Small molecule activator of LYN improved the outcome of islet transplantation in mice

by

Roozbeh Akbari Motlagh

A thesis submitted in partial fulfillment of the requirements for the degree of

Master of Science

in

Nutrition and Metabolism

Department of Agricultural, Food and Nutritional Science
University of Alberta

ABSTRACT

Islet transplantation can achieve insulin independence in individuals with type 1 diabetes. However, islets derived from multiple donors are often required, and functional β-cells are lost early after transplantation. Notably, up to 80% of newly transplanted islets are lost due to cell death during the acute post-transplant period, which can be attributed to poor vascularization and the instant blood-mediated inflammatory reaction. Thus, strategies are needed to improve graft survival and function, particularly in light of these challenges. Our lab has recently characterized LYN as a critical regulator of β-cell proliferation and survival. We herein sought to test the hypothesis that pharmacological activation of LYN improves the outcome of islet transplantation in mice.

Male BALB/c islets were isolated and transplanted (marginal mass of 125 islets) into syngeneic diabetic mice recipients under the left kidney capsule. Recipients were thereafter injected intraperitoneally once daily with a specific activator of LYN (MLR-1023) or vehicle for 7 days. An intraperitoneal glucose tolerance test was performed at days 8 and 28. The graft-bearing kidneys and pancreas were also harvested for immunohistochemical analysis.

A brief 7-day treatment with MLR-1023 was sufficient to stimulate β-cell proliferation in islet recipients. However, β-cell mass was not significantly altered, due to inter-individual variations. MLR treatment accelerated revascularization at day 8 compared to the control group. Remarkably, these results translated into better glucose tolerance in the MLR-treated group compared to controls at day 8, without increased insulin secretion. Although MLR-1023 exhibited a sustained effect on revascularization at day 28, the majority of its effects dissipated after drug withdrawal.

This finding could hold significant clinical implications, as MLR-1023 could potentially be utilized in clinical islet transplantation to reduce the required islet mass for achieving insulin independence or to expedite the attainment of normoglycemia. Moreover, our study demonstrated a notable increase in revascularization, although it did not reach statistical significance. Notably, we observed accelerated neovascularization independent of VEGF-A. These results suggest that MLR-1023 may potentially enable stimulating revascularization through a distinct pathway.

PREFACE

This thesis is an original work by Roozbeh Akbari Motlagh. No part of this thesis has been previously published. I hereby confirm in the preface that I have obtained the necessary publisher approval for the utilization of figures included in my thesis, which have been sourced from published materials.

For Mom

"Tough times never last, but tough people do."

- Robert H. Schuller

ACKNOWLEDGMENTS

I would like to extend my sincere appreciation to Dr. Jean Buteau for welcoming me to his laboratory and for providing me with the opportunity to work on my research project. His guidance and support have been invaluable throughout the course of my studies, particularly during the comprehensive review of my thesis. I am grateful for his expertise and thoughtful feedback, which have helped me to develop my skills as a researcher and to produce high-quality work.

I would like to express my heartfelt gratitude to the past and present members of Buteau's lab for their availability and for the good times we have spent together. Thanks to you, the atmosphere in the lab has always been joyful and supportive, and I am grateful for the sense of community and camaraderie that we have shared. I would like to extend a special thank you to Dr. Hui Huang and Dr. Qian Wang for their invaluable contributions to my research project. Their unshakable calm, technical help, and advice have been instrumental in shaping my work and helping me to achieve my goals. I would also like to thank Sucheta Solanki, a previous member of the lab, for her contributions and support during her time in the lab. Together, the contributions of everyone in the lab have been a driving force in my academic development, and I am truly grateful for all that you have done to support me in my research.

I would also like to express my gratitude to my supervisory committee members, Dr. Andrew Pepper , Dr. René Jacobs and Dr. Mourad Ferdaoussi for their priceless advice and support throughout my academic journey. Their contributions have been instrumental in shaping my research direction and refining my ideas. Furthermore, I would like to offer special thanks to Dr. James Shapiro for his collaboration and expertise in the field of islet transplantation. His guidance and insights have been integral to the success of my research project. I would also like to thank Emad Yuzbashian for his help with statistical analysis.

Finally, I would like to thank my family, especially my mother, for their unwavering support throughout my academic journey. Their words of encouragement and unwavering belief in me have been a source of strength and inspiration. My gratitude to them cannot be expressed in words and their support has helped me through tough times.

TABLE OF CONTENTS

Page

LIST OF FIGURES

Page

LIST OF TABLES

Page

LIST OF SYMBOLS

α-Alpha

β-Beta

δ-Delta

ε-Epsilon

LIST OF ABBREVIATIONS

ACE	Anterior chamber of eye
AJ	Adherent junction
AUC	Area under the curve
COX-1	Cyclooxygenase 1
DYRK1A	Dual-specificity tyrosine phosphorylation regulated kinase 1A
ER	Endoplasmic reticulum
ELISA	Enzyme-Linked Immuno Sorbent Assay
FAK	Focal adhesion kinase
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-Like peptide 1
GLUT2	Glucose Transporter 2
GLUT4	Glucose Transporter 4
GSK-3β	Glycogen synthase kinase-3β
HbA1c	Glycated blood hemoglobin
HIF	Hypoxia-inducible factor
HLA	Human leukocyte antigens
HPAECs	Human Pulmonary Artery Endothelial Cells
HTS	High-throughput screening
HUVECs	Human Umbilical Vein Endothelial Cells
IBMIR	Instant blood-mediated inflammatory reaction
IL-6	Interleukin 6
IPGTT	Intraperitoneal glucose tolerance tests

IRS-1	Insulin receptor substrate-1
JNK	c-Jun N-terminal kinase
MAPK	Mitogen-activated protein kinases
MHC1	Major histocompatibility complex class 1
MODY	Maturity-onset diabetes of the young
MCP-1	Monocyte chemoattractant protein-1
NAFLD	Non-alcoholic fatty liver disease
NFAT	Nuclear factor of activated T cells
NOD	Non-obese diabetic
OGTT	Oral glucose tolerance test
PP	Pancreatic polypeptide
PPAR	Peroxisome proliferator-activated receptors
PTP1B	Protein tyrosine phosphatase 1B
ROS	Reactive oxygen species
T1D	Type 1 Diabetes
T2D	Type 2 Diabetes
TZDs	Thiazolidinediones
SCID	Severe combined immunodeficiency disease
STAT-5	Signal transducer and activator of transcription 5
STZ	Streptozotocin
SFKs	Src family of kinases

CHAPTER 1. INTRODUCTION

1.1 Epidemiology and Diagnosis of Diabetes

Diabetes incidence is on the rise, afflicting 8–10% of the world population, with as many suspected of having pre-diabetes (Wild et al., 2004). This incidence will likely double in the next few decades due to widespread sedentary lifestyles and dietary indiscretions. According to the World Health Organization, 108 million adults lived with diabetes in 1980; by 2014, that number had increased to 422 million (WHO, 2016). This is especially concerning because diabetes represents a heavy burden on the patient's health. Diabetes can cause metabolic issues such as diabetic ketoacidosis, hyperosmolality, and hypoglycemia. Also, complications from diabetes can affect various tissues and organs, such as nerves, eyes, kidneys, and the cardiovascular system. The chance of developing heart failure is increased by diabetes, and globally, diabetes is a major contributor to the development of chronic renal disease (Deshpande et al., 2008). Moreover, it has been linked to an increased risk and early onset of cataracts and glaucoma (Kavya ChitraMekala, 2019). Finally, diabetes is expected to become the 7th leading cause of death by 2030 (American Diabetes, 2021).

1.1.1 Clinical Definition of Diabetes

Diabetic Mellitus is a metabolic disorder commonly manifested by hyperglycemia (elevated blood glucose); according to the American Diabetes Association and World Health Organization (American Diabetes, 2021), diabetes mellitus in adults is defined using the following criteria: Fasting hyperglycemia (plasma glucose ≥ 126 mg/dL (7.0 mmol/L) after at least 8 hours of fasting or glycated blood hemoglobin (HbA1c) greater or equal to 6.5% (48 mmol/mol),

Symptomatic patient with non-fasting plasma glucose greater than or equal to > 200 mg/dL (11.1 mmol/L), or blood glucose greater than or equal to 200 mg/dL after 75 g glucose loaded on an oral glucose tolerance test.

Blood glucose is mostly regulated by the antagonistic actions of two hormones produced in the pancreas, namely glucagon (which signals for a lack of glucose) and insulin (which is secreted after a meal, when glucose is abundant). This confers a crucial role to the pancreas in the regulation of glucose homeostasis.

1.2 Pathophysiology of The Endocrine Pancreas

1.2.1 The Endocrine Pancreas

The pancreas is a multifunction organ that contains both exocrine and endocrine glands. The exocrine part is made up of acinar cells, which release an alkaline juice full of enzymes through the Wirsung canal. These enzymes include proteases, ribonucleases, lipases, and amylases. The endocrine cells (Deshpande et al., 2008) are made up of many different types of cells that are spread out all over the pancreas and make up about 1% to 2% of the pancreas (Arrojo e Drigo et al., 2015). The islets of the pancreas are highly vascularized in comparison to exocrine pancreatic tissues and receive a disproportionately higher fraction of (10 to 20%) pancreatic blood flow (Lifson et al., 1985). Islets contain several types of cells, including β, α, and δ cells that produce insulin, glucagon, and somatostatin respectively (Fig 1.1). There is also a small proportion of ghrelin-producing ε-cells and pancreatic polypeptide (PP) cells in primate islets. In addition to producing hormones, the islet is home to immune cells, autonomic nerve system ends, and a dynamic vascular system (Arrojo e Drigo et al., 2015). Histological study of human pancreata revealed that human islets differ from rodent islets in cell arrangement. Whereas murine islets

contain a β-cells core and a non-β-cells mantle, human islets have intermingled β-cells (Kim et al., 2009).

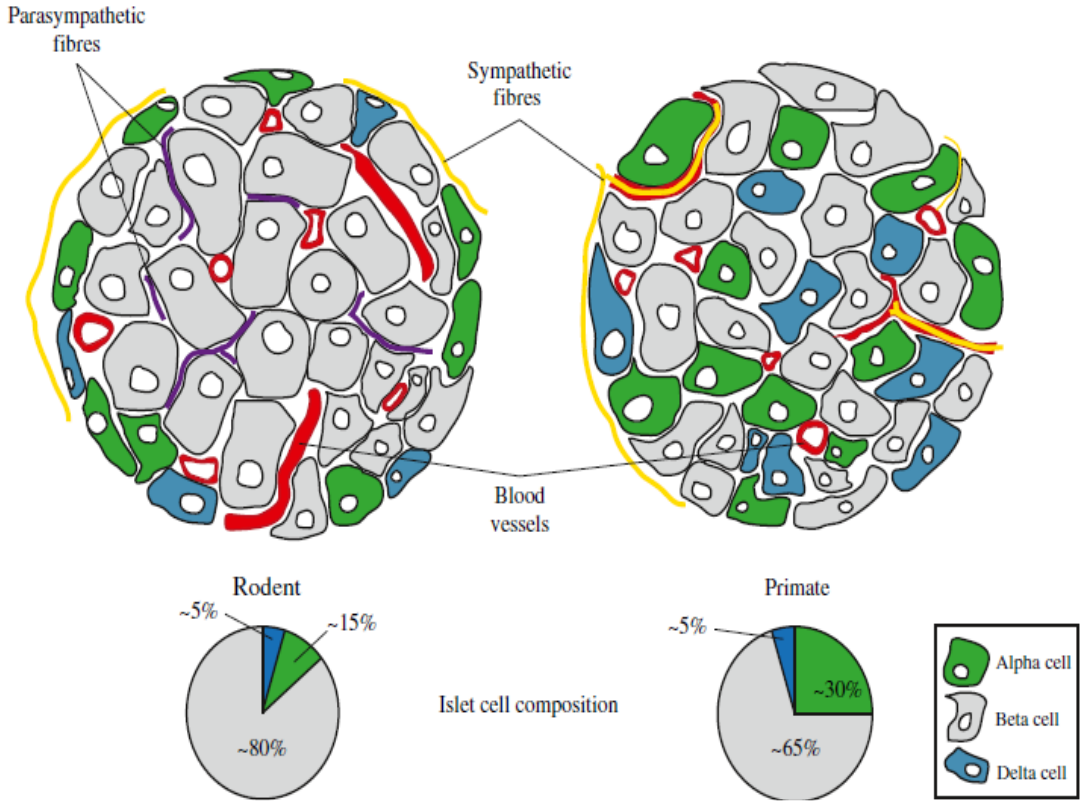


Figure 1.1 Differences in Islet Cytoarchitectures: Rodents vs Primates. Adopted from (Arrojo e Drigo et al., 2015) Used with Permission.

1.2.2 Glucose Homeostasis

Several hormones play a crucial role in controlling the concentration of plasma glucose in the blood, notably glucagon and insulin which are responsible for maintaining this concentration between 4 and 6 mmol/L (Roder et al., 2016). In general, the main stimulus for insulin production by β-cells is the elevated blood glucose levels following a meal. Upon secretion, insulin interacts and binds its receptor in target tissues to induce anabolic and hypoglycemic responses (Roder et

al., 2016). As a result of insulin binding to its hepatic tyrosine kinase receptor, a signalling cascade is activated. This cascade activates two processes (Fig 1.2); one inhibits glycogenolysis/gluconeogenesis and the other stimulates glycolysis/glycogenesis. Moreover, Insulin facilitates insulin-dependent glucose uptake in adipose and muscle tissues through the glucose transporter 4 (GLUT4). There are two main effects of insulin in adipose tissue: it increases lipogenesis and inhibits lipolysis.

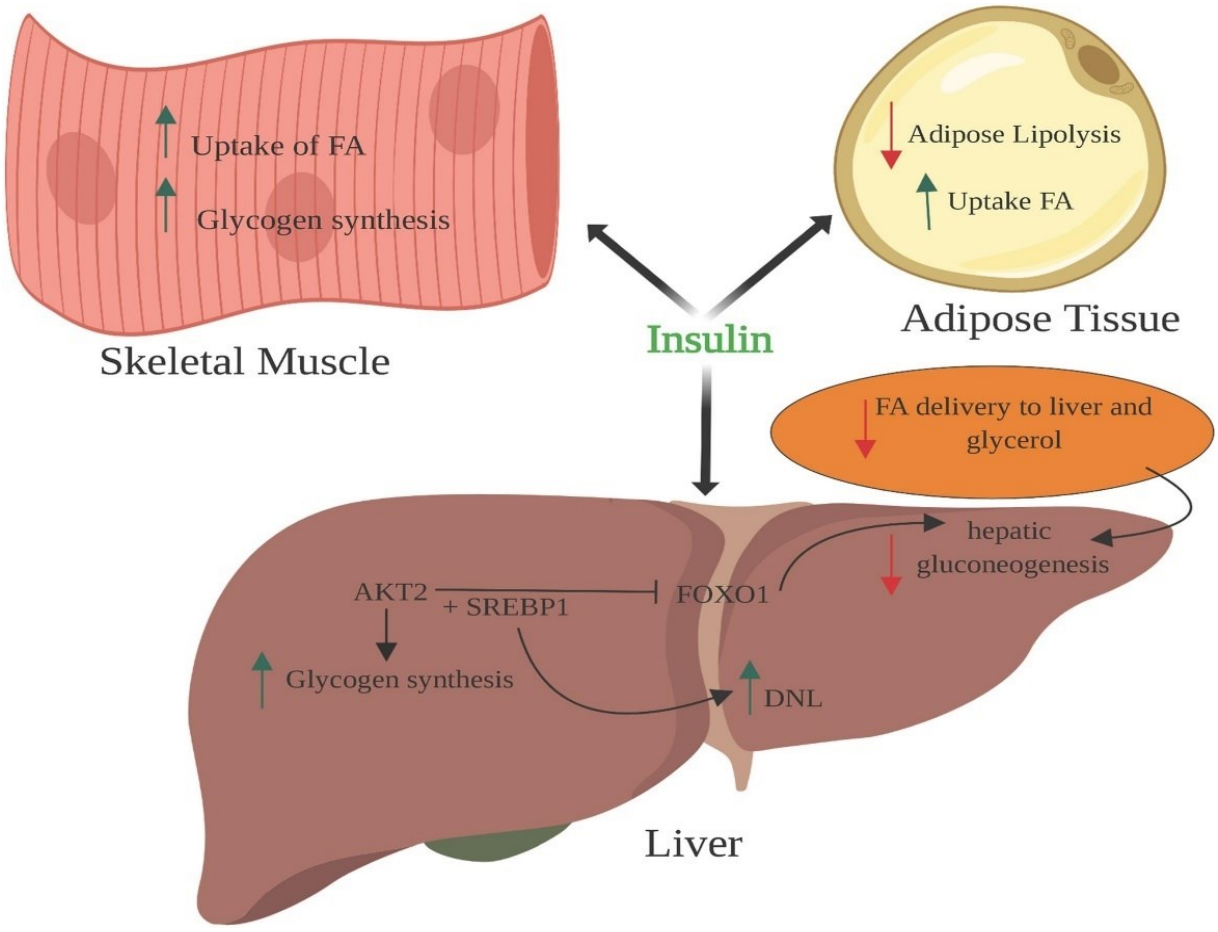


Figure 1.2 Effects of insulin on liver and peripheral tissues. Adopted from (Akhtar et al., 2019).Used with permission.

1.2.3 Insulin Secretion

Many different processes tightly regulate insulin secretion from β-cells, and a variety of insulin stimuli can trigger insulin release. Among them are glucose, fatty acids, amino acids, a small selection of hormones, and incretins (*incretins are hormones that are released from the intestine in response to food intake. They stimulate insulin secretion from the pancreas and reduce the amount of glucose produced by the liver, thereby helping to regulate blood sugar levels. Two of the most well-known incretins are glucagon-like peptide 1 (GLP-1*) and glucose-dependent insulinotropic polypeptide (GIP)). However, glucose is the central insulin stimulant and the predominant fuel in the diet (Fu et al., 2013). Two pathways involve glucose metabolism to stimulate insulin secretion: the triggering and amplifying pathways. The sequential steps of the triggering pathway are as follows: (Fig 1.3)

Transport of extracellular glucose to the cytoplasm by the Glucose Transporter 2 (GLUT2) of low affinity (Km ~ 15 mmol/L) and of high capacity. Phosphorylation of glucose to glucose-6-phosphate by glucokinase, a low-affinity hexokinase (Km ~ 11 mmol/L) which is not inhibited by glucose-6-phosphate (Henquin, 2000). Production of ATP from glucose-6-phosphate by glycolysis followed by oxidative phosphorylation in the mitochondria. An increased ATP/ADP ratio in β-cells s promotes plasma membrane depolarization by inhibiting ATP-sensitive potassium channels K+ ATP-sensitive channels. The depolarization leads to the simultaneous opening of voltage-dependent calcium channels, which increases intracellular calcium concentration ([Ca2+]i). As a consequence of this event, insulin-containing secretion granules are exocytosed (Henquin, 2000).

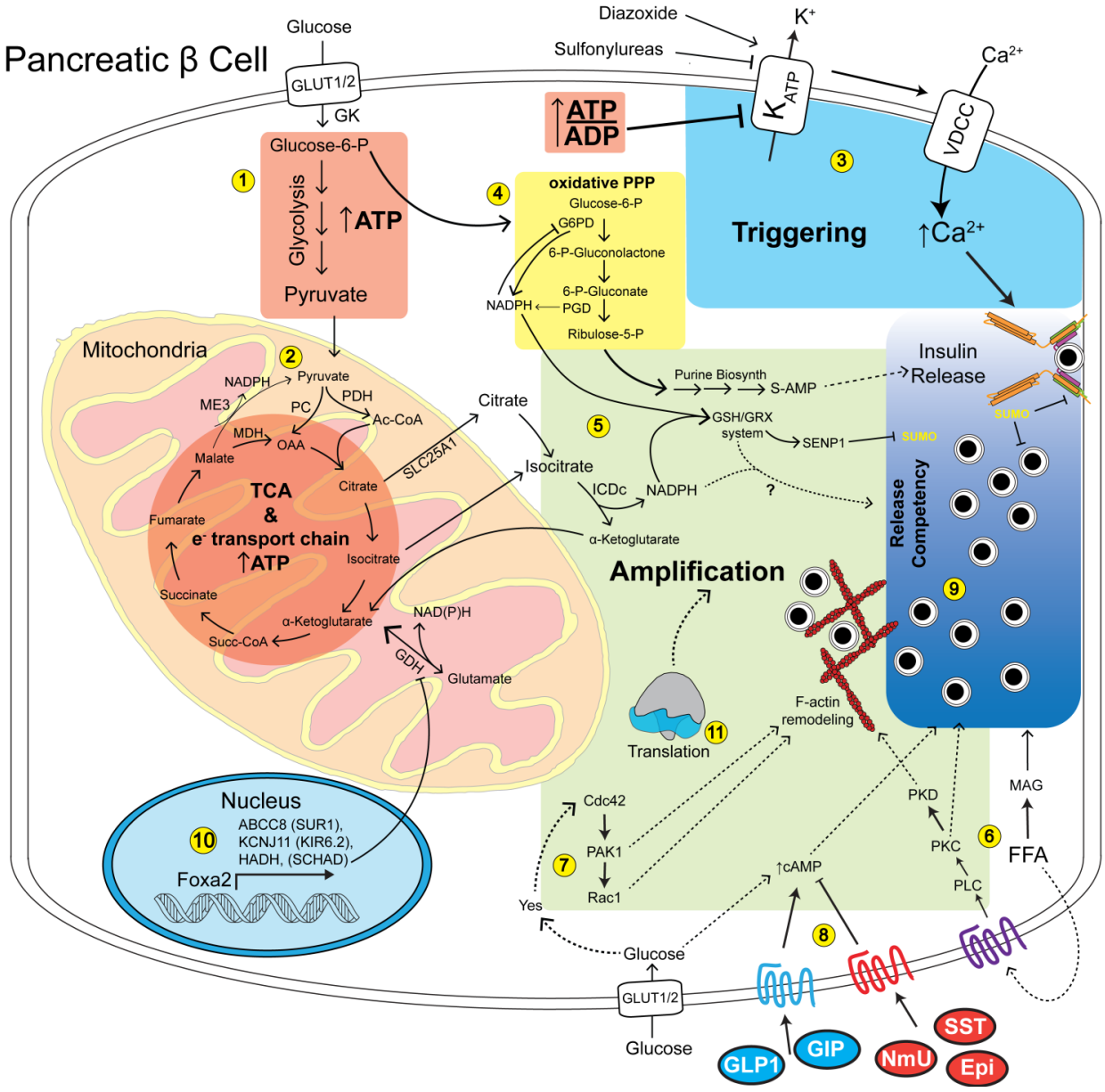


Figure. 1.3 Physiological mechanisms of nutrient-stimulated insulin release. Adapted from (Kalwat & Cobb, 2017).Used with permission.

1.2.4 Pathogenesis of Diabetes

Diabetes mellitus is a chronic metabolic disorder characterized by high blood glucose levels resulting from defects in insulin secretion, insulin action, or both. There are several types of diabetes mellitus, the most common of which are type 1, type 2, gestational diabetes and other specific types of diabetes such as maturity-onset diabetes of the young (MODY) (American Diabetes, 2010). Type 1 diabetes results from the autoimmune destruction of β-cells usually develop in childhood and account for approximately 5-10% of all diabetes cases (American Diabetes, 2010). Type 2 diabetes: this type of diabetes results from insulin resistance and/or impaired insulin secretion, accounting for approximately 90-95% of all cases. Gestational diabetes develops during pregnancy and typically resolves after delivery. Gestational diabetes occurs in approximately 2-10% of pregnancies, depending on the population studied (American Diabetes, 2010). Moreover, there are several forms of diabetes associated with monogenetic defects in β-cell function. Diabetes in these forms occurs frequently at an early age (generally before the age of 25) and is characterized by hyperglycemia (American Diabetes, 2010). The most common forms will be discussed in further detail below.

1.2.4.1 Type 1 Diabetes

Type 1 Diabetes (T1D) is characterized by the destruction of insulin-producing cells and the progressive loss of β-cell function, via an autoimmune reaction. Continuous exogenous insulin and islet transplantation are necessary to manage hyperglycemia in T1D (Zaccardi et al., 2016). Nevertheless, recent findings have challenged the perception that all β-cells are destroyed in T1D. Indeed, a large proportion of individuals with T1D maintain measurable serum C-peptide levels

even after long-duration diabetes (Oram et al., 2019). This unexpected discovery raises hopes that residual β-cells could be expanded to regenerate β-cell mass (Oram et al., 2019).

T1D is well established to be caused by genetic and environmental factors, as well as infectious triggers that β-cell deterioration (van Belle et al., 2011). Human leukocyte antigens (HLA) gene regions on chromosome 6p21 have been found to play a pivotal role in T1D susceptibility (van Belle et al., 2011). It has also been shown that enterovirus infections, such as Coxsackie virus B, mumps, Cytomegalovirus, and congenital Rubella syndrome, contribute to the appearance of islet antibodies that precede high glucose levels in T1D. Thus, a higher immune response is induced (Filippi & von Herrath, 2008; Sane et al., 2020). Inflammation caused by viruses may involve molecular mimicry and bystander T-cell activation mechanisms. Specifically, there has been strong sequence homology between Coxsackie virus B and the glutamic acid decarboxylase autoantibody in the islets (Filippi & von Herrath, 2008). The activation of autoreactive T cells is triggered by environmental triggers, which result in an increase in the expression of major histocompatibility complex class 1 (MHC1). Furthermore, pro-inflammatory stimuli such as CD4 T cells induce the production of β-cell-specific autoantibodies and the migration of CD8 T cells to destroy β-cells within islets. (van Belle et al., 2011).

Hypoglycemia is characterized by a sharp drop in blood glucose levels, which can be deadly if the patients are not aware. Severe hypoglycemia may induce shivering, shaking, sweating, heart palpitations, and cognitive issues. Hypoglycemia recurrently impairs the body's ability to recognize hypoglycemia, which leads to more frequent, severe episodes, and a reduced body's ability to detect hypoglycemia (Davis et al., 1997). The consequences of hypoglycemia can range from seizures to loss of consciousness to coma or death in the absence of rapid and effective intervention (Kalra et al., 2013). As another example, a patient may experience 'unstable' or 'brittle'

diabetes, in which blood glucose levels swing from high to low concentrations with unpredictable frequency, making it difficult to maintain any semblance of stability. These situations can lead to death and must be resolved urgently.

1.2.4.2 Type 2 Diabetes

Type 2 Diabetes (T2D) is a complex multifactorial disease characterized by both impaired insulin production by pancreatic β-cells (called "β-cell dysfunction") and blunted insulin signalling downstream of the insulin receptor (insulin resistance) (Butler et al., 2003). When there is visceral obesity accompanied by a subtle inflammatory syndrome, free fatty acids are accumulated in the blood and triglycerides accumulate ectopically, leading to peripheral insulin resistance (Mu et al., 2018). In addition, the liver becomes insulin resistant and plays a critical role in developing T2D. In the liver, insulin stimulates glycogen synthesis, inhibits glucose production, and suppresses lipid oxidation (Mu et al., 2018). In insulin-resistant states, the liver becomes less responsive to insulin, resulting in increased hepatic glucose production and decreased glycogen synthesis. This contributes to elevated blood glucose levels in individuals with T2D. Additionally, insulin resistance in the liver can lead to dysregulation of lipid metabolism, which contributes to the development of non-alcoholic fatty liver disease (NAFLD), a common complication of T2D (Mu et al., 2018).

1.2.4.2.1 β-Cells in Type 2 Diabetes

T2D is characterized by a progressive deterioration of both β-cell function and β-cell mass. Because it is difficult to isolate a single component and investigate if one precedes the other, we often refer to the expression "functional β-cell mass". β-cell deterioration is a key feature of the development and progression of T2D. This process typically occurs over several stages, with each

stage representing a further decline in the function and viability of these insulin-producing cells (Weir & Bonner-Weir, 2004). The first stage is characterized by insulin resistance, which results in increased insulin secretion by β-cells to maintain normoglycemia. In the next stage, the β-cells become exhausted, resulting in a decrease in insulin production. In the final stage of diabetes, β-cells are damaged or destroyed, severely impairing their function and resulting in a decline in β-cell mass (Weir & Bonner-Weir, 2004). Thus, β-cells are unable to produce sufficient insulin to control blood glucose levels, leading to the full manifestation of T2D. Generally, It is estimated that 60% of β-cells in T2D deteriorate, and the remaining cells are inefficient (Piciucchi et al., 2015). There are also inflammatory, immune, and microvascular abnormalities that cause clinical manifestations and widespread tissue complications in T2D patients (Folli et al., 2011).

1.3 β-Cell Mass in Diabetes

As discussed above, a common feature of T1D and T2D is the deterioration of functional β-cell mass. T1D is characterized by the massive destruction of cells by an autoimmune reaction, while T2D is characterized by a progressive deterioration of β-cells in the context of insulin resistance (Meier, 2008). It has been reported that bariatric surgeries can potentially reverse abnormal insulin secretion by improving glucose homeostasis. However, loss of β-cell mass is a more challenging condition to treat (Matveyenko & Butler, 2008). Hence, the relationship between β-cell mass and function is crucial to understanding the normal metabolic state and diabetes pathogenesis.

Pancreas specimens collected for histopathological studies showed (Butler et al., 2007) that 80 to 90% of the β-cells were destroyed when T1D was diagnosed and that the remaining β-cells were destroyed during the chronic phase (Matveyenko & Butler, 2008). However, upon further

investigation, researchers concluded that the β-cell number appeared to be less diminished at onset above the age of 15, and this remained the case during subsequent years. C-peptide secretion rapidly disappears in children after diagnosis, although in significant proportions of adolescent and adult onsets, it remains detectable for years (Meier, 2008). JJ Meier et al previously proposed the possibility of β-cell regeneration in individuals with long-standing T1D (Meier et al., 2005). It is generally accepted that the reduction of β-cell mass is necessary for T2D to occur (Weir et al., 2020). In this regard, some authors argue that the development of T2D is contingent upon a low β-cells mass (Matveyenko & Butler, 2008). According to several autopsy studies, both lean and obese patients who have T2D demonstrate a reduction of β-cell mass between 40-60% following an autopsy, regardless of whether they are lean or obese (Fig 1.4) (Weir et al., 2020). In another study, researchers found that post-challenge glycemia inversely correlated with β-cell mass in 82 patients undergoing pancreatic surgery (Meier et al., 2012). Moreover, they have shown that a reduction in β-cell mass of 21% results in glucose tolerance, and diabetic symptoms manifest when the β-cell mass is further reduced (Meier et al., 2012)

Taken together, several factors contribute to β-cells deficiency in both T1D and T2D, restoring or replacing β-cells mass is considered the logical long-term treatment option. As β-cells mass can be restored by islet transplantation. Therefore in this study, we focus on the magnitude of the maintenance of normoglycemia in patients with T1D by preserving or improving β-cells mass function after islet transplantation.

Consequently, these studies highlight the importance of studying the mechanisms that regulate pancreatic β-cell mass, a formidable puzzle of biology which we do not fully understand yet. The reasoning is that we could manipulate these mechanisms to protect and expand β-cells in order to prevent or cure diabetes.

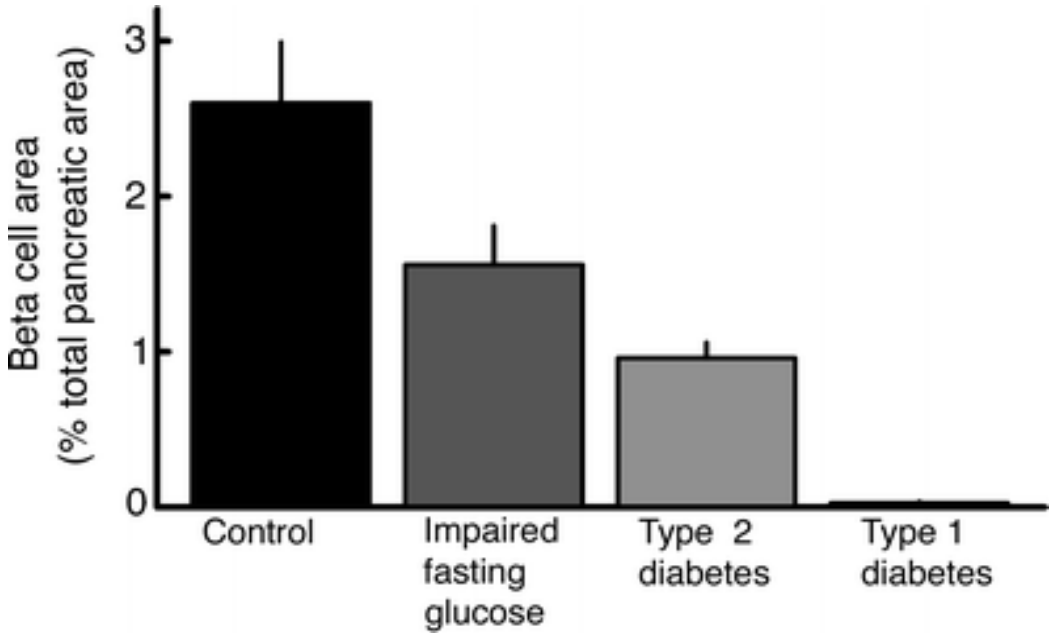


Figure 1.4 β-cell mass in type 1 and 2 diabetic patients vs non-diabetic control subjects. Adapted from (Meier, 2008).Used with permission

1.3.1 Physio-Pathological Regulation of β-cell Mass

There are many processes that seemingly act simultaneously to regulate β-cell mass. These processes include ***replication (proliferation)*** of pre-existing β-cells; the increase (hypertrophy) and the decrease (atrophy) of individual ***ß-cell size*** (Basile et al., 2019); ***neogenesis***, defined as a process in which precursor stem cells are differentiated into new mature β-cells; ***trans-differentiation*** of other cell types (such as pancreatic ductal epithelial or acinar cells) into functional β-cells (Aguayo-Mazzucato & Bonner-Weir, 2018). **Cell death** via necrosis and apoptosis leads to a deficit in β-cell number (Meier, 2008).

It must be highlighted that there are relatively few replication-capable β-cells in the adult human pancreas (one cell per 50 islets of 100 β-cells), approximately 0.5% of β-cell (Meier, 2008).

Thus, the importance of β-cell proliferation as a strategy to increase functional β-cell mass remains disputed for that reason. Be it as it may, β-cell regeneration is generally considered a promising avenue for the adjuvant treatment of diabetes.

1.3.2 β-Cell Proliferation-Stimulating Factors

β-cell development and maturation occur concurrently with normal human fetal development. In the third trimester of pregnancy, they become involved in controlling fetal growth, and they have the ability to respond to glucose (Basile et al., 2019). In this regard, in-depth research has been conducted on the replication of β-cells during the early postnatal period (Bonner-Weir et al., 2016). Indeed, β-cells have a high proliferation rate during the immediate postnatal period, but this rate gradually drops, and this decline occurs at a rapid rate until it eventually levels out (or stabilizes) (Bonner-Weir et al., 2016). In general, β-cell numbers present in the human pancreas at different stages of life can be estimated by the absolute number of β-cells generated during late fetal and early postnatal development, as well as the net loss of β-cells that occurs in a later lifetime (Wang et al., 2016). *There are noteworthy exceptions which lead to increasing β-cell replication and mass.*

*In **pregnancy***, insulin sensitivity declines, and therefore the β-cell mass increases to produce adequate insulin to meet the metabolic demands (Basile et al., 2019). In rodents, this requirement can be satisfied with increased β-cell proliferation (Basile et al., 2019), while in humans, the evidence is ambiguous. Human islet cells seem to be unresponsive to lactogenic hormones such as prolactin despite the expression of relevant signalling components (Basile et al., 2019). This is despite the fact that lactogenic hormones enhanced proliferation in rodents via the signal transducer and activator of transcription 5 (STAT-5) pathway (Basile et al., 2019). However, there are a few studies which have indicated that the rate of proliferation of existing β-cells has

been fairly stable (Butler et al., 2010), so this raises the question that improved neogenesis may be more significant than proliferation.

As mentioned in previous paragraphs, the β-cells have capacity to compensate for insulin resistance. It has been demonstrated that ***obese*** individuals can increase their β-cell mass in order to delay the onset of diabetes as a form of compensation (Saisho et al., 2013). This area of research has begun to yield some intriguing insights since the signals contributing to the expansion of β-cell mass are potentially many and may arise from a variety of metabolic organs that are resistant to insulin. As an example, ***SerpinB1*** has been identified as a peptide secreted by the insulin-resistant liver (El Ouaamari et al., 2016). In both rodents and humans, SerpinB1 levels have been determined to be altered in many diabetes models. The SerpinB1 protein has previously been implicated in a previous study by Ouaamarii et al. as a potential endogenous protein that can facilitate the enhancement of functional β-cells mass in diabetic patients. They have observed a twofold increase in proliferation in mice, zebrafish, and human β-cells (El Ouaamari et al., 2016). *Although extensive efforts have been made in recent years to promote the proliferation of adult human β-cells, most challenges still remain, such as human β-cell's poor ability to divide and differences between mice and humans in molecular pathways that control β-cell proliferation.*

This problem has been addressed by High-throughput screening (HTS), a technique whose versatility and ability to optimize assays are making it an increasingly important tool (Basile et al., 2019). ***Harmine*** was first reported as an active compound using HST to promote β-cell proliferation by Wang et al (Wang et al., 2015). Harmine is an alkaloid drug derived from plants that is capable of promoting cell proliferation in HepG2 cells through induction of the MYC promoter. Researchers found that Harmine mainly targets ***dual-specificity tyrosine phosphorylation-regulated kinase 1A*** (***DYRK1A***) (Wang et al., 2015). The nuclear factor of

activated T cells (NFAT) is phosphorylated and regulated by DYRK1A kinase. As NFAT is usually phosphorylated in the cytosol, Harmine inhibiting DYRK1A reduces its phosphorylation levels, causing it to translocate into the nucleus, improving mitogenic activity (Figure 1.5). Consequently, the authors confirmed that DYRK1A could stimulate proliferation of human β-cells both in *vitro* and *in vivo* as well as improve glucose homeostasis in mice (Wang et al., 2015). Kinome screening led to the identification of several inhibitors that inhibit NFAT/Cacinurin signaling, including, **GNF4877**, and **5-IT** (Basile et al., 2019). Recently, 5-IT was identified as another specific inhibitor of DYRK1A using HST (Dirice et al., 2016). Dirice et al. confirmed that the treatment of human isolated cells and transplanted human islet grafts under the kidney capsule in mice with 5-IT resulted in a significant increase in β-cell mass (Dirice et al., 2016). **TGF-β** is another signalling pathway which is considered to be related to β-cell division. Interestingly, Wang et al. have observed that the dual blockade of DYRK1A and TGF-β by the pharmacological inhibitors led to a 5-8% increase in β-cell proliferation in mice and humans (Wang et al., 2019). Previous reports have found that ablation or inhibition of ***Glycogen synthase kinase-3β*** (***GSK-3β***) stimulated DNA synthesis and increased β-cells proliferation in mice and humans (Basile et al., 2019). Shen et al. have indicated that inhibition of GSK3-β and DYRK1A with GNF4877 significantly increases islet mass in the grafted human islets. The authors also concluded that GSK-3β inhibition alone cannot explain GNF4877's proliferation-promoting effect (Shen et al., 2015).

Taken together, several studies have found that inhibition of DYR1A led to increased β-cells (Dirice et al., 2016; Shen et al., 2015; Wang et al., 2015; Wang et al., 2019), however, few studies have argued that inhibition of DYRK1A reduced β-cell development (Rachdi et al., 2014). Therefore, DYRK1A may be a promising option for promoting β-cell proliferation and growth and further investigation is needed for future drug discovery. Additional studies must be conducted to

determine whether the inhibitory effect of these compounds on β-cell proliferation is transient or not. Additionally, more efforts need to be made to identify the appropriate dose of inhibitor compounds that are tolerable in humans (Basile et al., 2019).

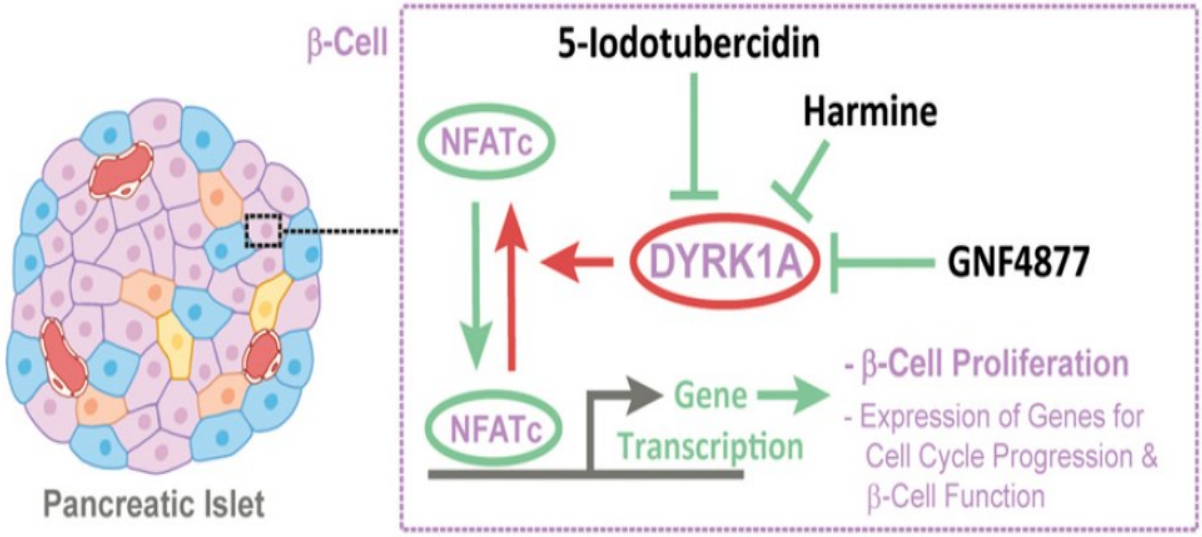


Figure 1.5 Inhibitors of DYRK1A in β-cells: mechanism of action.Adapted from (Belgardt & Lammert, 2016).Used with permission

1.3.2.1 Discovering Novel Determinants of β-cell Mass

The research program of our lab focuses on the identification of novel signalling molecules involved in the regulation of β-cell mass. Using GLP-1, a proposed β-cell growth factor, the Buteau lab has delineated novel signalling cascades that control β-cell proliferation and survival (Buteau, 2008; Buteau et al., 2003). The reasoning behind this strategy is that we might be able to harness therapeutically these newly identified molecules to maintain or expand residual β-cells in diabetes.

My own project follows up on these studies. Past lab members have identified the tyrosine kinase LYN as a critical regulator of β-cell proliferation and apoptosis (Buteau et al., 2003), and

unpublished results). The discovery of a specific activator of LYN (called MLR-1023) prompted us to ask the questions “Could MLR-1023 be used therapeutically to improve functional β-cell mass in diabetes?” and “Could MLR-1023 be used to improve the outcome of islet transplantation?”. I herein sought to answer the latter.

1.4 MLR-1023

MLR-1023, also known as Tolimidone, comprises 2 (1H)-pyrimidinone, 5-3-methyl phenoxy; a small active molecular compound originally developed as an antiulcer agent (Ochman et al., 2012; Saporito et al., 2012). According to previous studies, MLR-1023 has been shown to be an orally active insulin-sensitizing agent that decreases blood glucose levels in diabetic animals with a rapid-onset and durable effect (Saporito et al., 2012).

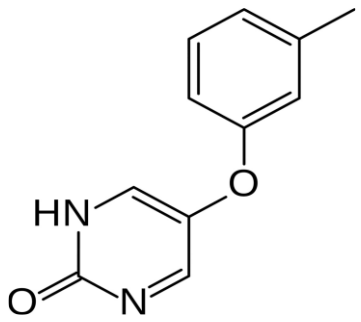


Figure 1.6 Structure of MLR-1023.Adopted from (Saporito et al., 2012).Used with Permission

The research found that there was an equivalent magnitude of reduction in blood glucose with MLR-1023 as with metformin or rosiglitazone (Ochman et al., 2012). In spite of this, the effects of rosiglitazone treatment were not significant until 10 days after initial administration. As MLR-1023 does not act as a PPAR agonist, the mechanisms by which insulin receptor sensitization is increased appear to be unique (Saporito et al., 2012). MLR-1023, unlike Thiazolidinediones

(TZDs), does not cause weight gain in mice [49]. Furthermore, it does not increase insulin receptor sensitivity through the PPAR pathway, thereby eliminating the risk of edema, weight gain, and cardiovascular disease associated with TZDs (Ochman et al., 2012; Saporito et al., 2012). As mentioned previously, the efficacy of MLR-1023 can be compared to that of metformin. However, unlike metformin, MLR-1023 did not demonstrate an impact on blood glucose levels in fasted mice. Furthermore, it appears that MLR-1023 does not inhibit gluconeogenesis in the liver, which is a mechanism of action associated with metformin (Saporito et al., 2012). In addition, MLR-1023 did not increase insulin secretion in RINm5F cells, thus it is concluded that MLR-1023 does not increase hypoglycemia as a result (Saporito et al., 2012). The results of the *in vitro* study were consistent with the *in vivo* findings that MLR-1023 administration reduced the blood glucose levels in mice that underwent an oral glucose tolerance test (OGTT) without increasing insulin secretion. Similarly, MLR-1023 lowered glucose levels and reduced HbA1c levels in db/db mice over 8 weeks (Saporito et al., 2012). The results of a four-week randomized; double-blind, placebo-controlled clinical trial indicated that 100 mg/day of MLR-1023 significantly reduced fasting plasma glucose after four weeks (Lee et al., 2020).

Taken together, MLR-1023 displays durable insulin sensitization and long-lasting blood glucose-lowering effects (Saporito et al., 2012). In addition, preliminary data from our lab indicates that MLR-1023 exhibits an anti-apoptotic effect in INS-1 cells and increases β-cells mass in non-obese diabetic (NOD) mice. The goal of this study was to determine the mechanism of action as well as the pharmacological effects of MLR-1023 on patients with T1D. This study will provide insights into the mechanism of action of MLR-1023, which may have a significant clinical impact.

1.5 Islet Transplantation

1.5.1 History of Islet Transplantation

It has been over a century since pancreas transplantations were first performed on patients with diabetes. A young boy with diabetic ketoacidosis received portions of a sheep pancreas subcutaneously by Watson Williams in 1893. The boy's glycosuria improved temporarily, but graft failure ultimately ended his life (Robertson, 2004). Younoszai et al. conducted the first transplantation in 1970 on rodents (Younoszai, 1970). Their experiments involved transplanting isolated rodent islets into a diabetic rat model; however, they were only able to achieve temporary normoglycemia (Downing, 1984). In 1972, Ballinger and Lacy reported the first successful reversal of hyperglycemia (Ballinger & Lacy, 1972); between 400-600 isolated rat islets were transplanted into the peritoneum or thigh muscle of streptozotocin-treated rats (Ballinger & Lacy, 1972). A major achievement for Reckard and Barker was the complete reversal of diabetes, achieved by injecting 800-1200 islets into the peritoneal cavity of hyperglycemic rats (Reckard et al., 1973).

A larger pancreas often contributed to poor yields and viability due to its difficulty in digestion. In the 1970s, sources of tissue such as unpurified pancreatic micro-fragments were investigated (Kretschmer et al., 1977; Mirkovitch & Campiche, 1976). It was Horaguchi and Merrell who introduced the concept of perfusing the pancreas with collagenase via the pancreatic duct to cleave the islets from the collagen matrix (Horaguchi & Merrell, 1981). However, a combination of complication factors, including portal hypertension and disseminated intravascular coagulation, led to the failure of pancreatic micro-fragments in clinical trials (Mehigan et al., 1980). In 1989, by using collagenase perfusion through the pancreatic duct, gentle mechanical

dissociation, and ficoll-gradient purification, Warnock et al. obtained purified human islets, which remains the standard procedure for islet isolation today (Warnock, Ellis, et al., 1989). The method has since been improved, including the intraductal infusion of Liberase (collagenase blend), the development of a semi-automated dissociation chamber, and using a COBE 2991 cell processor for islet purification (AM James Shapiro, 2007). In turn, this enabled large-scale isolation of islets, thus paving the way for islet transplantation to become a reality (AM James Shapiro, 2007). The first clinical islet transplant took place in 1977 (Najarian et al., 1977), and several subsequent transplants followed. It was reported by Warnock et al. that insulin requirements were reduced following islet-kidney allotransplants, as 5,000 IEQ/kilogram was infused into the portal vein. However, graft function was ultimately lost despite reduced insulin requirements (Warnock, Kneteman, et al., 1989). With the development of a low-temperature tissue bank, it is possible to provide patients with frozen islets, in addition to fresh islets, which increases the total number of islets per kilogram to over 10,000 (Warnock, Kneteman, et al., 1989). Some patients achieved sustained insulin independence as a result of this procedure, however, the majority of other patients did not achieve insulin independence within a year of receiving islet transplantation. According to reports, only 8.2% of the 267 islet allografts performed in the 1990s were able to maintain euglycemia for at least one year after their transplantation (Shapiro et al., 2000). Due to its success in solid organ transplantation, the initial islet transplants were generally performed in conjunction with renal transplants and immunosuppressive regimens including prednisone, azathioprine, and cyclosporine. In spite of this, the glucocorticoids in this regimen were toxic to the transplanted islets, limiting their success further (Ohneda et al., 1993; Zeng et al., 1993).

1.5.2 Edmonton Protocol

A pivotal study published in 2000 by the Edmonton group, showed that using the Edmonton Protocol, seven out of seven patients were able to achieve and maintain insulin independence for more than one year after transplant (Shapiro et al., 2000). This was an important milestone in this field. One aspect that explains the success of this particular study is the use of sufficient amounts of islets for transplantation: 11,546 equivalent islets were infused into the portal vein of recipients to help curb the progression of diabetes. In addition, a less toxic immunosuppressive regimen was used, including tacrolimus, sirolimus, and anti-interleukin-2 receptor antibody therapy (daclizumab) (Shapiro et al., 2000). There has been a five-year follow-up study done by the Edmonton Group, according to which over 140 islet transplants have been performed on 65 patients (Ryan et al., 2005). While 80% of patients were insulin-independent for one year, only 10% remained insulin-independent beyond that time period (Ryan et al., 2005). Nonetheless, glucose stability was improved, insulin requirements were reduced, and hypoglycemic episodes were avoided. It should also be noted that C-peptide levels were detected in 80% of the patients, indicating that there was some level of graft function (Ryan et al., 2005). In its recent study, the Edmonton group reported that post-transplantation graft survival for 10 and 20 years was 58% and 48%, respectively. The 20-year follow-up study also showed that 79% of patients had achieved insulin independence (Marfil-Garza et al., 2022). Even though islet transplantation is a very effective method of restoring glucose levels, it still has some challenges, which I will discuss in the next section.

1.5.3 Challenges to Islet Transplantation

In spite of the substantial improvement in clinical islet transplantation, insulin independence may not routinely be established due to several factors that result in reduced islet mass. These include limited availability of donor islets, instant blood-mediated inflammatory reaction (IBMIR), poor vascularization, hypoxia and toxicity from immune suppression (Fig 1.7) (Walker et al., 2022). Moreover, to prevent rejection-mediated cell loss, anti-rejection immunotherapies must be administered lifelong, which has prevented islet transplantation from universally being accepted as a therapy. In addition to immunosuppression concerns, there are also challenges associated with the islet isolation from the donor pancreas, which typically results in the need for more than one pancreas per islet recipient to achieve insulin independence. The most common type of challenge will be discussed in further detail below.

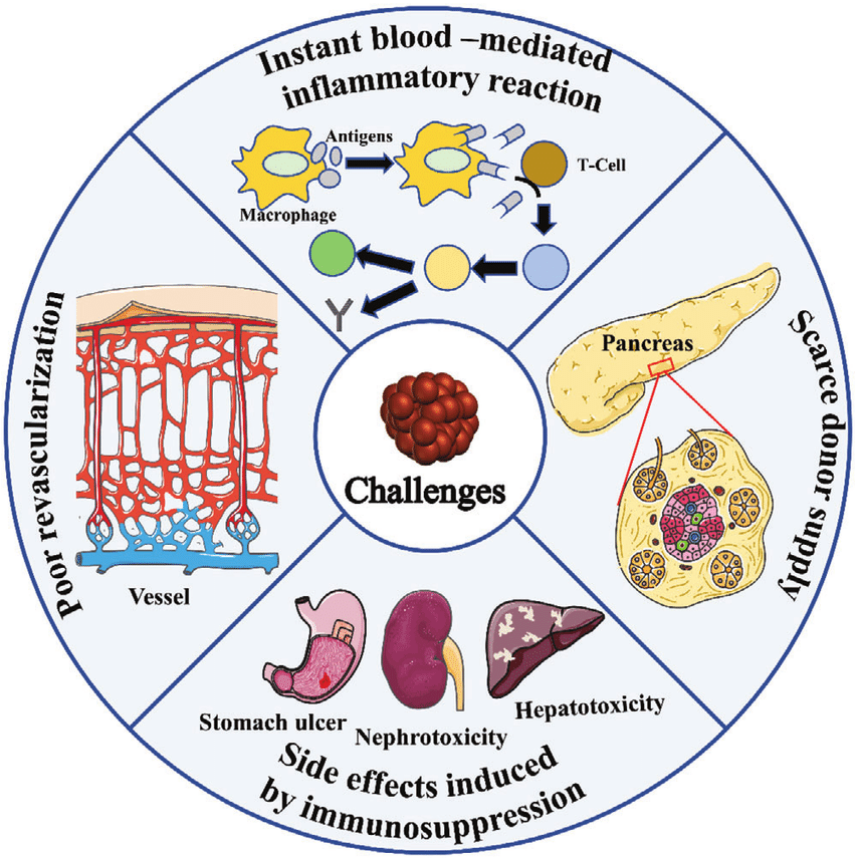


Figure 1.7 An overview of the current challenges associated with islet transplantation. Adopted from (Wu

et al., 2021). Used with permission.

1.5.3.1 Poor Vascularization

The islets of the pancreas are highly vascularized in comparison to exocrine pancreatic tissues and receive a disproportionately higher fraction of (10 to 20%) pancreatic blood flow (Lifson et al., 1985). The islet vasculature is integral to the islet's ability to secrete insulin and respond to blood glucose changes. Islet blood flow is regulated by both nervous, endocrine, and metabolic mechanisms to ensure appropriate blood flow when insulin secretion levels increase (Pepper et al., 2013; Wagner et al., 2022). The density of blood vessels within pancreatic islets lined with fenestrated endothelial cells is greater than that of blood vessels surrounding exocrine tissue, resulting in more significant partial oxygen pressure in the pancreatic islets compared to other organs and acinar tissue (Pepper et al., 2013). As the vasculature of the islet has been disconnected prior to transplantation, it is imperative that it is re-established following transplantation to achieve adequate graft function. After islet transplantation, metabolic exchange appears to be limited to passive diffusion. It is generally believed that intra-graft blood vessels appear within 3 to 5 days after transplantation and that blood flow returns within 7 to 14 days after transplantation. The maturity of islets is not achieved, at least as far as function is concerned, until several months have passed after implantation of aggregates of islets. Despite the fact that transplanted islets are restored to their native functions after blood flow is re-established, they still experience chronically low levels of vascular density and blood perfusion, and consequently, low levels of oxygen tension (5 mmHg), as compared with the native islets within the pancreas (40 mmHg). The vasculature of an area plays a crucial role in determining the success of an islet transplant. The kidney capsule and the anterior chamber of the eye (ACE) are highly vascularized

areas which provide favourable conditions for islet transplantation (Wagner et al., 2022). *Since ischemia and inflammation reduce the ability of islet cells to survive and function after transplantation, strategies that stimulate revascularization and dampen inflammation may provide a viable means of supporting the survival of islet cells following transplantation.*

1.5.3.2 Hypoxia

Human islets are generally isolated for transplantation in individuals with T1D. The negative effects of hypoxia are triggered during the pre-transplant and post-transplant phases (Komatsu et al., 2018). Hypoxic conditions pose a significant risk to pancreatic islets before transplantation, including the steps of pancreas procurement, islet isolation, and culture. It has been shown that hypoxic injury is associated with programmed cell death (Komatsu et al., 2018) and that intermittent hypoxia causes irreversible impairment of β-cell function in neonatal rats (Pae et al., 2013). In addition, other studies linked hypoxia-associated damage to oxidative stress since an increase in mitochondrial respiration results in an accumulation of reactive oxygen species (ROS). The expression of the Cyclooxygenase 1 (COX-1) and (Interleukin 6) IL-6 genes were upregulated (Brandhorst et al., 2016), and the release of Monocyte chemoattractant protein-1 (MCP-1) was increased in the cultured islets (Rodriguez-Brotons et al., 2016). β-cell function is impaired by hypoxia as a result of metabolic transition to anaerobic glycolysis. When islets are seeded in high density, hypoxic conditions can be induced *in vitro*. Culture vessels are supplied with oxygen from the surface of the culture medium, and the magnitude of oxygen tension decreases toward the bottom of the vessels where the islets are seated (Komatsu et al., 2018). The results of a 24-hour hypoxic condition with 3% oxygen in the islets and the culture medium demonstrated an increase in lactate levels, a classic indicator of hypoxia This result was concomitant with a lack of response

to high glucose levels (Garcia-Contreras et al., 2017). Molecular changes have been observed in hypoxia-inducible factor (HIF)-related pathways within islets exposed to hypoxia (Maillard et al., 2012). A mechanism independent of HIF-1 has also been suggested. In fact, when isolated mouse islets are exposed to hypoxia, their expression of genes associated with β-cells function and insulin secretion is suppressed independent of the change in HIF-1. Furthermore, hypoxia also led to downregulated genes critical for endoplasmic reticulum (ER) homeostasis, including those involved in adaptive unfolded protein response genes (Bensellam et al., 2016). The HIF-1 pathway is also not involved in the expression of these genes, but they are closely associated with pathways such as JNK (c-Jun N-terminal kinase) and p38 MAPK (mitogen-activated protein kinases) pathways (Pae & Kim, 2014). Moreover, hypoxia exposure both *in vitro* and *in vivo* down-regulated ZIP8 transporter, which is known as a zinc uptake transporter in β-cells (Pae & Kim, 2014).

1.5.3.3 Alternative Site of Transplantation

Currently, β-cells replacement is regarded as one of the most promising potential cures for T1D. The portal vein is currently accepted as a routine clinical site in humans where islet transplantations can take place, however, this method carries a few disadvantages including bleeding risks and the possibility of portal vein thrombosis (Stokes et al., 2017). Furthermore, due to the liver's exposure to both nutrients and insulin in the first place, it is a conceptually attractive place for the transplantation of islets (Shapiro et al., 2000). There are early studies that have shown success in using the portal vein site in mice (Contreras et al., 2003) and subsequent refinements have led to better results (O'Connell et al., 2013). Nevertheless, there are some disadvantages associated with the use of this site, including the possibility of complications caused by portal hypertension, bleeding, portal vein thrombosis, and hepatic ischemia (Stokes et al., 2017). In

addition to the rapid loss of islets after transplantation, there is another major problem associated with the portal vein. IBMIR, hypoxia, and immunosuppressive drugs absorbed through the gastrointestinal tract can all contribute to this process (Shapiro et al., 2005) (Deters et al., 2011). There was also reported a gradual functional decline in the majority of grafts and it is not feasible to perform routine biopsies to monitor the grafts (Ryan et al., 2005). Rodents commonly undergo renal subcapsular grafting which results in the reversal of diabetes within days, has a high success rate, and is relatively easy to do. For the maintenance of normoglycemia, 12-15% of the normal endocrine mass is required at the renal subcapsular site in adult rats (Merani et al., 2008). In an ongoing study conducted by Stokes (Stokes et al., 2017), the outcomes for several transplant sites were compared, including muscle, portal vein, kidney, liver capsules, and spleen-capsule transplant sites in mouse and human islets. In this study, they reported that transplanting mouse islets into the kidney subcapsular space resulted in the highest rate of success as compared with liver and spleen sites. Thus, considering the limited number of islets available for transplantation, it is of the utmost importance to achieve optimal engraftment. They also observed that spleen (47%) and liver (49%) grafts contained higher proportions of apoptotic cells than kidney (6-7%) grafts (Stokes et al., 2017). In addition to the ease of graft retrieval, the kidney offers many advantages, such as the possibility of excluding endogenous β-cells regeneration as well as histological evaluation. However, there is a marked difference in the oxygen tension between native and islets placed beneath the kidney capsule (Merani et al., 2008; Stokes et al., 2017). By continuing to refine this process, we will be able to develop techniques that will minimize the loss of endocrine cells after transplantation.

Chapter 2: Research Plan

2.1 Rationale

Diabetes incidence is on the rise, afflicting 8–10% of the world population, with as many suspected of having pre-diabetes (Wild et al., 2004). Globally, the prevalence of diabetes is increasingly alarming, leading to a public health crisis. In T1D, a disease caused by the destruction of insulin-producing cells by autoimmune reactions, continuous administration of exogenous insulin to control hyperglycemia is the only form of treatment considered. Along with this, technological advancements have allowed us to approximate physiological glucose monitoring and release with insulin types and treatment options. Although these advances have been made, there is still a minority of patients for whom these options are insufficient to maintain proper glucose levels.

Islet transplantation is proving to be an effective treatment for T1D (Shapiro et al., 2000). Even though islet transplantation is very effective for restoring glucose levels, it does have some limitations. Following a transplant, there is a significant amount of cell loss during the acute period. It is estimated that approximately 80% of the islets will be lost within the first 24 hours at least in part due to disruption of vascularization and IBMIR, thereby causing a shortage of nutrients and oxygen, as well as inflammation in the early stages of transplantation (Walker et al., 2022). As a result, recipients often require multiple transplants before achieving normoglycemia.

It is in this context that I tested the potential actions of MLR-1023, also known as Tolimidone, on the outcome of islet transplantation. MLR-1023 a small molecule activator of LYN was originally developed and tested by Pfizer as an antiulcer agent. However, the clinical trials were abandoned for lack of efficacy. Later, Melior Pharmaceuticals recognized (Ochman et al., 2012; Saporito et al., 2012) the anti-diabetic actions of MLR-1023 when trying to repurpose

that drug. In animals that were fed a high-fat diet, MRL-1023 led to a reduction in weight gain and an improvement in oral glucose tolerance. However, the exact mechanisms remain elusive. The authors posited that MLR-1023 increased insulin sensitivity (Saporito et al., 2012) and did not specifically test the actions of MLR-1023 in β-cells. The discovery that MLR-1023 acted as a growth factor resulted from the research efforts of the Buteau and prompted the original question: Could MLR-1023 be used to improve the outcome of islet transplantation in mice?

In humans, the results of a four-week randomized, double-blind, placebo-controlled clinical trial indicated that 100 mg/day of MLR-1023 significantly reduced fasting plasma glucose after four weeks (Lee et al., 2020). There is a dose-dependent and long-lasting lowering of glucose levels associated with MLR-1023, in addition to a reduction in levels of glycated hemoglobin (HbA1c) (Saporito et al., 2012) and preservation of β-cells in the pancreas. The preliminary data from our lab indicated that MLR-1023 had an anti-apoptotic effect on β-cells *in vitro*, as well as the ability to increase β-cell mass *in vivo*. However, the mechanism of action of MLR-1023 in T1D and its pharmacological effect on pancreatic islet transplantation is still unclear**.**

2.2 Hypothesis and Objectives

This study aims to test the hypothesis that MLR-1023, a β-cell growth factor, ameliorates glucose homeostasis in islet recipients as a result of improved graft function. To test this hypothesis, my thesis had the following objectives:1) determine if MLR-1023 can improve glucose control in islet recipient.2) determine if MLR-1023 can increase β-cell proliferation of the graft .3) determine if MLR-1023 can improve revascularization of the graft. To test this hypothesis, I examined whether a short MLR-1023 treatment of 7 days was sufficient to improve glucose homeostasis in islet recipients, and investigated the mechanisms involved. More precisely, I

conducted morphological assessments of the grafts to measure β-cell proliferation, β-cell mass and vascularization at day 8. We also repeated the same measurements at 28 days (thus 21 days after drug withdrawal) to determine whether the effects were transient or persistent. This was performed in a syngeneic islet transplant mouse model receiving a marginal (and thus suboptimal) mass of 125 islets under the kidney capsule. Typically, >200 islets are needed to normalize glycemia.

Chapter 3: Methods and Material

Animals. BALB/c male mice, aged 12-15 weeks, were selected as recipients for the islet transplantation study. The mice were acquired from the University of Alberta Health Science Laboratory Animal Service, and all animal use adhered to the guidelines set by the Canadian Council on Animal Care, with approval from the institutional animal ethics committee at the University of Alberta in Edmonton, AB, Canada. Measures were taken to minimize animal suffering and reduce the number of animals used in the study. The mice were kept in a temperature-controlled environment and maintained on a 12-hour light/dark cycle, with food and water provided ad libitum. The mice fasted for 12 hours before the glucose challenge**.**

MLR-1023 therapy. To conduct the *in vivo* study, MLR-1023 was obtained from Melior Pharmaceutical Company (Exton, PA, USA). Initially, a 15 mg/ml stock solution was prepared in phosphate-buffered saline (PBS; pH 4.5) to solubilize the compound and obtain a water-soluble concentration of 5 mg/ml. For control experiments, a solution containing 0.5% Cm-cellulose and Tween 80 (0.025%) in sterile PBS at the same concentration as MLR-1023 stock was used. The stock solution was stored at -20°C until use. The treatment group received MLR-1023 intraperitoneally (IP) at a dose of 30 mg/kg. For the *in vitro* study, a water-soluble concentration of 100 nM was achieved by diluting the stock solution into pure ethanol.

Islet transplantation studies. The isolation of male BALB/c islets was performed using established techniques (Gotoh et al., 1985). Recipient mice, BALB/c strain (in the case of syngeneic studies), were made diabetic through chemical induction using intraperitoneal streptozotocin 3 times one week before transplantation. To achieve this, 75 mg/kg of streptozotocin

(STZ; Sigma-Aldrich Canada Co., Oakville, Ontario Canada), dissolved in acetate buffer (pH 4.5), was administered intraperitoneally to induce diabetes in the recipient mice. Blood samples were collected from the tail to measure glucose using Freestyle Lite test strips (Abbott, Alameda, CA) until the animal became diabetic. Diabetes was confirmed when there were two consecutive readings of more than 15 mmol/L. Before transplantation, the islet preparations were split in half and incubated for 1 hour in either MLR–1023-containing medium (100nM in Dulbecco's modified Eagle's medium; Gibco, Waltham, MA, USA) or vehicle-containing medium. After incubation, the islets were washed, counted, and transplanted. Transplant recipients were injected with either MLR-1023 or vehicle (30 mg/kg body weight) on the day of transplant and for 6 days afterward (Fig 3.1). The transplantation of 125 islets under the left kidney capsule was carried out to study the renal subcapsular area. In some parts of the study, immunohistochemical analysis of the islet graft was performed after the graft-bearing kidney was harvested. Graft-bearing kidneys were collected at 8 and 28 days and fixed in 10% paraformaldehyde. Animals underwent a survival nephrectomy of the graft-bearing kidney at 8 and 28 days to determine whether euglycemia was graft-dependent. Following the nephrectomy, animals were monitored twice a week to assess whether hyperglycemia had returned to the animals. To determine regeneration markers, the pancreas was collected after sac removal.

Glucose tolerance test. To assess the metabolic capacity of the recipient, intraperitoneal glucose tolerance tests were performed at 8 and 28 days. The transplanted animals fasted for 12 hours before receiving injections of 25% Dextrose (3g/Kg) in Ringer's solution. Blood glucose levels were monitored at 0, 15, 30, 60, and 120 minutes following injection. Area under the curve (AUC) calculations were analyzed between transplant groups and untreated groups (naïve) using

GraphPad Prism 8.0 (GraphPad Software, La Jolla, CA, USA). Serum insulin levels were measured using a commercial Elisa kit (ALPCO, Salem, NH, USA) at 0 and 30 minutes after injection.

Mouse insulin assay. Blood was obtained through a tail puncture to evaluate insulin secretion stimulation. At 0 and 30 minutes, the procedures were repeated consecutively, and the collected blood was subjected to centrifugation. The resulting serum was stored at -80°C until further use. Serum insulin levels were measured using an Enzyme-Linked Immuno Sorbent Assay (ELISA) following the manufacturer's instructions (ALPCO, Salem, NH). Statistical analysis was performed using GraphPad Prism 8.0

Immunohistochemistry assay. Tissue sections were fixed in paraffin and sectioned into 5 µm slices for histological analysis. The sections were then deparaffinized and rehydrated before treatment with 3% hydrogen peroxide for 10 minutes. The sections were subsequently incubated in a blocking buffer containing 5% normal donkey serum in 1X PBS for 1 hour at room temperature. For immunostaining, a diluent antibody buffer was prepared using 0.05% normal donkey serum diluted in 1X PBS sterile. The slides were then incubated for 1 hour at room temperature with mouse anti-insulin (Sigma 1:500), followed by incubation for 1 hour with anti-mouse HRP (GE Healthcare, Little Chalfont, UK) for 30 minutes. Freshly prepared DAB substrate was then applied to the sections and counterstained with Hematoxylin solution. Finally, all images were analyzed using an Olympus BX61VS microscope (PerkinElmer, Guelph, ON) and the Olympus VS-Desktop imaging software.

Immunofluorescence assay. Islet grafts were fixed and embedded in paraffin, and 5 μm sections were deparaffinized and rehydrated. Microwave antigen retrieval in citrate (pH 6.0) was performed before incubating the sections overnight at 4°C with the following primary antibodies: guinea-pig anti-insulin (Dako, 1:500), rabbit anti-Ki67 (Abcam, 1:100), mouse anti-glucagon (Sigma, 1:1000), and rabbit anti-CD31 (Abcam, 1:50). To prepare the diluent antibody buffer, eBioscienceTM Foxp3 (Invitrogen, Waltham, MA, USA), was diluted to 5% normal donkey serum (normal donkey serum diluted in 1X PBS sterile). The following day, the slides were incubated for 1 hour at room temperature with anti-mouse Alexa Fluor 647, anti-guinea-pig Alexa Fluor 568, anti-rabbit Alexa Fluor 647 and anti-rabbit Alexa Fluor 488 secondary antibodies (1:250) (Life Technologies, Waltham, MA, USA) before mounting with Fluoromount-G® containing DAPI (Southern Biotech, Birmingham, AL).

For VEGFR2 staining, the islet graft sections were also fixed and embedded in paraffin, followed by deparaffinization and rehydration. Microwave antigen retrieval in citrate (pH 6.0) was performed, and the slides were blocked with 5% goat serum and 0.3% Triton™ X-100. The sections were then incubated overnight at 4°C with guinea-pig anti-insulin (Dako 1:500) and rabbit anti-VEGFR2 (Cell Signalling, 1:100) antibodies. The following day, the slides were incubated for 2 hours at room temperature with anti-rabbit Alexa Fluor 647 and anti-guinea-pig Alexa Fluor 568 secondary antibodies (1:250) (Life Technologies, Waltham, MA, USA),) before mounting with Fluoromount-G® containing DAPI (Southern Biotech, Birmingham, AL). All images were analyzed using an Olympus BX61VS microscope (PerkinElmer, Guelph, ON) and the Olympus VS-Desktop imaging software

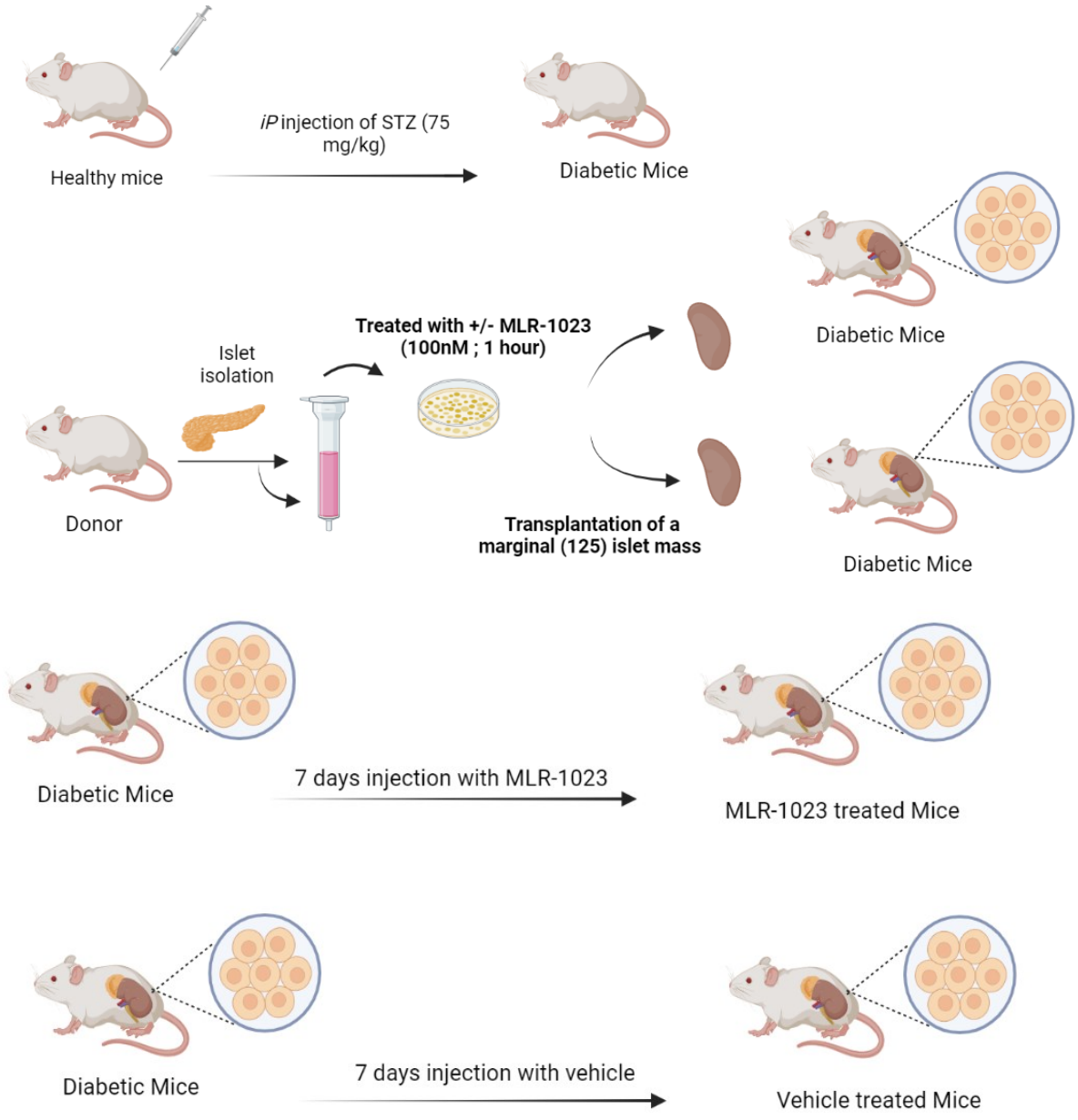
In Vitro assay. The INS-1 cell line (passage 50-70) was grown in RPMI 1640 complete medium (Gibco, Waltham, MA, USA), supplemented with 10 mM HEPES, 10% heat-inactivated fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, and 50 µM β-mercaptoethanol at 37ºC in a humidified 5% CO2 atmosphere. Cells were grown to reach 80% confluency in 24-well plates to assess VEGF-A secretion and expression. To investigate the impact of MLR-1023 on VEGF-A secretion and expression, the cells were treated with MLR-1023 at a concentration of 100 nM in a serum-free RPMI medium (Gibco, Waltham, MA, USA). The treatment duration was 24, 48, and 72 hours, with daily replacement of the serum-free RPMI medium with fresh medium containing MLR-1023 and vehicle. The cells were then incubated at 37°C with 5% CO2. After 72 hours of incubation, the serum-free medium was replaced with fresh medium containing MLR-1023 (100 nM) or vehicle, and the cells were incubated for an additional 4 hours. To assess VEGF-A secretion level, 100 µl of supernatant was collected and measured by VEGF-A Rat ELISA Kit (Invitrogen, Waltham, MA, USA). Simultaneously, the cells were lysed using RLT plus buffer (RNeasy Plus Kits, Qiagen) to evaluate VEGF-A expression.

qPCR. Total RNA was extracted using an RNeasy Plus Kit (Qiagen, Germantown, MD) and reverse-transcribed using a SuperScript First-Strand Synthesis System (Life Technologies, Waltham, MA, USA). The reagents used in this experiment were purchased from Life Technologies. Primers were designed for each gene of interest based on cDNA sequences from the GenBank database (http://www.ncbi.nlm.nih.gov/genbank) by Integrated DNA Technologies (Table 1). For real-time PCR, 7.5 µl of SYBR Green PCR Master Mix (Qiagen), 1.5 µl of cDNA, and 1 µl each of primers were used in a final volume of 20 µl. Duplicate or triplicate PCR reactions were performed for each sample. Gene amplification was performed with 40 cycles of denaturation

at 95°C for 15s, annealing and extension at 60°C for 1 min. An β-actin gene was selected as the reference gene, and relative quantification was carried out using the software Life Technology StepOneTM, according to the comparative $2(^{-\Delta\Delta Ct})$ method. Statistical analysis was conducted using GraphPad Prism 8.0.

Rat VEGF-A assay: After 72 hours of incubation, the serum-free medium was replaced with fresh medium containing MLR-1023 (100 nM) or the vehicle, and the cells were further incubated for 4 hours. To ensure the absence of cell debris, the supernatant samples were centrifuged for 5 minutes at 8000 rpm. For the assessment of VEGF-A secretion levels, 100 µl of supernatant was collected and stored at -80°C. The collected supernatants were measured using the VEGF-A Rat ELISA Kit (Invitrogen, Waltham, MA, USA) following the manufacturer's instructions. Statistical analysis was performed using GraphPad Prism 8.0.

Statistical analysis. Statistical analysis was conducted using GraphPad Prism 8 software (GraphPad Software, La Jolla, CA, USA). The data are represented as mean ± standard error of the mean (SEM). The data were analyzed using either a student's t-test or a two-way ANOVA, as appropriate. Results were considered significant at $p < 0.05$.


Healthy mice
iP injection of STZ (75 mg/kg)
Diabetic Mice
Diabetic Mice
Islet isolation
Treated with +/- MLR-1023 (100nM ; 1 hour)
Donor
Transplantation of a marginal (125) islet mass
Diabetic Mice
Diabetic Mice
7 days injection with MLR-1023
MLR-1023 treated Mice
Diabetic Mice
7 days injection with vehicle
Vehicle treated Mice

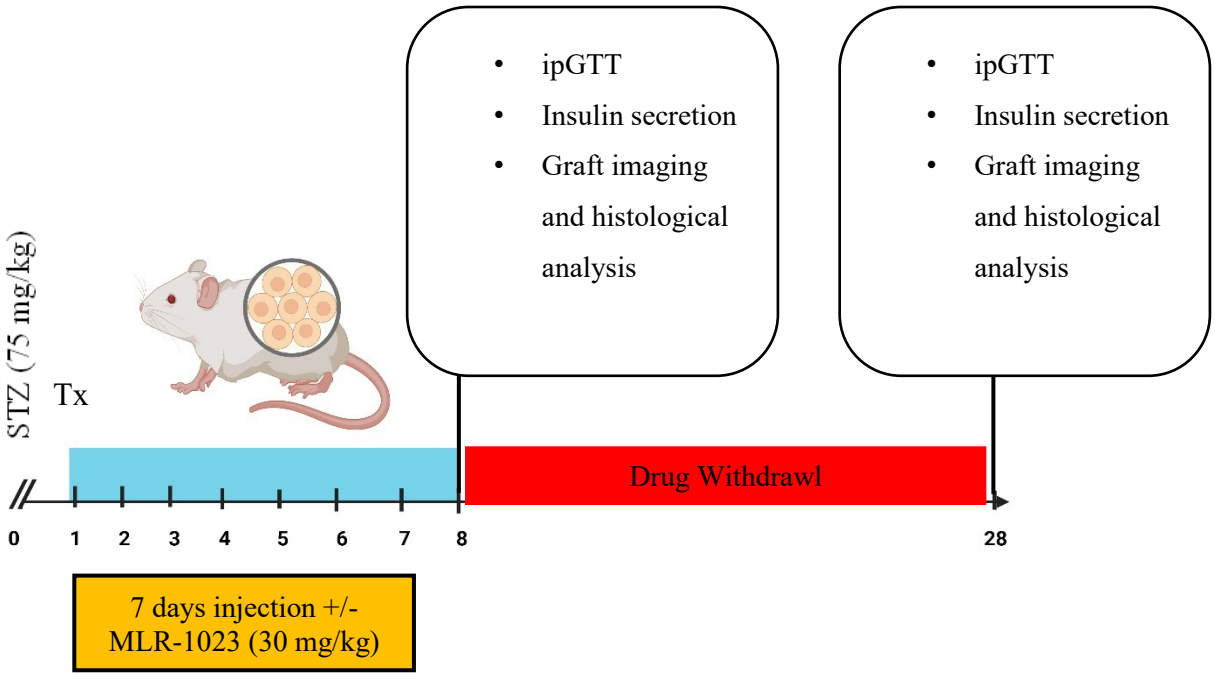


Figure 3.1: Study design. Islet preparations were incubated in MLR-1023-containing medium or vehicle-containing medium for 1 hour before transplantation. Transplantation of 125 islets under the left kidney capsule was performed to study the renal subcapsular area. One week prior to transplantation, recipients were induced with STZ (75 mg/kg) and then treated with MLR-1023 (30 mg/kg) or vehicle for 7 days. Pancreas section and islet grafts were retrieved and analyzed using immunohistochemistry at 8 and 28 days.

Table 1: List of Primers

rVEGFA-For	5'-ACTTCTGGGCTCTTCTCTCT-3'
rVEGFA-Rev	5'-CCCTCTCCTCTTCCTTCTCTT-3'
rβ-Actin-For	5'-ACAGGATGCAGAAGGAGATTAC-3'
rβ-Actin-Rev	5'-ACAGTGAGGCCAGGATAGA-3'

Chapter 4: Result

4.1 A short MLR-1023 treatment was sufficient to improve glucose tolerance in islet recipient mice

Our goal was to examine whether MLR-1023 administration could improve glucose tolerance after islet engraftment in diabetic mice. To do so, we first induced diabetes in mice via STZ injection (STZ is a β-cell-specific toxin that causes β-cell destruction) and subjected diabetic mice to islet transplantation under the kidney capsule. We selected a marginal dose of 125 islets, reasoning that this would allow us to detect any beneficial effects of MLR-1023 more easily.

Thus, (3-4 m.o.s old male BALB/c) mice received STZ to induce diabetes at day 0. Subsequently, diabetic mice were subjected to islet transplantation surgery and categorized into two distinct groups: The first group, designated as the MLR-1023-treated group, received a dose of 30 mg/kg MLR-1023 for 7 consecutive days following transplantation; The second group, referred to as the vehicle-treated group, was administered with the vehicle alone for 7 consecutive days. Finally, a third group, designated as the naïve (healthy control) group was included for comparison. The naïve group is comprised of mice not subjected to STZ injection nor transplantation.

All groups were subjected to an intraperitoneal glucose tolerance test (ipGTT) at day 8 (Fig 4.1). As expected, the vehicle group showed severely impaired glucose tolerance compared to the non-diabetic control (naïve) group. This demonstrates the effectiveness of our STZ treatment in inducing diabetes. Also, our results show that MLR-1023 treatment improved glucose tolerance in islet recipients. Specifically, the peak plasma glucose concentration in the vehicle group was higher than 20 mM, while the MLR-1023-treated and naïve groups remained similar and below 20 mM at 30 minutes. However, the differences were not significant. A significant improvement in

glucose tolerance was observed in mice treated with MLR-1023 at 90 min compared to vehicles (Fig 4.1A). We did not observe any significant statistical differences in blood glucose levels at 30, 60, or 120 minutes, despite noticing a tendency towards decreased blood glucose in mice treated with MLR-1023 compared to vehicles only. Interestingly, the area under the curve (AUC) analysis of blood glucose was significantly decreased in MLR-1023-treated mice compared to the vehicle group (Fig 4.1B). These observations suggest that a short treatment with MLR-1023 could be sufficient to ameliorate glucose tolerance and thus improve the outcome of islet transplantation.

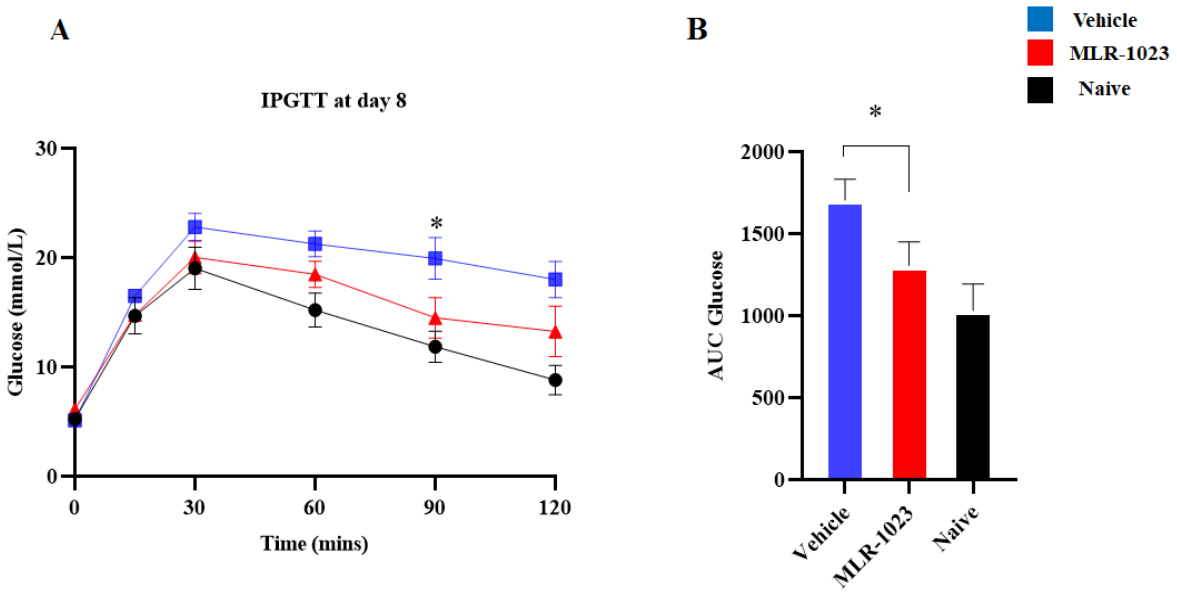


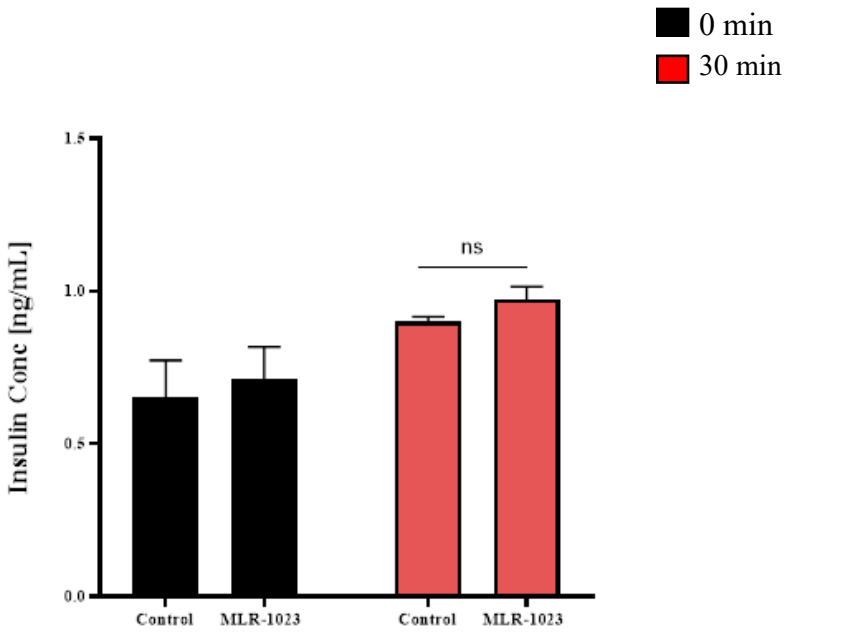
Figure 4.1: Short course MLR-1023 therapy improved glucose tolerance. A) (▲) MLR-1023 treated animals (n=5) maintained a tendency toward decreased blood glucose compared with vehicle controls (n=5) (■); (●) naive (n=7). B) AUC analysis of blood glucose revealed that blood glucose levels were significantly decreased by MLR-1023 compared with vehicle. All results are represented as (Two-way ANOVA; Bonferroni test, Means +/- SEM; *, $p < 0.05$).

The next step was to determine whether the enhanced efficacy of islet transplantation in MLR-1023-treated animals was associated with increased circulating insulin levels. We measured

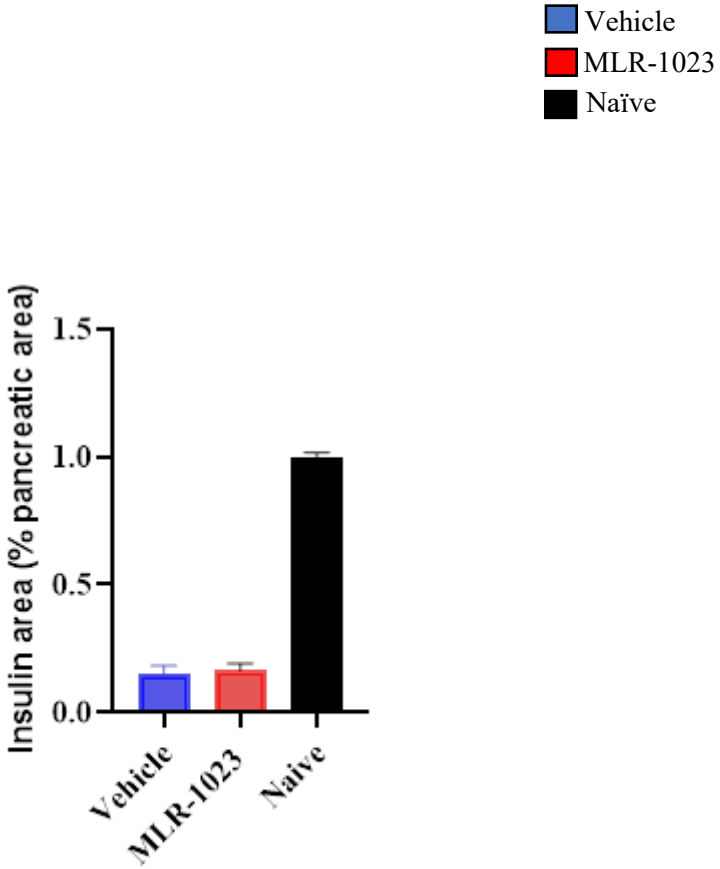
the serum insulin concentrations of mice in the vehicle and MLR-1023 groups using blood samples obtained 30 minutes after glucose injection during the 8 days. (Fig 4.2A). Surprisingly, we did not observe any additional increase in plasma insulin concentrations with MLR-1023, despite the glucose-lowering effect observed compared with vehicle-treated mice.

To better characterize the effectiveness of our STZ treatment, we performed a morphological assessment of pancreatic tissues at day 8. In brief, we harvested pancreases from each group to accurately measure β-cell mass and estimate β-cell loss. Our results revealed that STZ treatment caused a >85% reduction in β-cell area (Fig 4.2 B, C) compared to naïve controls. This severe reduction in β-cell mass mimics the pathophysiology of T1D. It is also worth mentioning that a loss of 65% of β-cell mass has been shown to cause diabetes (Meier et al., 2012). Our morphological analysis (Fig 4.2D) also shows that islet architecture is affected following STZ treatment, with islets being fragmented i.e. β-cells are dispersed. Finally, it can also be observed that MLR-1023 treatment did not change endogenous β-cell mass, suggesting that the improvement in glucose tolerance is probably not due to an effect in residual/surviving β-cells.

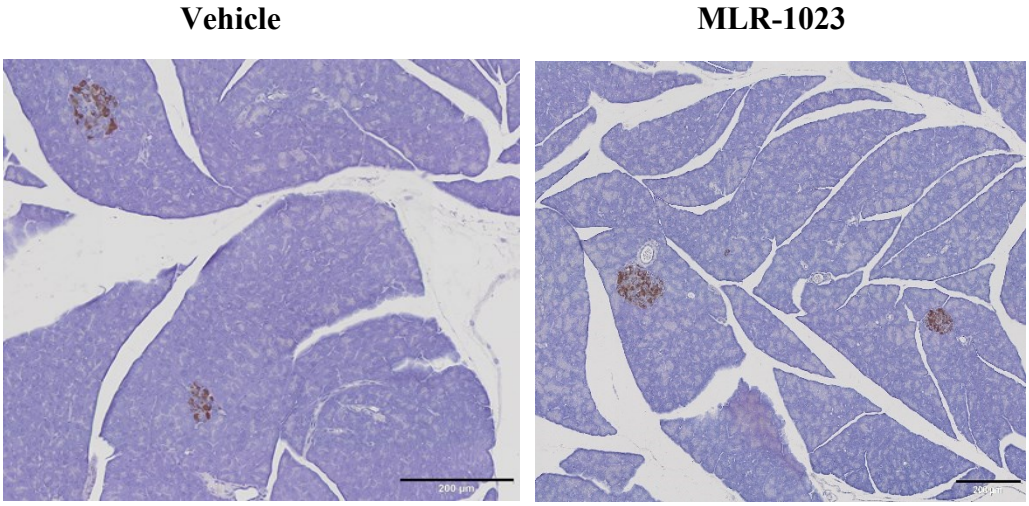
A


0 min
30 min
Insulin Conc [ng/mL]
1.5
1.0
0.5
0.0
ns
Control
MLR-1023
Control
MLR-1023

B


Vehicle
MLR-1023
Naïve
Insulin area (% pancreatic area)
1.5
1.0
0.5
0.0
Vehicle
MLR-1023
Naive

C



D

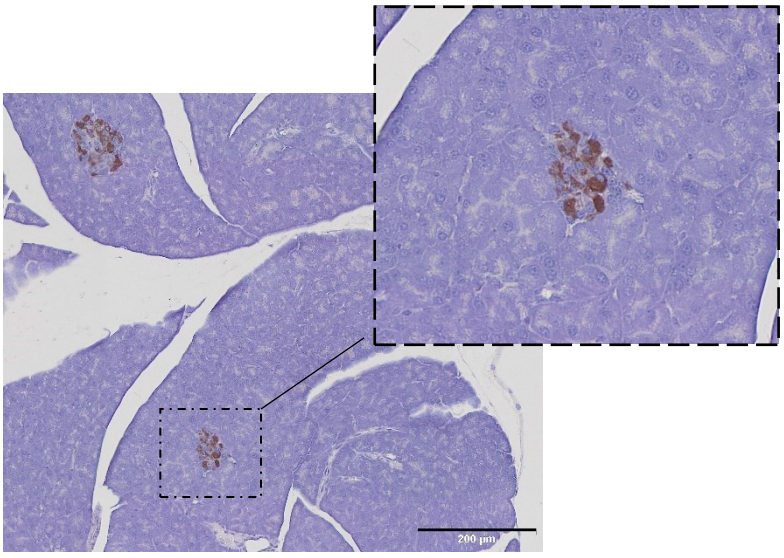


Figure 4.2: MLR-1023 improved islet transplant efficacy without increasing circulating insulin. A) Plasma

insulin levels 0 and 30 minutes after glucose injection at day 8. All results are represented as (Two-way ANOVA; Bonferroni test, Means +/- SEM; *, $p < 0.05$) B) Quantification of cross-sectional β-cell areas. C) Images show representative insulin immunostaining in the vehicle and MLR-1023 pancreata. D) The structure of islets is affected by STZ treatment. n=4 for each group. All results are represented as One-Way ANOVA,; Means +/- SEM; *, $p < 0.05$)

4.2 MLR-1023 induces β-cell proliferation in islet grafts

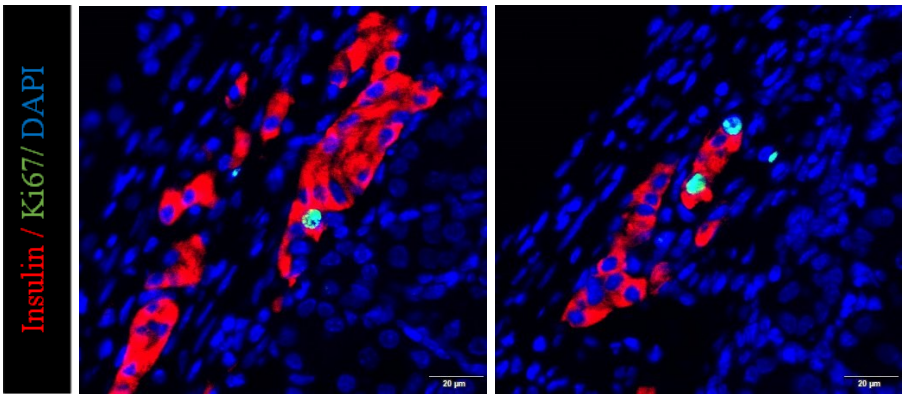
To investigate the beneficial effects of MLR-1023 on islet grafts, we harvested the islet graft at day 8 to perform morphological assessments. We specifically measured β-cell proliferation and β-cell mass in islet recipients at day 8. We performed co-immunostaining with insulin and Ki67 antibody to identify proliferating β-cells in the grafts. Interestingly, we observed an increase in the number of proliferating (Ki67-positive) β-cells in the MLR-1023 group compared to vehicle (Fig 4.3A). Indeed, β-cell proliferation increased ~ 4-fold in MLR-1023 treated versus vehicle animals (Fig 4.3B) suggesting that MLR-1023 acts as a β-cell growth factor in transplanted β-cells. It also shows that these cells retain proliferative capacities and that they could be amenable to expansion. However, our morphological study indicated that MLR-1023-induced β-cell proliferation did not translate into significant changes in β-cell mass (Figure 4.3.C, D) within 7 days of treatment.

Our laboratory had previously conducted studies on the anti-apoptotic effect of MLR-1023 *in vitro*. In these studies, we observed that MLR-1023 significantly reduced apoptosis in INS-1 cells as well as isolated human islets (unpublished data). These findings prompted us to investigate whether MLR-1023 could increase the survival of islet grafts. This was performed using an anti-active caspase-3 antibody, which would label cells undergoing apoptosis. However, we rarely detected apoptotic cells in the islet grafts at day 8. Apoptotic cells were too few to perform

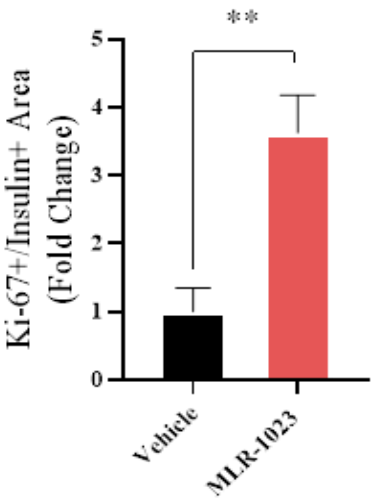
meaningful statistical analyses. It is possible that the burst of cell death occurs earlier than day 8 or that β-cells die by other mechanisms such as necrosis.

A

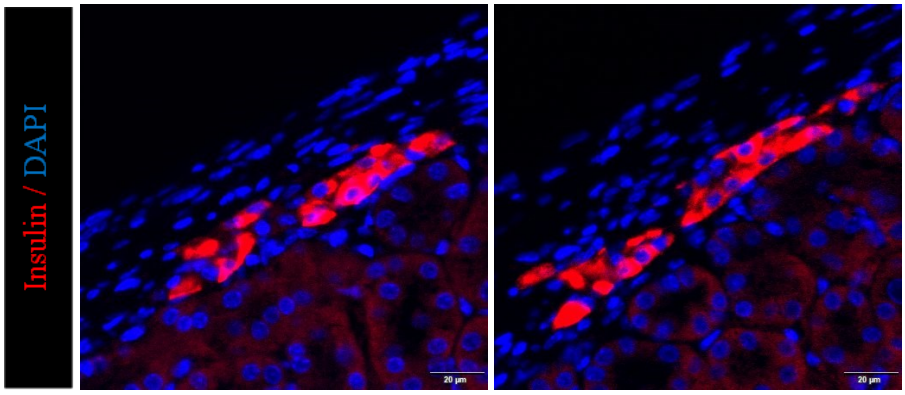
Vehicle **MLR-1023**



B



C



D

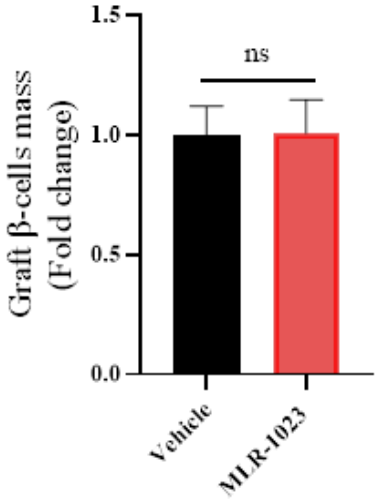


Figure 4.3: MLR-1023 significantly promoted β-cell replication in engrafted islets at day 8. A) Images show representative double immunofluorescence staining for insulin (red) and Ki67 (green) in vehicle and MLR-1023-treated mice. B) β-cell proliferation was measured by counting the number of Ki67 positive nuclei. Results are represented as fold-change over vehicle mice C) Images show representative insulin immunostaining in vehicle and MLR-1023-treated mice. D) Quantification of β-cell area of the engrafted islet. n=4 for each group. All results are represented as (t-Test, unpaired; Means +/- SEM; *, $p < 0.05$)

4.3 MLR-1023 accelerated the revascularization of islet grafts

Islet revascularization is critical for islet engraftment and function. To gain insight into the mechanisms by which MLR-1023 could improve the outcome of islet transplantation, we sought to explore a potential action of MLR-1023 on revascularization. Our reasoning is that MLR-1023 could enhance engrafted β-cell mass and function via acceleration of islet vascularization and microvascular remodelling. To do this, we retrieved islet grafts from both vehicle and MLR-1023-treated mice and performed immunofluorescence staining with insulin and CD31 antibodies to identify endothelial cells or vascular structures in engrafted islets (Fig 4.4 A). Although we observed an increase in the number of CD31-positive cells in the insulin-positive area of the engrafted islets in the MLR-1023 treated compared to the vehicle group, this increase was not statistically significant (Fig 4.4 B). Also, this finding suggests that MLR-1023 may positively affect islet vascularization, potentially contributing to the observed improvements in glucose homeostasis and β-cell replication.

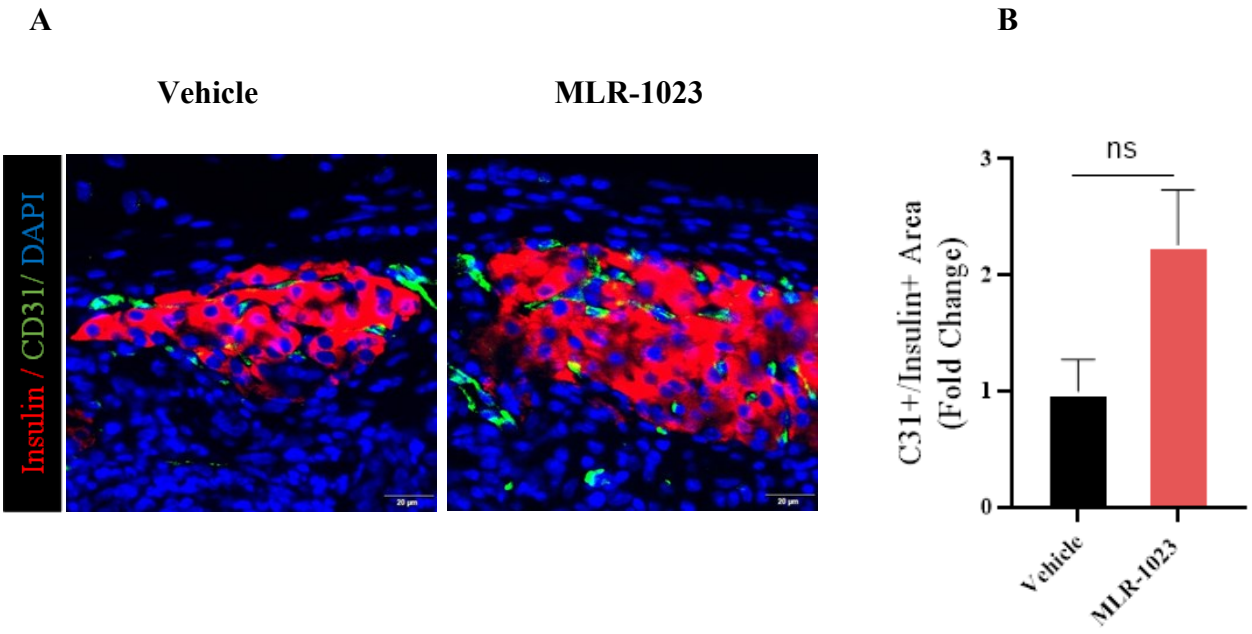


Figure 4.4: Short-term therapy with MLR-1023 accelerated remodelling of new vasculature. A) Images show representative double immunofluorescence staining for insulin (red) and CD31(green) in the vehicle and MLR-1023-treated mice. B) revascularization was measured by counting the number of CD31 positive nuclei in insulin-positive area. Results are represented as fold-change over vehicle mice. n=4 for each group. All results are represented as (t-Test, unpaired; Means +/- SEM; *, $p < 0.05$).

4.4 Some of the effects of MLR-1023 are long-lasting after drug withdrawal, but the improvement in glucose tolerance is not persistent

Next, we sought to test whether the effects of our short (7 days) treatment with MLR-1023 are permanent/persistent. We thus repeated our experiments at day 28, which corresponds to 21 days after drug withdrawal. We hypothesized that MLR-1023-induced neovascularization at day 8 would confer a significant advantage and translate into long-term improvements in islet graft function.

We first evaluated the effects of MLR-1023 on islet vascularization at day 28 to test whether the changes would persist 21 days after drug withdrawal. To do so, we repeated our

immunofluorescence staining with insulin and CD31 antibodies on islet grafts (Fig 4.5A). Our findings suggest that MLR-1023-induced revascularization of islet grafts is still observable at day 28. The amount of CD31 detected was approximately 2-fold greater in MLR-1023-treated mice vs vehicle-treated animals (Fig 4.5 B).

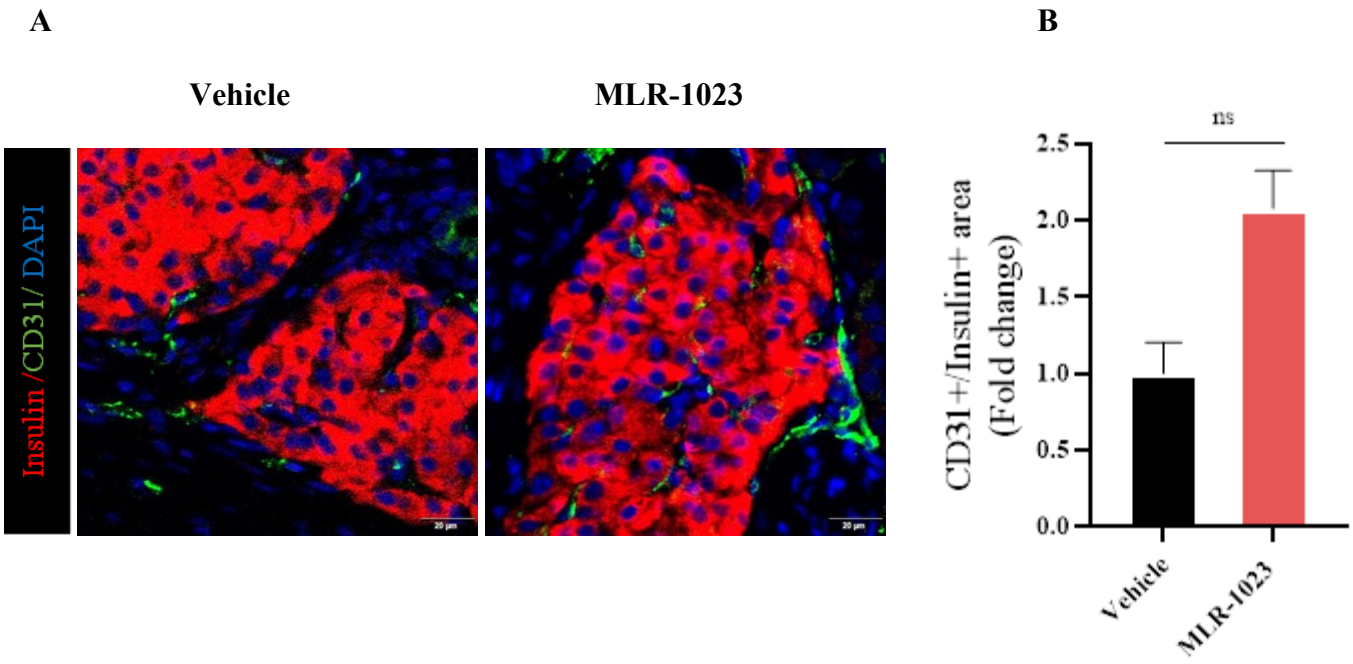


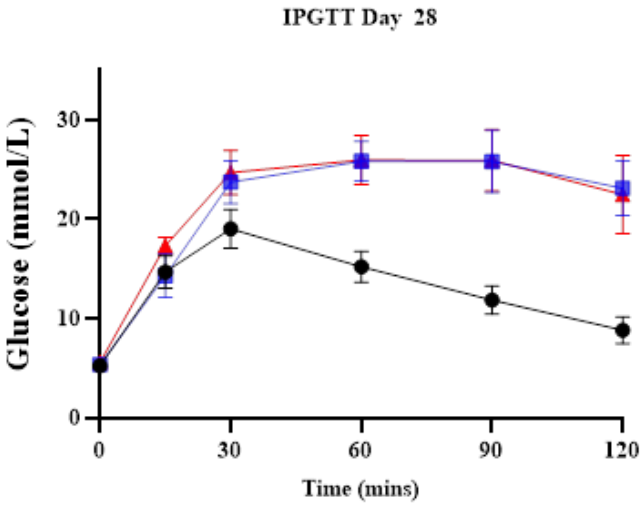
Figure 4.5: Long-Term effects of short-course MLR-1023 treatment on revascularization. A) Images show representative double immunofluorescence staining for insulin (red) and CD31(green) immunostaining in the vehicle and MLR-1023-treated mice. B) revascularization was measured by counting the number of CD31 positive nuclei in insulin-positive area. Results are represented as fold-change over vehicle mice. n=6 for each group. All results are represented as (t-Test, unpaired; Means +/- SEM; *, $p < 0.05$)

Next, we performed ipGTT at day 28 to test whether the improvement in glucose tolerance was still observable 21 days after drug withdrawal. Our results show that the changes in glucose tolerance were not persistent. Indeed, the MLR-1023-treated group displayed worsened glucose tolerance compared to day 8. Glucose values for the MLR-1023 and vehicle groups were

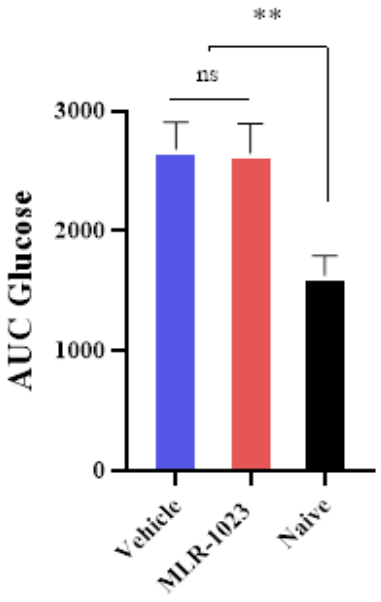
indistinguishable. In both groups, glucose concentrations failed to return to normal levels even after 120 minutes (Fig 4.6 A). Consistently, the AUC values showed no differences between the MLR-1023 and vehicle groups, both being higher than the naïve group (Fig 4.6 B).

Moreover, we measured insulin levels 30 minutes after glucose injection to assess islet function. No differences were observed between the MLR-1023 and vehicle groups. The median serum insulin concentration of mice in the vehicle group was 2.49 ng/ml, which is similar to that in the MLR-1023 group (2.36 ng/ml) (Fig 4.6 C).

A



B



C

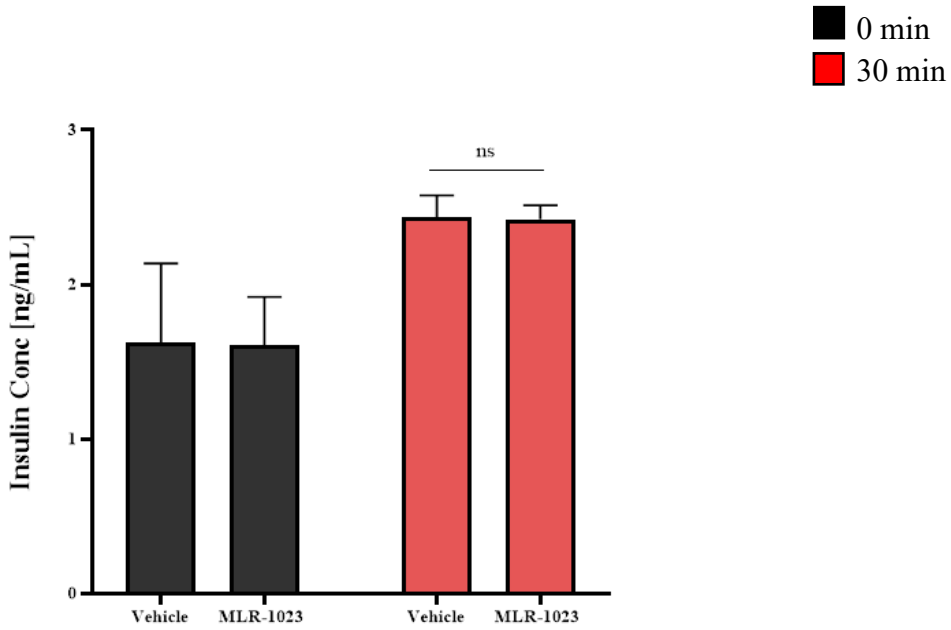
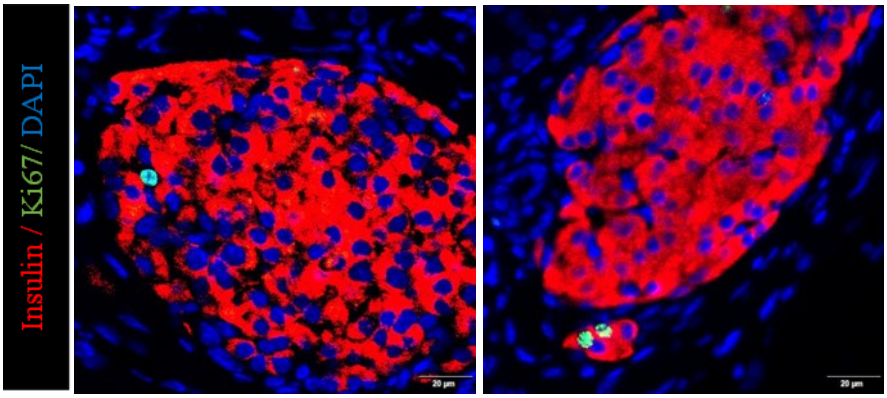


Figure 4.6: Short-course MLR-1023 treatment fails to maintain prolonged normoglycemia. A) The results showed severely impaired glucose tolerance in both the vehicle (■; n=5) and MLR-1023-treated (▲; n=5) mice compared with naïve groups (●; n=7). B) MLR-1023-treated and vehicle animals with 125 islet transplantation exhibited a significant increase in AUC at 28 days compared with naïve group. C) Plasma insulin levels 0 and 30 minutes after glucose injection at day 28. All results are represented as (Two-way ANOVA; Bonferroni test, Means +/- SEM; *, $p < 0.05$.)

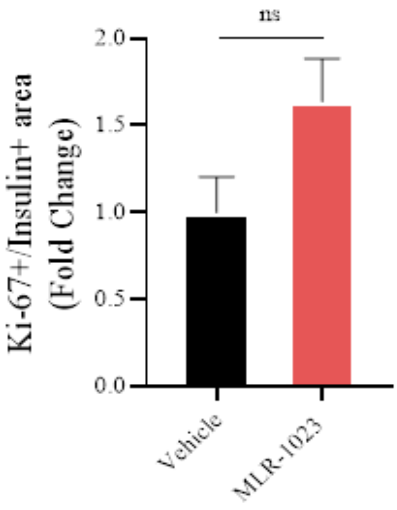
Together, these findings indicate that a short treatment with MLR-1023 did not have a long-lasting impact on glucose homeostasis and that its effects dissipated after treatment termination. We nonetheless pursued our investigation and examined the effects of MLR-1023 on engrafted islets at day 28 to detect potential changes in β-cell proliferation and/or mass. We thus harvested grafts from mice in vehicle and MLR-treated groups for immunohistochemical analysis. Specifically, we performed staining with insulin and Ki67 antibody to identify proliferating cells in the β-cell area of the engrafted islets (Fig 4.7 A). We detected a non-significant increase in β-cell proliferation in the MLR-1023-treated group compared to the vehicle group. In fact, β-cell

proliferation was approximate ~ 1.5-fold greater in MLR-1023-treated animals without achieving statistical significance (Fig 4.7 B). Our morphological study indicated that β-cell mass was similar between both groups, suggesting that MLR-1023 treatment did not affect the number of β-cells at day 28 (Fig 4.7 C, D).

A



B



C D

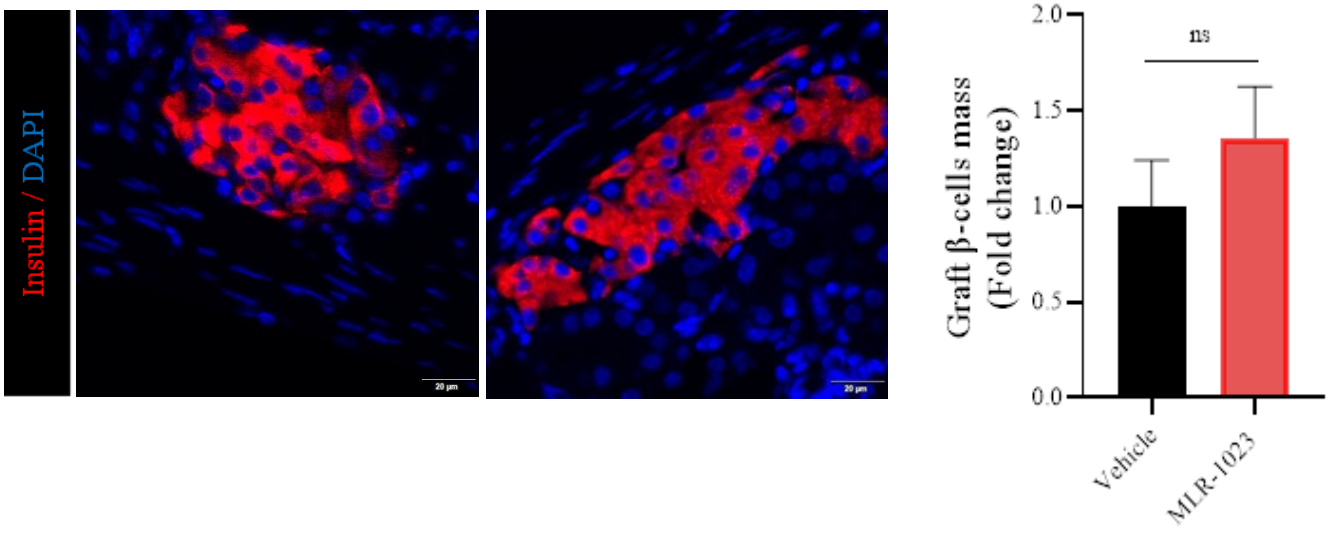


Figure 4.7: Long-Term effects of short-course MLR-1023 treatment on β-cell replication. A) Images show representative double immunofluorescence staining for inulin (red) and Ki67(green) in vehicle and MLR-1023-treated mice. B) β-cell proliferation was measured by counting the number of Ki67 positive nuclei. Results are represented as fold-change over vehicle mice C) Images show representative insulin immunostaining in vehicle and MLR-1023-treated mice. D) Quantification of β-cell area of the engrafted islet. n=6 for each group. All results are represented as (t-Test, unpaired; Means +/- SEM; *, $p < 0.05$.)

4.4 MLR-1023 action on islet vasculature is independent on islet VEGF-A

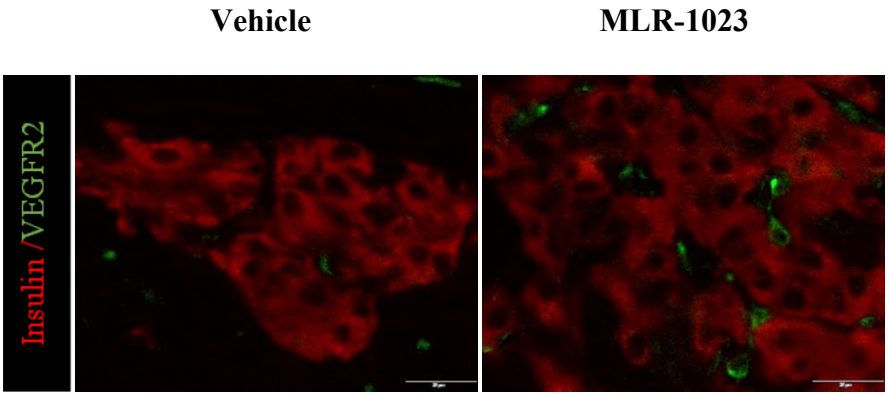
We next sought to gain insight into the mechanisms by which MLR-1023 could stimulate revascularization of islet grafts. Because the bulk of literature demonstrates the importance of VEGF in this process, we first evaluated expression of the VEGF receptor (VEGFR2) in engrafted islets via immunostaining. In brief, we stained harvested tissues at day 8 with insulin and VEGFR2 antibodies to identify intra-islet endothelial cells in the grafts (Fig 4.8 A). Our findings revealed a non-significant increase in VEGFR2-positive cells in the MLR-1023 group compared to our vehicle control group (Fig 4.8 A). In fact, the number of VEGFR2- positive cells was

approximately ~ 1.5-fold higher in MLR-1023-treated than in vehicle-treated animals but again, did not achieve significance (Fig 4.8 B).

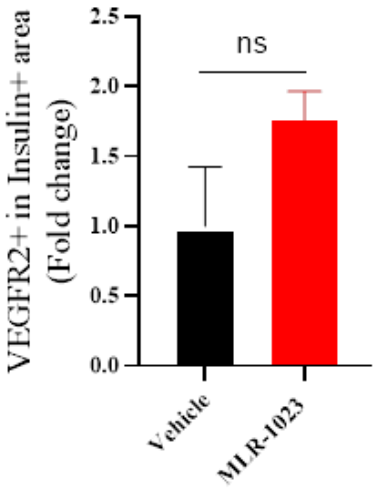
To directly test the hypothesis that MLR-1023 could promote islet revascularization via VEGF, we measured VEGF-A expression and secretion in a β-cell line. Our reasoning was that MLR-1023 could directly act on β-cells and stimulate the expression and/or secretion of VEGF-A to promote vascularization. We thus exposed INS-1 cells to 100 nM MLR-1023 or vehicle for different time points ranging from 24 to 72 hours. We then measured the expression of VEGFA by qPCR and its secretion in the culture medium by ELISA. Unexpectedly, we observed that MLR-1023 treatment did not increase the expression level of VEGF-A in INS-1 cells compared to vehicle-treated group (Fig 4.8 C). Similarly, VEGF-A secretion was mostly unaltered by MLR-1023 (Fig 4.8 D). In fact, the secretion of VEGF-A was even found to be decreased at 24h. These results suggest that MLR-1023-induced vascularization of the grafts are not mediated by β-cell-derived VEGF-A and must involve a different mechanism.

Altogether, our results suggest that MLR-1023 improves the outcome of islet transplantation. MLR-1023 was found to increase β-cell proliferation and accelerate islet revascularization, which translated into improved glucose tolerance. However, not all of these effects were long-lasting, and this warrants further studies with longer treatment duration.

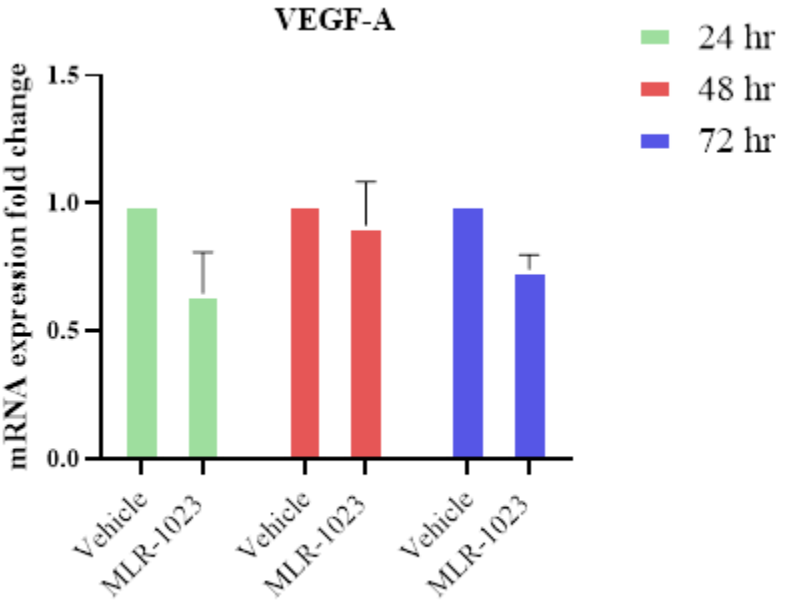
A


Vehicle
MLR-1023
Insulin /VEGFR2

B


VEGFR2+ in Insulin+ area
(Fold change)
2.5
2.0
1.5
1.0
0.5
0.0
ns
Vehicle
MLR-1023

C


VEGF-A
mRNA expression fold change
1.5
1.0
0.5
0.0
24 hr
48 hr
72 hr
Vehicle
MLR-1023
Vehicle
MLR-1023
Vehicle
MLR-1023

D

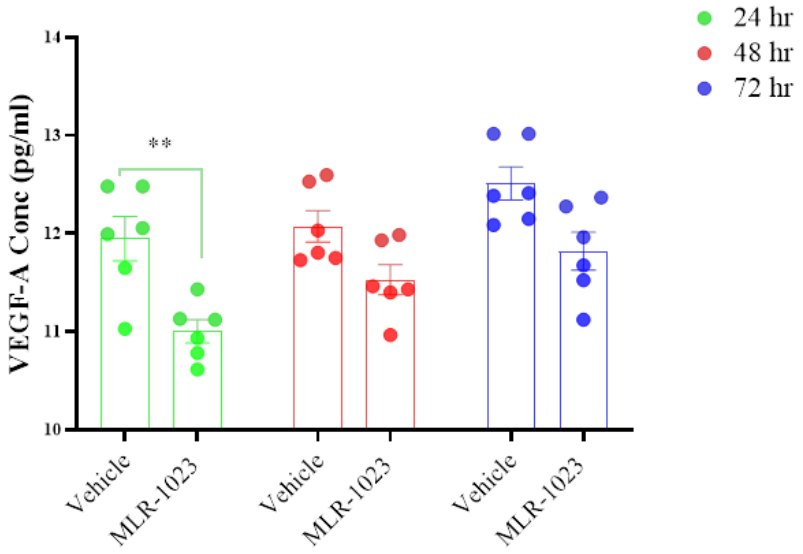


Figure 4.8: MLR-1023 does not induce VEGF-A in β-cell lines. A) Images show representative double immunofluorescence staining for inulin (red) and VEGFR2 (green) in vehicle and MLR-1023-treated mice. B) Intra-islet endothelial's number was measured by counting the number of VEGFR2 positive nuclei. Results are represented as fold-change over vehicle mice. n=4 for each group. All results are represented as (t-Test, unpaired; Means +/- SEM; *, $p < 0.05$). C) VEGF-A expression was evaluated by qPCR in INS-1 cells after treatment with MLR-1023 or vehicle at 24, 48 and 72 hours (n=3). D) β-cell lines were treated with either MLR-1023 or vehicle in G2.8/SF for various time points ranging from 24, 48 and 72 hours. After 72 hours, the amount of VEGFA secretion was measured by ELISA and analyzed. The VEGFA secretion data were then normalized to the control group for each treatment. The results indicate that there was not a significant difference between the MLR-1023 treated and vehicle groups at 48 and 72 hours, while VEGFA concentration in MLR-1023 vs vehicle at the 24-hour group, there was a significant decrease. The results are presented as (Two-way ANOVA; means +/- SEM (n=3), *, $p<0.05$)

Chapter 5: Discussion

5.1 General Discussion

Islet transplantation is considered a promising treatment option for individuals with T1D. As islet transplantation becomes more common, engraftment efficacy will need to be significantly improved and cell death will have to be reduced, particularly during the acute post-transplant period.

Recently, the use of small molecules has augmented the success of islet transplantation (Bruni et al., 2018; Denroche et al., 2013; Dirice et al., 2016; Emamaullee et al., 2007). Previously, MLR-1023 has attracted considerable attention for its potential impact on the treatment of T2D (Ochman et al., 2012). As a promising therapeutic candidate for diabetes, MLR-1023 is supported by a strong rationale for use as a monotherapy. Firstly, it has been observed that the magnitude of MLR-1023's effect on reducing blood glucose levels is comparable to that of metformin. In contrast to metformin, MLR-1023 did not reduce glucose levels in fasted mice. The second advantage of MLR-1023 is its lack of weight gain, a common side effect associated with another anti-diabetic drug, rosiglitazone, which is an activator of PPARγ (Ochman et al., 2012). The anti-diabetic effects of MLR-1023 are exerted through a unique mechanism that sets it apart from metformin and rosiglitazone. Indeed, MLR-1023 has been characterized as a direct, selective, and potent activator of LYN kinase (Ochman et al., 2012). Finally, it has been demonstrated that MLR-1023 can prevent the loss of pancreatic β-cells. Collectively, these findings suggest that MLR-1023 may be a promising monotherapy for diabetes, and its unique mechanism of action warrants further study. However, its potential effect of biological action on pancreatic β-cells remains largely unknown.

Our recent research effort has provided further insights into the potential therapeutic benefits of MLR-1023. Unpublished data from the Buteau lab have characterized MLR-1023 as a growth factor that enhances β-cell proliferation, survival and overall "health". Specifically, our preliminary findings indicated that MLR-1023 had an anti-apoptotic effect on β-cells *in vitro* in the presence of cytokines and toxic levels of glucose and/or fatty acids. *In vivo*, MLR-1023 expanded β-cell mass in animal models of both type 1 and type 2 diabetes, an effect that resulted from improved survival and proliferation.

Previous research (Denroche et al., 2013) has indicated that the transplantation of a marginal mass of 200 islets under the kidney capsule was inefficient in reversing hyperglycemia in STZ-induced diabetic mice. We reasoned that the beneficial actions of MLR-1023 on β-cell mass and function could perhaps confer a significant advantage and improve the outcome of islet transplantation in this setting. We thus evaluated the efficacy of MLR-1023 in enhancing the success of suboptimal islet transplantation, by transplanting 125 islets into diabetic mice subsequently treated with either MLR-1023 or vehicle. In the current study, treatment with transplantation of 125 islets alone, did not produce significant reductions in blood glucose levels. Although the synergic islet doses administered were deemed insufficient to alleviate hyperglycemia when administered alone, co-administration of MLR-1023 with islet transplantation led to a remarkably improved glucose tolerance during ipGTT at day 8.

To explore the potential mechanisms by which MLR-1023 could improve glucose tolerance in islet recipients, we harvested the graft and performed morphological assessments of islets. It was found that MLR-1023 stimulated β-cell proliferation in the grafts. However, these changes did not translate into a significant increase in β-cell area. We posit this could be due to different factors: 1) high inter-individual variations, 2) the difficulty in accurately measuring β-

cell mass in a marginal islet graft, and 3) the short treatment may not be long enough to allow for a significant increase in β-cell mass. Similarly, MLR-1023 treatment did not result in any significant increase in circulating insulin levels in recipients of islet transplantation. We postulated that the lack of increase in circulating insulin levels following administration of MLR-1023 in conjunction with islet transplantation may be attributed to the possibility that MLR-1023 does not directly stimulate insulin secretion. Nevertheless, it is important to point out that we did not examine the total insulin content of the graft, which may have contributed to our results. The results of our study support the notion that MLR-1023 leads to an immediate reduction in glucose levels, possibly by increasing glucose uptake without elevating insulin levels (Ochman et al., 2012; Saporito et al., 2012). The effect may be exerted by a variety of mechanisms, one of which was proposed previously through direct tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) in adipocytes (Ochman et al., 2012).

We observed that MLR-1023 significantly increased β-cell proliferation in engrafted islets in mice at day 8. Specifically, we observed an increase of nearly four-fold (~2%) in β-cells entering the cell cycle following MLR-1023 therapy as compared to vehicle, as determined by staining for Ki67, a marker of a proliferative cell. Furthermore, MLR-1023 has been shown to induce β-cell proliferation in humans by approximately ~ 2% *in vitro* setting, which is in contrast to GLPR agonists that only stimulate rodent β-cell proliferation and fail to stimulate β-cell replication in human β-cells (unpublished data, (Ackeifi et al., 2020; Parnaud et al., 2008)). Our findings are consistent with previous research indicating that DYRK1A inhibitors can induce β-cell proliferation in the range of 1-3% (Ackeifi et al., 2020). It has been suggested previously (Ackeifi et al., 2020) that any molecule that activates cAMP signalling in β-cells could potentially contribute to β-cell proliferation when coupled with a DYRK1A inhibitor (Ackeifi et al., 2020).

Our findings confirm that MLR-1023 can be used to induce β-cell proliferation, demonstrating an approximately 2% increase in β-cell proliferation *in vitro* and a similar increase in engrafted islets after treatment. There is a need for further research to clarify whether MLR-1023 inhibits DYRK1A or activates EPAC2 and PKA. To fully comprehend the therapeutic potential of MLR-1023, more research will be necessary. This will lead to the development of more targeted therapies for individuals who suffer from diabetes. Although our findings indicate that MLR-1023 has the potential to promote β-cell replication, we did not observe any changes in β-cell mass *in vivo*. we postulate several reasons why β-cell mass did not change despite the increase in Ki67. There is a possibility that a 7-day treatment period wasn't sufficient to cause significant changes in β-cell mass. It takes a considerable amount of time for changes in β-cell mass to manifest because β-cell turnover and proliferation are complex processes. It is also possible that the increased proliferation observed following treatment with MLR-1023 may have been counterbalanced by increased apoptosis, leaving the β-cell mass unchanged. There is also the possibility that there was some experimental error or variability among the treatment groups which may have influenced the results. In addition, it is important to note that these positive effects on β-cell proliferation and glucose homeostasis were observed during experiments lasting for 8 days; further research will be required to determine whether or how long these effects will persist.

The successful engraftment of islets requires a quick reestablishment of a functional vasculature, as islet grafts are avascular after isolation and must rely on the diffusion of oxygen and nutrients from the surrounding microenvironment for their maintenance (Narayanan et al., 2017). Endothelial cells play a critical role in regulating angiogenic functions during the formation of blood vessels, highlighting the importance of this process for the successful engraftment of islets (Narayanan et al., 2017).

We observed that MLR-1023 accelerated neovascularization in engrafted islets in mice at day 8. The results of our study indicated that MLR-1023 therapy led to a nearly two-fold increase in revascularization when compared to vehicle, as determined by staining for CD31, a marker of new vessels at day 8. It was found that the islet grafts had an adequately increased CD31-stained area and greater microvasculature. These findings were correlated with significantly improved blood glucose profiles in diabetic recipients treated with MLR-1023 after ipGTT at day 8. The utilization of small molecule compounds in islet transplantation has been studied previously (Figueiredo et al., 2019; Lee et al., 2018; Staels et al., 2018). Previous studies have demonstrated that the transfection of VEGF-A mRNA (Staels et al., 2018) into islets or treatment with resveratrol (Lee et al., 2018), as well as mice lacking the protein tyrosine phosphatase 1B (PTP1B) in their islets (Figueiredo et al., 2019), can improve islet revascularization by regulating VEGF-A production and ultimately enhance the success of islet transplantation. Our study is consistent with these previous findings, as we observed that MLR-1023 accelerates the revascularization of engrafted islets, however, through a distinct mechanism. Since we observed an adequately increased CD31-stained area and greater microvasculature in the islet grafts up to day 28. We postulate that MLR-1023 enhanced islet revascularization by regulating VEGF-A production. To investigate this hypothesis, we measured the expression level of VEGF-A in an *in vitro* setting. Surprisingly, we observed that MLR-1023 did not increase VEGF-A secretion. Instead, treatment with MLR-1023 resulted in a significant decrease in VEGF-A secretion after 24 hours in cells receiving an MLR-1023, and a non-significant decrease after 48 and 72 hours, suggesting impairment of VEGF-A secretion.

Several previous studies have reported LYN kinase expression in adult endothelial cells (Han et al., 2013). Additionally, it has been observed that LYN kinase is expressed in both Human

Pulmonary Artery Endothelial Cells (HPAECs) and Human Umbilical Vein Endothelial Cells (HUVECs), indicating the significant role of LYN in the function of these cells (Han et al., 2013). LYN kinase has been demonstrated to have a unique role in strengthening endothelial junctions and preventing an increase in vascular permeability, unlike other members of the Src family of kinases (SFKs) (Han et al., 2013). These mice were genetically engineered to lack only the SFK LYNgene, which results in a higher mortality rate when challenged with VEGF. LYN-deficient mice also displayed discontinuities in VE-cadherin distribution and inter-endothelial junctional gaps (Han et al., 2013). In addition, LYN deficiency reduced phosphorylation of Focal adhesion kinase (FAK) at tyrosine. The knockdown of FAK demonstrated that it was downstream of LYN and was involved in maintaining the integrity of the endothelial barrier through biochemical and cytoskeletal pathways. These findings suggest that LYN kinase plays an important role in regulating FAK activity in endothelial cells, as FAK is critical for maintaining the integrity of the endothelial barrier and the formation of VE-Cadherin at the endothelial borders, including adherent junction (AJ) (Han et al., 2013). We speculate that MLR-1023's ability to activate LYN kinase and strengthen endothelial junctions may be responsible for its effects on revascularization *in vivo*, which may explain the observed increase in CD31 staining in harvested grafts. Consequently, endothelial barrier function may be enhanced, and VE-Cadherin may be formed at the endothelial borders, which promotes survival of endothelial cells and inhibits apoptosis. VEGFR2, one of the main receptors for VEGFA, has also been reported to interact with and regulate the activity of VE-cadherin (Lampugnani & Dejana, 2007). Through this interaction, the permeability of the endothelial barrier can be modulated and the survival of the endothelial cells can also be affected (Giannotta et al., 2013). Consequently, it may be that the LYN kinase-mediated effects of MLR-1023 on the formation and function of VE-cadherin could also have an impact on VEGFR2 and

its downstream signalling pathways. The absence of an increase in VEGF-A observed in β-cell lines *in vitro* may be due to differences in the microenvironment and signalling pathways between *in vivo* and *in vitro* environments. There may be other types of cells and extracellular matrix *in vivo* that contribute to the observed revascularization effect of MLR-1023. Furthermore, cellular responses and signalling pathways in isolated β-cells *in vitro* may differ from those in intact islets *in vivo*. There may be further research required to elucidate the mechanisms involved in the observed effects of MLR-1023 on revascularization *in vivo* and to explain the lack of effect observed *in vitro*.

5.2 Conclusion

Islet transplantation is an effective treatment option for individuals suffering from T1D, however, engraftment efficacy and cell death must be reduced, especially in the acute phase following transplantation. The blood glucose-lowering effects of MLR-1023, the lack of weight gain, and the ability to prevent pancreatic β-cell loss have made it a potential therapeutic candidate for diabetes. Our recent studies have shed light on the potential benefits of short-term MLR-1023 therapy, including its promotion of β-cell proliferation, enhancement of β-cell health, and acceleration of neovascularization in engrafted islets at day 8. Even though MLR-1023 therapy increased revascularization nearly twofold at days 8 and 28, it did not increase VEGF-A secretion in β-cells *in vitro*, and the lack of an increase in circulating insulin levels following the administration of MLR-1023 along with islet transplantation may be attributed to the possibility that MLR-1023 may not directly stimulate insulin release. MLR-1023 may also affect revascularization *in vivo* because of its ability to activate LYN kinase and strengthen endothelial junctions. Further research is required to explain the lack of effect observed *in vitro* and the

mechanisms involved in the observed effects of MLR-1023 on revascularization *in vivo*. Overall, MLR-1023's unique mechanism of action and potential therapeutic benefit warrant further investigation as a monotherapy or in combination with other therapies.

5.3 Limitations

1) The study employed a brief 7-day treatment period with MLR-1023. The long-term effects and sustainability of the observed improvements in graft survival, revascularization, and glucose tolerance remain uncertain. Further investigation is needed to determine the durability of these effects.

2) Evaluation of insulin secretion was limited to specific time points and did not encompass a comprehensive assessment throughout the study duration.

3) One limitation of the study is the exclusive use of male BALB/c mice, which may limit the generalizability of the findings to diverse populations. Future research should include female mice to evaluate potential sex-specific differences in treatment outcomes. Incorporating both sexes would provide a more comprehensive understanding of MLR-1023's efficacy in islet transplantation.

4) Employing different transplantation models with varying islet masses would enable a more thorough exploration of MLR-1023's effects.

5.4 Future work

1) To determine the long-term efficacy of MLR-1023 as a therapeutic option for augmenting the success of islet transplantation, future studies could involve treating STZ mice

with either vehicle or MLR-1023 for 4-6 weeks using a subcutaneously implanted pump and evaluating its effects on engrafted islets.

2) To determine the effect of MLR-1023 as an activator of LYN on human islets, future studies could be conducted using a suboptimal dose of human islets transplanted under the kidney capsule of STZ mice.

3) One hypothesis is that the activation of LYN by MLR-1023 may enhance the survival and function of engrafted islets by modulating the immune response and suppressing immune cell activation and proliferation, potentially reducing the risk of rejection. To investigate this, future studies could involve treating mice with MLR-1023 for one week and compare the effects on engrafted islets in the anterior chamber of eye (ACE) with those in control severe combined immunodeficiency disease (SCID) mice, serving as a control.

4) To elucidate mechanisms underlying the effects of MLR-1023 on endothelial cells, studies should be conducted in both in vitro and in vivo settings to investigate the molecular processes contributing to endothelial cell function and survival. Integrin expression can be measured using immunofluorescence staining, while Endothelin-1 levels can be measured with ELISA or real-time PCR. Laminin levels can be quantified with ELISA or visualized with immunofluorescence staining, and V-cadherin expression can be visualized with immunofluorescence staining or measured with Western blotting.

REFERENCES

Ackeifi, C., Wang, P., Karakose, E., Manning Fox, J. E., Gonzalez, B. J., Liu, H., Wilson, J., Swartz, E., Berrouet, C., Li, Y., Kumar, K., MacDonald, P. E., Sanchez, R., Thorens, B., DeVita, R., Homann, D., Egli, D., Scott, D. K., Garcia-Ocana, A., & Stewart, A. F. (2020). GLP-1 receptor agonists synergize with DYRK1A inhibitors to potentiate functional human beta cell regeneration. *Sci Transl Med*, *12*(530). https://doi.org/10.1126/scitranslmed.aaw9996

Aguayo-Mazzucato, C., & Bonner-Weir, S. (2018). Pancreatic beta Cell Regeneration as a Possible Therapy for Diabetes. *Cell Metab*, *27*(1), 57-67. https://doi.org/10.1016/j.cmet.2017.08.007

Akhtar, D. H., Iqbal, U., Vazquez-Montesino, L. M., Dennis, B. B., & Ahmed, A. (2019). Pathogenesis of Insulin Resistance and Atherogenic Dyslipidemia in Nonalcoholic Fatty Liver Disease. *J Clin Transl Hepatol*, *7*(4), 362-370. https://doi.org/10.14218/JCTH.2019.00028

AM James Shapiro, J. A. M. S. (2007). *Islet Transplantation and Beta Cell Replacement Therapy*. New York: Informa Healthcare.

American Diabetes, A. (2010). Diagnosis and classification of diabetes mellitus. *Diabetes Care*, *33 Suppl 1*(Suppl 1), S62-69. https://doi.org/10.2337/dc10-S062

American Diabetes, A. (2021). 2. Classification and Diagnosis of Diabetes: Standards of Medical Care in Diabetes-2021. *Diabetes Care*, *44*(Suppl 1), S15-S33. https://doi.org/10.2337/dc21-S002

Arrojo e Drigo, R., Ali, Y., Diez, J., Srinivasan, D. K., Berggren, P. O., & Boehm, B. O. (2015). New insights into the architecture of the islet of Langerhans: a focused cross-species assessment. *Diabetologia*, *58*(10), 2218-2228. https://doi.org/10.1007/s00125-015-3699-0

Ballinger, W. F., & Lacy, P. E. (1972). Transplantation of intact pancreatic islets in rats. *Surgery*, *72*(2), 175-186. https://www.ncbi.nlm.nih.gov/pubmed/4262169

Basile, G., Kulkarni, R. N., & Morgan, N. G. (2019). How, When, and Where Do Human beta-Cells Regenerate? *Curr Diab Rep*, *19*(8), 48. https://doi.org/10.1007/s11892-019-1176-8

Belgardt, B. F., & Lammert, E. (2016). DYRK1A: A Promising Drug Target for Islet Transplant-Based Diabetes Therapies. *Diabetes*, *65*(6), 1496-1498. https://doi.org/10.2337/dbi16-0013

Bensellam, M., Maxwell, E. L., Chan, J. Y., Luzuriaga, J., West, P. K., Jonas, J. C., Gunton, J. E., & Laybutt, D. R. (2016). Hypoxia reduces ER-to-Golgi protein trafficking and increases cell death by inhibiting the adaptive unfolded protein response in mouse beta cells. *Diabetologia*, *59*(7), 1492-1502. https://doi.org/10.1007/s00125-016-3947-y

Bonner-Weir, S., Aguayo-Mazzucato, C., & Weir, G. C. (2016). Dynamic development of the pancreas from birth to adulthood. *Ups J Med Sci*, *121*(2), 155-158. https://doi.org/10.3109/03009734.2016.1154906

Brandhorst, D., Brandhorst, H., Mullooly, N., Acreman, S., & Johnson, P. R. (2016). High Seeding Density Induces Local Hypoxia and Triggers a Proinflammatory Response in Isolated Human Islets. *Cell Transplant*, *25*(8), 1539-1546. https://doi.org/10.3727/096368915X689929

Bruni, A., Pepper, A. R., Pawlick, R. L., Gala-Lopez, B., Gamble, A., Kin, T., Malcolm, A. J., Jones, C., Piganelli, J. D., Crapo, J. D., & Shapiro, A. M. J. (2018). BMX-001, a novel

redox-active metalloporphyrin, improves islet function and engraftment in a murine transplant model. *Am J Transplant*, *18*(8), 1879-1889. https://doi.org/10.1111/ajt.14705
Buteau, J. (2008). GLP-1 receptor signaling: effects on pancreatic beta-cell proliferation and survival. *Diabetes Metab*, *34 Suppl 2*, S73-77. https://doi.org/10.1016/S1262-3636(08)73398-6
Buteau, J., Foisy, S., Joly, E., & Prentki, M. (2003). Glucagon-like peptide 1 induces pancreatic beta-cell proliferation via transactivation of the epidermal growth factor receptor. *Diabetes*, *52*(1), 124-132. https://doi.org/10.2337/diabetes.52.1.124
Butler, A. E., Cao-Minh, L., Galasso, R., Rizza, R. A., Corradin, A., Cobelli, C., & Butler, P. C. (2010). Adaptive changes in pancreatic beta cell fractional area and beta cell turnover in human pregnancy. *Diabetologia*, *53*(10), 2167-2176. https://doi.org/10.1007/s00125-010-1809-6
Butler, A. E., Galasso, R., Meier, J. J., Basu, R., Rizza, R. A., & Butler, P. C. (2007). Modestly increased beta cell apoptosis but no increased beta cell replication in recent-onset type 1 diabetic patients who died of diabetic ketoacidosis. *Diabetologia*, *50*(11), 2323-2331. https://doi.org/10.1007/s00125-007-0794-x
Butler, A. E., Janson, J., Bonner-Weir, S., Ritzel, R., Rizza, R. A., & Butler, P. C. (2003). Beta-cell deficit and increased beta-cell apoptosis in humans with type 2 diabetes. *Diabetes*, *52*(1), 102-110. https://doi.org/10.2337/diabetes.52.1.102
Contreras, J. L., Smyth, C. A., Eckstein, C., Bilbao, G., Thompson, J. A., Young, C. J., & Eckhoff, D. E. (2003). Peripheral mobilization of recipient bone marrow-derived endothelial progenitor cells enhances pancreatic islet revascularization and engraftment after intraportal transplantation. *Surgery*, *134*(2), 390-398. https://doi.org/10.1067/msy.2003.250
Davis, S. N., Shavers, C., Mosqueda-Garcia, R., & Costa, F. (1997). Effects of differing antecedent hypoglycemia on subsequent counterregulation in normal humans. *Diabetes*, *46*(8), 1328-1335. https://doi.org/10.2337/diab.46.8.1328
Denroche, H. C., Quong, W. L., Bruin, J. E., Tuduri, E., Asadi, A., Glavas, M. M., Fox, J. K., & Kieffer, T. J. (2013). Leptin administration enhances islet transplant performance in diabetic mice. *Diabetes*, *62*(8), 2738-2746. https://doi.org/10.2337/db12-1684
Deshpande, A. D., Harris-Hayes, M., & Schootman, M. (2008). Epidemiology of diabetes and diabetes-related complications. *Phys Ther*, *88*(11), 1254-1264. https://doi.org/10.2522/ptj.20080020
Deters, N. A., Stokes, R. A., & Gunton, J. E. (2011). Islet transplantation: factors in short-term islet survival. *Arch Immunol Ther Exp (Warsz)*, *59*(6), 421-429. https://doi.org/10.1007/s00005-011-0143-0
Dirice, E., Walpita, D., Vetere, A., Meier, B. C., Kahraman, S., Hu, J., Dancik, V., Burns, S. M., Gilbert, T. J., Olson, D. E., Clemons, P. A., Kulkarni, R. N., & Wagner, B. K. (2016). Inhibition of DYRK1A Stimulates Human beta-Cell Proliferation. *Diabetes*, *65*(6), 1660-1671. https://doi.org/10.2337/db15-1127
Downing, R. (1984). Historical review of pancreatic islet transplantation. *World J Surg*, *8*(2), 137-142. https://doi.org/10.1007/BF01655128
El Ouaamari, A., Dirice, E., Gedeon, N., Hu, J., Zhou, J. Y., Shirakawa, J., Hou, L., Goodman, J., Karampelias, C., Qiang, G., Boucher, J., Martinez, R., Gritsenko, M. A., De Jesus, D. F., Kahraman, S., Bhatt, S., Smith, R. D., Beer, H. D., Jungtrakoon, P., Gong, Y., Goldfine, A. B., Liew, C. W., Doria, A., Andersson, O., Qian, W. J., Remold-O'Donnell,

E., & Kulkarni, R. N. (2016). SerpinB1 Promotes Pancreatic beta Cell Proliferation. *Cell Metab*, *23*(1), 194-205. https://doi.org/10.1016/j.cmet.2015.12.001
Emamaullee, J. A., Stanton, L., Schur, C., & Shapiro, A. M. (2007). Caspase inhibitor therapy enhances marginal mass islet graft survival and preserves long-term function in islet transplantation. *Diabetes*, *56*(5), 1289-1298. https://doi.org/10.2337/db06-1653
Figueiredo, H., Figueroa, A. L. C., Garcia, A., Fernandez-Ruiz, R., Broca, C., Wojtusciszyn, A., Malpique, R., Gasa, R., & Gomis, R. (2019). Targeting pancreatic islet PTP1B improves islet graft revascularization and transplant outcomes. *Sci Transl Med*, *11*(497). https://doi.org/10.1126/scitranslmed.aar6294
Filippi, C. M., & von Herrath, M. G. (2008). Viral trigger for type 1 diabetes: pros and cons. *Diabetes*, *57*(11), 2863-2871. https://doi.org/10.2337/db07-1023
Folli, F., Okada, T., Perego, C., Gunton, J., Liew, C. W., Akiyama, M., D'Amico, A., La Rosa, S., Placidi, C., Lupi, R., Marchetti, P., Sesti, G., Hellerstein, M., Perego, L., & Kulkarni, R. N. (2011). Altered insulin receptor signalling and beta-cell cycle dynamics in type 2 diabetes mellitus. *PLoS One*, *6*(11), e28050. https://doi.org/10.1371/journal.pone.0028050
Fu, Z., Gilbert, E. R., & Liu, D. (2013). Regulation of insulin synthesis and secretion and pancreatic Beta-cell dysfunction in diabetes. *Curr Diabetes Rev*, *9*(1), 25-53. https://www.ncbi.nlm.nih.gov/pubmed/22974359
Garcia-Contreras, M., Tamayo-Garcia, A., Pappan, K. L., Michelotti, G. A., Stabler, C. L., Ricordi, C., & Buchwald, P. (2017). Metabolomics Study of the Effects of Inflammation, Hypoxia, and High Glucose on Isolated Human Pancreatic Islets. *J Proteome Res*, *16*(6), 2294-2306. https://doi.org/10.1021/acs.jproteome.7b00160
Giannotta, M., Trani, M., & Dejana, E. (2013). VE-cadherin and endothelial adherens junctions: active guardians of vascular integrity. *Dev Cell*, *26*(5), 441-454. https://doi.org/10.1016/j.devcel.2013.08.020
Gotoh, M., Maki, T., Kiyoizumi, T., Satomi, S., & Monaco, A. P. (1985). An improved method for isolation of mouse pancreatic islets. *Transplantation*, *40*(4), 437-438. https://doi.org/10.1097/00007890-198510000-00018
Han, J., Zhang, G., Welch, E. J., Liang, Y., Fu, J., Vogel, S. M., Lowell, C. A., Du, X., Cheresh, D. A., Malik, A. B., & Li, Z. (2013). A critical role for Lyn kinase in strengthening endothelial integrity and barrier function. *Blood*, *122*(25), 4140-4149. https://doi.org/10.1182/blood-2013-03-491423
Henquin, J. C. (2000). Triggering and amplifying pathways of regulation of insulin secretion by glucose. *Diabetes*, *49*(11), 1751-1760. https://doi.org/10.2337/diabetes.49.11.1751
Horaguchi, A., & Merrell, R. C. (1981). Preparation of viable islet cells from dogs by a new method. *Diabetes*, *30*(5), 455-458. https://doi.org/10.2337/diab.30.5.455
Kalra, S., Mukherjee, J. J., Venkataraman, S., Bantwal, G., Shaikh, S., Saboo, B., Das, A. K., & Ramachandran, A. (2013). Hypoglycemia: The neglected complication. *Indian J Endocrinol Metab*, *17*(5), 819-834. https://doi.org/10.4103/2230-8210.117219
Kalwat, M. A., & Cobb, M. H. (2017). Mechanisms of the amplifying pathway of insulin secretion in the beta cell. *Pharmacol Ther*, *179*, 17-30. https://doi.org/10.1016/j.pharmthera.2017.05.003
Kavya ChitraMekala, A. G. (2019). *Epidemiology of diabetes mellitus* (Vol. Volume 1). Elsevier
Kim, A., Miller, K., Jo, J., Kilimnik, G., Wojcik, P., & Hara, M. (2009). Islet architecture: A comparative study. *Islets*, *1*(2), 129-136. https://doi.org/10.4161/isl.1.2.9480

Komatsu, H., Kandeel, F., & Mullen, Y. (2018). Impact of Oxygen on Pancreatic Islet Survival. *Pancreas*, *47*(5), 533-543. https://doi.org/10.1097/MPA.0000000000001050

Kretschmer, G. J., Sutherland, D. E., Matas, A. J., Steffes, M. W., & Najarian, J. S. (1977). The dispersed pancreas: transplantation without islet purification in totally pancreatectomized dogs. *Diabetologia*, *13*(5), 495-502. https://doi.org/10.1007/BF01234503

Lampugnani, M. G., & Dejana, E. (2007). Adherens junctions in endothelial cells regulate vessel maintenance and angiogenesis. *Thromb Res*, *120 Suppl 2*, S1-6. https://doi.org/10.1016/S0049-3848(07)70124-X

Lee, E. M., Park, I., Lee, Y. J., You, Y. H., Kim, J. W., Kim, M. J., Ahn, Y. B., Kim, P., & Ko, S. H. (2018). Effect of resveratrol treatment on graft revascularization after islet transplantation in streptozotocin-induced diabetic mice. *Islets*, *10*(1), 25-39. https://doi.org/10.1080/19382014.2017.1414764

Lee, M. K., Kim, S. G., Watkins, E., Moon, M. K., Rhee, S. Y., Frias, J. P., Chung, C. H., Lee, S. H., Block, B., Cha, B. S., Park, H. K., Kim, B. J., & Greenway, F. (2020). A novel non-PPARgamma insulin sensitizer: MLR-1023 clinicalproof-of-concept in type 2 diabetes mellitus. *J Diabetes Complications*, *34*(5), 107555. https://doi.org/10.1016/j.jdiacomp.2020.107555

Lifson, N., Lassa, C. V., & Dixit, P. K. (1985). Relation between blood flow and morphology in islet organ of rat pancreas. *Am J Physiol*, *249*(1 Pt 1), E43-48. https://doi.org/10.1152/ajpendo.1985.249.1.E43

Maillard, E., Juszczak, M. T., Langlois, A., Kleiss, C., Sencier, M. C., Bietiger, W., Sanchez-Dominguez, M., Krafft, M. P., Johnson, P. R., Pinget, M., & Sigrist, S. (2012). Perfluorocarbon emulsions prevent hypoxia of pancreatic beta-cells. *Cell Transplant*, *21*(4), 657-669. https://doi.org/10.3727/096368911X593136

Marfil-Garza, B. A., Imes, S., Verhoeff, K., Hefler, J., Lam, A., Dajani, K., Anderson, B., O'Gorman, D., Kin, T., Bigam, D., Senior, P. A., & Shapiro, A. M. J. (2022). Pancreatic islet transplantation in type 1 diabetes: 20-year experience from a single-centre cohort in Canada. *Lancet Diabetes Endocrinol*, *10*(7), 519-532. https://doi.org/10.1016/S2213-8587(22)00114-0

Matveyenko, A. V., & Butler, P. C. (2008). Relationship between beta-cell mass and diabetes onset. *Diabetes Obes Metab*, *10 Suppl 4*(0 4), 23-31. https://doi.org/10.1111/j.1463-1326.2008.00939.x

Mehigan, D. G., Bell, W. R., Zuidema, G. D., Eggleston, J. C., & Cameron, J. L. (1980). Disseminated intravascular coagulation and portal hypertension following pancreatic islet autotransplantation. *Ann Surg*, *191*(3), 287-293. https://doi.org/10.1097/00000658-198003000-00006

Meier, J. J. (2008). Beta cell mass in diabetes: a realistic therapeutic target? *Diabetologia*, *51*(5), 703-713. https://doi.org/10.1007/s00125-008-0936-9

Meier, J. J., Bhushan, A., Butler, A. E., Rizza, R. A., & Butler, P. C. (2005). Sustained beta cell apoptosis in patients with long-standing type 1 diabetes: indirect evidence for islet regeneration? *Diabetologia*, *48*(11), 2221-2228. https://doi.org/10.1007/s00125-005-1949-2

Meier, J. J., Breuer, T. G., Bonadonna, R. C., Tannapfel, A., Uhl, W., Schmidt, W. E., Schrader, H., & Menge, B. A. (2012). Pancreatic diabetes manifests when beta cell area declines by approximately 65% in humans. *Diabetologia*, *55*(5), 1346-1354. https://doi.org/10.1007/s00125-012-2466-8

Merani, S., Toso, C., Emamaullee, J., & Shapiro, A. M. (2008). Optimal implantation site for pancreatic islet transplantation. *Br J Surg*, *95*(12), 1449-1461. https://doi.org/10.1002/bjs.6391

Mirkovitch, V., & Campiche, M. (1976). Successful intrasplenic autotransplantation of pancreatic tissue in totally pancreatectomised dogs. *Transplantation*, *21*(3), 265-269. https://doi.org/10.1097/00007890-197603000-00014

Mu, W., Cheng, X. F., Liu, Y., Lv, Q. Z., Liu, G. L., Zhang, J. G., & Li, X. Y. (2018). Potential Nexus of Non-alcoholic Fatty Liver Disease and Type 2 Diabetes Mellitus: Insulin Resistance Between Hepatic and Peripheral Tissues. *Front Pharmacol*, *9*, 1566. https://doi.org/10.3389/fphar.2018.01566

Najarian, J. S., Sutherland, D. E., Matas, A. J., Steffes, M. W., Simmons, R. L., & Goetz, F. C. (1977). Human islet transplantation: a preliminary report. *Transplant Proc*, *9*(1), 233-236. https://www.ncbi.nlm.nih.gov/pubmed/405770

Narayanan, S., Loganathan, G., Dhanasekaran, M., Tucker, W., Patel, A., Subhashree, V., Mokshagundam, S., Hughes, M. G., Williams, S. K., & Balamurugan, A. N. (2017). Intra-islet endothelial cell and beta-cell crosstalk: Implication for islet cell transplantation. *World J Transplant*, *7*(2), 117-128. https://doi.org/10.5500/wjt.v7.i2.117

O'Connell, P. J., Holmes-Walker, D. J., Goodman, D., Hawthorne, W. J., Loudovaris, T., Gunton, J. E., Thomas, H. E., Grey, S. T., Drogemuller, C. J., Ward, G. M., Torpy, D. J., Coates, P. T., Kay, T. W., & Australian Islet Transplant, C. (2013). Multicenter Australian trial of islet transplantation: improving accessibility and outcomes. *Am J Transplant*, *13*(7), 1850-1858. https://doi.org/10.1111/ajt.12250

Ochman, A. R., Lipinski, C. A., Handler, J. A., Reaume, A. G., & Saporito, M. S. (2012). The Lyn kinase activator MLR-1023 is a novel insulin receptor potentiator that elicits a rapid-onset and durable improvement in glucose homeostasis in animal models of type 2 diabetes. *J Pharmacol Exp Ther*, *342*(1), 23-32. https://doi.org/10.1124/jpet.112.192187

Ohneda, M., Johnson, J. H., Inman, L. R., & Unger, R. H. (1993). GLUT-2 function in glucose-unresponsive beta cells of dexamethasone-induced diabetes in rats. *J Clin Invest*, *92*(4), 1950-1956. https://doi.org/10.1172/JCI116788

Oram, R. A., Sims, E. K., & Evans-Molina, C. (2019). Beta cells in type 1 diabetes: mass and function; sleeping or dead? *Diabetologia*, *62*(4), 567-577. https://doi.org/10.1007/s00125-019-4822-4

Pae, E. K., Ahuja, B., Kim, M., & Kim, G. (2013). Impaired glucose homeostasis after a transient intermittent hypoxic exposure in neonatal rats. *Biochem Biophys Res Commun*, *441*(3), 637-642. https://doi.org/10.1016/j.bbrc.2013.10.102

Pae, E. K., & Kim, G. (2014). Insulin production hampered by intermittent hypoxia via impaired zinc homeostasis. *PLoS One*, *9*(2), e90192. https://doi.org/10.1371/journal.pone.0090192

Parnaud, G., Bosco, D., Berney, T., Pattou, F., Kerr-Conte, J., Donath, M. Y., Bruun, C., Mandrup-Poulsen, T., Billestrup, N., & Halban, P. A. (2008). Proliferation of sorted human and rat beta cells. *Diabetologia*, *51*(1), 91-100. https://doi.org/10.1007/s00125-007-0855-1

Pepper, A. R., Gala-Lopez, B., Ziff, O., & Shapiro, A. M. (2013). Revascularization of transplanted pancreatic islets and role of the transplantation site. *Clin Dev Immunol*, *2013*, 352315. https://doi.org/10.1155/2013/352315

Piciucchi, M., Capurso, G., Archibugi, L., Delle Fave, M. M., Capasso, M., & Delle Fave, G. (2015). Exocrine pancreatic insufficiency in diabetic patients: prevalence, mechanisms,

and treatment. *Int J Endocrinol*, *2015*, 595649. https://doi.org/10.1155/2015/595649
Rachdi, L., Kariyawasam, D., Guez, F., Aiello, V., Arbones, M. L., Janel, N., Delabar, J. M., Polak, M., & Scharfmann, R. (2014). Dyrk1a haploinsufficiency induces diabetes in mice through decreased pancreatic beta cell mass. *Diabetologia*, *57*(5), 960-969. https://doi.org/10.1007/s00125-014-3174-3
Reckard, C. R., Ziegler, M. M., & Barker, C. F. (1973). Physiological and immunological consequences of transplanting isolated pancreatic islets. *Surgery*, *74*(1), 91-99. https://www.ncbi.nlm.nih.gov/pubmed/4146046
Robertson, R. P. (2004). Islet transplantation as a treatment for diabetes - a work in progress. *N Engl J Med*, *350*(7), 694-705. https://doi.org/10.1056/NEJMra032425
Roder, P. V., Wu, B., Liu, Y., & Han, W. (2016). Pancreatic regulation of glucose homeostasis. *Exp Mol Med*, *48*(3), e219. https://doi.org/10.1038/emm.2016.6
Rodriguez-Brotons, A., Bietiger, W., Peronet, C., Magisson, J., Sookhareea, C., Langlois, A., Mura, C., Jeandidier, N., Pinget, M., Sigrist, S., & Maillard, E. (2016). Impact of Pancreatic Rat Islet Density on Cell Survival during Hypoxia. *J Diabetes Res*, *2016*, 3615286. https://doi.org/10.1155/2016/3615286
Ryan, E. A., Paty, B. W., Senior, P. A., Bigam, D., Alfadhli, E., Kneteman, N. M., Lakey, J. R., & Shapiro, A. M. (2005). Five-year follow-up after clinical islet transplantation. *Diabetes*, *54*(7), 2060-2069. https://doi.org/10.2337/diabetes.54.7.2060
Saisho, Y., Butler, A. E., Manesso, E., Elashoff, D., Rizza, R. A., & Butler, P. C. (2013). beta-cell mass and turnover in humans: effects of obesity and aging. *Diabetes Care*, *36*(1), 111-117. https://doi.org/10.2337/dc12-0421
Sane, F., Bertin, A., Sioofy-Khojine, A. B., Oikarinen, S., Alidjinou, E. K., Veijola, R., Toppari, J., Ilonen, J., Knip, M., Engelmann, I., Hyoty, H., & Hober, D. (2020). Enhancing and neutralizing anti-coxsackievirus activities in serum samples from patients prior to development of type 1 diabetes. *Diabetes Metab Res Rev*, *36*(6), e3305. https://doi.org/10.1002/dmrr.3305
Saporito, M. S., Ochman, A. R., Lipinski, C. A., Handler, J. A., & Reaume, A. G. (2012). MLR-1023 is a potent and selective allosteric activator of Lyn kinase in vitro that improves glucose tolerance in vivo. *J Pharmacol Exp Ther*, *342*(1), 15-22. https://doi.org/10.1124/jpet.112.192096
Shapiro, A. M., Gallant, H. L., Hao, E. G., Lakey, J. R., McCready, T., Rajotte, R. V., Yatscoff, R. W., & Kneteman, N. M. (2005). The portal immunosuppressive storm: relevance to islet transplantation? *Ther Drug Monit*, *27*(1), 35-37. https://doi.org/10.1097/00007691-200502000-00008
Shapiro, A. M., Lakey, J. R., Ryan, E. A., Korbutt, G. S., Toth, E., Warnock, G. L., Kneteman, N. M., & Rajotte, R. V. (2000). Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *N Engl J Med*, *343*(4), 230-238. https://doi.org/10.1056/NEJM200007273430401
Shen, W., Taylor, B., Jin, Q., Nguyen-Tran, V., Meeusen, S., Zhang, Y. Q., Kamireddy, A., Swafford, A., Powers, A. F., Walker, J., Lamb, J., Bursalaya, B., DiDonato, M., Harb, G., Qiu, M., Filippi, C. M., Deaton, L., Turk, C. N., Suarez-Pinzon, W. L., Liu, Y., Hao, X., Mo, T., Yan, S., Li, J., Herman, A. E., Hering, B. J., Wu, T., Martin Seidel, H., McNamara, P., Glynne, R., & Laffitte, B. (2015). Inhibition of DYRK1A and GSK3B induces human beta-cell proliferation. *Nat Commun*, *6*, 8372. https://doi.org/10.1038/ncomms9372

Staels, W., Verdonck, Y., Heremans, Y., Leuckx, G., De Groef, S., Heirman, C., de Koning, E., Gysemans, C., Thielemans, K., Baeyens, L., Heimberg, H., & De Leu, N. (2018). Vegf-A mRNA transfection as a novel approach to improve mouse and human islet graft revascularisation. *Diabetologia*, *61*(8), 1804-1810. https://doi.org/10.1007/s00125-018-4646-7

Stokes, R. A., Cheng, K., Lalwani, A., Swarbrick, M. M., Thomas, H. E., Loudovaris, T., Kay, T. W., Hawthorne, W. J., O'Connell, P. J., & Gunton, J. E. (2017). Transplantation sites for human and murine islets. *Diabetologia*, *60*(10), 1961-1971. https://doi.org/10.1007/s00125-017-4362-8

van Belle, T. L., Coppieters, K. T., & von Herrath, M. G. (2011). Type 1 diabetes: etiology, immunology, and therapeutic strategies. *Physiol Rev*, *91*(1), 79-118. https://doi.org/10.1152/physrev.00003.2010

Wagner, L. E., Melnyk, O., Duffett, B. E., & Linnemann, A. K. (2022). Mouse models and human islet transplantation sites for intravital imaging. *Front Endocrinol (Lausanne)*, *13*, 992540. https://doi.org/10.3389/fendo.2022.992540

Walker, S., Appari, M., & Forbes, S. (2022). Considerations and challenges of islet transplantation and future therapies on the horizon. *Am J Physiol Endocrinol Metab*, *322*(2), E109-E117. https://doi.org/10.1152/ajpendo.00310.2021

Wang, P., Alvarez-Perez, J. C., Felsenfeld, D. P., Liu, H., Sivendran, S., Bender, A., Kumar, A., Sanchez, R., Scott, D. K., Garcia-Ocana, A., & Stewart, A. F. (2015). A high-throughput chemical screen reveals that harmine-mediated inhibition of DYRK1A increases human pancreatic beta cell replication. *Nat Med*, *21*(4), 383-388. https://doi.org/10.1038/nm.3820

Wang, P., Karakose, E., Liu, H., Swartz, E., Ackeifi, C., Zlatanic, V., Wilson, J., Gonzalez, B. J., Bender, A., Takane, K. K., Ye, L., Harb, G., Pagliuca, F., Homann, D., Egli, D., Argmann, C., Scott, D. K., Garcia-Ocana, A., & Stewart, A. F. (2019). Combined Inhibition of DYRK1A, SMAD, and Trithorax Pathways Synergizes to Induce Robust Replication in Adult Human Beta Cells. *Cell Metab*, *29*(3), 638-652 e635. https://doi.org/10.1016/j.cmet.2018.12.005

Wang, Y. J., Golson, M. L., Schug, J., Traum, D., Liu, C., Vivek, K., Dorrell, C., Naji, A., Powers, A. C., Chang, K. M., Grompe, M., & Kaestner, K. H. (2016). Single-Cell Mass Cytometry Analysis of the Human Endocrine Pancreas. *Cell Metab*, *24*(4), 616-626. https://doi.org/10.1016/j.cmet.2016.09.007

Warnock, G. L., Ellis, D. K., Cattral, M., Untch, D., Kneteman, N. M., & Rajotte, R. V. (1989). Viable purified islets of Langerhans from collagenase-perfused human pancreas. *Diabetes*, *38 Suppl 1*, 136-139. https://doi.org/10.2337/diab.38.1.s136

Warnock, G. L., Kneteman, N. M., Ryan, E. A., Evans, M. G., Seelis, R. E., Halloran, P. F., Rabinovitch, A., & Rajotte, R. V. (1989). Continued function of pancreatic islets after transplantation in type I diabetes. *Lancet*, *2*(8662), 570-572. https://doi.org/10.1016/s0140-6736(89)90701-0

Weir, G. C., & Bonner-Weir, S. (2004). Five stages of evolving beta-cell dysfunction during progression to diabetes. *Diabetes*, *53 Suppl 3*, S16-21. https://doi.org/10.2337/diabetes.53.suppl_3.s16

Weir, G. C., Gaglia, J., & Bonner-Weir, S. (2020). Inadequate beta-cell mass is essential for the pathogenesis of type 2 diabetes. *Lancet Diabetes Endocrinol*, *8*(3), 249-256. https://doi.org/10.1016/S2213-8587(20)30022-X

WHO. (2016). *Global report on diabetes*. https://www.who.int/publications/i/item/9789241565257

Wild, S., Roglic, G., Green, A., Sicree, R., & King, H. (2004). Global prevalence of diabetes: estimates for the year 2000 and projections for 2030. *Diabetes Care*, *27*(5), 1047-1053. https://doi.org/10.2337/diacare.27.5.1047

Wu, S., Wang, L., Fang, Y., Huang, H., You, X., & Wu, J. (2021). Advances in Encapsulation and Delivery Strategies for Islet Transplantation. *Adv Healthc Mater*, *10*(20), e2100965. https://doi.org/10.1002/adhm.202100965

Younoszai, R., Sorenson, R.L., Lindall, A.W. (1970). Homotransplantation of isolated pancreatic islets (abstract). . *Diabetes19,(Suppl.):406*

Zaccardi, F., Webb, D. R., Yates, T., & Davies, M. J. (2016). Pathophysiology of type 1 and type 2 diabetes mellitus: a 90-year perspective. *Postgrad Med J*, *92*(1084), 63-69. https://doi.org/10.1136/postgradmedj-2015-133281

Zeng, Y., Ricordi, C., Lendoire, J., Carroll, P. B., Alejandro, R., Bereiter, D. R., Tzakis, A., & Starzl, T. E. (1993). The effect of prednisone on pancreatic islet autografts in dogs. *Surgery*, *113*(1), 98-102. https://www.ncbi.nlm.nih.gov/pubmed/8417496