

REAGGREGATION OF PANCREATIC ISLET CELLS: IMPLICATIONS  
FOR ISLET HEALTH, STORAGE AND NEW DRUG DISCOVERY

By

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## **ABSTRACT**

Islets are clusters of cells in the pancreas that monitor and regulate blood glucose levels. In culture, single islet cells reaggregate into clusters, but the reaggregation process is random, resulting in large variation in the cluster numbers, sizes and cell composition. Our laboratory created a micromold, a specialized cell culture plate, to efficiently bioengineer small, uniformly-sized islet clusters that we called Kanslets. Currently, very little is known about the implications of islet cell clusters for the treatment of diabetes. The purpose of this dissertation was to optimize the experimental conditions necessary for islet storage (cryopreservation), drug discovery screening procedures, and islet health in culture utilizing Kanslets. The conventional methods of cryopreservation of islets use intact native islets, and the viability and function achieved after thawing with these methods is poor. In this dissertation work, we cryopreserved islets as single cells and, after thawing, reaggregated islet cells to form cryopreserved Kanslets using the micromold technology. We observed that the viability of cryopreserved Kanslets was superior to cryopreserved intact islets and cryopreserved Kanslets had the potential to lower blood glucose levels of diabetic rats for 10 months. Next, the effect of creating hybrid islets/spheroids using mixtures of cells was tested by combining cells from different human donors, or by combining mesenchymal stem cells (MSCs) and islet cells. In order to minimize the need for assay repetition during the drug screening procedures, multi-donor Kanslets, consisting of islet cells from different donors, were created. We observed that the multi-donor Kanslets were highly viable, but showed little response to drugs such as glibenclamide (insulin stimulator) and somatostatin (insulin inhibitor). To improve islet health in culture, islet cells were integrated with bone marrow MSCs and reaggregated into spheroids; these spheroids were highly viable even long-term in culture, but secreted less insulin irrespective of the proportion of MSCs and islet cells in the spheroids, when compared to the spheroids containing only islet cells. Overall, these findings greatly enhance the utilization of islets for islet transplantation, and

also provide valuable insight for future work in reaggregating islet cells with MSCs. The work provides a platform for future researchers to further explore the implications of islet cell reaggregation for diabetes research or therapy.

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## **Chapter 1**

### **Introduction**

## 1.1. Diabetes and its effect on pancreatic islets

Diabetes is a metabolic disease represented by increased blood glucose levels either due to defects in insulin production resulting from destruction of  $\beta$ -cells, or defects in insulin action from reduced responses of peripheral tissues to insulin (American Diabetes Association 2010). There has been a 390% increase in number of people suffering from diabetes since 1980 with numbers reaching over 400 million in 2014 and this number is expected to increase to 522 million by 2030 (World Health Organization 2016). As of 2012, in United States alone, 29 million people had diabetes (Center for Disease Control and Prevention 2014). Due to the various associated complications, life expectancy of people with diabetes is reduced by 7 years (Morgan, Currie et al. 2000).

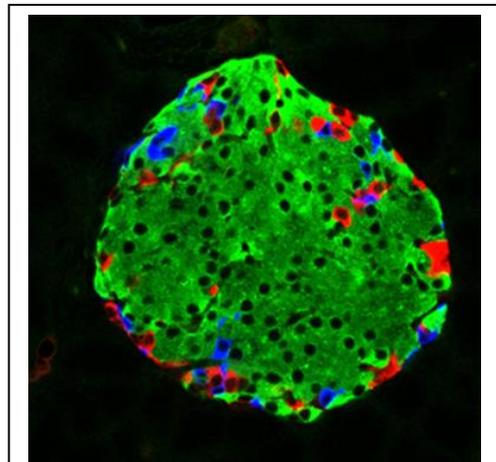
Previous studies, including ours, have found that diabetes damages the insulin producing structures of the pancreas, which are called islets. Diabetes not only affects the islet mass, it also disrupts islet morphology as well as reduces islet function (Kiraly, Bates et al. 2007, Rawal, Huang et al. 2013). Based on the etiopathogenesis, diabetes can be classified as type 1 or type 2 and both of these ultimately result in destruction or damage of  $\beta$ -cells. In type 1 diabetes there occurs destruction of pancreatic  $\beta$ -cells due to an autoimmune disorder (Vija, Farge et al. 2009). People with type 1 diabetes are symptomatic when they have lost more than 80% of  $\beta$ -cells and cannot regulate their blood glucose levels (Willcox and Gillespie 2016). Approximately 90-95% of the diagnosed cases of diabetes consists of type 2 diabetes while type 1 accounts for approximately 5-10% of cases (Colberg, Albright et al. 2010). Type 2 diabetes is characterized by initial systemic insulin resistance, which occurs when there is inadequate insulin uptake in the muscles.  $\beta$ -cells try to normalize the high blood glucose levels by hypersecreting insulin. Eventually, the  $\beta$ -cells fail to cope up with the increased demand (McCall, Toso et al. 2010). People with type 2 diabetes are symptomatic when  $\beta$ -cells can no longer produce enough insulin.

## 1.2. Islet of Langerhans

Islet function, including insulin release, is at the heart of preventing or reversing diabetes. Islets are housed in the pancreas, a gland located behind the stomach that lies transversely across the posterior abdominal wall. In humans, it is approximately 15-20 centimeters long and weighs about 90-100 grams. The pancreas is divided into the head, neck, body and tail. Within each section cells exist that are both exocrine and endocrine (Dolensek, Rupnik et al. 2015). The exocrine portion consists of connective tissue, blood vessels, nerves, acini and ductal cells and secretes pancreatic enzymes, which drain into the duodenum and help in digestion of proteins, carbohydrates and lipids. In contrast, 1-2% of the pancreatic mass consists of endocrine tissue containing islet of Langerhans that secrete hormones to help regulate blood glucose levels. There are approximately 1 million islets of Langerhans dispersed throughout the pancreas (Spencer and Peakman 2009).

### 1.2.1. Architecture of pancreatic islets

Islets of Langerhans are multicellular clusters of endocrine cells consisting of three major types of endocrine cells:  $\beta$ -cells that secrete insulin,  $\alpha$ -cells that secrete glucagon and  $\delta$ -cells that secrete somatostatin (Figure 1). Insulin helps to lower the blood glucose levels. In contrast, glucagon assists in increasing blood glucose levels. Somatostatin inhibits the release of insulin as well as glucagon. 65-80% of all endocrine cells are  $\beta$ -cells. 10-15 % of a pancreatic islet is made up of  $\alpha$ -cells while  $\delta$ -cells make up to 5-10% (El-Naggar, Elayat et al. 1993). Normally in rats, mice and rabbits,  $\alpha$ - and  $\delta$ -cells are situated in the periphery



**Figure 1. Immunofluorescence image of a typical rat pancreatic islet.**

Rat pancreatic islets consist of  $\beta$ -cells situated in the core (60-80%; green), surrounded by  $\alpha$ -cells (15-20%; red) and  $\delta$ -cells (less than 10%; blue).

while the islet center mostly consists of  $\beta$ -cells. In other species like horses and monkeys,  $\beta$ -cells occupy the periphery and  $\alpha$ - and  $\delta$ -cells are situated in the islet core. Human islets are more variable in arrangement of endocrine cells showing both types of arrangements and even random organization of the cells (Orci and Unger 1975, Orci 1976, Orci, Baetens et al. 1976, Murakami, Fujita et al. 1993).

Pancreatic islets are highly vascularized and have a rich blood supply from a dense capillary network which is ten times higher than that of the exocrine tissue (Vetterlein, Petho et al. 1987, Murakami, Miyake et al. 1997, Menger, Yamauchi et al. 2001, Zanone, Favaro et al. 2008, Schaeffer, Hodson et al. 2011). High vascularity of pancreatic islets is essential not only for the delivery of oxygen and nutrients to the endocrine cells, but also to serve as a pathway for chemical signals to reach the islets and for dispersion of islet hormones to the general circulation (Jansson and Hellerstrom 1986). Because of the high vascularity, there is higher oxygen tension in pancreatic islets than the surrounding exocrine tissue. Unfortunately, when these islets are isolated, they lose their blood supply and with subsequent islet cultures oxygen and nutrients are supplied to the pancreatic islets solely by the process of diffusion (Buchwald 2009).

### **1.2.2. Size of pancreatic islets**

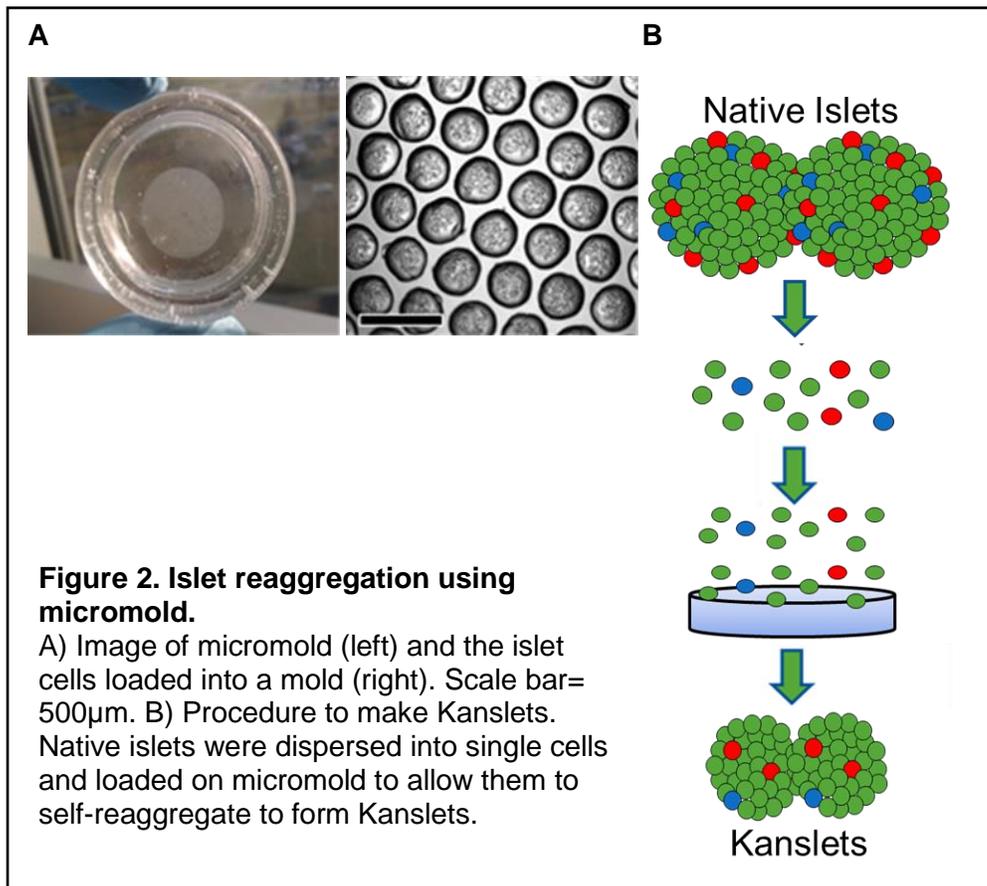
Islets must live on diffusion after removal from the body, therefore for survival, the size of a pancreatic islet (diffusion distance) becomes very important. Human islets can vary from 20 $\mu$ m to 400 $\mu$ m in diameter (Huang, Novikova et al. 2011). Our laboratory was the first to look at the differences between large and small islets. It was found that small size (<125 $\mu$ m in diameter) rat islets were more viable than large size islets (>150 $\mu$ m in diameter). Dead cells in large size islets were either scattered throughout the islets or present in the center of an islet. Small size islets also had significantly higher insulin secretion than large islets, and 80% of the diabetic rats transplanted with small size islets were successful while all diabetic rats

transplanted with large islets were unsuccessful in reversing diabetes (MacGregor, Williams et al. 2006). Based on the above mentioned paper, Lehman et al. in 2007 compared the differences between small size (50- and large size (150-300 $\mu$ m) human islets and had similar findings. It was found that insulin secretion (basal and stimulated) was higher with small size islets than the large size islets. The percentage of cell death was twofold higher during hypoxic culture than normoxic culture conditions in large and small size islets, and also, cell death was twofold higher in islets with diameter of > 100 $\mu$ m with respect to islets less than 50 $\mu$ m in diameter (Lehmann, Zuellig et al. 2007). Later, our laboratory found that center core cell death was present in all the large islets (> 150 $\mu$ m) within 7 days in culture with respect to the small size islets (< 100 $\mu$ m), which had 91% survival rate. In this study, core death was defined as an area in the center of an islet with more than 50% cells that were dead. The rate of glucose diffusion was approximately 1.5 times greater in the periphery of small islets with respect to the large islets (Williams, Huang et al. 2010). Another study in our laboratory found that small islets had greater cell density/area with respect to the large islets, and the insulin content of the large islets in the core was lower, which might possibly contribute to the fact that large islets secrete less insulin (Huang, Novikova et al. 2011).

### **1.3. Reaggregated islets: Kantslets**

Reaggregating islet cells and limiting the size of pancreatic islet seems to be one of the options for reducing the diffusion barrier and improving islet health. It is known that signals to reaggregate the islet cells are present in the neonatal (Falt, Odselius et al. 1985), fetal, (Masquelier, Amory et al. 1986) and adult (Halban, Powers et al. 1987) rat islets. Different methods of islet reaggregation have also been studied. In one study, the rat islet cells were dispersed into single cells and then cultured free floating in dishes for 3-4 days. (Hopcroft, Mason et al. 1985). Researchers found that the cells were able to morphologically organize themselves and also were responsive to dynamic (low followed by high) glucose challenges. In

another study islets were seeded in the polyglycolic acid scaffold to allow for spontaneous reaggregation (Weber, Hayda et al. 2008). Most of the studies published previously reported that islet cells reaggregated to form clusters, but neither these studies determined the cluster diameter, or there was great variation in the sizes of the reaggregated clusters formed (Halban, Powers et al. 1987, Matta, Wobken et al. 1994). Another group, reaggregated islet cells by the use of hanging drop method in which cells were seeded into single drops into the cell culture dishes (Cavallari, Zuellig et al. 2007). Islets reaggregated by the hanging drop method were uniform in size but the hanging drop method requires lot of labor and is not efficient if reaggregated islets are needed in larger quantities.



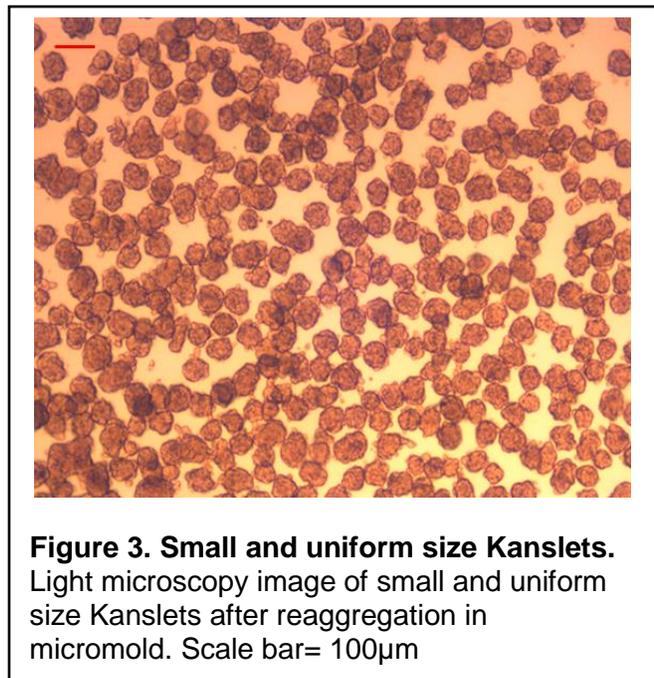
Looking at these previous observations, our lab created a micromold to bioengineer uniform small size islets (Ramachandran, Williams et al. 2013). The micromold consists of

conical shaped recesses (number of recesses depending upon the size of the micromold) each <math><200\mu\text{m}</math> in size (Figure 2A).

The uniform small size islets, called Kanslets, were engineered by dispersing fresh native islets into single cells, and then allowing them to reaggregate over a few days in the micromold, yielding small-size islets (Figure 2B). Large numbers of Kanslets of uniform size can be produced using this procedure, as shown in Figure 3.

Kanslets were tested for their viability, cellular composition, glucose diffusion, static and dynamic insulin secretion with respect to the fresh native islets. Kanslets have been shown to:

1. Have cellular composition similar to native islets
2. Have higher viability than native islets
3. Have higher glucose diffusion than native islets
4. Be responsive to increasing glucose concentrations by secreting insulin
5. Restore and maintain normoglycemia when transplanted into diabetic rats



In this dissertation, we will focus on how our technology of dispersing islets into single cells and later reaggregating can move diabetes research using three distinct approaches. First, we theorized that the reaggregation process would open new doors to solve the common problem of storage of islet cells, by allowing for the cryopreservation of the single cells and then reaggregation after thawing (Chapter 2). Such an approach had not been attempted before,

because the tools for reaggregation were inefficient and difficult to use. Next, we hypothesized that improved drug screening could occur if human islet cells were used in the three dimensional (3D) reaggregated form from multiple human donors. This hypothesis was tested and the results are described in Chapter 3. Finally, we built upon previous work by other labs indicating that the addition of mesenchymal stem cells (MSCs) to islet cells can increase their viability and their function. MSCs were loaded along with islet cells into the micromold to form spheroids and their morphology, viability and function was tested in Chapter 4.

#### **1.4. Reaggregating pancreatic islet cells for optimal cryopreservation**

##### **1.4.1. Need for optimal cryopreservation procedure for pancreatic islets**

Cryopreservation is defined as the process of freezing the cells/islets at deep subzero temperatures resulting in the seizure of chemical, physical and biological process (Taylor and Baicu 2009). Current preservation protocols of pancreatic islets are not optimal. Islets stored with short-term preservation techniques like tissue culture have a limited life-span and there is a high risk of contamination (Fraga, Sabek et al. 1998). Long-term storage can be achieved by cryopreservation of pancreatic islets at subzero temperatures. For more than 5 decades researchers have been trying to optimize the cryopreservation procedure for pancreatic islets (Kemp, Mullen et al. 1978, Rajotte, Ao et al. 1995), but there are no consistently successful cryopreservation techniques. Previous studies have reported reduced islet recovery, reduced viability, poor or abnormal insulin secretion after thawing and poor *in vivo* outcomes after transplantation of the cryopreserved islets (Piemonti, Bertuzzi et al. 1999, von Mach, Schlosser et al. 2003, Omori, Valiente et al. 2007). Hence, an improved method of long-term storage of islets would be advantageous both for essential, for research and islet transplantation.

Successful cryopreservation of pancreatic islet can offer several advantages. It will allow a sufficient transplantable mass of islets by pooling islets from pancreases of multiple donors

(Warnock and Rajotte 1988). Cryopreservation will also provide time for viability testing and human leukocyte antigen (HLA) matching of the islet preparation, as well as allowing time for quality control assays prior to transplantation (Gray, Reece-Smith et al. 1984, Lakey, Warnock et al. 1996). Furthermore, cryopreservation itself reduces the expression of major histocompatibility complex class 1 by 40% thus providing immunological benefits to the cryopreserved islets (Cattral, Warnock et al. 1993). Finally, the process of cryopreservation and thawing may help to improve islet purity (Rajotte, Warnock et al. 1983, Evans, Rajotte et al. 1987).

#### **1.4.2. Damage caused by cryopreservation**

There are four major steps in the cryopreservation process:

1. Addition of cryoprotective agent to cells/tissues
2. Cooling the cells/tissues
3. Thawing the cells/tissues
4. Removing cryoprotective agents

During these steps, cells/tissues can undergo damage. For 70 years, cellular injury during the process of cryopreservation and thawing has been an important focus of study (Storey 1990). Major factors causing cell/tissue damage during cryopreservation are thermal shock, the cytotoxic effects of the cryoprotectant, osmotic stress, and formation of intracellular and extracellular ice. As the temperature is decreased to allow cooling, the cells/tissues undergo cellular lesion because of thermal shock. Thermal shock can be avoided by the addition of a cryoprotectant in combination with slow cooling (Bakhach 2009). Dimethyl sulfoxide (DMSO) is the most commonly used as a cryoprotectant. It is an intracellular cryoprotectant with low molecular weight, capable of penetrating the cells. Once in the cells, it increases viscosity and reduces the amount of ice that forms at a temperature below 0°C (Fahy,

MacFarlane et al. 1984, Yu and Quinn 1994, Fuller, Dijk et al. 2003) (Fahy, MacFarlane et al. 1984). DMSO not only stabilizes lipid membranes but also protects cellular proteins from denaturation (Anchordoguy, Cecchini et al. 1991).

Despite several advantages of using a cryoprotectant during the cryopreservation process, cryoprotectants have been reported to cause toxicity if used in high concentrations (Giugliarelli, Urbanelli et al. 2016). Cryoprotectants can cause damage to cell membranes (Wang, Hua et al. 2007) as well as cause oxidative stress if used in higher concentrations (Sanmartin-Suarez, Soto-Otero et al. 2011). Furthermore, osmotic stress during the addition and removal of the cryoprotectant can also damage the cell. When a cryoprotectant is added to the solution, the cells shrink and then return to normal cell volume when the cryoprotectant permeates. Conversely, when the cryoprotectant is removed, cell volume expands. Cells undergo volume changes due to osmotic differences during cryoprotectant addition and removal during the process of cryopreservation and thawing, which causes cell injury and affects islet physical integrity (Mazur 1984, Gao, Liu et al. 1995).

Another important factor causing cell injury during cryopreservation and thawing is the formation of intracellular and extracellular ice. The temperature zone between -15 to -60°C is considered to cause the most lethal injuries to the tissue, and the samples have to pass through this zone twice: once during cryopreservation, and once gain during thawing (Mazur 1963). As the sample cools down, the temperature at which ice crystals are initiated is called nucleation temperature. Control of ice formation is a critical step during the cryopreservation process (Morris and Acton 2013). If no ice nucleating agents are present, ice generally starts to form at temperatures that are much below the freezing point of the solution. The lowest possible super cooling temperature in most biological systems is the point of homogeneous ice nucleation, around or at -40°C (Lee and Costanzo 1998). During nucleation, energy is released as the latent heat of fusion and this causes the temperature to rise near to the melting point of the

solution before it starts to cool down again. After the initial ice nucleation event, ice crystals have the capacity to grow into complex clusters forming networks that can grow exponentially as more and more water molecules participate in complex alignments with each other. Ice that forms at super cooled temperatures is spontaneous and can happen at random and at unpredictable temperatures. Ice nucleation affects the structural, osmotic and colligative integrity of cells, causing physical ruptures and mechanical injury.

Rapid ice crystallization at super cooled temperatures can also cause damage through formation of intracellular ice as there is increased retention of water. If extracellular ice formation occurs at much lower temperatures, then the chances of intracellular ice formation also increases. Therefore, in order to avoid uncontrolled extracellular and intracellular ice formation, it is recommended to initiate ice formation at temperatures not much below the melting point. There are many different ways to initiate ice nucleation, like touching the sample with super cooled needle, touching the exteriors of the sample with chilled rod or by mechanically agitating the sample (Stephen Butler and Pegg 2012).

Appropriate cooling rates are also important to minimize ice formation and cell/tissue damage. During the process of cryopreservation, when the temperature goes down to approximately  $-5^{\circ}\text{C}$ , cells as well as the surrounding medium are supercool. As the temperature lowers more, extracellular ice starts to form. At this time, to maintain osmotic equilibrium between the partially frozen outside of the cell and the unfrozen inside, the water molecules migrate to the extracellular area. During this process of freezing, cell injury depends upon the rate of cooling. Cell injury during freezing has been explained by Mazur in 1972 by using a two factor hypothesis. According to the hypothesis, when the cooling rates are too slow, cell injury occurs due to various solution effects like cell dehydration, extracellular ice formation and solute concentration. Specifically, with excessively slow cooling, cells can undergo an excessive amount of dehydration, volume shrinkage, and they will be in contact of high-solute

concentrations for long duration of time (Tyerman, Niemietz et al. 2002). If the cooling is too rapid, cell injury occurs due to intracellular ice formation (Mazur, Leibo et al. 1972). In this case, intracellular water is not lost fast enough to maintain equilibrium; the cells become increasingly super cooled, eventually attaining equilibrium by freezing intracellularly (Toner, Cravalho et al. 1993, Muldrew and McGann 1994). Extracellular ice formation also causes mechanical stress, leading to cell damage. Therefore, the cooling rate should be neither too rapid to allow intracellular ice formation, nor should it be too slow to allow excessive dehydration.

There are chances for cellular injury during the thawing phase as well. Warming rates are also considered crucial with respect to cell survival after thawing. Fast thawing is preferred as it can prevent the growth of small intracellular ice crystals into harmful large ice crystals (Gao and Critser 2000). Very small ice crystals, which are formed during the cryopreservation procedure, behave differently when the sample is warmed slowly or rapidly. With slow warming, intracellular ice crystals recrystallize and grow bigger and cause damage to the tissues whereas with rapid cooling rates, it is difficult for the ice crystals to recrystallize and grow as the ice crystals simply melt (Pegg 2007). It was shown that fetal islets thawed at a fast rate had almost normal insulin secretion and C-peptide content and these islets also had intact structural integrity with respect to the islets that were slowly thawed (El-Naggar, Al-Mashat et al. 2006).

Other causes of damage to the islets during cryopreservation are cold hypoxia, reoxygenation, and upregulation of inflammatory responses (Fuller, Dijk et al. 2003). Making the situation even worse, pancreatic islets possess poor antioxidant defense mechanisms (Modak, Parab et al. 2009), hence oxidative stress during the cryopreservation process can lead to extensive cellular damage.

### **1.4.3. Vitrification and microencapsulation**

Vitrification and microencapsulation of the islets have been suggested to prevent injury caused due to the cryopreservation process. Vitrification uses compounds like sugars that prevent the ice formation by keeping the water in the non-crystalline state (Armitage and Rich 1990), but the use of common vitrification compounds like poly vinyl pyrrolidone and poly ethylene glycol during the vitrification process can exert some toxicity. Also, vitrification requires the use of high concentrations of cryoprotective agents, which can be toxic to the cells (Fahy 1986, Jackson, Ungan et al. 1997). It was seen that insulin secretion of the islets vitrified in the presence of such compounds decreased by 45% after thawing (Agudelo and Iwata 2008). Cryopreservation also causes damage to the microencapsulated islets resulting in their low viability (Zhou et al 1997). In particular, cryopreservation damages the structure of the agarose gel microcapsules, thus affecting the diffusion of molecules like glucose and insulin within the gel (Agudelo and Iwata 2008).

### **1.4.4. Cryopreservation techniques described in the literature**

The conventional protocols described in the literature for cryopreservation of human islets are based on the results of the animal models and these protocols are based on the same principle of slow freezing and fast thawing with the use of DMSO, and sucrose dilutions. Researchers have stressed appropriate cooling and thawing rates to prevent intracellular ice formation (Taylor and Benton 1987, Miyamoto, Kenmochi et al. 1994). Most studies have shown that slow freezing to -40 °C and rapid thawing from -196 °C have provided more viability in rodent islets (Rich, Swift et al. 1993). As described previously, with a fast freezing rate there is not enough time for water to leave the cells; instead with slow freezing rates, water gets enough time to leave the cells, resulting in an increase in the intracellular solute concentration. Increased intracellular solute concentration lowers the equilibrium melting point of the solution, which reduces the temperature at which intracellular ice begins to form (Karlsson and Toner

1996). Slow freezing and rapid thawing has been suggested for human islets as well. 1.5M, or 10%, DMSO as opposed to 2M DMSO (Lakey, Anderson et al. 2001) has been suggested for human islets, as islet recovery was 74% approximately with 1.5M DMSO while recovery was only 62% in 2M DMSO. Our protocol for cryopreservation is based on slow freezing and rapid thawing to avoid intracellular ice formation.

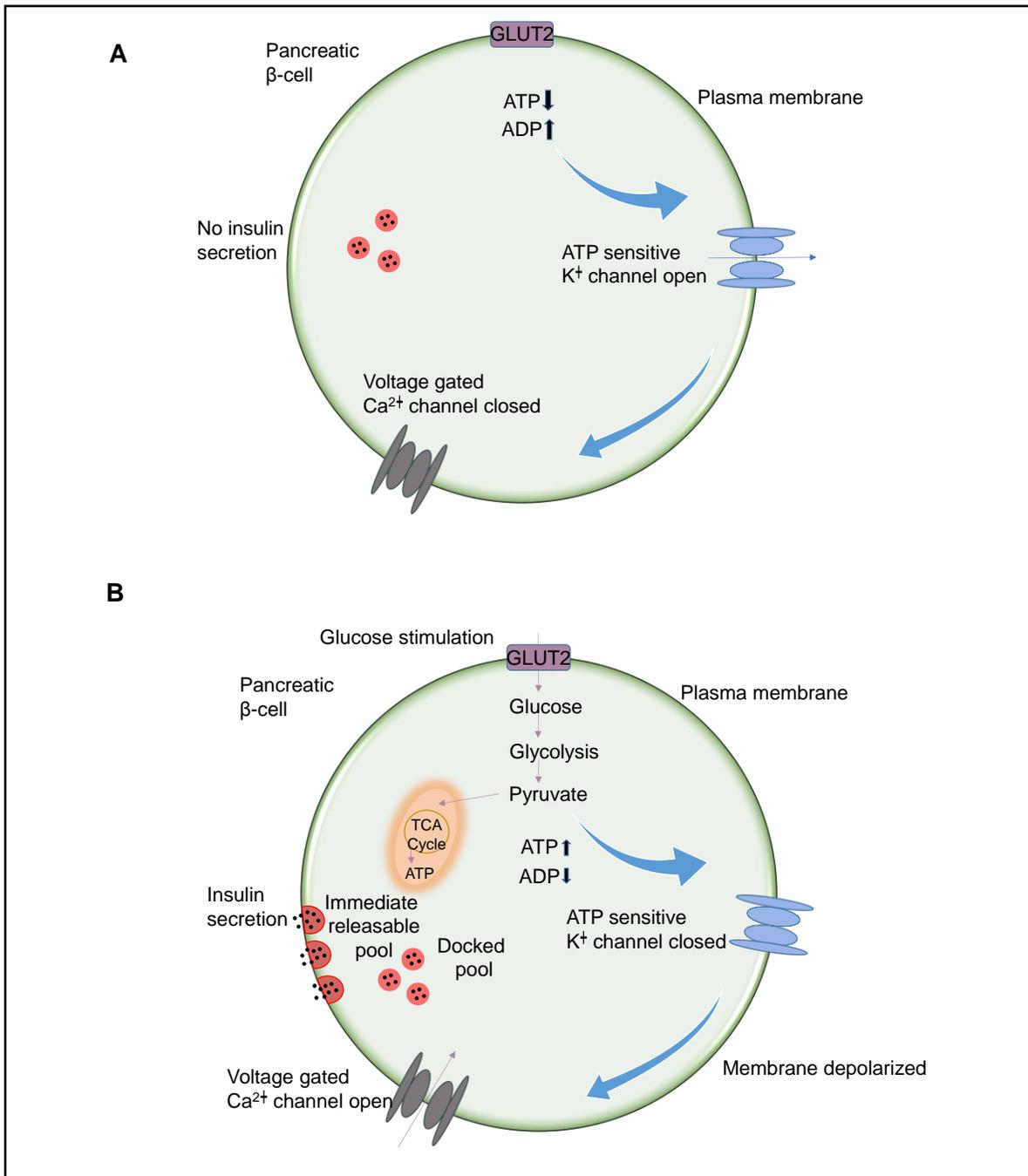
Conventional cryopreservation protocols described in the literature used intact native islets for cryopreservation. Native Islets are multicellular structures. Multicellular structures are more difficult to cryopreserve than single cells (reviewed in Chapter 2); hence, for experiments in Chapter 2, we cryopreserved islets as single cells, and after thawing, allowed them to reaggregate in the micromold to form Kantslets. We compared the outcomes of this form of cryopreservation to the conventional method used for cryopreservation of intact native islets.

#### **1.4.5. Glucose stimulated insulin secretion (GSIS)**

The primary function of pancreatic  $\beta$ -cells is to help monitor and regulate blood glucose levels. High concentration of blood glucose is the primary stimulus to  $\beta$ -cells to secrete insulin. Thus, glucose stimulated insulin secretion (GSIS) is the most common outcome to assess *in vitro* function of islets. GSIS is the increase in insulin levels with respect to the basal insulin secretion in response to external glucose levels. An increase in blood glucose levels causes increase in intracellular glucose levels as glucose diffuses into the  $\beta$ -cells with the help of glucose transporters (GLUT1 and GLUT2) (Newsholme, Gaudel et al. 2010). Subsequently, a series of downstream intracellular signaling events occur. During glycolysis, glucokinase phosphorylates glucose in  $\beta$ -cells forming fructose-1,6-biphosphate and two ATP molecules are converted to two ADP molecules. Fructose-1,6-biphosphate splits into 2 molecules, each containing 3 carbons with a phosphate group. Each of these molecule is converted into pyruvate and in the process 2 APT and one NADPH molecule are produced. In this whole process, a net of two ATP molecules are produced. Pyruvate then enters the tricarboxylic acid cycle in

mitochondria and gets oxidized generating another ATP (Muio and Newgard 2008). An increase in the ATP/ADP ratio leads to closure of ATP sensitive potassium ( $K^+$ ) channels in the plasma membrane. This further leads to membrane depolarization and the opening of calcium ( $Ca^{2+}$ ) channels, leading to exocytosis of a small fraction of insulin granules that reside in docked positions near the membrane called the immediately releasable pool.

In the first phase of insulin release there is a rapid release of insulin for approximately 4-10 minutes followed by a reduction of insulin secretion and then increased insulin secretion follows for 30 minutes until it reaches plateau, called the second phase. Although several hypotheses have been proposed to explain the mechanism behind the second phase of insulin release, there is no conclusive agreement (Straub and Sharp 2002). The second phase of insulin secretion is supported by the docked readily releasable pool. The rate limiting step during the second phase of insulin secretion is the conversion of the readily releasable pools into immediately releasable pools (Straub and Sharp 2002). With the first and second phases of insulin secretion as the major outcome measures for *in vitro* assays of islet function, it is important to understand all steps of the process when using GSIS to screen islet cells for the activity of new drugs. There are various drugs like glibenclamide, nifedipine and somatostatin that act through  $K^+$  and  $Ca^{2+}$  channels to either enhance or inhibit the insulin secretion. We tested glibenclamide and somatostatin for drug testing experiments in Chapter 3. Glibenclamide is a hypoglycemic drug, and binds to the  $K^+$  channel, leading to its closure, which results in opening of the  $Ca^{2+}$  channel and insulin secretion (Proks, Reimann et al. 2002). Whereas somatostatin inhibits the secretion by reducing the inflow of  $Ca^{2+}$  through the voltage gated  $Ca^{2+}$  channels. Somatostatin also reduces insulin synthesis. Somatostatin has also been shown to hinder the mobilization of insulin granules (Renstrom, Ding et al. 1996, Benali, Ferjoux et al. 2000, Farrell, Rankin et al. 2014).



**Figure 4. Mechanism of insulin secretion from  $\beta$ -cells after glucose stimulation.**

A) At rest, when there is low glucose concentration,  $K^+$  channel remain open, membrane is hyperpolarized and there is no electrical activity hence voltage gated  $Ca^{2+}$  channels remain closed and there is no insulin secretion. B) When there is higher levels of glucose in the blood, glucose enters the  $\beta$ -cells, is metabolized leading to  $K^+$  channel closure and membrane hyperpolarization which leads to  $Ca^{2+}$  influx and insulin secretion. Please note: other subcellular organelles such as nucleus, mitochondria, and endoplasmic reticulum are omitted from cells for clarity.

Modified from (Ashcroft 2007)

## **1.5. Drug testing: reaggregated 3D islets**

### **1.5.1. Drugs for diabetes and need to discover new drugs**

Drugs are used to help diabetics maintain blood glucose levels within the targeted range. Out of all the diagnosed cases of diabetes, in 2011, 50% of people were on oral medications, 17% were on insulin, and another 13% were on oral medications along with insulin (Center for Disease Control and Prevention 2012). Table 1 lists the various classes of drugs approved by Food and Drug Administration (FDA) for type 1 and type 2 diabetes and their mechanisms of action along with the adverse effects associated with them. Since the first insulin injections in 1920's, insulin has been the mainstay for people with type 1 diabetes. People with type 1 diabetes have to be on intensive lifelong insulin therapy and regular blood glucose level checks. According to the onset, peak time and duration, insulin formulations can be classified as rapid acting, short acting, intermediate acting or long acting. Insulin can be administered either by multiple chronic injections or by infusion pumps. Along with hypoglycemia, pain and redness at the injection site are common side effects of insulin injections. Infusion pumps can also cause diabetic ketoacidosis as well as subcutaneous skin infections (Chantelau, Spraul et al. 1989).

Type 2 diabetes is typically managed with oral medications (most commonly metformin) along with diet and exercise. Metformin is the only biguanide available in United States. It is one of the cheapest medications for people with type 2 diabetes. Metformin acts mainly by reducing the output of glucose by the liver and also by increasing the uptake of glucose in the skeletal muscles (Rojas and Gomes 2013). It is commonly associated with gastrointestinal side effects as well as rare but serious adverse effects of lactic acidosis, which is one of the causes of high mortality rate in these patients. As the disease progresses, people with type 2 diabetes might require an additional oral or injectable medication with or without insulin, and it is estimated that around 70% of people suffering from type 2 diabetes will be on more than one type of oral medications with or without insulin (Norris SL, Lee NJ et al. 2008). Sulphonylureas are preferred

as the second step when glycemic control cannot be achieved with exercise, diet and, metformin. Sulphonylureas have been used in the treatment of people with type 2 diabetes for more than 50 years. Sulphonylureas increase insulin secretion by closing potassium channel, leading to membrane depolarization and opening of calcium channel which leads to exocytosis and insulin release. Some studies reported cardiovascular adverse effects with first generation sulphonylureas like tolbutamide. Second generation sulphonylureas like glibenclamide and glimepiride are considered more potent and are still not free of adverse effects like hypoglycemia.

Addition of thiazolidinediones to the drug regime is also common. Thiazolidinediones were introduced in late 1990s and help improve the body's sensitivity to insulin (Consoli and Formoso 2013). Although it is well tolerated, it can also cause hypoglycemia and still needs to be used with caution in people with heart diseases. It can cause weight gain, which can increase the risk of mortality in this population (Maggio and Pi-Sunyer 2003). If avoiding hypoglycemia and weight loss is a priority, then injectable drugs like incretin mimetics and Dipeptidyl peptidase-4 (DPP-4) inhibitors are preferred. Incretin mimetics and DPP-4 inhibitors first came to market in 2005 and 2006, respectively. Incretin mimetics mimic the action of incretin hormone, glucagon-like peptide 1 (GLP1), which stimulates pancreas to produce more insulin and also slows gastric emptying and stimulates the satiety center in the central nervous system. DPP-4 inhibitors inhibit the action of the DPP-4 enzyme which shorten the half-life of GLP1 and thereby, DPP-4 inhibitors extend the action of GLP1. Incretin mimetics have been known to cause pancreatitis and renal dysfunction, and DPP-4 inhibitors have shown to be associated with neoplastic changes and increased risk of heart disease. Amylin analog is another injectable drug for people with type 2 diabetes, and their mode of action is very similar to incretin mimetics and DPP-4 inhibitor class of drugs. Alpha glycosidase inhibitors are another class of oral medication for type 2 diabetes. They inhibit the action of alpha glycosidase enzyme

and thereby cause delay in carbohydrate digestion and reduce postprandial hyperglycemia. This class of drugs is preferred for patients whose blood glucose levels rise after a meal containing complex carbohydrates. Gastrointestinal discomfort like flatulence, abdominal discomfort, and diarrhea caused by fermentation of carbohydrates by flora in the large intestine is one of the common side effects of alpha glycosidase inhibitors.

Despite availability of a number of drugs, it has been difficult to achieve tight glycemic control in people with diabetes. Studies suggest that approximately 60% of the people with type 2 diabetes have hemoglobin A1c levels still above 7 (Koro, Bowlin et al. 2004, Saydah, Fradkin et al. 2004). Moreover, even with intensive treatment people with diabetes still develop complications, which worsen over time (Ichii and Ricordi 2009). There is a need to discover new drugs with the hope that they will be more effective and with fewer and milder side effects.

**Table 1: FDA approved anti-diabetic drug for treatment of type1 and type 2 diabetes**

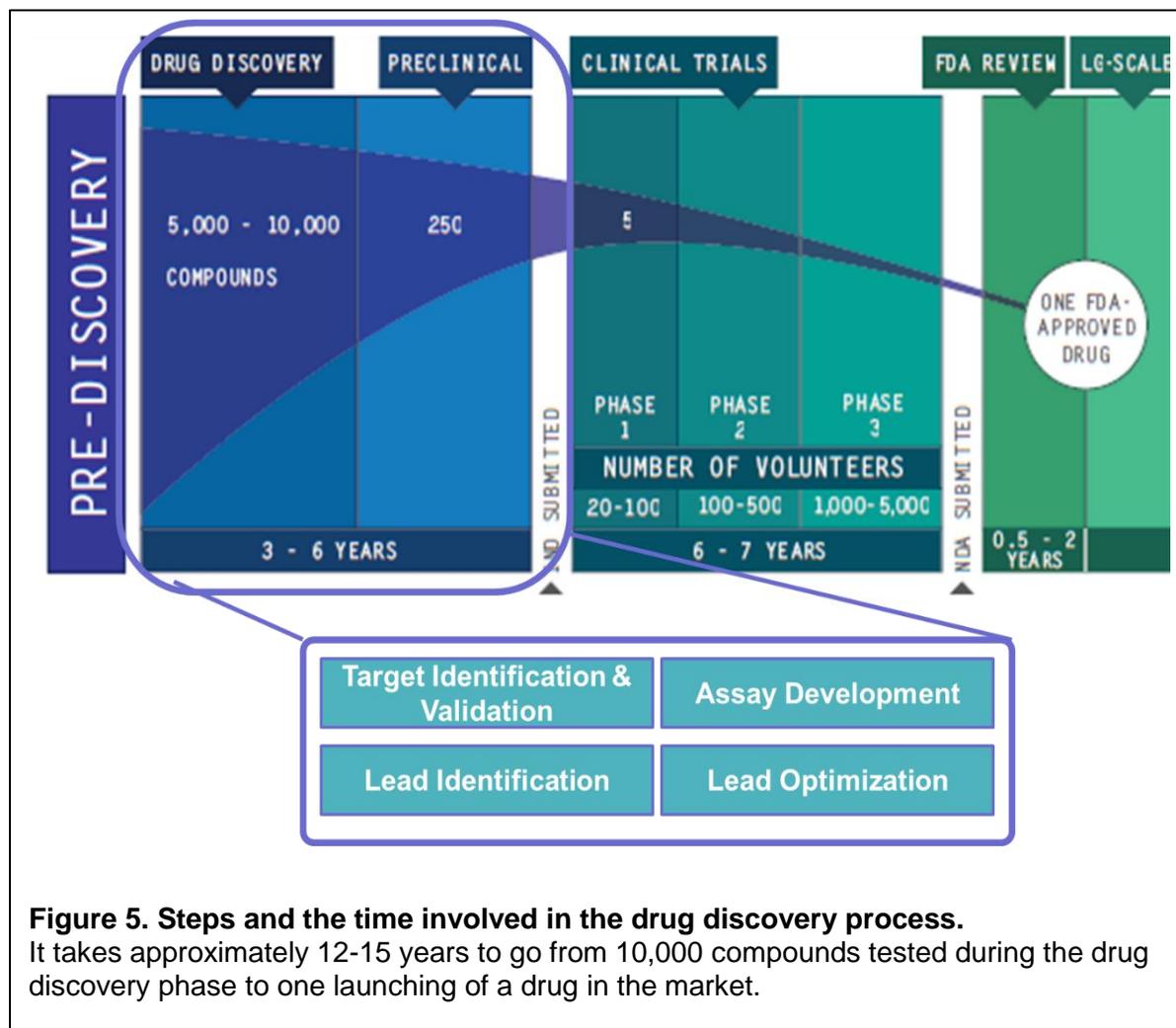
<b>Drug Category</b>	<b>Mode of action</b>	<b>Drug names</b>	<b>Side effects</b>	<b>References</b>
Insulin (rapid, short, intermediate, long acting)	Exogenous insulin	Novolog, Lantus	Hypoglycemia, redness, swelling at the injection site	(Lebovitz 2011)
Sulphonylureas	Increase insulin secretion by closing K <sup>+</sup> channels	Tolbutamide Chlorpropamide, Glimpiride, Glibenclamide	Nausea, vomiting, hypoglycemia, cardiovascular toxicity	(Sonnenblick and Shilo 1986)
Incretin mimetics	Increase insulin secretion by mimicking action of GLP1	Exanetide, Liraglutide	Vomiting, headache, upper respiratory tract infections, pancreatitis, pancreatic cancer, renal dysfunction	(Hinnen, Nielsen et al. 2006, Amori, Lau et al. 2007, Gale 2013)
Biguanides	Reduce hepatic blood glucose	Metformin	Gastrointestinal symptoms like diarrhea and nausea, lactic acidosis	(Hermann 1979, Berger 1985, Rojas and Gomes 2013)
Alpha-glucosidase inhibitors	Delay digestion and absorption of intestinal carbohydrate	Acarbose Miglitol	Gastrointestinal symptoms like flatulence and diarrhea	(Chiasson, Josse et al. 2003, Hanefeld, Josse et al. 2005, Dabhi, Bhatt et al. 2013)
Thiazolidinedione	Improve peripheral tissue sensitivity	Pioglitazone Rosiglitazone	Edema, heart failure, weight gain, hypoglycemia, increased incidence of fracture, myocardial infarction	(Rizos, Elisaf et al. 2009)
Amylin analog	Suppresses action of glucagon, prolong gastric emptying, suppresses appetite	Pramlintide	Nausea, vomiting, headache	(Ryan, Jobe et al. 2005, Adeghate and Kalasz 2011)
DPP-4 inhibitors	Inhibits the DPP-4 enzyme and prolongs the half-life of GLP1	Sitagliptin Saxagliptin Linagliptin	Headache, upper respiratory tract infections, pancreatitis, neoplastic changes	(Food and Drug Administration 2010)

### 1.5.2. Drug screening/ discovery process

New drug discovery is time consuming and requires around a billion dollars in investment (Dickson and Gagnon 2004). The steps involved in the drug screening discovery process are the drug discovery phase, preclinical phase, clinical trials and finally FDA approval. Figure 5 describes the time involved during each step of the drug screening and discovery process. The first step is the drug discovery phase, which involves target identification and validation, assay development, lead identification and lead optimization. Understanding the molecular mechanism of the disease is important even before the drug enters the drug discovery pipeline, this is carried out during the pre-discovery phase (Pharmaceutical Reserach and Manufacturers of America 2016). Intensive research is carried out to identify the drug-like molecules before the drug enters the preclinical phase. This initial basic step helps to develop a hypothesis that stimulation or inhibition of an identified target like a certain protein or a ribonucleic acid (RNA) will result in the desired effect in the diseased state. Target identification and validation is one of most important steps during the drug screening discovery process. Target validation can be performed in *in vitro* or *in vivo* models.

Once the target has been identified and validated, compound screening is performed to identify the molecule, called the hit molecule, which has desired activity on the target during the screening process. Various screening methods like high-throughput screening, focused screening or physiological screening can be performed to identify the molecules that are best to work with (Fox, Farr-Jones et al. 2006, Boppana, Dubey et al. 2009). Dose response curves are generated for each hit molecule in primary and then more complex secondary assays. When a drug is about to enter the lead phase, the primary focus is to identify if there are any structure-activity relationships, identify the magnitude of activity and selectivity of each compound, and also to collect data about how the compound is absorbed, distributed, metabolized, and excreted. All the compounds are then tested in rats/mice to study their pharmacokinetics. During

the lead optimization phase, deficiencies in the structure of the drug like molecule are improved and also metabolic profiling studies are carried out before the drug candidate makes it to the preclinical phase (Hughes, Rees et al. 2011).



Source: yalescientific.org

To begin with, thousands of compounds may be screened, but on average only 100's of compounds will make it to the preclinical phase and a very few of them enter the clinical phase. One out of 5 compounds in the clinical phase will get FDA approval and make it to the market (McKim 2010). Sometimes the failure even occurs during the phase 3 clinical trials. One of the biggest challenges faced by pharmaceutical industry is the failure of a drug candidate in the

preclinical and clinical trials. If failure occurs at the later stages, which leads to major time setbacks and tremendous financial loss. Therefore, it is crucial to identify the failures as early in the pre-discovery phase as possible to save time and avoid financial loss as well as to make the drug screening procedure more efficient. Molecules that do not behave when screened as they would in the human body are at the heart of failed drug screening procedures. An article by William Bians suggests that avoiding repetitions at various stages of drug development can substantially help bring down the cost and time involved with the drug testing procedure (William Bians 2004). Hence in Chapter 3, we created 3D Kanslets, consisting of human islet cells from various donors using our micromold and we hypothesized that multi-donor Kanslets would give an averaged dose response of individual donors in the study. If the hypotheses were correct, then the number of repetitions in the early drug screening process could be reduced.

### **1.5.3. Two-dimensional (2D) and three-dimensional (3D) drug testing**

In addition to decreasing the number of repetitions by pooling cells from multiple donors, screening drugs in a more natural condition, comparable to the human body, would also theoretically reduce the errors in drug screening. 2D monolayer culturing of cells has been used for drug testing and discovery for decades. 3D cell cultures more closely resemble the architectural microenvironment of natural tissue with respect to the standard 2D cultures (Graf and Boppart 2010). In 3D cell aggregates/spheroids, cell–cell interactions and cell–extracellular matrix interactions more closely mimic the natural environment found *in vivo*, so that the cell morphology resembles its natural shape in the body. Not only the spatial organization of the cell surface receptors engaged in interactions with surrounding cells is different in 3D structures than in 2D, but a 3D structure also induces physical changes to cells. These spatial and physical aspects in 3D cultures affect the signal transduction and ultimately influence gene expression and cellular behavior.

3D tissue culture has recently been extensively studied in the oncology field with the conclusion that 3D culturing of multicellular spheroids mimic the *in vivo* environment more than 2D culturing (Debnath and Brugge 2005, Meng 2010, LaBarbera, Reid et al. 2012, Alexander, Price et al. 2013). Different techniques for 3D culturing have been developed and multicellular tumor spheroids have been studied as an *in vitro* 3D model over the past decades to study tumor cell proliferation, apoptosis, differentiation, gene expression, and metabolism (Mueller-Klieser 1987). Tumor spheroids resemble the morphological as well as the physiological characteristics of the *in vivo* tumor and hence are considered an improved *in vitro* model. For example, cells grown in monolayer usually do not contain necrotic/dead cells, as the dead cells get detached from the monolayer. Also, cells in 2D culture are more flat and stretched out than those found *in vivo*. Further, 3D spheroids have a higher degree of morphological and functional differentiation than cells grown in monolayer culture (Sutherland, Sordat et al. 1986, Streuli, Bailey et al. 1991, Mayer, Klement et al. 2001), which can be important in testing drugs targeting mature cells. In 3D cultures, cells are often more resistant to treatment compared to those in 2D culture system, often being better predictors of *in vivo* drug responses. Due to this lack of resistance in the 2D models, drugs may appear to be effective, but when they get to *in vivo* studies they will lack efficacy. 3D spheroids have been shown to have similar growth kinetics, metabolic rates, and resistance to radiotherapy and chemotherapy as tumor cells *in vivo* (Sutherland and Durand 1976, Landry, Freyer et al. 1982, Nederman 1984). A study, looking at the difference in response of chemotherapeutic agents between leukemic cell lines in a 3D model in comparison to the 2D model found that the 3D model was more resistant to the drug induced apoptosis with respect to the 2D model and thus better predicted what happens in patients (Aljitawi, Li et al. 2014).

In regards to pancreatic islets, the cellular interaction between  $\beta$ -cells is considered important for proper islet function. Dispersed  $\beta$ -cells show increased basal and decreased

glucose-stimulated insulin secretion, but upon reaggregation these cells show normal secretory responses as seen in intact islets (Halban, Wollheim et al. 1982, Hauge-Evans, Squires et al. 1999) (Brereton, Carvell et al. 2006). This is not surprising, as it has been shown that in 2D culturing there is a lack of cell-cell interaction, which is important for normal cellular function (Weaver, Petersen et al. 1997, Bissell, Rizki et al. 2003). Several cellular mechanisms may explain this difference. Connexins and cadherins, involved in cell adhesion, affect insulin release. Specifically, gap junctions connect  $\beta$  cells and these gap junctions consist of connexins, which form intracellular channels. These intracellular channels are required for the exchange of cytoplasmic ions and metabolites, allowing for the coordinated response of islet cells. Thus, connexins are required for fine-tuning biosynthesis, storage and release of insulin (Nlend, Michon et al. 2006, Bavamian, Klee et al. 2007). In addition to the gap junctions, there are other mechanisms requiring cell-to-cell contact that help in the regulation of insulin release from the  $\beta$  cells. The neuronal cell-adhesion molecule present between the  $\beta$ -cells regulates the cortical F-actin network. In response to glucose, the F-actin depolymerizes and actin network becomes disrupted, which in turn leads to insulin secretion. Bi-directional signaling between  $\beta$ -cells is considered important in the regulation of insulin secretion as it also helps in depolymerization of F-actin network, which affects insulin secretion (Howell and Tyhurst 1982, Thurmond, Gonelle-Gispert et al. 2003). Thus, there are several possible molecular explanations for the improved response to drugs seen in 3D cell cultures compared to in 2D cultures.

#### **1.5.4. Primary cells or cell culture lines for drug testing**

The source of the cells is also important in drug screening. Either the primary cells (cells that are derived directly from the human donors) or the cell culture lines (cells originally derived from the donor tissue that can divide and grow, for a number of replications) can be utilized for research purposes (Pan, Kumar et al. 2009). The advantage of cell culture lines is that the

results obtained are consistent and can be reproduced, but there are disadvantages of cell culture lines too. Due to the long-term culture, the phenotypic characteristics of the cells can change (Borrell 2010, Lee, Nam et al. 2010, Philippeos, Hughes et al. 2012) and some cell lines have shown to have reduced insulin secretion with time in culture (Ulrich, Schmied et al. 2002). Several  $\beta$ -cell lines have been developed and none of these cell lines are truly representative of primary  $\beta$ -cells, as they cannot accurately imitate primary  $\beta$ -cell function and physiology, and most of these cell lines have inappropriate glucose sensitivity (Efrat and Hanahan 1987, Gilligan, Jewett et al. 1989, Wang, Beattie et al. 1997, Baroni, Cavallo et al. 1999), hence it is difficult to obtain the accurate drug test results using the cell current lines.

### **1.5.5. Kanslets for drug testing**

If one is to use 3D cell clusters of primary cells for drug testing, there are several challenges that must be overcome. In regards to pancreatic islets, there is a lot of variability in the insulin response of islets from different donors. It has been seen that age (Maedler, Schumann et al. 2006, Ihm, Moon et al. 2007) and race affect the insulin response of the islets. Out of the 20 times we have conducted GSIS for different experiments in our lab, we have measured wide variations in the GSIS curves from different donors. In addition, there is great variability within the same donor between different sizes of islets. When screening for drugs, the size of the islets that are placed in each well can alter the effects. For example, if drug A is placed in a well with 5 islets that are each 400 $\mu$ m in diameter, and drug B is placed in the neighboring well with 5 islets that are 50 $\mu$ m in diameter, drug A may have a larger effect, but in reality it may be less potent.

Our 3D clusters, Kanslets, created by dispersing the native islets into single cells and allowing them to reaggregate in the micromold for several days, are small and uniform in size. This uniformity along can eliminate some of the variability between the large and the small islets. We have tested known drugs prescribed for diabetes using the 3D Kanslets. A variety of

drugs like the calcium channel agonist, Bay K 8644; glibenclamide; tolbutamide; caffeine; carbachol; glucagon-like peptide-1; somatostatin; diazoxide; and sodium azide were tested on Kanslets. It was found that Bay K 8644, stimulated insulin secretion in native to rat islets and human Kanslets, but not in native human islets. The sulfonylureas (glibenclamide and tolbutamide) were also found to be more effective and potent in the Kanslets compared rat islets or human native islets. In addition, Kanslets were found to be more sensitive to somatostatin, diazoxide and sodium azide (Ramachandran, Peng et al. 2014). The results of the study showed that Kanslets can be utilized as a secondary screening tool for drug discovery and drug testing procedures.

In order to overcome the barrier of variability in islets from different donors, in Chapter 3 experiments aimed to determine whether dose response curves of multi-donor Kanslets (created by mixing of islet cells from two different donors) to insulin secretagogues, and an inhibitor are equivalent to average dose response curves of the same two single-donor Kanslets tested individually. Dose response curve to two insulin secretagogues (glucose and glibenclamide) and one inhibitor (somatostatin) were evaluated for both multi- and single-donor Kanslets.

### **1.6. Reaggregated hybrid Kanslets**

With the formation of multi-donor Kanslets, other cells can be incorporated into the Kanslets during reaggregation. Another important application of this approach is to improve culture conditions of pancreatic islets so that they can survive and function better in long term culture. Mesenchymal stem cells have helped maintain health of other cells in a variety of settings (Paschos, Brown et al. 2015, Rebelo, Costa et al. 2015).

### **1.6.1. Mesenchymal stem cells (MSCs)**

Stem cells are defined as a class of cells with the ability to self-renew and differentiate. According to the development stage at which the cells are isolated, stem cells can be classified into embryonic, fetal, neonatal, and adult stem cells (Sandner, Prang et al. 2012). Ethical concerns and tumor formation are the major problems associated with embryonic and fetal stem cells (Nandoe Tewarie, Hurtado et al. 2009, Riazi, Kwon et al. 2009). Another class of stem cells called induced pluripotent stem cells, obtained by the reprogramming of differentiated adult stem cells, have also been shown to cause tumors (Scheper and Copray 2009, Masip, Veiga et al. 2010, Liu, Fu et al. 2011, Volarevic, Ljubic et al. 2011). Adult stem cells derived from adult tissue have the advantage of autologous transplantation which can help with immune rejection and also does not result in tumor formation (Nandoe Tewarie, Hurtado et al. 2009).

MSCs can be derived from different adult tissues and have advantages as listed above. They were first discovered in the 1960s (Friedenstein, Piatetzky et al. 1966). MSCs can be isolated from bone marrow (Haynesworth, Goshima et al. 1992), adipose tissue (Zuk, Zhu et al. 2002), dental pulp (Sakai, Yamamoto et al. 2012) and many other different tissues throughout the body (da Silva Meirelles, Chagastelles et al. 2006). MSCs can self-renew and differentiate into many different cell types like osteoblasts, chondrocytes, and adipocytes (Pittenger, Mackay et al. 1999, Chamberlain, Fox et al. 2007, Phinney and Prockop 2007).

### **1.6.2. Properties of MSCs**

#### **1.6.2.1. Regenerative and anti-apoptotic properties**

Studies have shown that MSCs can help reverse apoptosis of different cell types like cardiomyoblasts, neurons, and lung fibroblasts. The release of interleukin (IL)-6, insulin-like growth factor 1 (IGF1), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), and transforming growth factor  $\beta$ 1 (TGF  $\beta$ 1) have been proposed to play role in the halt

of apoptosis in different tissues. In particular MSCs have been shown to help with islets survival and function (Boumaza et al 2009, Lu et al 2010, and Park et al 2009).  $\beta$ -cell expression of anti-apoptotic proteins increased when pancreatic islets were cultured with MSCs (Caja, Bertran et al. 2011, Lu, Lu et al. 2011). MSCs have been shown to reduce hyperglycemia by increasing  $\beta$ -cell mass. Specifically all the diabetic animals administered a single injection of MSCs were shown to have significantly increased  $\beta$ -cell mass compared to animals not injected with MSCs. All these findings suggest that MSCs may help with regeneration of  $\beta$ -cells (Sordi, Malosio et al. 2005, Lee, Seo et al. 2006).

#### **1.6.2.2. Angiogenic properties of MSCs**

Angiogenic properties of MSCs help in tissue repair and regeneration (Park, Kim et al. 2010). MSCs promote endothelial cell migration and up-regulate the expression of angiopoietin and VEGF in endothelial cells (Zacharek, Chen et al. 2007). When cultures consisted of human islets and endothelial cells in the presence of MSCs, endothelial cells migrated to the islet's surface. Islets with endothelial cells on their surface survived better and had improved insulin secretion (Johansson et al 2008). In the presence of MSCs endothelial cell density was higher in the transplanted islet graft. MSCs secrete VEGF, fibroblast growth factor (FGF), angiopoietin-1 (Ang-1), TGF  $\beta$ , IL-6, IL-8, HGF, and platelet-derived growth factor, all of which help with revascularization. One study found that increased VEGF expression at the islet transplant site was associated with significantly increased graft survival and function (Kinnaird, Stabile et al. 2004). When diabetic mice were co-transplanted with pancreatic islets and MSCs, euglycemia was achieved within two weeks and graft showed enhanced expression of VEGF. MSCs also secrete matrix metalloproteases, which help host-derived endothelial cells to migrate into the islets. It has been suggested that MSCs may help with islet vascularization by initiating the formation of vessel-like structures through secretion of proteases that degrade the islet extracellular matrix and allow the migration of endothelial cells into the islet.

### **1.6.2.3. Anti-inflammatory and immune modulatory properties of MSCs**

MSCs secrete anti-inflammatory proteins and soluble factors to suppress the activity of immune cells like T and B lymphocytes, dendritic cells and natural killer cells (Chamberlain, Fox et al. 2007, Phinney and Prockop 2007). T lymphocytes are the main effectors of the immune response and play a prominent role in transplant rejection. MSCs secrete factors like indoleamine 2, 3-dioxygenase (Li, Song et al.) TGF  $\beta$ 1 and nitric oxide suppressing T cell proliferation (Di Nicola, Carlo-Stella et al. 2002, Aggarwal and Pittenger 2005, Sato, Ozaki et al. 2007). Matrix metalloproteinase (MMP) 2 and 9 secreted by MSCs also suppress T cells. When MMP 2 and 9 were blocked, MSCs could not suppress the T cell proliferation (Ding, Xu et al. 2009). MSCs inhibit the proliferation of NK cells (Krampera, Cosmi et al. 2006) as well as alter the function of dendritic cells by inhibiting the production of tumor necrosis factor  $\alpha$  (TNF  $\alpha$ ) and up-regulating the production of IL-10 (Jiang, Zhang et al. 2005). Finally, MSCs also inhibit  $\beta$  cell proliferation (Glennie, Soeiro et al. 2005, Agudelo and Iwata 2008).

Therefore, in order to improve islet health during long term *in vitro* culture, in Chapter 4 we chose to integrate MSCs during the process of reaggregation of islet cells and formed hybrid spheroids consisting of islet cells and MSCs. We determined cell composition, diameter, viability and insulin secretion of these hybrid clusters, and compared those to spheroids containing only islet cells or MSCs.

### **1.7. Research Question**

The long term goal of our research is to better understand how bioengineered Kanslets can help advance the field of islet transplantation and diabetes research. Kanslets have previously been shown to be highly viable and functional. Kanslets have also been shown to reverse hyperglycemia in diabetic rats. The ability of pancreatic islet cells to self-reaggregate was utilized to create bioengineered Kanslets. In short, Kanslets were created by dispersing

pancreatic islets into single cells, and then allowing them to reaggregate in a micromold for 3-5 days. The purpose of this study was to optimize the experimental conditions for cryopreservation, drug testing, and islet transplantation procedures utilizing Kanslets. Success in all of these fields will greatly enhance the utilization of engineered islets for other applications such as drugs screening and transplantation.

In Chapter 2, “A simplified method of long-term cryopreservation of human pancreatic islets based on reaggregation” we determined whether pancreatic islets could be cryopreserved as single cells and later reaggregated to form highly viable and functional Kanslets. We compared the outcomes (yield, immediate viability, metabolic function, insulin secretion and extended viability) after cryopreservation and thawing of pancreatic islets using our novel cryopreservation technique to the conventional one. We also transplanted cryopreserved Kanslets into diabetic rats and monitored their blood glucose levels.

In Chapter 3 and 4, we examined the effect of creating hybrids using mixtures of cells, whether they were islet cells from different human donors, or cells from different tissues such as stem cells and islet cells.

In Chapter 3, “Reaggregated human islet cells from different donors for drug testing” we focused on creating multi-donor Kanslets consisting of islet cells from different donors and compared the average dose response curve of insulin secretagogues and inhibitors between two single-donor Kanslets versus multi-donor Kanslets made from the same two donors.

In Chapter 4, “Integration of islet cells and mesenchymal stem cells into spheroids improves viability but not long-term function” we created hybrid spheroids by reaggregating islet cells and mesenchymal stem cells (MSCs) in different proportions and studied their viability, survival, and function in culture with respect to spheroids containing only MSCs or islet cells.

In Chapter 5, we summarized all the findings from the work described here. We discussed the implication of this research to expand and/or advance research related to diabetes, drug discovery, and islet transplantation. In the last section of this Chapter we also suggested future directions.

## **Chapter 2**

# **A Simplified Method for Long-Term Cryopreservation of Human Pancreatic Islets Based on Reaggregation**

## 2.1. Abstract

Preservation of pancreatic islets would be beneficial for islet transplantation to improve the treatment of diabetes. Unfortunately, current methods of islet cryopreservation have poor outcomes in terms of islet survival and function. In general, single cells have been shown to tolerate the cryopreservation procedure better than tissues/multicellular structures like islets. Thus, we optimized a method of cryopreserving single islet cells and, after thawing, reaggregated them into islet spheroids. Human pancreatic islets were either cryopreserved as whole native islets or as single cells. Cryopreserved single islet cells reaggregated into islet spheroids, termed Kanslets, efficiently within 3-5 days ensuing thawing. Approximately 79% of islet cells were recovered following single-cell cryopreservation protocol. Viability after long-term cryopreservation (4 weeks or more) was significantly higher in the cryopreserved; CP Kanslets ( $97.4\pm 0.4\%$ ) compared to CP native islets ( $14.6\pm 0.4\%$ ). Moreover, cryopreserved Kanslets had excellent viability even after 7-10 days in culture ( $88.5\pm 1.6\%$ ). Metabolic activity was 4- 5 times higher in CP Kanslets than CP native islets at 24 and 48 hours after thawing. Diabetic rats transplanted with CP Kanslets were normoglycemic for 10 months, identical to diabetic rats transplanted with fresh islets. However, the animals receiving fresh islets required a higher volume of transplanted tissue to achieve normoglycemia compared to the CP transplanted tissue. Using our novel method of cryopreservation, we achieved highly viable and functional islets after thawing. By optimizing the cryopreservation and thawing methods for pancreatic islets, new options for storage, and subsequent treatment of diabetes may be available.

## 2.2. Introduction

Over a million adults and children are living with type 1 diabetes in the US and every year approximately 30,000 new cases of type 1 diabetes are diagnosed (VanBuecken, Lord et al. 2000, 2010, American Diabetes Association 2014). After cerebral palsy and asthma, it is ranked as the third most common chronic disease in children and adolescence (Gage, Hampson et al. 2004). Treatment for people with type 1 diabetes consists of insulin injections multiple times a day. For those unable to control their blood glucose levels including those with diligent insulin treatment, islet transplantation is an alternative treatment (Shapiro, Lakey et al. 2000). Until 2000, islet transplantation did not achieve much success. Only 10% of the patients who underwent islet transplants were insulin independent for 1 year (McCall and Shapiro 2012). With the advent of the Edmonton protocol in 2000, 7 patients were reported to have achieved insulin independence (Shapiro, Lakey et al. 2000). Since then, islet transplantation has been performed worldwide. A study in 2005 reported that approximately 70% of patients were insulin independent at 1 year after transplantation and the graft function was well maintained with 82% graft survival at 5 years (Ryan, Paty et al. 2005).

Despite such advances, one of the major challenges for islet transplantation is the limited availability of the pancreatic tissue (Malek and Davis 2015), and the difficulty in predicting the health and function of the graft tissue prior to the transplant. With respect to the number of Americans suffering from diabetes, pancreas from only a few thousand deceased donors are available each year, and not all of those are suitable for transplantation, as they fail the criterion of selection or are destroyed during processing (National data. Organ Procurement and Transplantation Network website, Kandaswamy, Skeans et al. 2015). Hence, only a limited number of islet transplants can be performed each year. To further complicate the situation, in order to achieve normoglycemia, the islet volume must be greater than or equal to 9,000-11,000 IEQ/kg of body weight for a single transplant procedure (Shapiro, Lakey et al. 2000). In order to

achieve that islet mass, islets from two or more donors are often needed, unlike other solid organ transplants, leading to greater inefficiencies (Naftanel and Harlan 2004, Froud, Ricordi et al. 2005, Shapiro, Lakey et al. 2005, Gangemi, Salehi et al. 2008). One of the major reasons for the multiple organ transplants is that the extraction of islets from the donated pancreas is not efficient and can cause stress on the islets during processing leading to low yield during the islet isolation process (Guignard, Oberholzer et al. 2004, Matsumoto, Noguchi et al. 2007). In addition to poor yield, which limits the number of islets available for transplant at any given time, the quality of the islets is difficult to judge in the short period of time available between receipt of the donor tissue and the scheduled transplant. Islets are typically transplanted within 48 hours after isolation from pancreas. Alternatively, islets may be cultured for an extended period of time so that multiple islet isolations can be performed, allowing for tissue to be pooled together once the sufficient number of islets collected for a single transplantation (Andersson, Borg et al. 1976). During culture, quality assurance assays on donor islets are conducted (Andersson, Borg et al. 1976). Islets stored with short-term preservation techniques, such as standard tissue culture, have a limited life-span and face a high risk of contamination (Fraga, Sabek et al. 1998). Therefore, a method to efficiently store islets to provide time for quality control testing, and allow pooling of islets from different donors, for a single transplant procedure would be beneficial.

Cryopreservation of pancreatic islets could offer a longer-term storage method. However, existing cryopreservation methods utilized are not optimal. Studies have reported reduced islet recovery (Lakey, Anderson et al. 2001, Lakey, Rajotte et al. 2001, Liu, Tian et al. 2014), decreased viability (von Mach, Schlosser et al. 2003, Miranda, Mohan et al. 2013) and impaired or reduced insulin secretion (Janjic, Andereggen et al. 1996, Omori, Valiente et al. 2007, Kenmochi, Asano et al. 2008) after thawing. Conventional cryopreservation protocols are based on slow freezing and rapid thawing of intact native islets. Native islets are complex multicellular structures containing five different types of hormone secreting cells. Islets have a

mean diameter of 150 $\mu$ m and are densely packed, each islet containing approximately 1000-10,000 cells (Bertram and Pernarowski 1998, Blodgett, Cura et al. 2014). In general, tissues/organs/multicellular structures are more difficult to cryopreserve than single cells (Hunt, Taylor et al. 1982). Heat transport limitations in tissues/multicellular structures lead to non-uniform temperature changes between the cells in the core and those at the outer layer. Because of the slower rates of temperature changes in the core during cooling phase, cells placed interiorly lose water at a slower rate than the cells on the outer layer and hence there are more chances of intracellular ice formation during the cooling phase and more chances of recrystallization during the warming phase (Levin, Cravalho et al. 1977). Of all the injuries caused during the cryopreservation procedure, ice crystallization can be most damaging to the cells/tissues as it causes chemical as well as mechanical stress during cryopreservation (Bakhach 2009). Moreover, extracellular ice formed during the cooling phase can cause damage to cellular suspensions, but only by exerting compressive forces on the cells, whereas in multicellular structures, extracellular ice forms within the three dimensional structure and can be detrimental to the tissues (Pegg 2007). Additionally, the inner and outer layer of cells may be exposed to different concentrations, and duration of DMSO. Previous publications have reported that intact pancreatic islets take a longer time to equilibrate with the cryoprotectant than individual islet cells, and in intact native islets equilibration times are dependent on the islet size (Benson, Liu et al. 1993, Benson, Benson et al. 2014).

It is critical to develop an effective method of islet cryopreservation that would yield highly viable and functional islets so that islet transplantation becomes a more feasible treatment option for people with diabetes. However, multicellular structures like islets pose multiple barriers towards successful cryopreservation. Therefore, we developed a new method to cryopreserve islets as single cells, and, after thawing, reaggregated cells into spheroids. This

study compares the outcomes of a novel cryopreservation technique to the conventional method following long-term cryopreservation.

### **2.3. Methods**

#### *Tissue procurement and group descriptions*

Native human pancreatic islets were ordered from the National Institutes of Health - Integrated Islet Distribution Program (IIDP). Pancreatic islets from human donors with obesity, diabetes or any other metabolic disorders were not included in the study. Only islet preparations with high purity (>85%) were included. As the islets were received, they were placed in tissue culture plates containing islet medium consisting of Connaught Medical Research Laboratories 1066 (CMRL; cat. no. MT5110CV; Thermo Fisher Scientific; Chicago, Illinois) medium supplemented with 10% fetal bovine serum (FBS; cat. no. 26140079; Thermo Fisher Scientific), 1% antibiotic/antimycotic (cat. no. 15240062; Thermo Fisher Scientific) and 1% glutamax (cat. no. 35050061; Thermo Fisher Scientific) and allowed to recover for 24 hours in an incubator at 37°C and 5% CO<sub>2</sub>. After 24 hours in culture, islets were divided into the native islet group (fresh or cryopreserved (CP)) or the reaggregated islet clusters, called the Kanslet group (fresh or CP).

#### *Dissociating islets into single cells*

Islets in the Kanslet groups were dispersed into single cells according to our previously published protocol (Williams, Wang et al. 2009). In short, islets were placed in a 50 ml tube and centrifuged at 1209 g for 5 minutes and the supernatant was discarded. The islets were washed twice with calcium and magnesium-free Hank's Balanced Salt Solution (cmf-HBSS). Subsequently, the digestion medium consisting of cmf-HBSS and 5 units/ml papain (Worthington, Lakewood, NJ) was added and the suspension was incubated on a rotator at 37°C for a maximum of 20 minutes. Islets were then dispersed using a pipette. Digestion was

stopped using culture medium containing calcium and magnesium. The cells were then washed twice with islet medium to remove the papain.

#### Seeding single cells in the micromold to form Kanslets

Single cells were seeded into the micromold as described in our previously published protocol (Ramachandran, Williams et al. 2013). In short, fresh or cryopreserved single cells, in 3 ml of islet medium were carefully plated into the micromold. Cells were allowed to settle into the wells of the mold for several minutes. The micromold was incubated at 37°C and 5% CO<sub>2</sub>. The cells self-reaggregated and formed Kanslets within 3-5 days. The culture media was changed every 24 hours.

#### Cryopreservation of single cells

For CP Kanslets, commercially-available products common for cryopreservation of other types of single cells were utilized. CryoStor CS10 (cat. no. C2874; Sigma-Aldrich, containing 10% dimethyl sulfoxide (DMSO)) has been used to cryopreserve different types of single cells to achieve better post-thaw results (Clarke, Yadock et al. 2009, Fortin, Azari et al. 2016). In detail, single cells were centrifuged at 1209 g for 5 minutes to form a pellet, and supernatant was removed. Cells were dispersed in 1 ml of CryoStor CS10 containing 10% DMSO, transferred to a 2ml cryovial and slowly cooled down at -1°C/ minute. In order to accomplish the slow cooling, single cells in a cryovial were placed in a CoolCell (cat. no. BCS-405; Biocision; San Rafael, CA) at -80°C. After 15-20 minutes, nucleation was performed to release the latent heat of fusion by mechanical agitation and returned to -80°C for 15 hours. Samples were then stored in liquid nitrogen at -196°C. At the time of thawing, cells were rapidly heated in a water bath at 37°C for approximately 2 minutes until the last ice crystal melted. The sample was then diluted with fresh islet medium. Cells were centrifuged at 1209 g for 5 minutes, fresh medium was added, and then the cell suspension was seeded in the micromold to allow reaggregation and formation of

CP Kanslets. Once CP Kanslets have reaggregated, they were transferred to a petri dish with fresh islet medium.

### Cryopreservation of native islets

There are no effective commercial products available to CP native islets, however various recommendations are provided in the literature with regard to cryopreservation. Keeping this in mind, pilot studies were carried out to determine the optimal cryopreservation conditions. Media tested included CMRL and Rosewell Park Memorial Institute (RPMI; cat No.11875093; Thermo Fisher Scientific) with and without curcumin (cat No. 458-37-7; Sigma-Aldrich) and zinc sulfate (cat No. 744620-0; Thermo Fisher Scientific). DMSO concentrations tested were 10% and 14%. Different equilibration times with DMSO included either adding DMSO in one step (incubated for 15 minutes in final concentration of 10% or 14% DMSO after single addition) or adding DMSO in a stepwise manner to get the desired final concentration of DMSO. Native islets were cryopreserved as intact islets according to Lakey's protocol as described in the literature (Lakey, Anderson et al. 2001) with some modifications. In short, native islets were centrifuged, and dispensed in media containing RPMI with curcumin (10 $\mu$ M) and zinc sulfate (16.8 $\mu$ M), and the sample was incubated with DMSO added in a stepwise increasing manner (incubated at 22 $^{\circ}$ C, first in 5% DMSO for 5 minutes, then in 7% DMSO for 25 minutes and, finally at final concentration of 10% for 15 minutes). Islets were moved to an ice bath at 4 $^{\circ}$ C for 15 minutes and then to an ethanol bath and nucleation was performed at -7 $^{\circ}$ C. After storing the samples in a CoolCell overnight (cools samples at -1 $^{\circ}$ C/ minute), samples were moved to -196 $^{\circ}$ C in liquid phase nitrogen. When thawing, samples were rapidly thawed at 37 $^{\circ}$ C in a water bath and DMSO was removed by stepwise sucrose dilution. 0.75 M sucrose solution was added for 30 minutes at 0 $^{\circ}$ C and then culture medium was added every 5 minutes over a period of 20 minutes. The suspension with CP islets was centrifuged at 1209g for 5 minutes and incubated at 37 $^{\circ}$ C with the fresh islet medium. CP native islets and CP Kanslets were thawed and allowed

to recover for 24 hours before they were assessed for viability, tissue recovery, metabolic function and glucose-stimulated insulin secretion (GSIS).

While optimizing the cryopreservation conditions, native human islets and single cells were cryopreserved for 1 week while for all other experiments native islets and single cells were cryopreserved for a minimum of 4 weeks.

### Viability

Cell death was analyzed using YO-PRO-1 and propidium iodide staining (cat. no. V13243; Thermo Fisher Scientific). YO-PRO-1 stain selectively passes through the plasma membranes of apoptotic cells and labels them with green fluorescence. Necrotic cells are stained red with propidium iodide (Gawlitta, Oomens et al. 2004). 1  $\mu$ l of YO-PRO-1 and PI each were added to 200 $\mu$ l of media containing fresh or CP native islets/Kanslets. Images were taken using an Olympus FluoView 300 confocal microscope. Later the images were analyzed using Adobe Photoshop CS 5 Extended. Total islet pixel number as well as the pixel numbers for the red and green staining of native islets/Kanslets was calculated. In order to calculate the percentage of apoptotic and necrotic cells, pixel numbers for green, and red staining were divided by the total pixel numbers of native islet/Kanslets and multiplied by 100. The proportion of live cells was defined as the islet area lacking green/red staining. Immediate viability was assessed at 24 hours while extended viability was assessed at 7-10 days in culture.

### Immunofluorescence Staining

To analyze the proportion of  $\alpha$ -,  $\beta$ - and  $\delta$ - cells in the fresh and CP Kanslets, the spheroids were stained for insulin ( $\beta$ ), glucagon ( $\alpha$ ) and somatostatin ( $\delta$ ) as described in our previously published protocol (Ramachandran, Williams et al. 2013). First, the fresh and CP Kanslets were washed in phosphate buffered saline (PBS) 3 times for 5 minutes each. Permeabilization was completed in 1% Triton X-100 in PBS for 30 minutes. Fresh and CP

Kanslets were washed in PBS again 3 times for 5 minutes each and incubated for 2 hours at room temperature with primary antibodies against insulin (1:100; cat no. ab7842; Abcam; Cambridge, MA), glucagon (1:300, cat no. 10988, Abcam) and somatostatin (1:300, cat.no. ab53165, Abcam). After incubating with primary antibody, samples were washed with PBS 3 times for 5 minutes each, followed by incubation in a mix of fluorophore conjugated secondary antibodies consisting of DyLight 488 (1:400, cat. no. 706-485-148 Jackson ImmunoResearch Laboratories Inc., West Grove, PA), Alexa 555 (1:400, cat. no. A31570; Thermo Fisher Scientific) and Alexa 647 (1:400; cat. no. A31573; Thermo Fisher Scientific) in a dark wet chamber for 1 hour. After incubation with secondary antibodies, samples were washed with PBS 3 times for 5 minutes each and mounted on microscopic slides with anti-fading agent Gel/Mount (Biomedex, Foster City, CA). The following solution was used to dilute primary and secondary antibodies: 1% BSA, 0.03% Triton X-100 and PBS. Images were captured on a Nikon C1Si or C1 Plus confocal microscopes. For cell composition analysis, the relative proportion of  $\alpha$ -,  $\beta$ -, or  $\delta$ -cells in each islet was evaluated by counting the number of individual types of cell and dividing it by the total sum of endocrine ( $\alpha$ -,  $\beta$ -, and  $\delta$ -) cells per islet and multiplying by 100. Image J was utilized to perform cell composition analysis.

### Metabolic function

Metabolic function was assessed by using the AlamarBlue assay (DAL 1100; Thermo Fisher Scientific). The active ingredient present in the AlamarBlue assay is resazurin, which is permeable through the cell membrane. The fluorophore acts as an intermediate electron acceptor in the electron transport chain. Fluorescence intensity was measured in a microplate reader at an excitation wavelength at 530 nm and an emission wavelength at 590 nm (Rampersad 2012). In our study, AlamarBlue dye was added to the culture media (10% volume) containing known volumes of native islets or Kanslets and the fluorescence reading was taken with a microplate reader (Spectramax M5, Molecular devices, Sunnyvale, CA) after 24 and 48

hours. Islet equivalents (IEQ) were used to normalize the fluorescence readings. Control wells consisted of culture media and the AlamarBlue dye without the Kanslets or native islets.

#### Glucose stimulated insulin secretion; GSIS

For static incubation, CP native islets/ Kanslets were handpicked with a micropipette and distributed in 96 well plates containing 100  $\mu$ l of Earle's balanced salt solution (EBSS) with 1.4mM glucose. CP native islets/ Kanslets were subjected to different glucose concentrations (1.4mM, 22.4mM, and 22.4mM +KCl) for an hour. Each dose was tested in quadruplets. After 60 min of static incubation at 37°C and 5% CO<sub>2</sub>, conditioned media samples were collected and frozen at -80°C until quantification using a human insulin enzyme linked immunosorbent assay (ELISA; cat. no. 80-INSHU-E10.1; Alpcoc; Salem, NH) was performed. During the time of quantification, standards as well as samples were added to the insulin specific monoclonal antibody coated microplate wells. Samples and standards were incubated with the detection antibody for an hour, the plates were washed with the wash buffer. Incubation was again carried out for 15 minutes after adding the substrate. The stop solution was added and optical density was measured at 450nm by a SpectraMax M5 Microplate Reader. Optical density values were divided by the respective number of IEQ's in each well for normalization.

#### Isolation of rat pancreatic islets

Pancreatic islets were isolated according to our published protocol (MacGregor, Williams et al. 2006, Williams, Wang et al. 2009, Williams, Huang et al. 2010), which was approved by the Institutional Animal Care and Use Committee. In brief, Sprague Dawley (SD) rats (Harlan; Indianapolis, Indiana) were anesthetized by intraperitoneal injection of ketamine (80 mg/kg; Vedco; St. Joseph; MO) and xylazine (10mg/kg; Lloyd Laboratories; Walnut, CA). The peritoneal cavity was opened and pancreatic main duct to the duodenum was clamped and distended with cold collagenase (CLS1, 450 units/mL; cat. no. LS004197 Worthington;

Lakewood, NJ). The pancreata were excised and incubated with gentle rotation for 20–30 min in a 37°C incubator. Pancreata were washed and strained through a 500µm mesh screen, and the contents were suspended in a 3:1 mixture of Histopaque 1077 and 1119 (density = 1.1085; Cat. No. 11191 and 10771; Sigma Aldrich; St. Louis, Missouri) and centrifuged beneath a layer of HBSS supplemented with 5% Bovine Calf Serum (BCS, Cat. No. SH3007303; GE Healthcare Lifesciences; South Logan, Utah). The islets, collected from the gradient, were sedimented and washed with HBSS supplemented with 5% BCS over a sterile 40-mm mesh cell strainer. Islets were placed into islet medium and allowed to recover for 24 hours in an incubator at 37°C and 5% CO<sub>2</sub>.

#### Rat islet transplantation

SD rats were injected with streptozotocin (55mg/kg; Sigma Aldrich), which induced diabetes 1 week prior to the surgery. During that week, their blood glucose was measured daily and animals were treated with long acting insulin (Lantus, 1-2 units/kg; SANOFI; Bridgewater, NJ) when glucose rose above 400mg/dl. Immediately prior to transplantation, animals were given ketamine (80 mg/kg; SANOFI)/ xylazine (10mg/kg; Lloyd Laboratories) and allowed to quiet. The surgical site was shaved with clippers and scrubbed with betadine (Medline; Mundelein, IL)/chlorhexidine (Xttrium Laboratories, Inc., Mount Prospect IL), followed by 10% povidone/iodine solution with a final wash of sterile warm saline. The animals were kept on an isoflurane vaporizer during surgery. An incision was made in the skin followed by a short upper abdominal incision of the linea alba to visualize the stomach/duodenal area. The stomach was grasped with blunt forceps and gently pulled through the incision. A non-adherent pad was moistened with warm saline, and placed beneath the omental area, and the omentum was gently expanded using forceps. CP Kantslets and fresh native islets (with two different IEQ ranges) were placed into the rat omentum using a hydrogel (HyStem-C, ESI Bio; Alameda, CA) to keep it in place as has been described previously (Rawal, Harrington et al. 2016). Basically, a

base layer of hydrogel was applied to the omentum surface and allowed to solidify. The Kanslets were mixed with more hydrogel and placed near the omentum vascularization on top of the base layer. After the gel solidified, the omentum was rolled and tacked in 2-3 places against the stomach using 5-0 prolene blue monofilament with RB-2 taper needle (AD Surgical; Sunnyvale, CA). The stomach/ omentum was gently placed in the abdominal cavity. Sterile warm saline was added to the cavity. The abdominal muscle was closed with simple interrupted technique using 3-0 braided polyglactin suture with a SH-1 taper needle. The skin was closed using a subcuticular pattern with buried knots using 4-0 braided polyglycolic acid with a reverse cutting P3 needle (AD Surgical), using the similar procedure as described.

Immediately after surgery, analgesia (Meloxicam, 2mg/kg; Norbrook Laboratories; Lenexa, Kansas) was given. The animals were placed under a heat lamp to allow the body temperature to rise for better recovery from the anesthesia. Rats were monitored daily and analgesia was given for up to 48 hours after surgery. Blood glucose was checked daily and long-acting insulin (Lantus 0.2 units/kg; SANOFI) was given until the blood glucose levels normalized. Once the blood glucose stabilized, daily checks were reduced to 3 times /week, and eventually to weekly, as appropriate.

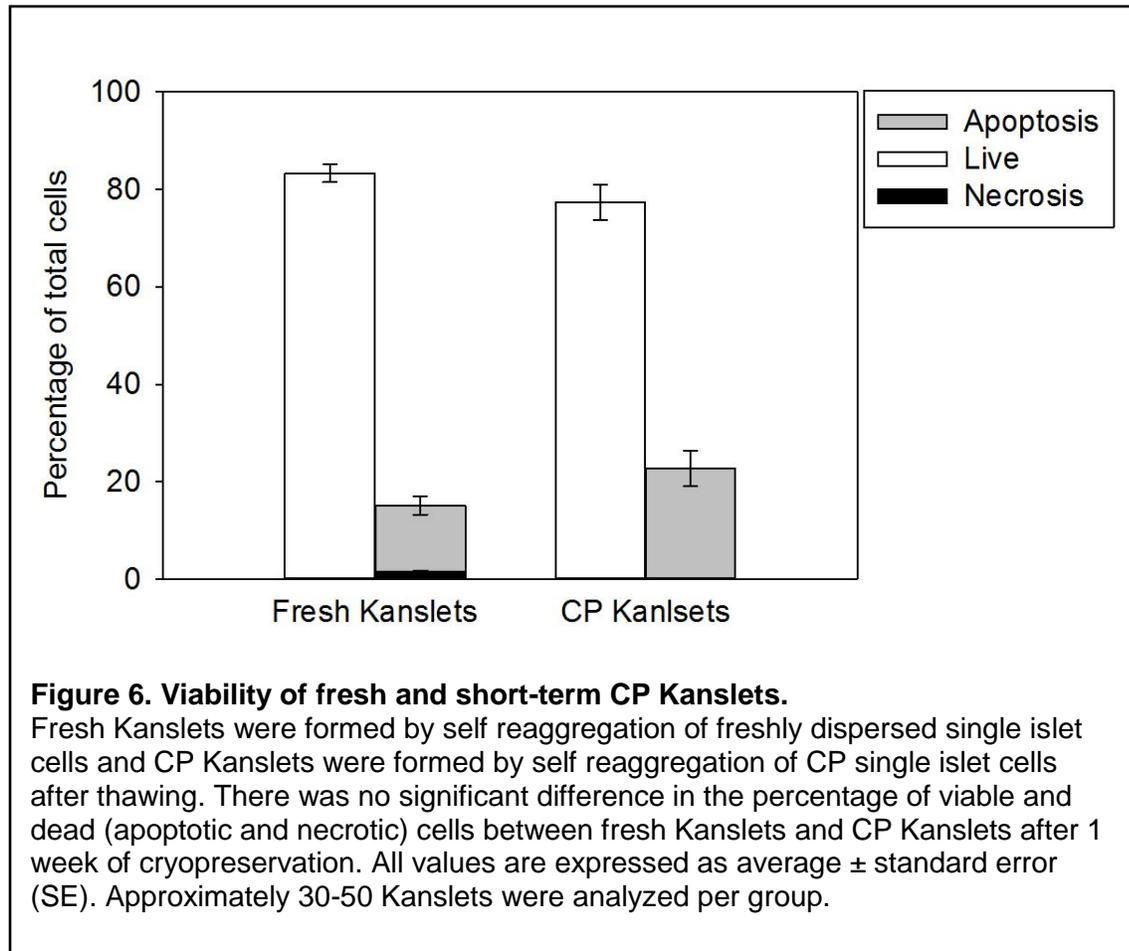
### Data Analysis

SigmaPlot software was used for data analysis. To determine significance between group differences for immediate viability, metabolic function, and extended viability, a student t-test was used. To determine the significance within group differences for immediate viability, extended viability, and GSIS, a one-way ANOVA was performed. An alpha level of 0.05 was utilized to determine the significance of these findings. All the values were expressed as average  $\pm$  standard error (SE).

## 2.4. Results

### Testing for optimal short-term cryopreservation conditions of native islets and Kanslets

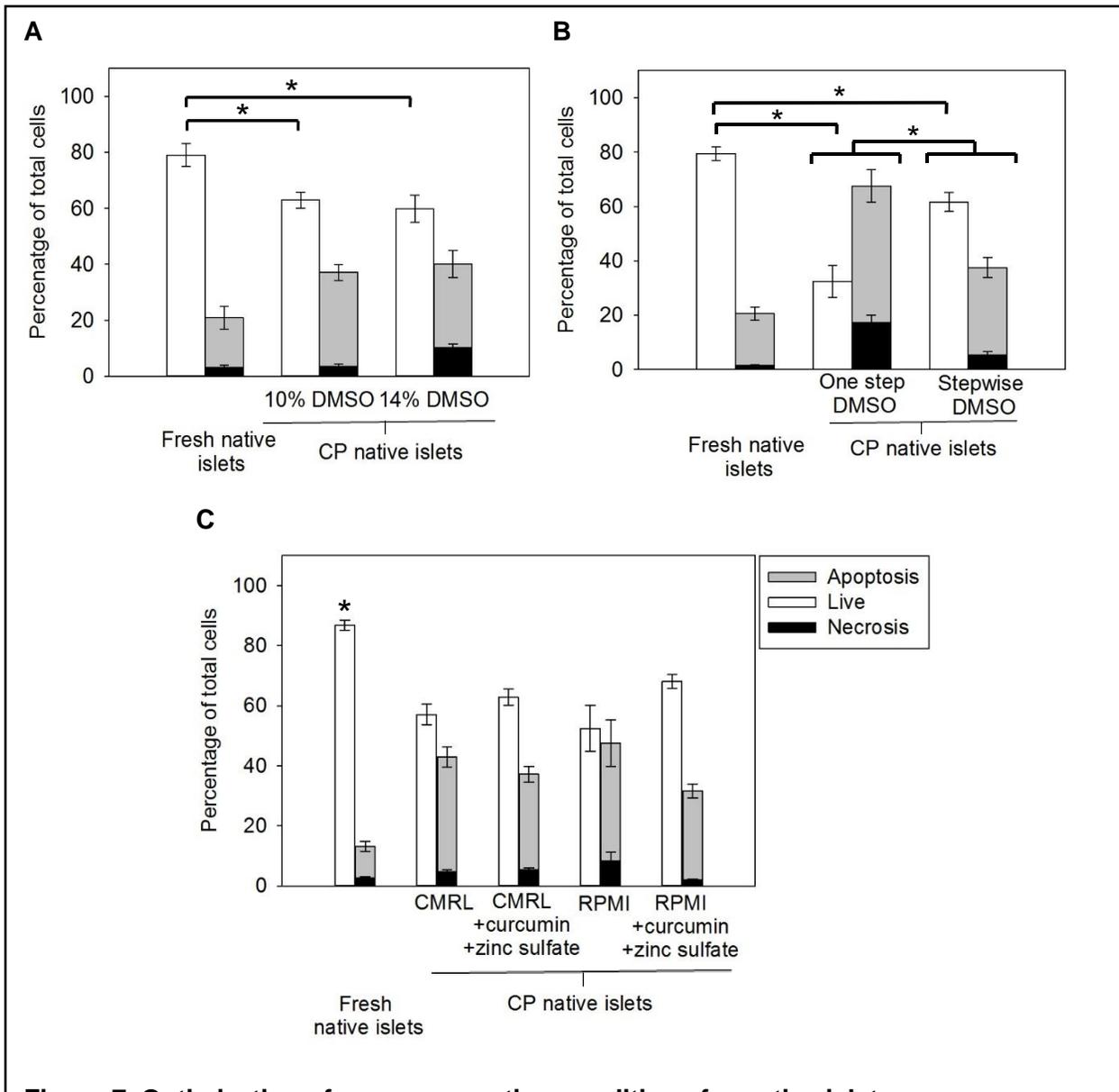
Optimal cryopreservation conditions of native islets as well as Kanslets were determined based on the results of the viability assessment after testing them with different conditions. Native islets were assessed for their viability after cryopreserving them with different percentages of DMSO, different equilibration times, and different media with additives. Kanslets were tested for their viability after cryopreserving them with CryoStor CS10.



Single islet cells were cryopreserved using a standard protocol designed for multiple cell types employing CryoStor solution with slow cooling and rapid thawing. After thawing, immediate viability was near 80% (Figure 6) and was not significantly different than fresh Kanslets. Likewise, the number of apoptotic or necrotic cells was low and there were no statistical differences in the percentages after cryopreservation compared to their fresh state.

For native islets, there was no standard protocol for cryopreservation so a series of optimization experiments were undertaken. First, using the step-wise cooling method, two different DMSO concentrations (10% and 14%) were compared. Both cryopreserved groups had reduced viability with respect to the fresh native islets, and the percentage of DMSO had no effect on the viability outcome (Figure 7A). Thus, next set of optimization studies were conducted using the lower DMSO concentration at 10%. When the one-step cooling method was compared to the step-wise method on native islets, there was a significant decrease in the percentage of viable and increase in percentage of dead cells (apoptosis and necrosis) using the one-step protocol (Figure 7B). A single addition of DMSO at the desired final concentration of 10% resulted in a low a viability of  $32.4 \pm 5.9\%$ . In contrast, the step-wise addition of DMSO resulted in significantly higher viability. However, both cryopreserved groups had significantly lower viability than the fresh native islets (Figure 7B). Based on this additional finding, the step-wise addition of 10% DMSO was used in the next set of experiments. The final optimization step was to test different media during the cryopreservation process. Again, viability of the groups cryopreserved using all the different media was significantly less than fresh native islets. There was no significant difference between the viable cells obtained after cryopreservation with different media. However, cryopreservation with the medium containing RPMI with curcumin and zinc sulfate resulted in higher proportion of viable cells (Figure 7C) with respect to the other media conditions tested (CMRL, CMRL with curcumin and zinc sulfate or RPMI alone). Thus, all

subsequent experiments with native islets used media containing RPMI with curcumin and zinc sulfate, 10% DMSO added in step-wise manner.



**Figure 7. Optimization of cryopreservation conditions for native islets.**

In all the tested conditions, fresh native islets had significantly higher proportion of viable cells than the CP native islets. A) Viable and dead cells after short-term cryopreservation using different percentages of DMSO showed no difference between the 2 DMSO concentrations. B) Fresh native islets had a significantly higher proportion of viable cells than CP native islets in both the tested conditions. There were significantly higher percentage of viable with fewer dead (apoptotic and necrotic) cells when DMSO was added stepwise rather than a one-step protocol. C) Fresh native islets had a significantly higher proportion of viable cells than CP native islets in all 4 the tested conditions (indicated by \*). The best results were obtained with cells in RPMI media enhanced with curcumin and zinc sulfate, compared to CMRL media with and without the same additive. All values are expressed as average  $\pm$  SE. Approximately 30-70 native islets were analyzed per group. \* indicates  $p < 0.05$ .

### Islet Morphology

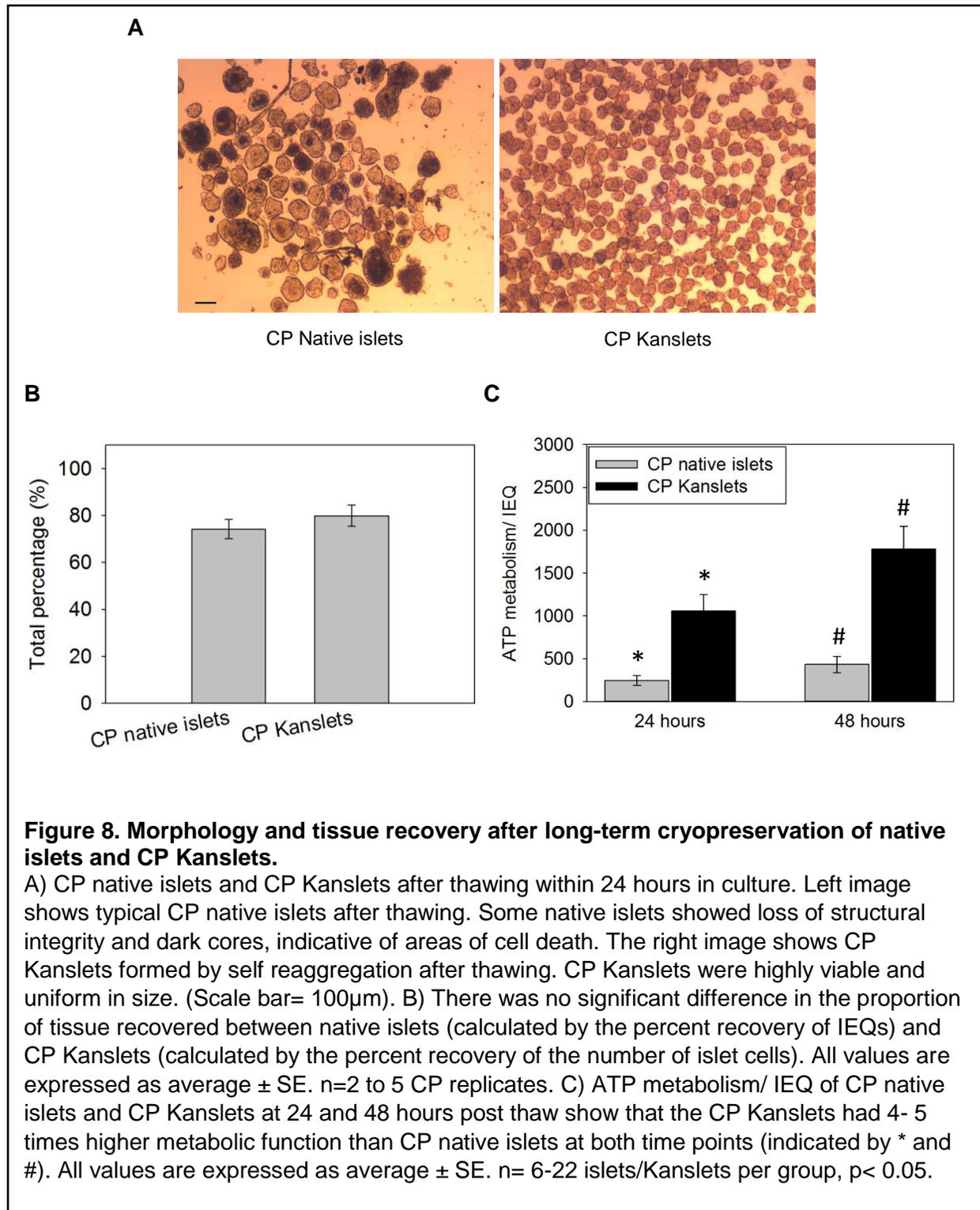
After optimizing islet cryopreservation using a short-term preservation protocol (1 week cryopreservation), the procedures were utilized to test the outcomes for long-term cryopreservation (4 weeks and longer). Figure 8A illustrates the non-uniform size and shape of the long-term cryopreserved native islets, as compared to the single cells that were cryopreserved and subsequently formed into CP reaggregated Kanslets after the thawing process. Some native islets showed a loss of structural integrity and dark cores, indicative of areas of cell death. The right image (Figure 8A) shows CP Kanslets formed by self-reaggregation after thawing. CP Kanslets were highly viable and uniform in size. Cryopreservation had no effect on the ability of the islet cells to self-aggregate.

### Tissue recovery

After having optimized the cryopreservation procedures for single cells and for intact islets for 1 week of storage, the outcome of these techniques was tested for longer-term cryopreservation (4 weeks and longer). Tissue recovery after long-term cryopreservation for the native islet group was based on the islet volume (measured by IEQ) recovered after thawing. Approximately 75% of native islets were recovered after thawing. Tissue recovery for CP Kanslets was measured by the single islet cells recovered immediately after thawing. On average, there was nearly an 80% recovery of single islet cells, with no difference between the two groups (Figure 8B).

### Metabolic function

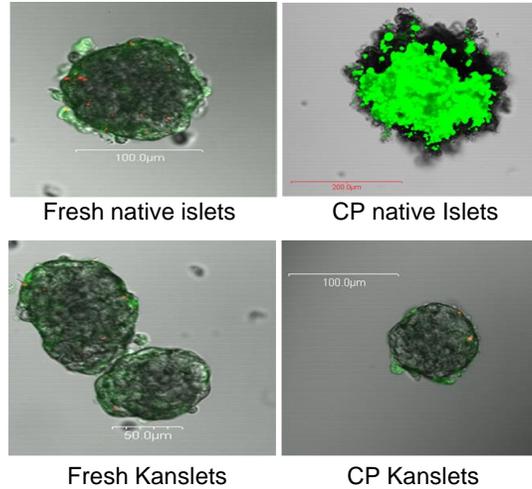
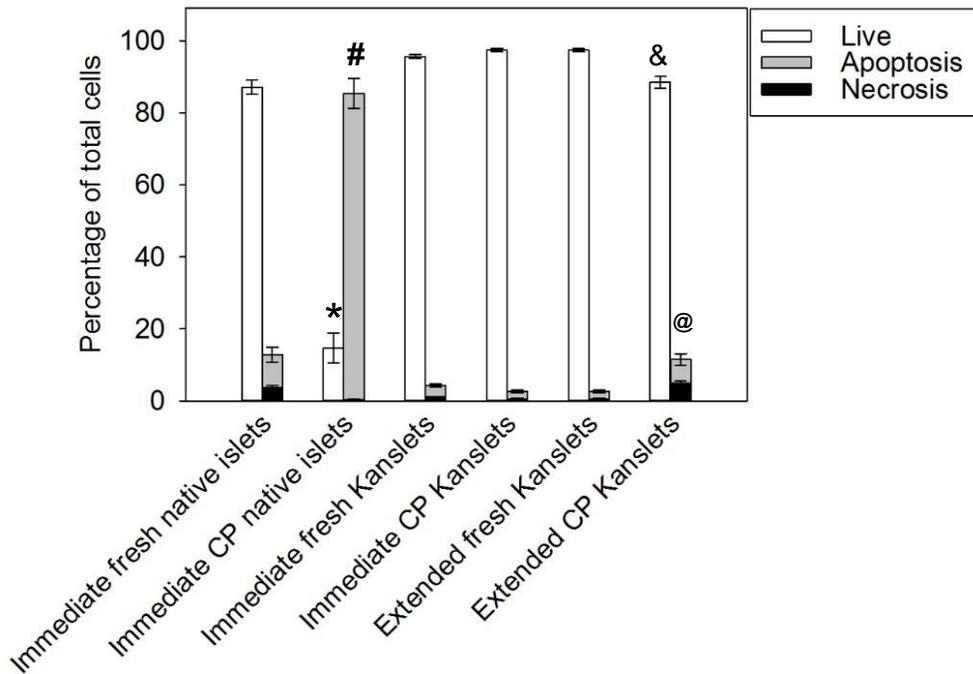
Interestingly, there was a significant difference in metabolic function of CP native islets and CP Kanslets at 24 hours as well as at 48 hours (Figure 8C). CP Kanslets were 4 to 5 times more metabolically active than CP natives at 24 as well as 48 hours.



## Cell Viability

Viability of all native islets, and Kanslets before and after long-term cryopreservation was assessed for apoptosis, and necrosis. Figure 9A illustrates representative images from both the native islet and Kanslet groups before and after cryopreservation. Red, and green in the images indicate cell death from necrosis, and apoptosis, respectively. The grey areas indicate regions of viable cells. Viability was high for native human islets prior to long-term cryopreservation with only a few dead cells on the surface ( $87.1 \pm 2.0\%$ ). However, after thawing, native islets had extensive cell death from apoptosis as indicated by the saturated green values ( $14.6 \pm 4.1\%$ ), which is in contrast to the short-term cryopreservation results shown in Figure 7.

Kanslets made from fresh or cryopreserved cells both demonstrated high viability. ( $95.6 \pm 0.4\%$  and  $97.4 \pm 0.4\%$  respectively). There was a significant increase in the percentage of apoptotic cells before and after long-term cryopreservation of native islets, but not for the Kanslets (Figure 9B). Due to the fact that the majority of cells in the intact native islets after long-term cryopreservation were dead, it was impossible to continue with experiments requiring longer culture. Therefore, only fresh and CP Kanslets were monitored to determine long-term survival in culture. Figure 9B summarizes the results showing that the immediate and extended viability of CP Kanslets was  $97.4 \pm 0.4\%$  and  $88.5 \pm 1.6\%$  respectively. There was no difference in the percentage of viable cells between fresh and CP Kanslets at 24 hours. However, there was 8% more decline in viable cells in the cryopreserved group compared to the fresh Kanslets with extended culture. Still the extended viability for the CP Kanslets group was over 88% (Figure 9B).

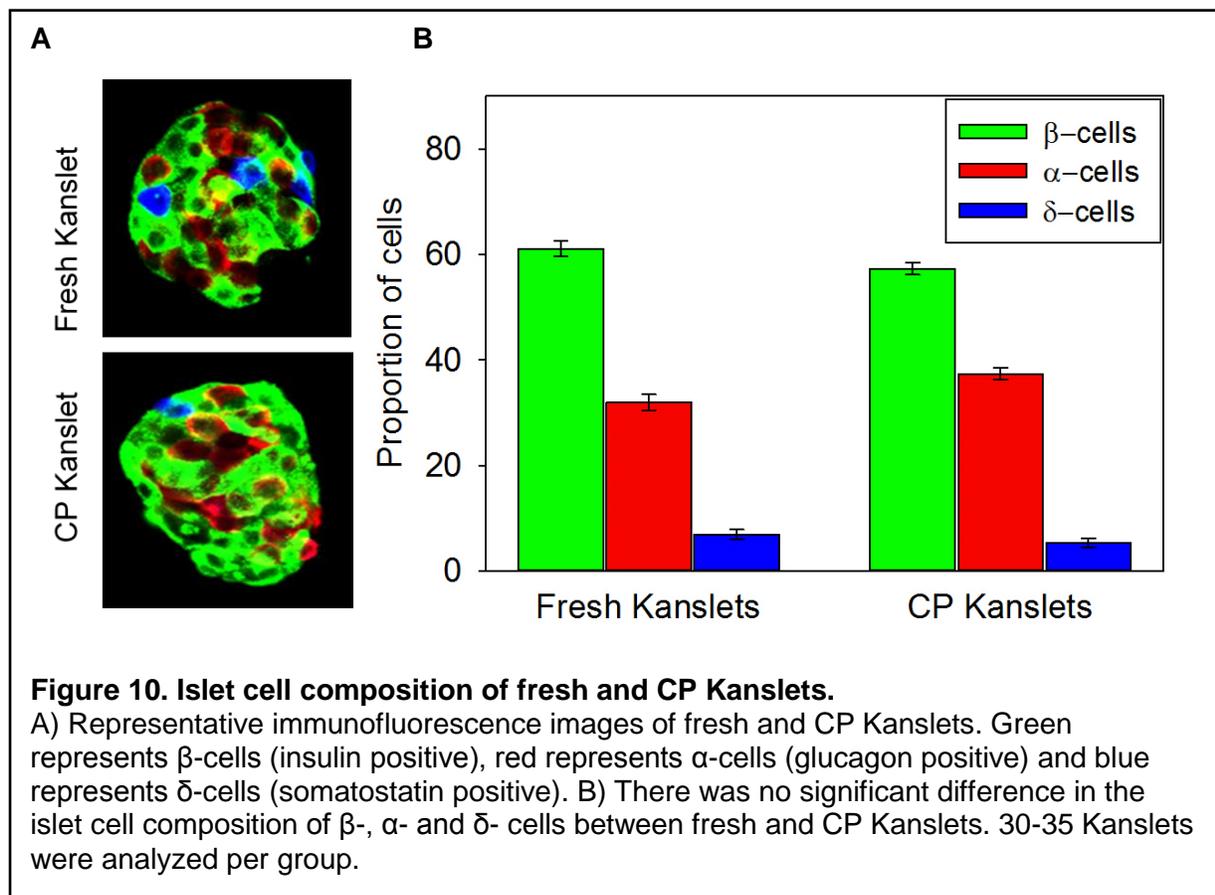
**A****B**

**Figure 9. Viability of native islets and Kantslets before and after cryopreservation.**

A) Representative islets from the native islets and Kantslets group before and after thawing. Gray areas indicate live cells, whereas red and green indicates cell death through necrosis and apoptosis, respectively. B) The percentage of viable and dead (apoptotic and necrotic cells) cells before cryopreservation and after thawing of native islets and Kantslets. There was a significant decline in the percentage of viable cells and significant increase in the percentage of dead (apoptotic and necrotic) in the native islets after thawing (indicated by \* and #), whereas CP Kantslets were as viable as fresh Kantslets. Extended viability after 7-10 days in culture had a significant decline in the viability of CP Kantslets (indicated by & and @) with respect to viability at 24 hours in culture and also with respect to extended viability of fresh Kantslets. Approximately 50-200 native islets/ Kantslets were analyzed per group.  $p < 0.05$ .

### Islet cell composition

Immunofluorescence staining was performed on fresh and long-term CP Kanslets to examine the proportion of  $\beta$ -,  $\alpha$ - and  $\delta$ -cells in the islet spheroids. No significant differences were found for each cell type between the fresh and CP groups.  $\beta$ -cells made up a majority of the cell types in fresh Kanslets as well as in CP Kanslets. The other endocrine cell types appeared to be randomly dispersed throughout the Kanslets (Figure 10A).  $\alpha$ -cells were the second most prevalent cell type in fresh Kanslets as well as CP Kanslets. Somatostatin-positive  $\delta$ -cells made up the smallest percentage of cells in fresh Kanslets and in CP Kanslets and there was no difference between the two groups (Figure 10B).

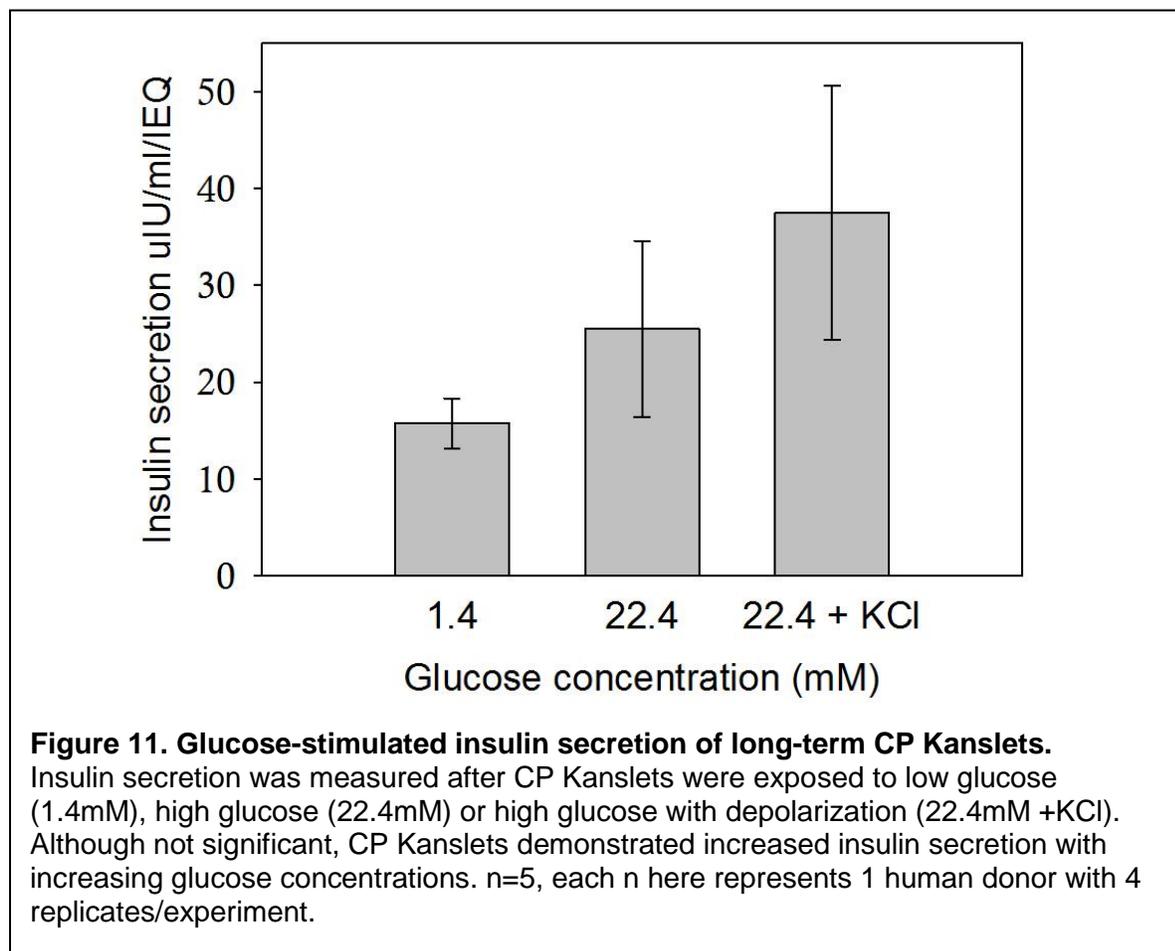


**Figure 10. Islet cell composition of fresh and CP Kanslets.**

A) Representative immunofluorescence images of fresh and CP Kanslets. Green represents  $\beta$ -cells (insulin positive), red represents  $\alpha$ -cells (glucagon positive) and blue represents  $\delta$ -cells (somatostatin positive). B) There was no significant difference in the islet cell composition of  $\beta$ -,  $\alpha$ - and  $\delta$ - cells between fresh and CP Kanslets. 30-35 Kanslets were analyzed per group.

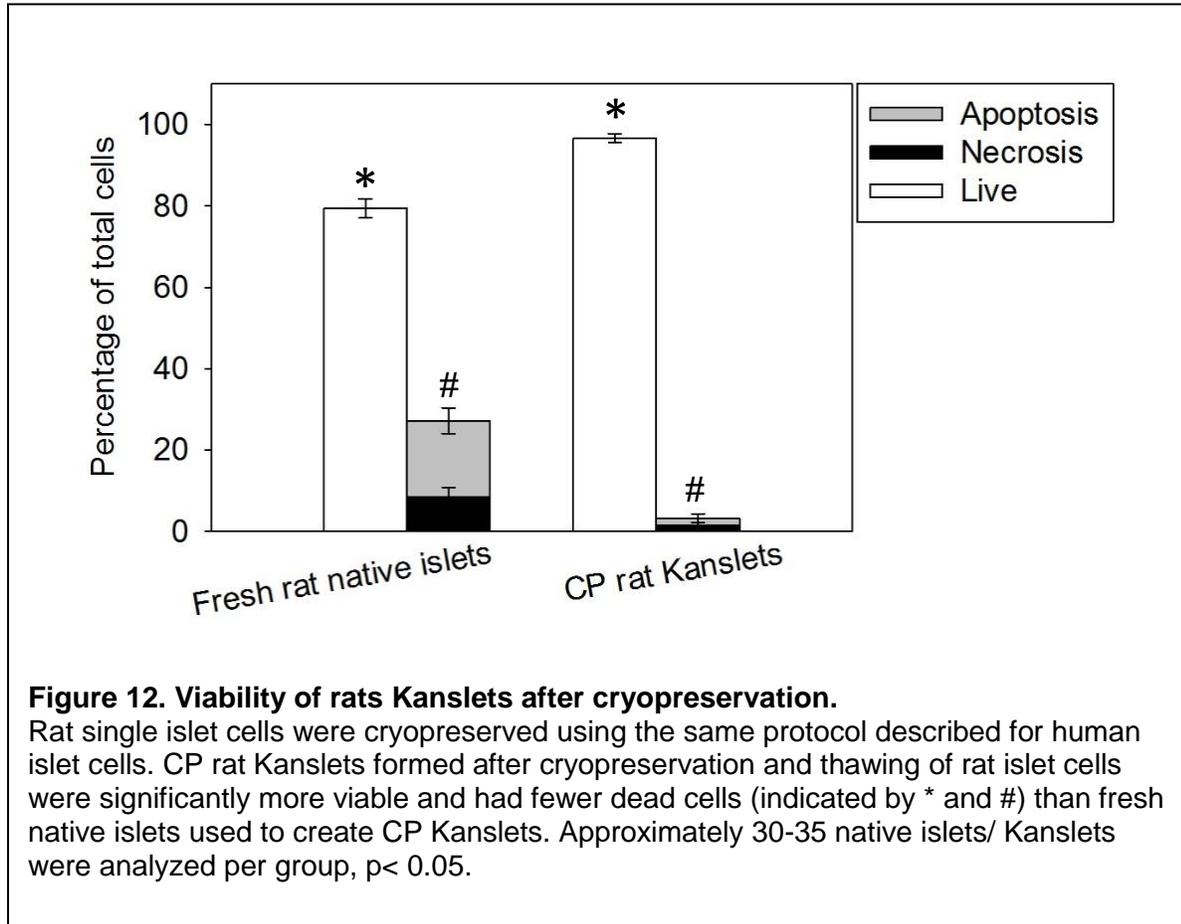
### Glucose stimulated insulin secretion (GSIS)

For GSIS, CP Kanslets and CP native islets were challenged with increasing concentrations of glucose (1.4mM, 22.4mM and 22.4mM with KCl) and the insulin secretion measured. Cell death in the long-term CP native islets was too high to measure insulin secretion after freezing; hence we only have insulin secretion data for the extended CP Kanslets. Figure 11 shows the amount of insulin secreted by CP Kanslets at different concentrations of glucose. Although there was no statistical significant difference between the insulin secretion values at different glucose concentrations due to the variability in responses at the higher concentrations, glucose sensitivity was noted in the CP Kanslets group.



### Viability of rat CP Kanslets

In order to determine whether CP Kanslets had benefit in a transplantation model, quality control experiments were repeated on the rat islets to be used in the transplant. Dispersed single cells from SD rat pancreatic islets were cryopreserved using the same protocol described in the methods section for human pancreatic islet cells. The rat CP Kanslets were greater than 95% viable. In fact, long-term CP rat Kanslets were 18% more viable than the native islets used to create CP Kanslets (Figure 12).

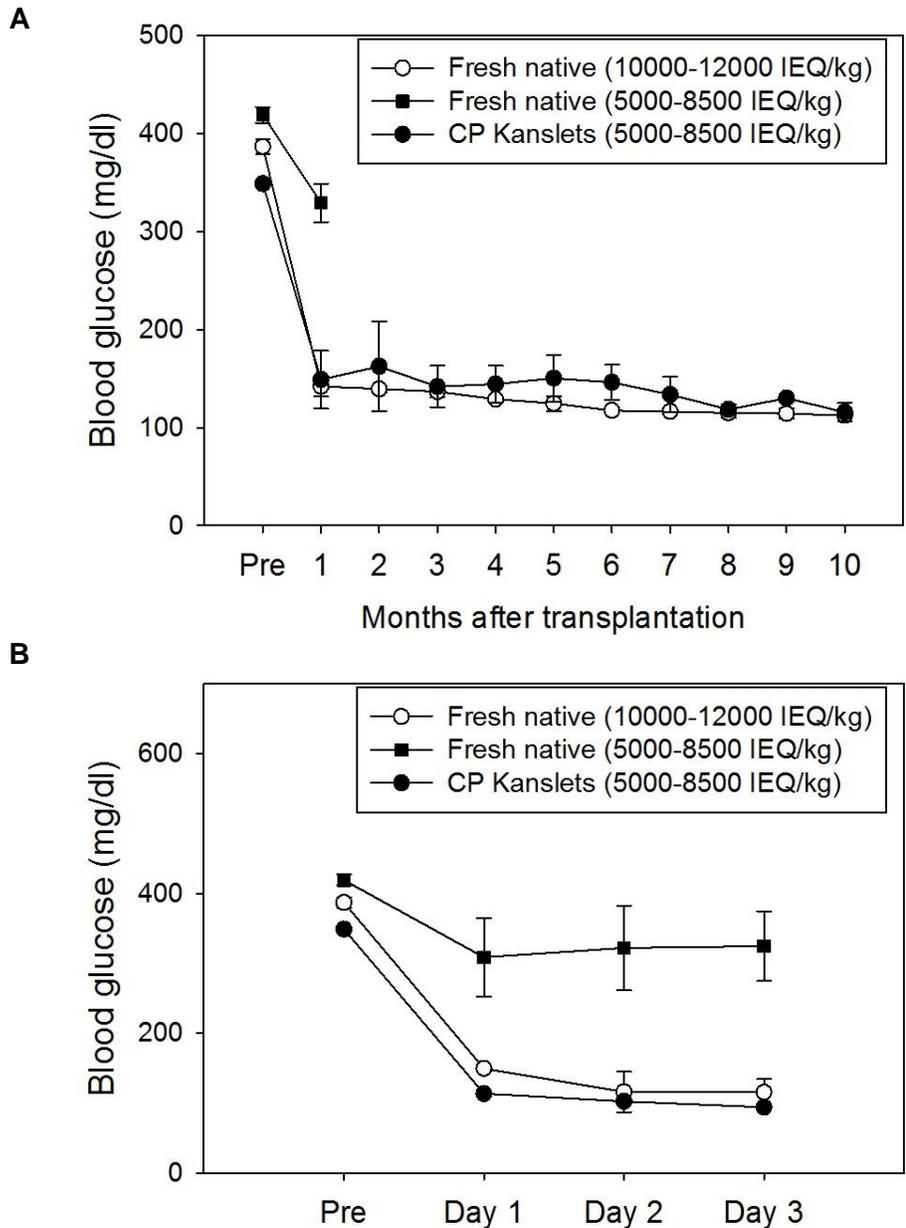


**Figure 12. Viability of rats Kanslets after cryopreservation.**

Rat single islet cells were cryopreserved using the same protocol described for human islet cells. CP rat Kanslets formed after cryopreservation and thawing of rat islet cells were significantly more viable and had fewer dead cells (indicated by \* and #) than fresh native islets used to create CP Kanslets. Approximately 30-35 native islets/ Kanslets were analyzed per group,  $p < 0.05$ .

### Rat islet transplantation

In order to determine whether the CP cells could function in a transplant situation, SD rat pancreatic islet cells that had been cryopreserved for 1-4 months were thawed, formed into CP Kanslets, and transplanted into diabetic rats. Diabetic rats transplanted with fresh native islets were used as controls. In order to determine the minimum amount necessary to reverse hyperglycemia, we first transplanted each diabetic rat with 5000-8500 IEQ/kg body weight of either fresh native islets or CP Kanslets. The average blood glucose levels pre-transplantation was approximately 390 mg/dl for both groups. Rats had an average blood glucose levels of 330 mg/dl during the first month after transplantation with fresh native islets, indicating that the low volume of transplanted islets failed to restore normoglycemia (Figure 13A). As fresh native islet failed to reverse hyperglycemia, the rats were euthanized and on termination the average blood glucose level was  $479 \pm 26.96$  mg/dl. In contrast, rats transplanted with similar IEQ of CP Kanslets had an average blood glucose of 150 mg/dl during the first month after transplantation, and rats remained non-diabetic for 10 months following transplantation (Figure 13B). At termination the average blood glucose levels of rats transplanted with CP Kanslets was  $111.5 \pm 17.50$  mg/dl. As 5000-8500 IEQ of native islets failed to reverse hyperglycemia, we subsequently transplanted diabetic rats with higher IEQs (10000-12000 IEQ/kg body weight) of fresh native islets. After transplantation, the average blood glucose of animals was 142 mg/dl during the first month. Rats maintained normoglycemia during the 10 month period. Animals transplanted with 10000-12000 IEQ/kg body weight of fresh native islets and animals transplanted with approximately 8500 IEQ/kg body weight of CP Kanslets not only were normoglycemic for 10 months but these group of animals also achieved normoglycemia within 24 hours of transplantation (Figure 13B).



**Figure 13. Transplantation of fresh native islets and long-term CP Kanslets into diabetic rats.**

A) Transplantation of fresh rat native islets (10000-12000 IEQ/kg body weight) and rat CP Kanslets (8500 IEQ/kg/body weight) lowered the blood glucose levels of diabetic animals. Normoglycemia could not be achieved in animals transplanted with 5000-8500 IEQ/kg body weight of fresh native islets within the first month after transplantation, hence animals in this group were euthanized. B) In the other 2 groups normoglycemia was achieved within the same day after surgery. Average blood glucose values show that normoglycemia was maintained until the rats were sacrificed at 10 months and blood glucose values of rats transplanted were below 200mg/dl at any given day after transplantation in both the successful groups. For long-term studies, N=2 rats/group [fresh native islets (10000-12000 IEQ/kg body weight) and CP Kanslets (8500 IEQ/kg/body weight)], N=3 [fresh native islets (10000-12000 IEQ/kg body weight)].

## 2.5. Discussion

Historically, cryopreservation of tissues has been challenging because of the stresses put on the tissue during the freezing process. In order to avoid non-uniform cooling, and warming and to minimize damage caused by extracellular ice formation, we cryopreserved pancreatic islets as single cells, and after thawing, reaggregated islets cells in micromolds to form CP Kanslets. We compared the outcomes of our single-cell cryopreservation protocol to the conventional protocol using intact native islets.

Previous studies have reported the reduced viability and function of intact pancreatic islets post-cryopreservation. Due to some of these poor results reported previously, process optimization suggestions have been made in the literature. We tested various suggestions alone and in combination to ensure that CP intact islets would yield the best possible outcomes after thawing. Reactive oxygen species are a major contributor towards cell death of different cell types during the cryopreservation procedure (Yard, Beck et al. 2004, Bucak, Atessahin et al. 2007). Curcumin has antioxidant properties (Liu, Chen et al. 2016) and in cryopreservation has been shown to improve islet viability and function (Kanitkar and Bhonde 2008). Additionally, zinc sulfate protects against deoxyribonucleic acid (DNA) damage during the cryopreservation procedure (Kotdawala, Kumar et al. 2012). Hence, during the optimization of the conditions of native islets, we chose to test the effects of curcumin, and zinc sulfate on viability of native islets after thawing. Even after mixing additives like curcumin and zinc sulfate and following published cryopreservation protocols with slight modifications, we continued to have significant levels of cell death in native islets after long-term cryopreservation. We identified less than 20% viable cells in native islets after 4 weeks of cryopreservation. That low viability is in contrast to the 60% viability that we measured using the same protocol for only 1 week. There is a possibility that even longer duration of storage might have resulted in greater decline in the viability of native islets. Most of the previous studies have cryopreserved islets for 3 days up to a maximum of 4

weeks. Out of those, many studies did not assess viability as one of their outcomes after thawing (Piemonti, Bertuzzi et al. 1999, Li, Xue et al. 2011, Xue, Luo et al. 2011). Additionally, studies reporting high viability post-cryopreservation have cryopreserved the native islets for around 1 week or failed to report the duration of cryopreservation (von Mach, Schlosser et al. 2003, Omori, Valiente et al. 2007). Only a few of studies have cryopreserved islets for more than a month. One recent study looked at islets that were cryopreserved for 16 years, and reported no differences in the proportion of apoptosis before and after cryopreservation but found only partial *in vivo* function of islets after thawing (Manning Fox, Lyon et al. 2015). To the best of our knowledge, the only other study that cryopreserved pancreatic islets for 3 months, which used the University of Wisconsin cryopreservation media along with ascorbic acid-2 glucoside, found that the highest viability achieved was around 68% (Arata, Okitsu et al. 2004).

As the native islets in our study had poor viability after long term cryopreservation, we chose not to freeze islets as intact and whole, but rather took the approach of freezing the cells individually. Single cells in our study were cryopreserved for 1- 6 months, a comparatively longer freezing period than most of the previous studies preserving intact islets. Even with a longer storage period, we obtained reaggregated CP Kantslets that were more viable as compared to CP native islets. In contrast to the significant loss of viability in the native islets after long-term cryopreservation, the new method of cryopreservation described here using single cells resulted in approximately 98% viable tissue after long-term cryopreservation. Further viability of CP Kantslets after 7-10 days in culture was still approximately 90%, which is much higher than what previous studies have reported (Miranda, Mohan et al. 2013).

Recovery of islets after cryopreservation, often referred to, as yield, has been shown to have great variability depending on the study, with values ranging from approximately 55-75% (Sandler, Kojima et al. 1986, Lakey, Rajotte et al. 2001, Liu, Tian et al. 2014) and few studies reporting islet recovery above 80% (Omori, Valiente et al. 2007). In our study, recovery of native

islets was 75%, which is similar to the results of the other laboratories (Lakey, Anderson et al. 2001). Following our novel cryopreservation protocol, recovery of islets cells after thawing was above 80%, comparable to other highly successful approaches.

Only one other study examined islet cryopreservation to the extent reported here including islet recovery, islet morphology, and both *in vitro* and *in vivo* function (Piemonti, Bertuzzi et al. 1999). In the study by Warnock et al., when DMSO was added in a stepwise manner, it was found that the basal insulin secretion was higher in cryopreserved islets than the fresh ones, but the responsiveness to a glucose challenge decreased after cryopreservation. Glucose control was also lost when these islets were transplanted into diabetic mice. Other studies have also reported decreased insulin secretion after cryopreservation (Janjic, Andereggen et al. 1996, Omori, Valiente et al. 2007, Kenmochi, Asano et al. 2008). In our study, CP Kanslets also demonstrated a blunted response to increasing glucose concentrations. However, more importantly, when transplanted into diabetic rats, they completely reversed the diabetes in less than 24 hours. In a similar study, blood glucose values returned to normal only after 7 days post transplantation (Ohnishi, Murakami et al. 2012). Most importantly, previous publications examining the ability of CP intact pancreatic islets to reverse diabetes after islet transplantation have followed only short-term success. When CP islets cultured with sertoli cells were transplanted into diabetic animals, graft survival averaged 35 days (Li, Xue et al. 2011) in contrast to our 300 days. Other studies also reported that normoglycemia could be maintained only for 3-5 months after transplantation (Nakagawara, Kojima et al. 1981) whereas in our rats transplanted with CP Kanslets were normoglycemic for 10 months until sacrifice.

Finally, one of the previous studies reported transplanting approximately 13,000 IEQ/ kg body weight of CP islets to reverse hyperglycemia (Lakey, Warnock et al. 1996). In another study that failed to reverse hyperglycemia after transplantation, an extremely large amount of cryopreserved islets (80,000- 160,000 IEQ/ kg body weight) were used (Manning Fox, Lyon et

al. 2015). In contrast, we were able to achieve long-term normoglycemia while transplanting only 8,500 IEQ/ kg body weight of CP Kantslets, which was even less than the IEQ required to reverse hyperglycemia when animals were transplanted with fresh native islets in our study. Results of this study showcase that small amounts of CP Kantslets can provide for quick return to normoglycemia post transplantation and CP Kantslets maintained their long-term functionality as compared to the best cases in the literature.

## **2.6. Conclusion**

Our study demonstrated that using the single-cell cryopreservation protocol, it is possible to obtain highly viable Kantslets after cryopreserving them for 4 weeks to 6 months in duration. CP Kantslets maintained their ability to reverse hyperglycemia in diabetic rats at a lower volume of transplanted tissue than fresh islets. The successful long-term cryopreservation technique presented here for storing pancreatic islet cells as compared to intact pancreatic islets provides advantages in several applications. It will not only provide additional time for quality control tests to be completed before islet transplantation, but also islets from different donors can be collected over time and pooled later to perform more successful islet transplantations.

## **Chapter 3**

### **Reaggregated Human Islet Cells for Drug Testing**

### 3.1. Abstract

People with type 2 diabetes are typically treated with a pharmacological therapy. However, the rate of diabetes-related complications continues to be high with diabetes as the leading cause of blindness, amputations and kidney failure. Finding a better pharmacological method to control blood glucose is key to improved outcomes. Currently, primary drug screening is performed on transformed  $\beta$ -cells, which fail to be predictive of the response *in vivo*. 3D human islets better predict the *in vivo* response to known diabetic drugs. However, when using human tissue there is great variability in the insulin response between donors, and hence the drug testing needs to be performed with a large number of human donors. To circumvent the need for assay repetition, we created multi-donor reaggregated human islets, called Kanslets. The purpose of this study was to determine whether drug screening with multi-donor Kanslets offered a superior platform for diabetes drug screening.

Islets from different human donors were dispersed and cryopreserved as single cells and subsequently used to create single-donor (reaggregated cells from single-donor) or multi-donor Kanslets (reaggregated cells from different donors). Viability and cell composition were measured for single- and multi-donor Kanslets. Insulin secretion was measured after exposure to increasing concentrations of known diabetic drugs, which either stimulate (glucose, glibenclamide) or inhibit (somatostatin) insulin secretion *in vivo*. Islet cells reaggregated within 3-5 days to form highly viable single- and multi-donor Kanslets. Within each cluster, the percentage of  $\beta$ -,  $\alpha$ -, and  $\delta$ - cells was not different. However, the cells within the multi-donor clusters were not equally comprised of cells from each donor. On average one donor comprised 60% of the cells while the second donor comprised only 40% of the cells. However, single-donor as well as multi-donor Kanslets showed little responsiveness to the different drugs tested, thus the hypothesis could not be fully tested.

### 3.2. Introduction

The prevalence of diabetes has been continuously increasing. In the US, 25.8 million people were suffering from diabetes in 2010 but this number rose to 29.1 million in 2012 (American Diabetes Association 2014). Type 2 diabetes accounts for 90-95% of all cases of diabetes (Center for Disease Control and Prevention 2011). According to a recent study, in the US every 2 in 5 adults are expected to suffer from type 2 diabetes throughout their lifetime (Gregg, Zhuo et al. 2014).

Pharmacological therapy is the most common therapy for type 2 diabetes. For more than 50 years, researchers have been trying to develop new drugs that would better control blood glucose levels in people with the disease (White 2014). Insulin and sulphonylureas have been the most commonly used drugs in past, but other classes of drugs like biguanides, incretins and thiazolidinediones are now available. Unfortunately, the new drugs, just like the older generations, can have severe side effects. Sulphonylureas are known to cause hypoglycemia (Bolen, Feldman et al. 2007, Barnett, Craddock et al. 2010). Metformin, a biguanide can cause gastrointestinal side effects as well as lactic acidosis (DeFronzo, Fleming et al. 2016). Drugs based on glucagon-like-peptide-1, which target the incretin system, have a causal relationship to pancreatic cancer and pancreatitis (Ryder 2013). Hence, there is a need to discover new, more effective drugs for diabetic patients, without the severe side effects.

The high cost associated with bringing the new drug to clinic and the high failure rate associated with clinical candidates argues for an urgent need for the development of suitable preclinical models that can efficiently and accurately predict the efficacy in humans. Currently, candidate drug screening is completed on models such as transformed pancreatic  $\beta$ -cells that fail to mimic the *in vivo* response reliably (Hohmeier and Newgard 2004). Moreover the phenotypic characteristics of a cell line can change in culture over time (Efrat 1999, Borrell 2010, Lee, Nam et al. 2010, Philippeos, Hughes et al. 2012). None of the current  $\beta$ -cell lines

used for screening truly represents the function and physiology of primary  $\beta$ -cells *in vivo* (Gilligan, Jewett et al. 1989, Wang, Beattie et al. 1997, Baroni, Cavallo et al. 1999). It is therefore difficult to get predictive results using these two dimensional (2D) cell line models and there is a need for better model systems for reliable drug screening.

It is widely believed that three dimensional (3D) cultures are far more superior to 2D cultures in predicting *in vivo* efficacy (Green and Yamada 2007, Walpita, Hasaka et al. 2012). While 2D culturing has been used historically to discover and test drugs, *in vivo*  $\beta$ -cells do not exist in a monolayer. Instead, they exist as 3D clusters together with other types of cells like  $\alpha$ - and  $\delta$ - cells (Li, Sun et al. 2013). Thus, it is not surprising that 3D human islets might better predict the *in vivo* response to known diabetic drugs (Gao, Wang et al. 2016).

Rodent islets are generally used as secondary screens as well, but there are disadvantages to using rodent islets too. There is huge variation in the size of the rodent islets, varying from 40-400 $\mu$ m (MacGregor, Williams et al. 2006). Also, there are some physiologic differences between rodent and human islets including enzyme activity, and protein level (Olson, Betton et al. 2000). With the use of human islets in the early drug discovery phase, species differences can be avoided, which can help to better predict the outcomes of a drug in the human body. A barrier to using human islet tissue is the inability to store islets for convenience and the requirement of repetitions on different donor islets to get an averaged response due to the great variability between the drug responses of different donors.

In this study we created multi-donor Kanslets (reaggregated islet cells from different donors) for the testing of reference drugs. This is the first study to create multi-donor Kanslets using banked human islet cells in order to minimize the need for repeated assays. This will help make the drug screening process more efficient in terms of time as well as money. The time and cost-savings to the pharmaceutical industry has the potential to successfully move more drugs through the clinical trials phase, and eliminate earlier the drugs that will likely fail in clinical trials.

We determined whether a mix of pancreatic islets cells from two different human donors will give an average dose/response to insulin secretagogues (glucose and glibenclamide), and an inhibitor (somatostatin).

### **3.3. Methods**

Our published cryopreservation technique was used to preserve islets as single cells and later reaggregated to form single- (reaggregated cells from single-donor) or multi-donor Kanslets (reaggregated islet cells from two different donors). Results from three different trials were averaged, and two human donors were utilized for each trial. We compared the average dose response curve to insulin secretagogues (glucose and glibenclamide), and an inhibitor (somatostatin). We also looked at the viability and cell composition of multi-donor and single-donor Kanslets.

#### *Tissue Procurement*

Native human pancreatic islets were ordered from National Institutes of Health-Integrated Islet Distribution Program (IIDP). Pancreatic islets from human donors with diabetes or any other metabolic disorders were not included for the study. Characteristics of human islet donors used in this study are shown in Table 2 and only islets with high purity (>75%) were included in the study. As the islets were received, they were placed in tissue culture plates containing Connaught Medical Research Laboratories 1066 (CMRL; cat. no. MT5110CV; Fisher Scientific; Chicago, Illinois) medium supplemented with 10% fetal bovine serum (FBS; cat. no. 26140079; Life Technologies; Chicago, Illinois), 1% antibiotic/antimycotic (cat. no. 15240062; Thermo Fisher Scientific; Massachusetts, USA) and 1% glutamax (cat. no. 35050061; Thermo Fisher Scientific). After allowing the islets to recover for 24 hours in an incubator at 37°C and 5% CO<sub>2</sub>, they were dissociated into single cells.

**Table 2. Donor Characteristics.**

<b>Number of donors</b>	<b>Age</b>	<b>Sex</b>	<b>Body Mass Index</b>	<b>Race</b>	<b>Cold ischemia time (minutes)</b>
6	48± 4.07	66.7% Males	30.78±3.32	83.3% Caucasian	921.67±237.20

*Islet cell dissociation*

Islets were dispersed into single cells according to our previously published protocol (Williams, Wang et al. 2009). In short, islets were placed in a 50 ml tube and centrifuged at 1209 g for 5 minutes. The islets were washed twice with calcium and magnesium-free Hank's balanced salt solution (cmf-HBSS; Cat. No. SH30031.02; GE Healthcare Lifesciences; South Logan, Utah). Subsequently, the digestion medium consisting of cmf-HBSS and papain (5 units/ml; Worthington, Lakewood, NJ) in ratio of 3:1 was added to the islets. The suspension was incubated on a rotor at 37°C for a maximum of 20 minutes. Islets were then dispersed using a pipette. Digestion was stopped using culture medium containing calcium and magnesium. The cells were washed twice at 1209 g for 5 minutes to remove the papain.

*Cryopreservation of single islet cells*

Dissociated single cells were cryopreserved according to our patent pending protocol described in our previous study (Rawal, Harrington et al. 2016). In short, single cells were centrifuged to form a pellet and the supernatant was removed. Cells were placed in 1 ml of CryoStor CS10 (cat. no. C2874; Sigma Aldrich, St. Louis, Missouri) and kept in a cool cell (cat. no. BCS-405; Biocision; San Rafael, CA), which freezes the sample at 1°C/minute. Samples were kept at 4°C for 5-7 minutes and then moved to -80°C. After 15-20 minutes, nucleation was

performed to release the latent heat of fusion by mechanical agitation and samples were moved back to -80°C. Samples were stored in liquid nitrogen at -196°C. For the preliminary trial, donor 1 and 2 were CP for 7-10 days whereas for the next two trials islet cells were CP for 5-7 months. At the time of thawing, cells were incubated in a water bath at 37°C until the last ice crystals melted. They were diluted with the fresh culture media containing CMRL supplemented with 10% FBS, 1% L glutamine and 1% antibiotic and antimycotic.

#### Seeding the cells into micromold

Single cells were seeded into the micromold as described in our previously published protocol (Ramachandran, Williams et al. 2013). In short, cells in 3 ml of culture media consisting of CMRL supplemented with 10% FBS and 1% antibiotic and antimycotic were carefully plated into the micromold. The micromold was incubated at 37°C and 5% CO<sub>2</sub>. Cells reaggregated and formed Kantslets within 3-5 days. The culture media was changed every 24 hours. After the Kantslets were formed they were removed from the mold by washing several times with the culture medium and moved to a petri dish.

#### Viability

Cell death was analyzed using Yo Pro 1 and propidium iodide staining. Images were captured under an Olympus FluoView 300 confocal microscope, and analyzed using Adobe Photoshop CS 5 Extended. The total islet pixel number as well as the positively stained pixels were used to calculate the proportion of live as well as dead (apoptotic and necrotic) cells.

#### Immunofluorescence staining of $\alpha$ -, $\beta$ - and $\delta$ -cells

In order to analyze the proportion of  $\alpha$ -, and  $\delta$ - cells, single-donor and multi-donor Kantslets were stained for insulin ( $\beta$ -), glucagon ( $\alpha$ -) and somatostatin ( $\delta$ -) cells as described in our previously published protocol. Briefly, permeabilization of the cells was completed in 1% Triton X-100 in PBS for 30 minutes. Kantslets were washed in PBS repeatedly and incubated for

two hours at room temperature with primary antibodies against insulin (1:100, cat. no. ab7842; Abcam, Cambridge, MA), glucagon (1:300, cat. no. ab10988; Abcam) and, somatostatin (1:300, cat. no. ab53165; Abcam). After incubating with the primary antibody, single- and multi-donor Kanslets were washed with PBS three times for five minutes each, and incubated in a mix of fluorophore conjugated secondary antibodies consisting of DyLight 488 (1:400, cat. no. 706-485-148; Jackson ImmunoResearch Laboratories Inc., West Grove, PA), Alexa 555 (1:400, cat. no. A31570; Thermo Fisher Scientific) and Alexa 647 (1:400, cat. no. A31573; Thermo Fisher Scientific) in a dark wet chamber for one hour. After incubation with secondary antibodies, Kanslets were washed in PBS three times for five minutes and mounted on microscopic slides with anti-fading agent Gel/Mount (Biomedex, Foster City, CA). The following solution was used to dilute primary and secondary antibodies: 1% BSA, 0.03% Triton X-100 and PBS. Images were captured on a Nikon C1Si or C1 Plus confocal microscopes. For each group, 20-25 islets were analyzed for the cell composition. For cell composition analysis, the relative proportion of  $\alpha$ -,  $\beta$ -, or  $\delta$ -cells in each islet was evaluated by counting the number of individual types of cell and dividing it by the total sum of endocrine ( $\alpha$ -,  $\beta$ -, and  $\delta$ -) cells per islet. Image J was utilized to perform cell composition.

#### *Proportion of cells from different donors in the multi-donor Kanslets*

Before mixing islet cells from two different donors to create multi-donor Kanslets, cells from different donors were labelled with unique markers; carboxyfluorescein diacetate, succinimidyl ester (CFDA-SE) and Dil according to the manufacturer's instructions. In short, after centrifugation and aspirating the supernatant, the islet cell pellet was re-suspended in a pre-warmed (37°C) PBS containing 10 $\mu$ m of CFDA-SE. Afterwards cells were incubated for 15 minutes at 37°C and then centrifuged. The supernatant was removed and cells were washed twice and re-suspended in pre-warmed fresh medium containing CMRL, 10% FBS, 1% glutamax and 1% antibiotic/ antimycotic until seeded on the micromold. In order to stain the

pancreatic islet cells with Dil, cells were resuspended in 1 ml of serum free culture media. 5 $\mu$ l of Dil at a working concentration of 5mM was added to the tube and mixed gently using a pipette and incubated for 15 minutes at 37°C. Cells were centrifuged at 1209 g for 5 minutes and re-suspended in fresh medium. Cells were washed again twice and re-suspended in fresh medium.

After labeling, the islet cells from the different donors were mixed thoroughly in equal proportions and were seeded into the micromold to allow them to reaggregate. After reaggregation, Kanslets were removed from the micromold by washing the mold and aspirating Kanslets using a pipette. After allowing the hybrid clusters to recover in fresh culture medium, Kanslets were seeded on microscopic slides and mounted with anti-fading agent Gel/Mount (Biomedex, Foster City, CA). Images were captured using the Nikon C1Si or C1 Plus confocal microscope. Images were analyzed using Image J to determine the percentage of islet cells from either donor in the multi-donor Kanslets.

#### *Response to insulin secretagogues and inhibitors*

We compared 7 half-log dose response curves to insulin secretagogues (glibenclamide) and an insulin inhibitor (somatostatin). Stock solutions for glibenclamide (10 $\mu$ M), and somatostatin (300nM) were made in dimethyl sulfoxide (DMSO) and water respectively. For static incubation, single- or multi-donor Kanslets were handpicked with a micropipette and a known quantity of islet equivalents (IEQs) were distributed in 96 well plates containing 200  $\mu$ l of Earle's balanced salt solution (EBSS), 11.2mM of glucose and corresponding drug. Each dose was tested in repetitions of six. After 60 min of static incubation at 37°C and 5% CO<sub>2</sub>, conditioned medium was collected and frozen at -80°C. The insulin concentration was later quantified using a human ELISA kit (cat. no. 80-INSHU-E01; ALPCO; Salem, NH) per the manufacturer's instructions. In short, standards and samples were added to the insulin specific monoclonal antibody coated microplate wells. After incubating the samples and standards with the detection antibody for an hour, the plates were washed with the wash buffer. The

microplates were subsequently incubated for 15 minutes after adding the substrate. The optical density was measured at 450nm by a spectrophotometer after adding the stop solution. Optical density values were divided by the respective number of IEQ's in each well for normalization.

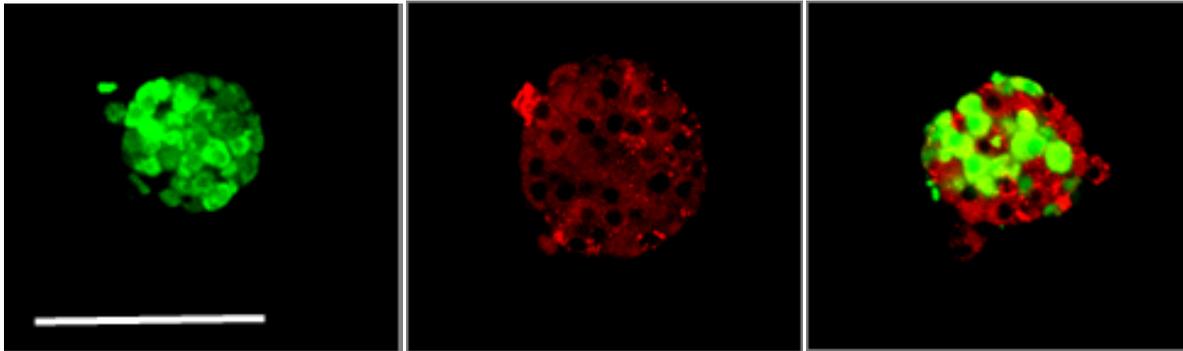
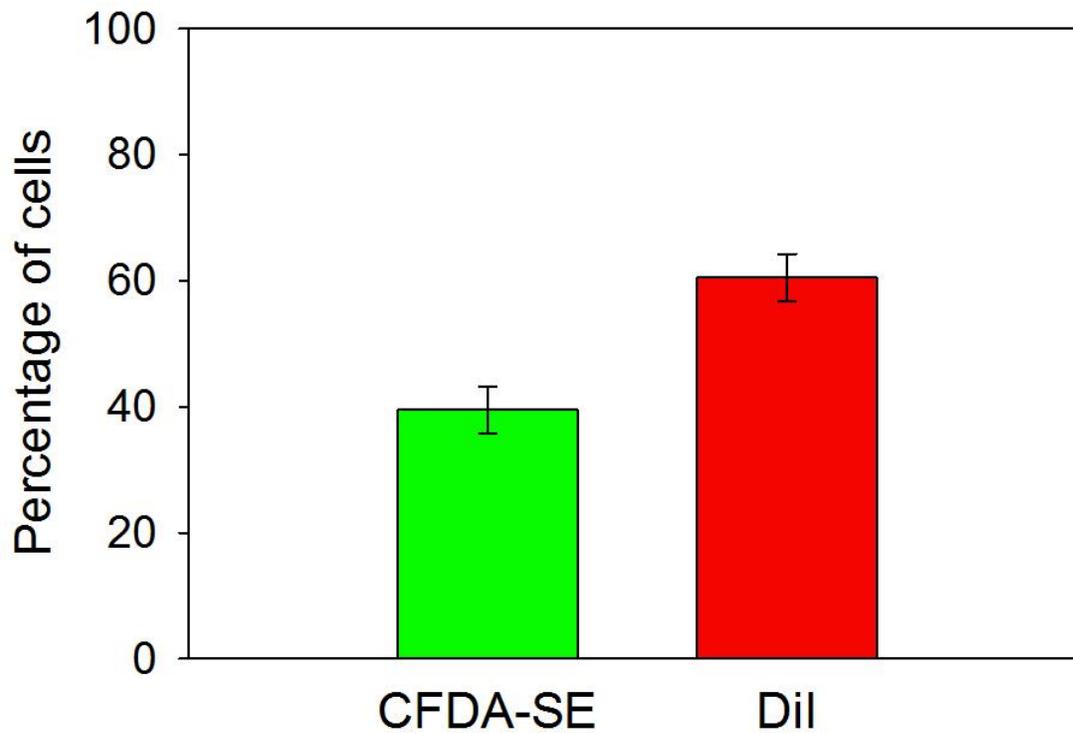
#### Data Analysis

Sigma plot was used for data analysis. To determine between group differences for cell composition and viability, a t- test was performed. To determine equivalence between groups (average of all single-donor Kanslets and multi-donor Kanslets) for dose response curve to insulin secretagogues, and inhibitor, we used the bioequivalence statistical test. Anything 25% above or 20% below the bound (difference between the mean of two groups) was considered a significant difference (<http://www.fda.gov/drugs/developmentapprovalprocess/ucm079068.htm> 2012, Li 2012). An alpha level of 0.05 was utilized to determine the significance of these findings. Results of all experiments were expressed as the average  $\pm$  SE.

### **3.4. Results**

#### Proportion of cells from different donors in multi-donor Kanslets

Islet cells from a donor were stained with CFDA-SE while cells from another donor were stained with Dil during each repetition. Representative images of single-donor Kanslets stained with CFDA-SE (green) or Dil (red) along with a multi-donor (containing cells from both donors) is shown in Figure 14A. As evident in the image, multi-donor Kanslets contained cells from both donors. However, the proportion of cells from each donor was not equal in the Kanslets, even though they were initially mixed in a 1:1 proportion (Figure 14B).

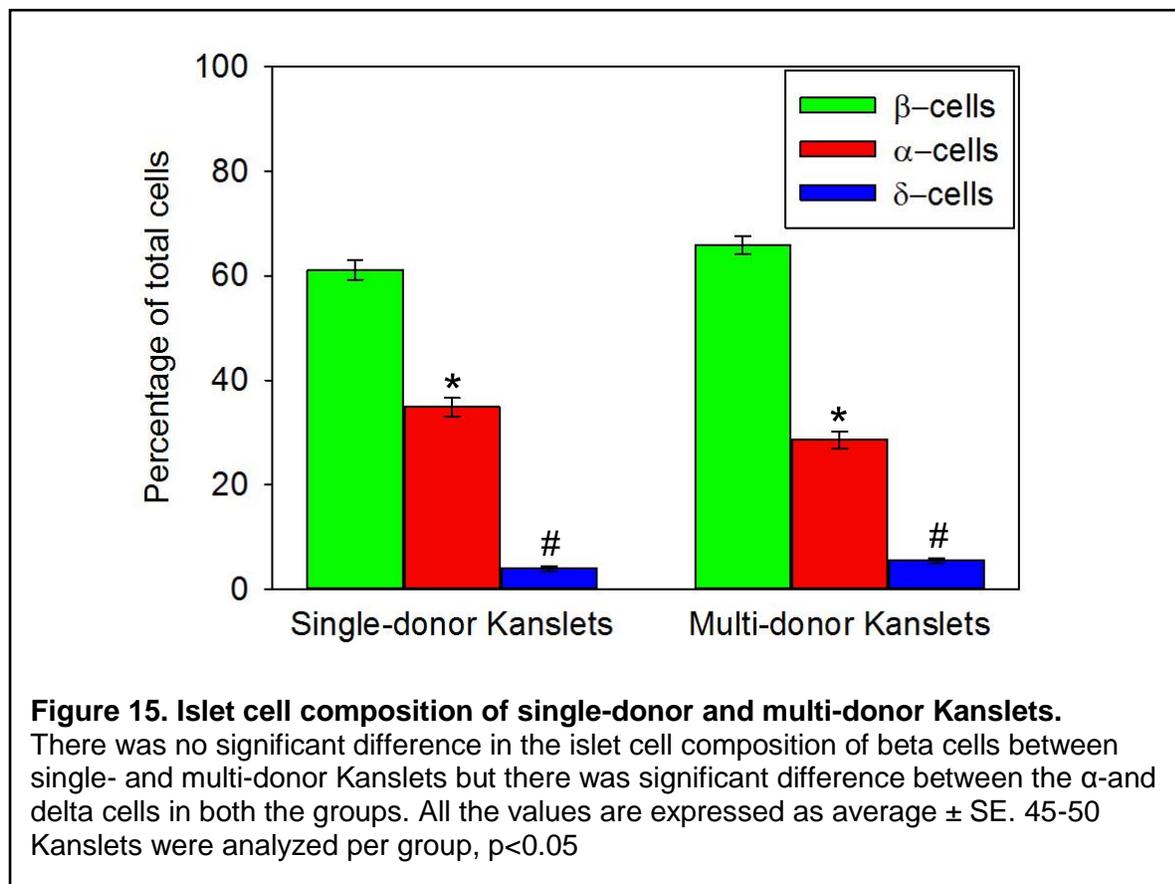
**A****B**

**Figure 14. Co-localization of islet cells from different donors in multi-donor Kanslets.**

A) In each trial cells from one donor were stained with CFDA-SE (green) and reaggregated into Kanslets. Cells from another donor were stained with DiI (red). Additionally, cells from the two different donors were mixed prior to reaggregation and the resulting multi-donor Kanslets show with cells from two different donors. Scale bar=100 $\mu$ m. B) The proportion of islet cells from different donors in the multi-donor Kanslets indicates that the multi-donor Kanslets did not contain an equal proportion of cells from both donors. Approximately, 50-70 Kanslets were analyzed per group.

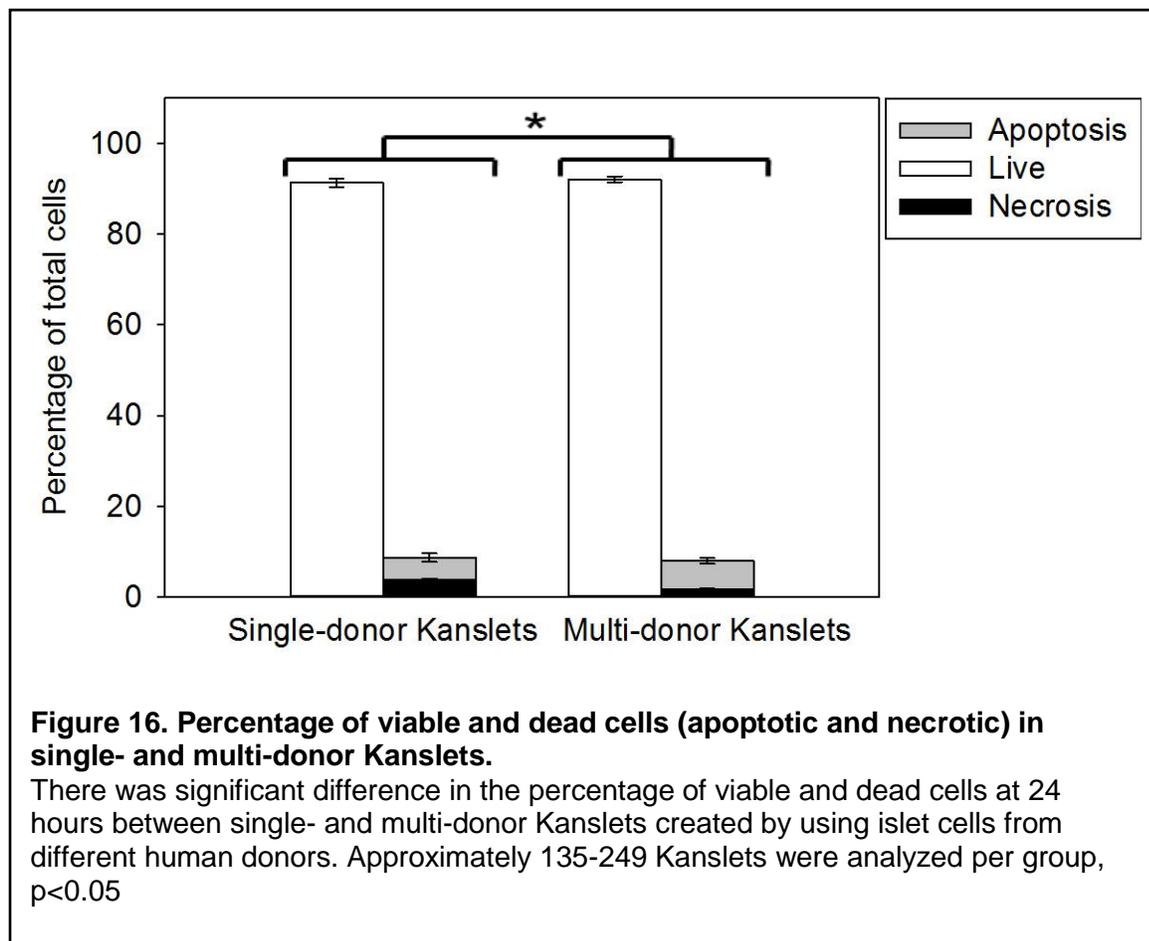
### Proportion of $\alpha$ -, $\beta$ - and $\delta$ -cells

The percentage of the different endocrine cells within the Kanslets was compared for the single- and multi-donor Kanslets. There was no significant difference in the proportion of  $\beta$ -cells between the single-donor and the multi-donor Kanslets.  $\beta$ -cells were the predominant cell type, comprising 60-65% of the cells, in all of the formed aggregates (Figure 15). By contrast, there was a difference in the percentage of  $\alpha$ -cells between the two groups with the single-donor Kanslets comprising statistically more  $\alpha$ -cells (Davalli, Ogawa et al.) compared to the multi-donor group (27%). Likewise, there was a significant difference in the proportion of  $\delta$ -cells in both the groups with  $\delta$ -cells constituting the third dominant cell type in both single-donor (4%) and multi-donor Kanslets (6%) (Figure 15).



## Viability

The viability of single- and multi-donor Kanslets from all the trials was assessed by Yo Pro 1 and propidium iodide staining, which measures cell death through apoptosis and necrosis. Figure 16 shows the percentage of viable and dead cells in the single- and multi-donor groups. Due to the low level of variance, there was significant difference in the proportion of viable and dead (apoptotic and necrotic) cells between the two groups with the multi-donor Kanslets showing a slightly better viability. However, both groups had excellent viability that was greater than 90%.



**Figure 16. Percentage of viable and dead cells (apoptotic and necrotic) in single- and multi-donor Kanslets.**

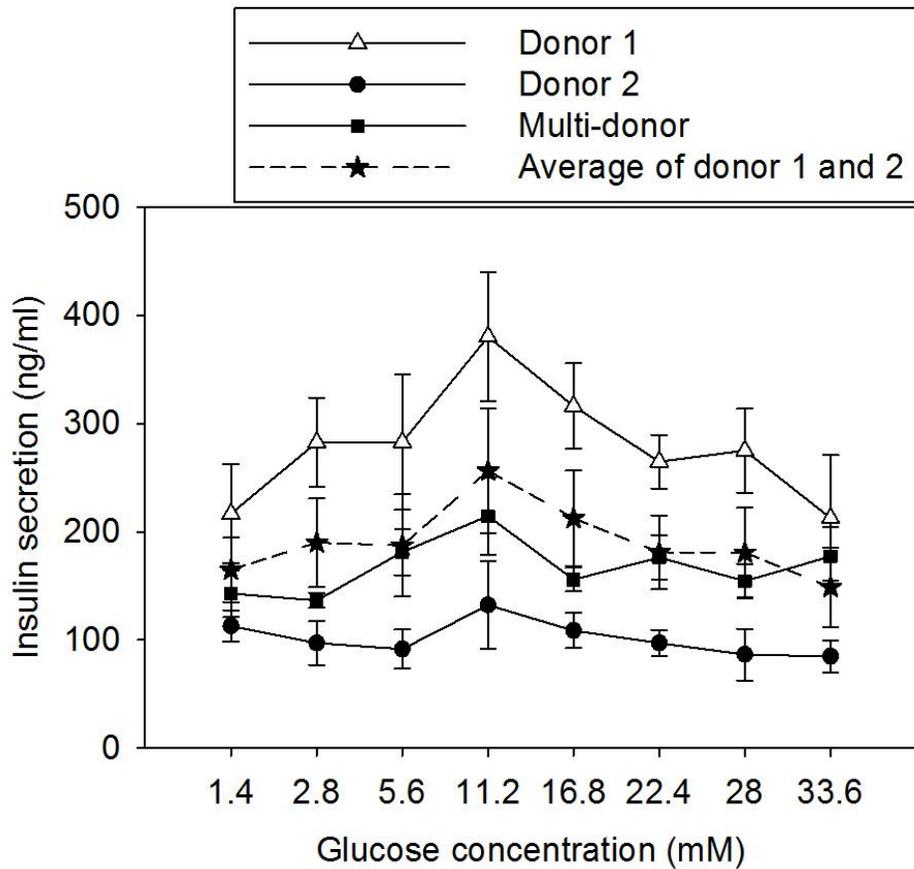
There was significant difference in the percentage of viable and dead cells at 24 hours between single- and multi-donor Kanslets created by using islet cells from different human donors. Approximately 135-249 Kanslets were analyzed per group,  $p < 0.05$

### Response to insulin secretagogues and inhibitors

Glucose:

$\beta$ -cells in the pancreatic islets function by releasing insulin in response to glucose stimulation (Haist 1971). We exposed single-donor as well as multi-donor Kantslets to increasing glucose concentrations and measured the amount of insulin secreted. Single-donor Kantslets from donor 1 and donor 2 as well the multi-donor Kantslets were challenged with different concentrations of glucose and secreted insulin was measured. Donor 1 had a peak insulin secretion level when exposed to 11.2mM glucose, which was 2 times greater than the basal insulin secretion at 1.4mM glucose (Figure 17). Donor 2 was unresponsive to different glucose challenges, illustrating the challenge of working with human donor tissues for drug screening.

The glucose sensitivity of the multi-donor Kantslets, composed of cells from these donors, fell between the individual donors' responses. A bioequivalence test of the preliminary data for the glucose dose response curve for multi-donor Kantslets and mathematical average of the two single-donor Kantslets suggested that the multi-donor Kantslets were bioequivalent to the averaged single-donor responses. This preliminary experiment supported the hypothesis that multi-donor Kantslets could provide an averaged drug response, but it was not superior to the mathematical average.



**Figure 17. Glucose stimulated insulin secretion of two single-donor (donor 1, donor 2) and multi-donor Kanslets (equal proportion of cells from donor 1 and donor 2).** The response of multi-donor Kanslets was approximately in the middle of the two single-donor Kanslets. Two single-donor Kanslets and multi-donor Kanslets had peak responses at 11.2mM glucose concentration. All values are expressed as average  $\pm$  SE, 4 replicates for each glucose concentration.

The results of repetitions of this experiment are summarized with the EC50 (effective concentration to elicit a 50% response), and the peak effect of single- as well as multi-donor Kanslets (Table 3). Both groups of Kanslets showed blunted increases in insulin secretion with exposure to higher glucose concentrations. This is demonstrated by the peak effect, which is the difference in the lowest insulin secretion value compared to the maximum insulin response. Compared to the responsive cells shown in Figure 17, which had a peak effect of 163 ng/ml, the subsequent trials elicited responses that were less than half as strong. The EC50 values indicated that the single-donor Kanslets were more responsive to lower concentrations of glucose than the multi-donor group.

Glibenclamide:

Glibenclamide acts by enhancing insulin secretion by binding to the SUR subunit of ATP sensitive potassium channel and thereby causing its closure and opening of calcium channel (Luzi and Pozza 1997, Proks, Reimann et al. 2002). In single- and multi-donor Kanslets, we again noted a blunted increase in insulin secretion when the glibenclamide concentration was increased. The EC50 of the multi-donor Kanslets was dramatically less than the single-donor group, which normally would point to a more potent assay. However, the peak effects for the multi-donor group was quite small and so no conclusions could be drawn.

Somatostatin:

Somatostatin inhibits the insulin secretion by activation of somatostatin receptors 1 and 5 (Schwetz, Ustione et al. 2013). When single- and multi-donor Kanslets were tested for somatostatin, yet again with increasing drug concentrations, an expected response towards decreasing insulin secretion was not seen. Single-donor Kanslets had a calculated theoretical EC50 value that was out of the range of tested concentrations, thus no conclusions could be

drawn. The multi-donor group had a reasonable EC50, but a very small peak effect, again leading to the inability to draw conclusions based on the results.

**Table 3. The EC50 and peak effect of single- and multi-donor Kanslets.**

	EC50 (mM-Glucose; nM-Glibenclamide, Somatostatin)		Peak effect (ng/ml)	
	Single-donor Kanslets	Multi-donor Kanslets	Single-donor Kanslets	Multi-donor Kanslets
<b>Glucose</b>	7.87 ± 3.15	37.05 ± 24.79	71.58 ± 26.68	51.24 ± 26.74
<b>Glibenclamide</b>	22462.56 ± 22176.03	88.05 ± 86.10	155.00 ± 70.14	41.52 ± 30.91
<b>Somatostatin</b>	53337.46 ± 5233.97	149.03 ± 43.28	102.66 ± 25.88	41.97 ± 11.63

Single- and multi-donor Kanslets were exposed to increasing concentrations of insulin secretagogues (glucose, glibenclamide) and an inhibitor (somatostatin). The EC50 was calculated based on the average insulin secretion response of all the trials after exposing the single-donor and multi-donor Kanslets to different drugs and their increasing concentrations. SigmaPlot was utilized to calculate the EC50 values. Single donor Kanslets (glucose: n=6, glibenclamide and somatostatin: n=4); multi-donor Kanslets (glucose: n=3, glibenclamide and somatostatin: n=2; each n represents one human donor).

### 3.5. Discussion

Currently, drug testing is performed on 2D cultures, which fail to mimic how the drug would respond *in vivo*. Previously, we have shown that Kanslets created from fresh human islet cells are more sensitive to different drugs, and their increasing concentrations than native human or rat islets (Ramachandran, Peng et al. 2014). In order to minimize the need for repetitious performance of candidate drug screening on  $\beta$ -cells from a large number of different human donors, we created multi-donor Kanslets, consisting of islet cells from different donors. We anticipated that the multi-donor Kanslets would give an average drug response of different single-donor Kanslets used to create the multi-donor spheroids in the study. We were successful in creating the multi-donor Kanslets, which were highly viable. The cell composition, comprised of  $\alpha$ -,  $\delta$ -, and  $\beta$ -cells, was similar to the cell composition of single-donor Kanslets and to the values reported for fresh native islets (Brissova, Fowler et al. 2005, Steiner, Kim et al. 2010). In the first trial, one donor responded to increasing glucose concentrations, while cells from the second donor failed to respond with an increase in insulin secretion. The mathematical average of the two curves was bioequivalent to the measured values from the multi-donor Kanslets. In the two subsequent trials with islet cells from four different donors, the Kanslets were responsive to glucose, but the peak effect was quite small, compared to donor 1. Interestingly, the single-donor Kanslets were more responsive than multi-donor with a lower EC50 and a larger peak effect. All Kanslets groups failed to respond to a stimulant drug, glibenclamide, or an inhibitor, somatostatin.

The most likely explanation for the poor responsiveness of the Kanslets tested in this study was the storage conditions. It is difficult to create multi-donor Kanslets using fresh human islet cells, as it is unlikely for a site to receive pancreatic islets from two or more human donors at the same time. Thus, the islet cells used in this study were cryopreserved using the optimized procedure described in Chapter 2. As soon as we received islets from human donors, we

cryopreserved them as single islet cells until we had another donor isolated. We have previously shown that Kantslets created using cryopreserved human islet cells are highly viable and diabetic rats returned to normoglycemia when transplanted with cryopreserved Kantslets (Rawal, Harrington et al. 2016). In our previous study, islet cells were typically cryopreserved for less than 4 months. In order to collect enough cells from qualified donors for this study, islet cells from all the donors were cryopreserved for 5-7 months. We have previously seen that islet cell death increases with the length of cryopreservation (Rawal, Harrington et al. 2016). All these observations suggest that duration of cryopreservation in this study may have adversely affected the outcome after thawing.

An additional explanation for the results revolves around the quality of the donor tissue. As described in table 1, we tried to exclude donors whose physical condition might adversely affect islet health, such as diabetes or other metabolic disorders. However, many human donors are overweight and we could not exclude all of them. Unfortunately, the islets used in the study were all from donors who were either overweight or obese. Long term exposure to fatty acids can lead to fat deposit within the islets and also exposure to fatty acids can lead to loss of co-localization of calcium channels and secretory granules which could have also made islets secrete less insulin (Hoppa, Collins et al. 2009, Ashcroft and Rorsman 2012).

Finally, the handling of the islets after removal from the donor could negatively affect the tissue quality. Cold ischemia time is that amount of time beginning with the chilling of the tissue after removal from the body to the warming of the pancreas to start the digestion process for removing the islets from the exocrine tissue. The average cold ischemia time for our donor tissue was 11-20 hours (Table 2), which is considered a longer duration. Previous studies have noted that cold ischemia times of greater than 8 hours lead to reduced quality of human islets (Hanley, Paraskevas et al. 2008). Thus the long cryopreservation and the quality of the donor islets, likely resulted in a lack of responsiveness shown in this study.

One of the most interesting findings of the study was the unequal distribution of cells from different donors. In each matched pair, islets from one donor appeared to outnumber the cells from the other donor by a ratio of 60 to 40%. However, on further review, the cells stained with Dil were always more prevalent than the cells stained with CFDA-SE. This leads one to the explanation of an artifact. In our experiments, Dil stain diffused to the neighboring cells staining the whole multi-donor Kantslet. In order to take this into consideration, cells that appeared to be double-labeled with CFDA-SE and Dil were counted as CFDA-SE only. However, there could have been cases where enough Dil was transported into CFDA-SE-positive cells that it overwhelmed the green fluorescence and the cells were misidentified as Dil-positive.

Improvements in the study would include; reduced cryopreservation time, more stringent donor criteria (including cold ischemic time), and different fluorophores used in the donor-specific staining. However, many of these improvements would also make the study extremely costly, if not impossible. With the current protocol, we had difficulty obtaining sufficient quantities of islets to be able to make single- and multi-donor Kantslets from the same donors. Thus, creating more stringent donor criteria would have exacerbated the problem. In addition, we, the end-user, have no control over the cold ischemia time at the donor site. Thus, requiring a shorter ischemic time would also have drastically limited the number of donors.

### **3.6. Conclusion**

In conclusion, the concept of multi-donor assays to reduce the time and cost of drug screening is appropriate in many situations. For example, in cancer drug screening where tumor cells are removed from patients and transformed into cell lines, because of the inherent characteristic of cancer cells to grow and divide, multi-donor tumors work well for candidate screens. Islet cells are more challenging, because they rarely proliferate *in vivo* in adult humans and they do not create cell lines without significant genetic modifications. Thus, the application

of the concept of multi-donor tissues for diabetes drug screenings does not appear to work at this time.

## **Chapter 4**

### **Integration of Mesenchymal Stem Cells and Islet Cells into Spheroids**

#### **Improves Viability, but not Long-Term Function**

#### 4.1. Abstract

Pancreatic islets, especially the large islets (>150µm in diameter) have poor survival rates in culture. Co-culturing with mesenchymal stem cells (MSCs) has been shown to improve islet survival and function, especially when the different cell types are in close contact, defined as islets cultured in media containing MSCs. The purpose of this study was to determine whether islet survival and function was improved, when the two populations of cells were intermingled with each other in a defined geometry. Hybrid spheroids containing 50% or 75% islets cells with appropriate number of MSCs were created along with spheroids comprised of only islet cells or only MSCs. All the groups were tested for the number of spheroids, proportion of spheroid type, proportion of islet cells and MSCs, viability, diameter, glucose stimulated insulin secretion (GSIS) and glucose stimulation index (SI). Spheroids were formed within 3-5 days. All the groups were highly viable with average diameters under 80µm at formation. Both the hybrid spheroid groups preferred to cluster in islet-only spheroids. Over a 2 week period, spheroids in all the groups significantly increased in their diameter but not their number. SI (calculated as insulin secretion/ islet cell at high glucose challenge to insulin secretion/ islet cell at low glucose exposure) was significantly less in the 75% islet cell ( $1.3\pm 0.8$ ) compared to spheroids containing only islet cells ( $4.1\pm 0.7$ ) at day 1 whereas there was no significant difference in the SI of 50% islet cell group ( $1.7\pm 0.8$ ) compared to the spheroids containing only islet cells. Our study showed that it was possible to create highly viable hybrid spheroids consisting of MSCs and islet cells intermingled, and in direct contact with each other, but this architectural arrangement did not improve islet cell function.

## 4.2. Introduction

Islets of Langerhans, containing the insulin-producing cells of the body, are multicellular clusters of endocrine cells found in the pancreas that vary in size from 20  $\mu\text{m}$  to more than 400  $\mu\text{m}$  in diameter (Huang, Novikova et al. 2011). Pancreatic islets in the human body have a rich blood supply from a dense capillary network (Zanone, Favaro et al. 2008), which is ten times higher than that of the surrounding exocrine tissue. However, when these islets are isolated, the clustered cells must survive solely on diffusion (Carlsson, Liss et al. 1998, Williams, Huang et al. 2010).

Traditional cell culture approaches for islets often yield poor results with cells generally dying after 7 days in culture (Chun, Huang et al. 2008). In large size islets ( $> 150\mu\text{m}$ ) core cell death has been measured within 24 hours in culture (Li, Zhao et al. 2014). For decades researchers have attempted to improve the culture conditions for isolated islets, with little improvement in long-term culture outcomes. Further, the clinical islet transplantation procedure requires extended periods of islet culture prior to transplantation while quality assurance assays are conducted on the donor islets (Andersson, Borg et al. 1976). Thus, it is important to maintain the islets in culture so that they can be utilized effectively for research purposes or islet transplantation.

Co-culture is a popular procedure in which different populations of cells are cultured together either to investigate the cellular interaction between different cell types or to help improve the cultural conditions (Goers, Freemont et al. 2014). It has been known for decades that certain cell types are difficult to maintain in a monoculture, but do better in a co-culture environment (Xu, Roberts et al. 1982, Harcombe 2010, Wu, Huang et al. 2010, Park, Kerner et al. 2011, Hesselman, Odoni et al. 2012). Cultured islets are already a co-culture, as there are at least 4 primary endocrine cell types, and a number of supporting cells in each islet. Loss of some of the cell types in the islet (namely endothelial and  $\beta$ -cells) can partially account for

reduced efficiency of islets in culture (Wang and Rosenberg 1999, Nyqvist, Kohler et al. 2005, Kin, Senior et al. 2008). Recently, the concept of adding extra support cells to the pancreatic islet culture has been investigated with a number of different cell types including hepatocytes and mesenchymal stem cells (MSCs) (Verga Falzacappa, Mangialardo et al. 2010). The goal of the new approaches has been to improve islet cell function, and long-term viability by mixing additional cells into the culture media.

MSCs are a reasonable option for supporting islet health. MSCs can be derived from various different adult tissues like bone marrow, adipose or peripheral blood.(Brunt, Hall et al. 2007, Hass, Kasper et al. 2011) MSCs have the ability to self-renew and self-proliferate (Via, Frizziero et al. 2012). MSCs help with repair and regeneration of tissues of different origins like neuronal (Park, Lee et al. 2008), cardiac (Takahashi, Li et al. 2006) and skeletal (Lee, Seo et al. 2006, Wu, Chen et al. 2007). Release of anti-apoptotic (Xu, Zhang et al. 2007), anti-inflammatory (Iyer and Rojas 2008), anti-oxidant (Ayatollahi, Hesami et al. 2014), immunosuppressive (Aggarwal and Pittenger 2005, Uccelli, Moretta et al. 2008) and angiogenic (Tang, Zhao et al. 2004, Zacharek, Chen et al. 2007, Ito, Itakura et al. 2010) factors by stem cells has been shown to help with tissue repair and regeneration. Further, MSCs may be protective to islet cells, because when pancreatic islets were co-cultured with MSCs, their survival and function improved (Chao, Chao et al. 2008, Boumaza, Srinivasan et al. 2009, Park, Kim et al. 2009, Karaoz, Genc et al. 2010, Lu, Jin et al. 2010, Scuteri, Donzelli et al. 2014).

Many techniques have been attempted to successfully co-culture islets with MSCs. Transwell plates, 3D scaffolds, and microfluidic platforms have been used for co-culturing. In indirect co-culture, islets are separated from MSCs by a semi-permeable membrane or an insert (Rackham, Dhadda et al. 2013). In direct co-culture, islets are allowed to contact the MSCs, either attached to a plate or in suspension. A few studies have reported that direct co-culture is superior to indirect co-culture as defined by improvement in insulin secretion (Jung, Kim et al.

2011). Direct contact with MSCs appears to help islets maintain their structural integrity (Luo, Badiavas et al. 2007). Yet, even with direct contact, the islet cells remain in a separate spheroid, typically with MSCs attached to the outer layer (Jung, Kim et al. 2011). The goal of this study was to determine if intermingling MSCs with islet cells in the same spheroid would further improve islet cell survival and function.

Hybrid islet and MSC spheroids of small uniform sizes were created. In order to know the right ratio of MSCs and islet cells needed to form viable and functional hybrid spheroids, we created hybrid spheroids consisting of different proportions of islet cells and MSCs. In this study, we aimed to look at the number of spheroids formed, viability (days 1 and 14), morphology and stimulation index of these hybrids containing different proportion of MSCs and islet cells.

### **4.3. Methods**

#### *Isolation of rat pancreatic islets*

Pancreatic islets were isolated and dispersed according to our published protocol (MacGregor, Williams et al. 2006, Williams, Wang et al. 2009, Williams, Huang et al. 2010). The protocol for isolation of rat pancreatic islets was approved by Institutional Animal Care and Use Committee at University of Kansas Medical Center. In brief, 32 Sprague Dawley rats (Harlan; Indianapolis, Indiana) were anesthetized by intraperitoneal injection of ketamine and xylazine. After opening the peritoneal cavity, the pancreatic main duct to the duodenum was clamped and distended with cold collagenase (CLS1, 450 units/mL; Cat. No. LS004197 Worthington; Lakewood, NJ). The pancreata were excised and incubated with gentle rotation for 20–30 min in a 37°C incubator. After washing and straining the contents of the tube through a 500µm mesh screen, the pellet was mixed with 3:1 mixture of histopaque 1077 and 1119 (density = 1.1085; Cat. No. 11191 and 10771; Sigma Aldrich; St. Louis, Missouri) and centrifuged beneath the layer of HBSS supplemented with 5% Bovine Calf Serum (BCS, Cat. No. SH3007303; GE

Healthcare Lifesciences; South Logan, Utah). The islets, collected from the gradient, were sedimented and washed with Hank's Balanced Salt Solution (HBSS, Cat. No. SH30030.02; GE Healthcare Lifesciences) supplemented with 5% BCS over a sterile 40-mm mesh cell strainer. Islets were placed into Connaught Medical Research Laboratories (CMRL; Cat. No. MT5110CV; Fisher Scientific; Chicago, Illinois) medium supplemented with 10% FBS, 1% antibiotic/antimycotic (Cat. No. 15240062; Thermo Fisher Scientific; Massachusetts, USA), and 1% glutamax (Cat. No. 35050061; Thermo Fisher Scientific) and allowed to recover for 24 hours in an incubator at 37°C and 5% CO<sub>2</sub>.

#### *Dispersing islets into single cells*

Native rat islets were dispersed into single cells according to our previously published protocol (Ramachandran, Williams et al. 2013). In short, islets were collected in 50 ml tubes and centrifuged. Then the islets were washed twice with calcium and magnesium free (cmf) HBBS (Cat. No. SH30031.02; GE Healthcare Lifescienc. After that, the digestion medium, consisting of cmf-HBSS supplemented and papain (3 units/ml; Cat. No. 9001-73-4; Worthington), was added to the islets and the suspension was incubated on a rotator at 37°C for a maximum of 20 minutes. Islets were further dispersed using a pipette until the media primarily contained single cells. Digestion was stopped using CMRL medium containing calcium and magnesium. The cells were subsequently washed to remove the papain. Cells were brought up in CMRL medium containing 10% FBS, 1% antibiotic/ antimycotic and 1% glutamax before mixing them with MSCs in appropriate proportion. Hemocytometer counts were performed to determine the number of islet cells.

#### *Establishing and passaging MSCs*

Bone marrow MSCs from Sprague Dawley rats (Cat No. RASM-01001; Cyagen Biosciences Inc; Santa Clara, California) were thawed according to manufacturer's instructions.

In brief, the cryovial containing MSCs was quickly thawed at 37°C in a water bath. Cells were transferred to a 50 ml centrifuge tube containing MSC medium supplemented with 10% FBS, 1% penicillin/ streptomycin and 1% glutamine (Cat. No. GUXMX-90011; Cyagen Biosciences Inc.). After centrifugation, the pellet of cells re-suspended in the fresh MSC medium supplemented with 10% FBS, 1% penicillin/ streptomycin and 1% glutamine, were seeded into a T25 flask and incubated at 37°C and 5% CO<sub>2</sub>. The medium was changed every 2-3 days and cells were trypsinized when they were 80-90% confluent. Adherent MSCs cells were detached with trypsin-ethylenediaminetetraacetic acid (trypsin- EDTA; 0.05% trypsin-EDTA in cmf-HBSS; Cat. No. 15400054; Thermo Fisher Scientific) solution for 5-10 minutes at 37°C and 5% CO<sub>2</sub>. Trypsin-EDTA was neutralized by addition of culture medium containing calcium and magnesium. The cell suspension was centrifuged at 435 g for 5 minutes. The supernatant was removed and the pellet was distributed in 75 cm<sup>2</sup> flasks with the MSC medium supplemented with 10% FBS, 1% antibiotic/ antimycotic and 1% glutamine. After the cells reached 70-80% confluency, they were passaged again and cells were reseeded. Once the appropriate number of cells was obtained, MSCs were trypsinized and dispersed in MSC growth media before mixing them with islet cells in defined proportions.

#### *Seeding the cells in the micromold to form hybrid spheroids*

After performing the cell count for islet cells and MSCs using hemocytometer, single cells were mixed in appropriate proportions and seeded into a micromold, as described in our previously published protocol (Ramachandran, Williams et al. 2013). The cells were plated in the micromold with 3ml of culture media, incubated at 37°C and 5% CO<sub>2</sub>. Cells reaggregated and formed hybrid spheroids within 3-5 days, with daily media changes. Subsequently, they were removed from the mold by washing multiple times with the culture medium. Table 4 describes the groups and the proportions of islet cells and MSCs loaded in each spheroid group. Another group of spheroids with 25% islet cells and 75% MSCs were attempted, but too

few spheroids were created to be able to conduct tests with confidence. Thus, only data from the groups shown in Table 4 were utilized for the study.

**Table 4. Proportion of islet cells and MSCs loaded in each group.**

<b>Groups</b>	<b>Proportion of islet cells</b>	<b>Proportion of MSCs</b>
<b>0%</b>	0	100
<b>50%</b>	50	50
<b>75%</b>	75	25
<b>100%</b>	100	0

Groups were named according to the proportion of islet cells loaded in each group. The 50% and 75% groups had both cell types. The 0% group had only MSCs and no islet cells, whereas the 100% group had only islet cells and no MSCs

*Viability assessment using Yo-Pro-1 and propidium iodide staining*

Cell death was determined by double-labeling cells with Yo-Pro-1 and propidium iodide nucleic acid stains (Cat. No. V13243; Thermo Fisher Scientific). Yo-Pro-1 stain selectively passes through the plasma membranes of apoptotic cells and labels them with green fluorescence. Necrotic cells stained red-fluorescent with propidium iodide (Gawlitta, Oomens et al. 2004). 1µl of Yo-Pro-1 and propidium iodide stain each was added to 200µl of culture media containing spheroids from different groups. Images were taken using an Olympus Fluo View 300 confocal microscope, later the images were analyzed using Adobe Photoshop CS 5 extended. 30-150 images were analyzed per group. Total pixels, as well as the red and green pixels were calculated. The percentages of necrotic and apoptotic cells was calculated by dividing the pixels for red/ green staining to the total pixels and multiplying by 100. The proportion of dead cells was assessed on day 1 after the spheroids were formed and at day 14 in culture.

### Glucose stimulated insulin secretion (GSIS)

For static incubation, spheroids were distributed in 96 well plates containing 100  $\mu$ l of Earle's balanced salt solution (EBSS) with 2.8mM glucose. Spheroids from different groups were exposed to glucose concentrations of 2.8mM (low) 16.8mM (medium), 22.4mM (high) and 22.4mM + 60mM KCl (high glucose along with depolarization at high potassium levels) for an hour in replicates of 6. After 60 min of static incubation at 37°C and 5% CO<sub>2</sub>, conditioned media samples were collected and frozen at -80°C. The insulin concentration was later quantified using a rat insulin enzyme linked immunosorbant assay kit (ELISA; Cat No. 80-INSRTH-E01; AlpcO; Salem, NH) as per the manufacturer's instructions. In short, standards and samples were added to the insulin specific monoclonal antibody coated microplate wells. After incubating the samples and standards with the detection antibody for two hours, the plate was washed with buffer. The microplate was again incubated for 15 minutes after adding the substrate. Optical density was measured at 450nm by a spectrophotometer after adding the stop solution. Optical density values were normalized by the number of spheroids as well as number of islet cells for each group. Glucose stimulation index (SI) was also calculated and was defined as insulin secretion per islet cells at 22.4mM glucose to insulin secretion at 2.4mM glucose challenge.

### Morphology of hybrid spheroids

In order to track the interspersions of the islet and MSC in the spheroids, the different cell types were labelled with distinct markers. Cells from pancreatic islets were labelled with Dil (1, 1'-dioctadecyl-3, 3', 3'-tetramethylindocarbocyanine perchlorate; Cat No. 42364, Sigma-Aldrich) and MSCs were labelled with CFDA-SE (carboxyfluorescein diacetate, succinimidyl ester; Cat. No. V12883; Thermo Fisher Scientific) according to the manufacturer's instructions. In short, after centrifugation and aspiration of the supernatant, the MSC pellet was re-suspended in a pre-warmed (37°C) phosphate buffered saline containing 10 $\mu$ M of CFDA-SE. After a 15 minute incubation, the supernatant was removed following centrifugation at 435 g for

5 minutes, and cells were re-suspended in pre-warmed fresh MSC medium with 10% FBS, 1% penicillin/ streptomycin and 1% glutamine for 30 minutes, pelleted and re-suspended in MSC medium with 10% FBS, 1% penicillin/ streptomycin and 1% glutamine. Dil is a lipophilic membrane stain that diffuses laterally to stain the entire cell. In order to stain the pancreatic islet cells with Dil, cells were re-suspended in 1 ml of serum free culture media with 5mM Dil and incubated for 15 minutes at 37°C. Cells were then centrifuged at 1209 g for 5 minutes and re-suspended in CMRL supplemented with 10% FBS, 1% antibiotic/ antimycotic and 1% glutamax.

After labeling, the islet cells and MSCs were mixed thoroughly in different proportions and were seeded into the micromold to allow them to reaggregate and form hybrid spheroids as described previously. Upon removal from the mold, the spheroids were placed on the microscopic slides (Cat. No. 12-550-12; Fisher Scientific) and mounted with anti-fading agent Gel/Mount (Biomedex, Foster City, California). Images were captured using a Nikon C1Si or C1 Plus confocal microscope. Interspersion data could only be obtained from spheroids less than 4 days after formation, because the fluorophores are depleted after that time. Images were analyzed using Image J to determine the percentage of islet cells and MSCs in each hybrid spheroid. In addition, analysis was also done to determine how many spheroids were comprised of a combination of islet cells and MSCs and how many consisted only of MSCs or islet cells alone.

The diameter of each spheroid was measured using Adobe Photoshop CS 5. Light microscopic images were utilized to analyze diameter. Two perpendicular diameter measurements were recorded and averaged for each spheroid. 350- 2000 spheroids were analyzed for each group at days 1 and 14.

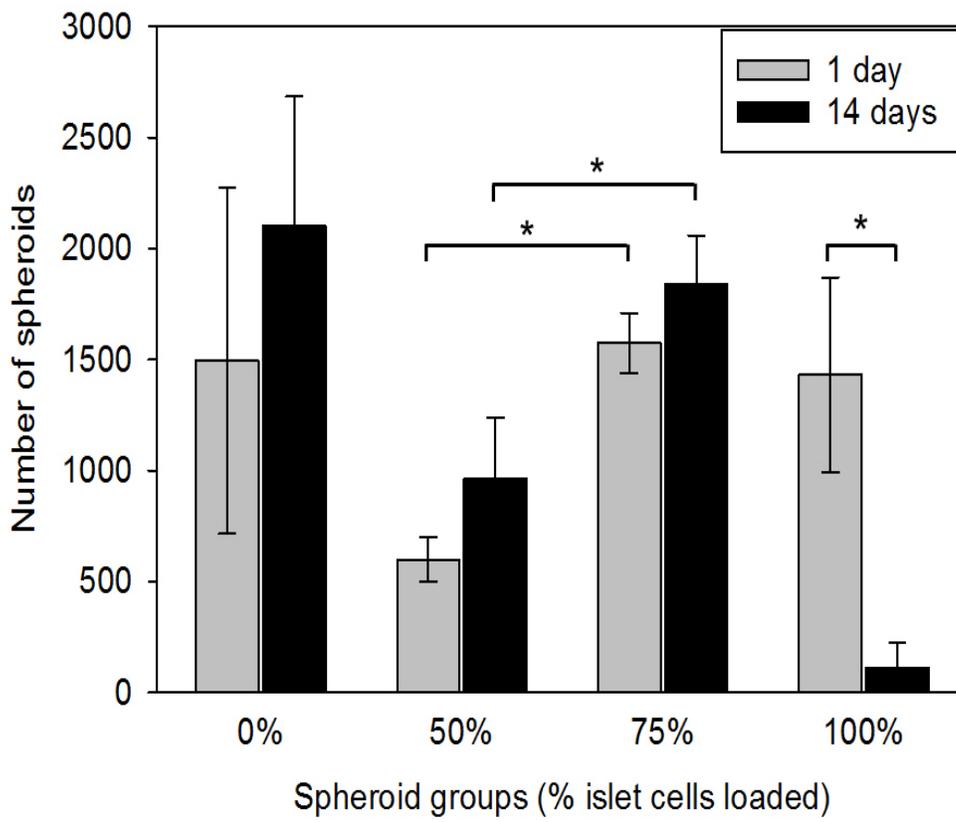
### Data Analysis

SigmaPlot software was utilized for data analysis. To determine significant differences between groups for the number of spheroids, proportion of spheroid type, proportion of islet cells and MSCs, viability, diameter GSIS and SI, one way ANOVA was performed. To determine the significant within group differences at day 1 and day 14 for number of spheroids, viability, diameter and SI, t test was performed. An alpha level of 0.05 was utilized to determine the significance of these findings.

## **4.4. Results**

### Yield

Spheroids were formed within 3-5 days of co-culturing of MSCs and islet cells in the micromold. While approximately the same number of cells was loaded into the molds to form spheroids in each group, the ability of the different cell mixtures to self-aggregate varied widely between the hybrid spheroid groups. The number of spheroids formed at day 1 for the 50% islet cell group was approximately 500, which was nearly 3 times less than the mixtures that had 75% islet cells (Figure 18). Further, another group with 25% islet cells was attempted, but formed too few spheroids for subsequent studies (results not shown). There was no significant difference in the number of spheroids formed in groups 0%, 75% and 100% at day 1. The 0% group (all MSCs) could form clusters in low adhesive petri dishes even without the molds. The number of spheroids present in the 100% islet cell group dropped dramatically over the course of 2 weeks (Figure 1B). In contrast, all groups that contained MSCs (groups 0, 50, and 75%), had no significant difference in spheroid numbers formed day 1 to 14 (Figure 18).



**Figure 18. Group differences in spheroid formation and maintenance.**

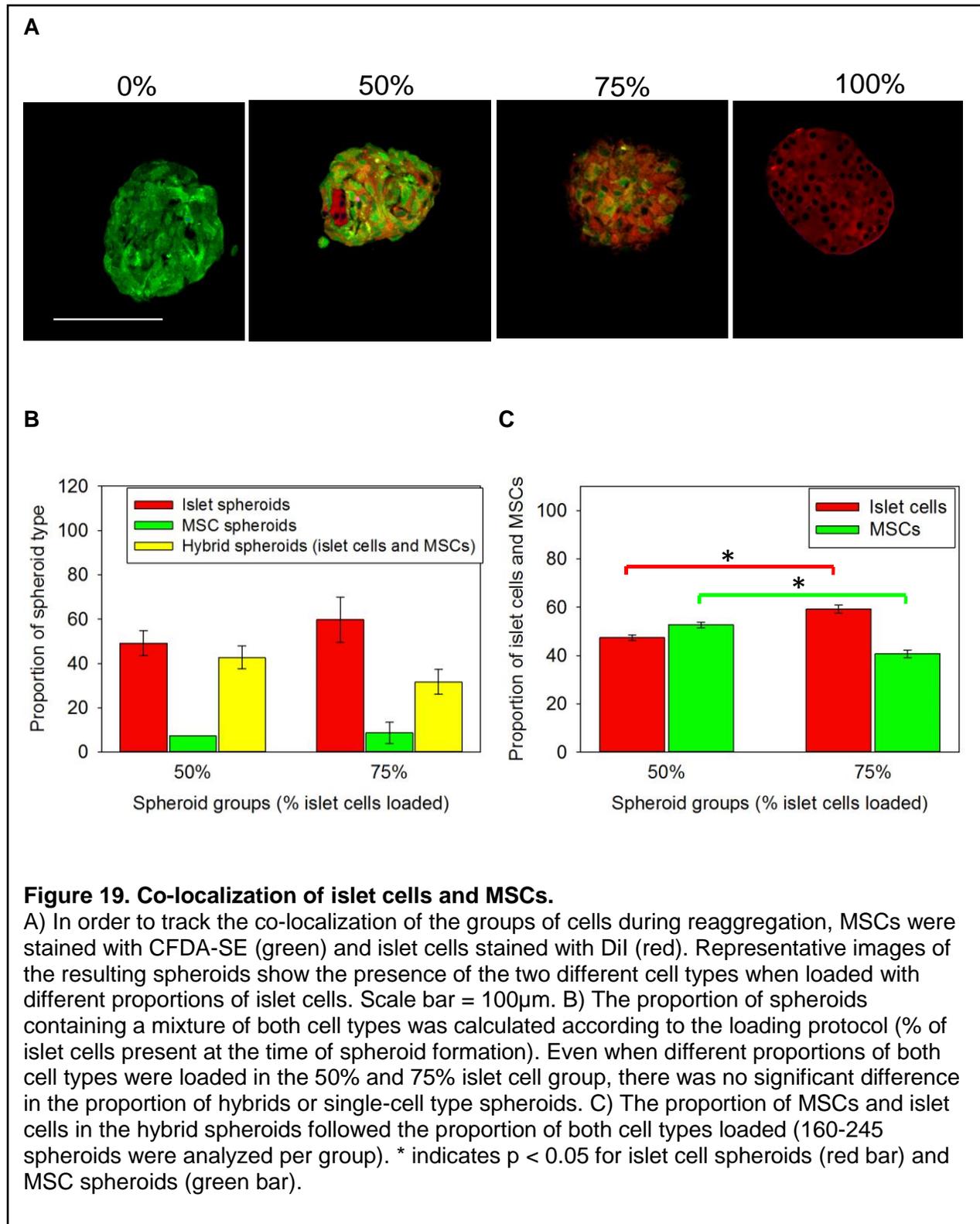
The total number of clusters in each group on day 1 and 14. There were more spheroids formed as the proportion of islet cells increased in the hybrid spheroid groups. There was no significant change in the number of spheroids within groups at day 14 except for the 100% islet cell group. \* indicates  $p \leq 0.05$ .

### Cell interspersion

An image of a representative spheroid from each group is shown in Figure 19A. As evident in the images, more islet cell staining (red) was present as the ratio of islet cells to MSCs increased from the 0% group to 50%, 75% and 100%. Within the hybrid groups, the MSCs and islet cells were either randomly distributed as shown in the example for the 75% group or collected together in regions found within the spheroids as shown in the 50% group example. These differences in cell organization appeared randomly.

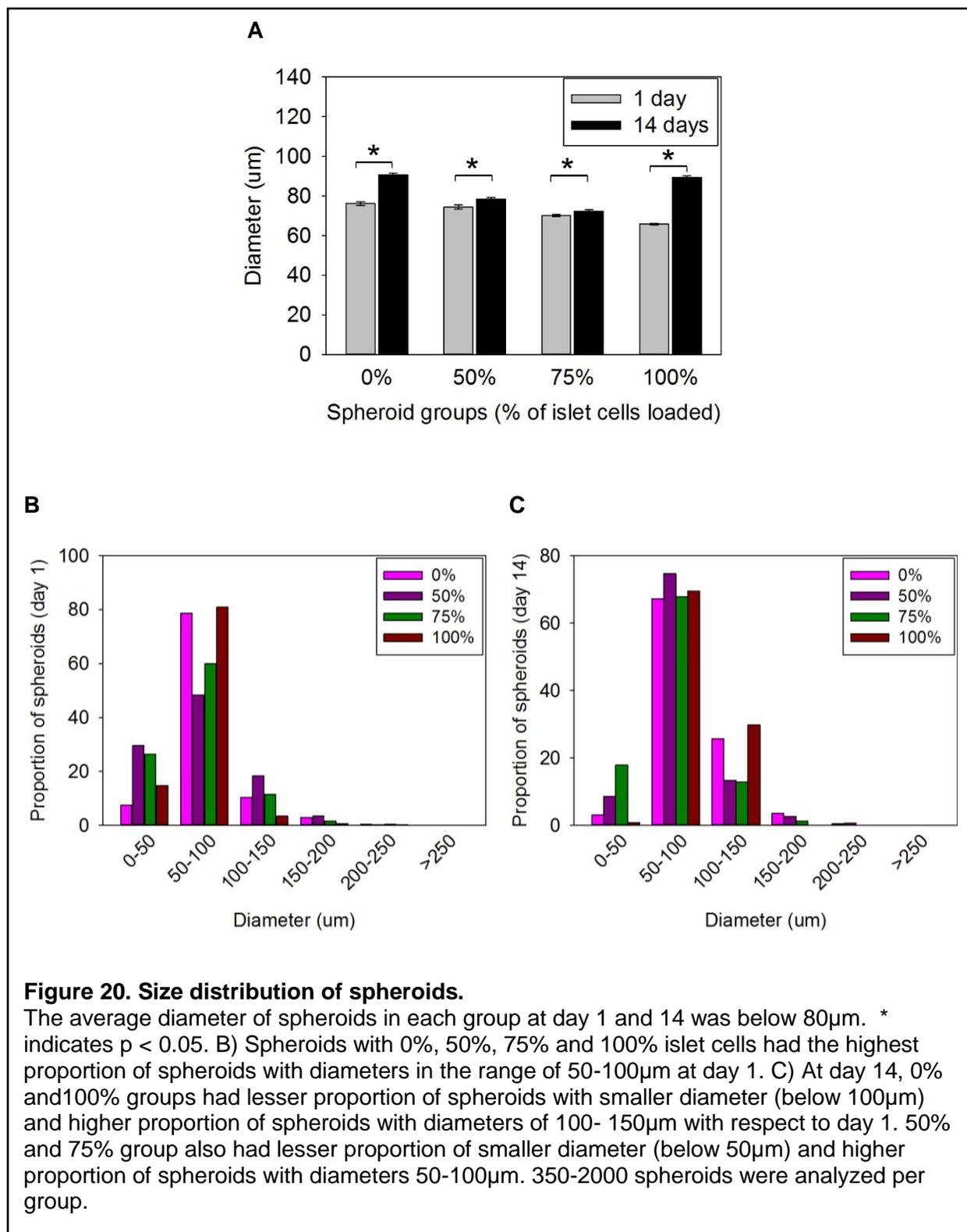
Although cells in the hybrid groups were thoroughly mixed in given proportions prior to loading in the micromolds, some spheroids contained only MSCs or islet cells. The percentages of spheroids consisting of a hybrid of islet cells and MSCs versus islet cells or MSCs alone were calculated (Figure 19B). There was no significant difference in the percentages of spheroids consisting of only islet cells, only MSCs or both between the 50% and 75% hybrid groups even though different proportions of islet cells and MSCs were loaded in the two groups.

Due to the fact that the cells were more likely to form single type spheroids (either MSC or islet-only spheroids), we completed additional analysis on the hybrids. As shown in Figure 19C, the proportion of MSCs or islet cells in the hybrid spheroids (containing both cell types) followed the loading proportions. There were more islet cells and fewer MSCs in the group loaded with 75% islet cells with respect to the group loaded with 50% islet cells.



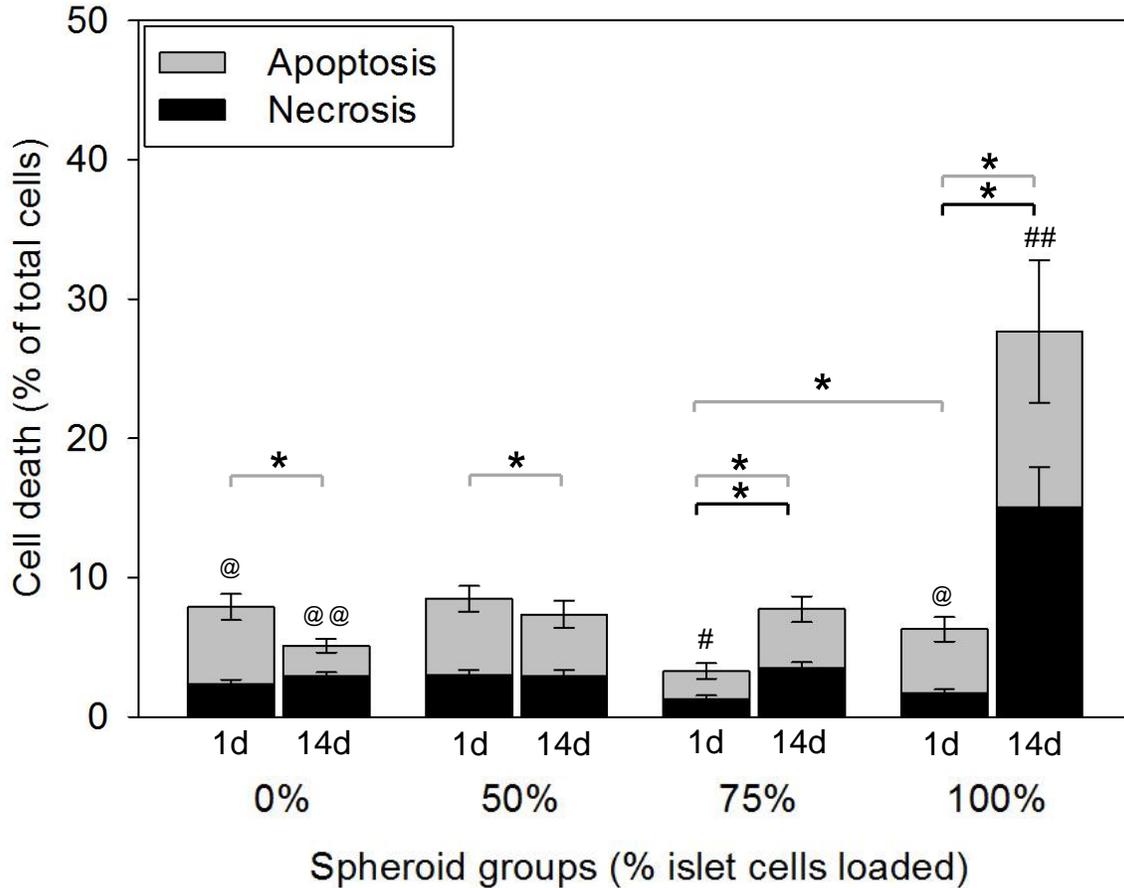
### Spheroid diameter

The potential change in spheroid size over the 14 day culture was important to determine. Figure 20A shows a field of spheroids and the possible time-induced changes in spheroid diameter. The average diameter of the spheroids increased slightly over the 14 day period in all the groups (Figure 20A). A significant increase in the diameter of the 100% islet cell group was surprising and warranted further investigation, because islet cells do not proliferate. On day 1, the majority of spheroids in all the groups were between 50-100 $\mu$ m in diameter (Figure 20B). On day 14, the 100% islet cell group showed a decline in the number of small islet spheroids (under 100 $\mu$ m), but also showed an increase in the number of larger spheroids (over 100 $\mu$ m diameter; Figure 20C). We hypothesized that an apparent increase in the mean diameter at day 14 was either the result of loss of small islet spheroids during subsequent media changes or due to fusion of spheroids as they were in culture. However, other spheroid groups also illustrated a decline in the number of small spheroids and an increase in the percentage of spheroids in the 50-100 $\mu$ m size range.



## Viability

The percentage of dead cells (apoptotic and necrotic) from 1 to 14 days in the different groups is summarized in Figure 21. Initially, each group had less than 10% dead cells. Of all the groups, the 75% group had the lowest proportion of dead cells at day 1, with significantly less necrotic cells in comparison to the 0% and 50% groups and significantly less apoptotic cells in comparison to the 0%, 50% and 100% islet cell groups. Interestingly, there was higher percentage of cell death through apoptosis at day 1 for the 0% and 100% groups compared to the 50% and 75% hybrid groups. Both the 75% and 100% groups had statistically significant increases in the percentage of apoptotic and necrotic cells at day 14 with respect to day 1, with the 100% islet cell group showing significantly higher percentages of both apoptotic and necrotic cells with respect to all other groups at day 14. In contrast, the 0% and 50% group had significant decline in the proportion of apoptotic cells at day 14 with respect to day 1. MSCs alone (0% group) had significantly less apoptotic cells at day 14 with respect to 50%, 75% and 100% groups. Thus, viability was maintained over time when MSCs were incorporated into the spheroids, regardless of the proportion.



**Figure 21. Percentage of cell death (apoptotic and necrotic cells).**

Cell death by necrosis and apoptosis was calculated for each group on days 1 and 14. All the groups had low initial cell death. Cell death was split almost evenly by apoptosis and necrosis in the two hybrid spheroid groups. @ indicates that within group 0% and 100% groups had higher proportion of cell death through apoptosis at day 1 ( $p < 0.05$ ). # indicates that 75% group had significantly less apoptotic and necrotic cells than 0% and 50% groups at day 1 ( $p < 0.05$ ). The 75% group also had significantly less apoptotic cells than the 100% group on day 1. ## indicates that 100% islet cell group had the highest proportion apoptotic and necrotic cells with respect to the 0%, 50% and 75% groups at day 14 ( $p < 0.05$ ). Cell death was evenly split between apoptosis and necrosis in all the groups at day 14, except in the 0% islet cell group, which had fewer apoptotic cells (indicated by @@ with  $p < 0.05$ ). 30- 150 islets were analyzed per group. \* indicates  $p < 0.05$ .

### Glucose Stimulated insulin secretion

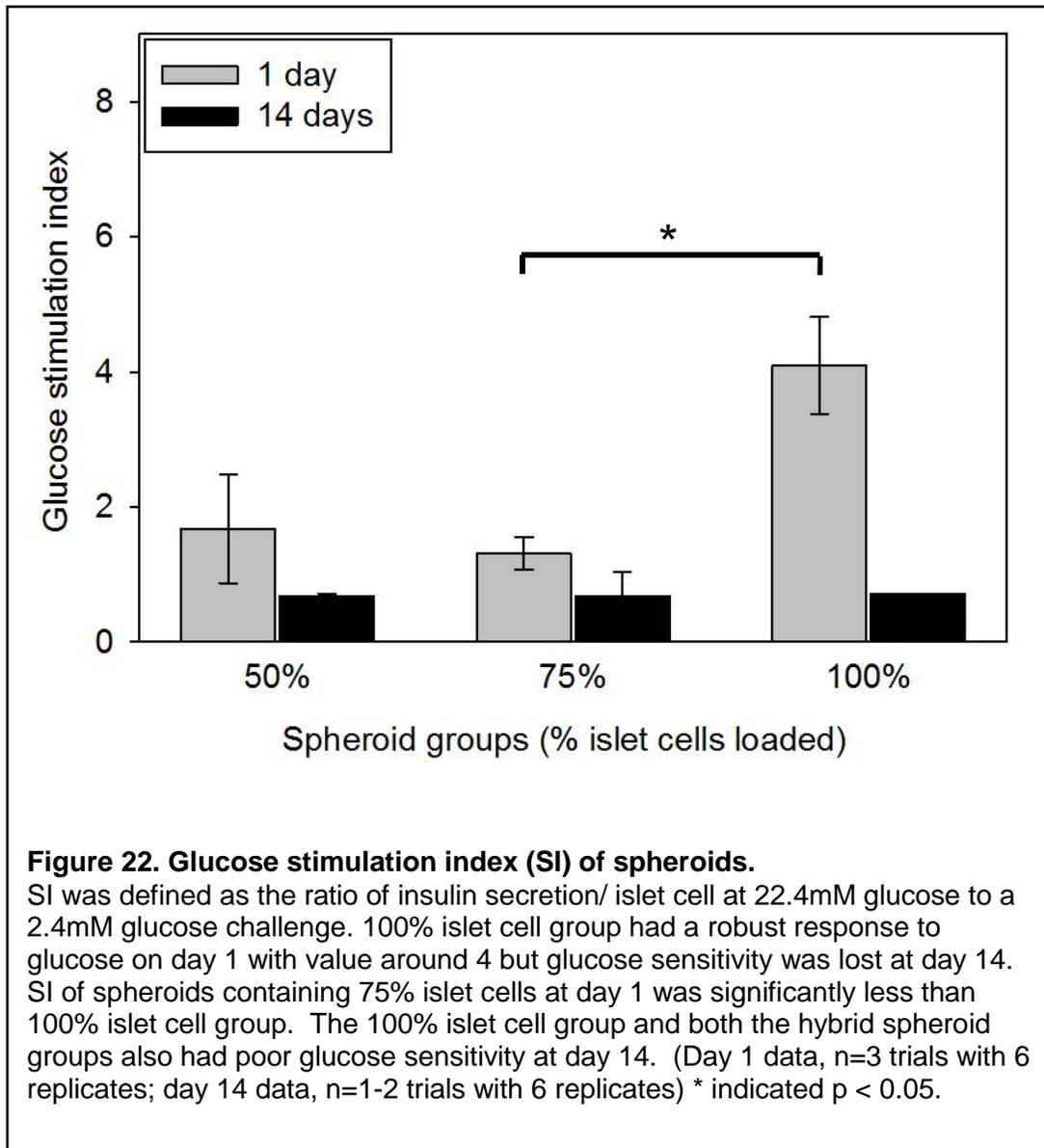
Insulin secretion in response to different glucose concentrations was normalized to the number of spheroids in the assay to assess function (Table 5). Although not significantly different, all three groups of spheroids containing islets cells showed a trend towards greater insulin release with increased glucose concentrations and in response to depolarization with high potassium levels (Table 5). However, at the low glucose concentrations (2.4 and 16.8mM) the 50% islet cell group secreted approximately 1/2 the amount of the 100% group. Insulin secretion/spheroid for the high glucose and depolarization conditions was 1/4 and 1/8 of the level secreted by the spheroids with 100% islet cells. Spheroid groups with lower numbers of islet cells could not be expected to secrete the same amount of insulin as spheroids with only islet cells. Thus, we normalized the insulin secretion to the number of islet cells loaded into the mold in each group. After normalization with the islet cells loaded, a trend towards increased release of insulin to the increasing glucose concentrations was seen again in the 100% islet cell group but not in the other groups (Table 5). In fact, in the 50% islet cells group insulin secretion values at 22.4mM and 22.4mM glucose with depolarization were 5 and 22 times less than the 100% islet cell group, respectively. Whereas in 75% group, insulin secretion values at 22.4mM and 22.4mM with depolarization were approximately 1.5 and 5.5 times less than 100% islet cell group. Thus, normalization by islet cell number still showed a decrease in insulin secretion in all hybrid spheroids with mixed MSCs and islet cells. GSIS was also performed for MSCs spheroids alone, and they had no detectible insulin secretion (results not shown).

**Table 5. Insulin secretion for groups loaded with 50%, 75% and 100% islet cells.**

Spheroid groups (% islet cells loaded)	Insulin secretion (ng/ml/ spheroid)				Insulin secretion (pg/ml/ islet cell)			
	2.8mM glucose	16.8mM glucose	22.4mM glucose	22.4mM glucose+ KCl	2.4mM glucose	16.8mM glucose	22.4mM glucose	22.4mM glucose +KCl
<b>50%</b>	0.27±0.18	0.37±0.31	0.44±0.37	0.58±0.48	2.14±0.67	6.14±5.26	4.61±3.32	3.52±2.64
<b>75%</b>	0.62±0.52	0.59±0.49	0.93±0.83	0.96±0.83	10.9±9.71	4.29±3.11	11.8±10.4	12.0±10.5
<b>100%</b>	0.55±0.23	0.79±0.23	1.71±0.67	4.52±2.29	5.85±3.52	8.17±5.01	19.6±11.0	65.5±41.4

Insulin secretion/ spheroid showed a trend towards greater insulin secretion to increasing glucose concentrations for the group with 100% and 50% islet cells. 100% islet cells had an average of 2-8 times the amount of insulin secretion/ spheroid than 50% and 75% group when exposed to different glucose doses. When insulin secretion was normalized to islet cell, the trend towards greater insulin secretion to increasing glucose concentrations was lost in the 50% islet cell group.

The glucose sensitivity was calculated, based on the stimulation index (SI). SI was defined as the ratio of insulin secretion/ islet cell at 22.4mM glucose to a 2.4mM glucose challenge. The SI of spheroids with 100% islet cells demonstrated a superior response to a high glucose with values reaching slightly above 4 on day 1 (Figure 22). In contrast, the 75% groups had significantly less SI than 100% islet cell group with value slightly above 1, indicating no glucose sensitivity. Although not statistically different than the 100% islet cell group, the 50% group was approximately 1.7, which was still at least 2 times less than the spheroids with 100% islet cells. In analyzing the 14 day data, there was a significant decline in the number of spheroids in the 100% group. Therefore, the 14 day data point for that group consisted of only 1 of the 3 trials, because the others trials did not have enough spheroids left in the culture. SI at day 14 demonstrated a lack of glucose responsiveness in all of the groups (Figure 22).



## 4.5. Discussion

Previous studies have used a variety of techniques to co-culture islet cells either directly or indirectly with MSCs. Rather than indirect co-culture using a transwell system (Karaoz, Genc et al. 2010, Scuteri, Donzelli et al. 2014), or direct co-cultures (Jung, Kim et al. 2011, Rackham, Dhadda et al. 2013), the current study attempted to co-culture different proportions of islet cells and MSCs by incorporating them into the same spheroids. We found that islet cells preferred to reaggregate in spheroids with other islet cells rather than in mixed hybrid spheroids with MSCs. To the best of our knowledge, the only other study attempting to create hybrid spheroids also found that without manipulating the cellular interaction to improve cohesiveness between MSCs and islet cells, MSCs and islet cells preferred to reaggregate with their own cell type (Hoffecker and Iwata 2014).

The results of our study suggested that it is possible to create highly viable hybrid spheroids consisting of MSCs and islet cells. Hybrid spheroids containing different proportion of MSCs and islet cells maintained high viability even after 14 days in culture. These results are in agreement with the previous studies, which have shown that islet co-culture with MSCs improves the islet cell viability and survival (Park, Kim et al. 2009, Park, Kim et al. 2010, Scuteri, Donzelli et al. 2014). Other studies have reported that the viability of islets in direct co-culture was higher than islets in indirect co-culture or islets alone. However, in the published direct co-culture study, viability was approximately 60% at 2 weeks (Jung, Kim et al. 2011), whereas our spheroids were approximately 90% viable at 2 weeks. The improved long-term viability in our study may have been due to the intermingling between the MSCs and the islet cells, as that has been shown to improve function (Jung, Kim et al. 2011, Rackham, Dhadda et al. 2013). However, size may also explain the improved viability over other studies. The only other published study that created hybrid spheroids failed to report their size, and they utilized a gel base to form reaggregated islets with MSCs (Hoffecker and Iwata 2014). The majority of the

hybrid spheroids in our study had diameters less than 100 $\mu$ m, hence our hybrid spheroids were under the size limitation shown to cause core cell death (Williams, Huang et al. 2010).

Unfortunately, the hybrid spheroids tested in this study had a diminished insulin secretion in relationship to glucose concentrations, even when insulin secretion/islet cell was estimated (Table 5). Our GSIS results are in contrast to previous studies that support the concept that MSCs improve insulin secretion of islets (Park, Kim et al. 2010, Jung, Kim et al. 2011). Namely, Park et al. showed that when islets were in indirect co-culture with MSCs, there was no significant increase in GSIS at 60mg/dl (3.3mM) glucose, but at 300mg/dl (16.7mM) glucose, islets with MSCs showed a modest (37.5%) increase in the insulin secretion compared to islets cultured alone (Park, Kim et al. 2010). In another study, islets co-cultured with MSCs indirectly, showed no significant increase in insulin secretion, whereas islets directly co-cultured with MSCs had approximately 69% increase in insulin secretion at 2 weeks (Jung, Kim et al. 2011). Cell death was prevented when MSCs were co-cultured with islets previously exposed to Further, a few studies have found that co-culturing of MSCs along with islets previously exposed to either cytokines or streptozotocin prevented cell death in islets and also maintained their SI/ glucose secretion (Karaoz, Genc et al. 2010, Yeung, Seeberger et al. 2012). In contrast to these positive results, we and others report a lack of enhanced glucose sensitivity with co-cultured MSCs. In one study, islets and MSCs were incubated for 3 hours in suspension resulting in adherence of the MSCs to the exterior of the islets. In this condition, the SI of the islet-MSCs spheroids was not significantly different than islets alone (Duprez, Johansson et al. 2011). In another study, when human islets were coated with MSCs and endothelial cells, the insulin secretion of these spheroids was not different than the islets alone (Johansson, Rasmusson et al. 2008). Karaoz et al. found that when islets were indirectly co-cultured with MSCs for 14 days and challenged with low and high conditions, there was no significant difference in the amount of insulin released with respect to islets cultured alone. However, when the islet/MSC co-

cultures were first stressed by exposure to streptozotocin, their insulin secretion was similar to normal non-stressed islets and better than stressed pure islets (Karaoz, Genc et al. 2010). Finally, even when spheroids formed by manipulating the cellular interaction in order to improve cohesion between islet cells and MSCs were exposed to 0.1 g/dl (5.55mM), 0.3g/dl (16.65mM) and then again to 0.1g/dl (5.5mM) glucose concentrations, there was no significant difference in the amount of insulin secreted at different glucose concentrations with respect to the non-manipulated islets, hence showing that co-culturing did not improve glucose sensitivity (Hoffecker and Iwata 2014). In contrast, intermingling the islet cells and MSCs actually resulted in a decline in insulin secretion per cell.

A major difference in our approach compared to the previous co-culture publications lies in our method for incorporating the MSCs into the islet spheroids in a way that altered the normal islet-to-islet cell interaction. While such a configuration dramatically improved islet viability, it failed to improve insulin secretion. It is quite possible that the 3D cell-to-cell interaction in an islet cell spheroid is essential to normal glucose sensing and insulin secretion. This theory is supported by different studies emphasizing the importance of cell-to-cell interactions between  $\beta$ -cells for normal functioning of islets (Halban, Wollheim et al. 1982, Pipeleers, in't Veld et al. 1982, Hopcroft, Mason et al. 1985, Hauge-Evans, Squires et al. 1999). The results of this study provide strong evidence supporting the importance of cell-to-cell interactions in islets for glucose-sensitive insulin secretion.

#### **4.6. Conclusion**

The purpose of this study was to measure changes in the morphology, survival and function of hybrid spheroids comprised of islet cells and MSCs in different proportions with the goal of improving islet culture. We show that hybrid spheroids have improved survival but islet cells could not maintain their function when they were randomly combined with MSCs in

spheroids. Thus, co-culturing islets and MSCs without disruption of the islet architecture may be a superior method for islet culture.

## **Chapter 5**

### **Summary of findings, discussion and future direction**

## 5.1. Summary of findings

Kanslets, created by reaggregating islet cells, have previously been shown to have higher viability and greater *in vitro* insulin secretion than native islets. Furthermore, they have been successfully used for maintaining blood glucose levels in diabetic rats after transplantation (Ramachandran, Williams et al. 2013). Previous data on drug testing has shown that Kanslets can be used as an alternative tissue for drug screening/testing (Ramachandran, Peng et al. 2014). To advance research in the field of diabetes, in this research, I worked on (1) optimizing the cryopreservation procedure of pancreatic islets, (2) optimizing the drug screening/discovery process for diabetic drugs as well as (3) optimizing the islet culture conditions utilizing Kanslets.

Conventionally, islets have been cryopreserved as intact native islets (Rajotte, Warnock et al. 1983, Lakey, Rajotte et al. 2001). Many single cell types have shown to be more tolerant to cryopreservation procedure than multicellular structures like pancreatic islets. Accordingly, I proposed that pancreatic islets be cryopreserved as single cells and reaggregated in the micromold following thawing to form cryopreserved Kanslets. Cryopreserved Kanslets were highly viable and metabolically active. Cryopreserved Kanslets were able to restore and maintain blood glucose levels in diabetic rats until sacrificed at 10 months post transplantation. This technological advancement opens opportunities for building cell banks of islet cells with subsequent reaggregation into viable islets when needed for either islet transplantation or research.

With cryopreservation techniques optimized, new applications for islets in research could be introduced. Bringing a new drug to the market is a lengthy and an expensive procedure. Much of the failure lies in the inability to reproduce the results in preclinical studies and in clinical trials. In addition, the number of repetitions at each stage of the drug screening discovery process creates added cost to the procedure. Hence, I proposed to create multi-donor Kanslets containing a mix of cells from two different human donors, hoping that the multi-donor

method would provide an average drug response potentially eliminating the need for assay repetition. Thawing and mixing cells from two cryopreserved donors generated highly viable Kantslets, but neither the multi-donor nor did the single-donor Kantslets used in the experiments were highly responsive to the drugs tested.

We also combined different cell types into the micromold to create hybrid Kantslets. Specifically, we co-seeded mesenchymal stem cells (MSCs) along with islet cells in the micromold, as MSCs have recently been of interest for their potential benefits to islet viability and function. Culturing islets in direct contact with MSCs has been shown to be superior to indirect co-culture where islets and MSCs are separated by inserts. In order to have MSCs in even more proximity to islet cells, I proposed to integrate MSCs with islets cells during reaggregation to create hybrid spheroids of MSCs and islet cells. I bioengineered hybrid spheroids containing different proportion of MSCs and islet cells, and performed *in vitro* studies to look at their architecture, diameter and stimulation index. Hybrid spheroids were viable irrespective of the proportion of MSCs incorporated. However, functionally hybrid spheroids were not sensitive to the increasing glucose challenges when compared to spheroids containing 100% islet cells, providing the evidence that the cellular interaction between  $\beta$ -cells is important to maintain its function.

## **5.2. Discussion**

### **5.2.1. The implications for islet transplantation**

Cryopreservation of pancreatic islets is necessary to make islet transplantation a feasible option for more people with diabetes. Long-term storage provides enough time for organization of islet transplantation, for tissue matching, and to accumulate the minimum number of islets from multiple donors, needed for a successful transplant. Thus, the findings in this dissertation may have direct and significant implications to the world of islet transplantation.

Islet transplantation is the isolation of pancreatic islets from a donor, and placement into either the recipient's hepatic portal system or under the kidney capsule or omentum. Since the initial attempts in 1882, the field of islet transplantation has evolved significantly. With the advent of the Edmonton protocol in 2000 through 2012, more than 750 islet transplantations had been performed worldwide (McCall and Shapiro 2012). It has been reported multiple times that the number of islets transplanted is one of the most important factors in determining the success of the transplantation (Shapiro, Lakey et al. 2000, Clayton, Davies et al. 2003, Morgan, Theruvath et al. 2012). For a successful transplantation, it is recommended to that at least 9000IEQ/kg of body weight of the recipient be transplanted, and this often requires islets from 2-4 donors (Markmann, Deng et al. 2003). However, because of the limited availability of donors at any given time, typically the recipient must undergo repeated transplant procedures to eventually get to the minimum necessary islet volume.

In the clinic, islet transplantation is performed immediately after isolation of the pancreatic islets from the donor. If the islets do not qualify for islet transplantation or if a sufficient amount of islets cannot be obtained from the isolation, then islets are shipped for research purposes all over the country. In this research, I optimized the cryopreservation

procedure of pancreatic islets so that islets can be pooled from different donors and it would also allow clinicians more flexibility in scheduling the islet transplantation.

In addition to the shortage of islets from donors and the inability to pool them into one transplant procedure, another important confounding factor in islet transplantation is the loss of functional islets during the early post transplantation period. This loss of function has detrimental effects on the outcome of individual grafts (Davalli, Scaglia et al. 1996). Previous studies have shown that a decline in islet function and survival is highest within the first week after islet transplantation (Davalli, Ogawa et al. 1995, Jirak, Kriz et al. 2009). Inadequate blood supply is considered as one of the reasons for immediate islet loss and graft failure. Newly transplanted islets experience limited blood supply for several days before the blood vessels start to form. Even when the new blood vessels have formed, islets still receive a diminished nutrient supply compared to islets in the native pancreas. Notably, small diameter islets have shorter diffusion distance than large diameter islets. Indeed, small islets have shown to be more viable in culture and more successful in islet transplantation. Our micromold restricts the size of the Kanslets to 125-150 $\mu$ m in diameter and Kanslets created by reaggregating islet cells in micromold have shown to be highly viable and were also successful in reversing hyperglycemia in diabetic rats (Ramachandran, Williams et al. 2013). Cryopreserved Kanslets were created by reaggregating cryopreserved and thawed single islet cells in the micromold. Cryopreserved Kanslets were highly viable and reversed hyperglycemia in diabetic rats even when 15%-30% less islet volume was transplanted with respect to fresh native islets. Multiple previous studies reported that cryopreservation improves the purity of islets. Although we did not assess the purity of islets after cryopreservation in our study, but one of the factors affecting our success could be the improved purity of the CP Kanslets. The efficiency of small CP Kanslets with their high viability is an important finding of my dissertation.

One of the major limitations of islet transplantation is the limited availability of pancreatic tissue, which does not proliferate in culture. Researchers are currently looking at different sources like bone marrow stem cells, hepatocytes or intestine epithelial cells, pancreatic acinar or ductal cells to generate pancreatic islets (Todorov, Omori et al. 2006). Alternatively, strategies for expanding human islets in culture are also being investigated (Kayali, Flores et al. 2007). Some of these studies have shown promising results in expanding and then differentiating ductal cells into islet like tissue (Bonner-Weir, Taneja et al. 2000). Our novel and successful cryopreservation technique, enables long term storage of expanded or differentiated cells, which can subsequently be thawed and reaggregated into Kanslets using our micromold whenever needed for islet transplantation.

In the study described in the 4th Chapter, I integrated MSCs with islet cells into spheroids. These hybrid spheroids were highly viable in long term culture. However, the hybrid spheroids could not maintain *in vitro* function of insulin secretion. *In vivo* studies might provide more insight into possible overall effects of the integrating of MSCs and islet cells. As I showed with the cryopreservation study, there was a less than impressive *in vitro* response to glucose. Yet, the CP Kanslets impressively reversed diabetes in rats for 10 months. As MSCs are integrated with islet cells in spheroids, these hybrid spheroids have continuous exposure to the benefits of MSCs. It would be important in future studies to see if hybrid spheroids would provide better transplantation outcomes than islets alone, and if they could help with islet engraftment, circumventing the need for islet re-transplantation.

### **5.2.2. The implications for drug testing and diabetes research**

The ability to bank human islet cells, and subsequently reaggregate them into viable islets, opens new opportunities for the utilization of human islets, for drug screening and therapeutics. Utilizing the approach of dispersing native islets and allowing them to reaggregate, provides the opportunity to create Kanslets containing islets from different donors. With the

significant increase in the number of people suffering from type 2 diabetes, there is definitely a need to discover new anti-diabetic drugs. Traditionally, 2 dimensional (2D) cell cultures have been used to perform drug testing for anti-diabetic drugs. In 2D cell cultures, cells are cultured on flat, rigid plastic substrates, which are very different from cell's *in vivo* architecture.

Bioengineered Kanslets created using our micromold are 3D structures that offer a practical alternative to 2D cell culture. Kanslets have been tested previously for different diabetic drugs, and the results have been compared with native human islets, and rat islets. Kanslets were responsive to different drugs, and in some cases, were an even better predictor of a drug response (Ramachandran, Peng et al. 2014). According to the Tufts Center for the Study of Drug Development, currently it takes more than 10 years, and approximately 2.6 billion dollars from drug discovery stage to get that drug to the market (Avorn 2015).

During the preclinical phase when *in vitro* drug studies are conducted, the assays typically need to be repeated to estimate an average response, whether it is for testing efficacy or toxicity. In this project, I desired to determine whether it was possible to combine the islet cells of two different donors, and form multi-donor Kanslets, and determine whether these multi-donor Kanslets could give an average dose response compared to the two individual donors used in the study. Unfortunately, our single- and multi-donor Kanslets were not as responsive to the different drugs tested as was expected.

It took several months to collect islets from 4 different donors, and because of that slow retrieval process, islet cells had to be cryopreserved for 5-7 months. I believe that the longer duration of cryopreservation could have affected the results of our drug studies. However, without repeating the same drug studies with Kanslets formed from cells cryopreserved for only a short length of time, this hypothesis can't be confirmed at this time. The multi-donor concept could be one of the ways to minimize repetitions and make the drug testing procedure more

efficient. However, it is possible that this concept works only with fresh Kanslets instead of cryopreserved ones.

While testing fresh Kanslets is sound theoretically, the implementation of such a plan would be very difficult. There is always a gap of days or months between the availability of islets from different donors through national consortium that supplies the tissue for research. Working with fresh islets to create multi-donor Kanslets would require the arrival of a minimum of two donors meeting the inclusion criteria within a 3 day period. That would only be possible, if the source of tissue was a company like Prodo Laboratories (Irvine, CA). However, their charges for islets are nearly 4 times higher than those of the non-profit national consortium. This same problem would prevent researchers from simply mixing intact native islets from different donors for a drug screen. In addition, using mixed intact native islets adds the complication of varying islet sizes that require an additional, and often ineffective step, of normalizing all results to islet volume or cell number (Ramachandran, Huang et al. 2015).

In our drug testing experiments, we tested glibenclamide, an insulin secretagogue, and somatostatin, an insulin secretion inhibitor, which act through potassium and calcium channels, respectively. In my 3rd Chapter, I did not look at the integrity of these channels after cryopreservation and thawing. It is possible that the process of cryopreservation left the cells viable, but damaged the individual channels, however until and unless further analysis is completed on the integrity and function of channels, we cannot conclude anything. It would be an interesting, and useful future study to investigate the functioning of channels after cryopreservation and thawing.

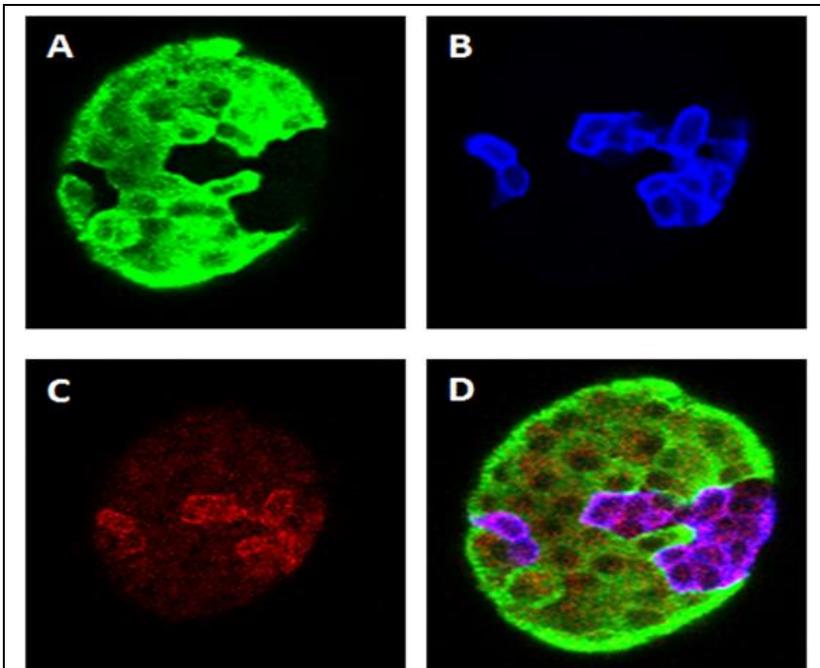
As an alternative to the CP islet clusters, there are multiple  $\beta$ -cell lines that have been discovered and used for drug testing. Although it is known that the  $\beta$ -cell lines do not fully mimic the *in vivo* insulin secretory response to glucose, they can still serve as an early (1<sup>st</sup> stage) screen. A few of the most appropriate cell lines,  $\beta$ --hyperplastic islet-derived cells ( $\beta$ HC-9),

transgenic C57BL/6 mouse insulinoma cell line (MIN6) and insulinoma cell line (INS-1) retain normal glucose regulation of insulin release (Miyazaki, Araki et al. 1990, Efrat 1999). Our own laboratory has a manuscript in press using a humanized version of INS-1 in 3D culture to test known diabetes drugs (Amin, Ramachandran et al, 2016). Other than our own laboratory work, other groups have used these cell lines to create 3D clusters by allowing cells to reaggregate in medium droplets using either low attachment plates or agarose gels. With these techniques, the diameters of the final clusters are quite variable and tend to be larger than the Kantslets tested here (Longati, Jia et al. 2013, Wen, Liao et al. 2013). 3D clusters of  $\beta$ -cell lines made using our micromold can be an alternative way to perform drug testing.

Another challenge for drug testing using human islets has been the length of the study. Many drugs are not designed to induce the release of insulin upon ingestion. Rather, the target of the drug is designed to enhance islet function over a long period of time. Thus, exposing islets or Kantslets to a single application of the drug and examining immediate effects may result in false negative findings. Yet, functional islets cannot be cultured for the amount of time necessary to uncover long-term effects. Maintaining the viability and function of islets during extended culture has been a challenge that researchers have attempted to overcome. Co-culturing islet cells with MSCs, either by separating both cell types using inserts, or by allowing both cell types to be in contact with each other, is one way to attempt to extend the life of the islet cells (Park, Kim et al. 2010, Jung, Kim et al. 2011). I integrated MSCs along with islet cells in different proportions into spheroids so that MSCs are in close proximity to islet cells. Hybrid spheroids had excellent viability but, surprisingly, could not maintain their *in vitro* function. We hypothesize that the loss of the endocrine cell-to-cell contact may have been the cause of the decreased function. The findings emphasize the importance of the interaction among the natural cells of the islet for their function. It is possible that this model can be utilized for further

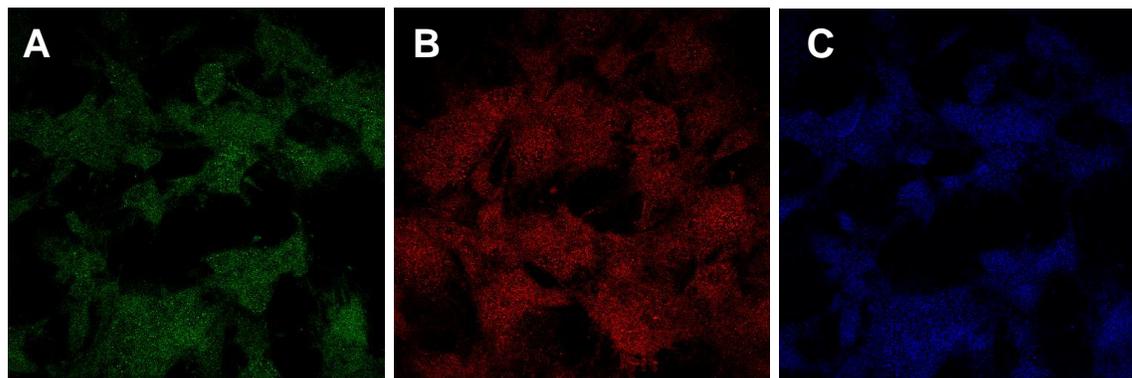
functional islet studies, but would not be appropriate as a therapeutic agent, such as for transplantation.

MSCs obtained from other sources like placenta or umbilical cord have higher proliferative and differentiation capabilities than MSCs derived from adult tissues like bone marrow (Hass, Kasper et al. 2011). One of the possible ways that could have helped the hybrid spheroids secrete more insulin would have been if the bone marrow MSCs in our study had differentiated into  $\beta$ -cells. Due to difficulties faced with staining MSCs, I could not determine whether MSCs differentiated into  $\beta$ -cells or any other endocrine cell type. The challenge came because the antibodies normally used to identify MSC's, also co-stained the islet cells. When hybrid clusters were stained for MSCs,  $\beta$ -cells and  $\alpha$ -cells, MSCs co-stained with  $\alpha$ -cells (Figure 23). In order to confirm if MSCs are differentiating into  $\alpha$ -cells, we stained MSCs grown on microscope slides for insulin ( $\beta$ -cells), glucagon ( $\alpha$ -cells) and somatostatin ( $\delta$ -cells), MSCs stained for all the three cell types (Figure 24).



**Figure 23. Immunofluorescence image of hybrid clusters.**

Hybrid clusters containing islet cells and MSCs were stained for  $\beta$ -cells (A, green),  $\alpha$ -cells (B, blue), MSCs (C, red) and  $\beta$ - $\alpha$ -MSC cells (D).



**Figure 24. Immunofluorescence image of MSCs.**

MSCs stained for  $\beta$ -cells (A, green),  $\alpha$ -cells (B, red) and  $\delta$ - cells fluorescing blue, C.

### **5.3. Future directions**

Although not ideal, when research is initiated in an area where little previous data exists, extrapolation from work in related areas may be necessary. The present study is one of the only studies which attempted to examine how the property of reaggregation of islet cells can be used to advance different fields of diabetes research such as cryopreservation of pancreatic islets, drug screening discovery for diabetic drugs and pancreatic islet health.

#### **5.3.1. Refinement of steps in our cryopreservation protocol**

We looked at how islet cells could be reaggregated to form highly viable and functional islets after cryopreservation. However we had approximately 21% islet cell loss during cryopreservation and thawing even under our optimized conditions. Although this loss is comparable to previous cryopreservation procedures, it still would be wise to work on the refinement of the steps in the single cell cryopreservation protocol so as to have even less tissue loss. Possible ways through which this could be achieved could be by making some modifications in our single cell cryopreservation protocol, like trying to minimize the steps involved in the cryopreservation process. It would be interesting to test whether a controlled rate of cooling using a controlled rate freezer could help to achieve even better outcomes than cooling using a Coolcell.

#### **5.3.2. Determining the appropriate length of time for cryopreservation of islet cells**

Our studies were the first to suggest that the duration of cryopreservation has a strong influence on islet function for both intact islets and single islet cells. In Chapter 2, when native islets were cryopreserved for 4 weeks or longer their viability was reduced by approximately 80%, whereas islets cryopreserved as single cells were highly viable when cryopreserved for up to 4 months. However, in work described in Chapter 3, when we cryopreserved islet cells for 5-7 months for our drug testing studies, our single-donor and multi-donor Kanslets were minimally

responsive to increasing glucose concentrations, suggesting that there may be a cut off time point for cryopreservation when the cells may still be alive, but not functional. In future studies, it will be imperative to determine the maximal time that cells can be cryopreserved without losing their function. This will add to data currently obtained in this dissertation, and in the future, will help clinicians and researchers determine how long islet cells can be cryopreserved, so that viable and functional tissue could be obtained after thawing.

### **5.3.3. Damage to potassium or calcium channels during cryopreservation and thawing**

Our single-donor and multi-donor Kanslets, although being highly viable, did not respond to different drugs tested. Potassium and calcium channels play an important part in regulating insulin secretion upon glucose stimulation. One of the other possible reasons for islets not responding to different drugs being tested could be damage to the ion channels of  $\beta$ -cells. Electrophysiological studies, such as patch clamp, could help determine whether the cryopreservation procedure damages the ion channels.

### **5.3.4. Multi-donor drug testing with CP Kanslets (cryopreserved for short length of time), fresh Kanslets and native islets**

Our cryopreserved single-donor/multi-donor Kanslets were not responsive to different drugs tested. Although not tested, but we assume that longer duration of cryopreservation of our single cells could have affected the results of drug testing experiments; repeating this experiment using Kanslets cryopreserved for shorter duration would be important. In the future, it would be worthwhile to experiment using fresh islet cells, and if at all possible, to get the islets from different donors at the same time. If the multi-donor concept does not work with fresh Kanslets also, I think performing multi-donor drug testing by simply mixing intact native islets from different donors would also be a worthwhile experiment.

### **5.3.5. Integrating lower proportions of MSCs into spheroids**

Irrespective of the proportion of MSCs and islet cells in the spheroids, all spheroids groups had excellent viability even long term in culture. A major difference in our approach was the incorporation of the MSCs into the islet clusters in a way that disturbed the normal islet-to-islet cell interaction. While such a configuration dramatically improved islet viability, it failed to improve insulin secretion. It is quite likely that the 3D cell-to-cell interaction in an islet cell cluster is essential to normal glucose sensing and insulin secretion. In future studies, it would be interesting to determine if lower proportions of MSCs (i.e well below 25%) incorporated into the islet spheroids would maintain or improve insulin secretion in long term culture.

### **5.3.6. Analysis of immune modulatory, angiogenic, anti-apoptotic and trophic factor effects of hybrid spheroids**

When we created these hybrid spheroids of MSCs and islet cells, we completed *in vitro* analysis of their diameter, viability as well as insulin secretion. Performing additional experiments on the culture supernatant to identify if hybrid clusters released trophic molecules like interleukin (IL)-6, vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), transforming growth factor-  $\beta$  (TGF-  $\beta$ ) and tumor necrosis factor  $\alpha$ -(TNF  $\alpha$ ), would have provided us more information on the function of the MSCs. Looking at the expression of various molecules like Ki67 and anti- apoptotic molecules like heat shock protein would have helped us provide more information about the behavior of MSCs when they are contained in a defined geometry of a hybrid cluster.

### **5.3.7. Performing transplantation studies with hybrid clusters**

Our data suggested that hybrid spheroids produced with 50% MSCs and 50% islet cells had a stimulation index not statistically significantly different from spheroids containing 100% islet cells at day 1. In the future studies, it would be vital to examine if these hybrid spheroids

could restore normal blood glucose levels after transplantation in diabetic rodent models. Also, it would be interesting to elucidate the potential benefits of islet-MSC hybrid spheroids for grafts survival and function. Future research could examine possible mechanisms for graft survival, which may include prevention of islet loss during the early days of transplantation, prevention of immune rejection, or promotion of neovascularization.

#### **5.4. Overall summary**

This dissertation looked at the implications of islet cells reaggregation to different areas like cryopreservation, drug testing and islet health in culture. This project is innovative because this is the first study to cryopreserve human islets as single cells and later reaggregate them to form viable islets. The work resulted in a US patent-pending. Results of this study will help optimize islet storage techniques. The ability to bank the human islets cells will open up new opportunities for utilization of human islets for diabetes research and islet transplantation. This is also the first study to create multi-donor islet clusters containing islet cells from two different human donors for drug testing procedures. Furthermore, to the best of my knowledge, this is the first study to successfully create hybrid spheroids containing islet cells and MSCs without any chemical manipulation of any of the two cell types. With this dissertation, we have obtained a greater understanding of implications of islet cell reaggregation to advance the field of diabetes research. The results of this study will have a significant positive impact on the current islet transplantation research. I also expect that all the work in this dissertation will not only unfold the complexities concerning drug testing and islet culturing, but will also be a helpful guide to researchers working in this or related fields.

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