Isolation and Characterization of Mesenchymal Stromal Cells from the Visceral Adipose Tissue in Peripancreatic Region

by

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Abstract

Type 1 Diabetes Mellitus (T1DM) is a multigenic autoimmune disorder that leads to the destruction of insulin producing β-cells of the pancreas by the host immune system. This can lead to chronic hyperglycemia, diabetic ketoacidosis (DKA), retinopathy, nephropathy, neuropathy, serious cardiovascular complications, severe hypoglycemic unawareness and glucose instability. Conventional management therapy includes daily glucose monitoring and exogenous insulin injections. Islet transplantation is an attractive alternative to conventional therapy. First attempts of islet transplantation dates to 1972. Clinical feasibility and efficacy of islet transplantation was first demonstrated by Edmonton protocol (EP) developed by the Islet Transplant Group in Edmonton in 2000. However, some of the limitations of this approach include limited islet supply, gradual graft loss, and harmful chronic immunosuppression regimen. Even with marked improvements, at its current state, the procedure it is reserved for specific subset of T1DM patients that have unstable T1DM and hypoglycemia unawareness, severe hypoglycemic episodes and glycemic lability that cannot be controlled with intensive insulin therapies.

Some of the major limitations of islet transplantation that need to be overcome for it to be widely available are limited islet supply, chronic immunosuppression to prevent allograft rejection, and gradual graft loss. The last two could be potentially be addressed with Mesenchymal Stromal Cells (MSCs) which are multipotent stem cells found in the stroma of most of the tissues in the body. These cells have self-renewal capacity and can be differentiate into adipocytes, osteoblasts, and chondrocytes. MSCs can suppress the inflammation and promote tissue repair and regeneration through the secretion of cytokines, anti-oxidants, pro-angiogenic factors, anti-apoptotic factors, antimicrobial factors, and trophic molecules.

Current literature suggests that there are source-dependent differences in MSCs with respect to cell yield per mass of tissue, transcriptome and secretome profiles, and proliferative and mitotic capacities. This thesis examined the ideas of microenvironment-dependent differences among different types of MSCs and that MSCs that are ontologically and anatomically closer to the islets might be more beneficial to them. Therefore, in this study we isolated and characterized cells from the visceral adipose tissue specifically in the peripancreatic region. In accordance with International Society for Cellular Therapy (ISCT), my data demonstrate that cells I prepared attached to the plastic, expressed a MSC-defining cell surface markers, and differentiated into adipocytes but not chondroblasts and osteoblasts. Reduced differentiation potential could be explained by the fact that the donors were elderly and obese, and the cells have undergone many mitotic divisions before undergoing differentiation protocols. Given the results from multiply analyses we denoted them as ppaMSCs. This newly characterized cells could be used in the future studies to assess their effects in islets transplantation.

Preface

This thesis is an original work by Osmanmyrat Hojanepesov. The research project, of which this thesis is a part, received ethics approval from the University of Alberta Research Ethics Board (HREB; Pro00001416)

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1.1.1 Normal Physiology of Glucose Homeostasis

Glucose is the main source of energy for cells in human body. Under aerobic conditions, all cells in the body harvest energy stored in glucose to produce adenosine triphosphate (ATP) molecules, which are used in almost all cellular processes. After a meal, macromolecules such as proteins, polysaccharides, lipids in food which have entered the intestines are broken down into smaller absorbable constituents: amino acids, monosaccharides, and free fatty acids (FFA), respectively. Liberated glucose residues are then absorbed into blood mainly by collaborated action of SGLT1 and GLUT2 transporter proteins located in the small intestinal epithelium cells [1,2]. Islets of Langerhans in the pancreas regulate blood glucose levels through complex mechanisms by constantly monitoring and responding to it. Human islets contain approximately 30% α-cells that produce glucagon, 60% β-cells that produce insulin, and 10% are made up of δ-cells that produce somatostatin, pancreatic polypeptide (PP) cells that produce PP, and ε-cells that produce ghrelin [3].

When the blood glucose levels are relatively high glucose enters into β-cells through GLUT1, GLUT2, and glucokinase; next, glucose is metabolized into ATP molecules that result in closure of ATP-sensitive K^+ channels; this leads to membrane depolarization and opening of voltage-gated L-type calcium channels; increased influx of calcium results in the exocytosis of insulin-containing vesicles [3]. Secrete insulin into the blood stream signals body cells to take up the blood glucose from the blood. Blood glucose then comes to a normal range which is defined by Diabetes Canada 2018 Clinical Practice Guidelines as either fasting plasma glucose (no caloric intake for at least 8 hours) of less than 6.1 mmol/L but more than 4.0 mmol/L, plasma glucose of less than 7.8 mmol/L but more than 4.0 mmol/L after 2-hour postprandial glucose test, or plasma

glucose of less than less 11.1 mmol/L but more than 4.0 mmol/L at any time of day [4]. Insulin secretion is enhanced by incretin hormones and inhibited by somatostatin of δ-cells, adrenaline, galanin, ghrelin of ε-cells, leptin [3].

During the first 8-12 hours of fasting when the blood glucose levels are low, β-cells are not stimulated to secrete insulin; therefore, α-cells are relived from inhibitory paracrine/endocrine effects induced by β-cells and δ-cells and they are stimulated by autonomic inputs which are stimulated by hypoglycemia; this culminates in α-cells secreting glucagon, which signals liver to release glucose into the bloodstream by breaking down its glycogen stores, in the process called glycogenolysis [5,6]. Blood glucose then comes to a normal range. If more glucose is needed and glycogen stores are depleted, as at a times of relative short-term starvation, the liver tries to meet the glucose needs of peripheral cells by synthesizing it from non-carbohydrate molecules mainly from amino acids and lactic acids, but also from glycerol and pyruvate (gluconeogenesis) [5,7]. If these mechanisms do not satisfy energy needs of the body as in the case of long-term starvation, liver starts breaking down fatty acids and release ketone bodies through the process of ketogenesis which can then be utilized by other cells in the brain and muscles to meet the energy needs. All these mechanisms are important since they provide means to keep the maintain glucose homeostasis.

1.1.2 Etiology and Pathophysiology of T1DM

The pathophysiological model of T1DM is a multigenic autoimmune disorder, which is often precipitated by an exogenous factor, which culminates in the destruction of insulin producing β-cells of the pancreas by the host immune system. Neither the initial trigger for autoimmunity nor the progression of self-destruction is well understood so far. However, it is widely acknowledged

that there is a significant genetic component to T1DM pathogenesis. Twin studies presented 30%-50% concordance rate for monozygotic twins and 6%-10% for dizygotic twins [8]. To date more than 40 genetic loci that are related either to immune function, insulin expression, or β-cell function have been linked to T1DM [8,9]. Noteworthy ones are *HLA region*, *CTLA-4*, and *PTPN22*.

CTLA-4 which encodes cytotoxic T-lymphocyte associated protein 4, plays a role in T-cell development and functionality [10]. Failure of proper development or functionality of T-cells can result in overamplified immune responsiveness. *PTPN22* codes for a special phosphatase in T-cells that functions to down-regulate the signals coming from the T-cell receptor (TCR) [10]. It can be expected that mutations that cause diminished activity of this "braking system" in T-cells will cause T-cell hyperactivity, and, diminished self-tolerance.

The most important genetic region among all identified ones so far is the Human Leukocyte Antigen (HLA) region on chromosome 6 which includes HLA Class I genes, HLA Class II genes, and HLA class III genes. It is suggested that these genes, and, most importantly, HLA Class II genes contribute around 60% to the overall genetic susceptibility [8]. Implications of HLA genes in the pathogenesis of several diseases was suggested as early as in 1975 [11].

HLA gene complex encodes major histocompatibility complex (MHC) class I and class II cell-surface proteins. MHC class I proteins are present on almost all nucleated cells. These cells present fragments of proteins from inside the cell on these MHC class I proteins to activate T-cells. In cases when a virus gets into the cell and hijacks cellular machinery to make more viruses, presentation of virus particles on MHC class I proteins will flag this infected cell for the destruction by T-cells. MHC class II proteins, on the other hand, present only on specialized antigen-presenting cells (APC) such as macrophages and dendritic cells. These APCs detect extracellular antigens, and following engulfment and digestion, antigen particles are presented on

MHC class II proteins to T-cells. Furthermore, MHC proteins also play role in positive and negative selection of thymocytes (mature T-cell progenitors) in the thymus where T-cell development occurs [12]. Thymocytes are selected based on their interaction with MHC complexes of APCs. If the thymocyte TCR binds too strongly to self-peptide/self-MHC of APC then this cell die through apoptosis, the mechanism known as negative selection. This process helps to eliminate autoreactive T-cells that recognize self-antigens as foreign antigens, which can lead impaired self-tolerance and autoimmunity. If the thymocyte TCR binds to MHC with proper affinity, then this thymocyte will survive and mature, the mechanism known as positive selection. In the process of death by neglect, a thymocyte that does not interact with APC at all dies.

Given the function of MHC class II proteins change in the gene composition can enhance or diminish their binding to antigens and TCRs of T-cells. In fact, HLA class II genes polymorphism were found to affect the susceptibility from protection to strongly at-risk [13]. The DRB1*1501-DQA1*0102-DQB1*0602 haplotype is found in 20% of non-T1DM and only in 1% of T1DM patients, which suggests its protection from developing T1DM [13]. Polymorphism in HLA region was suggested to be responsible for 40-50% of the genetic risk in developing T1DM [14]. 90% of T1DM patients and only 40% of non-T1DM patients have either HLA class II HLA-DR3, DQB1*0201 (DR3-DQ2) or HLA-DR4, DQB1*0302 (DR4-DQ8) haplotypes [14]. These evidences suggest that patients with T1DM have APCs with MHC molecules that recognize β-cell-related proteins as non-self and activate autoreactive T-cells which will cause cascade of activation and proliferation to destroy self β-cells.

The concordance rate for monozygotic twins of 30%-50% highlights that the pathophysiology and etiology of T1DM cannot be explained solely by genetic factors. These rates suggest that

there might be environmental factors that contribute to the disease. Studies in which susceptible individuals were monitored for the appearance of the first diabetes-associated antibodies revealed temporal variation: mostly in fall and winter, and rarely in the spring and summer [15]. Then, one proposed environmental candidate that might act as a trigger in T1DM in a seasonal manner is a virus. This is suggested by the strong temporal relationship between enterovirus infections and the appearance of the first diabetes-associated autoantibodies [15]. Children that were exposed to rubella during fetal development are more likely to develop T1DM [14]. On the other hand, molecular mimicry hypothesis suggests that immune system is activated by autoantigens such as glutamic acid decarboxylase (GAD65) because of its close similarity to viral antigen such as P2-C [14].

Even though the viral infection might be an attractive piece in a pathogenesis puzzle, there are a lot of controversies and inconsistencies which require further research and investigation. In addition to viruses there are other proposed environmental factors in T1DM pathogenesis such as shortened period of breastfeeding and cow's milk exposure in infants [8]. Some studies have shown that T1DM and non-T1DM differ in their gut microbiota composition which suggests that gut microbiota may be involved in T1DM pathogenesis [14]. These findings combined strengthen the proposition that T1DM is a complex disease and etiology is dependent strongly on genetical predisposition, but also environmental factors may play big role.

1.1.3. Complications of T1DM

Diabetes is associated with many detrimental complications such as potentially fatal diabetic ketoacidosis (DKA); microvascular complications such as retinopathy nephropathy, and

neuropathy; and macrovascular diseases such coronary heart disease, cerebrovascular disease, or peripheral artery disease.

Most common acute complication is diabetic ketoacidosis (DKA), which is estimated to occur in about one-third of cases [16]. Patients usually have hyperventilation, tachycardia, altered mental state, and acetone smell in their breath. DKA occurs when the body cannot secrete enough insulin to allow glucose to be taken up by cells and metabolized to fuel vital cellular processes. Starving cells signal to body's regulatory mechanisms, which will induce the body to switch gears and start to burn fatty acids (FA) that are stored in adipocytes. The liver takes up FAs released from adipocytes and metabolizes them into ketone bodies, which are transportable form of Acetyl-CoA that can be utilized by other parts of the body. This metabolic state is known as ketosis and it helps the body to survive through relatively short periods of fasting [17]. However, ketone bodies are very acidic and prolonged periods of elevated blood ketone bodies as in DKA can cause acidic blood; hence the term acidosis in DKA. DKA is responsible for ~50% of all deaths in diabetic patients younger than 24 years of age [18]. Even though DKA is more common in T1DM, patients with T2DM can have it too.

Another serious acute complication is hypoglycemia (low blood glucose). This can be a common side effect of insulin injections in T1DM. If a T1DM patient overshoots insulin injection, excessive insulin will cause clearance of "more-than-needed" amount of glucose from the blood, which can lead to very low levels of glucose. In healthy individuals, this acute hypoglycemia is sensed by alpha-cells and hypothalamus which will unleash the cascade of counter-regulatory mechanisms, including autonomic nervous system (ANS) activation, throughout the body to normalize the blood glucose. ANS activation produces many neurogenic symptoms such as palpitations, sweating and tremor. Healthy individuals can recognize these internal signs of

hypoglycemia. However, a subset of diabetic patients seem to have dampened counter-regulatory mechanisms which leads to neuroglycopenia before the appearance of ANS symptoms, or so-called hypoglycemia unawareness (HU) [19]. This ANS malfunction can kill without any warnings. One case study described a 23-year-old man with T1DM who was found dead in his bed [20]. Postmortem data in continuous glucose monitoring system (CGMS) revealed severe hypoglycemia around time of death, with appearance of minimal counter-regulatory response, which are sings of HU.

Chronic hyperglycemia leads to glycation of hemoglobin (Hb) in circulating red blood cells (RBC). Glycated Hb (HbA1c) is degraded by erythrocyte proteolytic enzymes which leads to the release of heme and ferrous iron (II) in association with free radicals; released iron reacts further to form ferric iron (II) and hydroxyl radicals, which lead to oxidative stress and RBC fragility [21]. Furthermore, oxidative stress promotes cellular damage and release of potent oxidative agents from RBCs into the bloodstream that are harmful to endothelial cells of the blood vessels [21]. Damage to the endothelial lining will promote local inflammation. Increased HbA1c lowers RBC's oxygen-carrying capacity and flexibility, increases their aggregation tendency, and ultimately leads to increased blood viscosity and impaired blood flow which will further exacerbate local inflammation and vascular damage [22]. Since the lifespan of RBC is about 8-12 weeks, HbA1c is clinical diagnostic marker of long-term glycemic control [4,23,24]

Microvascular complications are thought to be the result of those cells being unable to downregulate glucose influx. Normally, most cells in the body can regulate the rate at which glucose is taken up into the cells. They can downregulate transport rate under conditions of hyperglycemia. However, there are some cells such as capillary endothelial cells in the retina, mesangial cells in the renal glomerulus, and neurons with their glial cells in the periphery, that

are vulnerable to hyperglycemia [25]. Build-up of glucose in endothelial cells results in its metabolism through the polyol pathway, hexosamine pathway, advanced glycation end-products (AGE) pathway, and activation of PKC pathway. Activation of the polyol pathway results in sorbitol accumulation and consumption of NADPH, which is also essential cofactor in production of reduced glutathione, an important intracellular antioxidant. Therefore, reduced glutathione levels may increase cell susceptibility to oxidative stress [25]. Also, since sorbitol is an osmotically active molecule, sorbitol accumulation may increase osmotic stress on cells [26]. On the other hand, production of AGE precursors from glucose molecules results in glycation of intracellular proteins including transcription factors (TFs), which are important in regulation of gene expressions [25]. Intracellular hyperglycemia stimulates the synthesis of diacyl glycerol (DAG), the important activating cofactor for protein kinase C (PKC). Activation of PKC compromises barriers formed by endothelial cells and increases the permeability of small capillaries to albumin and other macromolecules [27].

In the eyes, all these intracellular changes negatively impact retinal cells, capillary endothelial cells, and pericytes, and lead to the development of diabetic retinopathy. Pericytes are thought to be responsible for structural integrity of capillaries. Loss of pericytes and endothelial cells with their junctions leads to disruption of blood flow to the retinal cells, hemorrhage, and retinal edema [28]. Since diabetic retinopathy is a progressive disease, it starts off without any symptoms, following with progressive vision loss, and culminating with retinal detachment and complete vision loss.

Similar scenarios take place in the kidneys. Chronic hyperglycemia leads to accumulation of intracellular glucose in podocytes and epithelium cells in Bowman's capsule of nephrons. Activation of different detrimental pathways such as the polyol pathway, AGE pathway, and

PKC pathway partially contributes to cell death and compromised filtration. The initial stages of diabetic nephropathy are characterized by glomerular hyperfiltration and some loss of podocytes and epithelial cells, which allows small amounts of albumin to be excreted in the urine (microalbuminuria) [26,29]. Later stages are characterized by thickening of the basement membrane, decreased glomerular filtration rate (GFR), increased podocyte and epithelial cell loss which leads to impaired glomeruli filtration and increased protein excretion [26,29]. If unmanaged, this can result in end-stage renal failure [30,31].

Not only is chronic hyperglycemia detrimental for eyes and kidneys but also for nerves, since capillaries that provide blood to peripheral neurons get damaged. The true cause of diabetic neuropathy is still not well understood [26]. However, ischemic injury caused by microcirculatory impairment and metabolic injuries through the polyol pathway, AGEs, and PCK activity were indicated in the pathogenesis of diabetic neuropathy [32,33]. There is a wide spectrum of clinical presentation of diabetic neuropathy: chronic neuropathic pain, limb ulcerations which can lead to gangrene and limb loss, sensory dysfunction, and impairment of autonomic nervous system (ANS) functions [33,34]. These neurogenic problems can contribute to reduced quality of life. Therefore, proper management and treatment will be required.

T1DM patients are at increased risk of developing cardiovascular disease (CVD) and CVD-related mortalities [35,36]. The age-adjusted relative risk (RR) for CVD such as coronary heart disease, cerebrovascular disease, or peripheral artery disease in T1DM is about 10 times more than general populations. Diabetes is associated with dyslipidemia, a condition in which there is high serum triglyceride levels, VLDL and IDL, and decreased HDL [37]. This process of plaque build-up underneath the endothelium cells continues and over the years can progress into life-threatening conditions. Atherosclerosis can occur in arteries of the body. When it occurs in

carotid arteries, which supply oxygenated and nutrient-rich blood to the brain and neck, it has potential to cause a stroke. Atherosclerosis occurring in the coronary arteries, which supply blood to the heart can lead to angina (chest pain) and myocardial infarction, where smooth muscles of the heart die from ischemic attack. Peripheral artery disease, caused by atherosclerosis of the abdominal aorta, iliac, and lower-extremity arteries can cause ischemic rest pain, ischemic ulcerations and possibly limb loss through gangrene development and amputation [38].

There are few proposed models to explain the mechanism of how diabetes causes chronic macrovascular complications [39-41]. In addition to the previously described mechanisms, chronic hyperglycemia damages vascular endothelial cells by inhibiting the production of nitric oxide (NO, an important vasodilator), NO-activated tissue plasminogen activator (anti-clotting protein), and causing oxidative stress through the accumulation of reactive oxygen species [42,43]. On top of that, inflammatory leukocytes (primarily monocytes and T cells) migrate and accumulate in the deeper layers (intima media) of blood vessels [42]. Once these monocytes get into intima media, they differentiate into macrophages and scavenge the atherogenic lipoproteins (especially LDL) until they die and fatty necrotic tissue remains .

Given these microvascular, macrovascular, and metabolic complications T1DM is a serious and potentially fatal disease that requires strict medical management for a patient to achieve good quality and higher life expectancy.

1.1.4 T1DM compared to other types of diabetes

Diabetes mellitus (DM) is a term used to describe a spectrum of related metabolic disorders with impaired blood glucose levels resulting in increased food intake (polyphagia), water intake (polydipsia) and urine production (polyuria). Chronic impaired blood glucose levels are associated with detrimental complications such as diabetic retinopathy (damage to the retina in eyes), nephropathy (damage to the nephrons in kidneys), neuropathy (injury to neurons) and major cardiovascular diseases. Etiologically DM can be subdivided into several groups such as type 1 diabetes mellitus (T1DM), type 2 diabetes mellitus (T2DM), gestational diabetes (GD), and other genetically defined conditions such as neonatal diabetes mellitus (NDM) and maturity-onset diabetes of the young (MODY). Given that there are differences in etiologies and progression mechanisms in different types of diabetes, diagnosing subjects with proper subset is crucial in providing proper therapeutic care.

While T1DM is an autoimmune disorder that results in absolute insulin insufficiency, T2DM is considered as complex metabolic disorder that results in peripheral insulin resistance and relative insulin insufficiency. Literature suggests that lifetime risk of developing T2DM for an individual if one or both parents have diabetes is 40% or 70%, respectively [44]. Furthermore, the concordance rate for T2DM in monozygotic twins is about 70% and for dizygotic twins is 20-30% [44]. On the other hand, T2DM is also associated with physical inactivity, sedentary lifestyle, and obesity [45]. In non-T2DM subjects insulin signals to the body to uptake glucose and store it for later use. In the liver insulin inhibits glucose production (gluconeogenesis), glycogen breakdown (glycogenolysis), lipids breakdown (lipolysis) and promotes glycogen synthesis (glycogenesis) and lipid synthesis (lipogenesis). However, in T2DM patients this mechanism seems to be impaired. These patients have chronically increased basal hepatic glucose production

(HGP) which dumps glucose into the bloodstream and elevates blood glucose levels [46]. Furthermore, peripheral muscle cells usually account for 80% of total glucose take up from the blood [46]. However, muscle cells in T2DM patients are insulin resistant and they do not take up glucose to the same extent as non-T2DM muscle cells [46]. This prevents blood glucose clearance and further exacerbates chronic hyperglycemia. Also, in obese non-T2DM patients β-cells seem to be able to compensate for peripheral insulin resistance by increasing the insulin output; however, this β-cell compensation mechanism is impaired in T2DM patients (relative insulin insufficiency) [46]. Therefore, given these differences in pathophysiology of T1DM and T2DM, they differ in the management strategies: for T1DM it is mainly insulin therapy to compensate for insulin insufficiency, for T2DM it is mainly change in life style, diet, and oral hypoglycemic agents to decrease chronic hyperglycemia and re-sensitize cells to endogenous insulin actions.

GD refers to glucose intolerance during pregnancy in women. Because the pregnancy is accompanied with a shift in hormone balance that affect glucose homeostasis, progressive insulin resistance (IR) develops from around mid-pregnancy and advances during the third trimester [47]. Normally, β-cells compensate for IR and increased insulin needs; however, if they do not secrete enough insulin then gestational diabetes occurs [47]. GD occurs only during pregnancy and resolves after the childbirth. Besides negatively impacting a mother, GD also affects the offspring. Intrauterine exposure to hyperglycemia permanently changes fetal metabolism, results in increased risk for high BMI in the offspring, and may increase the chances of the offspring in developing diabetes later in life [48,49]. Even though T1DM and GD differ in pathogenesis and progression the management strategy is similar. Initially, GD patients are prescribed medical nutritional therapy and exercise aimed to maintain desired glycemic control [47]. If GD patients do not respond to those therapies then they are prescribed insulin therapy as in T1DM [47].

MODY is a group of monogenic autosomal dominantly inherited disorders that are distinct from other forms of diabetes [50]. To date at least 9 genes have been indicated in MODY etiology which most of them result in diabetes primarily through β-cell dysfunction [51] . The four most common ones are *GCK* (MODY2)*, HNF1A* (MODY3)*, HNF4A* (MODY1)*,* and *HNF1B* (MODY 5) [51]. *GCK* encodes for a protein that catalyzes rate-limiting step of glucose phosphorylation in β-cells and hepatocytes, which lets to sense and respond blood glycemia appropriately; heterozygous inactivating mutations in this gene raises the sensing threshold in β-cells [51]. *HNF1* gene plays role in glucose transport and metabolism and mutation in this gene leads to progressive β-cell failure [51]. Mutations in *HNF4A* gene cause similar progressive β-cell failure as in *HNF1* gene-caused MODY [51]. *HNF1B* gene is involved in the regulation of gene expression and embryonic development in kidneys and pancreatic islets [51]. Therefore, mutations in this gene can cause impaired glucose tolerance. Treatment methods for MODY is dependent on etiology of the disease.

1.1.5. Epidemiology

Estimates suggest that 87-91% of all diabetes cases in high-income countries are T2DM, 7-12% are T1DM, and 1-3% other forms of diabetes [52]. According to the International Diabetes Federation (IDF), an estimated 30 million people had some form of DM in 1964 [52]. In 2000 this number climbed to 151 million and in 2013 this number increased further to 382 million [52]. In 2015 the world population which lives with DM is 415 million as estimated by (IDF) [52]. The IDF predicts 642 million people will suffer from diabetes by 2040 [52]. With modern medicine and its treatment approaches diabetes and higher-than-optimal blood glucose together led to the deaths

of 3.7 million people in 2012 [53]. History shows that the incidence of diabetes is on the rise and the given reduced quality of life and shortened life expectancy, diabetes should be taken seriously, and better treatment options are needed.

1.2 Treatment Methods for T1DM

1.2.1 The search for a cure—discovery of islets of Langerhans and insulin

In 1869, Paul Langerhans published a dissertation in which he described in detail the microscopic anatomy of the pancreas and identified what is now-called "islets of Langerhans" surrounded by pancreatic acinar cells [54]. However, he was not able to identify function of islets yet [54]. In 1889 Oscar Minkowski reported his findings that pancreatectomized dogs developed diabetes following few days after the surgery and persisted in that state for a few weeks until death has occurred [55]. This is one of the major turning points in the history of diabetes care since it was clearly shown that removal of the pancreas produced diabetic symptoms suggesting that it had not only exocrine but also endocrine function; and this allowed investigators to concentrate their research on the role of pancreas in pathogenesis of diabetes. In the early 1900s American pathologist Eugene Lindsay Opie while conducting postmortem examinations on patients, identified morphological changes in pancreatic islets of patients with DM [56]. In 1907, M.A. Lane distinguished the cells of islets as either A cells or B cells; the latter later became known as β-cells [56]. In 1921, Banting and Best obtained pancreas extracts from dogs and fetal calves and showed their efficacy in correcting hyperglycemia in dogs [57]. The same year James Collip helped to purify active protein from the extracts that Banting obtained and this protein later was named as insulin [56]. Starting from 1922 Collip's purified insulin mixture was used in clinics to treat

patients with T1DM for whom the disease meant a death sentence [58]. In 1923, Banting and Macleod won the Noble Prize "for the discovery of insulin". This was a revolutionary discovery since it alleviated a death penalty associated with T1DM and gave a hope for T1DM patients to manage severe daily symptoms of diabetes. In 1957, Lucy and Davies using immunohistochemical methods showed that insulin was produced by β-cells [56].

1.2.2 Exogenous Insulin Therapy for T1DM

In 1922, 14-year-old diabetic patient was successfully treated with Collip's purified bovine insulin and later the same year Banting and his colleagues reported clinical improvements in seven patients with diabetes when administered with the same purified insulin [58]. They did not know how lucky they were by injecting bovine insulin into human patients, since molecules from different species can cause immune reactions in immunocompetent humans [59]. Only with later development of amino acid sequencing by Frederick Sanger was it possible to sequence and compare insulin from different species—now we know that bovine and human insulin differ in three amino acids, but it is still weakly allergenic to humans [15,59,60]. This is issue was partly resolved by genetic engineering with introduction of recombinant human insulin in 1980—it was then that the human insulin coding gene was inserted into *E. coli* to express mature insulin protein [61]. However, even with this synthetic human insulin there are major issues that need to be addressed.

Insulin secretion in non-diabetic individuals has two components: basal insulin secretions to suppress lipolysis and balance hepatic gluconeogenesis with glucose demands, and prandial insulin secretions to inhibit hepatic gluconeogenesis, lipolysis and to promote glucose uptake and storage [62]. Insulin release is a very fine-tuned process that allows the body to keep blood glucose

levels in a narrow range. Therefore, the purpose of exogenous insulin therapy is to mimic this natural release. Human recombinant insulin injections cannot satisfy these criteria. Therefore, different modifications and strategies are being explored.

Today, there are different types of insulin analogues that are broadly divided into 4 categories based on their pharmacokinetic and pharmacodynamic profiles: rapid-acting analogues and short-acting insulins to mimic prandial insulin release, and intermediate-acting and long-acting insulins to mimic basal levels of insulin [63]. Over the years new insulin analogues have been developed that have better clinical profiles than their predecessors, and their integrating into standard care.

The Diabetes Control and Complication Trial (DCCT), 10-year controlled clinical trial involving 1441 T1DM patients with average follow-up of 6.5 years, was one of the major clinical studies conducted to test so-called "glucose hypothesis", which states that chronic hyperglycemia plays major role in the pathogenesis of long-term complications [64]. Specifically, the intensive-therapy regimen which was designed to maintain blood glucose at concentrations as close to non-diabetic levels as possible (with three or more insulin injections) was compared with conventional diabetes therapy which consisted of one or two insulin injections to maintain safe asymptomatic glucose levels with respect to long-term microvascular complications [65]. Results were clear and bold: at the end of follow-up intensive blood glucose control reduced risk of retinopathy by 76%, microalbuminuria by 34%-43% and neuropathy by 69%-57% [64]. Following DCCT, another observational study called Epidemiology of Diabetes Interventions and Complications (EDIC) was initiated that recruited 96% of DCCT subjects to assess the incidence and predictors of cardiovascular events and microvascular complications. Results showed that progression of

atherosclerosis in the intensive-therapy group was slowed, as were the incidence of fatal and nonfatal myocardial infarctions and strokes [39].

Even though the introduction of insulin and insulin analogous into clinical practice allowed better control of blood glucose levels and improved quality of life of many patients, insulin therapy is far from ideal. Insulin-induced hypoglycemia is the major severe side-effects of this therapy [63]. It can be so severe that it can cause confusion, coma and seizure, and in the worst-case scenario it can cause nocturnal hypoglycemia with lethal outcome [63]. In addition to that, insulin injections can cause allergic reactions ranging from local reactions to severe generalized anaphylactic reactions [66]. In the real world, patient adherence to his or her recommended treatment plan is another important factor that makes insulin therapy less favorable [67]. The fact that patients have to constantly monitor their blood glucose, predict their meal intakes and level of physical activity in order to match that with insulin injections makes exogenous insulin far from ideal. Even if insulins are perfected in their PK/PD profile, they are still going to remain as a "management" and not a "cure" for diabetes since continuous monitoring, calculation and prediction, and injection will be required from a patient. Better therapies needed to improve quality of life of diabetic patients. Furthermore, within the population of T1DM patients there is a subgroup that continue experiencing severe hypoglycemia, impaired awareness of hypoglycemia, and excessive glycemic variability regardless of effective education and intensive insulin therapy [68]. Recurrent hypoglycemia increases the risk of morbidity and mortality; in fact, 4-10% of mortality in T1DM patients is attributed to severe hypoglycemia [68]. This group of T1DM patients that respond poorly to intensive insulin therapy require better management options in order to survive and have a better quality of life.

1.2.3 Transplantation Approaches for T1DM

1.2.3.1 Whole Pancreas Transplantation for T1DM

Transplantation therapy is an alternative which can address major drawbacks of insulin therapy. In 1894, Watson-Williams and Harsant attempted to treat 13-year-old diabetic boy with a sheep pancreas transplanted subcutaneously; temporary improvement was noted and the boy died 3 days later rejecting the xenograft [69]. The first human pancreas transplantation attempts with some success date back to 1966, when Kelly and Lillehei developed a protocol for whole pancreas transplantation in conjunction with a kidney transplantation in patients with diabetes and renal failure, a procedure called simultaneous pancreatic kidney (SPK) transplantation, using organs from a deceased donor [70]. Their first surgery allowed the 28-year-old patient to be insulin-free for six days, and with further modifications of protocol better results were achieved [70]. In 1971, pancreas was transplanted using urinary drainage via the native ureter; in 1973 segmental pancreas transplantation with end-to-side ductoenterostomy [71]. In 1983, Hans Sollinger performed segmental pancreas transplantation with bladder drainage technique [71]. Even though whole pancreas transplantation was a big step towards finding a cure for T1DM, the procedure is far from ideal. Whole pancreas transplantation requires major invasive surgical procedure and it is associated with surgical morbidity [72]. It is associated with perioperative risks, graft thrombosis, hemorrhage, urologic complications and pancreatitis [71,73]. Patients require life-long immunosuppression. To outweigh the harm and risks associated with pancreas transplantation, it is reserved for specific population of patients: 72% of transplants are simultaneous pancreas-kidney (SPK) for patients who have detrimental nephropathy, 17% of transplants are pancreas after kidney (PAK) for patients who already had kidneys transplanted and are on immunosuppressive drugs, 4% of transplants are pancreas combined with other organs, and only

7% are pancreas transplant alone (PTA) procedures [74]. Given that pancreas transplantation requires major laparotomy, is associated with surgical morbidities and complications, and that life-long immunosuppression is required, the procedure is not available for all T1DM patients. Furthermore, due to listed and additional factors such as lack of primary referral source, lack of endorsement of pancreas transplants alone by the American Diabetes Association, associated high mortality rates the annual number of pancreas transplants performed has been declining in the United States [75].

1.2.3.2 β-cell transplantation for T1DM

1.2.3.2.1 Islet Allotransplantation

A significantly less invasive alternative to whole pancreas transplantation is isolated islet transplantation which is much safer and faster. First successful attempts of transplanting isolated islets date back to 1972 when Ballinger and Lacey transplanted islets from rats into the peritoneal cavity of diabetic rats which resulted in significant reduction of hyperglycemia. [76-78]. In 1973, Kemp et al. demonstrated that the infusion of islets into the portal vein achieved better results than the transplantation into the peritoneal cavity [78] The first clinical human trials of isolated islet transplantation in T1DM patients with optimistic results were performed by Najarian et al. at the University of Minnesota in 1974 [79]. In 1980, 10 patients with chronic pancreatitis underwent pancreatectomy and isolated autologous islets were infused back into those patients; 3 of the patients were insulin-independent for 1, 9, and 38 months, respectively [76]. In 1990, Scharp et al. achieved for the first time clinical insulin independence with islet transplantation for nearly a month [80]. This success was conceivable because of the advances in islet isolation and purification methods, including the development of Ricordi chamber [80,81].

According to International Islet Transplant Registry, out of 245 islet allograft recipients between 1990 and 1998 only 20 (8%) were insulin-independent at ≥ 1 year [82].

In 2000, Shapiro and his colleagues demonstrated the ability of islet transplants to regulate blood glucose in all seven transplanted patients for one year with improved islet transplantation procedure which later became known as the Edmonton Protocol (EP) [83]. The major difference between previous attempts and EP was that latter used glucocorticoid-free immunosuppressive regimen which consisted of sirolimus, tacrolimus, and daclizumab and increased islet mass transfusion (11,547±1604 islet equivalents per kg of recipient's body weight) [83]. This was one of the major breakthroughs in islet transplantation field and it gave high hopes and promised to solve problems of diabetic patients. However, recent results from the Collaborative Islet Transplant Registry (CITR) indicates that prevalence of insulin independence is ~70% at 1 year and ~40% at 5 year post last islet infusion [84].

Even with progress and refinements in different aspects of islet transplantation, such as organ procurement, islet isolation, blood type matching, and choice of immunosuppressive drugs longer-term patient follow-ups revealed some shortcomings of this approach [83,85]. Islet transplantation recipients usually require islets from more than one donor because islets die and diminish in function during isolation, culture, and after transplantation [86]. This one of the major drawbacks since it raises the question of the supply of good quality islet. Strategies of isolating high-yield and good quality islets are desirable. Islet transplantation, as with any other type of transplantation procedure, requires chronic immunosuppression to prevent acute graft rejection and gradual graft damage, that can cause loss of graft function over time. It is suggested that one of the major contributors to the initial graft rejection is a process called instant blood-mediated inflammatory reaction (IBMIR) caused by incompatibility between islets and the blood interface

[87-89]. In IBMIR, coagulation system, complement system, and innate cells are activated to induce clotting, platelet aggregation, attraction of immune cells, cell lysis, inflammation, and rejection [89].

The current immunosuppression scheme consists of three phases induction, anti-inflammatory and maintenance. Enhanced protocol for induction phase using alemtuzumab, tacrolimus, mycophenolate mofetil is designed to deplete T-cell before the surgery [90]. Anti-inflammatory therapy using etanercept (anti-TNF) and anakinra (interleukin 1 receptor antagonist), and maintenance therapy using tacrolimus and MMF yielded better results compared to earlier immunosuppressive regimens [90,91]. Even with these improvements, chronic immunosuppression is undesirable since it is harmful to the recipient, increases the susceptibility of individuals to opportunistic infections, and therefore, limits the patient inclusion [86,92].

1.2.3.2.2Porcine Islet Xenotransplantation

Another promising avenue that may solve the organ shortage problem is xenotransplantation which is a transplantation of organs between two different species. When recipient and donor are from two closely related species this is called concordant xenotransplantation[93]. When they are from two very different species, it is called discordant xenotransplantation [93].

There are major advantages of using porcine islets for xenotransplantation. Pigs have internal organs that are comparable to humans' in terms of morphology and size; they are phylogenetically closer to primates than rodents; and physiology of pigs is very similar to humans [94]. Furthermore, porcine insulin is very similar in structure to human insulin and differs only in one amino acid (alanine in pigs and threonine in humans at B30 position). With its initial

introduction by Banting in 1922 and further purification by Collip the same year, porcine insulin has been used in clinics and was a major source of therapeutic insulin before the introduction of recombinant technologies. With further purifications of porcine insulin injection site and systemic reactions have decreased, and insulin resistance was seen in less than 0.1% recipients [95]. Retrospective analysis shows that injection site reactions were seen in 3.9% of purified porcine insulin recipients and 2.4% of human recombinant DNA (rDNA) insulin recipients [95]. This shows that porcine insulin is only slightly less favorable than human rDNA insulin and a good therapeutic for clinical use. Furthermore, since pigs are an acceptable source of food, they must be an ethically acceptable source for major life-saving or life-enhancing surgeries such as islet transplantation in diabetic patients who have a suffer from insulin insufficiency. Pigs also have the potential to serve as unlimited source of reproducible, genetically controlled, and high-quality islets since they have large litters and can be bred in controlled manner. In addition, if ethics concerns are addressed, pigs can be cloned and genetically modified for desirable traits. Recently, investigators from China reported that they were able to edit the *INS* gene in pigs so they produce human insulin [96].

There are few factors in pig islet transplantation that need to be considered and addressed. The first factor is donor age. Adult pigs can offer large number of large-sized islets and start secreting insulin right-away. However due to many factors adult porcine islets are very fragile and sensitive, and therefore are not suitable for clinical settings [97]. Furthermore, adult islet isolation is relatively challenging process. Fetal and neonatal pigs islets are easier to isolate and they are more resistant to ischemic and inflammatory damage [98]. However, fetal porcine extracts have their limitations too—generally, they have a poor response to glucose [97]. Dr. Korbutt's lab have developed and over time refined relatively easy and optimized for large-scale protocol for

isolation of neonatal porcine islets (NPIs). Both fetal porcine islets and NPIs do not start functioning as fast as adult pig islets. However, NPIs start functioning significantly sooner than fetal porcine islets, > 4 weeks versus 8 weeks, respectively [99]. NPIs have growth potential post-transplant and induce lower T-cell response than adult pig islets in T1DM patients [100,101]. Some investigators worked with embryonic pig islets tissues as possible avenue; however, these cells require about 6 months of growth before they start effectively restoring normoglycemia and embryonic cells have a risk of tumorigenesis [98,102]. This long maturation time and risk of developing tumors are major drawbacks of using embryonic tissue. *In vitro* and *in vivo* studies demonstrated that NPIs are better candidates than adult and fetal porcine islets for islet source in xenotransplantation since they are viable, resistant to isolation steps, reproducible and relatively cheap [97,103].

Another aspect of porcine xenotransplantation that needs to be addressed is immunological rejection. In the first 24-hour porcine xenograft is rejected immediately by the host through the hyper acute rejection (HAR) due to the pre-existing anti-pig antibodies which upon binding to pig cells will initiate complement-mediated damage to the endothelial cells causing thrombosis, interstitial hemorrhage and edema. [102,104]. The most important among many immunogenic epitopes is galactose-α-1,3-galactose (Gal) which is found on the pig vascular endothelium [104]. Two promising strategies to get around HAR are genetic modification of pigs to knock out the immunogenic genes, induction of expression of complement regulatory proteins on the surface of islet cells (eg. hCD46, hCD55, hCD59) [102,104] Next, within a few days or weeks another immune mechanism, acute humoral xenograft rejection (AHXR), mediated through antibody deposition and complement activation leads to infiltration of the graft by innate immune cells that destroy the graft [104]. When knockout islets were combined with complement-regulatory proteins early

graft rejection was prevented [104] Finally, within 24 hours to 20 days adaptive immune response (or cellular rejection) causes further graft damage and rejection [102,104]. This process is mainly mediated by CD4+ T-cells, CD8+ T-cells, and anti-pig antibodies [102,104]. Since T-cell activation needs co-stimulatory molecules, blocking these specific proteins such as CD870/86-CD28 and/or CD40L (CD154)-CD40 is one way to prevent adaptive immune attack, which will further facilitate graft survival and function [102]. Another approach to allow porcine islets to function but prevent xenograft rejections is to encapsulate the islets into biocompatible material such as alginate and polytetrafluoroethylene [105]. There have been clinical studies that assessed the safety and efficacy of alginate encapsulated pig islet xenotransplantation [106]. The results from one of the clinical trials in New Zealand indicated low graft function and inconsistent efficacy of encapsulated pig islet xenografts [106]. Different clinical trial in Argentina showed partial graft function and significant reduction in the number of unaware hypoglycemic episodes [106].

Another very important aspect of xenotransplantation that needs to be addressed is the risk of transmission of pathogenic microorganisms from animals to humans. International Xenotransplantation Association suggests that most pathogens can be eliminated by breeding pigs in designated pathogen-free (DPF) facilities which contains Cesarean section, closed containment, feed and waste management, staff training, and other safety procedures [107]. However, even with DPF there is a risk of transmission of viral infections such as hepatitis E virus (HEV), herpes viruses, and porcine endogenous retrovirus (PERV) [107]. With current methods even these obstacles could be overcome. For example, studies have shown that HEV can be treated with ribavirin and in certain regions up to 56% of the adult population already have been exposed to HEV and have protective antibodies against it [107]. Clinical relevance of the risk of PERV transmission is debated. Islet xenotransplantation is already in clinical trials in

some countries and no transmission of PERVs have been reported from more than 200 recipients or up to 35 years of post-xenotransplantation [107]. Furthermore, recently emerging genetic editing technique CRISPR/Cas9 might provide a solution for selectively eliminating genome-integrated viruses such as PERV, HEV, and herpesviruses [108,109]. In fact, feasibility of this approach has been shown with the selective removal of 62 genomic copies of PERV in pig cell lines [107]. Given these optimistic results, porcine islet xenotransplantation can provide cost-effective and unlimited supply of islets for clinical transplantation.

1.2.3.2.3 Human Embryogenic Stem Cells (hESCs) derived β-cells

Stem cell-based therapies offer another approach to address organ shortage problem in the treatment of T1DM. Pluripotent stem cells (PSCs) are the cells that can renew and differentiate into three primary groups of cells that form the human being: ectoderm, mesoderm, and endoderm [110]. Ectoderm gives rise to skin cells and cells of nervous system [111]. Mesoderm gives rise to bone cells, heart cells, and skeletal muscle cells [112]. Endoderm gives rise to cells of gastrointestinal and respiratory tracts, endocrine glands, liver and pancreas [113]. Two major sources of PSCs are human embryonic stem cells (hESCs) and induced pluripotent stem cells (iPSCs). hESCs can be derived from inner cell mass of embryonic blastocyst that were created through *in vitro* fertilization [114]. There are few advantages of using these cells in the treatment of T1DM. First, hESCs potentially can provide unlimited supply of high-quality insulin-producing cells for regenerative medicine if ethical concerns are addressed. Second, hESCs-derived insulin-producing cells are allografts and they will not be as immunogenic as xenografts. hESCs-derived insulin-producing cells transplantation in combination with immunosuppressive regimen should provide comparable results as conventional islet transplantation.

The field is relatively new and some of the challenges with hESCs that need to be addressed are refinement of the differentiation protocol, prevention of the possibility of tumorigenesis, and addressing immuno-compatibility to avoid the use immunosuppressants. The first documented report isolating hESCs was in 1998 [115]. Since then many laboratories have tried to develop and refine the protocol to produce β-cells from hESCs [116-123]. In 2006, D'Amour and colleagues presented a 5-stage (11-18 days) protocol differentiating hESCs into insulin secreting cells; however, only 7% were insulin-positive and these cells were minimally responsive to glucose *in vitro* [118]. In 2006, Kroon and colleagues presented a 4-stage (12 days) protocol that resulted in relatively low insulin-positive cells *in vitro*; however, *in vivo* studies demonstrated that these cells after maturing *in vivo* can correct STZ-induced hyperglycemia in 92% of transplanted mice [119]. In 2004, Pagliuca and colleagues demonstrated that their 6-stage (27-34 days) differentiation protocol yielded 33% of cells co-expressing β-cell markers NKX6-1 and C-peptide [123]. Studies *in vivo* demonstrated the ability of these cells reverse hyperglycemia [123] In 20014, Rezania and colleagues demonstrated that their refined 7-stage (27-43) *in vitro* differentiation protocol produced one insulin-positive cell from every two hESCs and the expression of key β-cell markers such as *INS*, *MAFA*, *G6PC2* were indistinguishable from human islets [122]. Furthermore, their cells reversed diabetes by day 40 post-transplantation [122]. Given that the field has achieved these optimistic results in only about 14 years, it can be expected with further research and trials, researchers will elucidate complex differentiation mechanisms and will be able to efficiently differentiate pluripotent stem cells, including hESCs, into insulin producing β-cells.

Another important aspect of stem cell-based therapies that needs to be addressed is tumorigenicity potential of the stem cells. hESCs spontaneously form teratomas when transplanted into mice [124]. If cells are infused into the bloodstream, complex issue of

biodistribution that relates to cell localization, migration, survival, and differentiation becomes important for consideration [125]. One way to solve this issue would be to develop a therapeutic device that localizes, limits the migration of the stem cells, and allows safe immediate retrieval if needed. One example of this approach that addresses tumorigenicity and immunogenicity problems is the VC-01™ manufactured by ViaCyte is designed to be implanted subcutaneously. This combination technology includes insulin-secreting pancreatic endoderm cells (PECs) that showed promising pre-clinical results and have been optimized for large-scale manufacturing encapsulated into biocompatible and semi-permeable barrier (Encaptra®) [126]. Even though there are some challenges and shortcomings with stem cell therapies, this field is gaining strong momentum and gives us hope to treat diabetes. In fact, in 2014 ViaCyte has launched prospective, multicenter, open-label, first-in-human Phase 1/2 clinical studies to evaluate the safety, tolerability, and efficacy of VC-01™ in patients with T1DM (ClinicalTrials.gov identifier is NCT02239354). In 2017, ViaCyte launched an open-label, first-in-human Phase 1/2 clinical trial to evaluate the safety, tolerability, and efficacy of VC-02™ in patients with T1DM and hypoglycemic unawareness (ClinicalTrials.gov identifier is NCT03163511). No preliminary data is available as of right now, but the fact that clinical trials are happening today provides hope that stem cell therapies will help to cure T1DM in very foreseeable future.

However, one aspect of hESCs that raises many ethical questions and may potentially limit their wide-spread use is the that hESCs harvesting requires the destruction of the embryo [127]. Some examples of ethical issues related with hESCs use and research are destruction of an embryo, informed and voluntary donation of materials, confidentiality of donor information, medical risks, protecting the reproductive interests of women in infertility treatment [127]. Even though the hESCs field has shown great success in relatively short period of time, it is important to

acknowledge and address some of deficiencies its such as protocol efficiency, risk of tumorigenesis, immunogenicity, and ethical concerns.

1.2.3.2.4 Induced Pluripotent Stem Cells (iPSCs) derived β-cells

Ethical and immunogenicity concerns can be partly addressed with the use of induced pluripotent stem cells (iPSCs). In contrast to hESCs, iPSCs can be created from virtually any human somatic cell through the introduction and expression of four factors, Oct3/4, Sox2, c-Myc, and Klf4 into the cell [128,129]. iPSC-based therapies will allow not only to have unlimited supply of stem cells but also to have a solution for allograft rejection since transplants will be derived from the same donor. As mentioned previously, one of the limitations of clinical islet transplantation is the use of life-long immunosuppression to reduce the allograft rejection. Even though autologous iPSCs-derived β-cells would probably be destroyed by the same mechanisms that destroyed native β-cells in autoimmune T1DM, the body should be able to recognize the tissue as self and do not attack through the mechanisms used in xenograft and allograft rejections. Macro- or micro-encapsulation of β-cells into special semipermeable membrane made from non-immunogenic material that allows nutrient exchange but prevents immune cell infiltration may help overcome the autoimmunity problem [129]. Also, genetic editing of iPSCs-derived β-cells such as removing or modifying antigenic genes of β-cells may potentially reduce autoimmune rejection. Furthermore, autologous iPSCs-derived β-cells could be effectively used in non-autoimmune diabetes such as T2DM. Combination of iPSCs-derived β-cells with gene editing technologies such as CRISP/Cas9 would help to deliver personalized therapies for MODY patients.

The fact that iPSCs do not require embryo sacrifice relieves them from many ethical questions such as destruction of an embryo and protecting the reproductive interests of women in infertility

treatment that are applicable hESCs. This will simplify the barriers of using human iPSCs in research and therapeutics and help to accelerate the development of the field.

The field is even newer than hESCs field. Only with Yamanaka's and Takahashi's revolutionary work in 2006 in elucidating necessary transcription factors to induce pluripotency the field has gained a momentum [128]. In 2009, Maehr and colleagues reprogrammed fibroblast cells from T1DM patients into iPSCs and subsequently into insulin-positive and glucose responsive cells *in vitro* [130]. Similarly, in 2013, Thatava and colleagues induced epidermal cells from T1DM patients to become iPSCs, and the subsequently into islet-like clusters which expressed insulin, glucagon and somatostatin [131]. In 2012, Jeon and colleagues successfully converted mouse somatic cells into iPSCs and then into insulin-producing β-cell-like cells; these cells expressed β-cell markers such as *IAPP*, *INS*, and *GLUT2*, responded to glucose stimulation *in vitro*, and corrected hyperglycemia *in vivo* [132]. These results are remarkable because they are showing that the field is moving forward. In 2013, Takakashi and her team converted skin cells from patients with age-related macular degeneration into iPSCs, and then they converted these cells into retinal pigment epithelium (RPE) sheets; in 2014, these autologous RPE sheets were transplanted into one of the patients to treat the condition [133]. Patient's macular degeneration was halted and her vision brightened [133]

Given these results and achievements iPSC-derived therapies would allow personalized, on-demand, and targeted therapies if certain aspects are addressed. Specifically, in T1DM autologous iPSC-derived β-cells have the potential to cure the diabetes if protocol is optimized, and immunogenicity related to β-cells and ethical concerns are addressed.

1.3 Mesenchymal Stromal Cells (MSCs) for β-cell Transplantation

Another approach that can be taken to improve clinical islet transplantation is to harness the beneficial properties of Mesenchymal Stromal Cells (MSCs) to improve islet survival and function. These stem cells that are found in the stroma of most the tissues in the human body have self-renewal, differentiation, angiogenic, antimicrobial, and immunomodulatory capacities [134,135]. Our group have previously showed that MSCs from the bone marrow and pancreas protected the islets from the effect of pro-inflammatory cytokines by secreting cytoprotective factors *in vitro* [136]. Furthermore, we showed that co-transplantation of the bone-marrow derived MSCs resulted in mice reaching normoglycemia faster, having better glucose tolerance, and having more graft-derived cellular insulin [137]. Given these optimistic results it is worthwhile to discuss in details what exactly are the MSCs.

1.3.1 Characterization of MSCs

Friedenstein's pioneering work in 1970's lay the groundwork in the field of, which later became knowns as, MSCs [138-140]. In one of the studies, he plated bone marrow aspirates in plastic culture dishes and removed non-adherent cells after about 4 hours of seeding. Friedenstein observed that some cells tightly adhered to the plastic and started rapidly multiplying after about 2-4 days. The colonies of these spindle shaped plastic-adherent cells resembled small deposits of bone or cartilage. Further studies revealed the ability of these cells to differentiate into osteoblasts, chondroblasts, and adipocytes [138].

Initially referred to as a colony-forming unit fibroblast (CFU-F) by Friedenstein et al, these multipotent stem cells spiked an interest by the researchers from different laboratories [141,142]. However, with an increased and widespread interest the isolation, processing protocols and

defining characteristics of MSCs became inconsistent among different investigators [143]. Aimed to tackle this problem and provide common framework for researchers to come to consensus in terminology and characteristics, the International Society of Cellular Therapy (ISCT) proposed minimal criteria to define MSCs [143]. According to ISCT's 2006 position statement, these cells should be called multipotent Mesenchymal Stromal Cells (MSCs), they must adhere to the plastic, express panel of positive and negative markers, and have the ability to differentiate into adipocytes, chondroblasts, and osteoblasts.

1.3.1.1 Plastic adherence

First criterion of ISCT in defining MSCs is plastic adherence. Friedenstein used plastic-adherence as a characteristic to differentiate between adhering MSCs and non-adhering Hemopoietic Stem Cells (HSCs). Since then most of the investigators have been focusing on adherent cells for further investigation of MSCs. However, many researchers have demonstrated the possibility of deriving MSCs from the non-adherent portion of bone marrow aspirates [142]. Therefore, despite the restrictive criterion of ISCT one should be mindful about the possible existence of MSCs that do not necessarily adhere to the plastic after the isolation from the tissue. Furthermore, plastic-adherence alone is not sufficient criterion to identify MSC from a population of cells since functionally dissimilar cell type, fibroblast, morphologically is very similar to MSC. Fibroblasts are specialized cells in a tissue that secrete extracellular molecules to form stroma, structural part of a tissue; they also play role in tissue development, maintenance, and repair [144]. Following seeding and under standard cell culture conditions both MSCs and fibroblasts adhere to the plastic and take on spindle-shaped morphology with long protrusions [145]. Therefore, under phase contrast microscopy MSCs and fibroblasts are indistinguishable.

1.3.1.2 Cell Surface Markers

Second criterion of ISCT in defining MSCs is the expression of specific cell surface markers. Since there is no one single known marker that can be used to identify MSCs, ISCT proposed a panel of positive and negative markers which should help to reveal MSCs from the mixture of different cells [146]. To satisfy the second criterion, at least 95% of the MSC population must express positive markers such as CD105 (known as endoglin), which is predominantly expressed in angiogenic endothelial cells and acts as co-receptor for transforming growth factor (TGF)-β1 and TGF- β2; CD73 (known as ecto 5' nucleotidase, Ecto5'NTase) which catalyzes the extracellular dephosphorylation of AMP to anti-inflammatory adenosin; and CD90 (known as Thy-1) which is expressed in various cell types and specifically in MSCs it may play an important role in maintaining stemness [146-149]. Furthermore, less than 2% of the MSC population can express negative markers such as CD45, which is pan-leukocyte marker; CD34, which marks primitive hematopoietic progenitor and endothelial cells; CD14 or CD11b which mark monocytes and macrophages; CD79α and CD19 which mark B-cells; and HLA-DR is a member of the MHC class II family proteins which are expressed on professional antigen-presenting cells (APCs) such as dendritic cells, B cells, and macrophages [150-152].

1.3.1.3. Trilineage Differentiation

Third criterion of ISCT in defining MSCs is their multipotent differentiation potential. To induce adipogenesis, cells are cultured in adipogenic cocktail which usually contains insulin, dexamethasone, and 3-isobutyl-1-methyl xanthine (IBMX). Insulin activates ERK and Akt/PKB pathways which are essential for adipogenesis *in vivo*; dexamethasone inhibits the expression of Preadipocyte factor 1 (Pref-1), which is known to be inhibitor of adipocyte differentiation; and IBMX by inhibiting cAMP- and cGMP-degrading phosphodiesterases it causes increased levels

of cAMP and cGMP, which results in activation of adipogenic promoting cAMP-responsive element binding proteins and Epac pathway [153,154]. There are many publications showing the adipogenic potential of MSCs; however, there is no one standard protocol used by everybody .[155]. Regardless of that, as long as the cocktail contains the important trio of insulin, dexamethasone, and IBMX MSCs differentiate into adipocytes containing lipid droplets, which is a hallmark of adipogenesis [156]. Following the culturing period, to visualize lipid droplets in adipocytes and confirm adipogenesis, Oil red O (ORO) staining is used. This molecule is a lysochrome (fat-soluble) diazol dye that stains neutral lipids and cholesteryl esters but not biological membranes [157]. Hydrophobic ORO only minimally dissolves in the solvent and upon contact with lipid droplets ORO readily associates with lipids; under the phase contrast microscope ORO stain appears as red [157,158].

To induce osteogenesis, cells are cultured in osteogenic cocktail which usually contains dexamethasone, ascorbic acid, and β-glycerophosphate. Dexamethasone causes increased transcription of TAZ, MKP1, and FHL2 which in turn result in the transcription and activation of the master osteogenic transcription factor Runx2 [159]. Ascorbic acid acts as a cofactor for enzymes that hydroxylate proline and lysine in pro-collagen; therefore, it is needed for proper helical structure formation in collagen chains and subsequent secretion of collagen type 1 into the extracellular matrix (ECM) [159]. It is suggested that the interaction between ECM and osteoblast precursor is crucial for the induction of osteoblast phenotype in these cells [160]. Lastly, β-glycerophosphate plays a crucial role in osteogenic differentiation. It serves as a source of phosphate needed for hydroxylapatite mineral formation in bones and intracellular signaling to activate the expression of many genes related to osteogenesis [159]. Alizarin red and von Kossa's

staining protocols are commonly used to stain calcium depositions and thereby to assess osteogenic differentiation [161].

To induce chondrogenesis, cells are condensed into pellets and cultured in chondrogenic cocktail that usually contains dexamethasone, ascorbic acid, insulin, transferrin, selenous acid and transforming growth factor (TGF)-β [162]. Conditions are selected in an effort to mimic the nature. For example, it is known that *in vivo* cell condensation is an important step in cartilage formation, yet the full mechanism is to be worked out; therefore, pellet culture is adopted *in vitro* in order to increase the cell-to-cell contact and mimic the natural chondrogenic process [163]. Pellet formation is also important because it mimics hypoxic environment of chondrocytes and hopoyxia has been shown as a potent promoter of chondrogenesis[163]. The family of TGF-β proteins play in important role in chondrogenesis such as condensation, proliferation, terminal differentiation, and maintenance of articular chondrocytes [164]. It is suggested that TGF-β family members promote the expression of cartilage-specific genes expressions through complex regulation of intracellular pathway molecules such as receptor-regulated Smad proteins (R-smads), mitogen-activated protein (MAP) kinases, Rho-like GTPase, and phosphatidylinositol-3-kinase (PI3K)/AKT pathways [164,165]. Synthetic glucocorticoid, dexamethasone, acts through the intracellular glucocorticoid receptors to induce the expression of collagen XI and enhance TGF-β-dependent expression of aggrecan, collagen II, COMP, which are the hallmarks of chondrogenesis [166]. Ascorbic acid leads to hydroxylation and processing of procollagen so that it can form collagen fibrils and be secreted from the cells for extracellular matrix assembly [167]. Extracellular matrix is composed of proteoglycans (PGs) such as aggrecan and fibrous proteins such as collagens, elastins, fibronectins and laminins [168]. Safranin O is a dye molecule used to

stain sulfated glycosaminoglycans which are structural components of PGs and the intensity of staining is directly proportional to the PG content in the sample [169].

1.3.2 Sources of MSCs

MSCs are specialized multipotent stem cells that are found in the stroma of many body tissues. Following their initial isolation from the bone marrow, it has been shown that MSCs can be isolated from many other body tissues such as adipose tissue, placenta, umbilical cord, amniotic fluid and membrane, peripheral blood, the lung, heart, synovial fluid, dental tissue, foreskin, endometrium and menstrual blood [170,171]. Even though it has been shown that these cells were isolated from many different body tissues and labeled as MSCs according to the ISCT's minimal criteria, current research progress indicates that there is significant source-dependent differences in their cell yield per mass of tissue, transcriptome and secretome profiles, and proliferative and mitotic capacities [170,172-174]. Exploring and investigating these differences may help the regenerative medicine to harness those differential characteristics for more effective clinical applications.

1.3.2.1 Bone-Marrow Derived MSCs (BM-MSCs)

Friedenstein discovered MSCs from bone-marrow in 1970's. Since then bone-marrow derived (BM) MSCs were extensively studies and well characterized. These cells are considered as a gold standard in the field with which newer MSCs are compared. To isolate BM-MSCs, bone marrow biopsy and aspiration is performed from the posterior, anterior superior iliac crest, or sternum; and this procedure is associated with significant pain and discomfort [175]. Generally, once the bone marrow is obtained mononuclear cells are isolated using Ficoll-Paque density

gradient and seeded into plastic tissue culture-treated dish in culture medium containing 10% FBS [176,177]. Following 48-hour culture MSCs attach to the plastic and unwanted cells are washed away with media change; from there after media is changed regularly until confluency is achieved. Once confluent, cells are detached using trypsin solution and passaged at a desired density. Cells are passaged up until desired passage.

BM-MSCs has been shown to possess lower proliferative capacities compared to embryonic-stem cells-induced MSCs (ESC-MSCs) [178]. This can be explained by the fact that ESC-MSCs have longer telomeres and enhanced upregulation of the genes important in control of DNA replication and repair when compared with BM-MSCs [178]. Likewise, BM-MSCs has been shown to possess lower population doublings compared placenta-derived MSCs, 12 versus 64, respectively [179]. Mohammed-Ahmed et al. (2018) showed that BM-MSCs had lower proliferative capacity and adipogenic potential compared to AD-MSCs [180]. However, they also showed that BM-MSCs possessed higher osteogenic and chondrogenic potential. Similarly, Xu et al. (2017) demonstrated that BM-MSCs had higher osteogenic and lower adipogenic potential compared to AD-MSCs [181]. These results support the notion that the cells are affected by their microenvironments and possess source-dependent characteristics and potential.

In the context of islet transplantation, we showed that co-transplantation of islets with human BM-MSCs enhanced graft maturation resulting in mice reaching normoglycemia significantly faster and having better glucose tolerance [137].

1.3.2.2 Adipose Derived MSCs (AD-MSCs)

Fat depots are broadly divided into two main categories: subcutaneous depots, which include fat under the skin in the buttocks, thighs, and abdomen regions, and intra-abdominal fat depots, which include mesenteric, omental, and perirenal fat deposits [182]. Subcutaneous adipose tissue is an attractive alternative to bone marrow for harvesting MSCs since obtaining subcutaneous fat is relatively easier and less painful procedure for a donor. To isolate MSCs from adipose tissue, the tissue must be harvested and finely minced with a scalpel or scissors to increase the surface area for the enzyme digestion; then, the sample is digested with collagenase solution [176]. Resulted mixture is centrifuged to condense MSCs into pellet and subsequently seed them into plastic tissue culture-treated dish. Once confluent, cells are detached from the dish using trypsin and passaged at a desired density; and this cycle is repeated up until desired passage. Senescence

According El-Badawy et al. (2016), rat inguinal fat pad-derived AD-MSCs and BM-MSCs showed similar *in vitro* profiles with respect to morphology, differentiation potential, and cell surface marker expression. However, *in vivo* AD-MSCs transplants caused enhanced vascularization which was evidenced by CD31 (an endothelial cell marker), vascular endothelial growth factor (VEGF; a potent angiogenic factor), SMA (a marker for vascular smooth muscle cells) and MMP9 [183]. El-Badawy et al. (2016) also showed that when exposed to superoxide stress and ischemia AD-MSCs showed better resistance than BM-MSCs. These results suggested that AD-MSCs can probably better withstand hypoxic environment and oxidative stress when transplanted into avascular site. Furthermore, it was found that *in vitro* BM-MSCs reached the onset of replicative senescence earlier than AD-MSCs obtained from the same donor [184].

1.3.3 MSCs in tissue repair and regeneration

One of the most important reasons that interest in studying MSCs has increased greatly from its fist discovery is that MSCs play an important role in tissue repair and regeneration.

Normally, when the tissue gets damaged due to various factors immune system responds by activating inflammatory pathways in order to deal with injury-causing stimuli and facilitate the tissue healing. Initially, to minimize the blood loss from the injured site local coagulation and local vasoconstriction occurs. At the site of injury vascular permeability increases to allow leukocyte recruitment and accumulation. Then, neutrophils, mast cells, monocytes, and macrophages gets attracted to the injured site by various chemokines and chemotactic agents such TGF-β, formylmethionyl peptides produced by bacteria, platelet-derived growth factor (PDGF), elastin and collagen breakdown products [185]. These leukocytes secrete various inflammatory mediators such as interleukin (IL)-1β, IL-6, IL-8, TNF-α, and IFN-γ to enhance the inflammation [186]. Macrophages, one of the most important regulatory cell, secretes TGF- β, TNF- α, heparin binding epidermal growth factor, fibroblast growth factor (FGF), which are key molecules in activating keratinocytes, fibroblasts, and endothelial cells [185]. These factors lead to increased synthesize of extracellular matrix to and epithelialization. Once the injury gets resolved, complex processes occur to end the inflammation and if the injury does not get resolved or abnormality occurs then acute inflammation transitions into chronic inflammation [186]. This can lead to devastating effects.

MSCs can minimize the tissue damage by suppressing inflammation and promoting regeneration, enhance the survival of cells by secreting trophic and angiogenic factors, induce bactericidal effects. Current literature indicates that MSCs induce their effects through direct cell-to-cell interaction and secretion of paracrine factors locally or systemically [187]. MSCs secrete

cytokines, anti-oxidants, pro-angiogenic factors and trophic molecules to alleviate stress response and apoptosis and enhance the repair [187]. Specifically, at the site of inflammation MSCs can sense the environment and respond to the stimuli by secreting at least 11 soluble cytokines: TSG-6, hepatocyte growth factors (HGF), transforming growth factor (TGF)-β, prostaglandin (PG) E2, IL-6, IL10, IL-1 receptor antagonist (IL-1RA), inducible nitric oxide (NO) synthase, indoleamine 2,3-dioxygenase (IO), galectin, and human leukocyte antigen (HLA)-G [188]. TSG-6 inhibits inflammation by binding to hyaluronic acid (HA) which attracts CD44+ leukocytes and promotes leukocyte migration; IL-1RA prevents IL-1α and IL-1β function in macrophage activation and prevents T-cell activation; PGE2 suppresses T-cells; IDO is another T-cell repressor; NO is macrophage regulator and T-cell suppressor; TGF-β1 inhibits T-cell activation, macrophage activation, and dendritic cell migration [189]. It has been shown that MSCs can attenuate the release of pro-inflammatory TNF-α and IFN-γ, and augment the production of anti-inflammatory IL-10 and IL-4 [185]. Anti-microbial effects are mediated either by secretion of anti-microbial factors such as LL-37, or through the secretion of immunomodulatory factors that enhance immune cell-mediated killing [185].

MSCs secrete anti-apoptotic and trophic factors that can help reduce the tissue damage and enhance the healing process. It is important to acknowledge that these molecular processes are complex. However, researchers are actively working to unveil and elucidate these complex biochemical interactions. Block et al. (2009) demonstrated that MSCs reduced apoptosis of UV-irradiated skin fibroblasts by secreting stanniocalcin-1 (STC-1), a peptide hormone that controls calcium metabolism and increases cell resistance to damage [190]. Kwon et al (2016) found that Wharton's jelly-derived MSCs secreted XCL-1, an important anti-apoptotic factor, that prevented stress-induced cell death in skeletal muscles both *in vitro* and *in vivo* Similarly,

Sholugu et al (2018) showed that cells which were pre-treated with MSC conditioned media had significantly preserved the cell viability when exposed to hypoxic stress [191].In addition to decreasing cellular apoptosis, MSCs has shown to secrete various trophic factors. It has been shown that *in vitro* MSCs induce proliferation of keratinocytes, dermal, fibroblasts, and endothelial cells. Keratinocytes are the cells that will form new epithelium and restore barrier; fibroblasts are considered as the workhorse for ECM reconstruction; endothelial cells are needed to rebuild the vasculature and provide the access for the local tissue with nutrients and oxygen. Kalinina et al (2011) suggested that MSCs can secrete neurotrophic growth factors such as basic fibroblast growth factor (bFGF), hepatocyte growth factor (HGF), nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), and glial cell line-derived neurotrophic factor (GDNF) [192].

Finally, MSCs have been shown to induce blood vessel formation which can further enhance wound repair and tissue regeneration. It is important to understand that blood vessel formation is divided into three mechanisms: vasculogenesis (*de novo* blood formation); angiogenesis is the sprouting of existing blood vessels which occurs under conditions of ischaemia and hypoxia; and arteriogenesis is the growth of smaller collateral vessels which happens in response to larger vessel blockage and sheer stress in endothelial cells of vascular wall [193]. Numerous studies have shown that MSCs enhance vasculature formation in each of described mechanisms [193]. Pro-angiogenic effects are attributed to the factors secreted by MSCs which include bFGF (FGF2), VEGF, TGF-β, PDGF, angiopoitin-1, placental growth factor (PGF), IL-6, monocyte chemotactic protein-1 (MCP-1), HGF, and stromal-cell-derived factor-1 (SDF-1) [194]. VEGF elicits its effects by binding to VEGF receptor, which then activates intracellular protein kinase B to inhibit apoptosis, the mitogen-activated protein kinase (MAPK) pathway to induce

proliferation, src kinase, focal adhesion kinase, and p38 MAPK to cause cell migration [195]. FGF2 bins to FGF receptor and results in the activation of many intracellular pathways such as Ras, Src, PI3K and PLC [196]. The main action by which FGF2 stimulates angiogenesis is through the induction of VEGF expression in endothelial and stromal cells, and regulation of VEGF signaling.

Given these immunomodulatory, bactericidal, anti-apoptotic, and pro-angiogenic properties of MSCs, they could be used as a co-cellular therapy to improve islet transplantation.

1.4 Summary

T1DM is a multigenic autoimmune disorder caused by destruction of insulin producing β-cells that reside in the islet of Langerhans of the pancreas. This leads to insulin insufficiency, impaired blood glucose control, and body starvation, which can be fatal if not managed. Islet transplantation is an attractive alternative to daily insulin monitoring and injections. However, at this stage this procedure is reserved for small subsets of T1DM patients and requires some major improvements for it to be widely available. Major limitations of islet transplantation are gradual loss of a graft caused by hypoxic environment and IBMIR, limited islet supply, and the requirement for life-long immunosuppression, which opens the door for opportunistic infections and side-effects. MSCs are specialized and easily-obtained stem cells found in almost all body tissues. They have the capacity to regulate immunity, promote angiogenesis and tissue regeneration, and induce bactericidal effects. MSCs from different body tissues appear to behave differently suggesting that cell ontology and cell microenvironment may play certain roles in their characteristics and functions. We have previously demonstrated the cytoprotective effects

of human bone-marrow-derived and pancreatic-derived MSCs on human islets from pro-inflammatory cytokines *in vitro* [135]. Furthermore, our group have also showed that islets co-cultured with bone-marrow-derived MSCs had greater intracellular insulin and glucose-stimulated insulin secretion; co-transplantation of islets with bone-marrow-derived MSCs resulted in significantly earlier correction of hyperglycemia than transplantation of islet alone in mice [137].

Current research suggests that not all MSCs are the same and that there is source-dependent differences in their cell yield per mass of tissue, transcriptome and secretome profiles, and proliferative and mitotic capacities . We think that there are microenvironment-dependent differences among different types of MSCs and that MSCs that are ontologically and anatomically closer to the islets might be more beneficial to them. To-date there is no reported study on visceral fat-derived MSCs specifically from peripancreatic region. Therefore, aim of this thesis was to isolate and examine MSCs derived from human peripancreatic adipose tissue.

Chapter 2: Isolation and Characterization of Mesenchymal Stromal Cells from the Visceral Adipose Tissue in Peripancreatic Region

2.1 Introduction

Type 1 Diabetes Mellitus (T1DM) is a multigenic autoimmune disorder that leads to the destruction of insulin producing β-cells of the pancreas by the host immune system [8]. This can lead to chronic hyperglycemia, diabetic ketoacidosis (DKA), retinopathy, nephropathy, neuropathy, and serious cardiovascular complications [26,35,36]. Edmonton protocol (EP) developed by the Islet Transplant Group in Edmonton is a promising cure for T1DM; however, at its current state it is reserved for specific subsets of T1DM patients that have unstable T1DM and hypoglycemia unawareness, severe hypoglycemic episodes and glycemic lability that cannot be controlled with intensive insulin therapies [197].

Some of the major limitations of EP that need to be overcome for it to be widely available are chronic immunosuppression to prevent allograft rejection, limited islet supply, and graft loss [86,198]. Chronic immunosuppression therapy caused mouth ulcers, diarrhea, anemia, and ovarian cysts in females; it is also opens the door for opportunistic infections [198,199]. Some of the factors that can contribute to islet graft loss are instant blood-mediated inflammatory reaction (IBMR), intrahepatic hypoxic environment, slow revascularization, detrimental effects of immunosuppressive drugs, and chronic allograft rejection [200,201].

Mesenchymal Stromal Cells (MSCs) are multipotent stem cells found in the stroma of most of the tissues in the body [202]. These cells have self-renewal capacity and can be differentiate into adipocytes, osteoblasts, and chondrocytes [143]. Furthermore, MSCs can suppress the inflammation, and promote tissue repair and regeneration through the secretion of cytokines, anti-oxidants, pro-angiogenic factors, anti-apoptotic factors, antimicrobial factors, and trophic molecules [187,189,190,193,203].

The International Society for Cellular Therapy (ISCT) formulated minimal criteria for defining multipotent mesenchymal stromal cells (MSCs). First, they must adhere to the plastic [143]. Second, these cells must highly express (≥ 95%) positive cell surface markers such as CD105, CD73, CD90, and minimally express (≤ 2%) negative cell surface markers such as CD45, CD34, CD14 (or CD11b), CD79α (or CD19), and HLA-DR [143]. Third, MSCs must differentiate into adipocytes, osteoblasts, and chondrocytes [143].

We have previously demonstrated the cytoprotective effects of human bone-marrow-derived and pancreatic-derived MSCs on human islets from pro-inflammatory cytokines *in vitro* [135]. Furthermore, our group have also showed that human islets co-cultured with bone-marrow-derived MSCs had greater intracellular insulin and glucose-stimulated insulin secretion; co-transplantation of new born pig islets (NPI) with human bone-marrow-derived MSCs resulted in significantly earlier correction of hyperglycemia than transplantation of NPIs alone in mice [137].

Current research suggests that not all MSCs are the same and that there is source-dependent differences in their cell yield per mass of tissue, transcriptome and secretome profiles, and proliferative and mitotic capacities [172-174]. We think that there are microenvironment-dependent differences among different types of MSCs and that MSCs that are ontologically and anatomically closer to the islets might be more beneficial to them. To-date there is no reported study on visceral fat-derived MSCs specifically from peripancreatic region. Therefore, aim of this study was to isolate and characterize MSCs derived from human peripancreatic adipose tissue.

2.2 Material and Methods

2.2.1 Isolation of Peripancreatic Adipose Tissue-Derived MSCs

Peripancreatic adipose tissue was prepared from pancreata acquired by the Clinical Islet Laboratory at Alberta Health Services or IsletCore University of Alberta. Four deceased multi-organ donors were processed for either clinical or research islet isolation with appropriate ethical approval and research consent obtained from next-of-kin of the donor (**Table 2.1**). This study was approved by the Health Research Ethics Board (HREB; Pro00001416). Prior to peripancreatic adipose and islet isolation processing, procured organs were stored in University of Wisconsin (UW) solution on ice between 3-18 hours depending on the time and location procurement. During pancreas processing from islet isolation, peripancreatic adipose tissue was trimmed and utilized for ppaMSCs isolation.

Peripancreatic adipose tissue specimens with a wet weight of 11-14 grams were equally split into two Falcon 50 ml conical centrifuge tubes (Thermo Fisher Scientific, Massachusetts, USA) containing 20 ml of 0.15 w/v collagenase Type XI (>1200 CDU/mg; Sigma-Aldrich, Ontario, Canada). Specimens were minced and incubated in a shaking water bath (37 ^{0}C, 40 rpm, 1 hour), and filtered using 100-µm cell strainer (Fisher Scientific, Ontario, Canada). Filtrate was mixed with 20 ml of supplemented Minimum Essential Media α (α-MEM; Thermo Fisher Scientific) and centrifuged for 10 minutes at 1,200 rpm. Supplemented α-MEM contained 10% v/v FBS, 2.5 ng/ml basic fibroblast growth factor (FGF-2; EMD Millipore, Ontario, Canada), 1 mmol/L sodium pyruvate (Thermo Fisher Scientific), 10 mmol/L HEPES (Thermo Fisher Scientific), 100 units of penicillin/1,000 units streptomycin (Thermo Fisher Scientific). Next, supernatant was aspirated, and the pellet was washed 2 more times with the same parameters. Following last

aspiration, pellets from 2 tubes were combined and resuspended in 2-5 ml of α-MEM depending on the pellet size for the subsequent cell counting.

Donor #	Sex	Age (years)	BMI	A1C
D1	F	74	35.8	5.9
D2	M	69	27.2	N/A
D3	M	66	30.1	5.4
D4	F	61	30.8	N/A
Average		67.5	31.0	5.6
S.E.M		2.7	1.8	0.2

Table 2.1 Table showing available donor information such as sex, age, BMI, and A1C. Average donor age was 67.5 years of age; average BMI was 31.0; and average A1C was 5.6.

2.2.2 Cell counting using Crystal Violet and Initial Seeding

To count mononucleated cells, 20 µL of cell suspension obtained from the previous collagenase digestion step was mixed with 20 µL of 1:50 Crystal Violet (CV; Sigma-Aldrich) and incubated for 5 minutes at room temperature. Next, 12 µL of the cell suspension mixture was pipetted onto hemocytometer and the cells at 4 corner squares of the hemocytometer were counted. Total mononucleated cell number was calculated using the **Formula 2.1**. Once the cells have been counted, they were seeded into T175 flasks at a density 2,600-29,048 cells/cm^2 (Sigma-Aldrich). The flasks were incubated 1-3 weeks depending on confluency and the media was changed during the second week post-isolation.

$$Total\ cells = \frac{(Total\ cell\ count)}{4}\ x\ (dilution\ factor)\ x\ (resuspension\ volume)\ x\ 10{,}000$$

Formula 2.1 Formula for calculating total cells from hemocytometer counts.

2.2.3 Passaging ppaMSCs

Once the cells reached confluency, they were detached from the plastic with 0.25% Trypsin-EDTA (Corning, Massachusetts, USA). To stop the enzymatic digestion, mixture was diluted with warm supplemented α-MEM and transferred into 50 ml conical tubes (1 tube per 1 flask). Tubes were spun at 1,200 rpm for 5 minutes and supernatant was removed. Next, the cells from all tubes were combined and resuspended in 10-40 ml of supplemented α-MEM, depending on the pellet size. Counting was performed as described previously but CV was replaced with 0.4% w/v trypan blue (Sigma-Aldrich). Cells were re-seeded into T175 flasks at a density 11,429-27,500 cells/cm^2 and incubated at 37^{0}C/ 5% CO_2 till the cells became confluent again. Media was refreshed every 3-4 days. Cells were passaged 3 times in total (**Figure 2.1**).

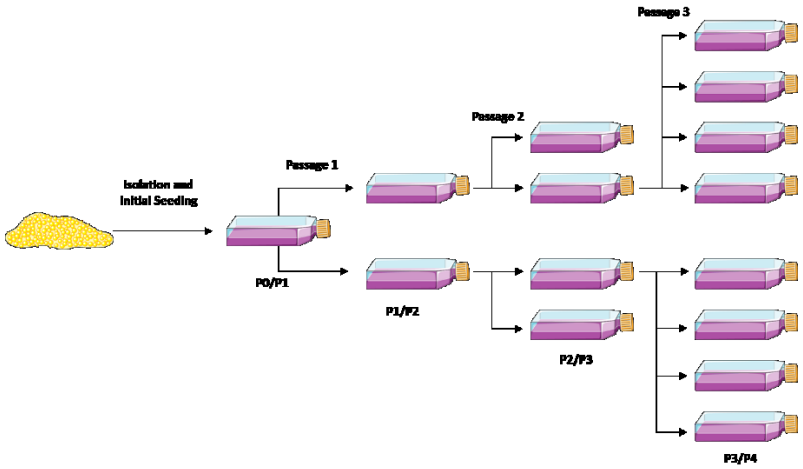

Figure 2.1 ppaMSCs were isolated and passaged 3 times in total**.** Cells were designated as P0 cells at the initial seeding, as P1 cells at the end of initial incubation period and after first passaging (Passage 1), as P2 cells at the end of second incubation period and after second passage (Passage 2), as P3 cells

at the end of third incubation period and after third passage (Passage 3), as P4 cells at the end of fourth incubation period.

2.2.4 Colony Forming Unit (CFU) assay and Population Doublings (PD)

CFU assay was performed for D1 and D4 to estimate the fraction ppaMSCs in the isolated cell mixture of mononucleated cells following ppaMSCs isolation. Predefined number of cells (500 or 320 cells) were seeded in three 100 mm culture treated plates (Sigma-Aldrich) at the same time of initial ppaMSCs seeding in T175 flasks. They received the same treatment and conditions as the flasks used for initial seeding. When the cells were passaged the first time (Passage 1), CFU plates were stopped and analyzed for the number of colonies formed. Briefly, the media was aspirated, the plates were washed twice with PBS (Thermo Fisher Scientific), fixed with Z-fix (Thermo Fisher Scientific) for 5 minutes, Z-fix was aspirated, and the plates were washed with PBS. To stain the cells, plates were incubated with 5 ml of CV for 10 minutes; then, the CV was removed, and the plates were washed with PBS. After, the plates dried the number of colonies formed in each plate was counted and using the **Formula 2.2** ppaMSCs fraction was calculated.

$$ppaMSCs\ fraction = \frac{Average\ number\ of\ colonies\ from\ 3\ replicates}{Number\ of\ mononucleated\ cells\ seeded\ in\ each\ plate}$$

Formula 2.2 Formula used to calculate the fraction of ppaMSCs from total number of mononucleated cells.

Results from the CFU assay were used to estimate the number of cells seeded into the flasks at initial seeding. Using this data and cell counts during passaging Population Doublings (PD) and Cumulative Population Doublings (CPD) were calculated for D1 and D4. PDs were calculated using formula described by Solchaga et al. (2010). For D2 and D3 CFU was not performed. Therefore, for these two donors PDs at P0 and CPDs were not possible to calculate. However, to provide two possible PDs at P0 and CPDs for D2 and D3 we used ppaMSCs fraction values from D1 and D4.

2.2.5 Flow Cytometry for Cell Surface Markers Expression

To assess the cell surface markers expression and cellular phenotypes, P4 ppaMSCs (n=4) were spun at 500g for 5 minutes to pellet the cells. Supernatant was removed and the pellet was resuspended in 5 ml of cold PBS. Sample was spun again at 500g for 5 minutes. Supernatant was removed and the pellet was resuspended in cold flow buffer (1%FBS in PBS; 100 µL/1x10^6 cells). 100 µL of cell suspension was added per test tube and mixed with 2.5 µg human Fc blocker (BD Biosciences, Ontario, Canada). Next, 10 µL/tube of CD73-PE, 20 µL/tube of CD90-FITC, 5 µL/tube CD105-PC7, 5 µL/tube of CD14-APC750, 10 µL/tube CD45-KO, and 10 µL/tube CD34-APC (all from Beckman Coulter, Ontario, Canada) were added to the appropriate tubes. Test tubes were kept in the fridge for 30 minutes. Cells were washed with 500 µL of cold flow buffer 3 times by centrifugation at 500 g for 5 minutes at 2-8 ^{0}C. Finally, cells were resuspended in 500 µL of cold flow buffer and analyzed on the CytoFlex Flow Cytometer (Beckman Coulter).

2.2.6 Trilineage Differentiation Assay

2.2.6.1 Adipogenesis

P4 ppaMSCs (n=4) were induced to become adipocytes. Procedure have been described previously by Bornes et al. (2015). Cells were seeded in a 6-well plate (Thermal Scientific, Texas, USA) at a density 5 x 10^3 cells/cm^2 and cultured in 3 ml of supplemented DMEM high glucose (sDMEM) 2-3 days until confluent. sDMEM was DMEM high glucose (Sigma-Aldrich) with 10% v/v Inactivated FBS (Sigma-Aldrich), 100 mM HEPES (Sigma-Aldrich), 100 U/mL penicillin/100 µg/mL streptomycin/029 mg/mL L-glutamine (PSG; Thermo Fisher Scientific). Next, the experimental group wells (n=4) were cultured in of Adipogenic Induction Medium (AIM) and control group wells (n=2) in of sDMEM EM for 72 hours. AIM was sDMEM with additional 1 µM dexamethasone (Sigma-Aldrich), 100 µM indomethacin (Sigma-Aldrich), 0.5 ml insulin-transferrin-selenium+1 (ITS+1; VWR), 500 µM isobutyl-1-methylxanthine (IBMX; Sigma-Aldrich). After 72-hour induction period, experimental group was cultured in Adipogenic Culture Medium (ACM) and control group in sDMEM for 24 hours. ACM was sDMEM supplemented with 0.5 ml ITS+1. Induction and culture cycle were repeated 3 more times. Then, cells were cultured in sDMEM for another week and at the end of culture period 2 experimental wells and 1 control well were stained; the wells were used for RNA extraction. To stain, the cells were fixed with Z-fix for 10 minute and washed with distilled water (DW). Then, cells were incubated with 0.3% w/v of Oil Red-O (Sigma-Aldrich) for 60 minutes at room temperature on a plate shaker. After, Oil Red-O was aspirated and the wells were washed with DW to remove residual stain. Images were taken using with Nikon Eclipse Ts2 diascopic illumination mode with 4X objective (Nikon Instruments Inc., NY, USA).

2.2.6.2 Osteogenesis

P4 ppaMSCs were induced to become osteoblasts. Procedure have been described previously by Bornes et al. (2015). Cells were seeded in a 6-well plate at a density 5 x 10^3 cells/cm^2 and cultured in sDMEM 2-3 days until confluent. Next, experimental group wells (n=4) were cultured in Basic Osteogenic Media (BOM) and control group wells (n=2) were cultured in sDMEM for 21 days with media refreshed twice a week. BOM was sDMEM with 0.1 mM L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate (Sigma-Aldrich), 10 nM β-glycerophosphate (Sigma-Aldrich), 10 nM dexamethasone (Sigma-Aldrich). At the end of culture period, 2 experimental group wells and 1 control group well were stained with 1% Alizarin Red S (Sigma-Aldrich); the rest were used for RNA extraction. To stain, the cells were fixed with Z-fix for 10 minutes and subsequently washed with DW. Then, the cells were incubated with 1% Alizarin Red S solution for 60 minutes at room temperature on a plate shaker. Finally, stain was removed, and the wells were washed with DW. Images were taken using with Nikon Eclipse Ts2 diascopic illumination mode with 4X objective (Nikon Instruments Inc.).

2.2.6.3 Chondrogenesis

P4 ppaMSCs were induced to become chondrocytes. Procedure have been described previously by Bornes et al. (2015). Briefly, cells were resuspended in Chondrogenic Medium (CM) and transferred into 1.65 ml conical tubes (n=6; 5 x 10^5 cells/tube). CM was DMEM High Glucose supplemented with 100 mM HEPES, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 µg/mL streptomycin, 0.29 mg/mL L-glutamine, 0.1 mM ascorbic acid 2-phosphate, 0.1 µM dexamethasone, 1x ITS+1, and 10 ng/mL transforming growth factor (TGF)-β3 (Neuromics Inc.). To form a pellet, cells were spun at 1,500 rpm for 5 minutes. Cells were incubated at 37

^{0}C/5% CO_2 for 3 weeks in total with media changes twice a week. Following the culture period, cells were used for histological analysis, glycosaminoglycan (GAG)/DNA assay, and RNA extraction. To stain the cells, pellets were fixed with Z-fix at least overnight and subsequently embedded into 2% agarose. Next, pellets were embedded into paraffin and sectioned at 5 μm thickness. Slides were stained with Safranin O (company). Images were taken using with Nikon Eclipse Ts2 diascopic illumination model with 4X objective (Nikon Instruments Inc.).

2.2.7 RNA Extraction and Reverse Transcription-Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR) Analysis of Differentiation Samples

Total RNA was extracted with 1 ml of Trizol (Thermo Fisher Scientific) and purified with RNease Mini Kit (Qiagen, Ontario, Canada). Briefly, extracted RNA was washed with 200 μL of chloroform (Sigma-Aldrich). Chondrogenic pellets were grinded with a pestle after adding the Trizol to facilitate the extraction. Total Aqueous phase was collected, mixed with equal volume of 75% EtOH (Thermo Fisher Scientific), and transferred into RNEase column (Qiagen). Then, the tube was spun at 12,000 rpm for 30 seconds. Flow-through was removed and the procedure was repeated for any remaining RNA-containing EtOH solution. Column was washed with 700 μL of RW1 and RPE (Qiagen). RNA was eluted off the column with RNase free water and quantified using the NanoDrop 2000c Spectrophotometer (Thermo Fisher Scientific).

Total RNA (500 ng) was reverse transcribed to cDNA using the High-Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific). qPCR analysis was performed as described in Bornes et. al (2015). To assess adipogenesis the gene expressions of lipoprotein lipase (LPL) and peroxisome proliferator activated receptor gamma (PPARγ) were studied; gene expression levels

were normalized to the mean expression of β-actin, YWHAZ, and SHDA. To assess osteogenesis the gene expressions of alkaline phosphatase (ALPL), runt-related transcription factor 2 (RUNX2), and osteopontin (OPN) were studies; gene expression levels were normalized to the mean expression of B2M, HMBS, and SDHA. Finally, to assess chondrogenesis the gene expressions of aggrecan (ACAN), collagen type 1 alpha 2 chain (COL1A2), collagen type 2 alpha 1 chain (COL2A1), collagen type 10 alpha 1 chain (COL10A1), and SRY-Box 9 (SOX9) were studied; gene expression levels were normalized to the mean expression of RPL13A, YWHAZ, and B2M.

2.2.8 Glycosaminoglycan (GAG) and DNA Quantification of Chondrogenic Pellets

In vitro cultured chondrogenic pellets were assessed for GAG and DNA amount present. Procedure have been described previously by our group (Bornes et al 2015). Briefly, pellets were washed in PBS and digested in proteinase K (1 mg/mL in 50 mM Tris supplemented with 1 mM EDTA, 1 mM iodoacetamide, and 10 mg/mL pepstatin A; Sigma-Aldrich) for the duration of 16 hours at 56 ^{0}C. GAG levels were measured with 1-9-dimethylmehtlene blue stain (Sigma-Aldrich); chondroitin sulfate (Sigma-Aldrich) was used as a standard. DNA levels were measured with CyQUANT Cell Proliferation Assay Kit (Life Technologies); supplied bacteriophage λ DNA was used as a standard.

2.2.9 Statistical Analysis

Both descriptive and inferential statistical analyses were performed using GraphPad Prism 8 (San Diego, CA, USA). Unpaired two-tailed t test or one-way ANOVA multiple comparisons with

Tukey's correction were performed, and statistical significance was tested at $P < 0.05$. Data are represented as mean ± standard error of mean (s.e.m.); range.

2.3 Results

2.3.1 Morphological Analysis of ppaMSCs

Qualitative morphological analysis of the flasks following the initial seeding revealed that our collagenase-facilitated digestion of peripancreatic adipose tissue yielded highly populated cell suspension at 0 hours and the confluency was 0% (**Figure 2.2**). At 48 hours post-initial seeding it became apparent some cells adhered to the plastic and display spindle-shaped morphology. Cells reached ~1% confluency at this point. At 72 hours post-initial seeding cells reached ~5% confluency and displayed long protrusions from the cell body. At 8 days post-initial seeding cells reached ~25% confluency and the cells appeared to be increased in size.

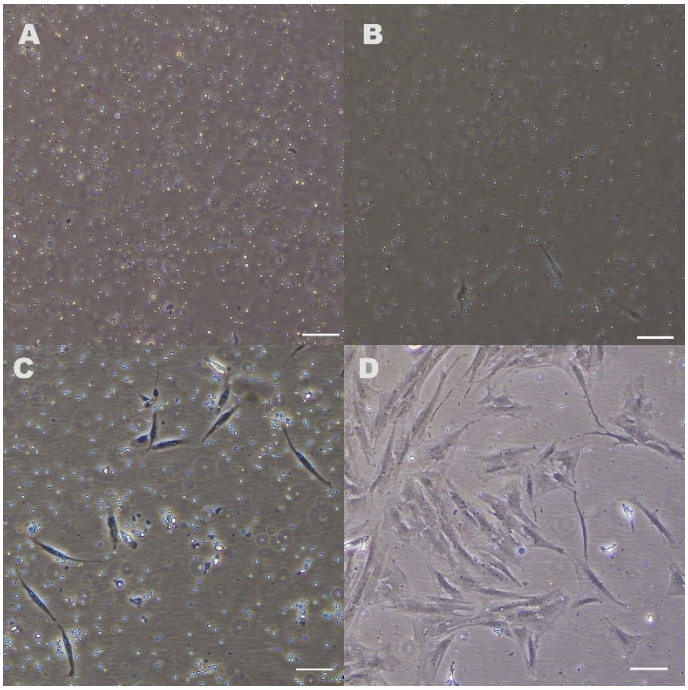

Figure 2.2 Qualitative analysis for morphological changes ppaMSCs using phase-contrast microscopy throughout 9 days reveals gradual adherence of the cells to the plastic and appearance of spindle-shaped morphology. (A) At 0 hours post-initial seeding highly populated cell-mixture contains floating but no attached cells. (B) At 48 hours post-initial seeding most of the cells are still floating while only

few have attached to the plastic. (C) At 72 hours post-initial seeding more cells attach to the plastic and display spindle-shaped morphology. (D) At 8 days post-initial seeding there are very few floating cells and adherent cells occupy greater area. Scale bar represents 100 μm.

2.3.2 Clonogenicity of ppaMSCs

To assess the fraction of mononucleated (MNCs) that were ppaMSCs, colony-forming unit assay was performed. From 500 D1 MNCs seeded on average 34.33 of those cells formed colonies (6.9%) (**Figure 2.3 A**). From 320 D4 MNCs seeded on average 3.33 of those cells formed colonies (1.0%) (**Figure 2.3 B**).

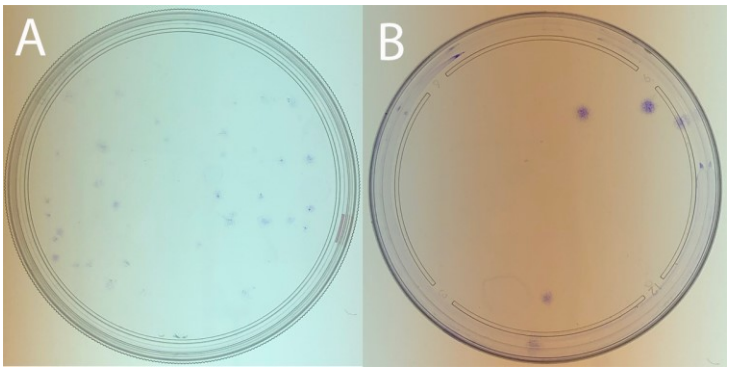

Figure 2.3 Images of representative CFU plates showing formed colonies that were stained with crystal violet (CV; blue). Seeded 500 D1 MNCs yielded on average 34.33 colonies (or 6.9%) as indicated (A). Seeded 320 D4 MNCs yielded on average 3.33 colonies (or 1.0%) (B).

2.3.3 Growth Kinetics

At each of 3 passages harvested cells were counted and known number of cells were seeded (n=4). P2 cells had 1.9 ± 0.6; 0.9-3.2 population doublings (PDs), P3 cells 1.1 ± 0.1; 0.8-1.4 PDs, P4 cells 1.15 ± 0.3; 0.5-1.7 PDs. PDs for D1 P1 and for D4 P1 cells were 6.9 and 10.0, respectively. PDs for D2 P1 and D3 P1 cells were estimated using CFU results from D1 (6.9%

ppaMSCs of total MNCs) and D4 (1.0% ppaMSCs of total MNCs) (**Figure 2.4**). If 1.0% was assumed, then PDs for D2 P1 and D3 P1 cells were 10.4 and 10.0, respectively. If 6.9% was assumed, then PDs for D2 P1 and D3 P1 cell were 7.6 and 7.2, respectively. CPDs for D1 cells were 9.5 at P2, 10.8 at P3, and 11.3 at P4. CPDs for D4 cells were 10.9 at P2, 12.3 at P3, and 14.0 at P4. CPDs for D2 and D3 varied depending on which PDs for P0 cells were used; both possibilities were displayed in **Figure 2.4**. If 1.0% was assumed, then CPDs for D2 were 11.2 at P2, 12.1 at P3, and 12.9 at P4. Similarly, if 1.0% was assumed then CPDs for D3 were 13.2 at P2, 14.1 at P3, and 15.8 at P4. If 6.9% was assumed, then CPDs for D2 were 8.5 at P2, 9.3 at P3, and 10.1 at P4. If 6.9 was assumed, then CPDs for D3 were 10.4 at P2, 11.4 at P3, and 13.0 at P4.

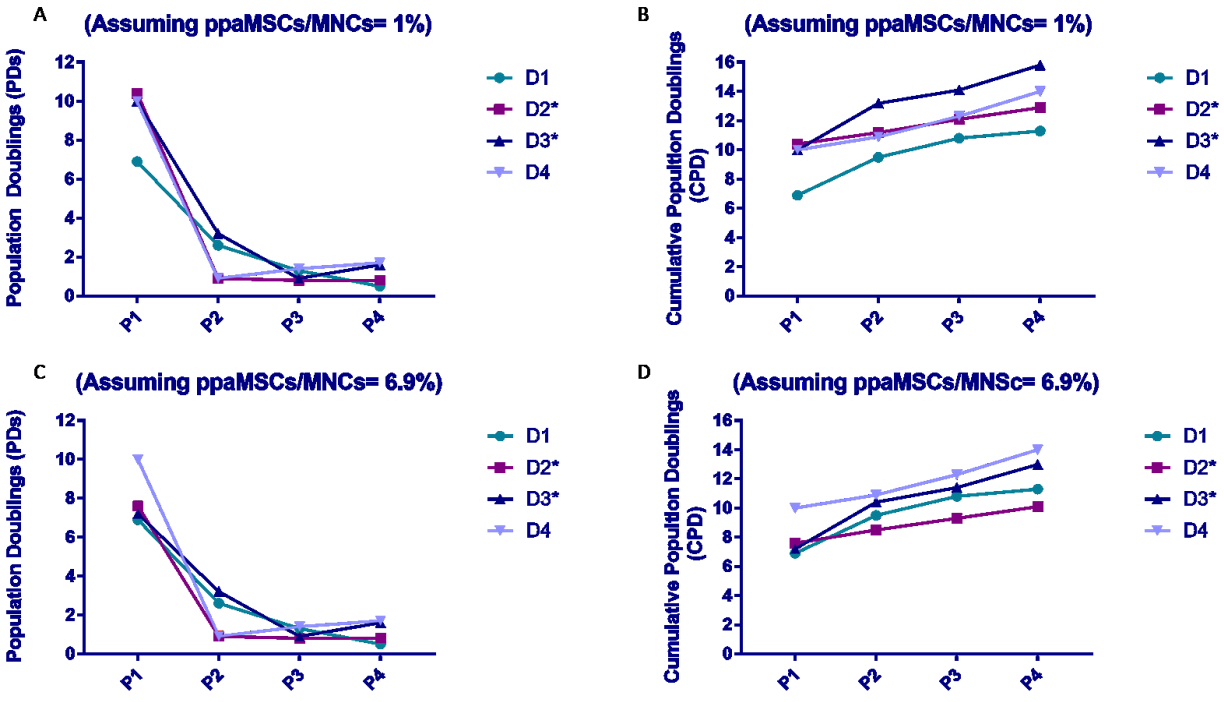

Figure 2.4 Growth Kinetics in terms of population doublings (PD) and cumulative population doublings (CPD) of ppaMSCs (n=4). Cells were isolated and seeded into flasks, and after that cells

were passaged 3 times. Using the data from CFU assay and cell counts during each passage, PDs (A, C) and CPDs (B, D) were calculated for D1 and D4; for D2 and D3 CFU assay was not performed; for these cells two possible PDs (A, C) and CPDs (B, D) outcomes are presented using CFU results from D1 (6.9%) and D4 (1.0%) (*).

2.3.4 Cell Surface Marker Expression

Flow cytometry analysis revealed P3 cells from four different donors (n=4) expressed comparable cell surface markers (**Figure 2.5**). On average, 99.6 ± 0.3%; 98.8-99.9% of the cell population expressed CD73. On average, 96.2 ± 1.2%; 92.9-98.4% of the cell population expressed CD90. Similarly, 98.6 ± 0.6%; 96.9-99.2% expressed CD105. Contrary, only 0.3 ± 0.2%; 0.1-1.0% of the cell population expressed CD14. Next, 1.2 ± 0.6%; 0.1-2.2% of the cell population expressed CD45. Finally, 0.3 ± 0.1%; 0.1-0.5% of the cell population expressed CD34.

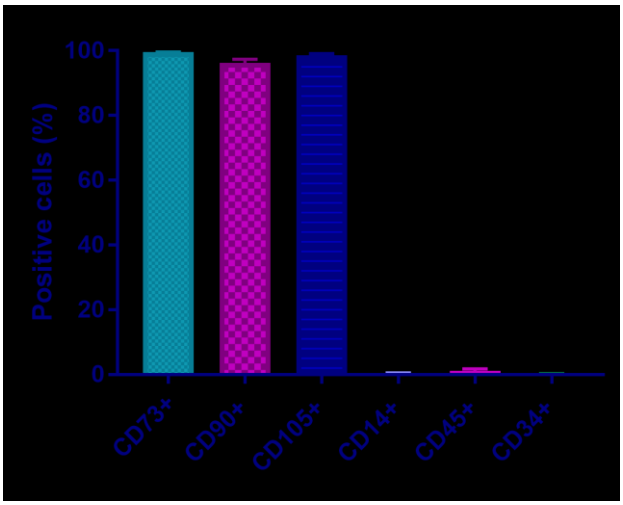

Figure 2.5 Cell surface expression of MSC-characterizing markers in ppaMSCs (n=4). Cells were analyzed for the MSC surface markers such as CD73, CD90, CD105, CD14, CD45, and CD34. ppaMSCs after third passage consistently expressed high levels of positive cell surface markers for

MSCs (CD73, CD90, CD105) and expressed very low levels of negative markers (CD14, CD45, CD34). Data are represented as bar graphs with the mean percentage ± standard error of the mean.

2.3.5 Mesoderm Differentiation Potential

P4 ppaMSCs (n=4) were assessed for their mesoderm differentiation potential. Specifically, cells were cultured in adipocyte-, obsteoblast-, and chondrocyte-induction conditions. Qualitative assessment showed that ppaMSCs differentiated into adipocytes but not osteoblasts and chondrocytes (**Figure 2.6**).

Quantitative gene expression analysis showed that LPL mRNA expression in adipocyte induction samples from D3 (n=2) and D4 (n=2) were higher than in their control samples D3 (n=1) and D4 (n=1) (**Figure 2.7 A**). Expression of LPL in D4 induction was significantly higher than in D3 induction, 0.31±0 and 0.05±0 (p= 0.0009; unpaired t test), respectively. Similarly, induction samples from D1 (0.02; n=2), D2 (0.02; n=2), D3 (0.04; n=2), and D4 (0.14; n=2) expressed higher PPARg mRNA than their respective control samples (**Figure 2.7 B**). Induction group from D4 expressed PPARg significantly higher than from D1 (p=0.0003; one-way ANOVA with multiple comparisons and Tukey's correction), D2 (p=0.0002; one-way ANOVA with multiple comparisons and Tukey's correction), and D3 (p=0.0005; one-way ANOVA with multiple comparisons and Tukey's correction). This complements qualitative studies where D4 induction samples had more lipids depositions compared to other samples.

Interestingly, in control groups the gene expression related to osteogenesis such as OPN, Runx2, and ALPL had higher or similar expression levels than in induction groups (**Figure 2.7 C-E**). Statistical analysis was not performed between induction and control groups as control samples had only one replicate.

Cells that underwent chondrogenesis induction expressed higher collagen type I (5.72 ± 0.9) relative to collagen type II (0.006 ± 0.003) and collagen type X (0.06 ± 0.01). D2 cells expressed significantly higher collagen type II (0.03 ± 0.004 versus 0.007 ± 0.001), collagen type X (0.123 ± 0.01 versus 0.06 ± 0.0005), and ACAN (0.002 versus 0.0005) compared to D1 cells. However, the relative expressions of ACAN, COL2A1, COL10A1, and SOX9 were very low compared to BM-MSC differentiated chondrocytes [204]. Even though Safranin O staining did not detect any proteoglycans, our pellets produced 9.7 ± 0.5 µg of GAG. Analyzed cells had 5.1 ± 0.5 GAG/DNA ratio. D1 had significantly higher GAG/DNA ratio compared to D4, 7.0 ± 0.7 compared to 4.2 ± 0.2, respectively.

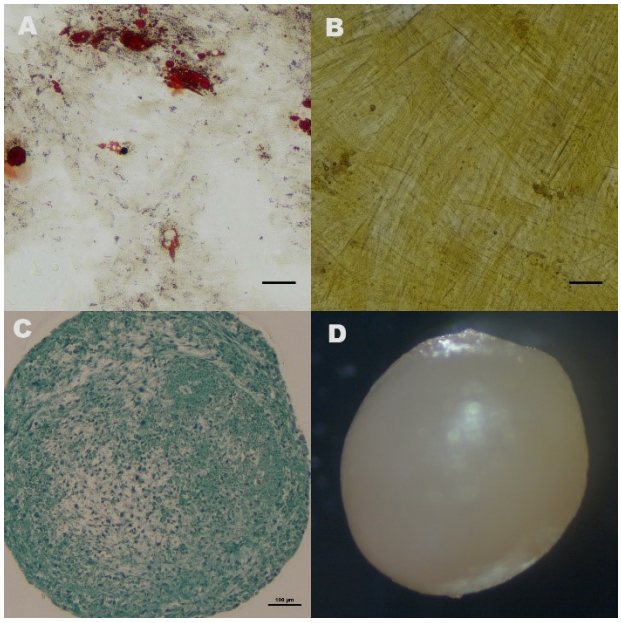

Figure 2.6 P4 ppaMSCs differentiated into adipocytes but not chondrocytes and osteoblasts. P4 ppaMSCs from each donor (n=4) were cultured in adipogenic, osteogenic, and chondrogenic induction conditions. (A) Cells consistently differentiated into Adipocytes as evidenced by Oil Red-O (red). (B) Cells did not differentiate into osteoblasts as evidenced by the lack of Alizarin Red S

staining. (C, D) Cells formed a pellet similar to chondrogenic pellet; however, these pellets did not stain for Safranin O (green is fast green). Scale bar in (A-C) represents 100 µm.

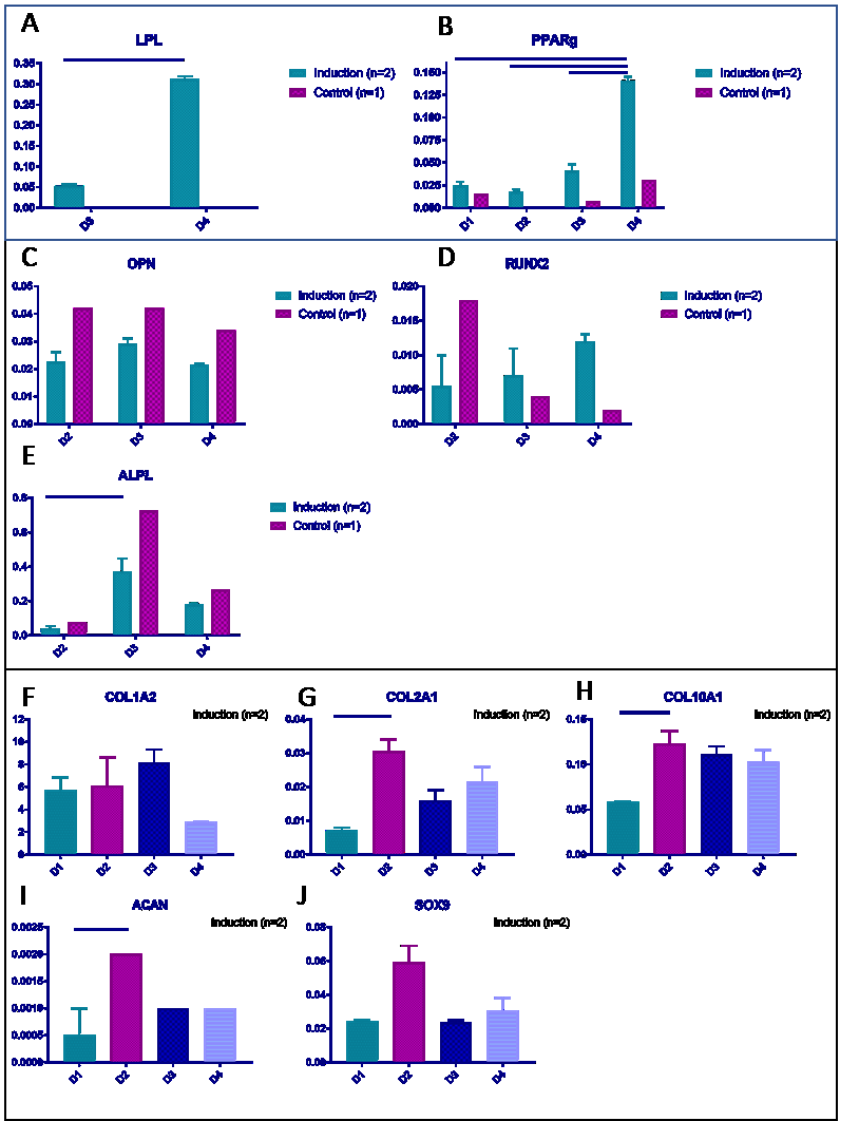

Figure 2.7 Reverse Transcription-Quantitative Real-Time Polymerase Chain Reaction (RT-qPCR) analysis of differentiation samples. Top box (A,B) shows the results for adipogenesis: LPL (n=2) and PPARg (n=4). Middle box (C-E) shows the results for osteogenesis: OPN (n=3), RUNX2 (n=3), and ALPL (n=3). Bottom box (F-J) shows the results for chondrogenesis: COL1A2 (=4), COL2A1 (n=4), COL10A1 (n=2), ACAN (n=4),

and SOX9 (n=4). Data represented as mean ± s.e.m. Statistical significance between groups (at $P < 0.05$) is represented with a bar.

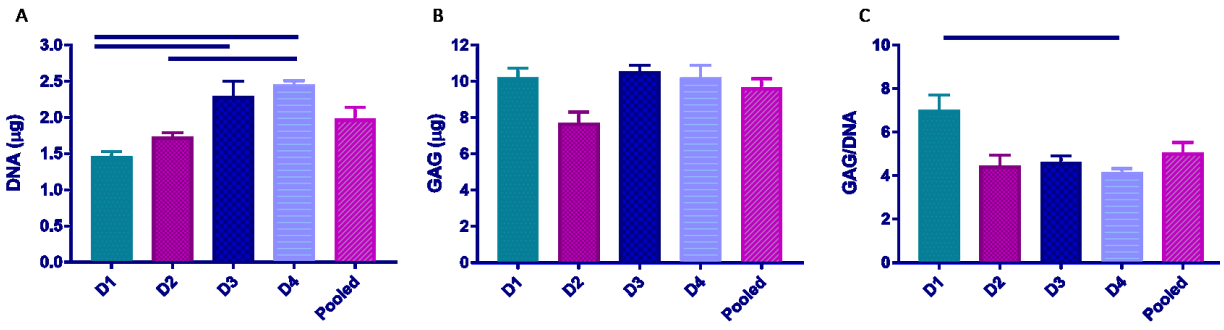

Figure 2.8 Glycosaminoglycan (GAG) and DNA quantification of chondrogenic pellets (n=4). For each donor 2 chondrogenic pellets were formed and analyzed. Last column in each panel represents data pooled from all donors (n=4). Data represented as mean ± s.e.m. Statistical significance (at $P < 0.05$) was tested between donors and it is represented with a bar.

2.4 Discussion

In this study we characterized the cells isolated from the visceral fat in the peripancreatic region. Based on morphological analyses, growth kinetics, cell surface marker expressions, and differentiation data these cells satisfied plastic-adherence and cell surface marker expression criteria but not fully differentiation criterion set by ISCT. Therefore, these cells were denoted as peripancreatic adipose-derived mesenchymal stromal cells (ppaMSCs). Current literature on visceral fat-derived MSCs is scarce and we believe that our study is first to isolate and characterize MSCs from visceral adipose tissue specifically in peripancreatic region.

In accordance with The International Society for Cellular Therapy (ISCT), our cells attached to the plastic, formed colonies, and displayed characteristic spindle-shaped morphology [143]. To estimate the fraction of ppaMSCs in a given population of ppaMNCs, we performed CFU assay which suggested that the yield was 1.0% for D4 and 6.9% for D1. In comparison, it is suggested that 1% of adipose tissue cells and 0.001%- 0.002% of BM aspirate cells are MSCs [205]. For D2 (Male; 69 y.o.) and D3 (Male; 66 y.o.) ppaMSCs that did not undergo CFU assay, we provided two possibilities using the ppaMSC fractions obtained from D1 (Female; 74 y.o.; 6.9%) and D4 (Female; 61 y.o.; 1.0%). Previous literature, suggest that age is inversely related to MSC frequency, colony-forming efficiency, functionality, and proliferation abilities possibly due to progressive telomere shortening, DNA damage, free radicals, and epigenetic alterations [206,207]. *In vitro* aging of MSCs is another very import factor that affects MSCs function and potential [144,207]. Furthermore, MSCs cannot expand indefinitely; it was suggested that MSCs can undergo 15-30 PDs at maximum [207]. Our cells expanded after each of 3 passages. The expansion potential rapidly declined and plateaued after passage 1. P1 cells from D1 and D3 had already undergone 6.9 PDs and 10 PDs, probably reaching their growth plateau. This could partly explain why PDs

of our cells at P2 and thereafter were so low. Jin et al. (2013) expanded their bone marrow-derived MSCs and adipose-derived MSCs up to P14 and their PDs for P1 cells were around 2.5 and 1.2, respectively [208].

In our study, CFU analysis showed that the older donor with higher BMI had higher ppaMSCs yield per mass of adipose tissue than younger donor with lower BMI. It is proposed that age is negatively correlated with cell yield [206,207]. This suggests that there might have been other donor characteristics that could have affected ppaMSCs yield; or one or both donors could have been the outliers. However, ppaMSCs from younger donor (D4) had higher population doublings at P1 and cumulative population doublings throughout P2, P3, and P4 than older donor (D1) which is in accordance with general theory of age and proliferation capacities of cells [206]. Our propositions with other donors did not yield any correlation. Therefore, more investigation with more donors is required to further elucidate the relationship between age and proliferation capacities in ppaMSCs.

There is no established one single cell surface marker that exclusively identifies MSCs from other types of cells and MSCs differ in the panel of cell surface markers expression depending on their origin and culture conditions [209]. However, ISCT suggested a minimal panel of positive and negative cell surface markers for identifying MSCs [143]. In accordance with ISCT, on average, ppaMSCs expressed more than 95% of positive cell surface markers such as CD73, CD90, CD105 and less than 2% of negative cell surface markers such as CD14, CD45, and CD34. This clearly satisfied the ISCT's second criterion.

Plastic adherence criterion for MSCs came from early studies of Friedenstein where he plated bone marrow aspirates onto plastic culture dishes and removed non-adherent cells after about 4 hours of seeding [138-140]. He observed that attached cells rapidly proliferated and some colonies

resembled small deposits of bone or cartilage [138]. Later studies discovered the ability of these cells to differentiate into osteoblasts, chondroblasts, and adipocytes, and they were named as MSCs [138]. However, fibroblasts which are specialized cells that secrete extracellular molecules to form stroma also attach to the plastic and are phenotypically are indistinguishable from MSCs [145]. Furthermore, similar to MSCs, fibroblasts can be expanded *in vitro* [210,211]. Fibroblasts expressed "MSC-defining" positive cell surface markers such as CD73, CD90, and CD105 and lacked the expression of negative cell surface markers such as CD14, CD45, and CD34 [145,212,213]. Future studies could screen for further cell surface markers such as CD106, CD146, ITGA11, SSEA-4, and GD-2 that are suggested to be MSC-specific [144].

Schneider et al. (2017) reported that were able to isolate MSCs from adipose tissue from liposuction (subcutaneous fat) and resected fat during implantation of a hip endoprosthesis [214]. Their morphological analysis results and flow cytometry results are very comparable to ours and they concluded that their cells were MSCs. It should be noted that abdominal subcutaneous fat and intra-abdominal visceral fat are two different distinct fat depots. Schneider et al. (2017) designated their cells as MSCs without differentiation studies endoprosthesis [214]. However, unlike them we did further differentiation studies to assess differentiation capacity of our newly isolated cells. A group from Singapore reported that they were able to isolate adipose-derived stem/stromal cells (ASCs) from visceral fat (VS) mainly based on their flow cytometry data and adipogenic differentiation capacity of their cells; both of these analyses were comparable to ours [215]. They reported that ASCs derived from VS had poor or very poor adipogenic potential; and these VS-ASCs had lower adipogenic potential than SC-ASCs [215]. Similarly, Potdar et al. (2019) reported that they were able to isolate MSCs from visceral fat mainly based on the expression of specific positive (CD105, CD13, SOX2, OCT4, NONG, LIF) and negative (CD45, CD34)

molecular markers [216]. However, they did not perform differentiation studies to show that the cells they were working with were truly "MSCs".

To satisfy ISCT's third criterion newly isolated cells underwent differentiation protocols for adipocytes, osteoblasts, and chondroblasts. As expected, ppaMSCs readily differentiated into adipocytes as confirmed by hydrophobic Oil Red-O (ORO) staining which stains for lipid droplets in cells. Youngest donor (D4; 61 y.o.) expressed highest levels of adipogenesis related genes and had the most lipid droplets. This is in accordance with general theory of the relationship between the MSCs age and their functional potential [144,207]. To our surprise, the cells from all donors did not differentiate into osteoblasts when they underwent our osteogenic differentiation protocol and chondroblasts under chondrogenesis differentiation protocol as evidenced by qualitative and quantitative analyses. Articular cartilage is composed of chondrocytes and extracellular matrix (ECM), which is predominantly composed of collagen type 2, aggrecan, and hyaluronic acid [217]. Gene expression analysis of chondrogenic pellets showed that our pellets expressed very low aggrecan, collagen type II and collagen type X, which is a marker of late stage chondrocyte hypertrophy associated with endochondral ossification [218,219]. Sox9 is known to be expressed in all chondroprogenitors and differentiated chondrocytes but not in hypertrophic chondrocytes; it is important regulator of chondrocyte differentiation [220]. Sox9 was very minimally expressed in our cells, which suggests that ppaMSCs did not undergo chondrogenesis. However, these cells expressed relatively high levels of type I collagen, which is found in skin, tendon, bone, ligaments, dentin, interstitial tissues [221]. In bones, type I collage is the most abundant protein, accounting for up to 90% of the organic matrix [222,223]. Furthermore, GAG/DNA ratio found in our study with ppaMSCs was higher than that previously described with BM-MSCs [204]. This discrepancy between absence of proteoglycan

staining by Safranin O and the presence of GAG detected by RT-qPCR could be explained by fact that samples where monoclonal antibodies detected chondroitin sulphate and keratan sulphate Safranin O staining was not detectable [169]. This could mean that our cells produced chondroitin sulphate or keratan sulphate which are found in bones too [224,225]. Then one possibility arises where our cells started forming bone ECM instead of cartilage ECM.

Baglioni et al. (2009) reported that they were able to successful induce osteogenesis and chondrogenesis in MSCs from the visceral fat as evidenced by Alizarin Red S and Toluidine Blue staining, respectively [226]. It should be noted that donor populations in our study and Baglioni et al. (2009) differed with respect to their BMI; their donors had normal weights with BMI ~23.4, whereas our donors were obese with BMI ~31.0 [226,227]. Visceral adipose tissue is hormonally active tissue that secretes different bioactive molecules and hormones such as adiponectin, leptin, tumor necrosis factor, resistin, and interleukin 6 (IL-6) [228]. Furthermore, abdominal obesity has been linked to pathological conditions such as impaired glucose and lipid metabolism, insulin resistance, increased predisposition to cancers of the colon, breast, and prostate [228]. It was reported that even gold-standard bone-marrow-derived MSCs from high BMI donors had impaired osteogenic and diminished adipogenic differentiation capacity [229]. In addition it was shown that MSCs from older patients have compromised chondrogenic and osteogenic but not adipogenic differentiation potentials [207]. Our results may confirm those findings as our donors were elderly patients (mean age was 67.5) and had high BMI (mean BMI was 31.0). Furthermore, the cells that we used for differentiation studies have undergone 11.3 PDs (D1) and 14 PDs (D4). As mentioned, it was suggested that human MSCs can undergo approximately 15-30 PDs [207]. Therefore, impaired osteogenic and chondrogenic ability of our cells may be explained by these cumulative factors such as *in vivo* age, *in vitro* age, and donor

BMI. On the other hand, since the visceral fat is a big tissue and we specifically isolated ppaMSCs from the adipose tissue adjacent to the pancreas, the difference in differentiation capacities observed in our study and by Baglioni et al. (2009) might confirm the acknowledged effect of microenvironment on MSCs [170,172-174].

If more vigorous examination of these cells show that they are not MSCs then another possibility is that our cells are adipocyte progenitor cells (APCs) since the cells from all donors where of adipose origin and readily differentiated into adipocytes. Phenotypically APCs are characterized by the expression of CD29, CD34, Sca-1, PdgfRα, and CD24, and the lack of expression of CD45 and CD31 [230]. Therefore, our cells could be screened for APC markers to assess whether the cells are APCs or MSCs.

Future studies could examine the effects of age and BMI on ppaMSCs. For example, ppaMSCs from younger versus older donors, and from high BMI versus low BMI donors could be compared with regards to their yield, proliferation, secretome profiles, and differentiation capacities. Furthermore, ppaMSCs from different donor categories could be compared with respect to their effects on islets *in vitro* and *in vivo*. If the age- and BMI-related differences exist, then testing ppaMSCs from the best donor group and comparing their effects on islets compared with MSCs from different sources from matched donors could elucidate the effect of ontology and microenvironment of the cell on its function and potential. This could support or reject the hypothesis that MSCs that are ontologically and anatomically closer to the pancreas could enhance the islet function better than MSCs that are further away.

In conclusion, we isolated and characterized ppaMSCs from peripancreatic adipose tissue. To our knowledge this is the first documented study to examine MSCs from visceral adipose tissue in the area specifically adjacent to the pancreas. Our cells expressed MSC markers and showed

the ability to differentiate into adipocytes but not osteoblasts and chondroblasts. Our group have previously shown that MSCs from bone-marrow and pancreas protect islets from harmful effects of inflammatory cytokines *in vitro* [135]. Furthermore, we also showed that bone-marrow derived MSCs improved islet function *in vivo* [137]. Literature indicates that microenvironment effects the potential of MSCs. This motivated to study newly characterized MSCs that are similar in the microenvironment to the pancreatic islets. Characterizing and establishing ppaMSCs will help to transition studies involving the effects of ppaMSCs on islet function.

Chapter 3: Conclusion and Discussion

3.1 General Discussion and Future Direction

Multipotent Mesenchymal Stromal Cells (MSCs) could potentially address some of the limitations of islet transplantation. MSCs are multipotent stem cells that are found in the stroma of most tissues in human body; these cells have self-renewal, differentiation, angiogenic, antimicrobial, and immunomodulatory capacities [134,170,171,193]. When the tissue gets damaged, MSCs can minimize this damage by suppressing inflammation, promoting regeneration and angiogenesis, enhancing cell survival [187-190,193].

Our group have previously demonstrated that human bone marrow-derived MSCs (BM-MSCs) protected human islets from the detrimental effects of cytokines by preserving glucose-stimulated insulin secretion in the islets and preventing β-cell apoptosis [135]. Encouraged by results *in vitro*, our group have moved on to test MSCs *in vivo*. Diabetic immunocompromised mice were transplanted with porcine islets or islets with human BM-MSCs under the kidney capsule, and were observed for over 30 weeks [137]. The group of mice that received co-transplants reached normoglycemia significantly earlier than islet-alone group and were more tolerant during oral glucose tolerance test [137]. Furthermore, analysis of extracted grafts revealed that BM-MSC/islet grafts had significantly more vasculature marker and more insulin content than islet-alone grafts [137].

Even though MSCs have been isolated from many different body tissues, current literature indicates that there is significant source-dependent and donor-dependent differences in MSC yield per mass of tissue, transcriptome and secretome profiles, and proliferative and mitotic capacities [170,172-174]. For instance, it has been suggested than only 0.001-0.002% of BM aspirate cells are MSCs; but, in adipose tissue 1% of digested cells are MSCs [205]. Xu et al. (2017) showed that BM-MSCs possessed stronger osteogenic and lower adipogenic differentiation potentials

compared to adipose tissue-derived MSCs and DNA methylation status of transcription factors controlling MSC fate was responsible for the differences [181]. Furthermore, adipose tissue is distributed throughout the body and they can be broadly divided into white versus brown fat, and subcutaneous versus intra-abdominal fat depots [182]. Depending on their type and depot location, fat tissues have differences in function and characteristics [182]. This led us to hypothesize that there might be microenvironment-dependent differences among different types of MSCs and that MSCs that are ontologically and anatomically closer to the islets might be more beneficial to them.

In chapter 2, we isolated and characterized cells from visceral adipose tissue specifically in peri-pancreatic adipose (ppa) region and given the results we denoted them as ppaMSCs. International Society for Cellular Therapy (ISCT) in their 2006 statements, proposed minimal criteria for defining MSCs [143]. First, they must adhere to the plastic; second, these cells must highly express (≥ 95%) positive cell surface markers such as CD105, CD73, CD90, and minimally express (≤ 2%) negative cell surface markers such as CD45, CD34, CD14 (or CD11b), CD79α (or CD19), and HLA-DR; and third, MSCs must differentiate into adipocytes, osteoblasts, and chondrocytes [143]. Our cells adhered to the plastic and displayed spindle shaped morphology and expanded after each passage. Next, our cells highly expressed panel of positive markers and very minimally expressed negative markers. Other labs denoted their cells as MSCs based on plastic adherence and cell surface markers that were comparable to ours [214,215]. To characterize further, we performed differentiation studies with our newly isolated cells. Our cells readily differentiated into adipocytes but not osteoblasts and chondroblasts. Our donors were elderly donors (mean age was 67.5 y.o.) and had high BMI (31.0). These results could be partly

explained by the fact that age and BMI is negatively correlated with MSC yield, function, and potential [206,207,229].

In the future we could study the effects of age and BMI on ppaMSCs. For example, ppaMSCs from younger versus older donors, and from high BMI versus low BMI donors could be compared with regards to their proliferation and differentiation capacities. Furthermore, ppaMSCs from different donor categories could be compared with respect to their effects on islets *in vitro* and *in vivo*. If the age- and BMI-related differences exist, then testing ppaMSCs from the best donor group and comparing their effects on islets compared with MSCs from different sources could be performed. This could support or reject the hypothesis that MSCs that are ontologically and anatomically closer to the pancreas could enhance the islet function better than MSCs that are further away.

Diabetes mellitus (DM) is serious metabolic disorder that results in impaired blood glucose homeostasis. Etiologically DM is subdivided into several subgroups such as type 1 diabetes mellitus (T1DM), type 2 diabetes mellitus (T2DM), gestational diabetes (GD), neonatal diabetes mellitus (NDM), and maturity-onset diabetes of the young (MODY). T1DM is multigenic autoimmune disorder, often precipitated by an exogenous factor, and which culminates in the destruction of insulin producing β-cells [8]. T2DM is a complex metabolic disorder that results in peripheral insulin resistance and relative insulin insufficiency; it is associated with physical inactivity, sedentary lifestyle, and obesity [45,231]. GD is disorder during pregnancy accompanied with a shift in hormone balance resulting in insulin resistance and impaired glucose regulation [47]. MODY is a group monogenic dominantly inherited disorders that are dissimilar to other types of diabetes [50].

Diabetes is associated with devastating conditions such as diabetic ketoacidosis (DKA), retinal damage (retinopathy), kidney damage (nephropathy), neural damage (neuropathy), coronary heart disease, cerebrovascular disease, peripheral artery disease [16,26,28,35,37]

Diabetes is on the rise: in 1964 an estimated 30 million people had DM, in 2000 this number went up to 151 million had DM, in 2015 there are estimated 415 million people with DM, and in it is predicted that by 2040 there will be 642 million people suffering DM [52]. Current estimates suggest that in high-income countries 87-91% of all diabetes cases in are T2DM, 7-12% are T1DM, and only 1-3% are other forms of diabetes [52].

Even though T2DM accounts for majority of DM cases, intensive lifestyle and pharmacological interventions have been shown to be effective in preventing or delaying the onset of diabetes, and in improving diabetes symptoms and complications [232-234]. On the other hand, since there is no endogenous insulin production in T1DM patients, they require life-long blood glucose monitoring and exogenous insulin injections. Medical expenditures are very costly to a patient with T1DM. Estimated discounted lifetime medical spending of a DM patient was $124,600 USD, $91,200 USD, $53,800 USD, and $35,900 USD diagnosed at ages 40, 50, 60, and 65 years, respectively [235]. In U.S.A, total yearly medical expenditure attributable to T1DM was estimated to be $6.9 billion USD [236]. Clearly, T1DM presents major economic burden in addition to decreasing quality of life of a patient. Strict blood glucose control with intensive insulin therapy showed to reduce the risk of developing retinopathy, microalbuminuria, neuropathy, atherosclerosis [39,65]. However, insulin therapy is a management and not a cure and the major severe side-effect of a therapy is hypoglycemia, which can be potentially fatal [63]. Furthermore, within the population of T1DM patients there is a subgroup of patients who have so-called brittle diabetes. These patients continue experiencing severe hypoglycemia, impaired awareness of

hypoglycemia, and excessive glycemic variability regardless of effective education and intensive insulin therapy [68]. These patients require attention the most.

Islet transplantation is a potential treatment option for T1DM. In 2000, the Islet Transplant Group in Edmonton infused islets isolated from deceased donors into the portal vein of 7 patients at 4,000 islet equivalents per kg of a patient with a combination steroid-free immunosuppressive therapy and achieved 1-year insulin-independence in all of them [83]. This was major milestone in islet transplantation and gave good grounds for expecting possible cure of T1DM in a future. However, 2017 reports from Collaborative Islet Transplant Registry (CITR) showed that insulin-independence rate in patients with common favorable factors was ~70% at 1 year, and ~40% at 5 years following last infusion [237]. Some of the factors that may contribute to islet damage and subsequent graft loss are islet damage during isolation and processing, acute and chronic rejection in the recipient, metabolic stress, hypoxic environment of the liver, and potential detrimental effects of immunosuppressive drugs on islets [86,238]. Since recipients require life-long immunosuppression, which opens the door for opportunistic infections and graft gradually fails the islet transplantation in its current form is reserved for brittle diabetic patients [197]. For it to be widely-available the procedure needs some major improvements to address graft failure, life-long immunosuppression, and potential limited islet supply.

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