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Functional Validation of Human Multipotent Stromal Cell-secreted Islet Regenerative Proteins

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Physiology and Pharmacology

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Abstract

Diabetes affects ~537 million people worldwide. Although insulin can help manage blood glucose, 80% of patients suffer severe complications, prompting a need for novel therapies. Human bone marrow-derived multipotent stromal cells (MSC) expanded under Wnt-pathway stimulation secrete islet regenerative factors into conditioned media (Wnt+ CM). Proteomic analyses identified 8 secreted proteins (FAM3C, PSAP, SOD1, PPIA, GAL1, CTSB, TGM2, CALU) as top candidates; however, the islet regenerative functions of these proteins have not yet been functionally validated. We tested the islet regenerative capacity of these MSC-secreted proteins *in vitro* using human islet cultures and *in vivo* using streptozotocin-treated mice. Although the proteins had minimal effects on islet viability and proliferation *in vitro*, intrapancreatic injection of the 8-protein combination showed islet regenerative effects comparable to Wnt+ CM *in vivo*, including decreased hyperglycemia, improved glucose tolerance, and increased beta cell mass. This study provides proof-of-concept for the development of protein-based therapies for diabetes.

Keywords

Diabetes, Multipotent Stromal Cells, Islet/Beta Cell Regeneration, Proteomics, Wnt Signalling

Summary for Lay Audience

Inside the islet of Langerhans in the pancreas, beta cells are responsible for producing insulin to decrease blood glucose levels by promoting glucose uptake and storage in tissues. Diabetes is a chronic disease in which the patient's beta cells are destroyed, or the body stops reacting to insulin properly, resulting in elevated blood glucose levels. Although diabetes can be managed with insulin injection, there is no cure, and 80% of patients develop severe complications in the heart and kidney, which negatively impact their quality of life. Development of strategies to regenerate beta cells may be a possible solution. Multipotent stromal cells (MSC) isolated from the bone marrow secrete regenerative proteins that drive tissue repair. Our previous research has shown that proteins made by MSC can promote human beta cell growth and reduce elevated blood glucose when directly injected into the pancreas of diabetic mice. However, specific proteins that stimulate beta cell regeneration are currently unknown. We have identified 8 proteins highly implicated in islet regeneration. Our goal is to document the regenerative functions of the 8 MSC-secreted proteins using human islets and in mice with diabetes.

Although the 8-protein combination did not promote human beta cell survival or growth in lab, we found that 8-protein combination had potent effects in the mouse model, including decreased blood glucose levels and improved glucose tolerance compared to mice that did not receive any treatment. In addition, analyses on the mouse pancreas showed that injection of the 8-protein combination increased islet size, islet number, and overall beta cell mass, confirming that islets were regenerated after protein injection.

To conclude, our data confirmed the islet regenerative functions of the 8 proteins secreted by MSC. These proteins may have great therapeutic potential since a single injection was able to improve glucose control. In the future, we will need to test 8-protein combination in other diabetes models, determine the right dosage of the proteins, and test their long-term effects. Overall, these data may lead to the development of islet regenerative therapies for the many patients with diabetes.

Co-Authorship Statement

The following thesis contains material from a manuscript in preparation co-authored by Xin Yue Xie, Miljan Kuljanin, Tyler Cooper, Yina Tian, Ajaya Sharma, Caleb Podgers, Gillian Bell, and Dr. David Hess.

Xin Yue Xie designed and performed all experiments, and interpreted data presented in this thesis in the lab of Dr. David Hess. Gillian Bell assisted with technique training, experimental conceptualization, and animal care. Miljan Kuljanin and Tyler Cooper assisted with the generation and analysis of the proteomic screens. Yina Tian assisted with the analysis of beta cell mass, islet size and islet number at Day 14. Ajaya Sharma and Caleb Podgers assisted with image quantification for beta cell/alpha cell ratio. Dr. David Hess contributed to the design and interpretation of all experiments included in this thesis.

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List of Abbreviations

ADAMTS13/18 – a disintegrin and metalloproteinase with a thrombospondin motif 13/18

ADP – adenosine diphosphate

Akt – protein kinase B

ALDH – aldehyde dehydrogenase

ALS - amyotrophic lateral sclerosis

APC – adenomatous polyposis coli

ATP – adenosine triphosphate

AUC – area under the curve

BM – bone marrow

BMI – body mass index

BrdU – bromodeoxyuridine

CAB – citric acid buffer

CALU – calumenin

CaM – calmodulin

CK1 – casein kinase

CK19 – cytokeratin 19

CM – conditioned media

COVID-19 – coronavirus disease 2019

CsA – cyclosporine A

CTSB – cathepsin B

CXCL2/3/5 – chemokine ligand-2/3/5

CypA – cyclophilin A

DAPI – 4′,6-diamidino-2-phenylindole

DMEM – Dulbecco's Modified Eagle Medium

DMSO – dimethyl sulfoxide

DNA – deoxyribonucleic acid

DYRK1 – tyrosine-phosphorylation regulated kinase 1

EdU – 5-ethynyl-2´-deoxyuridine

EGF – epidermal growth factor

EMT – epithelial to mesenchymal transition

ERK1/2 – extracellular signal-regulated kinase 1/2

ESC – embryonic stem cells

FAM3C – FAM3 metabolism regulating signalling molecule C

FBS – fetal bovine serum

FK506 – tacrolimus

FLT4 – fms-related tyrosine kinase 4

FMO – Fluorescence Minus One

GLP1 – Glucagon-like peptide 1

GLUT2 – insulin-independent glucose transporter

GLUT4 – insulin-dependent glucose transporter

GMP – good manufacturing practice

GOBP – gene ontology biological processes

GSK3 – glycogen synthase kinase

GWAS – genome-wide asssociation studies

HbA1C – glycated hemoglobin

HLA – human leukocyte antigen

HSF1 – heat shock transcription factor 1

IEQ – islet equivalents

IGF2 – insulin-like growth factor 2

IIDP – integrated islet distribution program

IL-1B/6/8 – cytokine interleukin-1β/6/8

iPan – intrapancreatic

iPSC – induced pluripotent stem cells

IRS1 – insulin receptor substrate 1

KDR – kinase insert domain receptor

KIT – proto-oncogene c-KIT

LGALS1/GAL1 – galectin 1

LIFR – leukemia inhibitory factor receptor alpha

MFI – mean fluorescent intensity

MHC – major histocompatibility complex

MMP3/10/12 – matrix metalloproteinases 3/10/12

MNC – mononuclear cells

MSC - multipotent stromal cells

MWCO – molecular weight cut-off

NADPH – nicotinamide adenine dinucleotide phosphate

NFBG – non-fasting blood glucose

NGN3 – neurogenin-3

NKX6.1 – homeobox protein NKX-6.1

NOD/SCID – non-obese diabetic/severe combined immunodeficiency

PBS – phosphate-buffered saline

PDX1 – pancreatic and duodenal homeobox 1

PI3K – phosphoinositide 3-kinase

PP – pancreatic polypeptide

PPIA – peptidylprolyl isomerase A

PSAP – prosaposin

PTF1A – pancreas transcription factor 1 complex

RNA – ribonucleic acid

ROS – reactive oxygen species

SGLT2 – sodium-glucose cotransporter 2

SNP – single nucleotide polymorphism

SOD1 – superoxide dismutase 1

SOX9 – transcription factor sox-9

SPON2 – spondin 2

STAT3 – signal transducer and activator of transcription 3

STZ – streptozotocin

T1D – type 1 diabetes

T2D – type 2 diabetes

TGF-beta – transforming growth factor beta

TGM2 – transglutaminase 2

TXNIP – thioredoxin interacting protein

UCB – umbilical cord blood

UK – United Kingdom

US/USA – United States of America

Wnt+ – Wnt pathway stimulated

Wnt- – Wnt pathway inhibited

WISP2 – WNT1-inducible-signaling pathway protein 2

List of Appendices

Chapter 1

1 Introduction

1.1 Diabetes Mellitus

1.1.1 Pancreatic regulation of glucose homeostasis

The human body uses tight control of blood glucose levels to ensure normal physiological function, as glucose is the primary source of energy for cellular respiration. Glucose homeostasis is achieved through sophisticated interactions between various hormones released from the pancreas, to maintain blood glucose levels within a very narrow range (4-6 mmol/L)[1].

The human pancreas is located behind the stomach within the upper left abdominal cavity, and has essential exocrine and endocrine functions. The exocrine pancreas consists of acinar and ductal cells that secrete enzymes to regulate macronutrient digestion and metabolism. The endocrine cells of the pancreas are clustered together to form islets of Langerhans, which are small, island-like structures dispersed throughout the acinar tissue. While comprising ~2% of the entire pancreas, the islets secrete important hormones into the blood to regulate glucose homeostasis, particularly glucagon and insulin[2]. During sleep or in between meals, glucagon is released from alpha cells to promote glycogenolysis and gluconeogenesis to increase blood glucose levels. In contrast, insulin is secreted from beta cell to decrease blood glucose levels after a meal via glucose uptake and storage into tissues[3].

In beta cells, circulating glucose after a meal is taken up by the insulin-independent glucose transporter, GLUT2. Glucose catabolism increases ATP/ADP ratio inside the cell, leading to a series of electrochemical events that will increase intracellular calcium concentrations, eventually triggering the fusion of insulin-containing granules with the cell membrane and the subsequent release of insulin into the blood stream via islet-derived microvessels[3].

Adipose, liver, and muscles cells take up glucose from the blood through the insulin-dependent glucose transporter, GLUT4. When insulin binds to its receptor, a downstream

phosphorylation cascade occurs, leading to the synthesis and recruitment of GLUT4 to the cell surface for glucose uptake[4]. Insulin also shifts the balance toward anabolism by promoting glycogenesis and inhibiting gluconeogenesis to decrease blood glucose levels[1].

Together, insulin and glucagon work in concert to maintain the optimum blood glucose levels in the body. However, disturbances in glucose homeostasis may lead to metabolic disorders such as diabetes mellitus[5].

1.1.2 Definition, diagnosis, and treatment of diabetes

Dysregulation of glucose homeostasis is a primary feature of diabetes mellitus, a heterogeneous metabolic disease characterized by hyperglycemia due to impaired insulin secretion, defective insulin action, or both. Diabetes may be diagnosed based on blood glucose or glycated hemoglobin (HbA1C) levels. Specifically, diabetes is diagnosed with any one of the following criteria: a fasting blood glucose $\geq$ 7.0 mmol/L, a random blood glucose $\geq$ 11.1 mmol/L, a 2-hour plasma glucose value $\geq$ 11.1 mmol/L in 75 g oral glucose tolerance test, or a HbA1C $\geq$ 6.5%[5].

Diabetes can be generally classified into the following categories: (1) type 1 diabetes (T1D) caused by autoimmune beta cell destruction leading to insulin deficiency; (2) type 2 diabetes (T2D) caused by insulin resistance and progressive defects in insulin secretion; (3) gestational diabetes caused by insufficient insulin production to meet the increased metabolic demand during pregnancy; and (4) specific types of diabetes due to other causes such as monogenic diabetes syndromes, diseases of the exocrine pancreas, and drug-induced diabetes[6].

T1D is commonly known as “insulin-dependent diabetes” or “juvenile-onset diabetes”. It accounts for approximately 5-10% of all diagnosed cases and is due to cellular-mediated autoimmune destruction of pancreatic beta cells. The presence of autoimmune markers, such as anti-insulin or anti-islet cell autoantibodies may be helpful in identifying T1D. The rate of beta cell destruction can be variable so T1D can occur at any age, but onset during childhood and adolescence is most common. In latter stages of T1D, there could be little or no insulin secretion occuring[5,6]. T1D is a complex multifactorial disease. Genetic and

environmental factors both play a role in the development of T1D. Genome-wide association studies (GWAS) have identified >50 loci that contribute to the risk of developing T1D, with HLA genes having the largest effect size[7]. While the environmental contributors remain poorly defined, diet during childhood, human enteroviruses, and socioeconomic status have all been correlated with T1D incidence[8]. Exogenous insulin therapy with continuous glucose monitoring is the first-line treatment to help patients reach their glycemic targets. Medications for high blood pressure and high cholesterol as well as aspirin can be prescribed with insulin to help improve patient outcomes[9].

T2D is commonly known as "non-insulin-dependent diabetes" or "adult-onset diabetes", but the incidence of T2D is increasing in adolescents, paralleling with the increasing rate of obesity in this age group. It accounts for 90-95% of all diagnosed diabetes. T2D may range from predominant insulin resistance with relative insulin deficiency to a predominant defect in insulin secretion with insulin resistance[5,6]. Due to insulin resistance, which is when cells in the muscles, adipose tissues, and liver fail to response efficiently to insulin, beta cells must overproduce insulin to regulate insulin-dependent glucose uptake. Eventually, beta cells become exhausted and subject to glucotoxicity, and insulin secretion becomes insufficient to compensate for insulin resistance, leading to the development of hyperglycemia. As a result, early stage T2D may remain undiagnosed for many years as hyperglycemia is often not severe enough for the patient to detect[10]. Prediabetes refers to a condition where blood glucose levels are higher than normal but not severe enough to be diagnosed as diabetes, defined by a fasting blood glucose of 6.1-6.9 mmol/L or an HbA1C of 6.0-6.4%. If left unmanaged, prediabetes may develop into T2D[5]. The etiology of T2D involves a complex interplay between genetic and environmental factors. In contrary to the traditional belief that T2D is solely caused by poor management of diet and exercise, T2D actually has a stronger genetic link than T1D. Approximately 75-90% of T2D patients have a family history of diabetes[11]. GWAS of multiple populations have linked single nucleotide polymorphisms (SNP) at >250 loci to the predisposition of T2D[12]. Yet, T2D also depends on environmental factors. The risk of developing T2D increases with age, obesity, and lack of physical activity. The first-line treatment includes lifestyle modifications and non-insulin antihyperglycemic agents such as metformin, sodium-glucose cotransporter 2 (SGLT2) inhibitors, and glucagon-like peptide-1 (GLP1) receptor agonists. Exogenous

insulin therapy may be needed during later stage of T2D in order to control blood glucose fluctuations[5,13].

1.1.3 Diabetes Epidemiology

Diabetes is becoming a global epidemic for people from all age groups. Currently, diabetes affects over 537 million adults worldwide, representing 10.5% of the world's adult population. This number is expected to increase to 784 million by 2045. Diabetes prevalence is similar in men and women and is highest in those aged 75-79 years[14]. In contrast, an estimated 149,500 children and adolescents under 20 years of age were diagnosed with T1D in 2021[15]. Prevalence in 2021 was estimated to be higher in high-income (11.1%) compared to low-income countries (5.5%), but the greatest relative increase in prevalence between 2021 and 2045 is expected to occur in middle-income countries (21.1%)[14]. However, when considering the prevalence of undiagnosed diabetes, it is possible that these statistics are underestimated. In 2019, 8.5 million adults who met laboratory criteria for diabetes were not aware of having diabetes, representing 23% of all adults with diabetes in the US[16]. Likewise, approximately 96 million or 38% of US adults have prediabetes, and nearly 80% of them are unaware of the condition[17].

Diabetes is not only costly to the quality of life of patients suffering from the disease, but is also a major burden to the global health care system. In 2021, the global diabetes-related health expenditures were estimated at 966 billion US dollars, and financial cost of diabetes is projected to reach 1.05 trillion US dollars by 2045[14]. In Ontario, 70% patients with diabetes reported that it is difficult to pay for their treatments, and about 1/3 of them had to pay out-of-pocket for required treatments[18]. The financial burden of the disease also contributes to poor medication adherence. At least 45% of patients with T2D fail to achieve adequate glycemic control (HbA1C<6.5%), which negatively impact patient outcomes[19].

1.1.4 Comorbidities of diabetes

Sustained hyperglycemia can have serious effects to the human body, with comorbidities focused on cardiovascular system, nerves, kidney, and eyes. In response to hyperglycemia, reactive oxygen species (ROS) production is stimulated via a protein kinase C-dependent

activation of NADPH oxidase, leading to the accumulation of ROS and thus damaging endothelial cells and vascular smooth muscle cells[20,21].

Although blood glucose levels can be managed using various medications, patients with diabetes have higher risk of developing severe cardiovascular comorbidities that shorten their life expectancy. Indeed, patients with T1D diagnosed prior to 10 or 20 years of age had a decreased life expectancy of 16 or 10 years, respectively[22], and overall mortality rates were twice as high for diabetic patients compared to individuals without diabetes[23]. Comorbidities that have traditionally been associated with diabetes include macrovascular conditions including coronary heart disease, stroke, and peripheral arterial disease, and microvascular conditions including diabetic kidney disease, retinopathy, and peripheral neuropathy. Vascular diseases once accounted for > 50% of deaths amongst people with diabetes[24].

More recently, advances in the management of diabetes lead to longer life expectancy but revealed a different set of lesser-acknowledged complications. Diabetes is associated with an increased risk of various cancers, especially gastrointestinal and female-specific cancers[25]. Hospitalization and mortality from infections including COVID-19, pneumonia, foot and kidney infections, are increased in people with diabetes[26,27]. Cognitive and functional disability, nonalcoholic fatty liver disease, and depression are also common comorbidities associated with diabetes[28,29].

1.1.5 Limitations of exogenous insulin therapy

Constant monitoring and maintenance of blood glucose within the ideal range is essential to decrease risk of diabetes-induced complications. For patients requiring exogenous insulin therapy, automated insulin pumps can continually sense blood glucose and inject insulin on demand throughout the day. However, there are still many challenges and uncertainties involved in insulin therapy. Despite the use of newly developed insulins, it is still difficult to obtain optimal glycemic control in patients with diabetes[30]. While an HbA1C below 5.7% is considered normal, the overall mean HbA1C was 8.4% in patients with T1D[31]. In addition, patients with stable HbA1C values may still experience wide

glycemic variability, which is associated with increased oxidative stress, arrhythmias, endothelial dysfunction, inflammation, and mortality[32].

Nonetheless, painful injection of insulin and limitations in daily activities further degrade the patient's quality of life[33]. In the event of improper dosage, exogenous insulin can lead to life-threatening hypoglycemia. Additionally, the inability of insulin pumps to accurately predict caloric needs further contributes to poor glucose control and uncontrolled peaks and troughs in blood glucose levels, leading to increased risk of complications[34].

Taken together, there exists a compelling need to develop curative therapies for diabetes, which would eliminate the immense burden of disease and stress faced by patients due to the development of comorbidities. Improving glycemic control through a therapeutic intervention that permits tightly controlled insulin delivery via the restoration of physiological beta cell function hold great therapeutic promises for patients. To date, islet replacement via transplantation and islet regenerative strategies *in situ* are being tested both pre-clinically and clinically, which are discussed in more details in 1.3 and 1.4.

1.2 Cells in the Pancreas

1.2.1 Pancreatic embryology and development

The pancreas is part of the gastrointestinal system that makes and secretes digestive enzymes into the intestine, and also part of the endocrine system that makes and secretes hormones into the blood to control energy metabolism[35].

The pancreas initially develops around the 5th weeks of gestation as two outpouchings of the endodermal lining of the duodenum, which eventually fuse together[36]. Exocrine and endocrine cell types derive from tri-potent progenitor cells that are PDX1+ and NKX6.1+ located within the pancreatic epithelium. In the developing pancreas, cellular differentiation and lineage selection are regulated by a cascade of transcription factors and signaling molecules. Maintenance of PTF1A expression specifies the acinar cell lineage in pancreas tip progenitors, whereas pancreas trunk progenitors maintain NKX6.1 expression to generate ductal cells, while transient NGN3 expression in trunk progenitors generates cells in the endocrine lineages. In humans, endocrine cells begin to bud from the pancreatic

ducts at around the 10th week of gestation[37,38]. A subset of early glucagon-positive cells co-express insulin, suggesting that the expression of glucagon and insulin is selectively up or down-regulated as endocrine lineage selection occurs[39]. The functional endocrine pancreas continues to develop throughout pregnancy followed by a phase of islet remodeling up to 4 years of age[37,38].

1.2.2 The exocrine pancreas

Comprising more than 95% of pancreatic mass, the exocrine pancreas generates and secretes digestive enzymes into the duodenum, and includes acinar and ductal cells associated with connective tissue, blood vessels, and nerves. The production of pancreatic enzymes such as trypsin, carboxypeptidases, and elastase occur predominantly in the acinar cells. These enzymes are secreted into the duodenum via a network of pancreatic ducts, lined with cuboidal ductal epithelial cells. The ductal cells secrete a bicarbonate-rich fluid that is needed to solubilize enzymes and carry them to the duodenum[40].

1.2.3 The endocrine pancreas

Endocrine cells of the pancreas are organized into islets, which are micro-organs with a dense capillary network, allowing hormones to be directly secreted into the blood. The human pancreas contains 1-2 million islets located throughout the exocrine tissue. Islets vary in size from 50-400 μm, with a mean diameter of 200 μm. Islets consist of 5 hormone-secreting cell types: (1) beta cells that secrete insulin, (2) alpha cells that secrete glucagon, (3) delta cells that secrete somatostatin, (4) pancreatic polypeptide cells that secrete pancreatic polypeptide, and (5) epsilon cells that secrete ghrelin[41].

Beta cells are the most abundant endocrine cell type and are responsible for decreasing blood glucose through the secretion of insulin. In diabetes, beta cell death can occur through apoptosis, necrosis, autophagy, and potentially ferroptosis. A decrease in beta cell mass ranging from 70-100% and 0-65% have been reported in T1D and T2D, respectively[42]. Beta cell death in T1D is mediated by autoimmune attack, whereas in T2D, beta cell dysfunction and apoptosis are contributed by the complex interplay of glucotoxicity, lipotoxicity, cytokines, oxidative stress, endoplasmic reticulum stress, and inflammation[43]. Adult human beta cells have a long lifespan and are considered “static” or

non-proliferative under normal physiological condition. Islet mass grows until adulthood to match increased hormonal demand. Beta cell population is largely established by the age of 20, and there is little evidence for continuous turnover or replication after this age[44,45].

Alpha cells are the second most abundance endocrine cell type in the pancreas and are responsible for increasing blood glucose through the secretion of glucagon. In response to systemic hypoglycemia, the ATP/ADP ratio in alpha cells decrease, triggering a series of electrochemical events that increase the intracellular calcium concentrations, resulting in the exocytosis of glucagon-containing vesicles. Interestingly, glucagon is also known to stimulate insulin secretion from beta cells, while insulin indirectly inhibits glucagon release via somatostatin, forming a negative feedback loop[46]. In diabetes, the loss of inhibitory signals from beta cells contributes to hyperglucagonemia[47]. Emerging evidence also showed that chronic hyperglycemia provokes changes in the islets cytoarchitecture, including alpha cell proliferation or hyperplasia, leading to an expansion in alpha cell mass[48,49]. As a result, alpha to beta cell ratio is increased in patients with diabetes[50]. Interestingly, inhibiting glucagon secretion using GLP1 receptor agonists is linked with improved glycemic control and preservation of beta cell mass[51,52].

Delta cells comprise ~5% of the islet cells. In response to glucose stimulation, delta cells secrete somatostatin, which acts locally within the islets as a paracrine inhibitor of insulin and glucagon secretion[53]. The association of somatostatin hypersecretion in diabetes has been suggested[54]. This may explain the loss of appropriate hypoglycemia-induced glucagon secretion in diabetic animals, which can be mitigated by somatostatin receptor antagonists. Therefore, agents that inhibit somatostatin secretion/action may also reduce the risk of insulin-induced hypoglycemia in patients with diabetes[53].

Pancreatic polypeptide (PP) cells account for <5% of islet cells, and it is the least studied pancreatic cell type. The cells are responsible for PP secretion, which regulates both pancreatic secretory activities via a paracrine mechanism as well as hepatic glycogen levels[55]. PP has a short biological half-life of ~7 minutes in human circulation[56]. PP secretion is regulated by circulating blood glucose, with hypoglycemia acting as a strong stimulus, and hyperglycemia suppressing hormone release. Somatostatin also acts as a

paracrine suppressor of PP secretion[55]. Conversely, PP has an inhibitory effect on insulin secretion, and it also has been reported to inhibit both glucagon and somatostatin release[57–59]. More recently, it was found that PP has protective effects against beta cell apoptosis, suggesting possible benefit in diabetes[57].

Finally, the epsilon cell is the least abundant endocrine cell type in the islet. It secretes ghrelin, one of the main hormones involved in stimulating appetite[60]. Pancreatic epsilon cells are an important source of ghrelin during human fetal development, but epsilon cell number decreases with age. This is opposite to the ghrelin-producing cells in the stomach, which are scarce during fetal development and increased in number in adulthood[61]. The effects of ghrelin on beta cells are complex. Ghrelin acts on delta cells to stimulate somatostatin release, which in turn inhibits insulin secretion, contributing to an increase in blood glucose levels[62].

1.2.4 Multipotent stromal cells (MSC) and pancreatic MSC

Multipotent stromal cells (MSC), also known as mesenchymal stem cells or mesenchymal stromal cells, are defined by their ability to adhere to tissue culture plastic and the potential to differentiate into restricted lineages such as adipocytes, osteoblasts, and chondrocytes[63]. To be classified as MSC, >95% must express CD105, CD73, and CD90 and <2% express hematopoietic markers such as CD45, CD34, CD14, and CD19[64]. MSC can be isolated from many organs and tissues, including bone marrow (BM), umbilical cord blood (UCB), placenta, liver, lung, and pancreas[65]. MSC are considered good candidates for cellular therapies and have been actively tested in both pre-clinical and clinical trials for several reasons: (1) MSC can be easily obtained and isolated from sources such as BM, (2) MSC can be efficiently expanded under normal culture conditions, (3) MSC have supportive functions via the secretion of various factors that mediate cell differentiation, expansion, migrations and survival, (4) MSC have immunomodulatory function and are well tolerated after allogeneic transplantation, and (5) MSC are part of the body's natural repair mechanisms and have been shown to home to sites of tissue injury and inflammation[66].

MSC can be isolated from both pancreatic exocrine and endocrine tissue culture[67] and they resemble BM-derived MSC in terms of surface markers expression and mesodermal

differentiation potential[68]. There is controversy in the source and function of pancreatic MSC, but epithelial to mesenchymal transition (EMT) has been suggested as a possible mechanism for the generation of MSC from cultured pancreatic tissues[69]. EMT is a process in which epithelial cells undergo phenotypic changes to become mesenchymal cells, marked by the decrease of epithelial markers such as E-cadherin and the increase of mesenchymal markers such as vimentin[70]. Lima *et al* reported that EMT occurs in cultured exocrine cells giving rise to MSC-like cells[69]. Lineage tracing studies conducted on human islets showed that beta cells undergo dedifferentiation and EMT in culture[71,72]. Some studies have also shown that pancreatic MSC can differentiate into pancreatic endocrine cells, suggesting their possible role as a target to replenish the beta cell population in diabetes[71,73].

1.2.5 Comparing human and mouse pancreas

Mice remain the most common animal model used in diabetes research. A clear understanding of the similarities and differences of pancreatic cytoarchitecture and function between human and mouse is required to better extrapolate findings from this animal model[74].

The human pancreas is a well-defined solitary organ that can be divided into 3 major parts, the head, body, and tail. In comparison, the mouse pancreas is not as well-defined, but it can also be separated into 3 major parts: duodenal, splenic, and gastric lobe. The pancreas is loosely attached to other organs of the gastrointestinal system such as spleen, stomach, and small intestine. Overall, there is a 1000-fold difference in pancreas size between human and mouse[74].

The exocrine pancreas accounts for 96-99% of total pancreas volume in both species, the endocrine pancreas accounting for the remaining 1-4% of total pancreas volume. Islet size is similar, varying between 50-400 µm in humans and 100-200 µm in mice. Mouse pancreas contains ~1000-5000 islets whereas there are 1-2 million islets in the human. Beta cells make up 50-70% or 60-80% of an islet in human and mice, respectively. Human islets have higher proportion of alpha cells, which make up 20-40% of islet cells, whereas alpha cells only account for 10-20% of islet cells in mice. Therefore, the ratio between beta and

alpha cells is higher in mice compared to humans. Delta cells and PP cells represent <10% of islet cells in humans, and <5% in mice. The epsilon cells represent <1% of islet cells in both species. In mice, beta cells are mainly located in the core of an islet, with alpha cells and other endocrine cells locating on the periphery, forming a "mantle-core pattern". In humans, beta cells are arranged in clusters, and more dispersed throughout the islet in an unorganized manner. Commonly, islet architecture become more complex with increasing islet size[74]. Interspecies differences in beta cell function have been reported, including different electrical behavior, glucose threshold for insulin secretion, and the ionic channels expressed[75]. Moreover, it is known that mouse beta cells have greater regenerative potential than human beta cells[74].

Many experimental mouse models for T1D and T2D have been developed. Diabetes and hyperglycemia can be modeled in mice via: (1) chemicals such as alloxan and streptozotocin (STZ) to inhibit insulin secretion or delete beta cells, respectively, (2) spontaneous autoimmune-induced beta cell death, (3) genetic mutations and engineering, (4) high fat diet, (5) surgeries such as partial pancreatectomy, and (6) viruses[76].

1.3 Islet Replacement Therapy

Beta cell replacement therapies involve the transplantation of insulin-producing beta cells to restore glucose homeostasis.

1.3.1 Whole pancreas transplantation

The proof-of-concept for beta cell replacement was first demonstrated in the late 1960s, with the first successful case of whole pancreas transplantation. Although the initial result was disappointing with the patient dying 3 months post-surgery, the success rate of pancreas transplantation increased over the years with improvements in surgical technique, patient selection criteria, and availability of new immunosuppressive drugs such as cyclosporin[77]. The transplantation of whole pancreas obtained from healthy donors could normalize blood glucose in patients with severe T1D, especially those with end-stage renal disease also requiring concomitant kidney transplantation. Hyperglycemia can be controlled if alloimmune and autoimmune responses are prevented using immunosuppressive drugs. The national 5-year patient survival rate in the UK was 88% for

simultaneous pancreas and kidney transplantation, and 78% for pancreas only transplantation, respectively[78].

Whole pancreas transplantation is a major abdominal surgery, and carries a risk of surgical complications including graft thrombosis, hemorrhage, pancreatitis, wound infection, peripancreatic abscesses and duodenal stump leakage[79]. The use of long-term immunosuppressive agents is also associated with many side effects such as increased risk of infection, malignancy, cardiovascular disease, and BM suppression[80]. Nonetheless, the widespread use of whole pancreas transplantation is limited by a severe shortage of donor organs. Currently, only patients with insulin dependent diabetes, normal insulin sensitivity, with severe metabolic instability and failure of insulin-based management, are eligible for pancreas transplant. Even with these strict inclusion criteria, only 30% of qualifying patients received a pancreas transplant in Canada[81].

1.3.2 Islet transplantation

Most patients with T1D have normal pancreatic exocrine function and did not require whole pancreas transplantation. Therefore, the concept of islet transplantation was proposed as a less invasive replacement for whole pancreas transplantation[82]. In 2000, Shapiro *et al* established the Edmonton protocol for islet transplantation, which involves infusing islets isolated from cadaveric donors into the hepatic portal vein of patients with T1D. Islets were isolated using enzymatic and mechanical digestion followed by density gradient purification. Transplanted islets become lodged in the portal sinusoidal capillaries and secreted insulin in response to glucose elevation. The 7 patients in the initial study each received ~11500 islet equivalents (IEQ)/kg body weight, and the procedure induced insulin independence and corrected HbA1C levels in all the patients[83].

However, out of 65 patients who received islet transplantation via the Edmonton protocol showed that only ~10% of the patients remained insulin independent at 5 years, possibly due to poor long-term engraftment and autoimmune or alloimmune destruction despite the use of immunosuppressive drugs. The mean duration of insulin independence was 15 months, and most patients required islets from 2 or 3 pancreas donors. Complications of immunosuppressive therapy included mouth ulcers, diarrhea, anemia, and ovarian cysts[84].

Although advancements in islet preparation and immunosuppressive therapies have been made in recent years, the 2020 report from the Collaborative Islet Transplant Registry showed re-infusion has been performed in about 73% of all allograft recipients, indicating complete or partial loss of graft function, and only 50% of participants were insulin independent at the 1-year follow-up[85].

Overall, islet transplantation was not effective in maintaining long-term insulin independence. Although islet transplantation is a less invasive alternative to whole pancreas transplantation, it has similar limitations such as side effects from immunosuppressive therapies and severe shortage of donor tissue. Each patient requires islets from multiple donors, but less than half of the donated pancreata are suitable for islet isolation. Moreover, there are only a few islet isolation centers around the world, further limiting the widespread application of this approach. Thus, there remains a need for a renewable source of islets for transplantation[86].

1.3.3 Alternative sources of beta cells for replacement therapy

Recent advances in stem cell technology have led to the development of new beta cell replacement strategies used for the treatment of diabetes. Embryonic stem cells (ESC) and induced pluripotent stem cells (iPSC) have self-renewal abilities and the potential to differentiate into all cell types from the 3 germ layers: ectoderm, endoderm, and mesoderm. Therefore, inducing pluripotent stem cells to become glucose-responsive insulin-producing beta cells presents a new therapeutic strategy for beta cell replacement. Currently, ESC and iPSC can be differentiated into pancreatic progenitors or insulin-producing beta-like cells *in vitro*, and transplantation of these cells reduced blood glucose in diabetic mouse models[87–90]. However, the efficiency of differentiating human ESC or iPSC into beta cells has been met with many challenges. One of the main drawbacks with this approach is that direct differentiation into mature beta cells was inefficient, and strict multistage differentiation protocols must be followed. Yet, most differentiation protocols result in immature beta cells, or a heterogenous population of beta-like, alpha-like, and enterochromaffin-like endocrine cells as well as ductal cells[91].

In 2014, a clinical trial involving transplantation of human ESC-derived pancreatic progenitor cells for T1D was led by ViaCyte Inc. Pancreatic progenitor cells were encapsulated in a semi-permeable immune-protective device to be transplanted subcutaneously. Upon transplantation, the cells were expected to mature into insulin-producing beta cells. However, the trial was quickly suspended due to inconsistencies in cell survival and poor cell engraftment, primarily caused by foreign body response, leading to cyst formation that prevented vascularization of the transplanted device[92]. In 2017, ViaCyte introduced an alternative device with a modified membrane that did not provide immune protection and allowed for graft vascularization. With concomitant use of immunosuppressive therapy, stimulated C-peptide was detected in 35% of participants, but none of the participants achieved insulin independence despite implantation of multiple units (~1,000,000 IEQ). The extracted device was found to be enriched with glucagon-expressing cells that did not expressing insulin[93,94]. Vertex Pharmaceutical is currently testing the transplantation of human ESC-derived islet-like organoids, both with and without an immuno-protective encapsulation device (VX-264 and VX-880). Although still early in the study, initial results from 2 patients who received VX-880 looked variable. Patient 1 was insulin independent and had 99.9% time-in-range for blood glucose at 270 days post-transplant. Patient 2 did not achieve insulin independence and had 51.9% time-in-range for blood glucose at 150 days post-transplant[95].

Overall, beta cell replacement therapies present a promising avenue to advance treatments for patients with severe diabetes. Nevertheless, there remain uncertainties with the reproducibility, long-term efficiency, and long-term safety of beta cell replacement therapies derived from human ESC or iPSC. More efficient differentiation protocols of the stem cells into beta cells as well as eliminating the need for immunosuppression will need to be established. Moreover, the cost and widespread accessibility of these therapies remain limited worldwide. However, these replacement therapies rely on the generation and/or delivery of exogenous insulin-secreting cells and fail to harness the innate endogenous regenerative potential of the pancreas.

1.4 Islet Regeneration *in situ*

Regeneration is the natural process of replacing and restoring cells and tissues damaged by age, diseases, or trauma. Some tissues have high regenerative capacity, such as liver, which can regrow to normal size after partial hepatectomy[96]. Therefore, regenerative medicine aims to develop therapies that promote endogenous islet regeneration in people with diabetes. Compared to replacement therapies, regenerative therapies may decrease the reliance on beta cell generation, transplantation, and immunosuppression, as well as alleviate some complications such as allorejection[97].

The human adult pancreas has limited regenerative capacity under normal physiological conditions, but evidence of beta cell regeneration has been observed during pregnancy, obesity, and metabolic stress[98]. The most compelling evidence of ongoing endogenous pancreas regeneration was presented in the Joslin Medalist Study[99]. The study evaluated the beta cell function of over 400 insulin-dependent patients that have had T1D for more than 50 years. Surprisingly, 67.4% of the participants had residual C-peptide (a by-product of insulin secretion) levels despite many years of autoimmune T1D. Post-mortem examination of pancreata obtained from multiple participants showed that islets contained proliferating beta cells as well as beta cells undergoing apoptosis, suggesting that residual beta cell mass undergoes a steady state turnover in the face of ongoing autoimmune destruction[99]. Therefore, therapies that could tip the balance to favour regeneration over destruction, may present a promising cure for diabetes.

While prevention of beta cell loss, including inhibition of beta cell apoptosis, necrosis, and dedifferentiation could augment beta cell survival, regeneration of beta cells is achieved by promotion of endogenous regeneration of new beta cells.

Several studies demonstrated that preventing beta cell death is therapeutically possible. Le May *et al* showed that estrogens protect beta cells from apoptosis and prevent insulin-deficient diabetes in mice. In brief, estradiol-deficient mice were more vulnerable to beta cell apoptosis and prone to diabetes after exposure to STZ, but administration of estradiol treatment was able to rescue STZ-induced beta cell apoptosis and help sustain insulin production[100]. Moreover, activation of thioredoxin interacting protein (TXNIP) triggers

apoptosis in beta cells when ER stress is prolonged[101]. In 2018, a phase II placebo-controlled clinical trial using daily Verapamil (TXNIP inhibitor) in recent-onset T1D in adult patients over 12 months demonstrated enhanced preservation of beta cell function, reduced hypoglycemic events and decreased insulin requirements, suggesting that preventing beta cell death may be an effective approach for diabetes treatment. [102]

Three central mechanisms have been proposed for the origin of new beta cells in the regenerating pancreas: generation of new beta cells from (1) pre-existing beta cells via cell replication, (2) pancreatic progenitor cells via neogenesis, and (3) other pancreatic cell types via transdifferentiation.

First, beta cells can dynamically respond to increased metabolic demand to expand their functional mass during physiological and pathological changes such as pregnancy and obesity[103,104]. To date, several human beta cell mitogens have been discovered, including harmine, GNF4877, INDY, and 5-IT. Many of these mitogens function to inhibit dual-specificity tyrosine-phosphorylation regulated kinase 1 (DYRK1)[105,106]. Specifically, Ackeifi *et al* showed that simultaneous silencing of both DYRK1A and DYRK1B using small molecules yielded greater beta cell proliferation than silencing either one individually[106]. Yet, beta cell adaptability is known to be governed by the age-related decrease of replication[107].

Secondly, whether a beta cell progenitor exist in the adult pancreas remains a controversy. Beta cell neogenesis from ductal regions has been suggested since fetal beta cells were derived from the differentiation of cytokeratin (CK) 19+ ductal cells. In 1995, Wang *et al* found that regeneration of beta and alpha cells was pronounced following partial pancreatic duct ligation in rats, and beta cell proliferation alone could not account for the expansion of beta cell population. Rather, they observed extensive proliferation and the expression of GLUT2 in ductal cells, suggesting that ductal proliferation and differentiation represents a major mechanism of beta cell neogenesis[108]. Using lineage tracing techniques, Zhang *et al* showed that Sox9+ pancreatic ductal cells can be induced into beta cells in mice, and gastrin and epidermal growth factors could augment this process, resulting in formation of new beta cells and reversal of diabetes[109]. In contrast, Kopp *et al* also traced Sox9+ ductal

cells, but they found that ductal cells did not differentiate into insulin-expressing cells during normal post-natal development nor after partial pancreatic ductal ligation[110]. Overall, more investigations will be needed to elucidate the identity and function of CK19+ beta cell progenitors in the adult pancreas.

Thirdly, pancreatic ductal, acinar, alpha, delta, and PP cells have been shown to have the capacity to transdifferentiate into beta-like cells that express insulin, to help replenish beta cells loss in diabetes. For instance, Bonner-Weir *et al* showed that human ductal cells could be cultured in Matrigel and form islet-like buds. These cells were detected using CK19 and insulin antibodies[111,112]. Kim *et al* observed that cotreatment of activin A and exendin-4 induced expression of beta cell specific markers in human CK19+ ductal cells, and transplantation of differentiated ductal cells normalized hyperglycemia in STZ-treated immunodeficient mice[113]. Acinar cells in the adult pancreas also demonstrate high plasticity after rapid and major beta cell loss. In response to pancreatic inflammation or injury, acinar cells can undergo transdifferentiation to a progenitor-like cell phenotype with ductal characteristics via acinar-to-ductal metaplasia[114]. Moreover, acinar cells have been shown to transdifferentiate into beta cells both *in vitro* and *in vivo*, with the generation of ductal cells as an intermediate step. Zhou *et al* demonstrated that the combination of 3 transcription factors, NGN3, MAFA, and PDX1, could directly reprogram pancreatic acinar cells into beta-like cells to secrete insulin and ameliorate hyperglycemia in diabetic mice[115]. In addition, alpha cells as a source for the replacement of beta cells has been explored in diabetes. Using lineage tracing, Thorel *et al* showed that loss of beta cells in adult mouse panaceas lead to the conversion of alpha cells into beta cells, with the appearance of cells that are double positive for insulin and glucagon as intermediate[116]. Similarly, Wei *et al* reported that an antagonistic glucagon receptor antibody promoted alpha cell proliferation, and subsequently increased beta cell mass in diabetic mice[117]. The pathway of alpha to beta cell transdifferentiation is often reported based on the epigenetic signatures of key phenotypic gene expression between the two cell types, and the ease with which these can be reversed[118]. Overall, alpha cells appear to be a promising target for the regeneration of beta cell mass in diabetes. Recent studies have suggested that beta cells pairing with delta cells benefit from stress protection, and there is evidence of conversion of mono-hormonal somatostatin+ cells to bi-hormonal cells that co-express somatostatin

with insulin following beta cell destruction[118,119]. Similarly, Perez-Frances *et al* discovered heterogeneity of the PP cell population, with the presence of bi-hormonal cells that share lineage-specific markers typical of other islet cell types. They showed that PP cells exhibited gene expression changes and some of those engaged in insulin production upon beta cell injury[120]. Last but not least, ghrelin produced by epsilon cells is involved in the growth and proliferation of beta cells, and the prevention of beta cell apoptosis and insulin insensitivity[121–123].

Overall, these findings suggest that both exocrine and exocrine cells in the pancreas have the potential to transdifferentiate into insulin-producing cells, and thus raising the possibility of targeting non-beta cells in the pancreas in regenerative therapies for diabetes.

The signaling factors responsible for initiating beta cell regeneration from proliferation, endocrine progenitors, or transdifferentiation from other pancreatic cell types require further characterization. It is unlikely that a single effector could be responsible for mediating this complex process and several pathways may act synergistically to restore or maintain beta cell mass and alleviate hyperglycemia.

1.4.1 Cell-based regenerative therapies for diabetes

Stem cell-based regenerative therapies for diabetes have been actively studied for many years. In 2003, Hess *et al* first demonstrated that the transplantation of murine BM-derived stem cells could initiate pancreatic regeneration via unknown paracrine mechanisms, rather than by the differentiation of transplanted cells into insulin-producing cells *in vivo*. It was found that transplantation of BM-derived stem cells ameliorated hyperglycemia and increased serum insulin levels. The transplanted cells did not express insulin at the onset of glucose reduction. Rather, transplanted cells engrafted into the pancreas and initiated islet cell proliferation, which resulted in increased insulin-expressing islets in the recipient mice[124]. Subsequent studies by Bell *et al* in the Hess lab purified progenitor cells from BM and UCB using high-aldehyde dehydrogenase activity (ALDH-hi), an enzymatic function conserved in hematopoietic, endothelial, and mesenchymal progenitor cell lineages. The purified ALDH-hi cell populations ameliorated hyperglycemia, increased beta cell mass, and induced islet cell proliferation and islet vascularization. Specifically, ALDH-hi cells

demonstrated more potent islet regenerative effects when they were delivered via direct intrapancreatic injection compared to intravenous injection[125,126]. Taken together, these studies demonstrated the therapeutic potential of stem cells in the promotion of beta cell regeneration.

Of the progenitor cell types that comprise human BM and UCB sources, MSC hold great promise to promote islet regeneration due to their immunomodulatory properties and supportive functions for tissue repair. In terms of treatment for diabetes, initial studies using MSC focused on differentiating MSC into insulin-producing cells for transplantation. However, MSC-derived insulin-producing cells were generated at a lower efficiency than ESC or iPSC sources, and often lacked mature beta cell markers[127]. In 2006, Lee *et al* first showed that human BM-MSC alone were effective in improving hyperglycemia by promoting islet repair when delivered via intracardiac infusion into STZ-treated non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice, suggesting that the islet regenerative effects previously observed by Hess *et al* were partially due to the actions of BM-MSC[128]. To confirm this speculation, another study in the Hess lab conducted by Bell *et al* tested human BM-MSC transplantation in STZ-treated NOD/SCID mice via tail vein injection. The transplanted MSC did not differentiate into beta cells *in vivo*, but activated regeneration of the murine pancreas, indicated by an increased number of small islets associated with the ductal regions, to reduce hyperglycemia. However, it was noted that human BM-MSC induced islet regeneration in a donor-dependent manner. Approximately 25% of the MSC lines transplanted could revert hyperglycemia, suggesting that the regenerative capacity of MSC was cell line and donor cell specific. Therefore, BM-MSC were categorized into regenerative MSC and non-regenerative MSC[126]. Since MSC mediate regenerative functions via paracrine signalling, transplantation of MSC to stimulate beta cell regeneration *in situ* represents a promising therapeutic approach.

It has been proposed that MSC could improve human islet transplantation through their regenerative and immunomodulatory properties to address issues such as poor engraftment, poor vascularization, and inflammation[129]. Co-culture of human BM-MSC with human islets improved glucose-stimulated insulin secretion and prevented beta cell apoptosis in the presence of pro-inflammatory cytokines[130]. More recently, allogeneic islets

transplanted with autologous BM-MSC in STZ-treated cynomolgus monkeys showed that the co-transplantation enhanced islet engraftment, survival, and glucose stimulated insulin release[131]. A meta-analysis of 20 islet and MSC co-culture studies found that islet viability and glucose responsiveness were consistently improved when islets were co-cultured with MSC, and that the viability were higher for islets co-cultured indirectly with MSC than those in direct contact[132].

Up to 2015, 493 clinical trials based on the use of MSC, including the use of MSC for the treatment of diabetes, have either been completed or are currently ongoing according to the US National Institute of Health ClinicalTials.gov database[133]. A clinical trial of portal vein infusion of islets along with autologous BM-MSC was conducted by Wang *et al* in 2018. Three patients with chronic pancreatitis underwent total pancreatectomy and subsequently received MSC and islet co-transplantation. Patients showed improved glycemic control and no direct adverse events at 1-year follow-up, suggesting that MSC co-transplantation with islets provides therapeutic benefits over transplantation of islets alone[134]. The first randomized controlled trial using MSC for 20 patients with recent-onset T1D was conducted by Carlsson *et al* in 2015. Patients received autologous MSC at 2.75×10^6 cells/kg body weight intravenously. Analyses of residual beta cell function showed that transplanted recipients had increased or sustained C-peptide levels, suggesting that MSC may preserve beta cell function[135]. Recent results from another phase I/II clinical trial involving autologous MSC transplantation in newly diagnosed T1D patients showed that MSC transplantation is safe and effective in reducing hypoglycemia episodes and HbA1C levels. However, none of the participants achieved insulin-independence, suggesting the possible reduction in therapeutic efficacy of autologous MSC in diabetic patients[136]. Compared to MSC of healthy subjects, MSC of diabetic patients had different expression of markers, morphology, and increased susceptibility to apoptosis and senescence[137].

Overall, regenerative therapies involving transplantation of stem cells hold multi-faceted promise for diabetes treatment. However, cell-based therapies are associated with ongoing drawbacks including risks of alloimmune or continued autoimmune responses, teratoma formation from pluripotent cell sources, and barriers to long-term transplant viability and functionality, and the accessibility of therapy[138].

1.4.2 Cell-free regenerative therapies for diabetes

Autologous and allogeneic MSC have been shown to be safe for transplantation, but they can still trigger an immune response due to low expression of MHC class I molecules[139]. Therefore, the use of conditioned media or exosomes collected from MSC as a cell-free alternative could circumvent complications associated with administering allogeneic MSC to patients[140]. In addition to having less immunogenicity, a cell-free, protein-based therapy could allow for more efficient production, delivery, and storage of the therapeutic product, together with a lowered risk of tumorigenicity and undesired differentiation of transplanted cells[141].

In 2017, a previous study in the Hess lab by Kuljanin *et al*, generated conditioned media (CM) from regenerative MSC versus non-regenerative MSC, which allowed the complex secretome of MSC to be analyzed and compared by global mass spectrometry-based analyses. Using STZ-treated NOD/SCID mice, Kuljanin *et al* found that direct intrapancreatic injection of CM collected from human BM-MSC induced islet regeneration via mechanisms similar to the transplantation of BM-MSC, through an increased presence of small islets associated with pancreatic ductal regions[142]. Similarly, Sabry *et al* in 2020 showed that intraperitoneal injection of exosomes isolated from albino rat BM-MSC was equally effective in terms of the glucose-lowering capacity compared to MSC[143]. While both treatment groups demonstrated islet regeneration, immunohistochemical analyses revealed rats that received exosomes had increased expression of insulin and PDX1 in islets compared to the MSC-treated group[143].

Kuljanin *et al*. from the Hess lab used mass-spectrometry-based proteomic analysis to compare the CM from regenerative versus non-regenerative MSC. Generation of CM was achieved using a molecular weight cut-off (MWCO) filter at 3 kDa that is also known to concentrate MSC-secreted exosomes containing proteins, long non-coding RNAs, and micro-RNAs. Analyses were performed on both RNAs and proteins, revealing that residual RNAs in the CM was severely degraded during collection and concentration of the media without ribonuclease inhibition. They found that regenerative MSC uniquely upregulated proteins involved in angiogenesis (*KDR, FLT4*), regulation of cell growth (*EGF, IGF2, WISP2, KIT*), matrix remodeling (*MMP3/10/12, ADAMTS13/18*), and active canonical

Wnt signalling (*SPON2, Wnt5a/b, WISP2*). Non-regenerative MSC uniquely upregulated proteins involved in inflammation (*CXCL2/3/5, IL-1B/6/8*)[142]. Therefore, human BM-MSC may induce pancreatic regeneration through the secretion of proteins into the microenvironment that form a niche permitting endogenous islet regeneration and active Wnt signalling was found to play a central role in this regenerative process[142].

Wnt signalling is an evolutionarily conserved pathway that regulates aspects of cell migration, fate determination, proliferation, polarity, organogenesis as well as stem cell differentiation and self-renewal[144]. Aly *et al* cultured intact human islets with CM collected from L cells that expressed several Wnt ligands, and showed that activation of Wnt signalling significantly increased human beta cell proliferation while maintaining differentiated beta cell phenotype[145]. In addition, Wang *et al* showed rat BM-MSC secreted Wnt signalling activators to prevent stress-induced apoptosis in co-culture with a murine islet microvascular endothelium cell line[146]. These studies suggested the potential of active Wnt signalling in islet regeneration induced by MSC transfer.

There are 2 types of Wnt signalling, canonical Wnt signalling that is dependent on beta-catenin, and non-canonical Wnt signalling independent of beta-catenin. Beta-catenin is a protein involved in regulation and coordination of cell-to-cell adhesion and gene transcription. During inactive Wnt signalling, intracellular beta catenin is maintained at a low concentration by undergoing rapid turnover by the multi-unit destruction complex, consisting of the scaffolding protein Axin, adenomatous polyposis coli (APC) protein, disheveled, serine/threonine glycogen synthase kinase (GSK3), and casein kinase 1 (CK1). Phosphorylation of beta catenin by GSK3 is a key step to initiate beta-catenin degradation in the proteasome. During active Wnt signalling, the binding of Wnt ligands to the Frizzled receptor recruits disheveled to the receptor complex. Disheveled interacts with Axin and recruits the destruction complex to the Frizzled receptor. Once in proximity, phosphorylation mediated by CK1 and GSK3 binds Axin with the co-receptor, preventing it from dissociating from the membrane. As a result, the destruction complex cannot phosphorylate beta-catenin for degradation. Excess intracellular beta catenin accumulates and translocates to the nucleus to regulate gene transcription[144].

In 2019, another previous study in Hess lab by Kuljanin *et al* aimed to assess the role of active canonical Wnt signalling in MSC-stimulated islet regeneration. Wnt-stimulated (Wnt+) CM was generated by culturing MSC with a pharmacological Wnt-pathway activator, CHIR99021. CHIR99021 is a highly selective inhibitor for GSK3, preventing the phosphorylation-dependent degradation of beta-catenin. Wnt-inhibited (Wnt-) CM was also generated by culturing MSC with a pharmacological Wnt-pathway inhibitor, IWR1, which acts by stabilizing Axin. They found that Wnt+ CM augmented beta cell viability and proliferation in human islet cultures *in vitro*, and reversed hyperglycemia in STZ-treated NOD/SCID mice following iPan injection. Mice that received Wnt+ CM showed increased beta cell mass, beta cell proliferation and islet number. Wnt- CM did not induce islet regeneration, while untreated CM induced partial recovery of islet function. Therefore, active canonical Wnt signalling in MSC plays a central role in the ability of MSC to stimulate islet regeneration via paracrine secretions[147].

1.5 Rationale, Hypothesis, and Objectives

1.5.1 Rationale

Our previous studies by Kuljanin *et al* demonstrated the islet regenerative effects of "regenerative BM-MSC" as well as "Wnt+ MSC CM" in STZ-treated NOD/SCID mice[142,147]. However, the specific factors (i.e., proteins) within the CM that were responsible for mediating islet regeneration required further characterization.

In our first proteomic analysis using comprehensive mass-spectrometry, we identified 470 proteins that were differentially expressed between regenerative CM and non-regenerative CM using a 2-fold cut-off. Of the 470 proteins, 253 were upregulated in regenerative CM compared to non-regenerative CM[142].

We conducted a second mass-spectrometry-based proteomic analysis comparing the Wnt+ CM and untreated CM. Here, we identified 57 proteins that were upregulated in the Wnt+ condition using a 1.5-fold cut-off (unpublished data by Kuljanin *et al*).

Combining these two proteomic screens, 8 proteins were found to be significantly upregulated in both the regenerative CM and Wnt+ CM, and thus, may be implicated in

the induction of beta cell regeneration: FAM3 metabolism regulating signalling molecule C (FAM3C), prosaposin (PSAP), superoxide dismutase 1 (SOD1), peptidylprolyl isomerase A (PPIA), galectin 1 (GAL1), cathepsin B (CTSB), transglutaminase 2 (TGM2), and calumenin (CALU). Although the biological functions of these proteins have been studied in other contexts (Table 1.1), it is still unknown whether these proteins directly or indirectly affect islet regeneration. Therefore, the islet regenerative capacity of each individual protein requires functional validation. This project aims to assess the islet regenerative functions of these 8 MSC-secreted proteins in combination as well as individually, using both *in vitro* and *in vivo* model systems. We predict that the 8 proteins will act synergistically to induce islet regeneration that restores beta cell mass and alleviates hyperglycemia.

1.5.2 Hypothesis and Objectives

We hypothesize that the 8 MSC-secreted proteins commonly upregulated in the regenerative MSC CM and Wnt+ CM will promote human islet cell viability and proliferation *in vitro* and/or induce islet regeneration after iPan injection into STZ-treated mice *in vivo*.

To address this hypothesis, we proposed the following objectives:

(1) To determine the cell lineage-specific viability and proliferation of human islets cultured with Wnt+ CM, 8-protein combination, or individual proteins for 7 days.

(2) To assess islet regeneration in NOD/SCID mice following beta cell ablation with STZ and iPan injection of Wnt+ CM, 8-protein combination, or individual proteins.

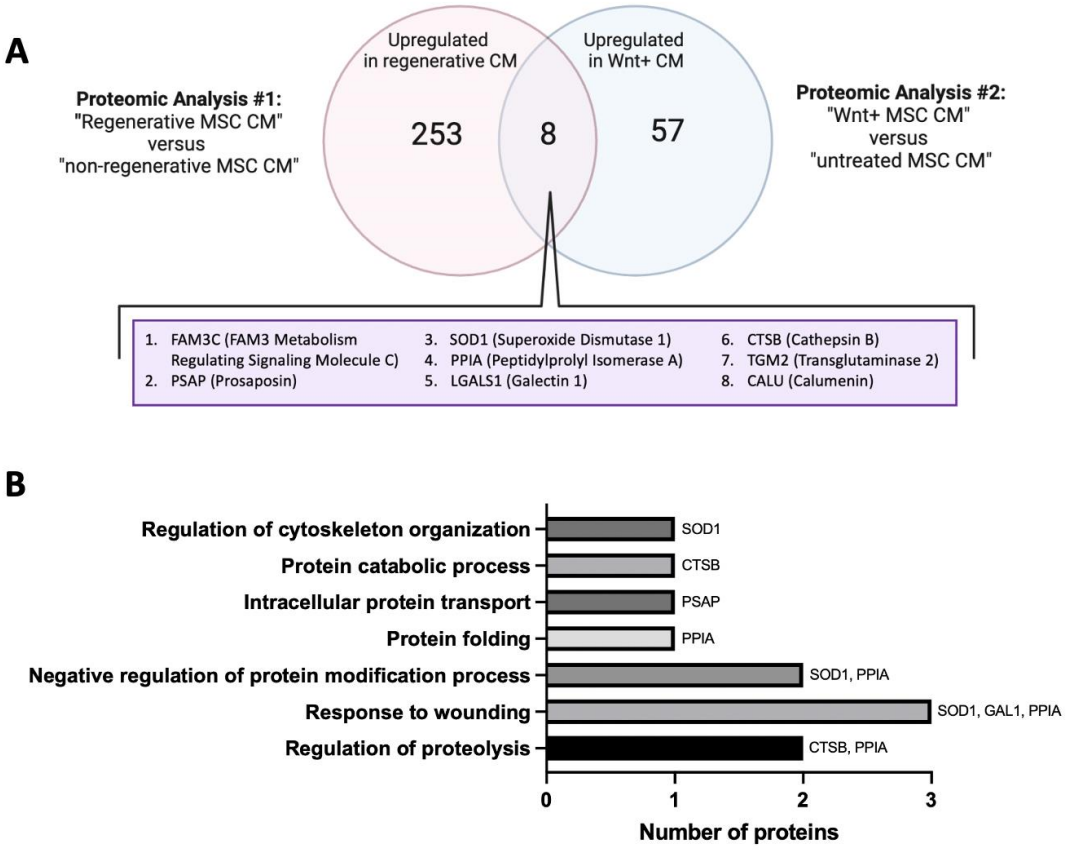

Figure 1.1 Proteomic analyses identified 8 MSC-secreted proteins as candidates to mediate islet regeneration.

(A) Mass-spectrometry-based proteomic analyses comparing the regenerative MSC CM to non-regenerative MSC CM and Wnt+ MSC CM to untreated MSC CM revealed 8 proteins that were commonly upregulated in regenerative CM and Wnt+ CM. **(B)** Gene Ontology Biological Process (GOBP) analysis revealed that the 8 proteins are commonly involved in some common biological processes that could potentially impact beta cell regeneration.

Table 1.1 Summary of the known biological functions and other implications of the 8 MSC-secreted protein candidates.

Gene symbol	Protein name	Biological functions	Other implications
FAM3C	FAM3 metabolism regulating signaling molecule C	Hepatic glucose and lipid metabolism	Tumour metastasis; epithelial to mesenchymal transition (EMT)
PSAP	Prosaposin	Lysosomal catabolism of glycosphingolipids	Development and protection of neurons; maintenance of biological systems
SOD1	Superoxide dismutase	Antioxidant defense against oxidative stress	Metabolism and gene transcription; REDOX signalling
PPIA	Peptidyl-prolyl cis-trans isomerase A	Protein folding	Cell signalling and recruitment; transcriptional regulation
LGALS1	Galectin 1	Cell-cell and cell-matrix interactions	Cell proliferation; cell differentiation; cell migration; apoptosis; inflammation; angiogenesis
CTSB	Cathepsin B	Lysosomal proteolytic degradation	Tumour metastasis; apoptosis; autophagy; cell proliferation; remodelling
TGM2	Transglutaminase 2	Multifunctional: protein modification	Angiogenesis; wound healing; cell adhesion; cell differentiation; apoptosis
CALU	Calumenin	Protein folding/sorting	Tumour metastasis; EMT; wound healing; immune response

1.5.3 Description of the 8 proteins implicated in islet regeneration

1.5.3.1 FAM3C

FAM3 metabolism regulating signaling molecule C (FAM3C) is a member of the FAM3 cytokine family along with FAM3A, FAM3B, and FAM3D. It is primarily expressed in secretory epithelial cells in humans, notably the pancreas, mammary gland, duodenum, and salivary gland. FAM3C has many biological roles including the regulation of hepatic glucose and lipid metabolism, osteogenic differentiation, retinal laminar formation, and brain amyloid beta peptide production in the brain[148].

In obesity, disruption of FAM3C signalling promotes the pathogenesis of diabetes[149]. In recent studies, FAM3C has been shown to improve insulin resistance and hyperglycemia in obese mice[150]. Liver analyses of STZ-treated mice showed repression of the FAM3C-HSF1-CaM signalling pathway while hepatic FAM3C overexpression activated the HSF1-CaM-Akt axis to downregulate gluconeogenic and lipogenic gene expression, and improve insulin resistance and hyperglycemia[149,150].

Importantly, FAM3C is also a known inducer of EMT in cancer, and is implicated in tumor formation, invasion, and metastasis. FAM3C promotes tumour formation by regulating associated proteins including Ras, STAT3, TGF-beta and LIFR. Increasing evidence suggests that FAM3C depletion improves survival outcomes by reducing tumorigenicity and lowering levels of proteins involved in tumour progression[148].

1.5.3.2 PSAP

Prosaposin (PSAP) is a highly conserved glycoprotein expressed on neuronal membranes and is cleaved via endosomal proteolysis into four homologous proteins: sphingolipid activator proteins (saposins) A-D. Mature saposins subsequently aid in the activation of lysosomal hydrolases for glycosphingolipid catabolism[151].

PSAP present on nervous tissue is implicated in a wide variety of neuroprotective and glioprotective processes. Addition of PSAP to Schwann cells enhanced phosphorylation of mitogen activated kinases ERK1 and ERK2, preventing cell death. Moreover, PSAP is found extracellularly in physiological fluids such as pancreatic secretions, bile,

cerebrospinal fluid, milk, and seminal fluid, suggesting a role in maintaining biological systems. In the testis and epididymis, it is involved in the maturation of sperm and enhancement of fertility[152]. Activity of PSAP specifically in the pancreas has not been reported.

Overexpression of PSAP is associated with tumorigenesis and cancer proliferation and progression. Specifically, high levels of PSAP were found to be an indicator of poor prognosis in patients with glioblastoma, prostate cancer, and breast cancer. In glioblastoma, PSAP was implicated in the invasion and migration of malignancy through the TGF-beta 1/Smad signaling pathway[153].

1.5.3.3 SOD1

Cu/Zn Superoxide Dismutase 1 (SOD1) is an antioxidant enzyme that functions to convert superoxide radicals, byproducts of cellular respiration, into oxygen and hydrogen peroxide. In the context of oxidative diseases, SOD1 dysfunction plays a role in several pathologies. SOD1 is primarily known for its contribution to amyotrophic lateral sclerosis (ALS). Mutations of SOD1 result in pathological misfolding and aggregation, ultimately leading to oxidative stress and neuron death[154,155]. Overexpression of SOD1 is seen in numerous cancer types, such as lung and breast cancers[154]. It is likely that protection against oxidative damage confers a growth advantage optimal for tumorigenesis.

The pro-survival mechanisms of SOD1 appears to be largely due to its antioxidant properties[154]. While abundant in the cytosol, beneficial effects are localized to the mitochondrial intermembrane space, where only a small fraction of total cellular SOD1 resides[156]. The majority of the SOD1 pool is responsible for non-canonical functions, namely peroxide-mediated redox reactions, acting as a nuclear transcription factor, zinc metabolism, and copper buffering[154].

Interestingly, deficiencies in protective molecules such as SOD1 have been reported in insulin resistance and diabetes. Studies have revealed that oxidative stress buildup in T2D targets beta cells, triggering dysfunction. Genetic ablation of murine SOD1 resulted in premature glucose intolerance, reduced phase I and II insulin secretion, and decreased beta

cell volume[157,158]. Moreover, increased SOD1 expression was associated with decreased fasting blood glucose, HbA1C, and the survival of hypertrophied beta cells during chronic hyperglycemia in multiple *in vivo* systems[158].

1.5.3.4 PPIA

Peptidylprolyl isomerase A (PPIA), also known as cyclophilin A (CypA), belongs to the class of cyclophilins that make up peptidylprolyl isomerases along with FK506 binding proteins, and parvulins[159]. These isomerases catalyze the cis-trans isomerization of proline peptide bonds to expedite protein folding. PPIA is also involved in cell signalling, protein trafficking, as well as acting as a receptor for cyclosporine A (CsA)[159,160]. Additionally, PPIA is responsible for the recruitment of immune cells, acting as a chemoattractant for monocytes, neutrophils, eosinophils, and T cells[161].

PPIA participates in many processes underlying the pathologies of an array of diseases. It is implicated during viral infections, cardiovascular diseases, disorders of the nervous system, and upregulated in cancers. Notably, elevated plasma and urine PPIA were found in patients with T2D, suggesting an association between PPIA and diabetes pathology[159,160]. Hyperglycemia-induced activation of inflammatory genes is considered to be responsible to amplify monocyte activation, leading to elevated secretion of PPIA[161]. Increased PPIA levels in early stages of T2D were found to be a reliable inflammatory biomarker of coronary heart disease and artherosclerosis[161,162].

1.5.3.5 GAL1

Galectin 1 (GAL1), encoded by the gene *LGALS1*, is a carbohydrate-binding protein belonging to the galectin family. Functions of GAL1 are multifaceted including cell adhesion, cell cycle progression, cell growth and proliferation, apoptosis, inflammation, cell migration, and angiogenesis, among others. Expression of GAL1 is highest in subcutaneous adipose tissue, vasculature, and female reproductive tissues[163].

GAL1 has also been proposed as a mediator associated with insulin resistance and diabetes. While the majority of evidence relating GAL1 to insulin resistance is focused on adipose tissue and inflammation, GAL1 is also altered in various cell types with the insulin resistant

state. Interactions between GAL1 and multiple metabolic signalling pathways downstream of the insulin receptor are proposed to contribute to insulin resistance. However, pathways with the highest clinical relevance have not been elucidated[163].

1.5.3.6 CTSB

Cathepsin B (CTSB) is a member of the cysteine cathepsin family responsible for driving proteolytic degradation in the lysosome and extra-lysosomal space. Cathepsins are generally implicated in autophagy, antigen presentation, cellular stress signaling, metabolism, and lysosome-dependent apoptosis. Increased levels of CTSB is a risk factor for tumor formation, proliferation, and migration, increasing cancer angiogenesis and resistance to chemotherapy[164].

CTSB has been extensively associated with diabetes and obesity[165]. An important role of CTSB is the conversion of proinsulin to insulin. It has been suggested that insulin resistance further disrupts insulin homeostasis in exhausted beta cells through the downregulation of CTSB[166]. Further, overexpression of CTSB in human skeletal muscle inhibits the degradation of IRS-1 and GLUT4, thereby decreasing insulin resistance[165].

1.5.3.7 TGM2

Transglutaminase 2 (TGM2) is a calcium-dependent enzyme with multiple roles, including apoptosis, wound healing, cell adhesion, and insulin release. In mice, dysregulation of TGM2 expression is associated with increased glucose levels and disrupted glucose-mediated insulin release. A SNP in the coding region of *LncTGM2* in pancreatic beta cells was associated with increased risks of T2D[167]. This is in part due to the regulatory functions of *LncTGM2*; *LncTGM2* is an activator of the *TGM2* promoter, thus modulating transcriptional activation. LncRNAs harboring the SNP associated with T2D results in disrupted TGM2 secondary structure, leading to decreased stability and expression in beta cells. Additional studies demonstrated that *Tgm2*$^{-/-}$ mice were glucose intolerant and had impaired insulin secretion, indicating the important role of TGM2 in glucose homeostasis[167,168].

Analyses of the *TGM2* gene in 205 patients with early-onset T2D demonstrated novel activity-impairing mutations not present in 300 normoglycemic control subjects, validating the importance of functional TGM2 in glucose metabolism[168].

1.5.3.8 CALU

Calumenin (CALU), a member of the CREC protein superfamily, is a multiple EF-hand calcium-binding protein localized to secretory functions of the endoplasmic reticulum[169,170]. Its primary functions include the maintenance of calcium homeostasis, protein folding, and secretory cargo sorting. Further influences of CALU consist of cell migration, invasion, and metastasis during tumorigenesis, wound healing, immune response, and blood coagulation[171].

In cancer, CALU expression is often altered. While downregulated in endometrial cancer and pancreatic carcinoma among others, CALU expression was increased in clinical colon cancer samples[173]. This contradiction may be explained by varying functions of different CALU isoforms. Regardless, CALU is routinely associated with invasiveness and metastasis in malignancies. Specifically, CALU was correlated with 3 EMT pathways: TGF-beta, PI3K/AKT, and hypoxia pathway[171].

Chapter 2

2 Methods

2.1 Human recombinant MSC-secreted proteins

The full-length recombinant human versions of the 8 MSC-secreted proteins were carefully selected and acquired from commercially available sources as detailed in Table 2.1. Proteins were aliquoted and stored according to manufacturers' instructions until use for *in vitro* and *in vivo* experiments.

2.2 MSC Culture and CM Collection

2.2.1 MSC isolation from human bone marrow

Human bone marrow (BM) was obtained from 7 independent healthy donors after informed consent at the London Health Science Center (Victoria Hospital, London, ON). The study using human BM-derived MSC for beta cell regeneration was approved by the Human Research Ethics Board at Western University (REB #12934, Appendix 1). Mononuclear cells (MNC) were isolated from BM aspirates following centrifugation on a Hypaque Ficoll gradient and erythrocyte lysis with ammonium chloride solution (StemCell Technologies, Vancouver, BC). Isolated MNC were plated at 270 000 cells/cm^2 in AmnioMAX^{TM} C-100 Complete Media (Thermo Fisher Scientific, Waltham, MA), that includes fetal bovine serum (FBS), gentamicin, and L-glutamine to establish MSC colonies adherent to plastic.

Table 2.1 Human recombinant proteins.

NA stands for not available.

Gene symbol	*FAM3C*	*PSAP*	*SOD1*	*PPIA*	*LGALS1*	*CTSB*	*TGM2*	*CALU*
Protein symbol	FAM3C	PSAP	SOD1	PPIA	GAL1	CTSB	TGM2	CALU
Company	Abcam	Novus Biologicals	Novus Biologicals	Ray Biotech	Bio-Techne	Abcam	Novus Biologicals	Novus Biologicals
Catalog #	ab151637	H00005660-P01	NBP2-34942	230-00764	1152-GA	ab283434	NBP2-52009	NBC1-26372
Purity	>95%	>80%	>95%	>95%	>97%	>95%	>85%	>90%
Protein length	Full	Full	Full	Full	Full	Full	Full	Full
Size (kDa)	23	81	40	21	15	36	80	37
Molar Quantity for 100 ng (pmol)	4.35	1.23	2.50	4.76	6.67	2.78	1.25	2.70
Endotoxin (Eu/µg)	<1	NA	<1	NA	<0.1	<0.005	NA	NA
Function tests	NA	NA	Potency: >1600 U/mg	NA	ED50: 0.5-3 µg/mL	Fully active: cleave Z-LR-AMC	NA	NA
Expression system	HEK 293 cells	Wheat Germ	*E. coli*	*E. coli*	*E. coli*	HEK 293 cells	*E. coli*	*E. coli*

2.2.2 Generation of MSC CM

Human BM-MSC were expanded until 80% confluency at passage 4. AmnioMAX^{TM} C-100 Complete Media was then removed, MSC were washed three times with phosphate-buffered saline (PBS) to remove residual proteins and growth factors contained within the media, and replaced with supplement-free AmnioMAX^{TM} C-100 Basal Media (Thermo Fisher Scientific). MSC were either treated with: (1) 10 μM CHIR99021 (AbMole Biosciences, Houston, TX), a GSK3 inhibitor, to generate Wnt+ CM by activating the canonical Wnt pathway; or (2) dimethyl sulfoxide (DMSO) vehicle control to generate untreated CM. After 24 hours of culture, CM was collected in 3 kDa centrifugal filter units with regenerated cellulose membrane (Millipore, Burlington, MA) by centrifuging at 2800 x g for 75 minutes. The resultant concentrated CM contained MSC-secreted proteins larger than 3 kDa and included microparticles and exosomal contents. The protein concentration was quantified using the Pierce 660 nm Protein Assay (Thermo Fisher Scientific) according to manufacturer's instructions, analyzed using a SpectraMax Plus 384 Microplate Reader and SoftMax Pro 7.0 (Molecular Devices, San Jose, CA). CM were either used immediately for *in vivo* experiments or frozen at -80°C in preparation for *in vitro* human islet culture experiments.

2.2.3 Flow cytometry on MSC: intracellular beta-catenin analysis

BM-MSC were treated with 10 μM CHIR99021 or DMSO vehicle control to generate Wnt+ MSC or untreated MSC, respectively. After CM collection, MSC were harvested and fixed for 5 minutes using 10% buffered formalin (Thermo Fisher Scientific) and permeabilized with 1% Triton X-100 (Thermo Fisher Scientific) for 15 minutes. MSC were incubated with anti-human beta-catenin antibody (Table 2.2) for 30 minutes at room temperature while protecting from light. Flow cytometry data were collected at the London Regional Flow Cytometry Facility (Robarts Research Institute, London, ON) using an LSR II flow cytometer (BD Biosciences, Mississauga, ON). Percentage increase in beta-catenin mean fluorescent intensity (MFI) was calculated for Wnt+ MSC compared to untreated MSC using FlowJo software (FlowJo LLC, Ashland, OR). An overview of the experimental procedures described under **2.2 MSC Culture and CM Collection** is shown in Figure 2.1.

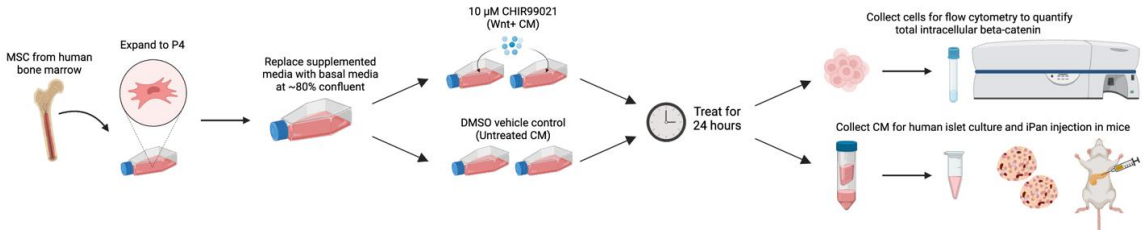

Figure 2.1 Overview of BM-MSC culture and generation of MSC CM.

MSC at passage 4 were treated with CHIR99021 to generate Wnt+ CM, or with DMSO vehicle to generate untreated CM. After 24 hours, CM were collected and concentrated using a 3kDa cut-off spin column for later experimental use. MSC were also collected to quantify total intracellular beta-catenin by flow cytometry. Figure created with BioRender.com.

2.3 Human Islet Culture and Flow Cytometry

2.3.1 Human islet isolation and culture

Fresh cadaveric human islets from 6 independent non-diabetic adult donors were obtained through the Integrated Islet Distribution Program (IIDP, USA) or the University of Alberta IsletCore (Alberta Diabetes Institute, Edmonton, AB). These studies included islet samples from both male (N=4) and female (N=2) donors. Islets from each donor were seeded in 6-well (9.6 cm^2) plates (Thermo Fisher Scientific) at 200 islet equivalents (IEQ) per well in 3 mL of DMEM basal media, supplemented with 5.5 mM glucose, 1.0 mM sodium pyruvate, and 4.0 mM L-Glutamate (Thermo Fisher Scientific). Each donor sample was divided into the following treatment groups: (1) DMEM basal media (vehicle control), (2) 10% FBS (positive control), (3) Wnt+ CM (4 μg/mL), (4) 8-protein combination (100 ng/mL/protein), (5) FAM3C (100 ng/mL), (6) PSAP (100 ng/mL), (7) SOD1 (100 ng/mL), (8) PPIA (100 ng/mL), (9) GAL1 (100 ng/mL), (10) CTSB (100 ng/mL), (11) TGM2 (100 ng/mL), and (12) CALU (100 ng/mL) and incubated at 37°C and 5% CO_2 for 7 days. Media change including recombinant human protein supplementation was performed every 2-3 days.

2.3.2 Analyses of human islets by flow cytometry

Human islet cell proliferation was assessed using Click-iT™ Plus EdU Pacific Blue™ Flow Cytometry Assay Kit according to manufacturer's instructions (Thermo Fisher Scientific). EdU (5-ethynyl-2´-deoxyuridine) is a thymidine analog that is incorporated into DNA during active DNA synthesis. Detection is based on click-it chemistry, which is a copper-catalyzed covalent reaction between an azide and an alkyne. In this application, the alkyne is found in the ethynyl moiety of EdU, while the azide is coupled to Pacific Blue™ dye. EdU reagent was added to each well 24 hours before preparation for flow cytometry at a concentration of 2 μL EdU (10 mM) per mL of media.

Cultured islets were harvested and dissociated using TrypLE™ Express Enzyme followed by vortexing. Viability of dissociated islets was assessed using Zombie Yellow™ Fixable Viability Kit according to manufacturer's instructions (Biolegend, San Diego, CA). Zombie Yellow™ is an amine-reactive fluorescent dye that is non-permeant to live cells

but permeant to cells with compromised membranes. Thus, it can be used to assess live versus dead status of mammalian cells by flow cytometry. Viability >40% under basal condition was used as a standard cut-off to be used to for analyses.

Following staining with Zombie Yellow for 20 minutes at room temperature. Cells were fixed with 10% buffered formalin for 5 minutes and permeabilized with 1% Triton X-100 for 15 minutes. To detect different islet cell types, cells were incubated with fluorescent antibodies for insulin (beta cells), glucagon (alpha cells), CK19 (ductal epithelial cells) and vimentin (stomal cells) for 30 minutes under room temperature while protected from light. All antibodies used and concentrations are detailed in Table 2.2.

Unstained, single stained and Fluorescence Minus One (FMO) controls were included for compensation and used to set gates for specific target cell populations. Flow cytometry data were collected at the London Regional Flow Cytometry Facility using an LSR II flow cytometer and data were analyzed using FlowJo software to determine overall islet cell viability/proliferation, cell type-specific viability/proliferation, and the percentage of cells that co-expressed insulin and other cell lineage-specific markers.

An overview of the experimental procedures described under **2.3 Human Islet Culture and Flow Cytometry** is shown in Figure 2.2.

Table 2.2 Antibody and fluorescent marker specifications for flow cytometry.

Antibody/Detector	Fluorophore/Dye	Company	Catalog #	Concentration
EdU	Pacific Blue	Thermo Fisher	C10418	1/800
-	Zombie Yellow	Biolegend	423103	1/250
Monoclonal mouse anti-beta-catenin	Alexa Fluor 488	Invitrogen	53-2567-42	1/40
Monoclonal rabbit anti-CK19	Alexa Fluor 488	Abcam	Ab192643	1/400
Monoclonal mouse anti- glucagon	R-Phycoerythrin (PE)	BD Biosciences	565860	1/133
Monoclonal mouse anti- insulin	Alexa Fluor 647	BD Biosciences	565689	1/133
Monoclonal mouse anti-vimentin	Alexa Fluor 594	Biolegend	677804	1/133

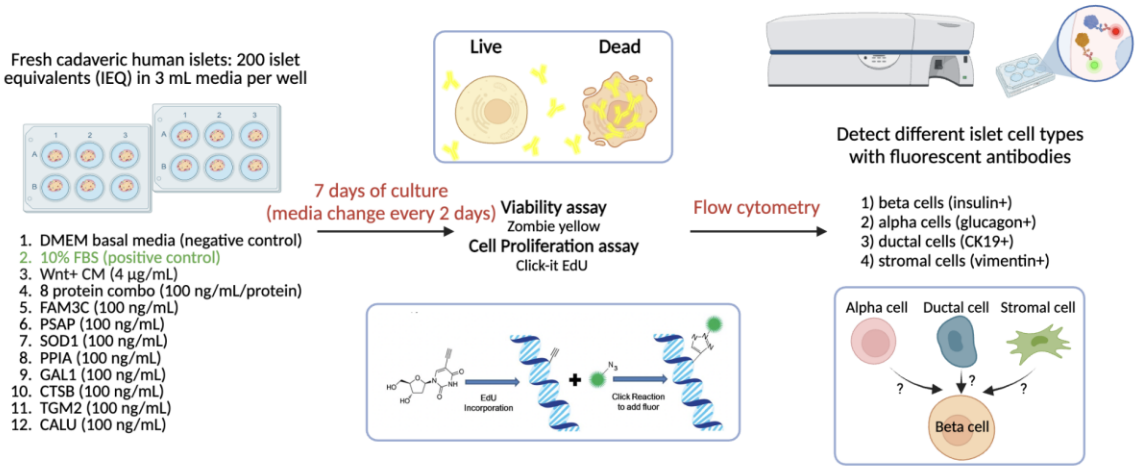

Figure 2.2 Overview of cultured human islet analyses in vitro.

Fresh cadaveric human islets obtained from adult non-diabetic donors were cultured under listed treatment conditions for 7 days at a seeding density of 67 islet equivalents/mL media. Cell viability and proliferation were quantified by flow cytometry using Zombie Yellow viability dye and Click-iT EdU proliferation assay. Figure created with BioRender.com.

2.4 Intrapancreatic (iPan) Injection of Wnt+ CM and 8 MSC-secreted Proteins

All experimental procedures were approved by the Animal Care Committee at Western University (AUP 2023-014, Appendix 2), and NOD/SCID mouse colonies were maintained following guidelines published by the Canadian Council for Animal Care.

2.4.1 Streptozotocin (STZ) administration in NOD/SCID mice

Both male and female immunodeficient NOD/SCID mice (Jackson Laboratories # 001303) 8-10 weeks of age, were treated with STZ (Sigma, St. Louis, MO), a beta cell specific toxin, to induce beta cell deletion and subsequent hyperglycemia. STZ was dissolved in citric acid buffer (CAB) at 3.75 mg/mL and was administered intraperitoneally into each mouse at 35 mg/kg/day for 5 consecutive days (day 0-4). A group of normoglycemic healthy control mice received daily injections of CAB vehicle control intraperitoneally using the same time course, instead of STZ. All mice were exposed to a standard 12-hour shift of the light-dark cycle, subjected to ad libitum supply of standard chow with access to unlimited water.

2.4.2 Intrapancreatic (iPan) injection

After STZ treatment, mice with non-fasting blood glucose (NFBG) levels lower than 13 mmol/L on day 7 and between the range of 13 to 25 mmol/L on day 10, were divided into the following treatment groups: (1) AmnioMAX^{TM} basal media (negative control), (2) Wnt+ CM (4-8 μg), (3) 8-protein combination (100 ng/protein), (4) FAM3C (100 ng), (5) PSAP (100 ng), (6) SOD1 (100 ng), (7) PPIA (100 ng), (8) GAL1 (100 ng), (9) CTSB (100 ng), (10) TGM2 (100 ng), and (11) CALU (100 ng). Mice were anesthetized with isoflurane and incisions were made in the skin and muscle to expose the pancreas and attached spleen. A single 20 μL dose of the corresponding treatments was directly injected into the pancreas of each mouse. Each treatment group included approximately equal numbers of male and female mice. Mice were weighed and non-fasting blood glucose levels were measured between 8-10 am using a FreeStyle Lite glucometer (Abbott Diabetes Care, Mississauga, ON) after tail vein puncture on days 0, 7, 10, 14, 17, 21, 28, 35, and 42.

2.4.3 Intraperitoneal glucose tolerance tests

Intraperitoneal glucose tolerance tests were performed on day 42. D-Glucose (Sigma) was dissolved at 0.25 g/mL in phosphate buffered saline. Mice were fasted for 4 hours prior to intraperitoneal injection of 2.0 g/kg sterile glucose solution. Blood glucose levels were measured by tail vein puncture at 0, 5, 10, 15, 30, 45, 60, 90, and 120 minutes post-injection.

2.4.4 Euthanasia and pancreas tissue preparation

Mice were euthanized by cervical dislocation under anesthetic on day 42. Pancreas tissues were resected, weighed, and frozen in optimal cutting temperature media (Thermo Fisher Scientific). Frozen samples were cryo-sectioned at 10 μm. Three different sections per mouse, >200 μm (>2 islet diameter) apart, were placed on the same microscope slide for subsequent immunohistochemical and immunofluorescent analyses.

2.4.5 Immunohistochemical analyses

Pancreas sections were fixed with 10% buffered formalin for 15 minutes, followed by blocking with 1% peroxidase block for 5 minutes and 5% horse serum (MJS Biolynx, Brockville, ON) for 1 hour. Sections were incubated with mouse anti-insulin primary antibody for 1 hour, followed with peroxidase anti-mouse secondary antibody for 30 minutes (Table 2.4). ImmPACT™ DAB (Vector Laboratories, Newark, CA) staining was performed to detect antibody binding, followed by a hematoxylin counterstain (Thermo Fisher Scientific) and slide mounting with VectaMount™ (Vector Laboratories).

Slides were scanned for islet quantifications at the London Regional Microscopy Facility (Robarts Research Institute, London, ON) using an 20X Aperio AT2 Digital Slide Scanner (Leica Biosystems, Wetzlar, Germany). Beta cell mass, islet size, and islet number were quantified using Aperio ImageScope software version 12.4.6 (Leica Biosystems) counting all insulin+ regions on 3 different sections per mouse. Beta cell mass, measured in mg, was calculated by: beta cell area ÷ total section area × pancreas weight. The circumference of each insulin+ region with > 5 insulin+ cells, measured in μm, was used to quantity islet

circumference or size. Islet number/mm^2 of total section area was also quantified by detection of insulin+ regions.

2.4.6 Immunofluorescent analyses

Pancreas sections were fixed with 10% buffered formalin for 15 minutes, and permeabilized with 1% Triton X-100 for 20 minutes, followed by blocking with 5% horse serum for 1 hour. Sections were incubated with mouse anti-glucagon primary antibody for 1 hour, followed with horse anti-mouse fluorescein secondary antibody for 30 minutes while protecting from light. After blocking with 5% goat serum (MJS Biolynx), sections were incubated again with rabbit anti-insulin primary antibody for 1 hour, followed with goat anti-rabbit Texas red secondary antibody for 30 minutes. Tissues were incubated with DAPI solution to detect nuclei. Antibodies and concentrations are detailed in Table 2.3. Slides were mounted with VectaMount™ and imaged using the Zeiss Axio Imager Z2 fluorescent microscope (Oberkochen, Germany). Images were analyzed using ImageJ software (National Institute Health, Bethesda, MD). Criteria for islet enumeration required a minimum cluster of 5 insulin+/glucagon+ cells. Total islet area was estimated using DAPI, insulin, and glucagon staining. The percentage of insulin+ and glucagon+ area within an islet was quantified. The ratio of beta to alpha cell numbers was estimated with the ratio of insulin+ area to glucagon+ area within all islets on 3 sections per mouse.

2.4.7 Short-term *in vivo* experiments

Short-term *in vivo* experiments were performed using the same procedures for STZ administration, non-fasting blood glucose monitoring, iPan injection, euthanasia, and pancreas preparation as described above. Mice were divided into 3 treatment groups to receive iPan injection: (1) AmnioMAX™ basal media (vehicle control), (2) Wnt+ CM (4-8 μg), or (3) 8-protein combination (100 ng/protein). Mice were euthanized on day 14. Immunohistochemical analyses for beta cell mass, islet size, and islet number using anti-insulin detection with DAB chromogen were quantified as described above for the 42-day (long-term) experiments.

An overview of all experimental procedures described under **2.4 Intrapancreatic (iPan) Injection of Wnt+ CM and 8 MSC-secreted Proteins** is shown in Figure 2.3.

Table 2.3 Antibody specifications for immunohistochemical and immunofluorescent staining.

Antibody	Company	Catalog #	Concentration
Monoclonal mouse anti-insulin	Sigma-Aldrich	I2018	1/333
Peroxidase labelled horse anti-mouse IgG	MJS Biolynx	VECTSK4105	1/250
Monoclonal mouse anti-glucagon	Abcam	AB10988	1/500
Monoclonal rabbit anti-insulin	Abcam	AB181547	1/1000
Fluorescein labelled horse anti-mouse IgG	MJS Biolynx	VECTFI2000	1/200
Texas Red labelled goat anti-rabbit IgG	MJS Biolynx	VECTTI1000	1/200
DAPI solution	Thermo Fisher	62248	1/1000

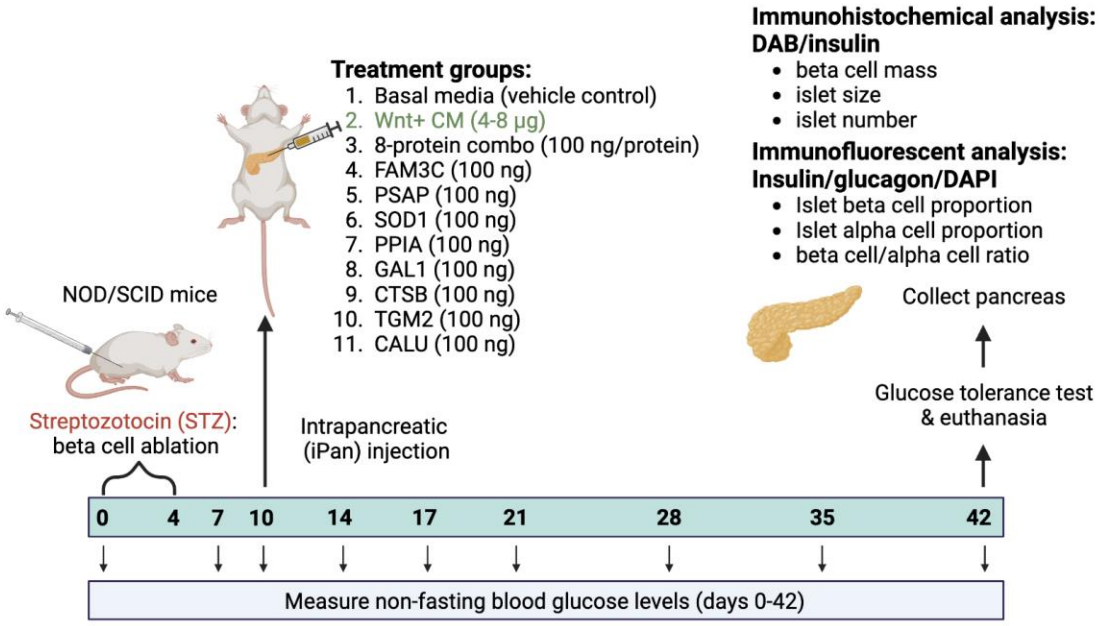

Figure 2.3 Overview of in vivo methodology.

Adult NOD/SCID mice received 35 mg/kg/day STZ from days 0-4 to induce hyperglycemia. On day 10, hyperglycemic mice were divided into the listed treatment groups for iPan injection. Non-fasting blood glucose levels were monitored over 42 days. Glucose tolerance tests were performed on day 42 prior to euthanasia. Mouse pancreata were collected for immunohistochemical and immunofluorescent analyses. Figure created with BioRender.com.

2.5 Statistical Analyses

Data were analyzed and graphed using GraphPad Prism version 9.2.0 (San Diego, CA). Values are represented as mean ± SEM unless otherwise stated. Two-way ANOVA with Dunnett's multiple comparisons test was used to analyze glucose data. Unpaired t test was used to compare between 2 treatment groups. One-way ANOVA with Dunnett's multiple comparisons test was used to compare 3 or more treatment groups. Comparisons were made against the negative controls unless otherwise stated. No outliers were identified using the ROUT method. Data were considered significant if $p < 0.05$ by the statistical analyses performed.

Chapter 3

3 Results

3.1 Treatment with CHIR99021 increased beta-catenin levels in human BM-MSC

To generate Wnt-stimulated (Wnt+) CM, human BM-derived MSC at passage 4 were treated with CHIR99021, a glycogen synthase kinase 3 (GSK3) inhibitor, to activate the canonical Wnt/beta-catenin pathway. Phosphorylation of beta-catenin by GSK3 triggers destabilization and degradation in the proteasome to maintain a low level of beta-catenin in the cytosol. GSK3 inhibition allows intracellular beta-catenin to accumulate, translocate to the nucleus, and subsequently regulate Wnt transcriptional pathways[144]. To confirm whether CHIR99021 could activate Wnt signalling, Wnt+ MSC were fixed and permeabilized to assess intracellular beta-catenin accumulation via flow cytometry. Untreated MSC were compared as control (Figure 3.1 A). Treatment with 10 μM CHIR99021 consistently increased mean fluorescence intensity (MFI) for beta-catenin compared to untreated MSC (21680.9±2847.4 vs 14171.4±1263.7, $p<0.01$, Figure 3.1 B). The mean percentage increase in beta-catenin MFI of Wnt + MSC relative to untreated MSC from 7 independent donors (N=7) was 50.6%±7.7% (Figure 3.1 C), suggesting that canonical Wnt signalling was upregulated in Wnt+ MSC.

To assess whether treatment with CHIR99021 would affect the amount of protein secreted by MSC, protein concentration for Wnt+ CM and untreated CM were quantified. No significant differences were observed in protein concentrations between Wnt+ CM and untreated CM (0.83±0.35 μg/μL vs 0.34±0.08 μg/μL, $p=0.2365$, Figure 3.1 D).

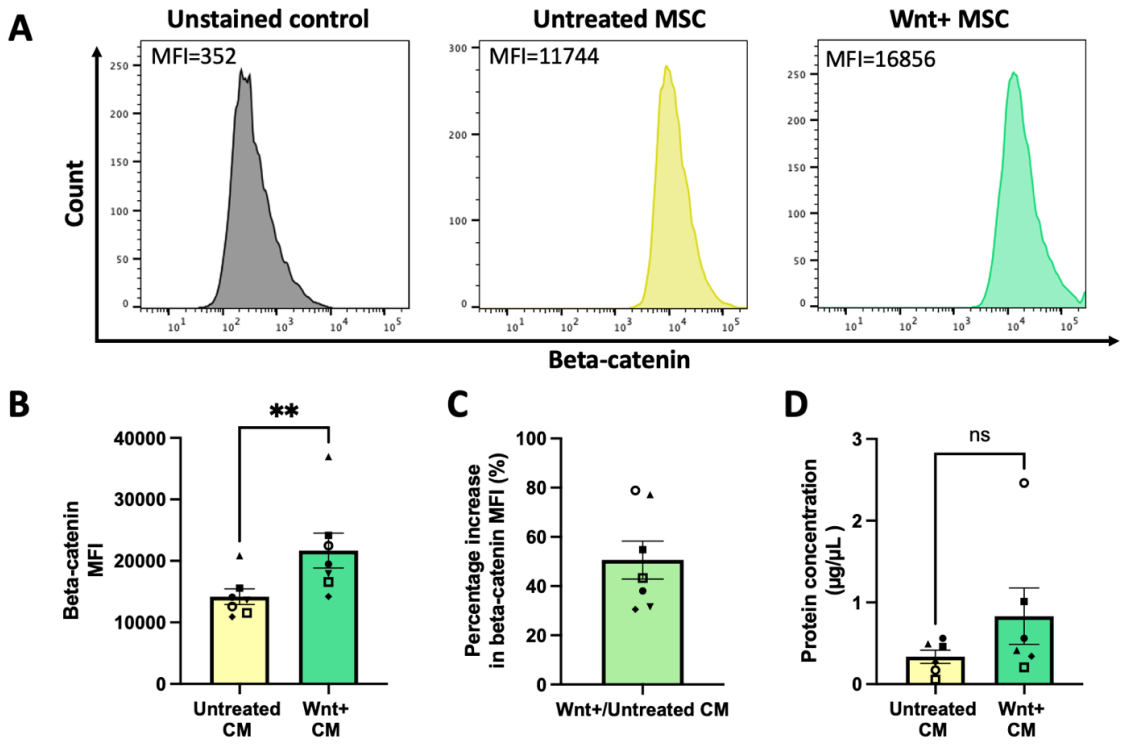

Figure 3.1 Treatment with CHIR99021 increased beta-catenin in human BM-MSC.

Intracellular beta-catenin was quantified by flow cytometry in 7 independent BM-MSC samples (N=7). **(A)** Representative flow cytometry plots of beta-catenin mean fluorescence intensity (MFI) in unstained MSC, untreated MSC, and Wnt+ MSC treated with 10 µM CHIR99021. **(B-C)** Wnt+ MSC showed increased intracellular beta-catenin levels compared to untreated MSC (** $p<0.01$). **(D)** No differences were found in the protein concentration of Wnt+ CM and untreated CM ($p=0.2365$). Data represent mean ± SEM. Paired t test was used for analysis of significance.

3.2 Culture with MSC-secreted proteins did not change human islet cell viability or proliferation *in vitro.*

To assess whether MSC-secreted proteins could impact human islet cell viability or proliferation *in vitro*, human islets from 6 independent donors (N=6) were cultured for 7 days in Wnt+ CM (4 µg/mL), 8-protein combination (100 ng/mL/protein), or with one of the individual candidate proteins (100 ng/mL). Multiparametric flow cytometry was used to analyze viability and proliferation of islet-associated cell types (Table 3.1).

Compared with basal media (negative control) and basal media + 10% FBS (positive control), there were no significant differences in the percentage of viable insulin+, glucagon+, CK19+, or vimentin+ cells in islets cultured with Wnt+ CM, 8-protein combination, or the individual candidate proteins (Figure 3.2 A-D). Large variability in the proportion of insulin+ cells and CK19+ cells was observed across islet samples from different donors, represented by different symbols (Table 3.1).

Cell viability and proliferation were assessed using Zombie Yellow and Click-iT EdU, respectively, and quantified by flow cytometry (Figure 3.2 E). Compared to basal media control, Wnt+ CM, 8-protein combination, or the individual candidate proteins did not significantly change overall islet cell viability or proliferation after 7 days of culture (Figure 3.2 F-G). Islets cultured in basal media and basal media + 10% FBS had comparable viability (62.8%±6.9% vs 55.0%±4.0%, $p=0.1450$, Table 3.1). Islets cultured with 10% FBS showed increased rates of cell proliferation compared to basal media control (2.98%±0.64% vs 0.30%±0.15%, $p<0.05$, Table 3.1).

Table 3.1 Summary of human islet donor information.

Sample preparations included age, sex, BMI, purity, cold ischemia time and pre-shipment time in culture. Sample #2 was excluded from analyses due to a viability in basal media condition of less than 40%. Data represent mean ± SEM. Paired t test was used for analysis of significance (* $p<0.05$).

Sample #	Donor information			Pre-shipment			After 7 days of culture			
	Age	Sex	BMI	Purity (%)	Cold ischemia time (hours)	Culture time (hours)	Overall viability (%)		Overall proliferation (%)	
							Basal media	10% FBS	Basal media	10% FBS
1 ●	39	M	33.4	80	13	55	61.2	49.6	0.86	3.55
(2)	(54)	(F)	(27.5)	(95)	(11)	(8)	(39.1)	(56.1)	(0.00)	(1.39)
3 ▲	42	F	29.2	95	10	68	75.3	54.7	0.19	0.82
4 ◆	72	M	30.1	95	20	43	44.4	47.8	0.05	2.32
5 ⬣	47	M	27.5	90	11	95	81.0	70.2	0.30	3.85
6 X	36	M	33.2	95	15	18	52.2	52.5	0.08	4.36
Mean	47.2	-	30.7	91.0	13.8	55.8	62.8	55.0	0.30	2.98 *
SEM	6.5	-	1.1	2.9	1.8	12.8	6.9	4.0	0.15	0.64 *

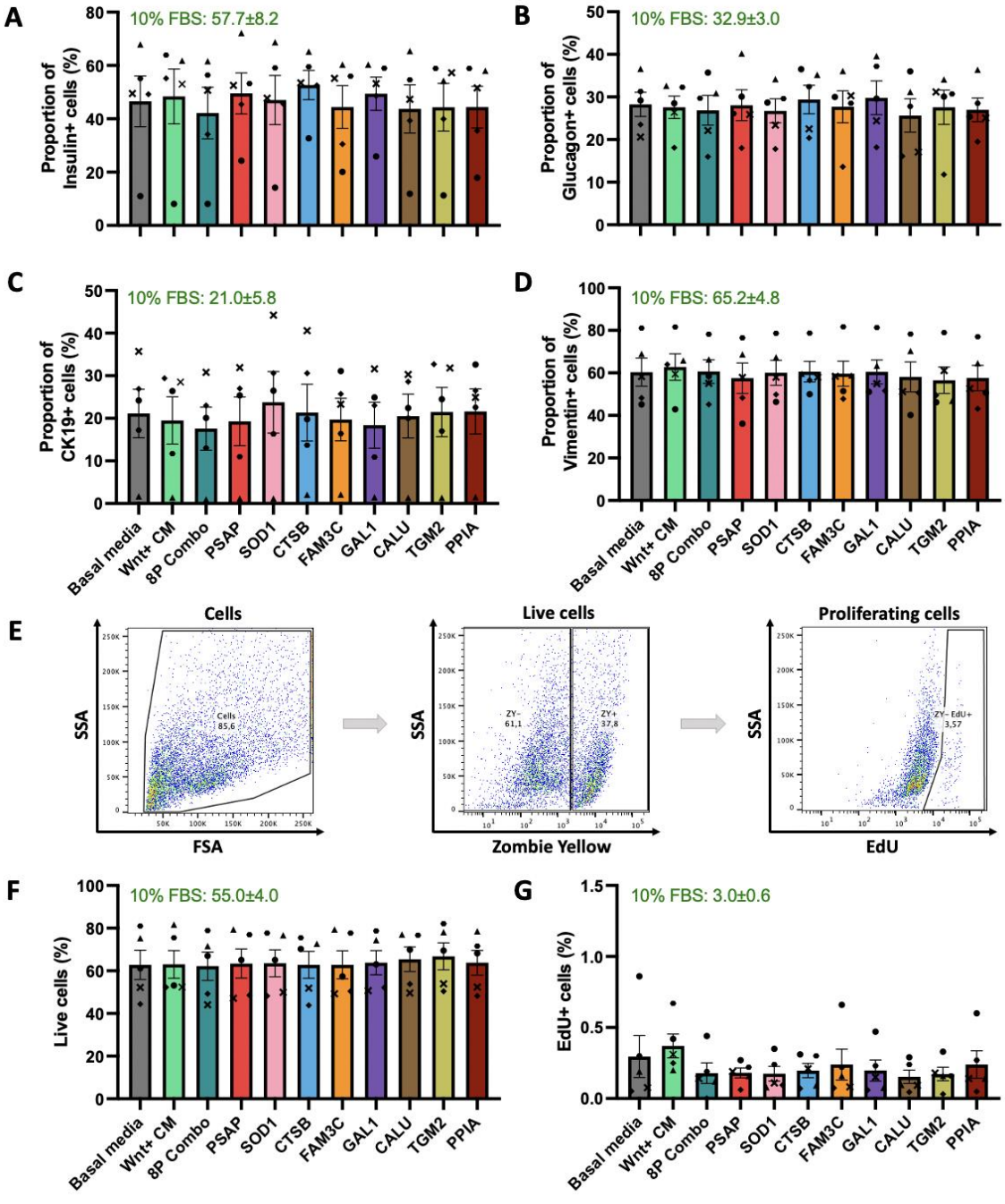
A
10% FBS: 57.7±8.2
Proportion of Insulin+ cells (%)
B
10% FBS: 32.9±3.0
Proportion of Glucagon+ cells (%)
C
10% FBS: 21.0±5.8
Proportion of CK19+ cells (%)
D
10% FBS: 65.2±4.8
Proportion of Vimentin+ cells (%)
Basal media
Wnt+ CM
8P Combo
PSAP
SOD1
CTSB
FAM3C
GAL1
CALU
TGM2
PPIA
E
Cells
Live cells
Proliferating cells
SSA
FSA
Cells 85,6
ZY- 61,1
ZY+ 37,8
Zombie Yellow
ZY- EdU+ 3,57
EdU
F
10% FBS: 55.0±4.0
Live cells (%)
G
10% FBS: 3.0±0.6
EdU+ cells (%)

Figure 3.2 Culture with MSC-secreted proteins did not affect human islet cell viability or proliferation.

Cadaveric human islets (N=5) were cultured with DMEM basal media, Wnt+ CM (4 µg/mL), 8-protein combination (100 ng/mL/protein), or individual proteins (100 ng/mL) for 7 days. No differences were observed in the percentage of **(A)** insulin+, **(B)** glucagon+, **(C)** CK19+, or **(D)** vimentin+ cells. **(E)** Representative flow cytometry plots showing the gating used to identify live (Zombie Yellow negative) and proliferating (EdU positive) cells. The treatment conditions did not significantly change islet cell **(F)** viability or **(G)** proliferation compared to basal media controls. Data represent mean ± SEM. Repeated measures one-way ANOVA was used for analysis of significance.

3.3 Culture with MSC-secreted proteins did not change islet cell lineage-specific viability or proliferation.

To assess whether MSC-secreted proteins had different effects on specific islet cell types, harvested cells were incubated with fluorescent antibodies for insulin, glucagon, CK19, and vimentin to identify beta cells, alpha cells, ductal epithelial cells, and stromal cells, respectively, and islet cell lineage-specific viability and proliferation were quantified by flow cytometry Figure 3.3 A).

Compared to basal media control, Wnt+ CM, 8-protein combination, or the individual proteins did not significantly change the viability of insulin+, glucagon+, CK19+ or vimentin+ cells (Figure 3.3 B, D, F, H).

Compared to basal media control, islets cultured with Wnt+ CM had an increased percentage of proliferating glucagon+ cells (0.18%±0.05% vs 0.52%±0.18%, $p<0.01$, Figure 3.3 E) and vimentin+ cells (0.33%±0.13% vs 0.56%±0.11%, $p<0.05$, Figure 3.3 I), but not insulin+ cells or CK19+ cells (Figure 3.3 C, G). The 8-protein combination or the individual proteins did not significantly change the proliferation of insulin+, glucagon+, CK19+ or vimentin+ cells (Figure 3.3 C, E, G, I).

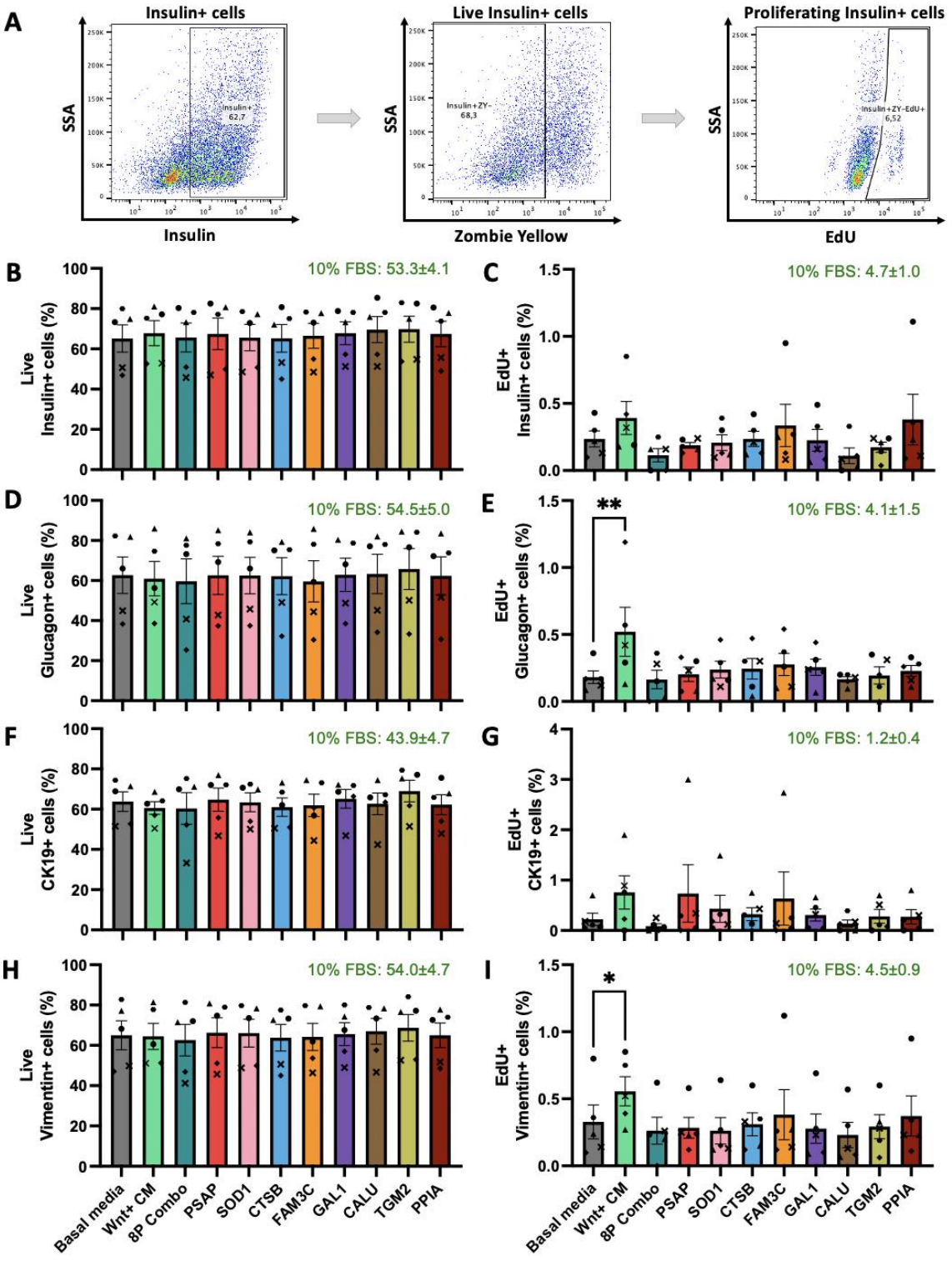
A
Insulin+ cells
Live Insulin+ cells
Proliferating Insulin+ cells
SSA
Insulin
Zombie Yellow
EdU
Insulin+
62,7
Insulin+ZY-
68,3
Insulin+ZY-EdU+
6,52
B
10% FBS: 53.3±4.1
Live Insulin+ cells (%)
C
10% FBS: 4.7±1.0
EdU+ Insulin+ cells (%)
D
10% FBS: 54.5±5.0
Live Glucagon+ cells (%)
E
10% FBS: 4.1±1.5
EdU+ Glucagon+ cells (%)
**
F
10% FBS: 43.9±4.7
Live CK19+ cells (%)
G
10% FBS: 1.2±0.4
EdU+ CK19+ cells (%)
H
10% FBS: 54.0±4.7
Live Vimentin+ cells (%)
I
10% FBS: 4.5±0.9
EdU+ Vimentin+ cells (%)
*
Basal media
Wnt+ CM
8P Combo
PSAP
SOD1
CTSB
FAM3C
GAL1
CALU
TGM2
PPIA

Figure 3.3 Culture with MSC-secreted proteins did not change islet cell lineage-specific viability and proliferation.

Cadaveric human islets (N=5) were cultured with basal media, Wnt+ CM (4 µg/mL), 8-protein combination (100 ng/mL/protein), or individual proteins (100 ng/mL) for 7 days. Fluorescent antibodies for beta cells (insulin), alpha cells (glucagon), ductal cells (CK19), and stromal cells (vimentin) were used to quantify cell type-specific viability and proliferation by flow cytometry. **(A)** Representative flow cytometry plots showing the gating of cells for insulin, followed by gating on Zombie Yellow negative (live cells) then EdU positive (proliferating cells). Islets cultured with Wnt+ CM, 8-protein combination, or the individual proteins did significantly change the viability of **(B)** insulin+, **(D)** glucagon+, **(F)** CK19+ or **(H)** vimentin+ cells compared to basal media control. Wnt+ CM increased the percentage of proliferating **(E)** glucagon + cells and **(I)** vimentin+ cells. However, the 8-protein combination or any of the individual proteins did not significantly change the proliferation of **(C)** insulin+, **(E)** glucagon+, **(G)** CK19+ or **(I)** vimentin+ cells compared to basal media control. Data represent mean ± SEM. Analysis of significance was performed by repeated measures one-way ANOVA followed by Dunnett's multiple comparisons test (* $p<0.05$, ** $p<0.01$).

3.4 Culture with MSC-secreted proteins did not change the percentage of live cells co-expressing insulin and other lineage-specific markers.

To identify cells in the islets that could potentially undergo transdifferentiation to give rise to new beta cells, the percentage of live cells co-expressing insulin and other lineage-specific markers was also quantified using flow cytometry.

Compared to basal media control, Wnt+ CM, 8-protein combination, and the individual proteins did not significantly change the percentage of cells co-expressing insulin and glucagon ($p=0.2632$, Figure 3.4 B), insulin and CK19 ($p=0.3678$, Figure 3.4 C), or insulin and vimentin ($p=0.4629$, Figure 3.4 D).

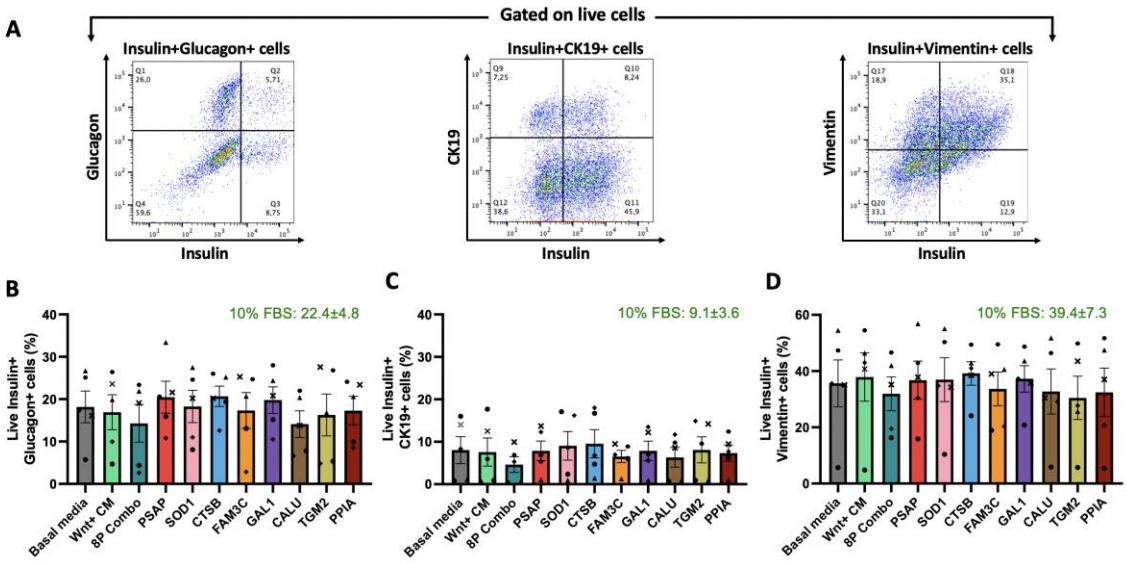

Figure 3.4 Culture with MSC-secreted proteins did not change the percentage of live cells co-expressing insulin and other lineage-specific markers.

Cadaveric human islets (N=5) were cultured with DMEM basal media, Wnt+ CM (4 µg/mL), 8-protein combination (100 ng/mL/protein), or individual proteins (100 ng/mL) for 7 days. Fluorescent antibodies for insulin (beta cells), glucagon (alpha cells), CK19 (ductal cell), and vimentin (stromal cells) were used to identify double positive cells by flow cytometry. **(A)** Representative flow cytometry plots showing the gating strategies used to identify cells co-expressing insulin and other lineage-specific markers. No differences were observed for the percentage of cells co-expressing **(B)** insulin and glucagon, **(C)** insulin and CK19, or **(D)** insulin and vimentin compared to islets cultured in basal media. Data represent mean ± SEM. Analysis of significance was performed by repeated measures one-way ANOVA.

3.5 Administration of STZ resulted in hyperglycemia, glucose intolerance, and decreased beta cell mass in NOD/SCID mice.

Adult NOD/SCID mice (8-10 weeks) were intraperitoneally injected with 35 mg/kg/day STZ from day 0-4 as previously described[147]. For comparison, a group of mice received citric acid buffer (CAB) vehicle control instead of STZ (n=6). STZ is an alkylating agent that kills insulin producing beta cells in the islet[172]. To assess the induction of hyperglycemia, a group of healthy control mice (CAB) and a group of STZ-treated mice (n=7) both received iPan-injection of supplement-free AmnioMAX^{TM} basal media on day 10, and non-fasting blood glucose (NFBG) levels were monitored for up to 42 days.

Compared to healthy control mice that remained normoglycemic (<6.9 mmol/L), STZ-treated mice demonstrated increased NFBG levels by day 7 and remained severely hyperglycemic (>30 mmol/L) until day 42 (Figure 3.5 A). The area under the curve (AUC) for NFBG was significantly increased in STZ-treated vs healthy control mice (201±8 vs 1006±32, $p<0.001$, Figure 3.5 B).

Intraperitoneal glucose tolerance tests were also performed on day 42 to assess whether STZ-treated mice could respond to glucose challenge. Compared to healthy control mice that showed glucose reduction back to baseline concentrations by 90 minutes, STZ-treated mice showed prolonged hyperglycemia that remained elevated throughout the 2-hour glucose tolerance test (>30 mmol/L, Figure 3.5 C), and AUC was significantly increased (713±64 vs 3353±58, $p<0.001$, Figure 3.5 D). To ensure that adverse weight loss did not affect glucose levels, mouse weights were also monitored weekly until day 42. Weight changes relative to day 0 were not significantly different between the healthy controls and STZ-treated mice (Figure 3.5 E)

Mouse pancreata were collected for post-mortem immunohistochemical analyses. Compared to the healthy controls, STZ-treated mice demonstrated a significant reduction in beta cell mass, islet size, and islet number (Figure 3.5 H-J).

Taken together, the results suggest that STZ treatment at 35 mg/kg/day for 5 consecutive days efficiently induced severe hyperglycemia by ablating beta cells in NOD/SCID mice.

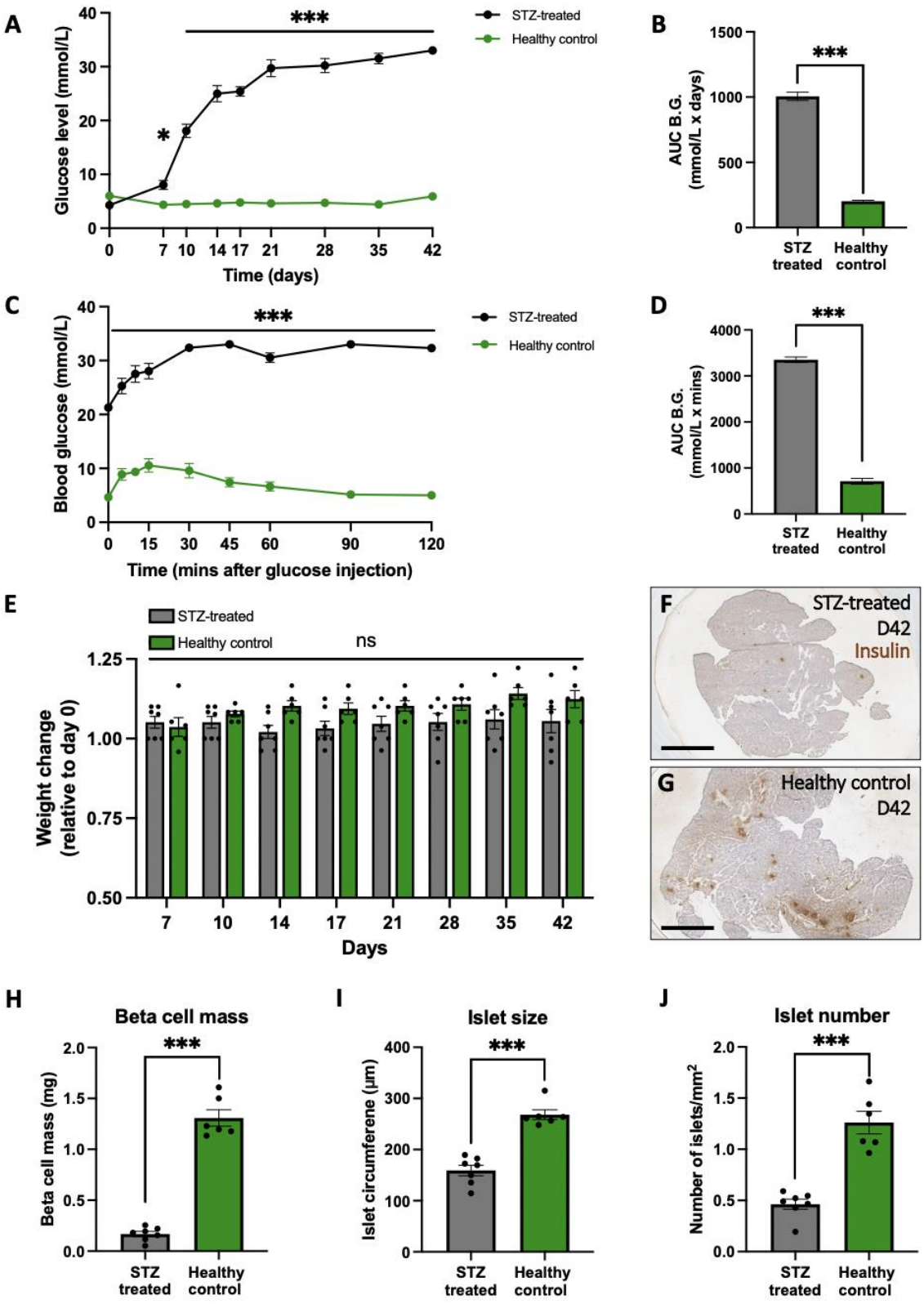
A
Glucose level (mmol/L)
Time (days)
STZ-treated
Healthy control
***
*
B
AUC B.G. (mmol/L x days)
STZ treated
Healthy control
***
C
Blood glucose (mmol/L)
Time (mins after glucose injection)
STZ-treated
Healthy control
***
D
AUC B.G. (mmol/L x mins)
STZ treated
Healthy control
***
E
STZ-treated
Healthy control
ns
Weight change (relative to day 0)
Days
F
STZ-treated
D42
Insulin
G
Healthy control
D42
H
Beta cell mass
Beta cell mass (mg)
STZ treated
Healthy control
***
I
Islet size
Islet circumferene (µm)
STZ treated
Healthy control
***
J
Islet number
Number of islets/mm²
STZ treated
Healthy control
***

Figure 3.5 Administration of STZ resulted in hyperglycemia, glucose intolerance, and decreased beta cell mass in NOD/SCID mice.

Healthy control (n=6) and STZ-treated (n=7) NOD/SCID mice received iPan injection of basal media on day 10. **(A)** Compared to the healthy controls, STZ-treated mice demonstrated increased non-fasting blood glucose (NFBG) levels by day 7 and remained severely hyperglycemic until day 42. **(B)** The area under the curve (AUC) for NFBG was increased in STZ-treated mice. **(C)** STZ-treated mice failed to respond to a glucose challenge, showing prolonged hyperglycemia up to 120 minutes and **(D)** increased AUC. **(E)** No differences in weight change were observed between groups. **(F-G)** Representative photomicrographs of insulin+ islets for healthy control mice and STZ-treated mice on day 42 (scale bar: 2 mm). STZ-treatment decreased **(H)** beta cell mass, **(I)** islet size and **(J)** islet number compared to healthy controls. Data represent mean ± SEM. Analysis of significance for glucose and weight change was performed by two-way ANOVA followed by Dunnett's multiple comparisons test. AUC, beta cell mass, islet size and islet number were compared using unpaired t test (* $p< 0.05$, *** $p<0.001$).

3.6 iPan injection of Wnt+ CM or 8-protein combination reduced hyperglycemia, improved glucose tolerance, and increased beta cell mass.

Kuljanin *et al* (2019) previously demonstrated the islet regenerative capacity of Wnt+ CM following iPan injection into STZ-treated NOD/SCID mice[147]. To validate the islet regenerative function of the 8 MSC-secreted proteins that were commonly upregulated in the regenerative CM and Wnt+ CM collected from MSC, hyperglycemic (13-25 mmol/L), STZ-treated NOD/SCID mice were iPan injected on day 10 with: (1) supplement-free AmnioMAX^{TM} basal media (n=7, vehicle control), (2) Wnt+ CM at 4-8 μg total protein (N=4, n=10), or (3) 8-protein combination at 100 ng/protein (n=7).

Compared to mice that received basal media, mice iPan-injected with Wnt+ CM or 8-protein combination both demonstrated significantly decreased NFBG levels from day 21-42 (Figure 3.6 A). While the basal media control mice developed severe hyperglycemia (>30 mmol/L), NFBG levels of mice that received Wnt+ CM or the 8-protein combination stabilized after iPan injection and plateaued at 23.8±1.3 mmol/L and 23.3 ±2.8 mmol/L at day 21-42, respectively (Figure 3.6 A). Injection of Wnt+ CM or 8-proten combination significantly reduced the AUC for NFBG levels compared to basal media controls over the 42 days (Figure 3.6 B). The AUC for NFBG was not significantly different between the two treatment groups (Wnt+ CM: 823±57 vs 8P combo: 789±82, p=0.9412, Figure 3.6 B), suggesting that Wnt+ CM and 8-protein combination had similar ability in improving hyperglycemia after iPan injection.

Following 4 hours of fasting for glucose tolerance tests on Day 42, mice that received Wnt+ CM or 8-protein combination showed similar glucose levels compared to mice that received basal media. Unlike the basal media controls that showed prolonged hyperglycemia following the glucose bolus, mice that received Wnt+ CM or 8-protein combination showed an initial increase in blood glucose following administration of the glucose bolus, followed by decreased glycemia by the 90- and 120-minute time points, similar to the time 0 concentrations. This suggested improved response to glucose challenge and better recovery of beta cell function in the treatment groups (Figure 3.6 C). The AUC calculated from the glucose tolerance test for both treatment groups were

significantly lower compared to basal media controls (basal: 3353±58), while not different from each other (Wnt+ CM: 2773±170 vs 8P combo: 2696±147, p=0.9257, Figure 3.6 D).

A ratio of starting (day 0) and final weight (day 42) showed that there were no significant changes in weight between the groups (Figure 3.6 I).

In post-mortem immunohistochemical analyses, Wnt+ CM or 8-protein combination increased beta cell mass (Figure 3.6 J), islet size (Figure 3.6 K), and islet number (Figure 3.6 L) compared to basal media controls, suggesting islet regeneration. Notably, no differences were observed between Wnt+ CM group and 8-protein combination group (Figure 3.6 J-L).

Taken together, mice that received iPan injection of either Wnt+ CM or 8-protein combination demonstrated sufficient recovery of beta cell mass and islet function to enable improved regulation of blood glucose levels.

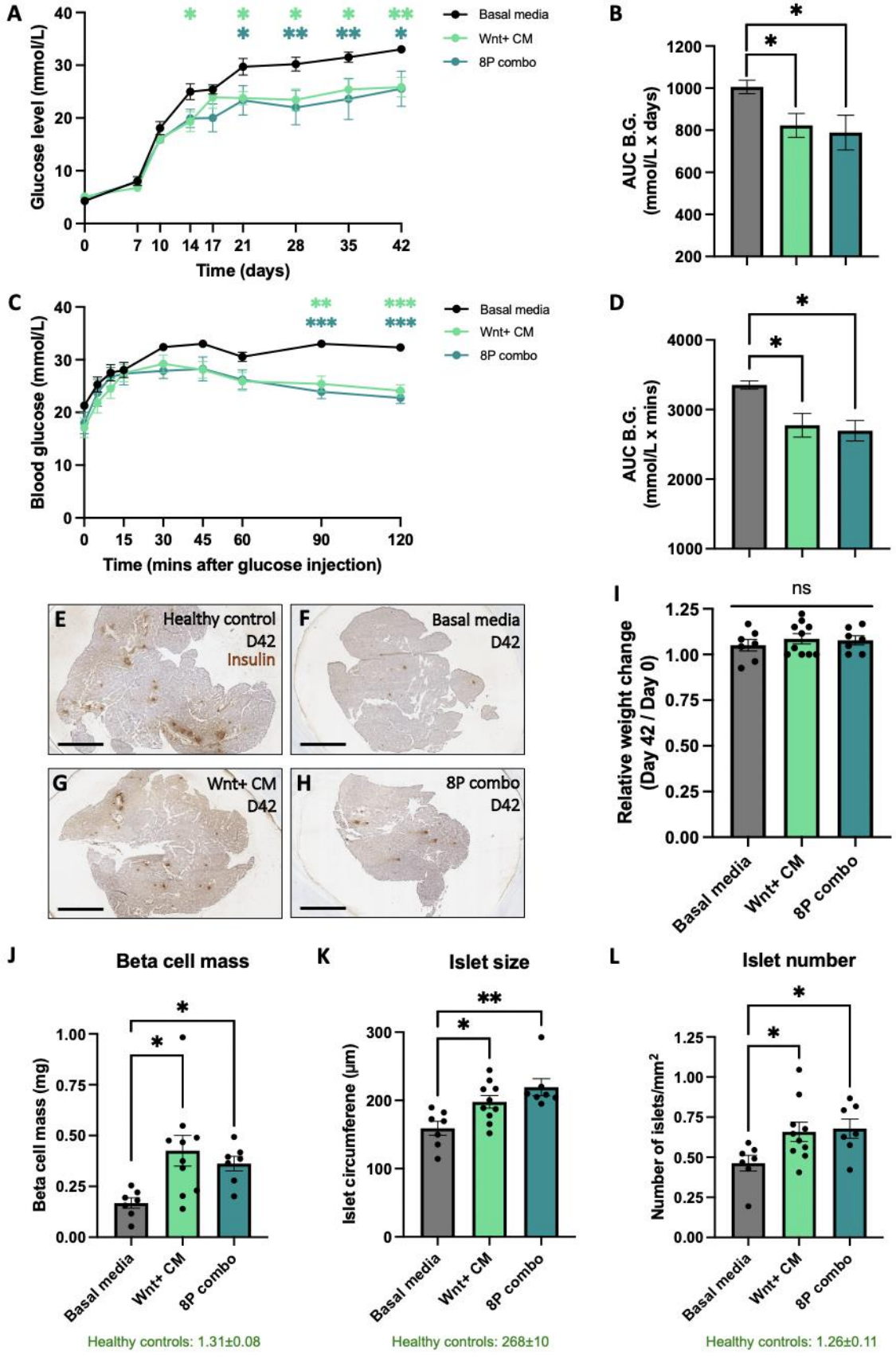
A
Glucose level (mmol/L)
Time (days)
Basal media
Wnt+ CM
8P combo
B
AUC B.G. (mmol/L x days)
C
Blood glucose (mmol/L)
Time (mins after glucose injection)
D
AUC B.G. (mmol/L x mins)
E
Healthy control D42 Insulin
F
Basal media D42
G
Wnt+ CM D42
H
8P combo D42
I
ns
Relative weight change (Day 42 / Day 0)
J
Beta cell mass
Beta cell mass (mg)
Healthy controls: 1.31±0.08
K
Islet size
Islet circumferene (µm)
Healthy controls: 268±10
L
Islet number
Number of islets/mm²
Healthy controls: 1.26±0.11

Figure 3.6 iPan injection of Wnt+ CM or 8-protein combination reduced hyperglycemia, improved glucose tolerance, and increased beta cell mass.

Hyperglycemic (13-25 mmol/L), STZ-treated NOD/SCID mice received iPan injection of basal media (n=7), Wnt+ CM at 4-8 μg total protein (N=4, n=10), or 8-protein combination at 100 ng/protein (n=7) on day 10. Blood glucose levels were monitored weekly and pancreata were harvested on day 42. **(A)** Compared to basal media controls, mice iPan injected with Wnt+ CM or 8-protein combination demonstrated decreased non-fasting blood glucose (NFBG) levels from day 21-42. **(B)** The area under the curve (AUC) for NFBG were reduced for both treatment groups. **(C)** Mice that received Wnt+ CM or 8-protein combination demonstrated lower glucose levels at 90- and 120-minute following glucose bolus, and **(D)** reduced AUC during glucose tolerance tests. **(E-H)** Representative photomicrographs of insulin+ islets on day 42 (scale bar: 2 mm). **(I)** No differences in weight change were observed between groups. iPan injection of Wnt+ CM or 8-protein combination increased **(J)** beta cell mass, **(K)** islet size and **(L)** islet number compared to basal media controls. Data represent mean ± SEM. Analyses of significance for glucose data was performed by ordinary two-way ANOVA followed by Dunnett's multiple comparisons test. AUC, weight change, beta cell mass, islet size and islet number were compared using ordinary one-way ANOVA followed by Dunnett's multiple comparisons test (* $p< 0.05$, ** $p<0.01$, *** $p<0.001$).

3.7 8-protein combination increased proportion of islet beta cells and improved beta cell/alpha cell ratio.

To assess the proportion of beta cells and alpha cells within mouse islets, mouse pancreatic sections collected at day 42 were stained for insulin, glucagon using fluorescent antibodies, and nuclei were stained using DAPI. The percentage of insulin+ and glucagon+ area within each islet was quantified for healthy control mice (n=6), and STZ-treated mice that received (1) supplement-free AmnioMAX^{TM} basal media (n=7, vehicle control), (2) Wnt+ CM (N=4, n=10), or (3) 8-protein combination (n=7).

The proportion of islet beta cells and alpha cells in healthy control mice were 79.8%±2.5% and 18.6%±3.8%, respectively (Figure 3.7 E-F). Compared to these healthy control mice, STZ-treated mice that received either basal media, Wnt+ CM, or 8-protein combination showed decreased proportion of beta cells within islets (30-60% of islet area) due to STZ-induced beta cell ablation ($p<0.01$, Figure 3.7 E). Conversely, mice that received basal media, Wnt+ CM, or 8-protein combination showed increased proportion of islet alpha cells after STZ treatment (~60% of islet area), compared to healthy control mice ($p<0.001$, Figure 3.7 F).

Compared to basal media controls, the proportion of islet beta cell increased in mice that received 8-protein combination (Figure 3.7 E). Additionally, mice that received 8-protein combination showed increased beta cell/alpha cell ratio compared to basal media controls (Figure 3.7 G).

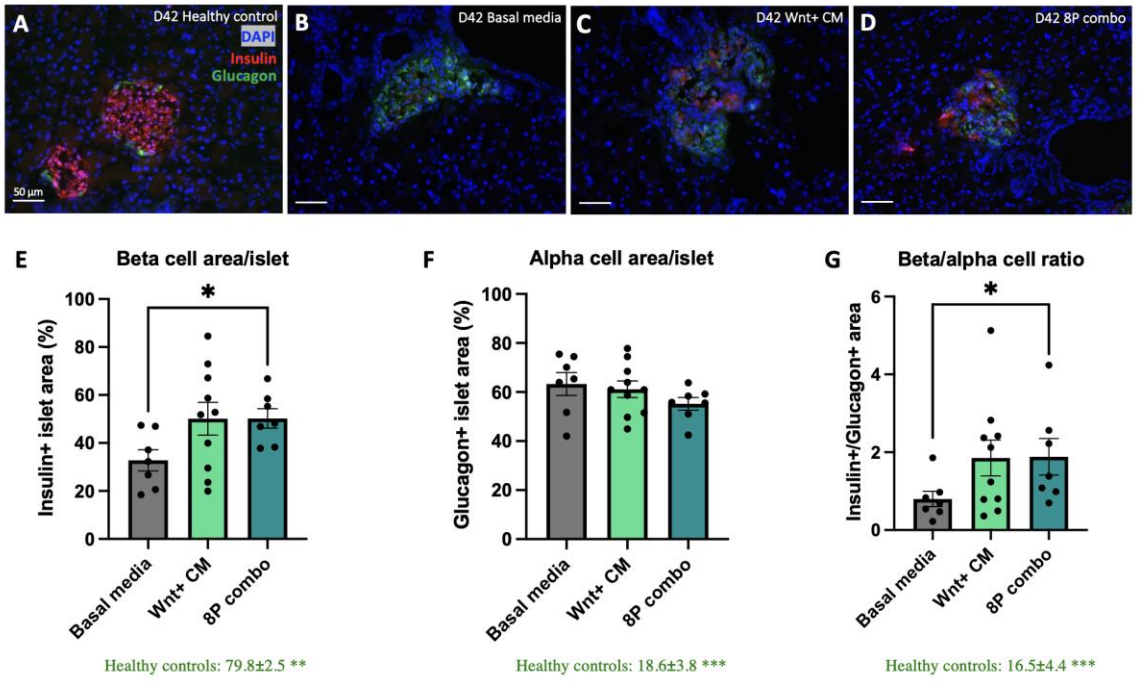

Figure 3.7 8-protein combination increased proportion of islet beta cells and improved beta cell/alpha cell ratio.

Hyperglycemic (13-25 mmol/L), STZ-treated NOD/SCID mice received iPan injection of basal media (n=7), Wnt+ CM at 4-8 µg total protein (N=4, n=10), or 8-protein combination at 100 ng/protein (n=7) on day 10. Pancreata were harvested on day 42 and stained for insulin and glucagon using fluorescent antibodies, and nuclei were stained using DAPI. **(A-D)** Representative photomicrographs of insulin+ and glucagon+ islets (scale bar: 50 µm). **(E)** Compared to healthy controls (n=5), STZ treatment decreased the proportion of islet beta cells in mice that received basal media, Wnt+ CM, or 8-protein combination. Mice that received 8-protein combination showed increased proportion of islet beta cells compared to basal media controls **(F)** Proportion of islet alpha cells was increased in groups that received STZ treatment. **(G)** Beta cell/alpha cell ratio were decreased in the STZ-treated groups, and mice that received 8-protein combination had increased beta cell/alpha cell ratio compared to basal media controls. Data represent mean ± SEM. Analysis of significance was performed by ordinary one-way ANOVA followed by Tukey's multiple comparisons test. (* $p<0.05$, ** $p< 0.01$, *** $p<0.001$).

3.8 Beta cell regeneration occurred within 4 days after iPan injection of Wnt+ CM or 8-protein combination.

To assess the efficiency of beta cell recovery induced by the 8 candidate proteins, a short-term experiment where mice were euthanized on day 14 was performed. Hyperglycemic, STZ-treated NOD/SCID mice were iPan injected on day 10 with: (1) supplement-free AmnioMAX^{TM} basal media (n=6, vehicle control), (2) Wnt+ CM at 4-8 μg total protein (N=3, n=9), or (3) 8-protein combination at 100 ng/protein (n=8).

STZ-treated mice had similar NFBG levels prior to iPan injection on day 10 (Figure 3.8 A). Compared to basal media controls, mice that received Wnt+ CM or 8-protein combination showed increased beta cell mass at 4 days after iPan injection (Figure 3.8 F). Interestingly, iPan injection of 8-protein combination increased islet size on day 14, whereas no significant differences in islet size were observed when comparing the Wnt+ CM group with basal media controls (Figure 3.8 G). iPan injection of Wnt+ CM or 8-protein combination did not significantly increase islet number by day 14 (Figure 3.8 H). Collectively, both the Wnt+ CM and 8-protein combination increased beta cell mass within 4 days post iPan injection, suggesting that the proteins initiate islet regeneration shortly after injection.

Interestingly, in the 8-protein combination group, beta cell mass was significantly reduced at day 42 (0.36mg±0.04mg, Figure 3.6 J) compared to day 14 (0.63mg±0.10mg, $p<0.05$, Figure 3.8 F). This suggests that islet regeneration induced by the 8-protein combination may be transient or unsustain by a single iPan injection of proteins.

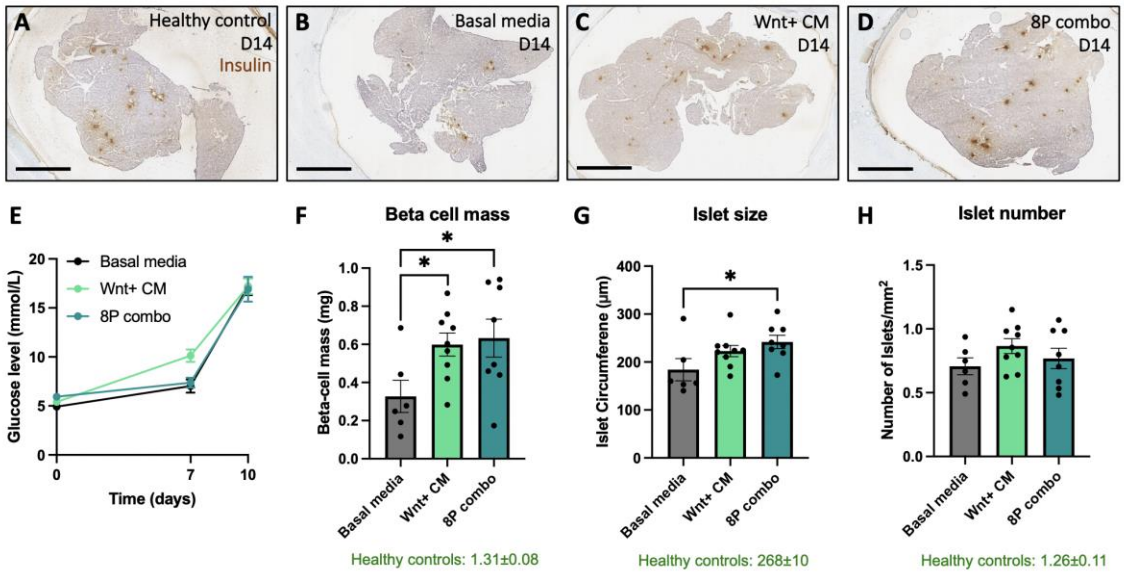

Figure 3.8 Beta cell regeneration occurred within 4 days after iPan injection of Wnt+ CM or 8-protein combination.

Hyperglycemic (13-25 mmol/L), STZ-treated NOD/SCID mice received iPan injection of basal media (n=6), Wnt+ CM at 4-8 µg total protein (N=3, n=9), or 8-protein combination at 100 ng/protein (n=8) on day 10. Pancreata were harvested on day 14. **(A-D)** Representative photomicrographs of insulin+ islets on day 14 (scale bar: 2 mm). **(E)** Mice had similar non-fasting blood glucose levels prior to iPan injection. Compared to basal media controls on day 14, iPan injection of Wnt+ CM or 8-protein combination increased **(F)** beta cell mass. **(G)** 8-protein combination but not Wnt+ CM increased islet size. **(H)** No differences in total islet number were observed. Data represent mean ± SEM. Analysis of significance for beta cell mass, islet size and islet number was performed by ordinary one-way ANOVA followed by Tukey's multiple comparisons test. Day 14 vs Day 42 were compared using ordinary two-way ANOVA followed by Sidak's multiple comparisons test (* $p < 0.05$).

3.9 Islet regenerative effects of SOD1, TGM2, CTSB, and CALU.

To determine which candidate protein(s) were responsible for the islet regenerative effects observed after iPan injection of 8-protein combination, STZ-treated, hyperglycemic (13-25 mmol/L) NOD/SCID mice were iPan injected on day 10 with: (1) 100 ng SOD1 (n=6), or (2) 100 ng TGM2 (n=8), or (3) 100 ng CTSB (n=7), or (4) 100 ng CALU (n=6), and compared to mice that received basal media (n=7, vehicle control).

Compared to basal media controls, mice iPan injected with SOD1 or CALU demonstrated significantly decreased NFBG levels from day 14 to day 42, which plateaued at ~20mmol/L shortly after iPan injection (Figure 3.9 A). In contrast, mice iPan injected with CTSB demonstrated reduced NFBG levels between days 21 to 28, and mice injected with TGM2 only demonstrated reduced NFBG at day 21 (Figure 3.9 A). Overall, iPan injection of SOD1 or CALU reduced the AUC for NFBG levels over the 42 days, whereas TGM2 or CTSB did not (Figure 3.9 B).

Intraperitoneal glucose tolerance tests were performed on day 42 to assess response to a glucose challenge. Compared to basal media controls which had sustained elevated glucose in response to a glucose bolus, mice that received SOD1 or TGM2 had reduced glycemic levels 90 minutes post-glucose bolus (Figure 3.9 C). Mice that received CTSB or CALU had reduced glycemic levels at ~60 minutes post-glucose bolus (Figure 3.9 C). Overall, the AUC measurements for 2-hour glucose tolerance test were significantly reduced for SOD1, CTSB, and CALU compared to basal media controls (Figure 3.9 D).

A ratio of the starting (day 0) and final weight (day 42) showed that there was no significant weight reduction for any of the treatment groups (Figure 3.9 I).

In post-mortem immunohistochemical analyses, iPan injection of SOD1, TGM2, CTSB, or CALU did not increase beta cell mass (Figure 3.9 J), or islet number (Figure 3.9 L) compared to basal media controls. However, mice that received SOD1 or CTSB showed increased islet size (Figure 3.9 K).

Taken together, while mice iPan injected with SOD1 or CALU demonstrated significant recovery of beta cell function to regulate blood glucose levels, there was no substantial recovery of beta cell mass. Likewise, TGM2 or CTSB showed marginal enhancement in beta cell function, but no significant restoration of beta cell mass.

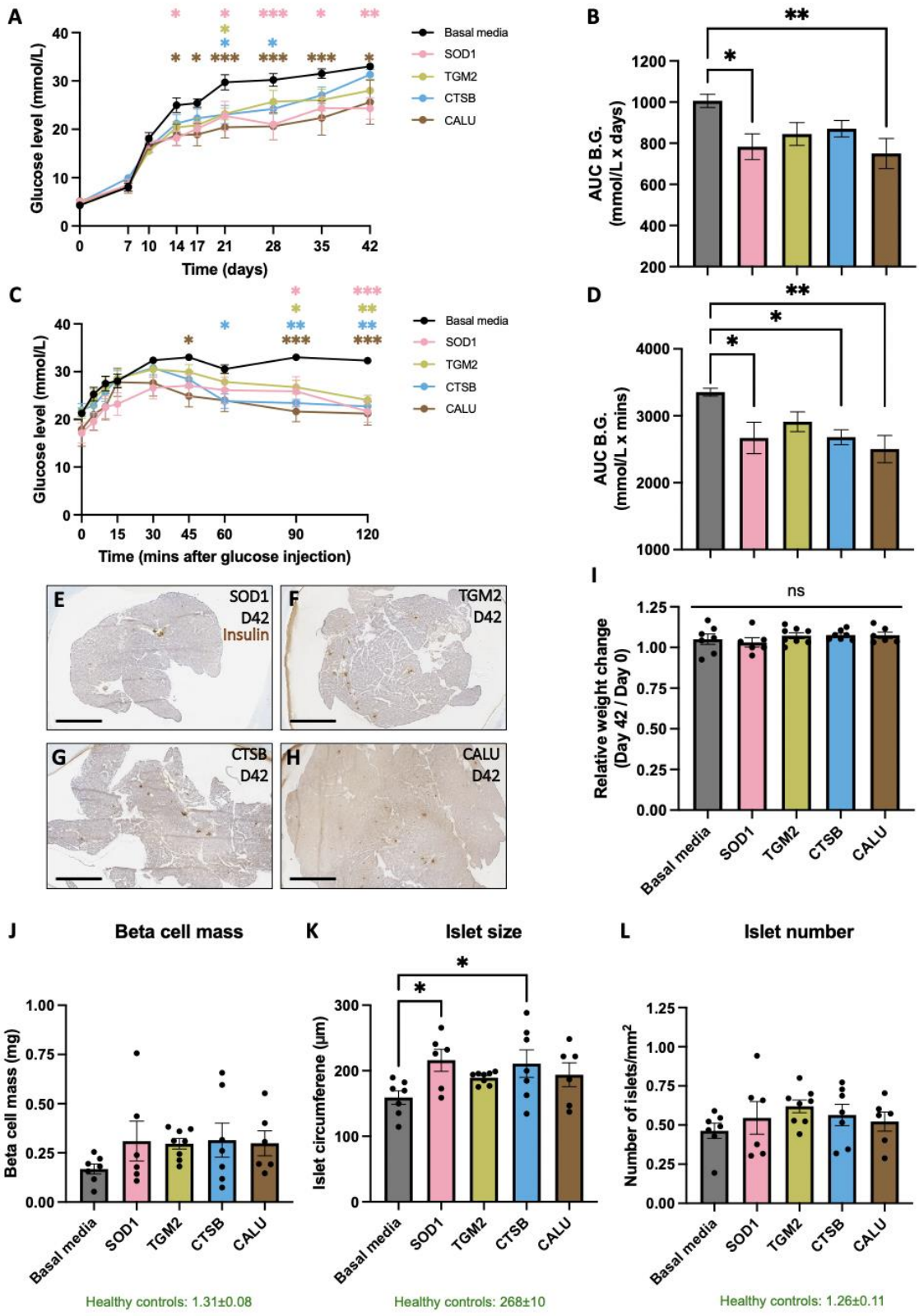
A
Glucose level (mmol/L)
Time (days)
Basal media
SOD1
TGM2
CTSB
CALU
B
AUC B.G. (mmol/L x days)
C
Glucose level (mmol/L)
Time (mins after glucose injection)
D
AUC B.G. (mmol/L x mins)
E
SOD1 D42 Insulin
F
TGM2 D42
G
CTSB D42
H
CALU D42
I
Relative weight change (Day 42 / Day 0)
ns
J
Beta cell mass
Beta cell mass (mg)
Healthy controls: 1.31±0.08
K
Islet size
Islet circumferene (µm)
Healthy controls: 268±10
L
Islet number
Number of islets/mm²
Healthy controls: 1.26±0.11

Figure 3.9 Islet regenerative effects of SOD1, TGM2, CTSB, and CALU.

Hyperglycemic (13-25 mmol/L), STZ-treated NOD/SCID mice received iPan injection of basal media (n=7), SOD1 (n=6), TGM2 (n=8), CTSB (n=7), or CALU (n=6) at 100 ng on day 10. Blood glucose was monitored weekly and pancreata were harvested on day 42. **(A)** Compared to basal media controls, mice iPan injected with SOD1 or CALU demonstrated reduction in non-fasting blood glucose (NFBG) levels from day 14-42, and the **(B)** area under the curve (AUC) for NFBG was reduced for both treatment groups. In contrast, mice that received TGM2 or CTSB only demonstrated a transient reduction in NFBG levels at Day 21. **(C)** Mice that received SOD1, CTSB or CALU showed improved response to glucose challenge and **(D)** reduced AUC during glucose tolerance tests. **(E-H)** Representative photomicrographs of insulin+ islets on day 42 (scale bar: 2 mm). **(I)** No differences in weight changes were observed between groups. iPan injection of SOD1, TGM2, CTSB, or CALU did not increase **(J)** beta cell mass or **(L)** islet number compared to basal media controls. **(K)** SOD1 or CTSB increased islet size. Data represent mean ± SEM. Analyses of significance for glucose data was performed by ordinary two-way ANOVA followed by Dunnett's multiple comparisons test. AUC, weight change, beta cell mass, islet size and islet number were compared using ordinary one-way ANOVA followed by Dunnett's multiple comparisons test (* $p< 0.05$, ** $p<0.01$, *** $p<0.001$).

3.10 Islet regenerative effects of PSAP, FAM3C, GAL1, and PPIA.

As described previously, hyperglycemic (13-25 mmol/L), STZ-treated NOD/SCID mice were iPan injected on day 10 with: (1) 100 ng PSAP (n=6), or (2) 100 ng FAM3C (n=6), or (3) 100 ng GAL1 (n=6), or (4) 100 ng PPIA (n=6), and compared to mice that received AmnioMAX^{TM} basal media (n=7, vehicle control).

Compared to basal media controls, mice iPan injected with PSAP, GAL1, or PPIA did not demonstrate reduction in NFBG levels over 42 days and remained severely hyperglycemic (>30mmol/L, Figure 3.10 A). In contrast, mice iPan injected with FAM3C demonstrated a transient decrease in NFBG levels on day 21 (Figure 3.10 B). Overall, iPan injection of PSAP, FAM3C, GAL1 or PPIA did not reduce the AUC for NFBG levels over the 42 days (Figure 3.10 B).

Intraperitoneal glucose tolerance tests were performed on day 42 to assess response to a glucose challenge. Compared to basal media controls which had sustained elevated glucose in response to a glucose bolus, mice that received FAM3C or PPIA had reduced glycemic levels 90 minutes post-glucose bolus, and GAL1 had reduced glycemic levels 120 minutes post-glucose bolus (Figure 3.10 C). In contrast, mice iPan injected with PSAP failed to respond to glucose challenge (Figure 3.10 C). Overall, the AUC measurements for the 2-hour glucose tolerance test were significantly reduced for FAM3C compared to basal media controls (Figure 3.10 D).

A ratio of the starting (day 0) and final weight (day 42) showed that there was no significant weight reduction for any of the treatment groups (Figure 3.10 I).

In post-mortem immunohistochemical analyses, iPan injection of PPIA increased beta cell mass (Figure 3.10 J) and islet size (Figure 3.10 K), but not islet number (Figure 3.10 L); whereas mice that received PSAP or GAL1 showed increase in islet size compared to basal media controls (Figure 3.10 K). Interestingly, iPan injection of FAM3C did not affect beta cell mass, islet size, or islet number at day 42 despite improved glucose tolerance (Figure 3.10 J-K).

Taken together, while mice that received iPan injection of PPIA demonstrated limited improvement in blood glucose management, there was a significant increase in beta cell recovery. Mice that received FAM3C showed improvement in glucose tolerance, but no significant restoration of beta cell mass.

To summarize, the 8 MSC-secreted proteins demonstrated variable potential in promoting islet regeneration when administered individually at a dose of 100 ng.

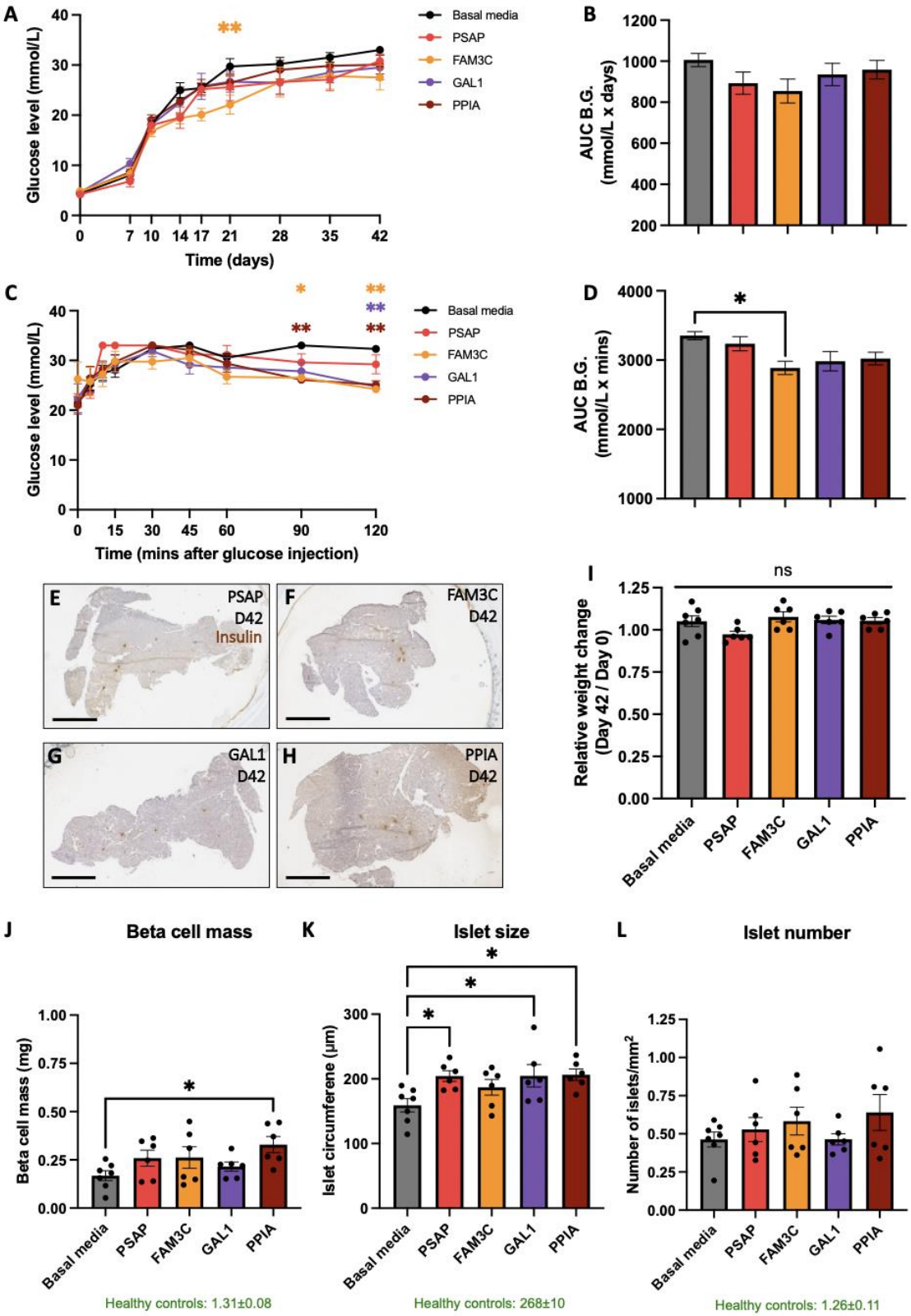
A
Glucose level (mmol/L)
Time (days)
Basal media
PSAP
FAM3C
GAL1
PPIA
B
AUC B.G. (mmol/L x days)
C
Glucose level (mmol/L)
Time (mins after glucose injection)
D
AUC B.G. (mmol/L x mins)
E
PSAP D42 Insulin
F
FAM3C D42
G
GAL1 D42
H
PPIA D42
I
ns
Relative weight change (Day 42 / Day 0)
J
Beta cell mass
Beta cell mass (mg)
Healthy controls: 1.31±0.08
K
Islet size
Islet circumferene (µm)
Healthy controls: 268±10
L
Islet number
Number of islets/mm²
Healthy controls: 1.26±0.11

Figure 3.10 Islet regenerative effects of PSAP, FAM3C, GAL1, and PPIA.

Hyperglycemic (13-25 mmol/L), STZ-treated NOD/SCID mice received iPan injection of basal media (n=7), or PSAP (n=6), or FAM3C (n=6), GAL1 (n=6), or PPIA (n=6) at 100 ng on day 10. Blood glucose levels were monitored weekly and pancreata were harvested on day 42. **(A)** Compared to basal media controls, mice iPan injected with PSAP, GAL1, or PPIA did not demonstrate any reduction in non-fasting blood glucose (NFBG) levels up to day 42. Mice that received FAM3C demonstrated a transient reduction in NFBG on day 21. **(B)** No differences in area under the curve (AUC) for NFBG were observed. **(C)** Mice that received FAM3C, GAL1 or PPIA showed slight improvement in glucose tolerance at 90- and 120- minute, whereas the PSAP group failed to respond to glucose challenge. **(D)** The AUC for 2-hour glucose tolerance test was reduced for FAM3C. **(E-H)** Representative photomicrographs of insulin+ islets on day 42 (scale bar: 2 mm). **(I)** No differences in weight change were observed. iPan injection of PPIA increased **(J)** beta cell mass and **(K)** islet size compared to basal media controls. Mice that received PSAP or GAL1 showed increased islet size. **(L)** No differences in islet number were observed between groups. Data represent mean ± SEM. Analyses of significance for NFBG was performed by ordinary two-way ANOVA followed by Dunnett's multiple comparisons test. AUC, weight change, beta cell mass, islet size and islet number were compared using ordinary one-way ANOVA followed by Dunnett's multiple comparisons test (* $p< 0.05$, ** $p<0.01$).

Chapter 4

4 Discussion

4.1 Summary of important findings

The following novel findings contributed to our knowledge of the islet regenerative capacities of 8 MSC-secreted proteins both individually and in combination:

Protein effects on islet regeneration after iPan injection *in vivo*

(1) iPan injection of 8-protein combination (100 ng/protein) in STZ-treated NOD/SCID mice reduced hyperglycemia, improved glucose tolerance, and increased beta cell mass compared to basal media controls. These effects were comparable to mice that received Wnt+ CM (4-8 µg).

(2) Islet beta cell area was increased, and beta/alpha cell ratio was improved in mice that received 8-protein combination (100 ng/protein) compared to basal media controls.

(3) Mice that received Wnt+ CM (4-8 µg) or 8-protein combination (100 ng/protein) showed increased beta cell mass within 4 days post-injection compared to basal media controls.

(4) At 32 days post-iPan injection, SOD1 (100 ng) or CALU (100 ng) decreased non-fasting blood glucose levels and improved glucose tolerance but did not significantly change beta cell mass compared to basal media controls.

(5) At 32 days post-iPan injection, PPIA (100 ng) improved glucose tolerance and increased beta cell mass but did not significantly change non-fasting blood glucose levels compared to basal media controls.

(6) iPan injection of TGM2 (100 ng), CTSB (100 ng), or FAM3C (100 ng) improved glucose tolerance, but did not significantly change non-fasting blood glucose levels or beta cell mass at 32 days post-injection, compared to basal media controls.

(7) iPan injection of PSAP (100 ng) or GAL1 (100 ng) did not affect non-fasting blood glucose levels, glucose tolerance, or beta cell mass at 32 days post-injection, compared to basal media controls

Protein effects on cultured human islets *in vitro*

(1) Human islets cultured for 7 days with Wnt+ CM (4 µg/mL) increased the percentage of proliferating glucagon+ cells and vimentin+ cells; but did not significantly change CK19+ or insulin+ cell proliferation or viability compared to basal media controls.

(2) Human islets cultured 7 days with 8-protein combination (100 ng/mL/protein), or the individual proteins (100 ng/mL) did not significantly change lineage-specific pancreatic cell viability or proliferation compared to basal media controls.

(3) Human islets cultured 7 days with Wnt+ CM (4 µg/mL), 8-protein combination (100 ng/mL/protein), or the individual proteins (100 ng/mL) did not significantly change the percentage of live cells co-expressing insulin with glucagon / CK19 / vimentin compared to basal media controls.

4.2 Wnt+ CM and 8-protein combination exhibited comparable efficiency stimulating beta cell regeneration after iPan injection into STZ-treated NOD/SCID mice.

One of the objectives of this project was to functionally validate the islet regenerative effects of the 8 MSC-secreted proteins *in vivo* using STZ-treated NOD/SCID mice, the model that we previously characterized the therapeutic potential of Wnt+ CM in promoting beta cell regeneration.

Our study showed that iPan injection of Wnt+ CM at 4-8 µg decreased NFBG, improved glucose tolerance, and increased beta cell mass in STZ-treated NOD/SCID mice, consistent with previous studies performed by Kuljanin *et al*[147]. Using the same *in vivo* model, our study demonstrated that a single injection of the novel 8-protein combination at 100 ng/protein stimulated endogenous islet regeneration without the need for cell transfer. The regenerated beta cells functioned to ameliorate STZ-induced hyperglycemia by lowering

NFBG levels from 11 days post-injection, and this effect was sustained until the end of experiment (32 days post-injection). Recovery of beta cell function was also demonstrated by intraperitoneal glucose tolerance tests at 32 days post-injection, in which mice that received 8-protein combination responded to the glucose challenge and showed improved ability to regulate a glucose bolus compared to basal media controls. The recovery of beta cell function resulted from increased beta cell mass, islet size, and islet number was revealed in post-mortem immunohistochemical analyses. Importantly, the islet regenerative effects observed in mice that received 8-protein combination were comparable to mice that received Wnt+ CM, suggesting that the 8 MSC-secreted proteins represented key players in stimulating islet regenerative effects within the Wnt+ CM.

However, beta cell regeneration induced by Wnt+ CM in studies by Kuljanin *et al* was more robust than in the current study. Following iPan injection, Kuljanin *et al* previously saw a drastic reduction in NFBG levels that plateaued at ~10-15 mmol/L, whereas mice that received Wnt+ CM plateaued at ~20 mmol/L in our study[147]. This discrepancy may be explained, in part, by lower protein concentrations collected in Wnt+ CM in this study, where some mice received 4 µg total proteins instead of 8 µg. Kuljanin *et al* demonstrated that the extent of glucose control was dependent on the concentration of secreted proteins delivered to the pancreas as they saw mice that received 4 µg Wnt+ CM only demonstrated partial recovery of beta cell function compared to mice that received 8 µg Wnt+ CM[147]. This could also explain the large variability we observed within the Wnt+ CM in our study. Due to the limitation of iPan injection, we were restricted by animal ethics considerations to deliver a single dose of 20 µL to the pancreas. Nonetheless, the generation of CM is known to collect other biological factors such as RNAs and lipid-based metabolites. We cannot rule out the possibility that other bioactive factors within CM, such as non-protein cargo contained in MSC-secreted extracellular vesicle or microparticles may also contribute to islet regeneration. Thus, future studies should focus on developing reliable methods to concentrate and standardize the biological molecules contained in MSC CM, and explore different routes of administration to ensure consistency and reproducibility of results.

Moreover, the current study only evaluated the islet regenerative effects of the 8 MSC-secreted proteins at 1 dosage (100 ng/protein). The dosage was determined using the average detector intensity to estimate the concentration of each effector in the 8 µg total proteins in the CM. Further optimization is needed to determine the optimal concentration of each protein to maximize islet regeneration.

4.3 Alpha-to-beta cell conversion as a mechanism of beta cell regeneration induced by 8-protein combination.

Our results showed that islet alpha cell area increased in proportion in all the treatment groups that received STZ compared to healthy controls, and this increase in alpha cell content was also evident in other studies[147,173,174]. For instance, Feng *et al* observed a significant increase in alpha cell mass in STZ-treated mice, and STZ treatment increased alpha cell proliferation in isolated islets. Feng *et al* also showed that giving STZ-treated mice GABA largely restored insulin secretion and decreased alpha cell mass, suggesting that the expanded alpha cell population may have implication in beta cell regeneration[174].

In our study, immunofluorescent analyses of mouse pancreatic sections at 32 days post-injection revealed increased beta/alpha cell ratio for mice that received 8-protein combination compared to basal media controls. Healthy mice typically contain a greater percentage of beta cells (60-80%) within an islet compared to alpha cells (10-20%)[74]. STZ treatment decrease beta/alpha cell ratio by ablating beta cells and inducing alpha cell hyperplasia/proliferation[172,174,175]. Thus, increased beta/alpha cell ratio represents another evidence for beta cell regeneration.

Furthermore, the increase in islet beta cell proportion is accompanied with a non-significant decrease in islet alpha cell proportion in the 8-protein combination group, suggesting that certain number of islet cells co-expressed insulin and glucagon after iPan injection of 8-protein combination. This finding provides indirect evidence supporting alpha-to-beta cell conversion as a possible mechanism for beta cell regeneration induced by these effectors. These results were consistent with several observations by Kuljanin *et al* that supported alpha-to-beta cell conversion as a putative mechanism for the recovery of beta cell mass after iPan injection of Wnt+ CM: (1) beta cell mass gradually increased with time while

the frequency of glucagon+ cells was diminished, (2) using NKX6.1 as a marker of functionally matured beta cells, the expression of endocrine marker NKX2.2 remained constant 1 day, 4 day and 32 days post-injection, while the frequency of NKX6.1+ cells/islet was significantly increased in mice that received Wnt+ CM, and (3) glucagon+ cell that co-expressed NKX6.1 or insulin were consistently detected after injection of Wnt+ CM. Although these finding support the hypothesis of alpha-to-beta cell conversion, Kuljanin *et al* reported that the endogenous mechanisms underlining the induction of beta cell regeneration by Wnt+ CM were multifactorial, including islet formation associated with ductal regions and increased beta cell proliferation at early time points (1, 3 and 7 days post-injection) [147]. In our study, we also observed small clusters of cells that were insulin+ and glucagon+ adjacent to ductal regions in the Wnt+ CM group and 8-protein combination group (data not shown), providing indirect evidence for the neogenic mechanism of beta cell regeneration. However, due to the limitations of the NOD/SCID model, lineage tracing experiments will have to be performed to further elucidate the actual mechanisms of beta cell regeneration induced by Wnt+ CM or 8-protein combination either from a CK19+ ductal cell or a glucagon+ alpha cell intermediate. These experiments are currently underway in other projects in our lab.

4.4 Beta cell regeneration occurred within 4 days after a single injection of Wnt+ CM or 8-protein combination.

Our results showed that increased beta cell mass was evident in mice that received Wnt+ CM or 8-protein combination at 4 days post-injection compared to basal media controls, consistent with what Kuljanin *et al* observed after iPan injection of 8 µg Wnt+ CM[147]. Kuljanin *et al* also showed increased beta cell proliferation at 4 days post-injection; however, we did not conduct a quantitative analysis of beta cell proliferation at this specific time point[147]. As a result, we were unable to draw definitive conclusions regarding the source of this increase in beta cell mass. It is plausible that Wnt+ CM or 8-protein combination may contribute to the prevention of ongoing beta cell death caused by STZ, and/or facilitate the regeneration of new beta cells through mechanisms such as proliferation, neogenesis, and/or transdifferentiation. Nevertheless, the results suggest that Wnt+ CM and the 8 MSC-secreted proteins activated beta cell regenerative programs in

the murine pancreas shortly after injection. Furthermore, important questions persist regarding the maturity of these beta cells and insulin secretion function of these beta cells as mice remained hyperglycemic at 4 days post-injection despite having increased beta cell mass.

We also observed that beta cell mass was significantly lower at 32 days post-injection compared to 4 days post-injection in the 8-protein combination group, suggesting that islet regeneration induced by the MSC-secreted proteins may be transient or unsustain by a single iPan injection. Due to ethnical concerns, it was impractical to perform multiple iPan injections on the same mouse. Moreover, the precise location where the needle dispenses the proteins into the pancreas may vary from injection to injection. Thus, proteins may be retained in the pancreas at different rates. For instance, injection of proteins into the duodenal lobe may not reach the splenic lobe. Overall, future studies should consider investigating the effects of prolonged exposure to these proteins *in vivo* using: (1) device such as osmotic pumps, (2) transplanting MSC that continually overexpress these proteins versus knockout of the protein of interest, or (3) by testing other routes of administration that are less invasive and permit multiple injections such as intravenous/intraperitoneal administration.

4.5 Individual proteins demonstrated variable potential to promote islet regeneration.

To assess which candidate protein(s) were responsible for the islet regenerative effects observed after iPan injection of 8-protein combination, we performed the 42-day experiments using the same *in vivo* model for each MSC-secreted proteins that were commonly upregulated in regenerative MSC CM and Wnt+ CM: FAM3C, PSAP, SOD1, PSAP, GAL1, CTSB, TGM2, and CALU.

Surprisingly, a single iPan injection of SOD1 or CALU at 100 ng/protein ameliorated hyperglycemia and improved glucose tolerance without significantly affecting beta cell mass at 32 days post-injection. One possible explanation is that SOD1 and CALU act to improve beta cell function, leading to more efficient secretion of insulin to decrease NFBG levels and to generate better response during glucose tolerance tests, without direct

evidence for beta cell mass expansion. Future investigation into the effects of SOD1 and CALU on serum insulin levels *in vivo* and glucose-stimulated insulin secretion *in vitro* are warranted.

An association between SOD1 and diabetes is evident in the literature. Previously, Hamasaki *et al* concluded that oxidative stress due to I113T SOD1 mutation combined with muscle atrophy and visceral obesity due to inactivity by familial amyotrophic lateral sclerosis may have been the causative factor for severe insulin resistance[176]. In animal models, increased SOD1 expression is associated with decreased fasting blood glucose, HbA1C, and the survival of hypertrophied beta cells during chronic hyperglycemia[177,178]. In contrast, genetic disruption of SOD1 gene caused hyperglycemia and impaired beta cell function[179]. The mechanism behind this impairment is multifold. Firstly, SOD1 modulates the expression of PDX1, a transcription factor that regulates pancreatic development and beta cell maturation, as well as the stimulation of pancreatic genes such as insulin, GLUT2, and glucokinase 5[179]. To increase PDX1 mRNA levels, SOD1 increases H3 acetylation and H3K4 methylation of the promoter region. Second, knockout of SOD1 reduces FOXA2 mRNA levels, a transcription factor that enhances PDX1 expression *in vivo.* Therefore, a lack of SOD1 decreased PDX1 levels through various molecular pathways, ultimately leading to decreased insulin secretion and beta cell volume[179].

On the other hand, the association between CALU and diabetes is less evident in the literature. CALU is ubiquitously expressed in human tissues, and it normally functions to maintain calcium homeostasis, protein folding, and secretory cargo sorting. Recent evidence has suggested that CALU may also have a role in modulating EMT[171]. This is particularly relevant during beta cell regeneration as alpha-to-beta cell conversion is often accompanied by EMT and hyperplasia in the ductal epithelium, which are believed to subsequently convert to endocrine cell types that expand to generate new beta cells through an islet neogenic cascade[180,181]. Overall, further investigations employing lineage tracing models specific for glucagon-Cre mice (to document alpha-to-beta cell transition), or CK19-Cre mice (to document ductal epithelial cell transition to endocrine cell types) are warranted to establish the precise mechanisms underlying islet regeneration associated with SOD1 and CALU.

Interestingly, iPan injection of PPIA at 100 ng also improved glucose tolerance and increased beta cell mass, but did not affect NFBG levels compared to basal media controls throughout the 32 days post-injection period. One possible explanation is that PPIA failed to stimulate the differentiation of newly generated immature beta cells to a more insulin secreting phenotype. Newly generated or immature beta cells are known to secrete lower levels of insulin in response to elevated blood glucose[182]. Therefore, future experiments testing the expression of functional and mature beta cell markers such as GLUT2, MAFA, and NKX6.1 will be important to elucidate the mechanisms of islet regeneration induced by iPan injection of PPIA.

In addition, iPan injection of TGM2, CTSB, or FAM3C at 100 ng improved glucose tolerance but did not significantly increase beta cell mass at 32 days post-injection, and only showed a transient reduction in NFBG levels at 11 days post-injection compared to basal media controls. The stability and longevity of these MSC-secreted proteins once injected into the pancreas is unknown, and it is possible that many of the proteins are degraded rapidly *in vivo* (likely within 24 hours) so only a transient reduction in NFBG levels is apparent. It has been previously reported that the half-life of TGM2 is ~10 hours in colorectal cancer HT29 cells, and that of FAM3C is ~18 hours in renal epithelial cells[183,184]. However, half-lives and functions within pancreas tissue remain to be elucidated. Overall, it is possible that multiple injections of Wnt+ CM or the MSC-secreted proteins are needed to achieve a more consistent and sustained reduction of NFBG levels.

Finally, the protein-protein interactions between the 8 proteins also remain unknown. It is unlikely that a single effector could be responsible for mediating the complex process of islet regeneration. The findings from this study suggest that multiple mechanisms are likely to act synergistically in the 8-protein combination group, facilitating the restoration or maintenance of beta cell mass, amelioration of hyperglycemia, and improvement in glucose tolerance.

4.6 MSC-secreted proteins did not change islet cell viability or proliferation at 7 days of culture.

Another objective of this project was to functionally validate the islet regenerative effects of the 8 MSC-secreted proteins *in vitro* using cultured human islets by assessing cell lineage-specific viability and proliferation. Although mice iPan injected with 8-protein combination or some of the individual proteins demonstrated potent islet regenerative effects *in vivo*, human islets cultured with MSC-secreted proteins did not change cell lineage-specific viability or proliferation at 7 days.

Human islets cultured for 7 days using established protocols exhibited comparable viability (~60%) among insulin+, glucagon+, CK19+, and vimentin+ cells across all treatment conditions, consistent with the negative (basal media) and positive (basal media + 10% FBS) controls. These findings suggest that islet cell viability was surprisingly well-maintained in culture with DMEM basal media without any serum supplementation. DMEM basal media contains a range of essential components, including amino acids, vitamins, and inorganic salts (i.e., glutamine, cysteine, and nicotinamide), which are known to contribute to the preservation of cell viability[185]. In a previous study conducted by Kuljanin *et al*, the effects of regenerative MSC CM on cultured human islets were evaluated, revealing a viability rate of ~40% for beta cells when cultured in RPMI basal media for 7 days. However, when the islets were treated with regenerative MSC CM, the viability of beta cells increased to ~53%[142]. These results suggest that islet cell viability in the current study was relatively high in DMEM basal media alone, and neither supplementation with 10% FBS nor the addition of the MSC-secreted proteins significantly increased islet cell viability. Supplementation with 10% FBS is commonly employed in islet research culture as serum contains numerous factors beneficial for overall islet viability[186]. Additionally, 10% FBS serves to neutralize endogenous pancreatic enzymes and exogenous enzymes remaining from the isolation process, thereby preventing further islet cell destruction or dissociation[187–189]. Therefore, it was unexpected to observe that supplementation with 10% FBS did not yield an anticipated increase in islet cell viability. However, future *in vitro* investigations regarding the islet regenerative effects of Wnt+ CM and the MSC-secreted proteins should consider utilizing basal media with a sub-optimal

composition to better elucidate potential effects of putative islet regenerative proteins. In addition, recombinant proteins could be supplemented into culture media daily (rather than every 2-3 days) to prolong any potential effects on islet cell viability.

We observed 2.98%±0.64% of human islet cells undergoing proliferation during the last 24 hours of culture for islets cultured with 10% FBS, measured by EdU incorporation and detected by flow cytometry. However, proliferation was minimal (<1%) for insulin+, glucagon+, CK19+ and vimentin+ cells in human islets cultured with basal media or MSC-secreted proteins. Islets cultured with Wnt+ CM showed a small but non-significant increase in the percentage of proliferating insulin+ cells compared to basal media controls (0.39%±0.12% vs 0.24%±0.06%). Similarly, Kuljanin *et al* previously showed that Wnt+ CM increased proportion of insulin+/EdU+ beta cells after 3 days of culture compared to islets cultured in basal media, but not at 7 days[142]. This observation suggests that cultured beta cells from human islets may lose their proliferative capacity with prolonged culture time and testing at early time points may be required to elucidate any potential effects of the proteins. Surprisingly, islets cultured with Wnt+ CM for 7 days demonstrated a significantly increased percentage of proliferating glucagon+ cells and vimentin+ cells. Human alpha cells were shown to proliferate at a 5-fold higher rate than human beta cells and express lower levels of cell cycle inhibitors CDKN1A and CDKN1C[190]. A study by Lam *et al* also demonstrated proliferation of alpha cells in human islets, and showed that replicative capacity of alpha cell decreased with age and diabetes states, suggesting that reprogramming of alpha cells may be a viable strategy to replenish beta cell pool in diabetes[191]. Bets cells from cultured human islets are known to undergo EMT and give rise to highly proliferative mesenchymal cells that retain the potential to re-differentiate into insulin-producing cells[71,192]. Similarly, CK19+ ductal epithelial cells and other endocrine cell types such as alpha cells, delta cells, and PP cells were shown to co-express vimentin in culture[193]. The presence of EMT in these cell types could be relevant to designing strategies aiming to re-differentiate the expanded islet cells towards a beta cell phenotype for the treatment of diabetes. However, whether these proliferating glucagon+ cells and vimentin+ cells induced by Wnt+ CM have implications in beta cell regeneration remained unknown since we did not observe an increased proportion of cells that co-expressed insulin and glucagon, nor insulin and vimentin. It is likely that optimal dosing was not

achieved, supplemented proteins were degraded rapidly in culture, or additional effectors (i.e., other proteins, RNAs, lipid-based metabolites) would be needed to complete the re-differentiation process.

Overall, our results did not demonstrate direct islet regenerative effects of MSC-secreted proteins using human islets *in vitro*; however, several factors could in part explain the disparity between *in vitro* and *in vivo* results: (1) decreased human islet regenerative capacity with age; (2) large inter-donor variability; and (3) microenvironment differences and interplay of additional factors/cell types in the pancreas. It will be crucial to consider these possibilities in order to better understand the findings of *in vitro* studies and their implications.

Firstly, decreased islet regenerative potential with age is documented in both humans and mice. Krishnamurthy *et al* showed that mouse survival after STZ-mediated beta cell ablation, which requires islet proliferation, declined with increasing age[194]. Greater than 25% of 5-9 weeks old mice survived more than 100 days after STZ treatment, whereas no mice older than 20 weeks demonstrated survival after STZ[194]. Similarly, Chen *et al* showed that exposure of juvenile human islets (mean age 3.0 ± 0.6 years) to platelet-derived growth factor-AA (PDGF-AA) stimulated beta cell proliferation, whereas exposure to adult human islets (mean age 44.3 ± 5.3 years) did not[195]. The human islets used in this study had a mean age of 47.2 ± 6.5 years (range: 36-74). Therefore, the age-dependent decrease in islet regenerative potential may be a contributing factor to the absence of regenerative effects observed. Secondly, we consistently observed large inter-donor variability for all parameters quantified from human islet experiments, which was likely due to differences in donor demographics (i.e., cause of death, BMI, sex, race), isolation centers, cold ischemia time (13.8±1.8 hours), and pre-shipment culture time (55.8±12.8 hours)[196]. For instance, the 2 islet samples with cold ischemia time >15 hours had decreased overall viability compared to other samples (49.8%±5.4% vs 70.1%±4.8%). Hence, large inter-donor variability may post limitations in data interpretation and a larger sample size may be required for future experiments using human islets. Finally, *in vitro* human islet culture provided a controlled and simplified environment that may not fully replicate the complex conditions present in the exocrine and endocrine pancreas *in vivo*. As discussed previously,

other cell types in the pancreas such as acinar cells, ductal cells, and stromal cells may be implicated in beta cell regeneration. Communication of the islets to the rest of the pancreas including exocrine tissue and extracellular matrix components, and peripheral organs may be important for the islet regenerative effects induced by Wnt+ CM and MSC-secreted proteins[197]. For instance, variation in FAM3C expression in the liver was found to affect insulin resistance and hyperglycemia in obese mice[150]; GAL1 is associated with insulin resistance in the adipose tissue[163]; expression of CTSB in human skeletal muscle was shown to affect insulin resistance[165]. Therefore, the proteins may directly or indirectly affect islet regeneration and the exact mechanisms of action are warranted for further investigation.

4.7 Summary of limitations and future directions

This study has several limitations and suggests several potential future directions:

(1) Optimal Protein Dosage: The current study evaluated the islet regenerative effects of the MSC-secreted proteins at a single dosage of 100 ng. Further experimentation is necessary to determine the optimal concentration of each protein in order to maximize islet regeneration and establish an appropriate dosage for clinical applications.

(2) Lineage Tracing Experiments: Due to the limitations of the NOD/SCID model, conducting lineage tracing experiments is essential to elucidate the signal receiving cells within the pancreas and to determine the mechanisms of beta cell regeneration induced by Wnt+ CM or the 8-protein combination. The proteins could directly or indirectly affect islet regeneration.

(3) Sustained Islet Regeneration: The transient and potentially unsustain islet regeneration induced by the MSC-secreted proteins suggests the need for multiple injections or continual targeted delivery. Future investigations could explore prolonged exposure to these proteins using devices like osmotic pumps, transplantation of MSC with reduced/elevated expression of target proteins, or testing different routes of administration to achieve consistent reduction of non-fasting blood glucose levels.

(4) Protein Half-lives and Functions: The specific half-lives and functions of the proteins within the pancreas remain to be elucidated. Investigating these aspects can contribute to a deeper understanding of their mechanisms of action in islet regeneration.

(5) Functional and Mature Beta Cell Markers: Further experiments should assess the expression of functional and mature beta cell markers and associated transcription factors to provide a more comprehensive understanding of the effects of the proteins on mature versus immature beta cell function.

(6) Future *in vitro* investigations could utilize different culture condition and examination at multiple endpoints, while considering factors such as decreased islet regenerative potential with age and large inter-donor variability across different islet samples.

(7) Testing in Other Diabetic Animal Models and Application with Islet Replacement: Additional preclinical testing in relevant models of diabetes need to be addressed before clinical translation. For example, in STZ-treated NOD/SCID mice, endogenous islet regeneration can occur unabated in the absence of inflammation or autoimmunity. Thus, injection into NOD mice, combined with strategies to dampen autoimmunity, requires investigation to determine if regeneration can occur in the face of ongoing autoimmunity. Furthermore, exploring the therapeutic potential of the MSC-secreted proteins in conjunction with beta cell replacement strategies using pancreatic progenitor cells or further differentiated beta-like cells derived from pluripotent stem cell sources is a promising avenue for future investigation.

(8) Investigation of proteins downregulated in Regenerative MSC CM and Wnt+ MSC CM: Current study only functionally validated the islet regenerative effects of proteins that were commonly upregulated in Regenerative CM and Wnt+ MSC. It is possible that the downregulation of certain proteins in the CM was important for the islet regenerative effects previously observed.

4.8 Overall significance

The development of a curative therapy for diabetes holds transformative potential for millions of patients impacted by this debilitating condition. Stimulating endogenous

regeneration using Wnt+ CM derived from BM-MSC holds a promising avenue to restore lost beta cells in individuals with diabetes. Notably, our study successfully demonstrated the regenerative capacities of the 8 MSC-secreted proteins, particularly when administered in combination. This research has significantly contributed to our understanding of the roles of these proteins within the pancreas, while also establishing proof-of-concept for the development of protein-based therapies for diabetes. Protein-based therapies offer distinct advantages over cell-based and MSC CM-based approaches, including more efficient production, storage, and delivery due to the complex procedures and stringent protocols involved in generating and collecting MSC CM under good manufacturing practice (GMP). Based on our findings, we firmly believe that the 8 MSC-secreted proteins will serve as pivotal mediators of islet regeneration or beta cell differentiation, and delivery of these proteins represents a compelling and novel therapeutic approach for drug development and the treatment of diabetes using protein-based therapy.

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Appendices

Appendix 1. Human Ethics Approval

Western Research

Date: 21 February 2023

To: David Hess

Project ID: 4050

Review Reference: 2023-4050-75896

Study Title: Transplantation of human stem cells for the induction of angiogenesis and the regeneration of beta-cell function (REB #12934)

Application Type: Continuing Ethics Review (CER) Form

Review Type: Full Board

REB Meeting Date: 21/Feb/2023 13:00

Date Approval Issued: 21/Feb/2023 15:08

REB Approval Expiry Date: 19/Dec/2024

Re-Opening of file at the request of the Principal Investigator

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Dear David Hess,

The Western University Research Ethics Board has reviewed the application. This study, including all currently approved documents, has been re-approved until the expiry date noted above.

REB members involved in the research project do not participate in the review, discussion or decision.

Western University REB operates in compliance with, and is constituted in accordance with, the requirements of the Tri-Council Policy Statement: Ethical Conduct for Research Involving Humans (TCPS 2); the International Conference on Harmonisation Good Clinical Practice Consolidated Guideline (ICH GCP); Part C, Division 5 of the Food and Drug Regulations; Part 4 of the Natural Health Products Regulations; Part 3 of the Medical Devices Regulations and the provisions of the Ontario Personal Health Information Protection Act (PHIPA 2004) and its applicable regulations. The REB is registered with the U.S. Department of Health & Human Services under the IRB registration number IRB 00000940.

Please do not hesitate to contact us if you have any questions.

Appendix 2. Animal Ethics Approval

Western

AUP Number: 2023-014
PI Name: Hess, David A
AUP Title: Transplantation of Novel Stem Cells for the Regeneration of B-Cell Function
Approval Date: 05/01/2023

Official Notice of Animal Care Committee (ACC) Approval:

Your new Animal Use Protocol (AUP) 2023-014:1: entitled " Transplantation of Novel Stem Cells for the Regeneration of B-Cell Function" has been APPROVED by the Animal Care Committee of the University Council on Animal Care. This approval, although valid for up to four years, is subject to annual Protocol Renewal.

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

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

1. This Animal Use Protocol is in compliance with:
    - Western's Senate MAPP 7.12 [PDF]; and
    - Applicable Animal Care Committee policies and procedures.
2. Prior to initiating any study-related activities—as per institutional OH&S policies—all individuals listed within this AUP who will be using or potentially exposed to hazardous materials will have:
    - Completed the appropriate institutional OH&S training;
    - Completed the appropriate facility-level training; and
    - Reviewed related (M)SDS Sheets.

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

Curriculum Vitae

Name: Xin Yue (Sharon) Xie

Post-secondary Education and Degrees:

The University of Western Ontario
London, Ontario, Canada
2017-2021
Bachelor of Medical Sciences
Honours Specialization in Physiology and Pharmacology

The University of Western Ontario
London, Ontario, Canada
2021-2023
Master of Science in Physiology and Pharmacology

Honours and Awards:

Western Scholarship of Excellence (2017)
Western's 125th Anniversary Alumni Awards (2019)
Dean's Honour List (2017-2021)
2021 Physiology and Pharmacology Research Day
Poster Award for New Student
2022 Robarts Research Retreat
Poster Award
2022 Till & McCulloch Meetings
Travel Award
Western Graduate Research Scholarship (2021-2023)
Canada Graduate Scholarship – Master (2022-2023)

Related Work Experience

Research Assistant
The University of Western Ontario
Department of Psychology, Dr. Paul Gribble
2018-2019

Research Assistant
The University of Western Ontario
Department of Medical Biophysics, Dr. Aaron So
2020-2021

Teaching Assistant
The University of Western Ontario
Department of Physiology and Pharmacology
2021-2023

Publications:

Journal Articles

So, A., Yang, Z., Li, L., Li, W., Pan, C., Vivekanantha, P., Yun, H. W., **Xie, X. Y.**, Yun, C. H., Huang, W. M., Hung, C. L., Gao, M., Zhang, X., Zha, Y., & Shen, J. (2022). Relationship between lung injury extent and phenotype manifested in non-contrast CT and cardiac injury during acute stage of COVID-19. International journal of cardiology. Heart & vasculature, 38, 100938.

Thesis/Dissertation

Xie, XY; Bell, GI; Hess, DA. (2021) Identification and functional validation of multipotent stromal cell-secreted islet regenerative proteins. Western University. Bachelor's Honours.

Conference Publications

Xie, XY; Bell, GI; Hess, DA. (October 2022). Functional validation of multipotent stromal cell-secreted islet regenerative proteins. Poster #65. Stem Cell Network. 2022 Till & McCulloch Meetings. Vancouver, Canada.