mated with C-fed males. Pups (F2) from all litters were euthanized at postnatal day 7. Stars demonstrate timepoints where an intraperitoneal glucose tolerance test was performed, the pancreas was removed for histology (n = 4–7 animals for each group), and serum was collected *via* cardiac puncture.

Pancreata were removed immediately following euthanasia and fixed in 4% paraformaldehyde for histology (PFA, Electron Microscopy Sciences, Hatfield, PA, USA). Fixed pancreas tissue was prepared and sectioned as previously described [34]. At least two 7 μm-thick cryosections (replicates) were cut from each pancreas with an interval between each section >100 μm representing at least 2 longitudinal slices through the pancreas. Sections included both the head and tail of the pancreas. All animal procedures were approved by the Animal Care Committee of Western University in accordance with the guidelines of the Canadian Council for Animal Care.

3.2.2. Glucose Tolerance Test

An IPGTT was performed on all animals prior to euthanasia as previously described [35] at the assigned day postpartum or age for the age-matched group (n = 4−7 C and LP animals per timepoint). Mice were fasted for 4-h with free access to water, injected intraperitoneally with 5 μl/g body weight of 40% glucose solution (2g/kg body weight glucose, Sigma Aldrich, St. Louis, Missouri, USA), and blood glucose measured from the tail at 0, 5, 15, 30, 60, 90, and 120 min using a One Touch Ultra2 glucometer.

3.2.3. Immunohistochemistry and Morphometric Analysis

Fluorescence immunohistochemistry was performed to localize insulin (β-cells) and glucagon (α-cells) for morphometric analysis as previously described [6]. Every insulin and glucagon-expressing cell was imaged at 20X with the observer being blind to tissue identity using a Nikon Eclipse TS2R inverted microscope (Nikon, Tokyo, Japan) with the program NIS elements. The microscope was equipped with an LED light source with emission bandwidths set to 460/50 nm for blue emission, 535/50 nm for green emission and 590/40 nm for red emission. For morphometric analyses, manual tracing of all islets for the tissue section was completed using ImageJ to quantitate fractional α- and β-cell area (sum of all glucagon or insulin-expressing areas divided by the whole pancreas surface area). Fractional areas were calculated from at least 2 sections (replicates) per pancreas (n = 4−6 C and LP animals per timepoint). Islets were counted per tissue section and further separated by size into small (< 5000 μm^2), medium (5000–10,000 μm^2) or

large (>10,000 μm2) islets as previously described [36]. ApopTag Plus *In Situ* Apoptosis Fluorescein Detection Kit (S7111, EMD Millipore, Billerica, MA, USA) was used to identify apoptotic β-cells using the terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) assay.

3.2.4. Protein Extraction and Cytokine Analysis

Posterior subcutaneous white adipose tissue (27-50 mg) was lysed for 30 min in an ice-cold buffer as previously described [37]. Cytokine levels in adipose tissue protein extracts (n = 3−4 C and LP animals per timepoint, PPD90 C n = 2) were determined by multiplexing in a Bioplex system using customized kits from R&D systems (Magnetic Luminex assay) for cytokines of interest (TNF-α, IL-6, IL-1β, and IFNγ). Values were normalized to weight of sample.

3.2.5. Statistical Analysis

Data are presented as mean ± SEM, with statistics analyzed using GraphPad Prism software (Version 5.0, La Jolla, CA, USA). To determine effects of pregnancy on pancreas morphometry postpartum (CP), a one-way ANOVA was used followed by Bonferroni's post-hoc test. For comparisons of LPP and CP groups over time, a two-way ANOVA followed by Bonferroni's post-hoc test was used. A repeated measures two-way ANOVA followed by Bonferroni's post-hoc test was used for comparison of the IPGTT curves between treatment groups (LP *vs.* C) at each timepoint after parturition. An unpaired two-tailed Student's *t*-test was used for comparison of IL-6 levels at PPD7 between dietary groups. Each animal presented as a single unit of analysis (n). Only the postpartum timepoints were included in statistical analyses to present novel data. To account for differences in litter sizes, the mean litter size for LPP and CP groups was determined. Litter sizes that were more than 2 standard deviations greater than the mean were considered outliers. This did not result in any outliers in the data set. Statistical significance was determined as $P < 0.05$.

3.3 Results

3.3.1. Mice with gestational glucose intolerance display altered pregnancy characteristics

Offspring born to dams fed a LP diet during gestation and lactation (F1, LPP) weighed less than controls (F1, CP) throughout gestation (Fig. 3.2A) and gained significantly less weight than CP females at GD17 and 19 (Fig. 3.2B). There were no significant differences in body weight between dietary groups after parturition (Fig. 3.2C). Body weight differed with time after parturition in both dietary groups ($P<0.01$, Fig. 3.2C).

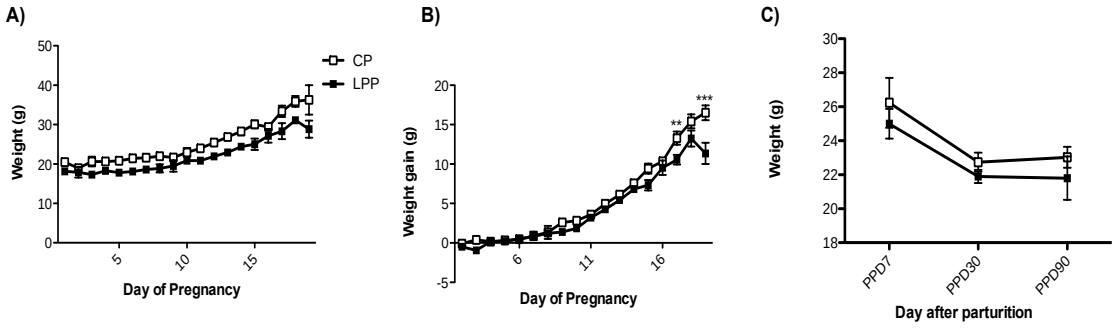


Figure 3.2. Mice with gestational glucose intolerance display altered pregnancy characteristics

(A) Pregnant female mice born to dams fed a low protein diet (LPP) weighed less and (B) gained less weight than pregnant female mice born to dams fed a control diet (CP) throughout pregnancy (n = 7 CP females, n = 10 LPP females). (C) No differences in body weight were found after parturition between dietary groups (n = 4–6 CP and LPP females). ***$P < 0.001$, **$P < 0.01$, LPP *vs*. CP.

3.3.2. Mice with gestational glucose intolerance demonstrate prolonged glucose intolerance after parturition

We compared glucose tolerance in LPP *vs.* CP mice after parturition. LPP females that were relatively glucose intolerant at GD18 continued to display glucose intolerance at PPD7 with higher blood glucose levels at 15 and 30 min into an IPGTT (Fig. 3.3A). The relative glucose intolerance persisted at 1 month postpartum (PPD30, Fig. 3.3B). However, by 3 months postpartum (PPD90, Fig. 3.3C) LPP females had a similar glycemic profile as that seen in controls. Furthermore, the glycemic profile at PPD90 was restored to that of a non-pregnant animal (Fig. 3.3D). There were no significant differences in fasting blood glucose levels between dietary groups or with time (Supplemental Fig. 3.1A). Additionally, area under the glucose tolerance curve was higher in the LPP group at PPD7 compared to controls (Supplemental Fig. 3.1B).

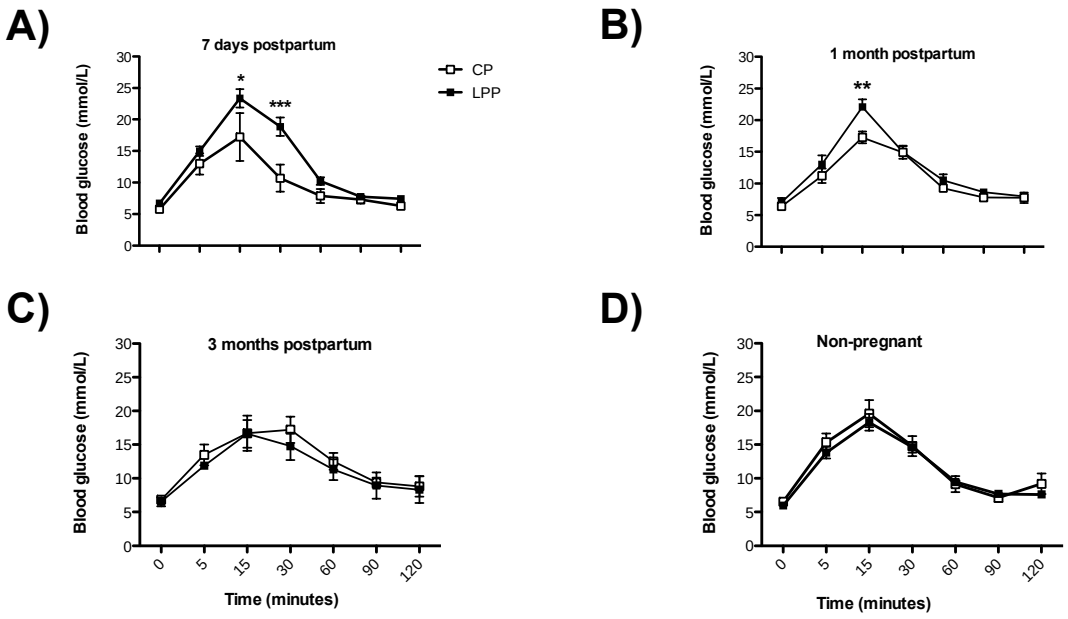


Figure 3.3. LPP females show prolonged glucose intolerance after parturition

Pregnant female mice born to dams fed a low protein diet (LPP) show glucose intolerance relative to pregnant female mice born to dams fed a control diet (CP) at (A) postpartum day 7 (PPD7, *n* = 6 CP females, 7 LPP females) and continue to be glucose intolerant at (B) 1 month postpartum (PPD30, *n* = 6 CP females, 5 LPP females). (C) However, by 3 months postpartum (*n* = 4 CP females, 4 LPP females) LPP females display a glycemic profile similar to that of a control and demonstrate a similar glycemic profile to that of a (D) non-pregnant animal (*n* = 6 C non-pregnant females, 5 LP non-pregnant females). ***$P < 0.001$, **$P < 0.01$, *$P < 0.05$, LPP *vs*. CP.

3.3.3. Pregnancy alters pancreas morphology postpartum after both control and hyperglycemic pregnancies

We investigated how endocrine pancreas morphology changed after parturition, both in CP and LPP groups. Pancreatic sections were immunostained for insulin (β-cells) and glucagon (α-cells) for morphometric analyses (Fig. 3.4). Since pancreas weight was relatively higher in the LPP group at PPD30 and PPD90 (Fig. 3.5A), β/α-cell fractional areas were used for histological analyses rather than BCM. Beta-cell fractional area was lower in LPP animals compared to CP animals at PPD7 (Fig. 3.5B). Notably, relative to PPD7, CP females had a ~30% reduction in BCM at PPD30 and a ~40% reduction by PPD90 (Fig. 3.5B), the latter resulting in a β-cell fractional area comparable to a non-pregnant animal. In contrast to CP females, β-cell fractional area did not further decrease postpartum in the LPP group; rather the values remained at a similar level from GD18 up until PPD90 (Fig. 3.5B). Alpha-cell fractional area was lower in LPP animals compared to CP at PPD7 (Fig. 3.5C). Furthermore, α-cell area was lower at PPD30 *vs.* PPD7 in CP females. There were no significant differences in the α- to β-cell ratio (Fig. 3.5D) between dietary groups or with time after parturition. No evidence of dual-stained insulin/TUNEL cells was found when investigating the contribution of β-cell apoptosis at PPD7.

Islet quantification demonstrated that the number of small, medium and large-sized islets varied with time after parturition (Table 3.1). At PPD90, there were significantly more small-sized islets in the LPP females compared to controls (Table 3.1). There were no significant differences in mean islet size after parturition between dietary groups (Table 3.1). However, the mean islet size was 1.8-fold higher at PPD90 (6373 ± 2065 μm^2) compared to PPD7 (3599 ± 452 μm^2) in the LPP group ($P = 0.147$, Table 3.1).

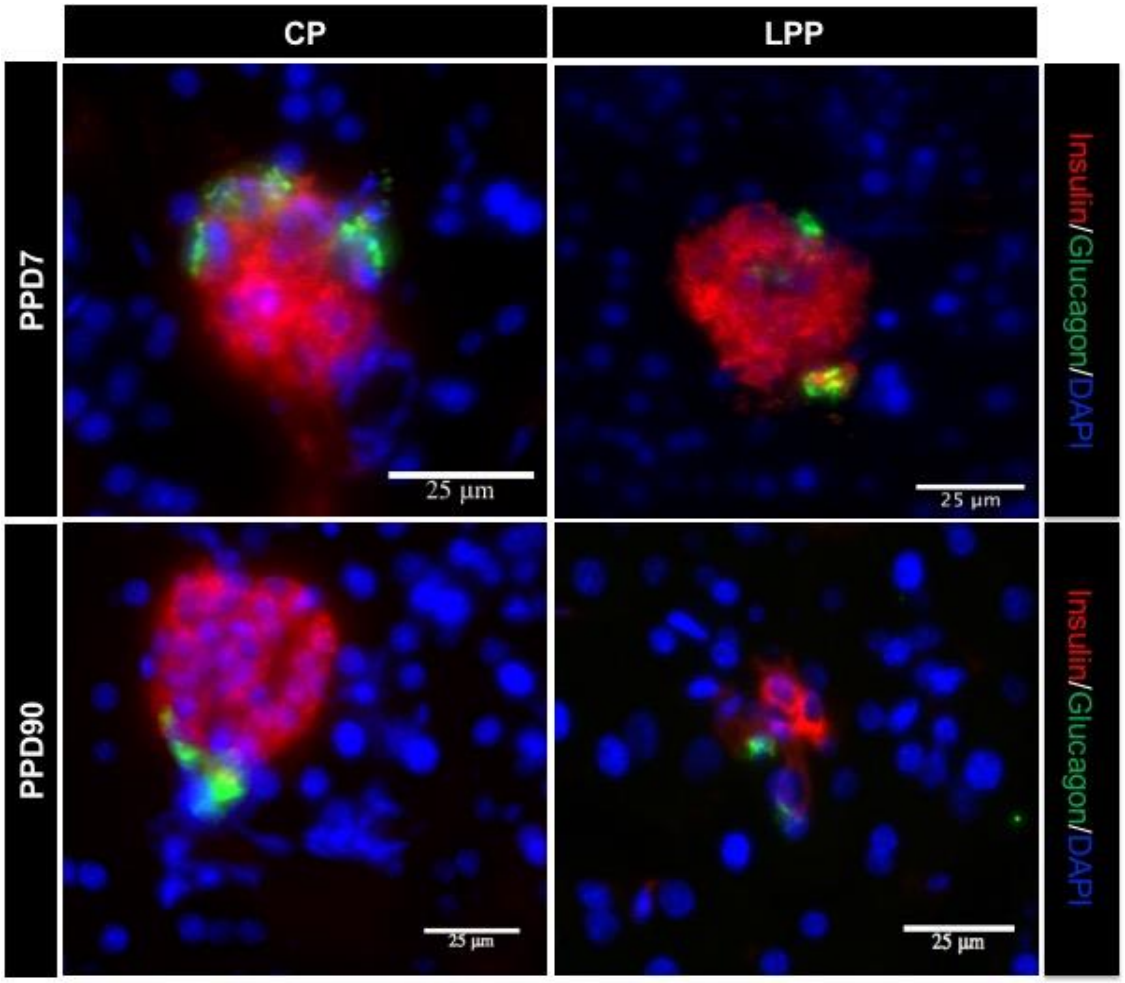


Figure 3.4. Representative immunofluorescent images of islets after parturition

Representative immunofluorescent images demonstrating β- (insulin, red) and α-cells (glucagon, green) in pregnant female mice born to dams fed a control diet (CP) and pregnant female mice born to dams fed a low protein diet (LPP) at postpartum days (PPD) 7 and PPD90. Nuclei were counterstained with DAPI (blue). Scale bar represents 25 microns.

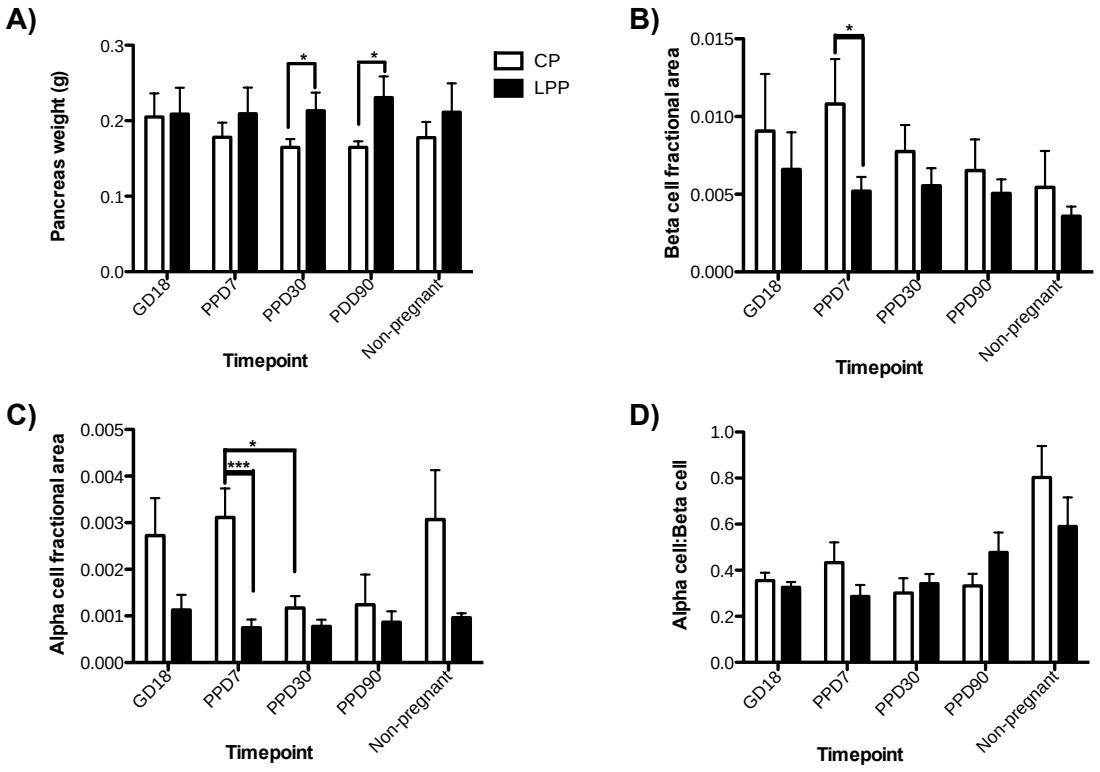


Figure 3.5. Pregnancy alters pancreas morphology postpartum after control and hyperglycemic pregnancies

(A) Pancreas weight was higher in pregnant female mice born to dams fed a low protein diet (LPP) at postpartum day (PPD) 30 and PPD90 relative to pregnant female mice born to dams fed a control diet (CP). Both the fractional (B) β-cell area and (C) α-cell area were lower in LPP animals vs controls at PPD7. Furthermore, α-cell area was lower at PPD30 vs PPD7 in CP females. (D) Alpha to β-cell ratio did not differ between dietary groups or timepoints after parturition (n = 4–6 CP and LPP females). ***$P < 0.001$, *$P < 0.05$, LPP *vs*. CP.

Table 3.1 Islet size distributions are altered postpartum after control and hyperglycemic pregnancies in mouse

The number of small, medium and large-sized islets varied with time after parturition in both dietary groups ($P<0.05$). There were more small islets at postpartum day (PPD) 90 in pregnant female mice born to dams fed a low protein diet (LPP) compared to pregnant female mice born to dams fed a control diet (CP). There were no significant differences in mean islet size after parturition between dietary groups. * $P<0.05$ LPP *vs.* CP.

Timepoint	Treatment	Small (< 5000 μm2)	Medium (5000–10,000 μm2)	Large (>10,000 μm2)	Mean islet size (um2)
Non-pregnant	Control	9.64	1.79	2.00	4974
	LP	7.50	1.42	1.33	4076
GD18	CP	17.88	3.00	5.00	7967
	LPP	7.90	1.30	1.60	4324
PPD7	CP	13.92	2.50	3.83	6394
	LPP	11.80	1.30	1.80	3599
PPD30	CP	17.20	4.50	6.70	6141
	LPP	13.73	2.63	4.00	5390
PPD90	CP	5.29	1.50	2.13	6403
	LPP	12.29 *	2.00	2.00	6373

3.3.4. Cytokines may contribute to glucose intolerance after parturition in mice experiencing hyperglycemic pregnancies

To investigate a potential contribution to prolonged glucose intolerance after parturition in the LPP group, we measured levels of representative cytokines in adipose tissue extracts by determining levels of TNF-α, IL-6, IL-1β, and IFNγ. TNF-α levels varied with time after parturition in both dietary groups and were 1.4-fold higher in the LPP group at PPD7 compared to controls (3.56 ± 1.10 pg/mL/mg tissue *vs.* 2.55 ± 0.98 pg/mL/mg tissue, P=0.534, Fig. 3.6A). Levels of IL-6 were higher in the LPP group at PPD7 compared to controls (Fig. 3.6B). There were no significant differences in levels of IL-1β with time after parturition or between dietary groups (Fig. 3.6C). However, IL-1β was 1.4-fold higher at PPD7 in LPP compared to controls (698.5 ± 254.7 pg/mL/mg tissue *vs.* 517.8 ± 274.2 pg/mL/mg tissue, P=0.654, Fig. 3.6C). Levels of IFNγ were 3.1-fold higher at PPD7 in LPP compared to controls (68.63 ± 24.94 pg/mL/mg tissue *vs.* 21.88 ± 4.51 pg/mL/mg tissue, P=0.139, (Fig. 3.6D).

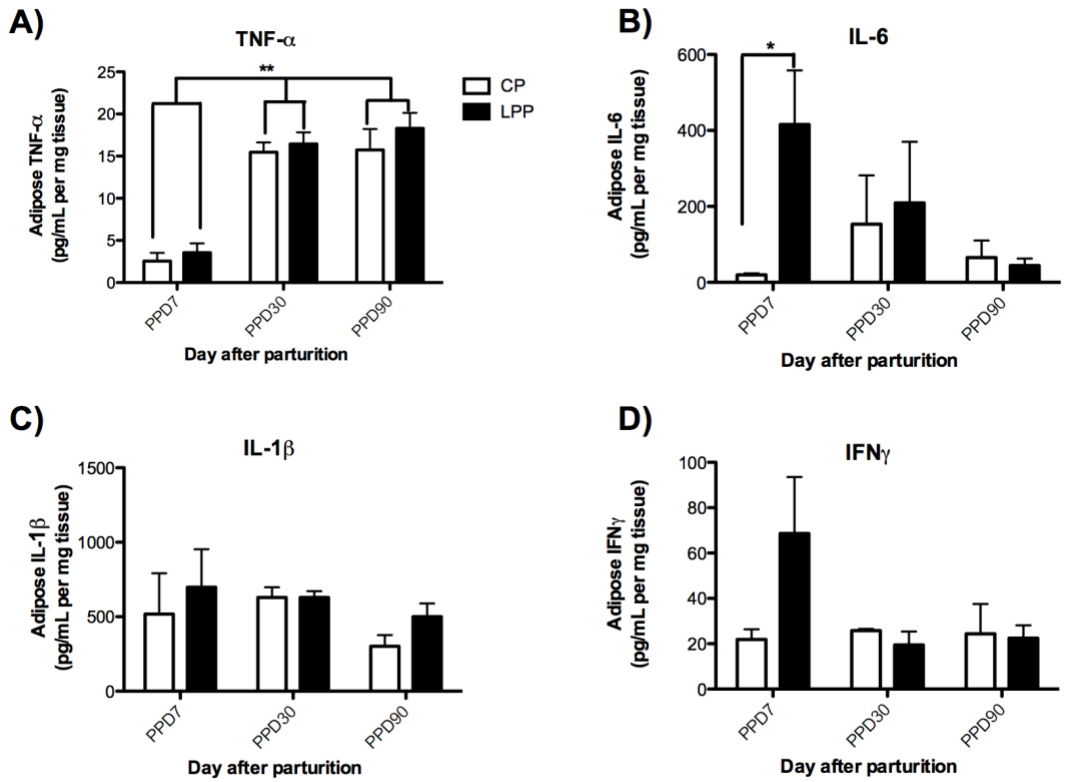


Figure 3.6. Altered cytokines may contribute to glucose intolerance in hyperglycemic mice after parturition

Adipose tissue content of: (A) TNF-α, (B) IL-6, (C) IL-1β, and (D) IFNγ for pregnant female mice born to dams fed a control diet (CP) and pregnant female mice born to dams fed a low protein diet (LPP) at postpartum days (PPD) 7, 30, and 90. (A) TNF-α content significantly increased with time postpartum but not between dietary groups. (B) IL-6 content decreased with time but was significantly higher in LPP animals. (C) IL-1β and (D) IFNγ content did not differ with time or between diets. n = 3–4, $^{**}P < 0.01$, $^{*}P < 0.05$, LPP *vs*. CP.

Next, circulating levels of PD-L1, insulin and glucagon in serum were quantified. There were no significant differences in levels of PD-L1 between dietary groups. However, values differed with time after parturition as levels of PD-L1 were higher at PPD90 compared to PPD7 in controls, but not in LPP mice (Fig. 3.7A). Of note, there was also a 2.7-fold higher amount of PD-L1 present in LPP animals at PPD7 compared to controls (145 ± 49.54 pg/mL *vs.* 54.76 ± 28.9 pg/mL, P=0.214, Fig. 3.7A). The ratio of serum insulin to glucagon varied with time after parturition in both CP and LPP mice (Fig. 3.7B) with a trend towards a higher ratio in the LPP group (P=0.071 LP *vs.* C, Fig. 3.7B).

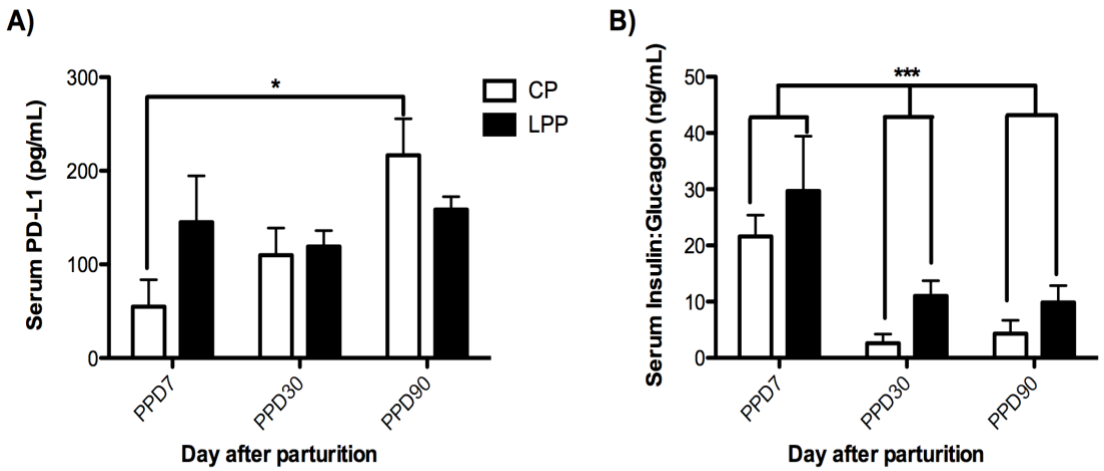


Figure 3.7. Pregnancy results in long-term alterations in serum levels of PD-L1, insulin, and glucagon after parturition

Serum levels of (A) PD-L1 and (B) insulin:glucagon ratio. (A) PD-L1 levels increased with time in pregnant female mice born to dams fed a control diet (CP) but not in pregnant female mice born to dams fed a low protein diet (LPP). (B) The serum insulin:glucagon ratio trended to be higher in LPP animals compared to controls (P = 0.0705) and varied with time in both groups. n = 3–5, ***P < 0.001, *P < 0.05, LPP *vs*. CP.

3.4 Discussion

GDM increases the subsequent risk of maternal dysglycemia or T2DM by up to 7-fold [17], although the relationship of this risk to longstanding changes in β-cell histology postpartum is unknown. Using a mouse model of gestational glucose intolerance, we addressed this knowledge gap by showing that dams continue to show glucose intolerance for at least a month after parturition (PPD30), and glycemic control did not normalize until 3 months postpartum (PPD90). This persistent glucose intolerance was associated with lower β- and α-cell fractional areas at PPD7 compared with control pregnancies representing an extension of the relative differences seen in late pregnancy for the glucose-intolerant dams.

Several studies have reported the presence of β-cell dysfunction postpartum clinically after GDM [18–21]. Progressive β-cell dysfunction is likely the predominant factor that drives the transition from impaired glucose tolerance to T2DM after GDM [38,39]. In this study, LPP animals displayed glucose intolerance at PPD7 and PPD30 relative to controls. We have previously shown that LPP animals exhibit β-cell dysfunction at late gestation (GD18), resulting in reduced GSIS [6, Chapter 2 of this thesis]. A previous clinical study classified β-cell dysfunction as the key factor in the development of postpartum dysglycemia amongst non-obese patients [40]; insulin resistance, however, was determined to be the driver of postpartum hyperglycemia in obese patients. Our animal model of gestational glucose intolerance, which represents the non-obese category, therefore accurately reflects the changes that occur during β-cell dysfunction after GDM, rather than another underlying cause (such as insulin resistance as seen in obese patients). Although pre-gestational obesity is a major driver of GDM, 20-30% of women that develop GDM do not fall into this category. Therefore, this implicates dysfunction at the level of the β-cell to GDM pathophysiology [12]. This study thus proves useful for revealing underlying mechanisms of glucose intolerance postpartum characterized by β-cell dysfunction. Since the animal model represents only a mild hyperglycemia, the animals in this study were able to normalize blood glucose levels by PPD90. Nonetheless, it is plausible that additional metabolic stress such as a second pregnancy, or an age-related decline in β-cell function [41], could precipitate T2DM.

Human studies of impaired glucose tolerance postpartum do not allow visualization of histological changes occurring in endocrine pancreas. Previous studies of healthy mouse pregnancy [7,8] report that offspring born to dams fed a control diet (CP) had a ~50% reduction in BCM at PPD7 compared to GD18. In this study, CP females had a ~30% reduction in β-cell fractional area at PPD30 compared to PPD7 and ~40% by PPD90 relative to PPD7, reducing β-cell fractional area to a level comparable to a non-pregnant animal. These differences could be attributed to the use of fractional area in this study instead of BCM. We used fractional area to exclude the effect of changes in exocrine tissue mass postpartum. Prolactin can alter exocrine tissue mass during lactation in mice [42] and we found differences in pancreatic weight postpartum in LPP mice. Thus, the use of fractional area provides a more accurate representation of endocrine changes in this study. These data support previous findings that β-cell apoptosis is occurring in controls after parturition in order to facilitate normalisation of BCM. This occurs in part due to a switch of β-cell serotonin receptor expression from *HTR2B* to *HTR1D*, mediating an inhibitory signal and promoting regression of BCM *via* increased β-cell apoptosis [9,43], in addition to higher levels of steroid hormones at late gestation which block lactogen-induced β-cell replication [44]. Insulin/TUNEL staining was negative when investigating the contribution of β-cell apoptosis at PPD7, suggesting that the apoptotic processes likely occur earlier postpartum since BCM was already reduced by PPD7. Nonetheless, a potential explanation for the retained β-cell fractional area at PPD7 in controls could be due to persistently higher expression of serotonin synthetic enzyme tryptophan hydroxylase-1, *Tph1*, which normally increases during pregnancy in β-cells in order to mediate BCM expansion. A previous study found that *Tph1* expression remained high at PPD7 until the end of lactation (PPD21) when levels returned to pre-pregnancy levels [43]. Further regression of β-cell fractional area at PPD30 and PPD90 could be due to regression of β-cell size, which was recently suggested as a contributor to BCM regression after pregnancy, prior to subsequently being increased during lactation. However, PPD30 and PPD90 mice in our study were not lactating, therefore, it is plausible that regression of β-cell fractional at these timepoints does indeed occur due to reduced β-cell size [45].

In terms of changes in pancreatic α-cell abundance, fractional area was lower at PPD30 compared to PPD7 in CP females. Thus, it is evident that there is a prolonged effect of pregnancy on the ontogeny of α-cells, as fractional area decreases to a level lower than the non-pregnant control at PPD90. These findings prompt interesting questions for future studies, especially concerning a second pregnancy and whether the pool of α-cells would increase and expand as observed on GD18 in a healthy pregnancy? [6,46]. Furthermore, would the pool of α-cells take longer to replenish, or is the α-cell complement fully depleted after a first pregnancy?

In contrast to CP females, β-cell fractional area was not further decreased postpartum in LPP mice; the values instead remained at a similar level from GD18 to PPD90. There were no differences in β-cell proliferation between dietary groups at PPD7 compared to non-pregnant animals (Supplemental Fig. 3.1C). These data confirm previous findings that lactogen-induced β-cell proliferation that normally occurs during pregnancy, which is also functionally linked to increased levels of progesterone and estradiol as pregnancy progresses [44], is arrested by PPD7. However, since levels of β-cell replication were comparable in CP and LPP groups postpartum this excludes β-cell replication as a mechanism for the sustained elevated fractional β-cell in LPP mice. As previously mentioned, we found no evidence of β-cell apoptosis at PPD7. These findings support clinical data that markers of β-cell loss were reduced in serum samples from women postpartum after GDM and reached levels seen in non-pregnant women [47]. Thus, our study is the first to provide histological evidence to support these clinical findings by demonstrating that less β-cell loss is occurring postpartum after mild GDM.

When comparing healthy and hyperglycemic pregnancies, both α- and β-cell fractional areas were lower in LPP mice at PPD7; a likely result of the insufficient endocrine pancreas adaptation previously found to occur at GD18, appearing to persist at PPD7. Importantly, α-cells have been identified as a target for serotonin action as a study in human islets showed that β-cell-derived serotonin inhibited glucagon secretion in response to high glucose [48]. Due to a reduced fractional β-cell area in LPP animals at PPD7 in this study, it is plausible that α-cells receive a reduced serotonergic input from β-cells and thus lose their ability to regulate glucagon secretion. This may result in

uncontrolled glucagon secretion and could contribute to the hyperglycemia seen in LPP animals at PPD7. This was shown to occur *in vivo* in women with GDM through a lack of suppression of plasma glucagon at late pregnancy that persisted postpartum [49]. These findings highlight an important role for pancreatic α-cells in pregnancy which has previously been overlooked.

Despite the lack of a placental hormone stimulus to endocrine cell expansion postnatally, the β-cell fractional area did not change in LPP mice. This might be a compensatory mechanism in attempt to attain euglycemia. Size stratification of islets varied at PPD7 and PPD30 in both control and LP groups, but there were no differences between dietary groups. This suggests that pregnancy itself causes re-modeling of islet populations after parturition. Additionally, there were more small islets in LPP females compared to controls found at PPD90, contributing to a recovered mean islet size comparable to that of a control. Thus, these data support an additional adaptive response in endocrine cells postpartum in LPP animals. These data could implicate β-cell neogenesis of small islets to facilitate normalization of mean islet size at PPD90 as a compensatory mechanism, thereby resulting in a rescue of glucose tolerance relative to controls. The LPP group had a higher ratio of insulin relative to glucagon after parturition compared to controls which provides further support of such an adaptive response.

Pro-inflammatory cytokines can modify insulin signaling pathways and can lead to β-cell dysfunction [50]. Consequently, overexpression of cytokines can accelerate inflammation and exacerbate insulin resistance. Women with GDM have been shown to have increased circulating levels of the pro-inflammatory cytokines TNF-α, IL-1β, and IL-6 all of which are associated with β-cell dysfunction [12,51]. Thus, it is plausible that prolonged glucose intolerance or T2DM after GDM could involve persistent inflammation postpartum. Both mouse and human placenta express multiple cytokines that contribute to the state of insulin resistance that occurs during pregnancy; for instance, TNF-α induces IRS-1 serine phosphorylation, which contributes to BCM expansion and insulin resistance in pregnancy [52,53]. In women with GDM, the decrease in insulin receptor tyrosine kinase phosphorylation does not improve postpartum as it does in women following a healthy pregnancy [33,54]. In the present model, levels of TNF-α were relatively higher in the

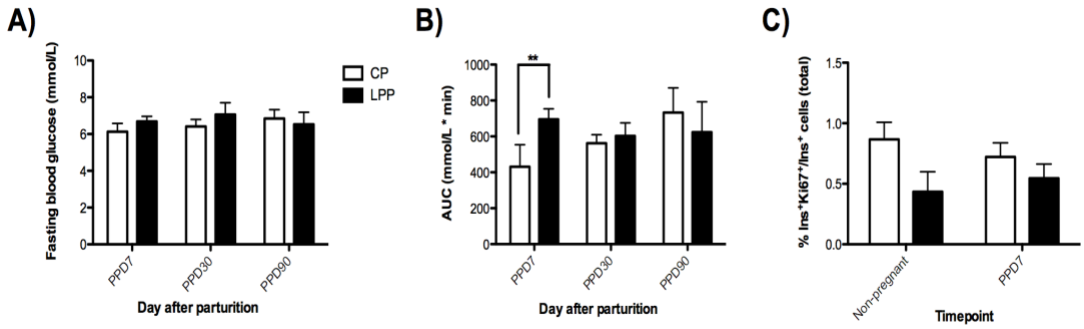
LPP animals compared to controls after parturition which could contribute, in part, to the glucose intolerance seen at PPD7 and PPD30. TNF-α levels in adipose increased with time, as seen in non-obese women in a study investigating longitudinal changes in serum pro-inflammatory markers across pregnancy and postpartum [55]. IL-6 has been found to be significantly higher in women with GDM, independent of adiposity [56] as observed in adipose samples from the non-obese animals used in this study where levels of IL-6 were higher in LPP mice at PPD7 compared to controls. Interestingly, IL-6 levels were reduced in both CP and LPP groups by PPD90 concomitant with the return of glucose tolerance to control values. Some studies have found that high concentrations of IL-6 could promote β-cell apoptosis and contribute to glucose intolerance [57]. However, since β-cell area did not change at any timepoint after parturition in LPP mice it is more likely that IL-6 is exerting effects on non-islet tissues. IL-6 has been shown to increase lipolysis in adipocytes, damage mitochondria and Glut2 function, and as a result decrease insulin sensitivity [58]. It is reasonable that higher IL-6 tissue levels contibuted to increased insulin resistance postpartum in LPP mice [52]. IL-6 can also induce production of IL-1β and TNF-α [59] further intensifying levels of pro-inflammatory cytokines postpartum and contributing to β-cell dysfunction and glucose intolerance. However, IL-1β levels in adipose tissue did not differ between dietary groups in the present studies. Collectively, these findings implicate a potential contribution of inflammation to insulin resistance postpartum in LPP mice, resulting in a maintaned β-cell area resistant to apoptosis postpartum.

An increase in cytokine production occurs in healthy pregnancies. However, the activity of PD-L1 also increases and attenuates the low-grade inflammatory immune response [27], potentially protecting maternal β-cells from cytotoxic damage. In the present study, levels of PD-L1 were relatively higher in LPP mice at PPD7 compared to controls. This may have helped to enhance β-cell survival in the face of a higher cytokine environment, as has been observed in autoimmune diabetes in NOD mice [29]. The PD-L1 ligand has also been shown to be expressed in β-cells of individuals with type 1 diabetes as a possible attempt to attenuate autoimmune attack [60]. However, PD-L1 was absent from islets of non-diabetic controls. Furthermore, the same study showed that IFNγ induced

PD-L1 mRNA expression in human pancreatic β-cells *in vitro*, potentially implicating IFNγ at PPD7 in LPP mice in this study as a mechanism for increasing PD-L1 levels. This pathway might also mediate β-cell neogenesis leading to a subsequent increase in the number of small islets observed at PPD90. This inflammatory pathway may also explain why β-cell fractional area remained elevated in LPP mice postpartum. Follow-up experiments treating LPP and CP mice at PPD7/30/90 with a PD-L1 inhibitor would be insightful to investigate potential differences in the ratio of serum insulin to glucagon. Interestingly, PD-L1 levels increased from PPD7 to PPD90 in controls. As a multitude of immunological changes involving both pro- and anti-inflammatory cytokines are occurring throughout pregnancy to the postpartum period, it is plausible that serum PD-L1 levels were elevated in controls in part to suppress maternal immunity, or, to mediate a protective effect on β-cells against higher levels of TNF-α at postpartum. Interestingly, since PD-L1 levels were ~25% lower in LPP mice at PPD90, perhaps these animals will be more prone to cytoxic β-cell damage and could be on the trajectory to dysglycemia or T2DM. PD-L1 has also been identified as a biomarker for GDM in humans [61] and our findings support a potential role as a marker for prolonged glucose intolerance after GDM.

In summary, we present novel findings of the ontogeny of α- and β-cell fractional areas of islet morphology and glucose tolerance postpartum in normal and hyperglycemic mouse pregnancies. The results demonstrate long-term pancreatic re-modeling after parturition involving both pancreatic α- and β-cells, which was associated with changes in the pro-inflammatory environment. These findings are informative in understanding the pathophysiology involved in the progression from GDM to glucose intolerance and T2DM.

3.5 Supplemental Figures



Supplemental Figure 3.1. Metabolic and pancreas histology parameters after a GDM and healthy pregnancy

A) Fasting blood glucose levels did not vary after parturition between dietary groups. Values represented are mean ± SEM analyzed by two-way ANOVA, $P>0.05$.

B) Area under the glucose tolerance curve was higher in LPP animals at PPD7 compared to controls. Values represented are mean ± SEM analyzed by unpaired two-tailed Student's *t*-test, ** $P<0.01$. **C)** There were no differences in proportion of beta-cell proliferation (visualized by cell counting of dual-stained insulin and proliferation marker, ki67, positive cells) found relative to all counted beta cells. Values represented are mean ± SEM analyzed by two-way ANOVA, $P>0.05$.

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Chapter 4

4 The Increased Alpha and Beta Cell Mass during Mouse Pregnancy is not Dependent on Transdifferentiation

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4.1 Introduction

Pregnancy is a physiological state characterized by relative maternal insulin resistance [1]. This has been linked to the presence of placentally-derived hormones and cytokines in the maternal circulation in the second half of pregnancy [2]. In preparation for the increased demand for insulin, adaptive changes occur in the endocrine pancreas in order to maintain euglycemia whilst also supplying the growing fetus with an adequate nutrient supply. A reversible expansion of β-cell mass (BCM) has been documented in both mice and humans and is maximal at late gestation (around gestational day (GD) 18.5 in mice) [3–8]. In situations where BCM expansion is suboptimal, gestational diabetes mellitus (GDM) can develop. This has been demonstrated in both clinical studies [9] and animal models of GDM [3,8,10–13] implicating β-cell failure as a major driver to metabolic pathogenesis. GDM is described as diabetes that first appears during pregnancy, which regresses postpartum in most cases. Nonetheless, GDM is associated with adverse short and long-term fetal and maternal health outcomes [14–18] necessitating the development of effective methods of intervention. Current treatments for GDM, such as lifestyle behavioural change or administration of insulin or metformin, aim to decrease hyperglycemia but do not treat the underlying causes including a suboptimal BCM. Thus, a better understanding of mechanisms of BCM expansion in pregnancy are needed in order to effectively target potential therapeutic interventions.

The adaptive mechanisms of BCM expansion during mouse pregnancy have been shown to involve a re-entry of normally quiescent pre-existing β-cells into cell replication, mediated in part through prolactin receptor signaling in response to lactogenic hormones [12,19,20], in addition to increased β-cell hypertrophy [10]. These processes are maximal around mid-gestation in mice to prepare the pancreas for enhanced glucose-stimulated insulin release in late pregnancy [7,8]. Additional mechanisms of BCM expansion are likely to include the expansion and subsequent differentiation of a multipotent β-cell progenitor pool expressing some insulin but low levels of glucose-transporter 2 ($Ins^{+}Glut2^{LO}$) [7]. $Ins^{+}Glut2^{LO}$ cells are able to differentiate into mature β-cells under metabolic stress [21,22]. Pregnant mice were shown to have a higher proportion of proliferating $Ins^{+}Glut2^{LO}$ cells at GD9.5, preceding maximal β-cell proliferation at

GD12.5. This was concurrent with increased *Pdx1* mRNA expression, marking endocrine progenitor and mature β-cells, implicating this progenitor pool to BCM expansion during pregnancy. The contribution of non-β-cell progenitors to gestational BCM expansion has also been proposed and could contribute up to 25% of new β-cells in pregnancy [23,24]. An increase in the number of islets during mouse pregnancy has also been documented, providing further support for a contribution of islet neogenesis [7,8,25]. Furthermore, there were fewer small-sized islets throughout pregnancy in glucose-intolerant pregnant mice, implicating a potential critical role for a deficiency of β-cell neogenesis in the development of glucose intolerance [8, Chapter 2 of this thesis]. Although evidence exists to support expansion of BCM in pregnant humans, due to the scarcity of human samples, the mechanisms involved in BCM expansion remain unclear and controversial [6,26]. As such, the scarcity of pregnant human pancreas implicates the reliance on animal models of diabetes in pregnancy.

Although there is evidence that α-cells contribute to hyperglycemia in patients with type 2 diabetes mellitus (T2DM) *via* hyperglucagonemia [27–31], the dynamics of pancreatic α-cells in pregnancy have only recently been explored [8,32]. The changes in α-cell abundance during the endocrine adaptation to pregnancy were described with an expansion in α-cell mass (ACM) at GD18.5 in mice [8,32] which was impaired in glucose-intolerant pregnancies [8, Chapter 2 of this thesis]. One source of new β-cells during pregnancy could derive from a molecular re-programming of glucagon-producing α-cells as part of dynamic changes in the α-cell population. Previously it was shown that α-cells can replenish β-cells following extreme β-cell loss or during β-cell stress by α- to β-cell transdifferentiation [33,34]. Quesada and colleagues suggested that a negligible amount of α- to β-cell transdifferentiation was occurring at GD18.5 in normal pregnancy compared to non-pregnant mice, however genetic lineage tracing of α-cells was not performed to confirm this, and earlier timepoints in pregnancy were not examined [32]. As this study was performed in normal pregnancy there remains a lack of information about α-cell plasticity in the development of GDM. In this study, we aimed to address these knowledge gaps by: (1) documenting changes in the balance of α- and β-cells in control compared to glucose-intolerant mouse pregnancy, and (2) elucidating any temporal changes in α- to β-cell transdifferentiation in normal mouse pregnancy using

genetic lineage tracing. We hypothesized that one of the putative mechanisms related to the endocrine adaptational increase in β-cells in pregnancy could be the transdifferentiation of α-cells into β-cells and that any disbalance in this process will predispose to GDM.

4.2 Methods

4.2.1. Animals and Sample Collection

All animal procedures were approved by the Animal Care Committee of Western University in accordance with the guidelines of the Canadian Council for Animal Care. Mice were housed in a temperature-controlled room with 12-h light:dark cycle at Lawson Health Research Institute, London, ON, Canada. Water and food were given *ad libitum.*

Aim 1: Adult (6-week-old) C57BL/6 male and female (F0) mice were obtained from Charles River Laboratories (Wilmington, MA, USA). Mice showing gestational glucose intolerance at GD18.5 were generated using a previously described protocol involving a dietary insult during early life [8, Chapter 2 of this thesis]. Briefly, F0 females underwent estrous cycling and were time-mated with males. Dams were randomly assigned to either a control (C, 20% protein, Bio-Serv, Frenchtown, NJ, USA) or a low protein (LP, 8%) diet similar to that described by Snoeck *et al.* [35]. The two diets were isocalorific, the deficiency in calories in the LP diet being compensated by additional carbohydrate [8]. F0 dams were fed either the LP or C diet throughout gestation and lactation, and female offspring (F1) were weaned onto C diet. At maturity (postnatal day, PND, 42), female offspring (F1) of LP and C diet-fed mothers were randomly allocated into two study groups: pregnant or non-pregnant. All pregnant-grouped females were time-mated (GD9.5, 12.5, 18.5) with C diet-fed males. Females (F1) were euthanized by CO_2 asphyxia for comparison to non-pregnant age-matched F1 females. The pancreas was removed at each assigned day of gestation (n = 4-6 C and LP animals for each timepoint

during gestation and for the non-pregnant groups). The pancreas was fixed in 4% paraformaldehyde for histology and embedded in optical cutting temperature compound.

Aim 2: Glucagon-Cre$_{iCre}$ mice (stock #030663, Jackson Laboratories, Bar Harbor, Maine, USA) that express Cre in 93-95% of α-cells, were crossed with a Rosa26-eYFP reporter mouse strain (stock #006148, Jackson Laboratories) to produce double transgenic Glucagon-Cre/Rosa26-eYFP (Gcg-Cre/YFP) mice. At maturity, double transgenic female offspring were randomly separated into 2 study groups: pregnant and non-pregnant. Pregnant-grouped females underwent estrous cycling in order to produce timed pregnancies [36]. Individual double transgenic female and wildtype C57BL/6 male mice (Charles River Laboratories, Wilmington, MA, USA) were housed together the morning of pro-estrous for mating and were separated the following morning. Females in the non-pregnant group were age-matched to animals in the pregnant group (GD9.5, 12.5, and 18.5). Animals were euthanized by CO_2 asphyxia and the pancreas was removed at each assigned day of gestation (n = 4 animals for each timepoint during gestation and n = 8 animals for the non-pregnant group) and prepared for histology as described above.

4.2.2. Immunofluorescence Staining

Fixed pancreas tissue was prepared and sectioned as previously described [37]. At least two 7 μm-thick cryosections (replicates) were cut from each pancreas for immunohistochemical analysis. The interval between each section was >100 μm, representing at least two longitudinal slices through the pancreas. Sections included both the head and tail of the pancreas. For aim 1, immunofluorescence immunohistochemistry was performed to localize insulin, glucagon and Ki-67 as described previously [7,8]. Antibodies against insulin (1:2000, anti-mouse, Sigma-Aldrich, St. Louis, MO, USA) and glucagon (1:200, anti-rabbit, Santa Cruz Biotechnology, Dallas, TX, USA) were applied to tissues and incubated overnight at 4°C. To investigate α-cell proliferation, antibodies against glucagon (1:200, anti-rabbit, Santa Cruz Biotechnology) and Ki-67 (1:50, anti-mouse, Biosciences, Mississauga, ON, Canada) were applied to tissues and incubated overnight at 4°C. The following day, secondary antibodies (1:500 Thermo Fisher Scientific Waltham, MA, USA) were applied against the primary antibody using 555 and

488 fluorophores, respectively, along with DAPI (1:500, Thermo Fisher Scientific) to counterstain nuclei. ACM data was retrieved from our previous studies [8, Chapter 2 of this thesis] and calculated by multiplying the fractional α-cell area (sum of all glucagon-expressing areas divided by the whole pancreas surface area) by pancreas weight. For aim 2, fluorescent immunohistochemistry was performed to localize insulin (phenotypic β-cells), glucagon (phenotypic α-cells) and YFP (α-cell origin) for cell counting analysis. Background Sniper (Biocare Medical, Concord, CA, USA) was applied to each tissue section for 8 minutes to reduce non-specific background binding. Subsequently, antibodies against insulin (1:50, anti-guinea pig, Abcam, Cambridge, UK), glucagon (1:2000, anti-mouse, Sigma-Aldrich) and YFP (1:1000, anti-rabbit, Abcam) were applied to tissue sections and incubated overnight at 4°C. The following day, secondary antibodies (1:400 Thermo Fisher Scientific) were applied against the primary antibody using 555, 647, 488 fluorophores, respectively, along with DAPI (4, 6-diamidino-2 phenylindole, dihydrochloride, 1:500, Thermo Fisher Scientific) to counterstain nuclei.

4.2.3. Cell Counting Analysis

Tissue sections were visualized by a blinded technician at 20x using a Nikon Eclipse TS2R inverted microscope (Nikon, Minato, Tokyo, Japan) with the program NIS elements (Nikon, Minato, Tokyo, Japan), and images were captured and analyzed using cell counter on ImageJ software. Every insulin, glucagon, and YFP expressing cell was imaged for each section and for each animal. In this study, an “islet” was considered to contain >5 β-cells, and an extra-islet endocrine “cluster” as containing 1-5 β-cells [37]. For aim 1, manual cell counting analysis determined the percentage of $Insulin^{+}Glucagon^{+}$ (insulin and glucagon double-positive) cells as a marker for α- to β-cell transitional cells [32,38–40]. Alpha-cell proliferation was determined by manually counting glucagon and Ki-67 double-positive cells. For aim 2, manual cell counting analysis determined the percentage of $Insulin^{+}YFP^{+}Glucagon^{+}$ cells as a marker for a possible intermediate, transitional cell type between an α-cell and a β-cell (Fig. 4.1). The percentage of $Insulin^{+}YFP^{+}Glucagon^{-}$ cells was also determined to identify phenotypic β-cells arising from an α-cell origin. These cells were further localized as either being in the islet core or mantle. Co-localized cells that were part of the outermost layer of $Insulin^{+}$ cells within

each islet were classified as being part of the islets' mantle. Any co-localized cells that were closer to the middle of the islet, and therefore surrounded by this outer layer of Insulin^{+} cells, were classified as being part of the islet core. While islets are large enough to be able to break down into either core or mantle components, clusters were not. As each cluster of cells is only made up of 1 to 5 Insulin^{+} cells, no definitive outer layer of cells exists within this structure. Therefore, the division of co-localized cells into core and mantle layers was only feasible in "islets", which are each composed of 6 or more Insulin^{+} cells. The core and mantle analysis was completed for both Insulin^{+}YFP^{+}Glucagon^{+} cells and Insulin^{+}YFP^{+}Glucagon^{-} cells. For the core and mantle calculations, the Insulin^{+}YFP^{+}Glucagon^{-} or Insulin^{+}YFP^{+}Glucagon^{+} cells that fell within either the core or the mantle were divided by the total number of Insulin^{+}YFP^{+}Glucagon^{+} or Insulin^{+}YFP^{+}Glucagon^{-} counted for the tissue section.

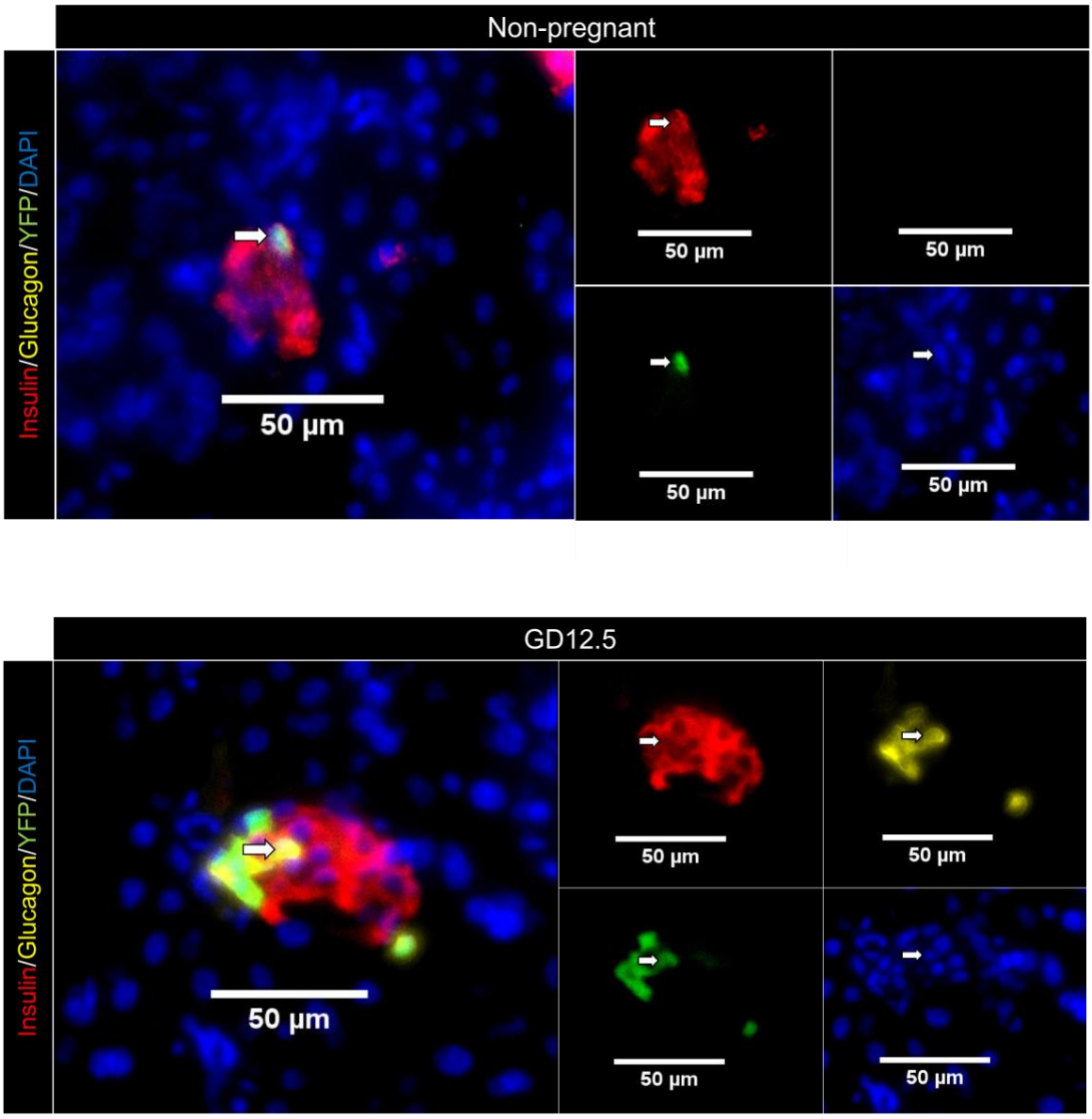


Figure 4.1. Representative images of islet populations

Representative images demonstrating staining for insulin (red), glucagon (yellow), YFP (green) and nuclei (DAPI, blue) in pancreatic sections from GlucagonCre-YFP transgenic female mice. The arrow in the non-pregnant islet represents a β-cell arising from an α-cell that no longer expresses glucagon (Insulin^{+}YFP^{+}Glucagon^{-}). The arrow in the GD12.5 islet represents an Insulin^{+}YFP^{+}Glucagon^{+} cell.

4.2.4. Serum ELISA Assays

For aim 1, maternal (F1) blood was collected *via* cardiac puncture after euthanasia in order to quantify serum insulin and glucagon using an Ultra-Sensitive Mouse Insulin ELISA kit (Crystal Chem, Downers Grove, IL, USA) or Mouse Glucagon ELISA kit (Crystal Chem), respectively. The insulin assay has a sensitivity of 0.05 ng/mL using a 5uL sample with precision CV ≤ 10.0%. The glucagon assay has a sensitivity of 1.1pg/mL using a 10uL sample with precision CV ≤ 10%. Samples were run in duplicate. Data were collected using a BioRad iMark plate reader and analyzed using Microplate Manager Software. Data throughout pregnancy was compared to non-pregnant animals, as a previous study [32] found that mice showed hypoglucagonemia as they entered pregnancy. Therefore, we compared the data as a percent change to non-pregnant animals, to determine how the animals adapt pancreatic α-cells in response to pregnancy.

4.2.5. Statistical Analysis

The sample size of four to six animals per variable in either the LP or C groups was calculated based on achieving a statistically significant difference with an expected standard deviation around mean values for BCM and glucose tolerance of 15% or less based on our previous studies [7]. Data are presented as mean ± SEM, with statistics analyzed using GraphPad Prism software (Version 5.0). An unpaired two-tailed Student's *t* test, one-way ANOVA or two-way ANOVA were applied according to the set of groups that were compared. A Tukey's post-hoc test or a Bonferroni post-hoc test was performed after one-way ANOVA or two-way ANOVA analysis, respectively. Non-parametric tests were performed when data did not meet the assumption of normality. Significant outliers were determined using Grubbs' test for each parameter. Each animal presented as a single unit of analysis (*n*). Statistical significance was determined as $P < 0.05$.

4.3 Results

4.3.1. Glucagon presence and α-cell proliferation in control *vs.* glucose-intolerant pregnancies

We examined the changes in α-cell presence and function during pregnancy, and particularly the cells co-staining for insulin and glucagon, comparing normal pregnancies and those previously shown by us to have impaired gestational glucose tolerance with a decreased BCM [8, Chapter 2 of this thesis]. During pregnancy, both control and LP mice exhibited hypoglucagonemia relative to non-pregnant animals (Fig. 4.2A). However, the LP diet group showed a significantly greater serum glucagon presence (Fig. 4.2A) and lower serum insulin (Fig. 4.2B) in late gestation compared to control-diet animals when expressed relative to the values in treatment-matched non-pregnant animals. Nonetheless, the overall serum insulin/glucagon ratio did not change during pregnancy between treatment groups, although values were higher throughout pregnancy compared to non-pregnant animals (Fig. 4.2C). This indicates that an increase in both circulating insulin and glucagon occurs during pregnancy but with relatively more insulin.

When the ontogeny of α-cell proliferation was examined during pregnancy, a significant increase was seen at GD9.5 across the whole pancreas compared to pre-pregnancy in control animals, although this subsequently declined (Fig. 4.3A). However, proliferating α-cells were significantly reduced in extra-islet clusters at GD9.5 in the LP diet group relative to controls (Fig. 4.3B). Alpha-cell mass changed across gestation in both control and LP groups ($P=0.01$, Fig. 4.3C). However, ACM was significantly reduced in the LP group compared to control animals at GD18.5 (Fig. 4.3C).

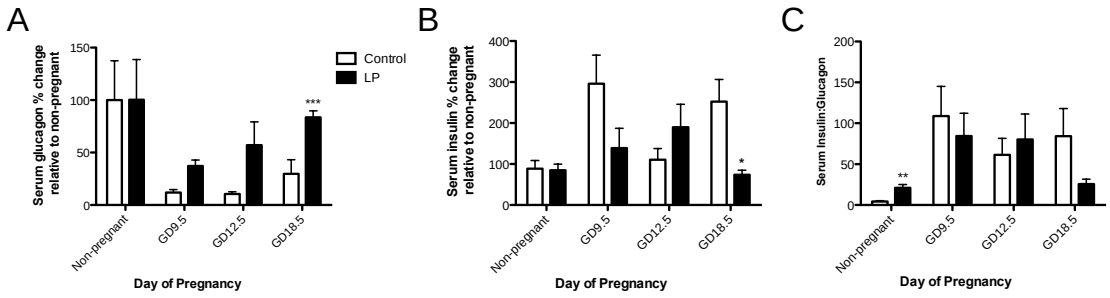


Figure 4.2. Circulating levels of glucagon and insulin during control and LP pregnancies, and changes in ratio of insulin to glucagon

(A) Serum glucagon and (B) insulin levels are shown as a percentage change relative to non-pregnant animals for the gestational days indicated. (C) Serum insulin to glucagon ratio throughout pregnancy. Samples were collected after euthanasia *via* cardiac puncture following an intra-peritoneal glucose tolerance test. n = 4-6 C and LP animals, *** P<0.001, ** P<0.01, * P<0.05, LP *vs*. C.

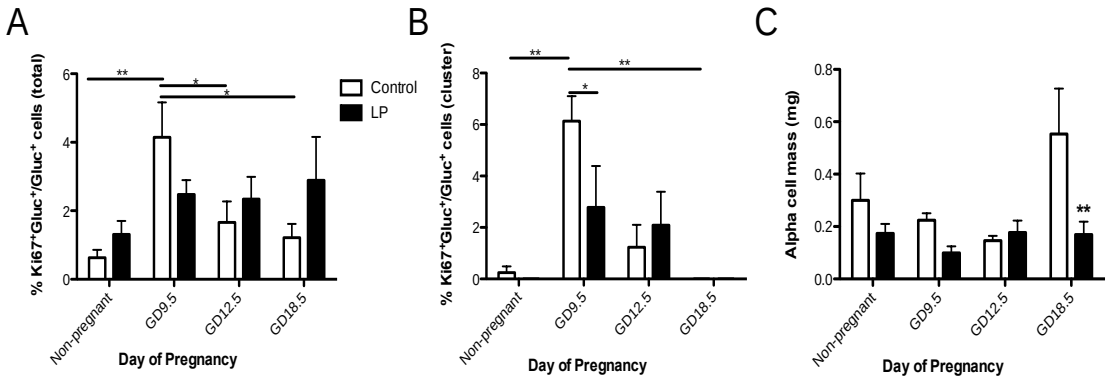


Figure 4.3 Gestational α-cell proliferation measured by the nuclear presence of Ki67 in control and glucose-intolerant (LP-treated) mouse pregnancy

The percentage of proliferating α-cells in (A) whole pancreas and (B) extra-islet clusters. is shown relative to all glucagon immunopositive cells. (C) α-cell mass in control and LP pregnancy. *n* = 4-6 C and 4-5 LP animals, ** *P*<0.01, * *P*<0.05, LP *vs*. C.

4.3.2. The balance of pancreatic α- and β-cells in control *vs.* glucose-intolerant pregnancies

The frequency of insulin-staining cells that also contained glucagon was approximately 15% in non-pregnant control diet mice (Fig. 4.4A) but the abundance of such bihormonal cells in whole pancreas, islets or extra-islet clusters did not change in control animals during pregnancy, and also did not differ in the LP diet group (Fig. 4.4A-C). However, the LP mice did enter pregnancy with a pre-existing reduction in the number of such cells compared to controls. Furthermore, there was a trend towards fewer dual-stained cells at GD12.5 in LP *vs.* control animals, suggesting that this potential lack of plasticity remained throughout pregnancy (Fig. 4.4A, $P = 0.087$). Notably, there was a transient decrease of ~50% of such cells in control pregnancies at GD9.5 (non-pregnant 16 ± 3% to GD9.5 7 ± 1%) prior to replenishment of these cells by GD12.5/18.5, potentially implicating a burst of α- to β-cell transdifferentiation at GD9.5. This relative decrease in dual-stained cells was absent in LP dams at GD9.5 and could implicate α- to β-cell transdifferentiation as a mechanism to increased BCM expansion gestation in control animals that was impaired in LP females.

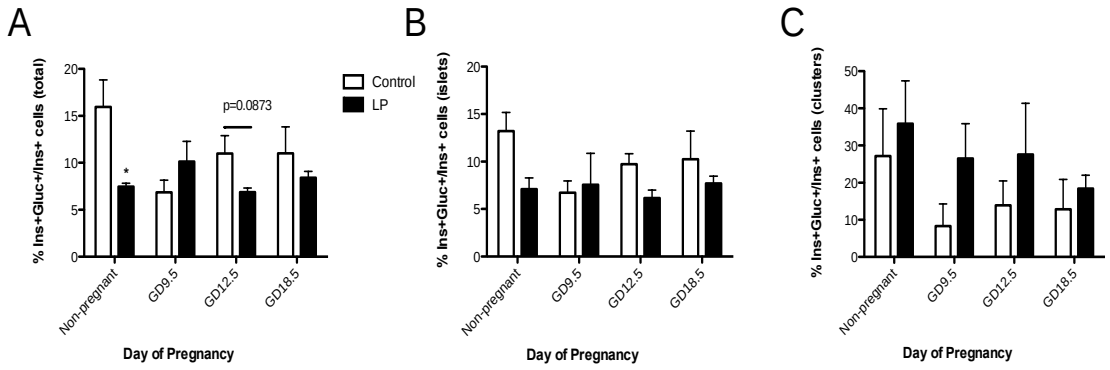


Figure 4.4. Ontogeny of bihormonal cells containing both glucagon and insulin in control and glucose-intolerant (LP-treated) pregnancies

The percentage of bihormonal cells is shown in (A) whole pancreas, (B) islets, and (C) extra-islet clusters relative to the total insulin immunopositive cells. *n* = 4-6 C and 4-5 LP animals, * *P*<0.05, LP *vs*. C.

4.3.3. The contribution of α- to β-cell transdifferentiation to new β-cells in control pregnancy

To address this question, we investigated the contribution of α- to β-cell transdifferentiation to BCM expansion in the pancreas during pregnancy by immunostaining histological sections of Gcg-Cre/YFP mouse pancreata for YFP, glucagon and insulin. By using Gcg-Cre/YFP transgenic mice, we were able to accurately lineage trace changes in the fate of glucagon-expressing pancreatic α-cells during the course of pregnancy to determine if some cells transdifferentiate to express insulin but not glucagon. First, co-localization of YFP with insulin in cells that did not contain glucagon ($Insulin^{+}YFP^{+}Glucagon^{-}$) was examined within the pregnant mouse pancreas at various timepoints throughout pregnancy. Co-localization was seen in a minority of cells in both islets and small extra-islet endocrine clusters. In non-pregnant mice approximately 8% of insulin-staining cells also expressed YFP and this did not alter significantly throughout pregnancy when examined for the whole pancreas (Fig. 4.5A) or considering islets (Fig. 4.5B) or extra-islet clusters alone (Fig. 4.5C). Furthermore, the pattern for fold change relative to non-pregnant animals was also negligible in whole pancreas (Supplemental Fig. 4.1). Whilst the relative number of insulin-YFP dual stained cells in islets did not change during pregnancy the distribution did alter, with a relative reduction being seen in late gestation in the outer mantle of the islets relative to the islet core (Fig. 4.5D).

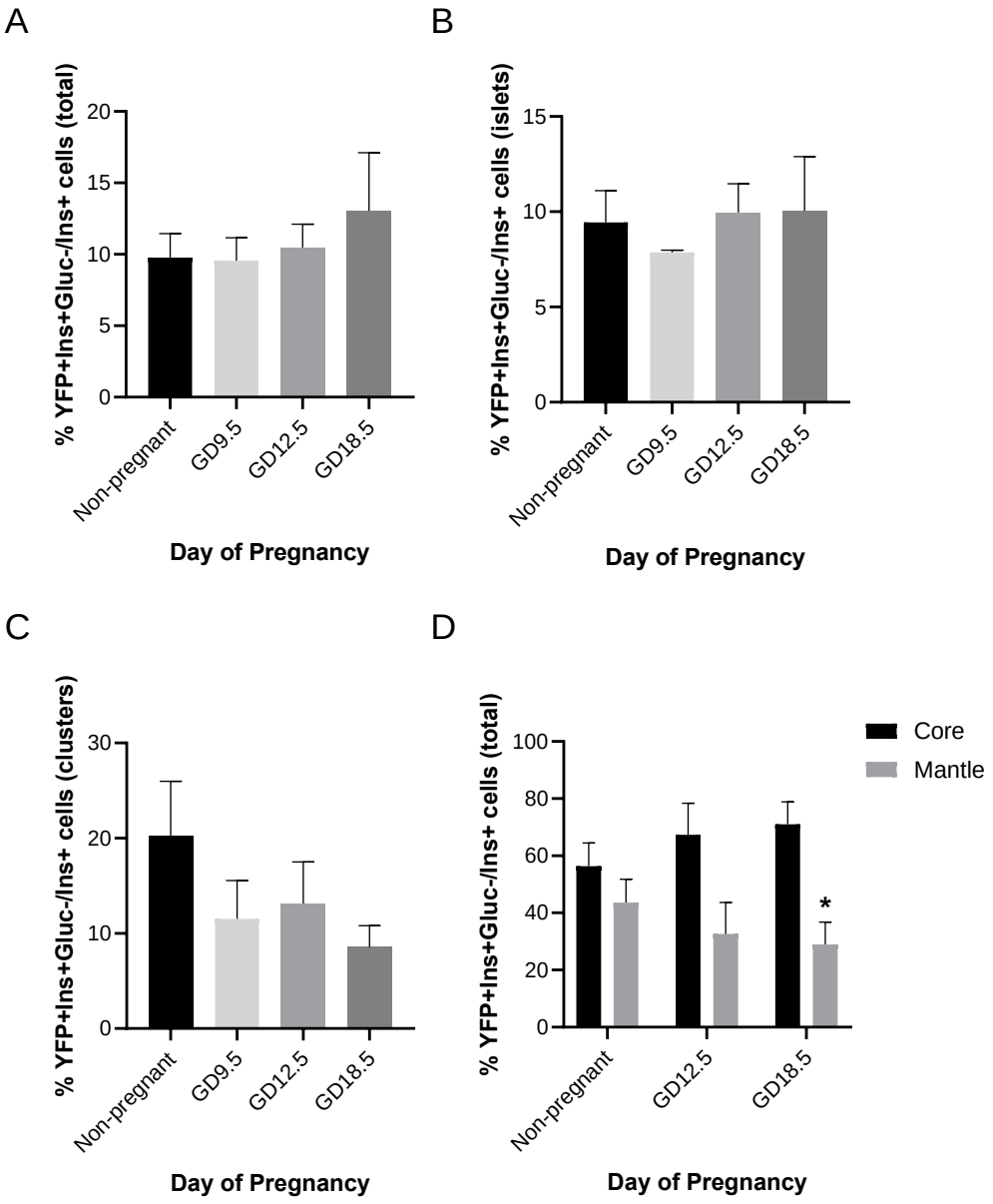


Figure 4.5. Alpha to β-cell transdifferentiation expressed as the percentage of phenotypic β-cells (Insulin^{+}YFP^{+}Glucagon^{-})

Alpha to β-cell transdifferentiation in non-pregnant mice and at various gestational ages during normal pregnancy as expressed by the percentage of phenotypic β-cells (Insulin^{+}YFP^{+}Glucagon^{-}) relative to the total insulin immunoreactive cells. The percentage of such cells in (A) the entire pancreas, (B) islets and (C) clusters are shown. (D) Localization of Insulin^{+}YFP^{+}Glucagon^{-} cells to the islet mantle *vs.* the core. $n = 8$ non-pregnant and 4 pregnant animals, * $P<0.05$, core *vs.* mantle.

4.3.4. Cells in a transitional stage of α- to β-cell transdifferentiation increase in the islet mantle at GD18.5

In addition to insulin-staining cells expressing YFP in the absence of glucagon (Insulin^{+}YFP^{+}Glucagon^{-}) an approximately equal number of cells co-stained for insulin, YFP and glucagon in non-pregnant animals (Fig. 4.6A). As observed above with the C and LP-diet animals, the relative abundance of these cells did not change during pregnancy in either islets or extra-islet clusters (Fig. 4.6B-C), but their relative anatomical distribution within islets did alter in late gestation with significantly more being observed in the islet mantle (Fig. 4.6D).

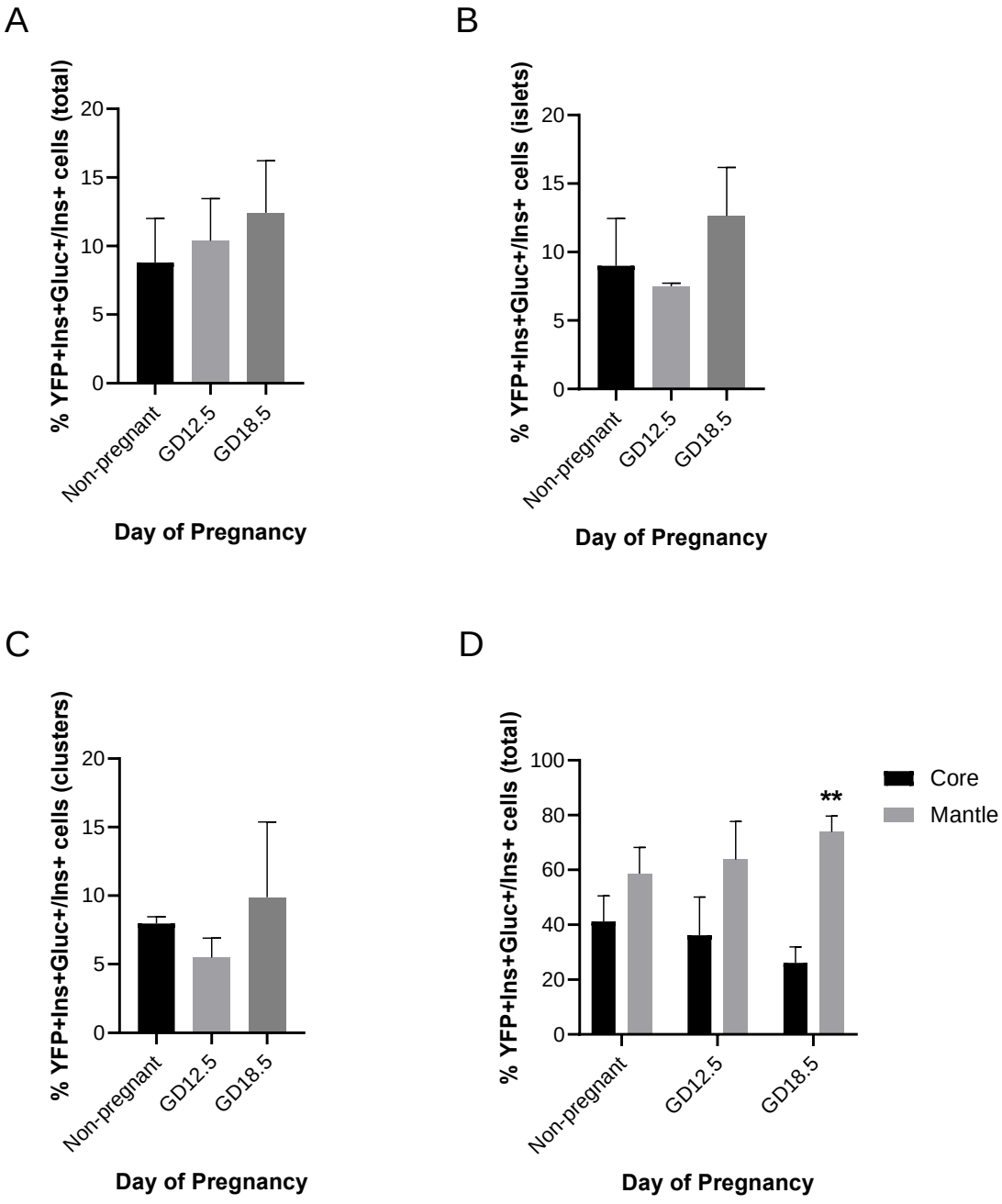


Figure 4.6. Presence of Insulin$_+$YFP$_+$Glucagon$_+$cells in the pancreas of non-pregnant and pregnant mice at gestational days 12.5 and 18.5

The percentage of cells present in (A) whole pancreas, (B) islets and (C) clusters is shown relative to all insulin immunoreactive cells. (D) Localization of Insulin$_+$YFP$_+$Glucagon$_+$cells to the islet mantle *vs.* the core. $n = 4$ non-pregnant and 4 pregnant animals, ** $P<0.01$, core *vs.* mantle.

4.4 Discussion

Pregnancy displays a remarkable reversible adaptation of BCM in order to maintain euglycemia, otherwise, pathologies such as GDM can arise. Although β-cells make up the majority of the islet, α-cells are the next most abundant cell type in the pancreas. These two endocrine cells play a critical role in maintaining glucose homeostasis by functioning in an antagonistic manner, whereby the intra-islet hypothesis states that insulin inhibits glucagon secretion [41]. The contribution of α-cells to hyperglycemia in patients with T2DM *via* hyperglucagonemia has been well-documented [27–31]. However, much less is known regarding the plasticity of pancreatic α-cells in pregnancy and this has yet to be investigated in glucose-intolerant pregnancy. Since α-cells can act as a reservoir to increase β-cell regeneration *via* α- to β-cell transdifferentiation in non-pregnant animals [42], it was also important to elucidate the role of this transdifferentiation in pregnancy.

We first investigated changes in pancreatic α-cells in glucose-intolerant pregnancy using a previously established mouse model involving a dietary (LP diet) insult [8, Chapter 2 of this thesis]. Both dietary groups (LP and C) exhibited hypoglucagonemia during pregnancy. This supports findings from a previous study that showed that pregnant mice exhibited hypoglucagonemia and impaired glucagon secretion at GD18.5 [32]. This likely occurs as a protective effect to prevent hyperglycemia in the presence of insulin resistance at late pregnancy. Although, in our study there was less suppression of serum glucagon in LP mice at GD18.5, contributing to glucose intolerance in these animals as has been shown to occur at late pregnancy in women with GDM [43,44]. Importantly, higher glucagon levels persisted after parturition in women with GDM and it has been shown that this can contribute to dysglycemia and eventual development of T2DM. While treatment for GDM currently focuses on administering blood glucose lowering agents, such as insulin, management of uncontrolled glucagon secretion in GDM could theoretically also serve as a mechanism to reverse blood glucose levels in hyperglycemic women, by means of suppressing these levels. In contrast, levels of insulin were lower in LP mice at GD18.5, further contributing to glucose intolerance in these animals, occurring due to reduced BCM and insulin secretion [8, Chapter 2 of this thesis]. These findings demonstrate the sophisticated integrative islet communication between

pancreatic α- and β-cells, functioning to balance levels of insulin and glucagon to accommodate metabolic homeostasis in pregnancy, which becomes dysregulated in GDM.

To further elucidate the role of pancreatic α-cells in control and glucose-intolerant (LP-diet mice) pregnancies, the ontogeny of α-cell proliferation throughout pregnancy was assessed. We found that α-cell proliferation was highest at GD9.5 in controls and subsequently declined. The decline in α-cell proliferation likely follows similar progesterone-mediated inhibition that has been shown to occur in β-cells at late pregnancy [32,45]. A previous study determined that α-cell proliferation is mediated by placental lactogens and prolactin, similarly to what has been observed in β-cells [32]. However, earlier timepoints were not examined in this study [32] which could have provided crucial information as pregnancy hormones have been shown to mediate changes in pancreatic β-cells at GD9.5 to prepare the pancreas for adaptive BCM expansion at GD18.5. Thus, our results demonstrate an earlier onset of α-cell proliferation during gestation in control diet animals at GD9.5, which is a significant temporal difference that could have important implications for therapeutics. This provides histological evidence that α-cells follow similar temporal dynamics to β-cells in early pregnancy, which also reach maximal proliferation early in gestation [7,8]. Proliferating α-cells were subsequently also localized to islets or clusters within the pancreas, as it has been shown that $Ins^{+}Glut2^{LO}$ β-cell progenitors are enriched in clusters [37]. In contrast to control-diet animals, glucose-intolerant animals (LP) exhibited less α-cell proliferation in clusters at GD9.5. These data could implicate a contribution for α-cell neogenesis from small endocrine clusters to the adaptive expansion of ACM at GD18.5, which has also been shown to be a mechanism of BCM expansion [7,8,25]. However, our data suggest that adaptive α-cell mechanisms were impaired in GDM.

Interestingly, we noted a high percentage of proliferating α-cells at GD9.5 relative to a non-pregnant animal (4.15% *vs.* 0.63%), providing speculation for a process of α- to β-cell transdifferentiation in pregnancy requiring subsequent α-cell renewal mechanisms. This represented a 6.6-fold increase in α-cell proliferation, in comparison to the 3.6-fold increase in β-cell proliferation that occurs at the same time during pregnancy in mouse

(3.5% *vs.* 1%) [7,8]. This data brings into question the purpose of such a high level of α-cell proliferation, which is greater than necessary simply to achieve the ACM expansion observed, which is only 2-fold higher at GD18.5 relative to a non-pregnant animal. In comparison, a 4-fold increase in BCM is achieved at GD18.5 with less β-cell proliferation [8, Chapter 2 of this thesis]. However, studies suggest that α-cells may serve as a reservoir for β-cell regeneration [42]. For example, GABA has been shown to cause α- to β-cell transdifferentiation and induces replacement of α-cells from duct-lining precursor cells that develop an α-cell identity prior to conversion into β-cells [46]. An additional study in mice with experimental type 1 diabetes mellitus found an increase in the proportion of glucagon^{+} cells that were positive for insulin or β-cell specific transcription factor *Pdx1* [39]. Together, these findings suggest that increased pancreatic α-cell renewal mechanisms are a strategy to replenish and maintain the α-cell reservoir and/or to increase β-cell regeneration *via* α- to β-cell transdifferentiation.

Consequently, we co-localized insulin and glucagon double-positive cells whose presence outside of pregnancy has been demonstrated previously [32,38–40]. Although insulin and glucagon double-positive cells have been suggested to be bihormonal cells in previous studies, better characterization of this population of cells would be important for future experiments. Data regarding secretion of insulin, glucagon or both by localization of hormones to granules using immune transmission electron microscopy would be insightful. It would also be interesting to determine if there is heterogeneity of function in bihormonal cell populations. Furthermore, additional questions remain, such as whether these bihormonal cells are a transitory type of cell, or a dedifferentiated type. A minority (~15%) of β-cells were bihormonal in non-pregnant, control-diet females suggesting that these cells are present as a normal feature of pancreas morphology and could represent functionally immature cell types. However, there was a transient decrease of bihormonal cells in controls at GD9.5 that was absent in LP dams, potentially implicating a burst of α- to β-cell transdifferentiation as a mechanism to increase BCM expansion during gestation in C animals that was impaired in LP females. Transdifferentiation may be reduced in these mice due to fewer Insulin^{+}Glucagon^{+} cells being present in the non-pregnant LP animal. Therefore, this dietary insult *in utero* may impair the plasticity of the α- and β-cell endocrine lineages and reduce α- to β-cell transdifferentiation [47].

However, there were also fewer Insulin+Glucagon+ cells at GD12.5 in LP animals *vs.* controls, potentially implicating a deficit of α- to β-cell transdifferentiation in glucose-intolerant pregnancy specifically at this time.

Accordingly, to elucidate the role of α- to β-cell transdifferentiation to BCM expansion in pregnancy, we used transgenic mice to lineage track α-cells. Our data suggested that α- to β-cell transdifferentiation does not significantly contribute to BCM expansion in pregnancy. Interestingly, a minority (~8%) of β-cells in non-pregnant females expressed an α-cell label (Insulin+YFP+) suggesting that these cells are present as a normal feature of pancreas morphology. These findings are in contrast to studies that investigated α- to β-cell transdifferentiation in unchallenged mice, where baseline values for transdifferentiation were only around 1% [33,48,49]. However, it is important to acknowledge that these studies used an inducible method for tagging α-cells that was initiated after pancreatic development, which would not take into account the significant pancreatic remodeling that occurs during postnatal development [50]. Importantly, studies suggest that it is possible that some β-cells undergo a bihormonal, glucagon-expressing progenitor stage during embryonic/postnatal development. One study used Gcg-Cre/YFP mice and reported that 10% of β-cells expressed an α-cell label at postnatal day 5, and 20% at postnatal day 21, which is comparable to values reported in the present study. Likewise, an additional study found comparable values, where 5-10% of β-cells were tagged with an α-cell label at postnatal day 1, and 12% at postnatal day 7-14, in a similar model using Gcg-Cre/YFP mice where α-cells were also labelled during pancreas development [51]. Although direct lineage tracing of transdifferentiated β-cells from an α-cell lineage would not be feasible in human samples, clinical data also suggests that it is possible that some β-cells undergo a bihormonal, glucagon-expressing progenitor stage during embryonic/postnatal development as bihormonal cells were also found in the developing human pancreas [52–54]. Since our model is a conditional Cre that is present from conception, the higher baseline values in our study compared to what has been published in many α- to β-cell transdifferentiation studies could be explained by the different lineage tracing models used. One way to address this discrepancy to elucidate the effects of the pregnancy time window would be to use an inducible Gcg/CreER model [55]. Alternatively, using the model in the present study the fold change can be calculated

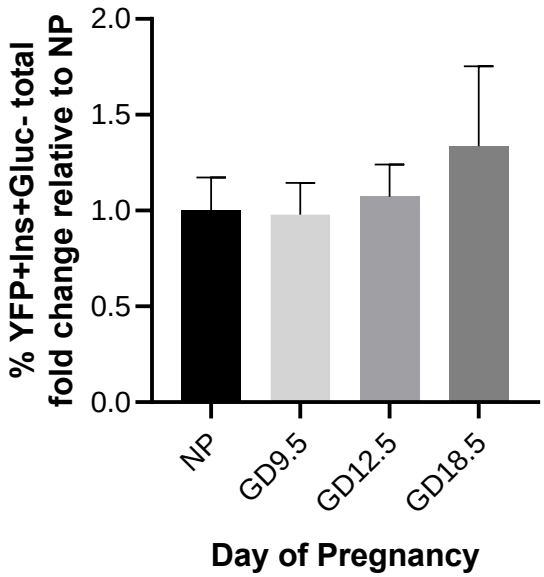
and compared to baseline in the non-pregnant animals. As we found no temporal differences in the percentage of β-cells that underwent transdifferentiation, the pattern for fold change was unsurprisingly also negligible.

Although the relative number of Insulin-YFP dual-stained cells did not change during pregnancy, we found that there were fewer of these cells in the islet mantle compared to the core at GD18.5. Previous studies have suggested that the mantle of the islet of Langerhans (where α-cells predominantly reside in mouse) contains a neogenic niche of β-cell progenitors [49]. It is suggested that this group of cells is persistent throughout life and could represent a transitional cell type between an α-cell and a β-cell phenotype, perhaps within a process of α- to β-cell transdifferentiation. If so, then it does not appear that the metabolic stress of pregnancy enables a further differentiation of these cells to become unihormonal insulin-expressing. Using the lineage tracking molecule YFP, subpopulations of cells were also identified within this model that co-expressed both insulin and glucagon. While their relative abundance did not change during pregnancy their anatomical distribution did. In contrast to phenotypic β-cells ($Insulin^{+}YFP^{+}Glucagon^{-}$) that were predominantly located in the islet core at GD18.5, the $Insulin^{+}YFP^{+}Glucagon^{+}$ cells were found predominantly in the mantle. This supports previous findings that lineage-flexible α-cells may be most abundant in the mantle of the islets of Langerhans [49], and that they are present during pancreatic remodeling and endocrine adaptation in pregnancy. It has been previously reported that β-cell maturation begins from the islet mantle and propagates to the islet core, being coordinated by islet vascularization [56]. Our data would support the notion that transitional endocrine cell types originate at the islet mantle and then likely propagate towards the centre of the islet once lineage committed. This process occurs as pregnancy progresses, in order to coordinate optimal islet function and facilitate cell-to-cell communication at GD18.5 when metabolic stress is highest [57].

In summary, we present novel data showing that there is an early onset of α-cell proliferation during pregnancy in controls, contributing to ACM expansion. This was impaired in glucose-intolerant pregnancies (LP) resulting in reduced ACM expansion and possibly fewer α-cells for α- to β-cell transdifferentiation to occur. However, using

lineage tracing, the process of transdifferentiation did not appear to dynamically alter during pregnancy. Nonetheless, both cell phenotypes examined (Insulin^{+}YFP^{+}Glucagon^{-}, Insulin^{+}YFP^{+}Glucagon^{+}) underwent anatomical changes in distribution within the islets in late gestation and in opposing directions. These data provide support for a potential transitional cell type in a pancreatic neogenic niche.

4.5 Supplemental Figures



Supplemental Figure 4.1. Transdifferentiation expressed as fold change relative to baseline

The fold change of β-cells arising from α-cells that no longer express glucagon (Insulin^{+}YFP^{+}Glucagon^{-}) relative to the baseline (non-pregnant animals). *n* = 8 non-pregnant and 4 pregnant animals. P>0.05.

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Chapter 5

5 Strategies to Improve Glucose Tolerance in Pregnancy

5.1 Introduction

Pregnancy presents as a physiological state of insulin resistance that requires compensatory adaptations in maternal endocrine pancreas to maintain euglycemia [1]. If this compensation fails, gestational diabetes mellitus (GDM) can develop, implicating pancreatic β-cell failure as a major driver to metabolic pathogenesis. GDM is described as diabetes that first appears during pregnancy, which regresses postpartum in most cases. Nonetheless, GDM is associated with adverse short and long-term health outcomes to the mother (birthing difficulties, T2DM) and offspring (pre-term birth, respiratory distress syndrome, obesity, type 2 diabetes mellitus, T2DM) necessitating the development of effective methods of intervention [2–6]. Current treatments for GDM, such as lifestyle behavioural change or administration of insulin or metformin, aim to decrease hyperglycemia but do not treat the underlying causes including a suboptimal β-cell mass (BCM). Thus, a better understanding of mechanisms of BCM expansion in pregnancy are warranted in order to effectively target potential therapeutic interventions.

In both mice and humans, a reversible expansion of pancreatic BCM and α-cell mass (ACM) has been documented and is maximal at late gestation (gestational day (GD) 18.5 in mice) [7–13]. In mice, these changes have been shown to be mediated by increased levels of placental lactogen and prolactin, initiating proliferation of pre-existing β- and α-cells [13,14]. Additional placental peptides, such as apelin and apela which signal through the apelin receptor (APJ), have been shown to alter β-cell number and function in non-pregnant animals and could also influence β-cell adaptations during pregnancy [15]. Although evidence exists to support expansion of BCM in pregnant humans, the mechanisms involved remain unclear and controversial due to limited samples [10,16]. An additional source of new β-cells during pregnancy could derive from

transdifferentiation of α-cells. Previous studies in non-pregnant animals reported that α-cells can replenish β-cells during metabolic stress by α- to β-cell transdifferentiation [17,18]. The use of structural analogs of artemisinins, a class of anti-malarial drugs, has been shown to stimulate α- to β-cell conversion *in vivo* and *in vitro* [19] and improve glucose tolerance in non-pregnant animal models of diabetes [20,21]. In rodent and zebra fish models, treatment with artemisinins increased $GABA_A$ signaling which led to transdifferentiation of α-cells into β-cells [19]. The increased BCM resulted in improved glucose homeostasis, which suggests a therapeutic effect of treatment with artemisinins in animal models of diabetes. Furthermore, based on studies investigating the safety of these compounds in pregnancy, the World Health Organization has deemed artemisinins as safe to be used during pregnancies complicated by malaria [22,23]. Some data in animals suggests that artemisinins are embryotoxic during first trimester and the use is thus discouraged in first trimester [23,24]. Nonetheless, recent human studies have found no adverse pregnancy outcomes when artemisinins were used in the first trimester [22] and additional studies are now recommending re-assessment of this guideline as the benefits of artemisinin use in the first trimester exceed any potential risks [25].

GDM severely impacts healthcare costs around delivery due to pregnancy complications and admission to NICU, as well as long-term health resources due to future T2DM in both the mother and offspring. Therefore, a safe method of prevention is needed. Replacement of β-cells as a strategy for diabetes treatment is limited by the shortage of islet supply from deceased donors, and the use of immune-suppressive drugs would not be safe during pregnancy [26]. Current data suggests antidiabetic effects of artemisinins in non-pregnant animals, however, no data exists in pregnant animals. In this study, we investigated the potential therapeutic effects of artemisinin treatment in an animal model of gestational glucose intolerance during and following pregnancy, and elucidated the underlying potential mechanisms involved leading to improved glucose tolerance.

5.2 Methods

5.2.1. Animals, Treatment, and Sample Collection

All animal procedures were approved by the Animal Care Committee of Western University in accordance with the guidelines of the Canadian Council for Animal Care. Mice were housed in a temperature-controlled room with 12-h light:dark cycle at Lawson Health Research Institute, London, ON, Canada. Water and food were given *ad libitum.* Adult (6-week-old) C57BL/6 male and female (F0) mice were obtained from Charles River Laboratories (Wilmington, MA, USA). Mice showing glucose intolerance at GD18.5 were generated using a previously described protocol involving a dietary insult during early life [12, Chapter 2 of this thesis]. Briefly, F0 females underwent estrous cycling and were time-mated with males. Dams were fed a low protein (LP, 8% protein, Bio-Serv, Frenchtown, NJ, USA) diet similar to that described by Snoeck et al. [27] throughout gestation and lactation. Female offspring (F1) were weaned onto a control diet (C, 20% protein) for the remainder of the study. At maturity (postnatal day, PND, 42), female offspring (F1) of LP diet-fed mothers were randomly allocated into two study groups: pregnant (GD18.5 or postpartum day (PPD) 7.5) or non-pregnant. We chose GD18.5 based on previous findings that this was the timepoint where glucose intolerance and reduced BCM were present. We also investigated mice after parturition at PPD7.5 due to a previous study showing that glucose intolerance persisted until 1 month postpartum [6, Chapter 3 of this thesis]. These animals were subsequently separated into an artemisinin-treated group, acetone vehicle group or non-treated group (non-treated non-pregnant and GD18.5 data retrieved from [12, Chapter 2 of this thesis], non-treated PPD7.5 data retrieved from [6, Chapter 3 of this thesis]). All pregnant-grouped females were time-mated with control diet-fed males. Initial experiments followed a protocol diluting the artemisinin, artesunate, in dimethyl sulfoxide vehicle (DMSO) [28]. However, pregnant mice treated with this mixture presented with pregnancy complications including preterm birth and embryolethality (Supplemental Fig. 5.1). Therefore, we adapted the protocol from an additional study where mice were treated with artesunate in an acetone vehicle diluted in drinking water daily [19]. A stock

solution of 250mg/ml artesunate (Cayman Chemicals, Ann Arbor, MI, USA) in acetone (Sigma-Aldrich, St. Louis, MO, USA) was prepared daily, 40 μl of which was diluted daily in 10mL drinking water for a final concentration of 1mg/mL artesunate. An equal concentration of acetone was used in the control group, and drinking water was provided *ad libitum*. Water bottles were covered with aluminum foil to prevent light penetration [19]. Vehicle or treatment was replaced daily from GD0.5-6.5, after which the solution was replaced with tap water for the remainder of the experiment (Fig. 5.1). Females were euthanized by CO_2 asphyxia at their assigned day for comparison to non-pregnant age-matched females. Maternal pancreatic samples were fixed in 4% paraformaldehyde for histology and embedded in optical cutting temperature compound. Maternal serum samples were collected *via* cardiac puncture. Placenta samples were collected in 1mL of RNAlater RNA Stabilization Reagent and frozen at -20°C (Qiagen, Hilden, Germany).

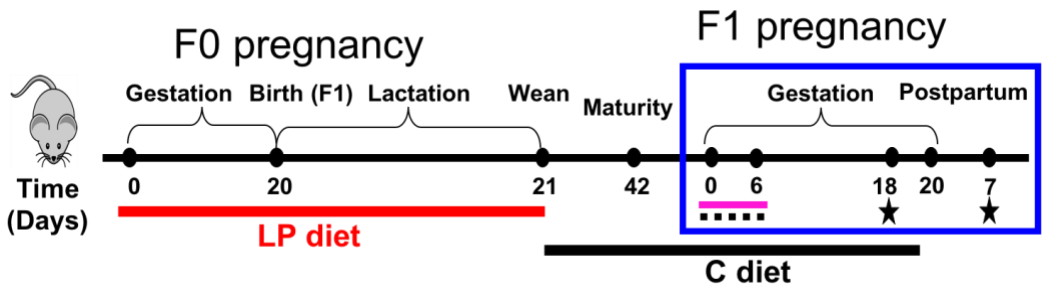


Figure 5.1. Animal model of treating glucose intolerance in pregnancy

F0 dams were fed a LP diet (8% protein) during gestation and lactation. Offspring (F1) were weaned onto control diet (C, 20% protein). At maturity, pregnant-grouped F1 females were time-mated with C-fed males. Artesunate-grouped pregnant females were treated (1mg/mL) *via* drinking water from gestational day (GD) 0.5-6.5 *vs*. vehicle-grouped females which were treated with the acetone vehicle alone, and non-treated females which were given regular tap water. The artesunate/acetone treatment group is represented by the pink bar, the acetone vehicle alone is represented by the black dashed bar. Non-pregnant animals were age-matched to females in the pregnant group. Stars indicate timepoints where an intraperitoneal glucose tolerance test was performed prior to euthanasia and the pancreas was removed for fluorescence immunohistochemistry. The blue box represents the F1 pregnancy experimental timepoints.

5.2.2. Intra-peritoneal Glucose Tolerance Test

Prior to euthanasia, an intra-peritoneal glucose tolerance test (2g glucose/kg body weight, IPGTT) was performed on all animals. Mice were fasted for 4 h. Blood glucose was measured from the tail at 0, 5, 15, 30, 60, 90 and 120 minutes using a One Touch Ultra2 glucometer.

5.2.3. Immunohistochemistry and Endocrine Pancreas Morphometry

Fixed pancreas tissue was prepared and sectioned as previously described [29]. At least two 7 μm-thick replicate cryosections were cut from each pancreas with an interval between each section >100 μm representing at least two longitudinal slices through the pancreas. Sections included both the head and tail of the pancreas. Immunofluorescence immunohistochemistry was performed to localize insulin and glucagon as described previously [12]. Antibodies against insulin (1:2000, anti-mouse, Sigma-Aldrich) and glucagon (1:200, anti-rabbit, Santa Cruz Biotechnology, Dallas, TX, USA) were applied to tissues and incubated overnight at 4°C. The following day, secondary antibodies (1:500 Thermo Fisher Scientific Waltham, MA, USA) were applied against the primary antibody using 555 and 488 fluorophores, respectively, along with DAPI (1:500, Thermo Fisher Scientific) to counterstain nuclei.

Tissue sections were visualized at 20x using a Nikon Eclipse TS2R inverted microscope (Nikon, Minato, Tokyo, Japan) with the program NIS elements (Nikon, Minato, Tokyo, Japan), and images were captured and analyzed using cell counter on ImageJ software. Every insulin and glucagon expressing cell was imaged for each section and for each animal. Manual cell counting analysis determined the percentage of bihormonal $Insulin^{+}Glucagon^{+}$ cells as a marker for α- to β-cell transitional cells [13,30–32].

To determine BCM and ACM, morphometric analysis was performed by manually measuring the total pancreas area for each tissue section, and the relative area of β-cells and α-cells [12,33]. BCM and ACM was calculated by multiplying total β or α-cell area

(sum of entire β or α-cell area/surface area of entire tissue section) by the pancreas weight. Islets were counted per tissue section and further separated by size into small (<5000 μm^2), medium (5000–10,000 μm^2), or large (>10,000 μm^2) islets as previously described [12,33].

5.2.4. Serum ELISA Assays

Maternal (F1) blood serum was used to quantify insulin and glucagon using an Ultra-Sensitive Mouse Insulin ELISA kit (Crystal Chem, Downers Grove, IL, USA) and Mouse Glucagon ELISA kit (Crystal Chem), respectively. The insulin assay has a sensitivity of 0.05 ng/mL using a 5uL sample with precision CV ≤ 10.0%. The glucagon assay has a sensitivity of 1.1pg/mL using a 10uL sample with precision CV < 10%. Data were collected using a BioRad iMark plate reader and analyzed using Microplate Manager Software.

5.2.5. Quantitative Polymerase Chain Reaction

Placenta samples (3-5mg) were minced with scissors in lysis buffer and Qiashredder spin columns (Qiagen) prior to total RNA extraction according to the RNeasy Plus Micro kit manufacturers' specifications (Qiagen). Sample yield and purity was quantified by absorbance at 260 and 280 nm (value 1.7-2) using a NanoDrop 2000c Spectrophotometer (ThermoFisher Scientific, Mississauga, ON). Total RNA (<1 μg) was extracted and reverse transcribed to cDNA using iScript Reverse Transcription Supermix (Bio-Rad Laboratories, Hercules, CA, USA). Quantitative PCR (qPCR) experiments were accomplished using the $2^{-\Delta\Delta CT}$ method after confirmation of parallel PCR amplification efficiencies. The mRNA levels of apelin receptor and apela were quantified using the TaqMan gene expression assay and the TaqMan Fast Advanced Master Mix (Invitrogen, Carlsbad, CA, USA) with the following Taqman primers: apelin receptor (Mm00442191_s1, Applied Biosystems, Forest City, CA, USA), apela (Mm04278372_m1, Applied Biosystems), with Cyclophilin A (Mm02342429_g1, Applied Biosystems) as the housekeeping gene. qPCR reactions were performed on triplicate samples with 20ng cDNA added per reaction using the QuantStudio Design and

Analysis Software. QuantStudio 5 Real-Time PCR System (Applied Biosystems) was programmed with the following thermal-cycling profile: polymerase activation step at 95ºC for 20 seconds, followed by 40 cycles of denaturation at 95ºC for 3 seconds, and annealing/extension at 60ºC for 30 seconds. Levels of mRNA expression were calculated relative to those of the housekeeping gene cyclophilin A.

5.2.6. Statistical Analysis

Data are presented as mean ± SEM, with statistics analyzed using GraphPad Prism software (Version 5.0). An unpaired two-tailed Student's *t* test, one-way ANOVA or two-way ANOVA were applied according to the set of groups that were compared. A Tukey's post-hoc test or a Bonferroni post-hoc test was performed after one-way ANOVA or two-way ANOVA analysis, respectively. Each animal presented as a single unit of analysis (*n*). $n = 4 – 7$ animals per treatment per timepoint. Statistical significance was determined as $P < 0.05$.

5.3 Results

5.3.1. Artesunate treatment in mid-gestation and pregnancy outcomes

Our hypothesis was that treatment with artesunate would improve glucose tolerance during pregnancy, as was observed in previous studies in non-pregnant diabetic mice [34]. We initiated these experiments by treating animals with artesunate in an acetone vehicle *via* drinking water between GD8.5-14.5. Artesunate/acetone treatment caused a reduction in weight gain (Supplemental Fig. 5.2A), food consumption (Supplemental Fig. 5.2B), and altered water consumption (Supplemental Fig. 5.2C) at the onset of treatment compared to a non-treated animal. However, fetal resorptions were observed implicating substantial embryonic lethality. Similar findings were observed in rats treated with artemisinins during organogenesis [23]. Nonetheless, in this study embryolethality was

only observed during organogenesis and not when the rats were treated during blastogenesis/pre-implantation (GD0.5-6.5) or during the fetal period (GD14.5-20.5). Thus, subsequent experiments were modified to treat mice with the artesunate/acetone intervention from GD0.5-6.5.

5.3.2. Artesunate treatment in early gestation and pregnancy outcomes

Weight gain was significantly reduced in both artesunate/acetone and the acetone vehicle group compared to non-treated animals (Fig. 5.2A). However, artesunate/acetone treated animals had a higher food consumption relative to non-treated animals (Fig. 5.2B). Both treatment groups drank an average of 4mL of solution a day during treatment (Fig. 5.2C), which is comparable to values for non-treated mice of 3-4mL depending on body weight [35]. There was no difference in the number of fetuses at GD18.5 (Fig. 5.2D). There were no significant differences in placental weight (Fig. 5.2E) or fetal weight between treatment groups at GD18.5 (Fig. 5.2F). However, artesunate/acetone treated animals trended to weigh more compared to non-treated animals (P=0.0618).

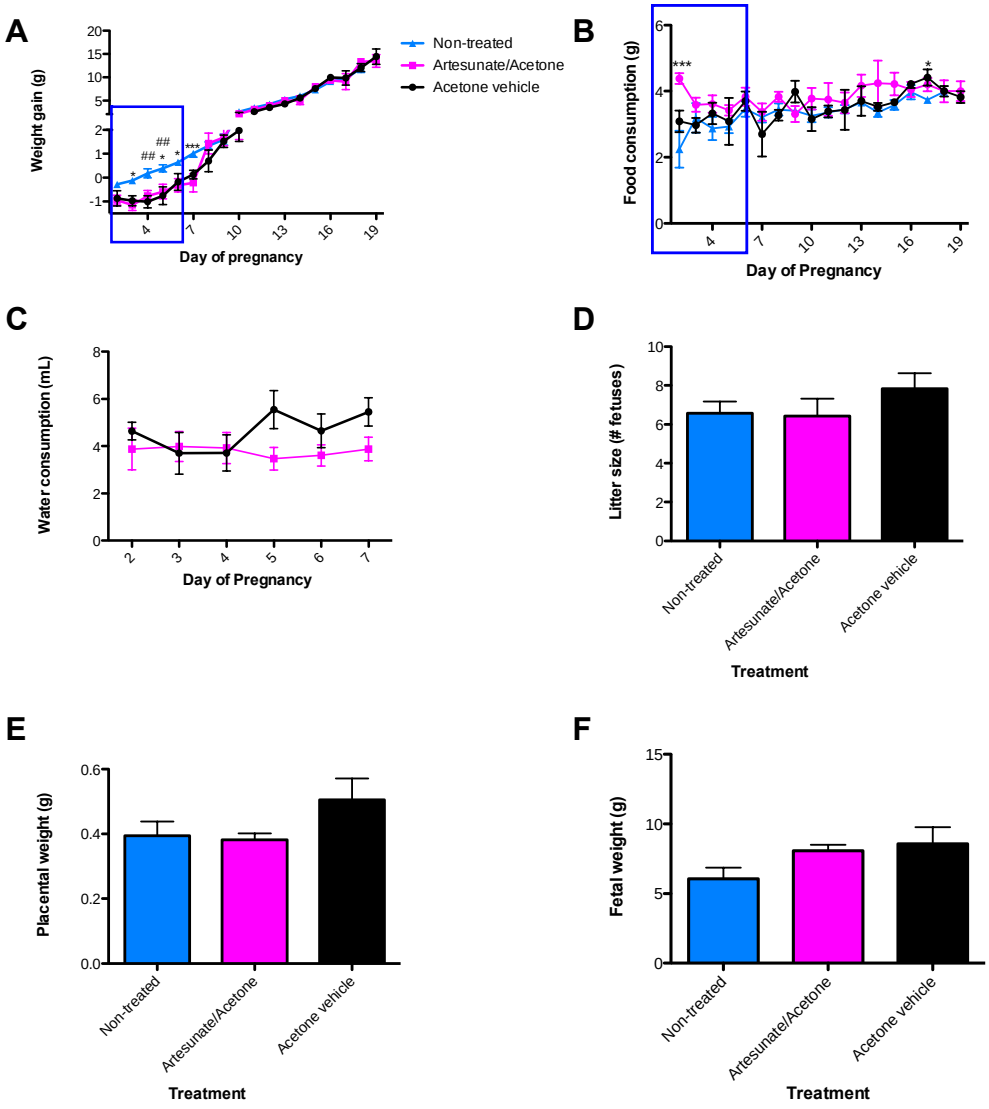


Figure 5.2. Pregnancy and model characteristics between treatment groups

A) Both treated groups gained less weight during pregnancy, and **B)** food consumption was higher in artesunate/acetone treated animals. **C)** Water consumption did not differ between treatment groups. There were no differences in **(D)** number of fetuses at GD18.5, **E)** placental weight, or **F)** fetal weight between treatment groups. *n* = 4-6 animals per treatment group. *** $P<0.001$, * $P<0.05$, non-treated vs. treatment group. ## $P<0.01$ non-treated vs. vehicle.

5.3.3. Both artesunate-treated and acetone vehicle-treated animals have improved glucose tolerance *vs.* non-treated females

Animals in both the artesunate/acetone and acetone vehicle group had significantly lower blood glucose levels at 5, 15 and 30 minutes during the IPGTT relative to non-treated mice at GD18.5 (Fig. 5.3A). At 120 minutes, the acetone vehicle-treated mice had significantly higher blood glucose levels compared to non-treated mice. Furthermore, the area under the glucose tolerance curve was significantly lower in the acetone vehicle group (Fig. 5.3B). IPGTT curves/glycemic curves were similar (as shown for those groups at GD18.5), and not significantly different between the artesunate/acetone vehicle and acetone vehicle alone for non-pregnant and PPD7.5 animals. This led us to postulate that the acetone vehicle was primarily responsible for improved glucose tolerance. Therefore, artesunate/acetone and acetone vehicles animals were pooled for further analysis. Blood glucose levels were significantly lower in non-pregnant acetone-treated animals compared to non-treated animals (Fig. 5.3C/D). Blood glucose levels were also lower in acetone-treated animals compared to non-treated animals at PPD7.5 (Fig. 5.3E/F).

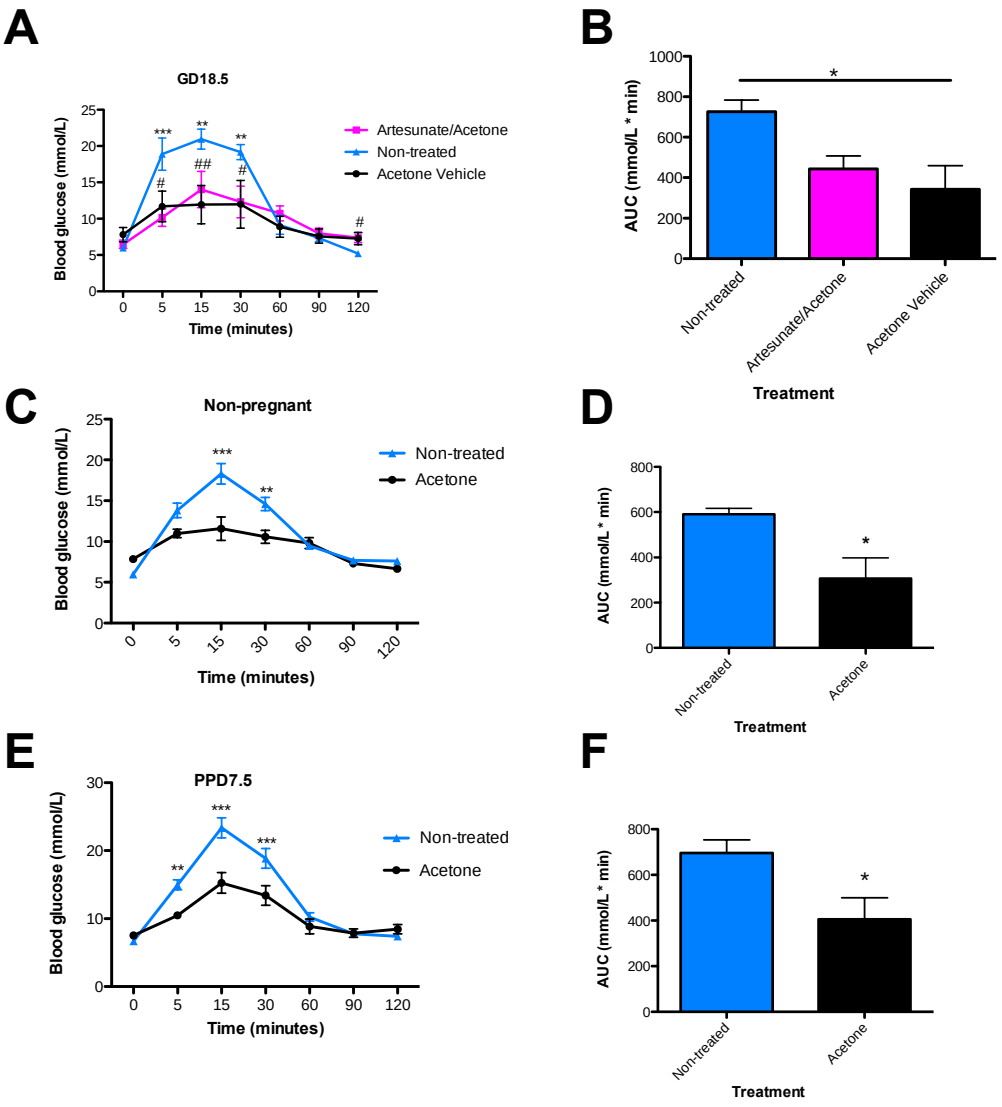


Figure 5.3. Artesunate-treated and vehicle animals have improved glucose tolerance *vs.* non-treated females

A) Artesunate/acetone and acetone vehicle-treated females had significantly reduced blood glucose levels compared to non-treated animals at GD18.5. **B)** Area under the glucose tolerance curve was significantly lower in acetone vehicle-treated animals vs. non-treated LP females at GD18.5. Similar trends were observed in non-pregnant (C, D) and PPD7.5 (E, F) animals. n = 4 animals at GD18.5 per treatment group, n = 4-7 animals per treatment in non-pregnant and PPD7.5 animals. *** P <0.001, ** P<0.01, * P<0.05, non-treated vs. treatment group. ## P<0.01, # P<0.05 non-treated vs. vehicle.

5.3.4. Acetone treatment alters pancreas histology during and after pregnancy

Again, there were no differences in BCM between artesunate and vehicle-treated animals, thus data was combined. Beta-cell mass was significantly higher in non-pregnant acetone-treated animals compared to non-treated animals (Fig. 5.4A). There were no significant differences in ACM (Fig. 5.4B) or mean islet size (Fig. 5.4C) between treatment groups over time. Islet sizes did not vary between treatment groups in non-pregnant (Fig. 5.4D) or GD18.5 (Fig. 5.4E) animals. However, acetone-treated animals at PPD7.5 had significantly more medium and large-sized islets compared to non-treated animals (Fig. 5.4F).

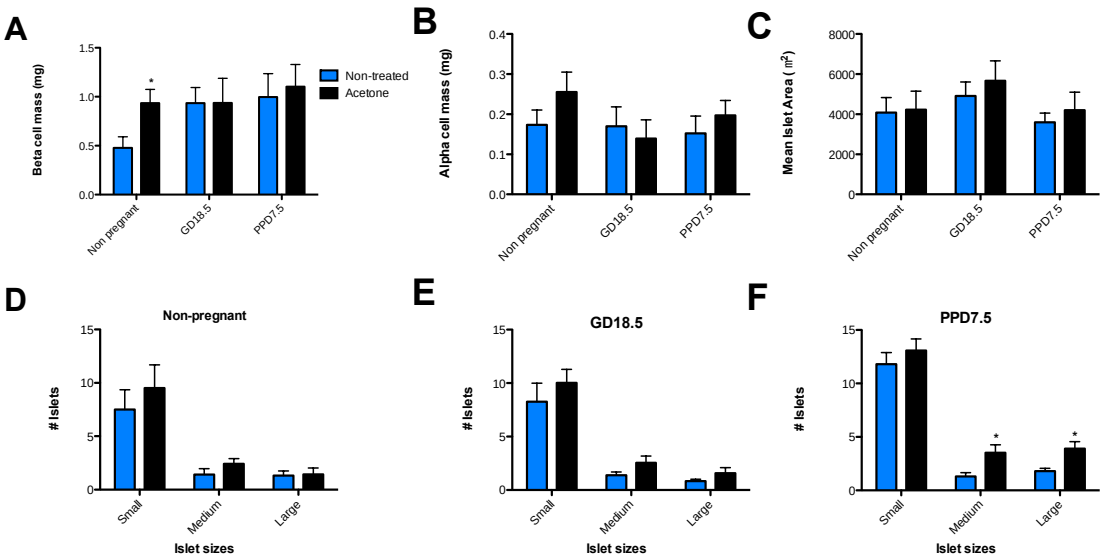


Figure 5.4. Acetone treatment alters BCM in non-pregnant animals but not during or after pregnancy

A) BCM, B) ACM, and C) mean islet size. Islet sizes did not differ in D) non-pregnant or E) GD18.5 animals. F) However, there were more medium and large islets at PPD7.5 in treated animals. *n* = 4-8 animals per treatment group. * *P* <0.05, acetone-treated vs. non-treated.

5.3.5. Acetone treatment causes hyperglucagonemia during and after pregnancy

Serum insulin and glucagon were quantified from blood collected *via* cardiac puncture at the end of the IPGTT (120 mins). There were no significant differences in serum insulin between treatment groups over time (Fig. 5.5A). However, serum glucagon levels were significantly higher at GD18.5 and PPD7.5 in acetone-treated animals compared to non-treated animals (Fig. 5.5B). There were no significant differences in serum insulin to glucagon ratio between treatment groups over time (Fig. 5.5C).

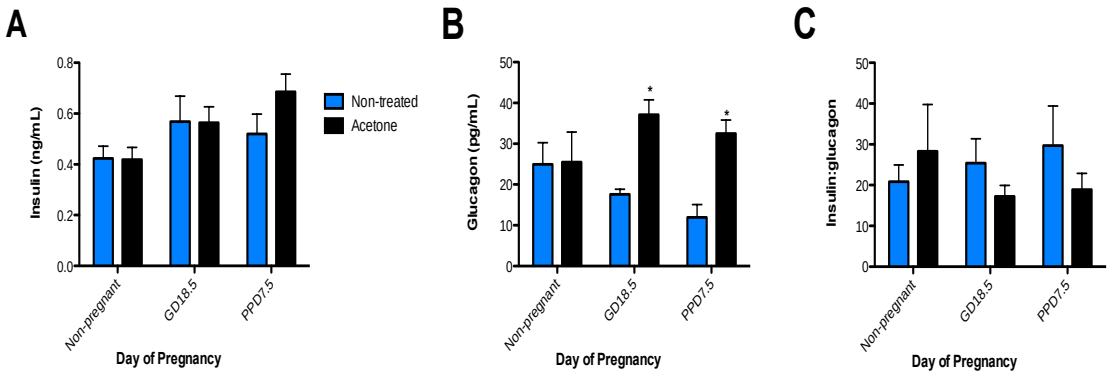


Figure 5.5. Acetone treatment alters glucagon levels during and after pregnancy

A) Serum insulin, B) serum glucagon, and C) serum insulin to glucagon ratio. *n* = 3-8 animals per treatment group. * *P* <0.05. acetone-treated vs. non-treated.

5.3.6. Acetone treatment increases bihormonal transitional cell number in islets

To investigate a potential mechanism of new β-cells observed in non-pregnant acetone-treated animals, we quantified the percentage of Insulin+Glucagon+ (insulin and glucagon double-positive, Fig. 5.6A/B) cells as a marker for α- to β-cell transitional cells [13,30–32]. Acetone-treated non-pregnant animals had significantly more bihormonal cells compared to non-treated animals (Fig. 5.6C). However, there were no significant differences in the percentage of bihormonal cells between treatment groups at GD18.5 or PPD7.5.

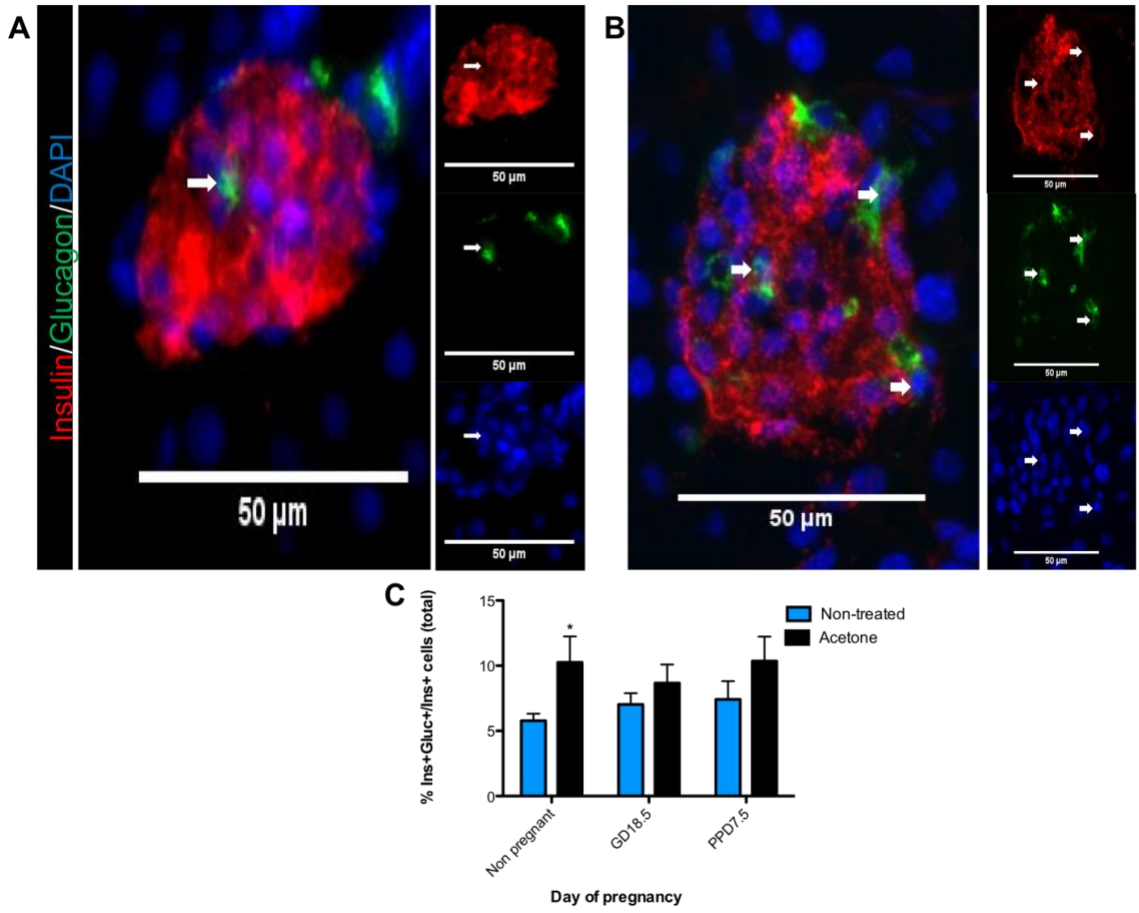


Figure 5.6. Representative micrographs and quantification of bihormonal cells

A) Bihormonal (Insulin$_+$Glucagon$_+$) cell of a non-treated non-pregnant animal. B) Bihormonal cell of an acetone-treated GD18.5 animal. C) Total percentage of bihormonal cells relative to all insulin$_+$ cells. n = 5-8 animals per treatment group. ** P <0.01, * P <0.05, acetone-treated vs. non-treated.

5.3.7. Acetone treatment leads to increased expression of the placental apelinergic system

To investigate a potential mechanism of improved glucose tolerance in pregnant acetone-treated animals, we analyzed the placenta, since placental weight was relatively higher in acetone-treated animals and body weight was recovered in acetone-treated animals despite reduced weight gain during treatment (Fig. 5.2E). Since the placenta secretes apelin, and it is known to alter both β-cell number and function we looked at the apeligneric system. Both APJ (Fig. 5.7A) and apela (Fig. 5.7B) mRNA levels were significantly higher in acetone-treated animals compared to non-treated animals.

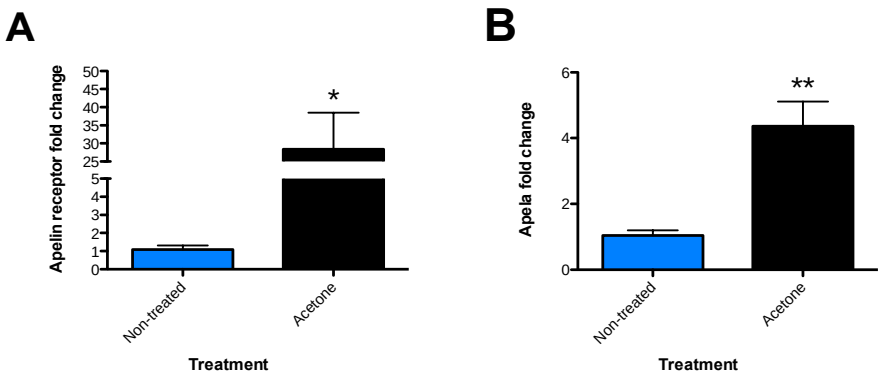


Figure 5.7. Acetone treatment increases placental apelinergic system

Placental mRNA expression of both A) apelin receptor, and B) apela were significantly higher in acetone-treated vs non-treated animals at GD18.5. The fold change in expression was measured relative to housekeeping gene cyclophilin A. n = 5 non-treated animals and 4 acetone-treated animals. ** P<0.01, * P<0.05, acetone-treated vs. non-treated.

5.4 Discussion

GDM is associated with adverse health consequences for both the mother and her child, implicating the need for an effective method of treatment. The initial experiments in this study sought to treat mice with gestational glucose intolerance with the artemisinin, artesunate *via* drinking water. Artemisinins have been shown to increase BCM *via* α- to β-cell transdifferentiation and improve glucose homeostasis in non-pregnant animal models of diabetes [19], although these findings are controversial and have been rebutted by some studies [28,36]. Our experiments revealed a high consumption of artesunate within drinking water and no indications of fetal resorptions to implicate embryolethality. Nonetheless, the initial objective of testing artesunate was negated as we noted that multiple parameters investigated in our study demonstrated similar findings between the treatment group (artesunate diluted in the acetone vehicle) and the vehicle (acetone alone). This led us to conclude that our findings could be primarily due to the use of the acetone vehicle. Thus, we rejected our initial hypothesis and suggest that artesunate had no effect on multiple parameters in this study, as compared to the vehicle alone. Subsequently, we re-adjusted our focus to determine the effects of acetone on glucose homeostasis and pancreas histology.

Weight gain was lower in acetone-treated animals during treatment, although body weight recovered by the end of the experiment. Acetone-treated animals consumed a comparable amount of food as non-treated animals, bringing into question whether there could have been a transient effect of acetone on nutrient uptake *via* the villi in the small intestine, resulting in reduced weight gain in treated animals. Although we did not collect gastrointestinal tissues in our studies, other studies have found that acetone abolished adhesion of F18-fimbriated (F18R) *E. coli* to isolated porcine intestinal villi *in vitro*, concluding F18R was a glycolipid [37]. Since glycosphingolipids (GSL) are a major component of intestinal enterocytes, it is possible that acetone could be breaking down these villi and preventing nutrient absorption. In an animal model with genetic deletion of the gene for the enzyme that catalyzes the initial step of GSL biosynthesis (*Ugcg*), newborn mice presented with growth retardation and loss of body fat deposits, due to a severe disturbance in uptake of nutrients [38]. The same study showed that adult mice

presented with a drastic decrease in body weight, as was observed in animals in our study during treatment with acetone. It was concluded that GSLs in the intestinal epithelium are essential for intestinal endocytic function to effectively absorb nutrients. These findings could provide an explanation for the reduced weight gain observed in acetone-treated animals in our study at a time where food consumption was unchanged. It is worth noting that reduced nutrient and glucose uptake for the time period of the treatment in our study could mimic a situation of fasting, which has been suggested to have protective effects on reducing oxidative stress and protects against many diseases in both rodents and humans [39,40]. Intermittent or periodic fasting has also been shown to improve glucose tolerance in part *via* adipose tissue remodeling [41,42], which could explain why glucose tolerance was improved in non-pregnant, GD18.5 and PPD7.5 acetone-treated animals in our study. Thus, acetone abolition of glycolipids on enterocytes could have affected nutrient absorption, mimicking a situation of fasting which led to improved glucose tolerance in the treated-mice in our study. It is important to note that the data for untreated controls used in this chapter were retrieved from chapter 2 and 3. Thus, we acknowledge the use of historical controls as a potential weakness in design which could be strengthened with an additional group of untreated animals.

Next, we sought to investigate a mechanism of improved glucose tolerance in acetone-treated animals and investigated changes in endocrine pancreas. BCM was higher in non-pregnant acetone-treated animals but this did not correlate with higher serum insulin levels. Serum glucagon levels were higher at the end of the IPGTT at both GD18.5 and PPD7.5, despite no differences being observed in ACM. The high glucagon levels at GD18.5 likely contributed to hyperglycemia in the acetone-treated animals compared to non-treated animals at the end of the IPGTT. In contrast, there were no differences in serum insulin levels at GD18.5 and PPD7.5. However, with the half-life of insulin being relatively short (~4-6 minutes) in comparison to the time span of the IPGTT and blood collection *via* cardiac puncture (~120 minutes), it is possible that there could have been differences in serum insulin levels at earlier timepoints (0, 5, and 15 minutes) when insulin secretion is highest (i.e. first phase insulin secretion). Indeed, analysis of the IPGTT curves of acetone-treated animals might implicate improved insulin secretion, as shown by the blunted blood glucose curve in response to the glucose bolus. Furthermore,

the L-cells in the distal ileum and colon secrete glucagon-like peptide 1 (GLP-1), an incretin hormone that is released in response to nutrient ingestion. GLP-1 increases insulin secretion and inhibits glucagon secretion [43]. Thus, a mechanism for improved glucose tolerance could also involve a regenerative response of the enteroendocrine cells post acetone treatment, resulting in greater GLP-1 production and improved insulin secretion. Therefore, acetone could be resulting in increased insulin secretion, although improved insulin sensitivity in peripheral tissues also remains to be explored. Interestingly, the finding of hyperglucagonemia at the end of the IPGTT at GD18.5 and PPD7.5 was not observed in non-pregnant animals which was likely due to BCM being higher and maintaining glucose homeostasis. There is much convincing data that α-cells play an essential role in regulating insulin secretion from β-cells [44]. For example, one study reported that insulin secretion was higher in response to glucose in paired α- and β-cells compared to single β-cells alone [45]. Furthermore, it was shown that α-cells were a target for serotonin in human islets, whereby β-cell-derived serotonin inhibited glucagon secretion in high glucose conditions [46]. Since BCM was higher in non-pregnant animals in our study, it is plausible that α-cells received an increased serotonergic input from β-cells to regulate glucagon secretion. However, BCM did not increase in acetone-treated animals at GD18.5 and was lower than BCM levels observed in a healthy pregnancy (~2mg). Thus, a potential explanation for the hyperglucagonemia observed at GD18.5 could be due to decreased serotonergic input from β-cells, resulting in hypersecretion of glucagon. Overall, these findings demonstrate the importance of the sophisticated integrative islet communication between pancreatic endocrine cells in order to effectively manage glucose homeostasis.

To investigate a potential mechanism for the generation of new β-cells resulting in an increased BCM in non-pregnant acetone-treated animals, we quantified bihormonal (insulin and glucagon double-positive) cells as a marker for possible α- to β-cell transitional cells. There was an increased percentage of bihormonal cells in non-pregnant acetone-treated animals compared with non-treated animals. Since the acetone ingestion in our study may mimic a situation of short-term fast, these findings agree with those observed following transient fasting where a greater number of transitional α- to β-cells were observed upon re-feeding [47]. In these non-pregnant mice, transient fasting

resulted in β-cell regeneration and rescue from type 1 and type 2 diabetes [47]. In this study, non-pregnant animals were fasted for 4 days followed by up to 10 days of re-feeding. This is comparable to our study where we postulate that the animals are fasted for at most 6 days, dependent on the length of time required for acetone to destroy villi and impair nutrient uptake.

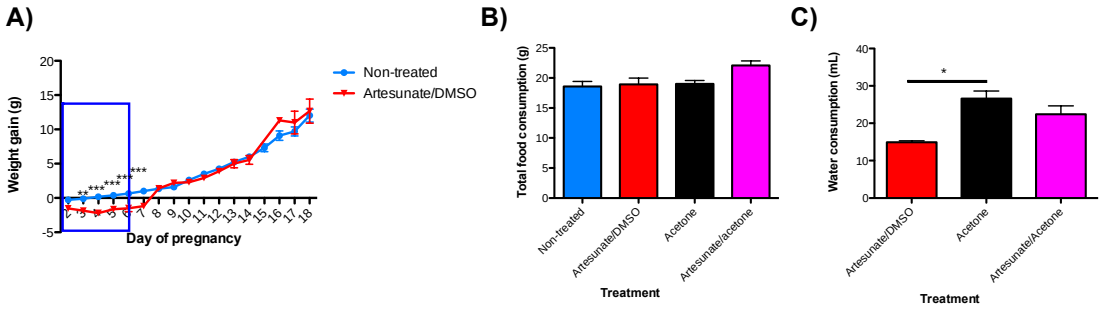
In terms of pregnancy and fasting, clinical studies on the effects of fasting on pregnancy outcomes are inconsistent. Some studies suggest that fasting during pregnancy results in adverse fetal outcomes [48] and a higher incidence of developing GDM [49], while others found no differences in pregnancy and fetal outcomes in fasting women [50–52]. A number of these studies also reported lower birth weight or intrauterine growth restriction. However, in our study there were no indications of growth restriction in acetone-treated animals. Therefore, because of the short duration of the treatment during the first week of pregnancy it is plausible that deleterious effects would not be observed. Nonetheless, to our knowledge, this has not been investigated in pregnancy in mouse and we are the first to show a beneficial effect of a likely pathologically-induced functional fasting in early gestation to improved glucose tolerance in mice with GDM at GD18.5 and glucose intolerance at PPD7.5 without adversely affecting fetal parameters.

Given that there were no differences in bihormonal cells or BCM in pregnant or lactating animals to account for the improved glucose tolerance observed, we investigated if a placental-specific mechanism might exist. To investigate a mechanism for improved glucose tolerance in acetone-treated animals during and after pregnancy, we analyzed the placenta for compensatory mechanisms since placental weight was relatively higher in acetone-treated compared to non-treated animals. For example, previous studies have shown increased deposition of glycogen in GDM placentas with the placenta acting as a buffer for excess glucose and thereby lowering blood glucose levels in the mother [53]. Interestingly, the apelinergic system was shown to promote transplacental transport of glucose from mother to fetus in rat dams injected intravenously with apelin-13 without changes to the expression of placental glucose transporters Glut1 and Glut3 [54]. Rather, it was reported that at mid to late gestation, apelinergic signaling increased vasodilation of fetal arterioles and glucose transport to the fetus. In the present study, acetone-treated

animals expressed increased placental apela and APJ at GD18.5 compared to non-treated animals. Therefore, transfer of glucose from mother to fetus in our study could be increased by the placental apelinergic system, resulting in improved glucose tolerance in the mother. The trend of higher fetal weight in acetone-treated animals further supports this hypothesis, as excess glucose is transferred to the fetus and subsequently stored in fetal tissues. Apelin has been linked to placental growth and efficiency due to observations that fetal apelin levels were reduced in studies with maternal food restriction during gestation [54]. In the present study, high levels of apelingeric system in the placenta could be responsible for the relatively larger placenta observed in acetone compared to non-treated animals. Apelin has also been shown to be involved in the regulation of food intake [55] and could explain the hyperphagia seen in acetone-treated animals at late gestation (GD17.5). Interestingly, apelin is a beneficial adipokine with anti-obesity and diabetic effects [55]. Despite reduced weight gain upon acetone treatment, the treated animals in this study recover in body weight. The subsequent hyperphagia could result in adipogenesis, and thus it could be insightful to determine levels of apelin in adipocytes in future studies to investigate if apelin is also secreted from adipocytes and causing an anti-diabetic effect in acetone-treated animals.

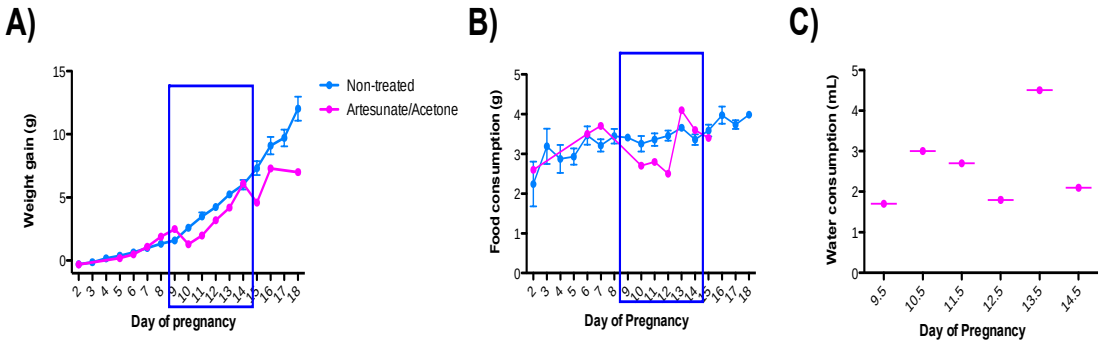
In conclusion, artesunate had no effect on multiple parameters investigated in this study. However, acetone treatment improved glucose tolerance in non-pregnant, GD18.5 and PPD7.5 LP-treated animals. In non-pregnant animals, improvements in glucose tolerance were due to an increased BCM, possibly involving α- to β-cell conversion. However, pregnant/lactating animals demonstrated overall improved glucose tolerance likely due to compensatory mechanisms in the placenta involving upregulation of placental apelinergic system, resulting in vasodilation and increased glucose transfer decreasing maternal blood glucose levels, and/or better insulin release dynamics during an IPGTT. Our findings provide a potential therapeutic glucose-lowering effect of acetone *via* mimicking a situation of short-term fasting to improve glucose tolerance, including a model of gestational glucose intolerance. Potential mechanisms include beneficial changes in pancreas histology and placental function.

5.5 Supplemental Figures



Supplemental Figure 5.1. The effects of artesunate intervention in a DMSO vehicle on a GD18.5 LP animal treated GD0.5-6.5

A) Weight gain was reduced in DMSO vehicle animals compared to non-treated animals. **B)** Food consumption during treatment did not vary between treatment groups. **C)** Treatment solution consumption in artesunate/DMSO was lower compared to non-treated animals throughout gestation.



Supplemental Figure 5.2. The effects of artesunate intervention on a GD18.5 LP animal treated GD8.5-14.5

A) Weight gain, **B)** food consumption, and **C)** artesunate/water consumption in an artesunate/acetone-treated dam throughout gestation.

5.6 References

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6 Summary and Perspectives

6.1 Summary of Major Findings

GDM is an increasingly prevalent pathology in pregnancy that is associated with adverse maternal and fetal health outcomes, necessitating the need for interventional strategies. There is currently no reliable method of prevention for GDM. Thus, we sought to better understand the mechanisms of impaired endocrine adaptability in GDM by creating a mouse model that can be used to establish novel therapeutics.

We hypothesized that a dietary LP insult during early development in mice would impair β-cell adaptability in pregnant offspring, resulting in glucose intolerance during pregnancy, which could be reversed with treatment. Within this thesis, we addressed four major objectives: first, we established a novel mouse model for study of suboptimal endocrine adaptations during pregnancy. Using this model, we then determined the long-term effects of GDM following parturition and mechanisms of suboptimal endocrine mass expansion. Finally, we used this model and our knowledge of impaired mechanisms in GDM from our previous work to propose a therapeutic intervention for GDM through the manipulation of BCM.

6.1.1 A mouse model of gestational glucose intolerance through exposure to a low protein diet during fetal and neonatal development

To implement targeted therapeutics, a better understanding of suboptimal endocrine adaptations in GDM is needed. However, as pancreatic samples from GDM patients are scarce, and no safe *in vivo* imaging modalities for endocrine cells in pregnancy exist at the present time, initial experiments involved the development of a novel mouse model of gestational glucose intolerance. This was accomplished using a dietary insult (LP diet) during fetal and neonatal development, previously shown to program impaired endocrine pancreas plasticity in offspring [1–3]. This model produced female offspring with glucose

intolerance restricted to GD18.5, as is observed in late pregnancy in human GDM. Glucose intolerance was attributed to reduced β-cell proliferation, leading to reduced BCM expansion and GSIS at GD18.5 relative to a healthy pregnancy. We also presented novel findings of reduced ACM at GD18.5 in glucose-intolerant mice, revealing the significance of the often overlooked pancreatic α-cell population to glucose homeostasis in GDM. A major strength of these findings was the ability to reproduce glucose intolerance in pregnancy that was restricted to late gestation, as other animal models of diabetes in pregnancy demonstrate pre-gestational obesity and/or diabetes [4–7], which is not comparable to a diagnosis of clinical GDM [8].

6.1.2 Altered pancreas remodeling following glucose intolerance in pregnancy in mice

GDM increases the risk of T2DM after parturition by up to 90% [9], yet no histological data existed comparing endocrine pancreata after healthy and GDM pregnancies. Next, we sought to use our animal model of gestational glucose intolerance to determine the long-term effects of GDM on glucose tolerance and pancreas histology after pregnancy. Analysis of pancreata at PPD7.5 revealed suboptimal pancreatic maladaptations in glucose-intolerant mice that persisted from GD18.5, resulting in prolonged glucose intolerance until 1 month postpartum. By 3 months postpartum, a compensatory increase in the number of small islets and a higher insulin to glucagon ratio likely enable euglycemia to be attained in the previously glucose-intolerant mice. Our findings demonstrated long-term pancreatic re-modeling after parturition involving both α- and β-cells, which were potentially associated with changes in the pro-inflammatory environment. These findings are important to understanding the mechanisms involved in the progression from GDM to T2DM after parturition.

6.1.3 The increased alpha and beta cell mass during mouse pregnancy is not dependent on transdifferentiation

It was evident that impaired endocrine adaptations during GDM were one of the key determinants of glucose intolerance not only during pregnancy, but also after pregnancy, resulting in long-term metabolic impairments. In order to prevent these adverse health outcomes through therapeutic interventions, it is essential to target the underlying causes of a suboptimal BCM in GDM. To provide some mechanistic insights of reduced BCM in GDM, we looked at the contribution of α- to β-cell transdifferentiation to BCM in pregnancy, and also α-cell plasticity in healthy *vs*. glucose-intolerant pregnancies. Alpha-cell proliferation was maximal at GD9.5 and resulted in increased ACM expansion at GD18.5 in control animals, but this was reduced in glucose-intolerant (LP) mice. However, LP mice displayed hyperglucagonemia at GD18.5 contributing to glucose intolerance at late gestation in GDM. Notably, hyperglucagonemia has also been observed in women with GDM which persisted after parturition, contributing to glucose intolerance [10]. Although there were trends in bihormonal transitional ($Insulin^{+}Glucagon^{+}$) cells in LP *vs.* control pregnancy, lineage tracing in control pregnancy revealed a negligible amount of α- to β-cell transdifferentiation contributing to BCM expansion. These findings further emphasized the importance of other islet cell types, other than just β-cells, to glucose homeostasis in pregnancy, a subject area that has previously been overlooked. Importantly, the dynamic changes in ACM that occurred during normal pregnancy were altered in glucose-intolerant pregnancies, providing an additional potential avenue for therapeutics by targeting hyperglucagonemia to reduce hyperglycemia in pregnancy.

6.1.4 Strategies to improve glucose intolerance in pregnancy

As the development of an effective intervention for GDM is clinically important, we sought to explore the use of artemisinins, which have been shown to increase BCM and

improve glucose tolerance in non-pregnant animal models of diabetes [11]. Importantly, artemisinins are safe for use in pregnancy as they are used to treat women suffering from malaria [12]. Glucose-intolerant animals were treated with the artemisinin, artesunate. While an improved glucose tolerance was found in non-pregnant, GD18.5 and PPD7.5 animals, this was shown to primarily result from the use of the acetone vehicle. In non-pregnant acetone-treated animals, this was attributed to a higher BCM, possibly involving α- to β-cell conversion. BCM did not differ between acetone-treated and non-treated animals at GD18.5. Instead, glucose tolerance in pregnant animals was improved possibly due to an upregulation of the placental apelingeric system [13], and/or improved insulin secretion. Additionally, acetone-treated animals in these studies demonstrated reduced weight gain during treatment despite unaltered food consumption. These findings could implicate a transient state of fasting, which could additionally be contributing to improvements in glucose tolerance through glucose uptake mechanisms in peripheral tissues. Thus, transient fasting could be particularly beneficial in preventing glucose intolerance during pregnancy.

Collectively, the data presented throughout this thesis implicate the importance of endocrine adaptations to successfully counter relative maternal insulin resistance during pregnancy. Although most research has focused on the importance of pancreatic β-cell adaptation in pregnancy, we presented many findings revealing the role that pancreatic α-cells simultaneously play in regulating glucose levels during pregnancy and demonstrated how this is altered in GDM. Although a therapeutic potential of artemisinins was not demonstrated, a mimicked state of fasting induced by dilute acetone treatment yielded a potential therapeutic, glucose-lowering effect. Nonetheless, further research is needed before the mechanisms of impaired endocrine adaptability presented in this thesis, and therapeutic effects of acetone in GDM, can be transferred to a clinical setting.

6.2 Limitations and Future Directions

The purpose of this section is to discuss some limitations of the experiments presented throughout this thesis and discuss potential future experiments that can strengthen our findings.

6.2.1 Use of animal models to study diabetes in pregnancy in humans

The rationale for establishing an animal model of gestational glucose intolerance was due to the very limited access to pregnant human pancreas samples, and samples from GDM women would be even more scarce. Although having human samples would be preferred, if samples were retrieved it would be highly likely that data from multiple gestational timepoints would need to be combined which could lead to inaccurate conclusions if time-specific physiological changes occur, as has been observed in mice. As such, studies thus far have highly relied on animal models of diabetes in pregnancy. Although mice are powerful models that recapitulate many aspects of human pregnancy, they are not without limitations. For example, it is difficult to directly demonstrate the multi-factorial nature of GDM pathogenesis in an animal (i.e. including both polygenetic and environmental factors). Of relevance to the findings in this thesis are the differences in the context of endocrine adaptations in humans compared to mice. The most controversial studied difference between mouse and human pregnancy is in regard to β-cell neogenesis and proliferation, as human β-cells are thought to rarely divide [14]. The role of β-cell proliferation in human pregnancy is unclear, as the only study investigating this phenomenon in humans showed a lack of replication from pre-existing β-cells [15]. However, these findings need to be taken with extreme caution, as samples were pooled over multiple gestational timepoints potentially diluting an effect of proliferation occurring in a timing-specific manner. Further contributing to a potential difference regarding β-cell replication as a major driver of BCM expansion in mouse pregnancy is the influence of lactogenic hormones to this process. In mice, strong evidence supports that β-cell replication is driven by PRLR signaling [16]. However, human studies report

conflicting results on the influence of lactogen treatment on mitogenic activity of β-cells [17,18] which could be due to lower PRLR expression on human β-cells than in mice [19]. Although it is premature to confirm that there are species differences in mechanisms of pancreas adaptations in pregnancy based on a reliance of *in vitro* data, it is important to acknowledge that studies support the presence of endocrine mass expansion in human pregnancy [15,20]. Furthermore, both mouse and human gestation implicate β-cell dysfunction and insulin resistance as a key driver to metabolic dysfunction in human and animal models of diabetes in pregnancy [21]. Therefore, these findings provide strong rationale for continued research efforts in this field.

An exciting methodology to deciphering these mechanisms would be non-invasive *in vivo* imaging to monitor BCM in humans. Indeed, many sophisticated studies have performed *in vivo* imaging of endogenous β-cells in humans and small and large animals using positron emission tomograph (PET), single photo emission computed tomography (SPECT) and magnetic resonance imaging (MRI) [22,23]. Each methodology presents with both strengths and limitations in terms of resolution and sensitivity, and specificities of radiotracers for β-cells. These studies are also limited by the small size and density of β-cells relative to the remainder of the exocrine pancreas, and/or potential uptake of tracers in peripheral tissues. Undeniably, important additional considerations in the context of pregnancy would involve technical issues as pregnancy progresses and the abdomen enlarges, as the pancreas is located deep in the abdomen. Furthermore, the requirement for non-toxic contrast agents that are safe for the fetus are of paramount importance. Thus, these considerations need to be elucidated in non-pregnant humans first, before implementation can be safely suggested to pregnant women.

In conclusion, it remains to be investigated whether the maladaptations in endocrine pancreas presented in this thesis occur in human GDM. As such, additional caution must be considered before extrapolating data in mice directly to humans. Further studies would need to be performed in a clinical setting to elucidate whether these mechanisms could provide new therapeutic opportunities to promote generation of new β-cells.

6.2.2 Influence of cytokines to β-cell dysfunction after GDM

Cytokines released from adipose tissue and from placenta influence metabolism during pregnancy, which often becomes dysregulated in GDM. GDM is characterized as an inflammatory state [24–26] which can impact successful β-cell adaptation during pregnancy. Imbalanced levels of cytokines can contribute to glucose intolerance in GDM by contributing to β-cell dysfunction [27,28] and insulin resistance *via* impaired insulin receptor signaling [29,30]. We showed that increased levels of the pro-inflammatory cytokine, IL-6, in adipose tissue could have contributed to glucose intolerance after parturition in GDM mice (**Chapter 3**). Since cytokines can act in a paracrine/autocrine manner, it would have also been valuable to measure levels of cytokines in the pancreas in order to elucidate if there was a local effect on the histomorphometric changes observed in fixed pancreas sections. Some preliminary qPCR experiments were performed in whole pancreas preparations; however, most values were below the levels of detection. Cytokines in serum samples were also quantified, but most samples were also below the levels of detection. In order to draw more precise conclusions, future experiments could quantify cytokines in isolated islets at postpartum, as levels of cytokines could be diluted by exocrine pancreas in whole pancreas samples since the endocrine portion only compromises 2% of the pancreas. Importantly, IL-6 has been shown to be involved in α-cell growth and function in rat neonates during suckling [31]. In the context of the LP model, undernourished rat neonates had impaired glucagon production and secretion. However, there could be species differences and these findings could differ in adult mice, such as the animals used in the present study. Nonetheless, these experiments further reinforce the importance of elucidating the impact cytokines, and specifically IL-6, on the endocrine pancreas in LP compared to control-diet exposed mice. It is also important to consider that the animal model of gestational glucose intolerance presented in this thesis presents with only a mild glucose intolerance. Thus, it is also plausible that these animals present with a mild pro-inflammatory state and therefore we might not anticipate observing elevated levels of pro-inflammatory cytokines in the pancreas implicating a potentially negligible effect on pancreatic endocrine cells in our model. Therefore, the low cytokine values in both serum and

pancreas samples could simply implicate a low level of inflammation in our model of GDM.

6.2.3 Discovering the contribution of non-β-cell endocrine cells in pregnancy

Despite the significant influence that pancreatic α-cells have on regulating glucose homeostasis by working antagonistically with β-cells, very little was known about the contribution of these cells in pregnancy. We investigated α-cell plasticity in healthy pregnancies and concluded that α- to β-cell transdifferentiation was negligible. However, it is important to acknowledge that the amount of β-cell loss can influence the extent of α- to β-cell transdifferentiation. Previous studies have reported that with mild β-cell ablation, less α-cell reprogramming occurred, and near-total β-cell ablation was required to trigger reprogramming [32]. In the case of the healthy animals in our study, there was no loss of β-cells, rather an adaptive increase in BCM expansion was observed in pregnancy. Therefore, the metabolic stress of pregnancy was likely insufficient to trigger reprogramming of α-cells and without the stressor of β-cell loss, α- to β-cell transdifferentiation will likely not occur. Nevertheless, it is plausible that in a situation of higher metabolic stress in pregnancy, such as in GDM, α- to β-cell transdifferentiation could occur. In our animal model of gestational glucose intolerance, we observed a ~50% reduction of BCM (**Chapter 2**). These findings provide rationale for genetic tagging of α-cells to provide mechanistic insights into whether α- to β-cell transdifferentiation could be occurring as a compensatory mechanism in GDM pregnancies with mild hyperglycemia. These studies would involve the combination of our established animal model of gestational glucose intolerance *via* dietary LP insult **(Chapter 2)** and transgenic Gcg-Cre/YFP mice **(Chapter 4)**. Although BCM was reduced in GDM compared to a control, β-cell proliferation was also reduced. Thus, α- to β-cell transdifferentiation could be occurring at a larger scale in GDM mice than in controls to contribute to the suboptimal BCM expansion that was still higher than in a non-pregnant animal.

At the same time, it is important to consider that our animal model of gestational glucose intolerance presented with only a mild glucose intolerance, which might not pose a high

enough metabolic stress to trigger conversion of α-cells into β-cells. Pregnancies with an additional metabolic stress, such as in obese mothers, are an example of where there could be a high enough metabolic stress to trigger transdifferentiation. Although, some of these models are limited by the presence of pre-gestational glucose intolerance [7]. An additional experiment that could be performed in subsequent studies would involve treating LP mice with a mild STZ intervention before mating, to only partially reduce BCM. This could theoretically ensure that glucose tolerance is maintained before mating and in early pregnancy. Additionally, future examination of subsequent pregnancies using our model could also provide an additional metabolic stress to trigger α- to β-cell transdifferentiation. GDM recurs in an estimated 30-69% of subsequent pregnancies following a pregnancy with GDM [33]. In our animal model of gestational glucose intolerance, glucose intolerance persisted until 1 month postpartum and normalized by 3 months postpartum (**Chapter 3**). Nonetheless, it is plausible that an additional metabolic stress such as a second pregnancy, could pose a large enough metabolic demand on the β-cells, triggering α- to β-cell transdifferentiation. Our findings provide a strong rationale for investigating a subsequent pregnancy, as we identified that α-cell fractional area was lower at 3 months postpartum in control diet mice relative to a non-pregnant animal. These findings prompt interesting considerations as to whether an adaptive expansion of ACM would occur in a subsequent pregnancy, or if perhaps the α-cell reservoir would be fully depleted after the first pregnancy. The investigation of subsequent pregnancies would be an invaluable area of future study. Bihormonal cells ($Insulin^{+}Glucagon^{+}$) have been identified in human pancreas sections, where it was reported that de-differentiation of β-cells into α-cells contributed to loss of BCM in patients with T2DM [34]. These findings suggest endocrine plasticity is possible in humans, however further studies are required to elucidate this in humans, which is limited with lineage tracing technology.

As our studies showed the critical contribution of pancreatic α-cells to endocrine adaptations in pregnancy, especially in the context of maladaptations of α-cells contributing to hyperglycemia in GDM, these findings provide a strong rationale to investigate additional endocrine islet cell types. An additional mechanism that could be contributing to hyperglucagonemia in GDM and would be worth exploring in future studies would be to assess δ-cell function. Eloquent studies have started to reveal the

precise mechanisms of somatostatin secretion from δ-cells, as was previously discussed **(Chapter 1)** [35–37]. It is postulated that defective somatostatin secretion can occur in diabetes [36]. Indeed, a recent study showed that reduced δ-cell function resulted in reduced inhibition of insulin and glucagon secretion, contributing to hyperglucagonemia in mice fed a high fat diet [38]. In pregnant mice, a novel contribution for δ-cells in early compensatory adaptations during pregnancy was also suggested [39]. Delta-cells were shown to reprogram to a β-cell identity, increasing insulin secretion to counter relative insulin resistance in pregnancy, mediated *via* less somatostatin-mediated inhibition. Therefore, it would be interesting to investigate whether this process is altered in GDM pregnancies using our animal model of gestational glucose intolerance. Maladaptations in δ-cells in GDM could be possible, contributing to reduced GSIS or hyperglucagonemia, however further studies are required to elucidate this. Evidently, continued research efforts to elucidate the integrative communication between multiple endocrine islet cell types in pregnancy are important, as the pathology of hyperglycemia in GDM could be much more complex than initially presumed if multiple endocrine cell types are involved.

6.2.4 Reversing glucose intolerance in pregnancy

Our findings in animals treated with artesunate/acetone present convincing data that the acetone vehicle was responsible for improvements in glucose tolerance. Although we posit some mechanistic insights to these improvements (**Chapter 5**), definitive mechanisms underlying the improved glucose tolerance remain elusive. As we postulate that acetone could be impairing nutrient intake in intestinal villi, subsequent studies examining histology or nutrient uptake of enterocytes would be of value to provide more precise conclusions about whether nutrient intake is indeed impaired. If proven to be true, these findings would provide strong evidence that a transient fast-mimicking situation could be contributing to improved glucose tolerance in our study. Because the IPGTT curve is drastically improved in acetone-treated animals, as shown by significantly reduced areas under the curve (**Chapter 5**), it would also be important to collect blood samples from animals at earlier timepoints during the IPGTT to assess if there is

improved insulin secretion. Finally, should acetone impair nutrient intake in enterocytes, it would be essential to follow animals to a longer time post-treatment to ensure long-term safety of this compound. Assessment of peripheral tissues could also prove insightful to assess for potential effects of toxicity. Moreover, long-term effects of acetone exposure *in utero* on parameters of health in the offspring, and even transgenerational effects, would be of interest to elucidate the safety of this compound in pregnancy.

6.3 Concluding Remarks

β-cell dysfunction has been described as a major driver of GDM, although based on the data presented in this thesis, the importance of α-cells has also come to light. Evidently, effective regulation of glucose homeostasis relies on sophisticated communication amongst both of these endocrine cell types. As such, effective treatments for GDM regulating both hormones could be pertinent. In summary, the work presented in this thesis advances our understanding of mechanisms involved in suboptimal endocrine adaptability and glucose intolerance in pregnancy (Fig. 6.1). While we were limited by the lack of GDM human pancreas samples for experimentation, the development of sophisticated endocrine pancreas imaging modalities to provide non-invasive monitoring of BCM/ACM in GDM would be essential to validate our findings. For the time being, the animal model and mechanisms explored in this thesis could lay the groundwork for evaluating new therapeutic opportunities to safely prevent and/or treat glucose intolerance in GDM.

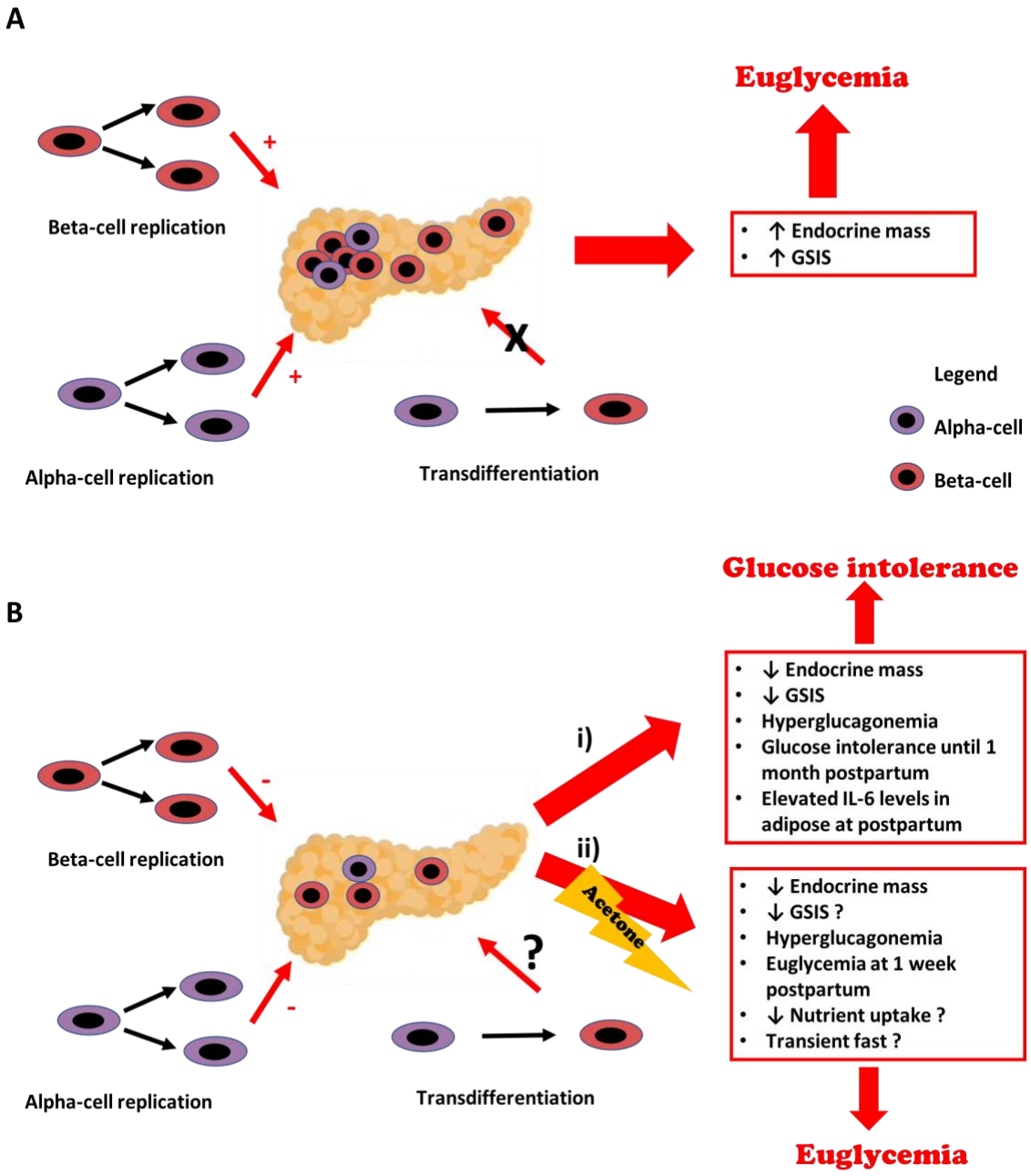

Figure 6.1. Summary of endocrine adaptations in a healthy pregnancy and maladaptations in GDM

A) <u>Healthy Pregnancy</u>: Alpha and BCM expansion occurred in response to increased insulin demand during the insulin resistant state of pregnancy. Endocrine mass expansion occurred due to increased replication of both α- and β-cells. Euglycemia was maintained during insulin resistance due to increased endocrine mass and increased insulin secretion.

There was a negligible contribution of α- to β-cell transdifferentiation to BCM expansion during pregnancy.

B) <u>GDM Pregnancy:</u> i). Both reduced α- and β-cell replication contributed to reduced ACM and BCM expansion. Insufficient compensatory endocrine adaptations, including decreased insulin secretion, led to glucose intolerance at late gestation which persisted until 1 month postpartum. Hyperglucagonemia also contributed to glucose intolerance at late gestation. The role of α- to β-cell transdifferentiation in GDM pregnancy remains to be determined *via* lineage tracing of α-cells during pregnancy.

ii). Treatment with acetone improved glucose tolerance at late gestation without increasing BCM, although these animals presented with hyperglucagonemia. Improvements in glucose tolerance persisted until 1 week postpartum. It remains to be determined whether nutrient uptake is reduced in intestinal villi, mimicking a transient state of fast, and/or whether insulin secretion is increased.

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Appendices

Appendix A 1. Animal Use Protocol Ethics Approval: Low Protein Pregnancy Study

AUP Number: 2015-097
PI Name: Arany, Edith
AUP Title: Control Of Regeneration In The Endocrine Pancreas
Approval Date: 06/17/2016

Official Notice of Animal Use Subcommittee (AUS) Approval: Your new Animal Use Protocol (AUP) entitled "Control Of Regeneration In The Endocrine Pancreas" has been APPROVED by the Animal Use Subcommittee of the University Council on Animal Care. This approval, although valid for four years, and is subject to annual Protocol Renewal.2015-097::1

1. This AUP number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this AUP number.
3. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

The holder of this Animal Use Protocol is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

Submitted by: Copeman, Laura
on behalf of the Animal Use Subcommittee
University Council on Animal Care [http://www.uwo.ca/animal-research/img/tr-signature.jpg]

[http://www.uwo.ca/animal-research/img/footer_auspc.png]

Appendix A 2. Animal Use Protocol Ethics Approval: Transgenics and Artemisinin Study

AUP Number: 2018-027
PI Name: Arany, Edith
AUP Title: Control of regeneration in the endocrine pancreas
Approval Date: 12/01/2018

Official Notice of Animal Care Committee (ACC) Approval:
Your new Animal Use Protocol (AUP) 2018-027:1: entitled " Control of regeneration in the endocrine pancreas"
has been APPROVED by the Animal Care Committee of the University Council on Animal Care. This approval, although valid for up to four years, is subject to annual Protocol Renewal.

Prior to commencing animal work, please review your AUP with your research team to ensure full understanding by everyone listed within this AUP.

As per your declaration within this approved AUP, you are obligated to ensure that:

1) Animals used in this research project will be cared for in alignment with:

a) Western's Senate MAPPs 7.12, 7.10, and 7.15

http://www.uwo.ca/univsec/policies_procedures/research.html

b) University Council on Animal Care Policies and related Animal Care Committee procedures

http://uwo.ca/research/services/animalethics/animal_care_and_use_policies.htm

2) As per UCAC's Animal Use Protocols Policy,

a) this AUP accurately represents intended animal use;

b) external approvals associated with this AUP, including permits and scientific/departmental peer approvals, are complete and accurate;

c) any divergence from this AUP will not be undertaken until the related Protocol Modification is approved by the ACC; and

d) AUP form submissions - Annual Protocol Renewals and Full AUP Renewals - will be submitted and attended to within timeframes outlined by the ACC.

e) http://uwo.ca/research/services/animalethics/animal_use_protocols.html

3) As per MAPP 7.10 all individuals listed within this AUP as having any hands-on animal contact will

a) be made familiar with and have direct access to this AUP;
b) complete all required CCAC mandatory training (training@uwo.ca); and
c) be overseen by me to ensure appropriate care and use of animals.

4) As per MAPP 7.15,
a) Practice will align with approved AUP elements;
b) Unrestricted access to all animal areas will be given to ACVS Veterinarians and ACC Leaders;
c) UCAC policies and related ACC procedures will be followed, including but not limited to:
i) Research Animal Procurement
ii) Animal Care and Use Records
iii) Sick Animal Response
iv) Continuing Care Visits

5) As per institutional OH&S policies, all individuals listed within this AUP who will be using or potentially exposed to hazardous materials will have completed in advance the appropriate institutional OH&S training, facility-level training, and reviewed related (M)SDS Sheets,
http://www.uwo.ca/hr/learning/required/index.html

Submitted by: Copeman, Laura
on behalf of the Animal Care Committee
University Council on Animal Care

AUC Chair Signature
Dr.Timothy Regnault,
Animal Care Committee Chair

The University of Western Ontario
Animal Care Committee / University Council on Animal Care
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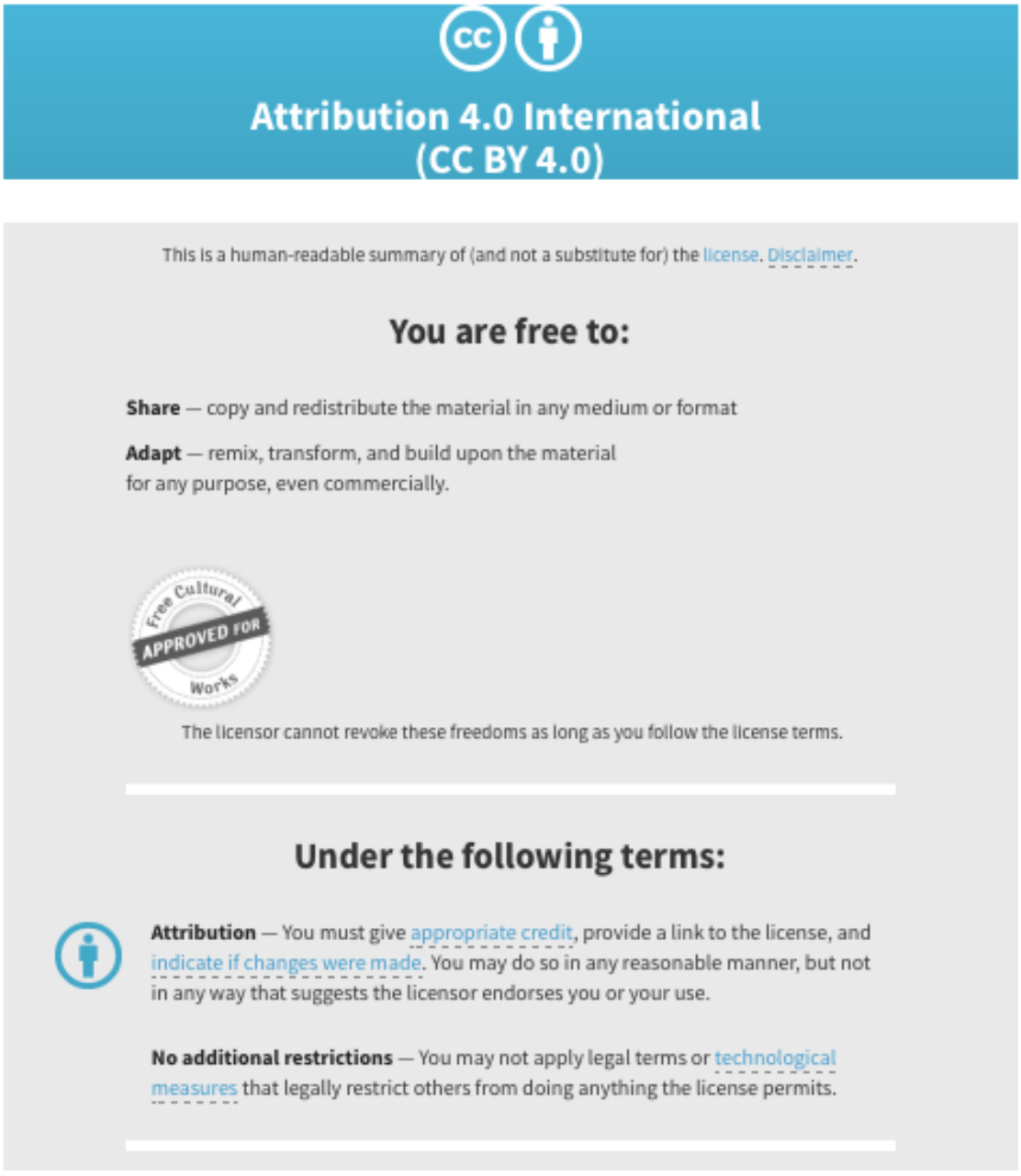

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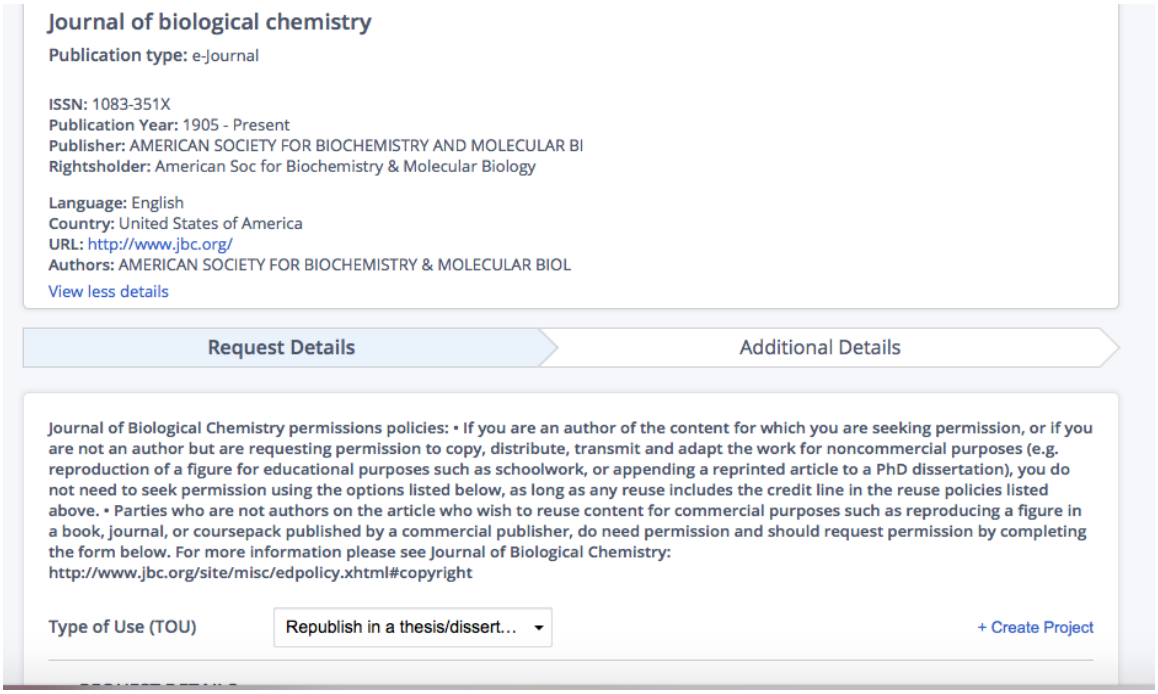

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3. 1st Place Prize in the Developmental Biology Category Graduate Student Poster Presentation Competition, Physiology and Pharmacology Research Day, Western University, 2018
4. Ontario Graduate Scholarship (OGS), Western University, 2018-2019, $15,000/year
5. Diabetes in Pregnancy Study Group Young Investigator Travelling Fellowship, 2019, €500
6. Children's Health Research Institute Trainee Award, 2019-2020, $10,000/year
7. Children's Health Research Institute Trainee Travel Award, 2019-2020, $1,500/year
8. Ontario Graduate Scholarship (OGS), Western University, 2019-2020, $15,000/year
9. Western Graduate Research Scholarship, Western University, 2015-present, $7,000/year

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2. Graduate Teaching Assistant, 3130z Human Physiology Laboratory, Western University, September 2016-April 2017
3. Graduate Teaching Assistant, 2130 Human Physiology, Western University, September 2017-April 2018

4. Graduate Teaching Assistant, 3140A Cellular Physiology, Western University, September 2019-December 2019

Peer-Reviewed Publications:

1. Beamish C, Zhang L, **Szlapinski S**, Strutt B, Hill D. 2017. An increase in immature β-cells lacking Glut2 precedes the expansion of β-cell mass in the pregnant mouse. PLoS ONE. 12(7): e0182256.
2. **Szlapinski SK**, King RT, Retta G, Yeo E, Strutt BJ, Hill DJ. A mouse model of gestational glucose intolerance through exposure to a low protein diet during fetal and neonatal development. J Physiol 2019;597:4237–50. doi:10.1113/JP277884.
3. **Szlapinski S**, Hill DJ. Metabolic Adaptations to Pregnancy in Healthy and Gestational Diabetic Pregnancies: The Pancreas - Placenta Axis. Curr Vasc Pharm 2020. E-pub ahead of print.
4. **Szlapinski S**, Botros AA, Donegan S, King RT, Retta G, Strutt BJ, Hill DJ. Altered pancreas remodeling following glucose intolerance in pregnancy in mouse. J Endocrinol 2020. doi:10.1530/JOE-20-0012.

Selected Presentations:

International

1. American Diabetes Association 77th Scientific Session, June 2017, poster, *San Diego, CA.*
2. 50th Annual Meeting of the Diabetic Pregnancy Study Group (DPSG), September 2018, oral, *Rome, Italy.*
3. American Diabetes Association 79th Scientific Session, June 2019, poster, *San Francisco, CA.*
4. American Diabetes Association 80th Scientific Session, June 2020, poster, *Chicago, IL*. Virtual Experience.

Local

1. London Health Research Day, March 2016, poster, *London, ON.*
2. Physiology and Pharmacology Research Day, November 2017, poster, *London, ON.*
3. London Health Research Day, March 2017, oral, *London, ON.*
4. Developmental Biology Research Day, Western University, May 2018, oral, *London, ON.*
5. 16th Annual Paul Harding Research Day – Obstetrics and Gynecology Research Day, April 2018, poster, *London, ON*
6. Endocrinology Grand Rounds, St. Joseph's Health Care London, May 2019, oral, *London, ON*
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