EVALUATION OF INSULIN SECRETION BY *IN VITRO* GENERATED HUMAN ISLET-LIKE CLUSTERS

by

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Abstract

Type 1 diabetes is an autoimmune disease in which patients' insulin-secreting beta cells in pancreatic islets are destroyed by their own immune system, leading to unregulated blood glucose levels and severe complications. Its only treatment is intensive insulin therapy, which carries the risk of hypoglycemic episodes and can result in seizures, coma, and even death. Islet transplantation has recently become an alternative, albeit experimental, treatment for type 1 diabetes patients. More than one donor graft is usually required to render recipients insulin independent, making the shortage of donor tissue an extremely important challenge in islet transplantation. Identifying the cell type that has the ability to differentiate into islet-like tissue is an important area of study.

In this study, I hypothesized that insulin secreting human islet-like clusters could be generated from pancreatic ductal cells, a potential pancreatic progenitor cell type. Islet-like clusters were generated using crude exocrine tissue from human cadaveric donors. This crude exocrine tissue contained a large number of ductal cells, as well as other pancreatic cell types. To evaluate insulin secretion by human islet-like clusters, a static incubation system was set up and tested using Min6 cells, a known insulin-secreting cell line. Using static incubation, significant increases in insulin secretion by islet-like clusters were observed when the clusters were exposed to higher glucose levels and GLP-1, a known insulin secretagogue. Presence of corresponding C-peptide secretion demonstrated that *de novo* insulin secretion occurred. Furthermore, basal insulin secretion increased as culture stages progressed. An attempt was made to generate islet-like clusters using ductal cells purified by fluorescent activated cell sorting or magnetic activated cell sorting. Nevertheless, it was difficult to ensure survival and proliferation of purified ductal cells. Further studies will be necessary to confirm the role of ductal cells in the generation of islet-like clusters using the crude exocrine tissue, as well as to identify factors that can promote ductal cells proliferation after cell sorting.

Table of Contents

List of Tables

List of Figures

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Chapter 1 Introduction

1.1 Diabetes, The World, and Canada

Diabetes is a disease with increasing worldwide prevalence. Generally speaking, it is caused by the lack of insulin producing beta cells (type 1 diabetes) or the inability of the body to respond to insulin (type 2 diabetes), both of which result in abnormally high blood glucose levels. Various research groups, along with the World Health Organization, estimated that the number of people with diabetes is likely to double by year 2025 (Wild *et al*, 2004; King *et al*, 1998). The International Diabetes Federation suggested that 3.8 million deaths were caused by diabetes in 2007. In Canada, over 2 million people suffer from diabetes. The cost associated with treating diabetes-associated complications has become an intensive burden to the Canadian health care system, with a projected cost of 8 million dollars in 2016 (Ohinmaa *et al*, 2004). With the increasing prevalence in both type 1 and type 2 diabetes, (Onkamo *et al*, 1999; EURODIAB ACE Study Group, 2000; Devendra *et al*, 2004; Yoon *et al*, 2006; Gregg *et al*, 2004; Singh *et al*, 2004), finding a long-term or permanent treatment for diabetes has been an area of intensive research.

1.2 Type 1 Diabetes

Type 1 diabetes is an autoimmune disease in which patients' insulin producing pancreatic beta cells are damaged or destroyed by their own immune system. Approximately 10% of all diabetes patients have type 1 diabetes. Type 1 diabetes is usually juvenile onset; patients with such an illness suffer from a life long requirement of insulin replacement therapy and severe short-term or long-term complications.

1.2.1 Cause and Progression

The exact cause of type 1 diabetes is currently unclear. Genetics appears to play an important role, with the involvement of genes including human leukocyte antigen (*HLA*) locus, insulin gene (*IDDM2*), LYP gene (*PTPN22*), cytotoxic T lymphocyte antigen-4 (CTLA-4) gene (*IDDM12*), and other IDDM genes (Jahromi *et al*, 2006; Redondo *et al*, 2001; Devendra *et al*, 2004). Most recently, genome wide screening has allowed the identification of *KIAA0350*, a gene that encodes a sugar-binding C-type lectin, which associates closely with type 1 diabetes (Hakonarson *et al*, 2007). Using this novel technique, it is hoped that more diabetes relevant genes can be identified in the future.

Environmental factors also play important roles in triggering the autoimmune attack of islet beta cells. Many studies attempted to establish a conclusive relationship between a specific environmental trigger and the development of type 1 diabetes (Devendra *et al*, 2004; Daneman, 2006). The best known are the DAISY studies, in which the associations between factors such as enteroviral infection as well as exposure to bovine protein and type 1 diabetes were studied (Norris *et al*, 1996; Graves *et al*, 2003). Nevertheless, most of these studies found no close association between type 1 diabetes with specific environmental factors with the exception of congenital rubella infection (Menser *et al*, 1978). The combination of genetic susceptibility and environmental triggers result in the destruction of pancreatic beta cells by autoimmune T-cells. The mechanism of such destruction is believed to arise from the recognition of beta cell specific auto-antigens by T-cells (Yoon and Jun, 2005). Presence of autoantibodies against several beta cell specific antigens, such as insulin (IAA), glutamic acid decarboxylase-65 (GAD-65), and insulinoma-associated antigen-2 (IA-2) can be detected in patients of type 1 diabetes (Gianani and Eisenbarth, 2005; Yoon and Jun, 2005). Recently, the role of insulin as an autoantigen has been emphasized (Nakayama *et al*, 2005; Kent *et al*, 2005). Presence of more than one antibodies

indicates a higher possibility of developing type 1 diabetes in the future (Gianani and Eisenbarth, 2005).

Upon the triggering of autoimmune attack, patients usually maintain normal glucose level for a period of time even though their beta cell mass is slowly decreasing. Based on animal studies, it was suggested that the symptoms of diabetes are established after more than 80-90% of the beta cell mass has been lost (Bonner-Weir *et al*, 1983). Results from early living donor pancreas transplantation studies also supported this suggestion (Humar *et al*, 1997, Sutherland *et al*, 1984). At this stage, patients must depend on insulin replacement therapy to maintain normoglycemia.

1.2.2 Complications and Treatments

Because of the lack of insulin-secreting beta cells, patients with type 1 diabetes suffer from hyperglycemia. Immediately, patients can feel hungry and tired because their body cannot utilize the circulating glucose as an energy source due to the lack of insulin. This will lead to drastic weight loss and many serious long-term complications including cardiovascular disease, neuropathy, blindness, and kidney failure (Sander and Giles, 2003; Jensen and Deckert, 1992; Vinik *et al*, 2003; Bronson *et al*, 2003; Strippoli *et al*, 2003). Without any treatment, none can live past childhood.

The only treatment for type 1 diabetes patients is insulin replacement therapy. Patients usually have to receive exogenous insulin injections once or twice throughout the day depending on the fluctuation in their blood glucose levels. Therefore, this treatment requires patients to constantly monitor their blood glucose levels. According to the Diabetes Control and Complications Trial (DCCT) Research Group and the Epidemiology of Diabetes Interventions and Complications (EDIC) Research Group, intensive insulin therapy and careful glycemic control could delay the development and progression of numerous complications (DCCT, 1993

and 1994; DCCT/EDIC, 2000 and 2003; EDIC, 2003; Nathan *et al*, 2005; White *et al*, 2001) and allow the maintenance of endogenous insulin secretion (DCCT, 1998). An extension to the intensive insulin therapy is the best medical therapy, in which intensive glycemic control is coupled with complication prevention therapy, including the use of ACE inhibitors and/or angiotensin II receptor to control blood pressure and protect renal function (Warnock *et al*, 2007). On the flip side, intensive insulin therapy is associated with an increased risk of hypoglycemia (DCCT, 1997). Hypoglycemic episodes can result in milder symptoms such as anxiety, mood changes, sweating, loss of orientation and severe symptoms such as convulsions, coma, or even long term intellectual decline (Frier, 2004). Frequent mild or asymptomatic hypoglycemic episodes can also result in impairment in glucose counterregulation, eventually lead to severe hypoglycemia (Fanelli *et al*, 2004). Because of the disadvantages associated with intensive insulin therapy, research has been focused on finding new treatments for type 1 diabetes patients to avoid the side effects of insulin injections.

1.2.3 Islet and Whole Pancreas Transplantation

Because the beta cells in pancreatic islets are destroyed in type 1 diabetes patients, the logical treatment is to reintroduce functional and viable pancreatic islets into patients. Beta cell replacement therapy eliminates the need for insulin injections and the possibility of hypoglycemic episodes, even though patients are still required to receive immunosuppressive therapy. Whole pancreas transplantation was first performed in 1967 (Kelly *et al*, 1967). Pancreas transplantation has been an effective way to reverse type 1 diabetes. The University of Pennsylvania recorded an insulin-free graft survival rate of 75% two years after whole pancreas transplantation (Frank *et al*, 2005). The two-year graft survival rate of whole pancreas transplantation (including simultaneous pancreas-kidney, pancreas alone, and pancreas after kidney transplantation), as indicated by insulin independence, can reach approximately 70-80%

according to the International Pancreas Transplant Registry (Gruessner and Sutherland, 2005). Nevertheless, since pancreas transplantation is a major organ transplantation and carries the risk of morbidity and mortality, it is usually only recommended for patients who have kidney failure and will receive a kidney transplantation (simultaneous pancreas-kidney transplantation) (Robertson *et al*, 2006).

In order to justify the use of transplantation as a treatment for type 1 diabetes, an illness that is not immediately life threatening, the transplantation procedure must be less invasive than whole pancreas transplantation. Pancreatic islet transplantation is therefore an ideal alternative. In 1967, Lacy and Kostianovsky first successfully isolated rat islets from intact rat pancreas using collagenase (Lacy and Kostianovsky, 1967). Later, islets isolated from rats and rhesus monkeys were subsequently transplanted and shown to ameliorate diabetes (Kemp *et al*, 1973; Scharp *et al*, 1975). These studies demonstrated the possibility of having islet transplantation as a treatment for type 1 diabetes. Human islets were first isolated in 1976 (Andersson *et al*, 1976). Although human islet transplantation was feasible in the 1980s, the success rate was extremely low. According to the International Islet Transplant Registry, 1-year islet graft survival rate was only 38% between 1990 and 1993 based on C-peptide secretion and reduced, but not elimination, of insulin requirement (Frank *et al*, 2005). In addition, only 10% of the recipients were rendered insulin independent (Brendel *et al*, 1999). In 2000, with improved islet isolation and transplantation techniques and new immunosuppressive regimens, the Edmonton Islet Transplantation Protocol markedly increased the success of islet transplantation (Shapiro *et al*, 2000). In this study, all seven patients were insulin independent immediately after transplantation. It was later noted that the three most experienced centres were able to achieve a 90% "insulin-free rate" (Shapiro *et al*, 2003). Pancreatic islet transplantation has since become an alternative, albeit experimental, treatment for type 1 diabetes patients.

In islet transplantation, pancreatic islets need to first be isolated from cadaveric donor pancreas. The pancreas is digested by collagenase or alternative enzymes and broken down mechanically. Pancreatic islets are then purified by density gradient purification. These islets enter the patients' liver by percutaneous injection through a catheter that is inserted into the portal vein. Transplanted islets regulate the patient's blood glucose level by secreting insulin upon glucose stimulation. If necessary, a subsequent transplantation can proceed quickly through the same catheter to allow an increase in total islet volume and amelioration of insulin requirement. Because type 2 diabetes is also related to the reduction in beta cell mass to some extent, patients with severe type 2 diabetes could also possibly benefit from islet transplantation (Hirshberg *et al*, 2003; Bernard-Kargar and Ktorza, 2001). In addition, a preliminary study demonstrated in an animal model that by transplanting islets, islet regeneration could be induced in pancreata of type 2 diabetic mice (Miao *et al*, 2005).

Even with the initial success of the Edmonton protocol, islet transplantation still faces several important challenges. The need for continued immunosuppressive therapy is one, since otherwise the patient's immune system will damage and destroy the transplanted islet tissue. Many of the non-steroid immunosuppressive drugs used in islet transplantation have been known to affect islet function and viability *in vitro* and in animal models (Reffet and Thivolet, 2006; Zhang *et al*, 2006; Uchizono *et al*, 2004). Evaluation of their effects on transplanted islets as well as utilizing alternative immunosuppressive therapy will be important subjects of study. Another issue is that when compared with whole pancreas transplantation, islet transplantation cannot seem to ameliorate long-term insulin requirement at the same level. In a study by Frank *et al*, 78% of the patients maintained insulin independence three years after whole pancreas transplantation, while only 23% of the patients maintained insulin independence two years after islet transplantation (Frank *et al*, 2004; Frank *et al*, 2005). If, however, a less stringent standard was used to measure graft survival rate (such as using maintenance of C-peptide presence as well

as a reduction in insulin requirement instead of amelioration of insulin requirement), there was no significant difference between the whole pancreas and the islet transplant groups. Similar results were found by the Alberta group (Ryan *et al*, 2005). While 82% of the patients remained C-peptide positive after 5 years, only 7.5% of the patients were insulin independent. These results suggested that despite the presence of transplanted islets, evident by C-peptide data, these islets could not achieve the insulin independence that was achieved by whole pancreas transplantation. More functional studies will be necessary to identify factors affecting the results of purified islet transplantation.

Another major challenge faced by islet transplantation is the lack of donor tissue. Typically, islets purified from two to three pancreata are required for each patient to reverse the need of exogenous insulin. It was estimated that less than 1% of all type 1 diabetes patients could receive islet transplants each year due to a critical shortage of tissue donor (Lechner and Habener, 2003; Hirshberg *et al*, 2003). This prevents islet transplantation to be available to all type 1 diabetes patients. Limitation in donor availability also prevents islet transplantation from being implemented at all as a type 2 diabetes treatment (Ratner, 1998; Hirshberg *et al*, 2003). Several attempts have been made to allow the use of available pancreata more efficiently. For example, the Minnesota group successfully treated several patients with single donor, marginal dose islet transplantation (Hering *et al*, 2005). The possibility of using pancreata from living donors has been investigated (Matsumoto *et al*, 2005), but the welfare of the donor must be carefully considered before such a procedure can be accessible (Pruett *et al*, 2006). Identifying an alternative source of islet tissue for transplantation will have important implication on the availability of islet transplantation.

1.3 Islets and Pancreas: Physiology and Development

1.3.1 Pancreatic and Islet Physiology

The human pancreas is located behind the stomach and sits slightly in the first loop of the duodenum. It weights approximately 80-120 grams, depending on the weight of the individual. It is innervated and vascularized extensively due to its complex structure and function. The pancreas consists of an exocrine component and an endocrine component. The exocrine component produces digestive juices and transports them into the digestive tract. The pancreatic acinar cells generate many digestive enzymes and enzyme precursors, including amylase (active), lipase (active), trypsinogen (precursor), and chymotrypsinogen (precursor). The ductal cells, on the other hand, produce a secretion rich in bicarbonate and many other ions, thus earning its alkaline nature. The alkaline secretion, together with digestive enzymes, are secreted through pancreatic ducts into the duodenum. In the duodenum, the alkaline secretion neutralizes the digested food coming from the stomach, hence providing a suitable environment for enzyme activities and preventing acidity in the chyme from damaging the intestine.

While the exocrine component takes up more than 98% of the pancreas, the remaining 1-2% endocrine component plays equally if not more important roles. The endocrine component of the pancreas consists of islets of Langerhans, each composed of approximately 1000-2000 cells. Their main function is to maintain proper glucose metabolism by secreting hormones such as insulin and glucagon. Because of this important function, pancreatic islets are penetrated by arterioles, which eventually branch into a complicated capillary network that is five times denser than that in the exocrine pancreas (Henderson and Moss, 1985). The capillaries in the islets differ in having thinner walls and larger diameters (Henderson and Moss, 1985). An islet has five cell types. Alpha cells secrete glucagon, which allows glucose to be released from glycogen stored in liver and promotes gluconeogenesis. Glucagon also prevents cells from utilizing glucose in the circulation. Beta cells secrete insulin, which promotes glucose uptake and storage. Generally

speaking, glucagon and insulin counter each other's activity. A third cell type, called the delta cells, secrete somatostatin, which inhibits the release of glucagon and insulin. The PP cells secrete pancreatic polypeptide, a hormone associated with food intake and exocrine pancreas secretion (Kojima *et al*, 2007). Last but not the least, there has been an intensive interest on the newly identified ghrelin producing epsilon cells (Sussel *et al*, 1998; Prado *et al*, 2004). Ghrelin is a hormone predominantly found in the stomach. It binds to growth hormone secretagogue receptor type 1a (GHS-R 1a), commonly expressed in the hypothalamus and pituitary, and subsequently regulates immediate hunger control as well as long-term weight control (De Vriese and Delporte, 2007). Genetic variation in ghrelin and ghrelin receptor genes are associated with obesity, anorexia, type 2 diabetes, lipid metabolism, etc (Higgins *et al*, 2007). Its role in pancreatic islets is yet to be determined.

It was previously believed that human islets, like mouse islets, maintain the structure of a beta cell core and an alpha cell mantle. The arterioles enter the core of the islets, branch out into capillaries, and eventually reach the periphery of the islets (Konstantinova and Lammert, 2004). The blood flow therefore reaches the beta cells first, bringing paracrine signals downstream to non-beta cells. Recently, many studies utilizing confocal microscopy challenged this idea (Carbera *et al*, 2006; Brissova *et al*, 2005). Instead, in human islets, alpha, delta, and pp cells are scattered throughout the islets. There also does not appear to be a beta cell core. Compared with mouse islets, human islets have less beta cells (55% compared with 77%) but more alpha cells (38% compared with 18%) (Carbera *et al*, 2006). Additionally, Carbera *et al* suggested that the blood flow through the islets does not appear to reach different islet cell types in any particular order, and thus the microcirculation in islets does not have relevance to the intercellular interactions between different islet cells.

Insulin, the most important hormone secreted by pancreas, is a polypeptide of 51 amino acids. It is first synthesized as preproinsulin, a preprohormone of 106 amino acids. 24 amino

acids are cleaved off in the endoplasmic reticulum, generating proinsulin. The maturation of proinsulin into the final insulin hormone occurs in the secretory granules, whose ionic composition and acidity allows activation of prohormone convertase 1/3 (PC1/3), prohormone convertase 2 (PC2), and carboxypeptidase E (CPE) (Hutton, 1994). Because all the enzymes are packaged into the secretory granules at once, there are two possible routes for insulin maturation. If PC1/3 cleaves proinsulin first, des31, 32 proinsulin is generated intermediately after CPE cleavage. On the other hand, if PC2 cleaves the proinsulin first, des 64,65 proinsulin is generated instead (Halban, 1994). In human, PC1/3 cleavage generally occurs first because PC2 exhibits higher efficiency in converting des 31,32 proinsulin than proinsulin (Goodge and Hutton, 2000). After both conversions, the mature insulin consisting of a shorter (21 amino acids) A chain and a longer B chain (30 amino acids) that are bound by two disulfide bonds, and its by-product C-peptide, are released into circulation. Because C-peptide is released simultaneously with insulin, it has been used as a reliable indicator for *de novo* insulin secretion. It is worth noting that a small amount of immature proinsulin does get released together with mature insulin and C-peptide. However, because proinsulin only has 5% of the activity of insulin, proinsulin does not play an important role in normal metabolism regulation (Boron and Boulpaep, 2005).

The secretion of insulin is mainly stimulated by glucose (Rhodes and Halban, 1987). Glucose enters beta cells through glucose transporter-type 2 (GLUT-2) and is then phosphorylated by glucokinase (GK). GK has a high Km of ~10mM, making the phosphorylation of glucose by GK a rate-determining step for glycolysis (Meglasson and Matschinsky, 1986). This also makes glucokinase commonly known as the "glucose sensor" of beta cells. The process of glycolysis generates NADH, ATP, and pyruvate. Pyruvate enters the tricarboxylic acid (TCA) cycle in mitochondria to generate more ATP, which, although not critical, contribute to the total increase in ATP level inside the beta cells (MacDonald *et al*, 2005). With the contribution of ATP from glycolysis and the TCA cycle, the ATP to ADP ratio

increases drastically, causing the ATP sensitive K^+ (K_{ATP}) channels to close. The blockage of K^+ efflux from beta cells to extracellular space results in depolarization, which causes the voltage dependent calcium channels to open. The influx of calcium eventually leads to the release of insulin by its secretory granules through exocytosis. Insulin release can also be stimulated by certain amino acids (particularly arginine and lysine), other gastrointestinal hormones (gastrin, glucagon-like peptide 1, gastric inhibitory peptide, etc), and other hormones (growth hormone, glucagon, etc), although by completely different mechanisms.

1.3.2 Genetic Signals Regulating Pancreatic Development and Function

In mice, pancreas organogenesis occurs at embryonic day E8.5 (Wilson *et al*, 2003). Signals from neighboring cardiac and notochord mesoderm induce the suppression of hedgehog signals *Shh* (sonic hedgehog) and *Ihh* (Indian hedgehog) at the pancreas anlage (the portion of cells that is to develop into pancreas) of foregut endoderm (Ball and Barber, 2003; Edlund, 2001). This is an important first step to the development of pancreas. Ectopic expression of *Shh* in developing pancreas tissue resulted in formation of intestine-like tissue (Apelqvist *et al*, 1997). *In vitro*, expression of *insulin* and another essential pancreatic marker *pdx-1* in isolated chick endoderm were lost with the concurrently increased expression of *Shh* (Hebrok *et al*, 1998). This was rescued by the addition of notochord. In addition, addition of SHH peptide resulted in the lack of insulin and pdx-1 expression, which could be subsequently rescued by the addition of SHH antibody (Hebrok *et al*, 1998). The notochord signals involved were identified to be fibroblast growth factor-2 and activin beta (Hebrok *et al*, 1998).

With the hedgehog signals suppressed, pancreas differentiation is dependent on the expression of *Ptf1a* (DNA-binding subunit of transcription factor Ptf-1) and *Hlxb9* (Hb9 transcription factor). In ventral pancreas, the differentiation of pancreas anlage is dependent on the additional absence of fibroblast growth factor signaling by the cardiac mesoderm. Ventral

endoderm exposed to FGF differentiates into liver (Deutsch *et al*, 2001), while that exposed to FGF with the lack of *Ptf1a* expression continues an intestinal fate (Kawaguchi *et al*, 2002). The expression of the pancreatic duodenal homeobox factor-1 (*pdx-1*) then follows. Cells positive for Ptf-1a and Pdx-1 are deemed the pancreatic progenitors that differentiate into all pancreatic cell types (Ball and Barber, 2003; Gu *et al*, 2002). Ductal fate is determined by E12.5 with a short-term expression of *pdx-1* between E9.5 and E12.5 (Gu *et al*, 2002). The exocrine lineage is committed by the suppression of neurogenin-3 (*ngn-3*), a necessary endocrine fate determinant (Gradwohl *et al*, 2000), and the continual expression of *Ptf1a* (Ball and Barber, 2003). All islet cells arise from Ptf-1a and Pdx-1 positive cells expressing transcription factor Ngn-3 (Gu *et al*, 2002). The expression of transcription factor Ngn-3 is controlled by the expression of *Hes* gene, which is maintained by Notch signaling. Suppression of Notch signaling leads to decreased expression of *Hes*, which prevents the suppression of Ngn-3 expression (Lee *et al*, 2001). Other than the removal of *Hes* expression, other regulators include hepatocyte nuclear factor 6 (HNF-6), HNF-1, and Foxa (Jacquemin *et al*, 2000; Lee *et al* 2001; Wilson *et al*, 2003). Activation of Ngn-3 leads to the downstream expression of NeuroD1, another important endocrine transcription factor (Wilson *et al*, 2003). Expression of Ngn-3 deems the cells an endocrine fate, but their exact islet subtype is possibly determined by factors other than Ngn-3. In fact, ectopic expression of Ngn-3 leaded to the generation of all alpha cells, demonstrating that other factors are necessary in the determination of islet subtype (Schwitzgebel *et al*, 2000). Although other factors, including early factors such as pax4, nkx2.2, nkx6.1 (co-express with Ngn-3) and late factors such as pax6, isl1, Brn4, HB9, and Pdx-1 (expressed in more mature endocrine cells), have been found important in endocrine fate determination, there is currently no evidence suggesting that they are sufficient in controlling endocrine fate (Wilson *et al*, 2003).

1.4 Islet Regeneration Using Pancreatic Cells

1.4.1 Evidence of Pancreas Regeneration

Physiologically, beta cell mass increases in obese or pregnant individuals to compensate for their increasing need for insulin to maintain normoglycemia (Bernard-Kargar and Ktorza, 2001). Pancreatic regeneration and islet regeneration can also be found in animal models of pancreatic injury, induced either chemically or physically (Holland *et al*, 2004; Hardikar *et al*, 2004). For example, when alloxan, a chemical that selectively destroys beta cells, is injected into the head of the murine pancreas, there appears to be an increase in beta cell mass in the tail of the pancreas (Waguri *et al*, 1997). In addition, although the injection initially renders the mice diabetic, blood glucose levels of the test animals eventually return to normal. Similar effects were found in animals injected with streptozotocin (Pour *et al*, 1990; Tomioka *et al*, 1991). Pancreas regeneration can also be observed in models of physical destruction such as partial pancreatectomy (Brockenbrough *et al*, 1988; Liu *et al*, 2001; Fong *et al*, 1992; Finegood *et al*, 1999; Hardikar *et al*, 1999), cellophane wrapping (Rosenberg *et al*, 1989; Rosenberg and Vinik, 1989), and duct ligation (Rosenberg, 1998; Page *et al*, 2000). This evidence for pancreas regeneration provides support for the possible use of pancreatic cells to generate islet tissue for the use of transplantation for diabetes treatment.

Beta cell mass in pancreas is kept at homeostasis possibly by several mechanisms: beta cell replication/proliferation, beta cell apoptosis, and islet neogenesis (from other pancreatic cell types or unknown stem/progenitor cells). In the animal models of pancreas recovery mentioned previously, beta cell mass could be increased by increasing beta cell replication, reducing beta cell apoptosis, or inducing islet neogenesis. However, the reason behind pancreas regeneration remains controversial and there has been no consensus on which pancreatic cell population is responsible for this recovery.

1.4.2 Beta Cell Replication

It was previously suggested that existing pancreatic beta cells have only limited ability to replicate (Teta *et al*, 2005; Bonner-Weir, 2001) and lose their ability to secrete insulin over time after extended period of replication *in vitro* (Beattie *et al*, 1999). However, a recent study demonstrated the importance of beta cell replication in beta cell mass regeneration using a lineage-tracing technique. The Melton group at Harvard University followed beta cell replication during pancreatic recovery after pancreatectomy using a Cre/lox linage tracing system driven by rat insulin promoter (Dor *et al*, 2004). Pancreatic beta cells arose after pancreatectomy were found to be progenies of pre-existing, insulin-expressing beta cells. In a later study, Georgia and Bhushan confirmed the importance of cyclin D2 in the maintenance of postnatal beta cell mass (Georgia and Bhushan, 2004). Since cyclin D2 is an important factor in the regulation of replication, this study thus suggested that replication is the major mechanism in maintaining beta cell mass.

The Melton study demonstrated the potential of beta cells to generate more insulin-secreting cells for diabetes treatments. It is currently unknown whether beta cell replication is also the recovery mechanism used by mouse in other models of pancreatic damage, whether long term damages induce the same response, whether there are insulin expressing progenitor cells mistakenly treated as beta cells, and what other mechanisms (de-differentiation or transdifferentiation) are involved in beta cell replication. Furthermore, it is too early to conclusively say that the same mechanism is employed in human pancreas or to eliminate the possibility that there exist other progenitor cells important in pancreatic recovery. Nevertheless, this study opened up the possibility to generate more insulin-secreting cells through beta cell replication. The reason why beta cell replication hasn't been observed might simply be that the replication process is inhibited under normal conditions *in vivo*. As long as the right conditions

and environment are given, beta cell culture *in vitro* might serve as a potential source for new beta cell or islet tissue source in the future.

1.4.3 Islet Neogenesis from Pancreatic Ductal Cells

Adult pancreatic ductal cells have long been speculated to have the potential to act as progenitor cells for pancreatic islet cells. Through histological observations, pancreatic ducts and islets were found to associate closely in human and rat pancreata (Bouwens *et al*, 1997; Leeson and Leeson, 1986; Bertelli *et al*, 2001; Bouwens and Pipeleers, 1998). It was interpreted from histological data that pancreatic beta cells arose from ductal tissue after pancreatic damage is induced by pancreatectomy or duct ligation (Bonner-Weir *et al*, 1993; Wang *et al*, 1995). In addition, adult ductal cells were found to express Pdx-1/Ipf-1, an important transcription factor associated with islet and pancreas development (Jonsson *et al*, 1994), either in tissue sections or *in vitro* culture of human pancreatic tissue (Heimberg *et al*, 2000; Gmyr *et al*, 2000). Cornelius *et al* first demonstrated that islet-like, insulin-secreting cells could be generated from pluripotent islet-producing stem cells (IPSC) in islet-associated ductal tissue (Cornelius *et al*, 1997). The same group later used ductal tissue from mouse model of type 1 diabetes (Non-obese diabetic mice, NOD mice) to generate islet-like clusters and reversed type 1 diabetes in NOD mice by transplantation of islet-like clusters (Ramiya *et al*, 2000). This demonstrated the possibility of using pancreatic tissue from diabetic patients to generate insulin-secreting cells. Similar results were obtained by Bonner-Weir *et al* using human ductal tissue (Bonner-Weir *et al*, 2000). In the Bonner-Weir study, human ductal tissue was obtained through density gradient isolation and ductal cells were selectively cultured using non-culture treated tissue flask to prevent islet cell contamination. In both animal and human cases, islet-like clusters containing cells that express islet hormones including insulin and glucagon were generated. The generation of islet-like clusters using similar or replicated protocols has been repeated by other groups using murine or

human ductal tissue (Katdare *et al*, 2004; Gao *et al*, 2003). Because of the success in generating islet-like clusters from pancreatic ductal tissue, the Bonner-Weir group further attempted to establish that pancreatic ductal cells could serve as progenitor cells in the pancreas (Bonner-Weir, 2004).

Even though it was possible to generate islet-like clusters from human ductal tissue, such studies have been criticized for the impurity of the initial "ductal tissue preparation." The ductal tissue used in the generation of islet-like clusters was purified by density gradient. This does not produce a highly purified ductal cell fraction and therefore the insulin positive cells might arise from beta cell contaminant, other pancreatic cell types (such as acinar cells), or other stem/progenitor cell types. A recent study by the Bonner-Weir group attempted to generate insulin positive cells using ductal cells purified by magnetic-activated cell sorting using a pan-ductal cell surface marker (Yatoh *et al*, 2007). The purified fraction, after re-aggregation, does not generate insulin positive cells. Nevertheless, by introducing pancreatic stromal cells into the *in vitro* culture, they were able to generate 0.1% insulin positive cells. After transplantation of these ductal cells into the kidney capsule of murine pancreas, the percentage of insulin positive cells increased to 1% after engraftment. Although this demonstrated the potential of ductal cells to differentiate, the small percentage of differentiation suggested that the majority of insulin positive cells generated by the impure ductal fraction might be contributed by pre-existing beta cells. The requirement of stromal cells suggested that other extracellular stimulations might be necessary for ductal cell differentiation. Furthermore, other similar protocols have been used to generate insulin-secreting clusters from purified pancreatic islets, showing that the culture conditions used by Bonner-Weir protocol can possibly induce pancreatic islet proliferation or differentiation, and therefore islet-like clusters generated might originate from other islet cells types (Lechner *et al*, 2005; Banerjee and Bhonde, 2003).

1.4.4 Islet Neogenesis from Pancreatic Acinar Cells

Previously, it has been demonstrated that acinar tissue can differentiate into cells that possess ductal phenotypes (Rooman *et al*, 2000; Gmyr *et al*, 2001). It is therefore logical to propose that acinar cells have the potential to differentiate into beta-like cells. This was first demonstrated by two studies using impure acinar tissue (Song *et al*, 2004; Baeyens *et al*, 2005). Subsequently, a lineage tracing study confirmed that insulin positive cells could be generated *in vitro* from amylase/elastase expressing acinar cells (Minami *et al*, 2005). Semi-quantitative PCR results also suggested a progression from acinar phenotypes to islet cell phenotypes, reinforcing the idea of acinar to islet cell differentiation. Later, the same group further demonstrated that insulin positive cells could be generated using acinar cells from streptozotocin treated diabetic mice as well as Komeda diabetes-prone rats (another animal model of type 1 diabetes) (Okuno *et al*, 2007). Because lineage-tracing technique was used in these studies, their results conclusively suggested the possibility of acinar to islet transdifferentiation in murine models. Further studies using human tissue are required to confirm the feasibility of using acinar tissue to generate human islets for transplantation. If that is proven to be possible, the large amount of acinar tissue collected during the process of islet purification for transplantation might become an excellent alternative source of islet tissue.

1.4.5 Other Pancreatic Non-Islet Cells

It is possible that a phenotypically less defined pancreatic progenitor cell population might exist in adult pancreas (Petropavlovskaia and Rosenberg, 2002). Many groups suggested potential markers to purify islet progenitors from pancreas (Zhang and Sarvetnick, 2003). Some markers studied include nestin that is commonly used to identify neural stem cells (Abraham, 2004; Lechner *et al*, 2002; Zulewski *et al*, 2001), vimentin that is used for isolation of mesenchymal stem cells (Ko *et al*, 2004), and the hepatocyte growth factor receptor/c-met

(Suzuki *et al*, 2004). Nestin was later found to be associated with microvasculature generation but not islet cell generation (Treutelaar *et al*, 2003; Klein *et al*, 2003). The Suzuki group followed up with a study demonstrating that CD133 positive ductal cells (which also express c-met) purified using fluorescent-activated cell sorting can differentiate into cells with islet phenotypes (Oshima et la, 2006).

In 2004, the van der Kooy group at the University of Toronto used clonally diluted pancreatic cells obtained from ductal and islet tissue of mice and successfully demonstrated that such a progenitor cell population does exist in adult mouse pancreas (Seaberg *et al*, 2004). This pancreatic cell population, named "pancreas-derived multipotent precursors" (PMPs), was isolated from mouse pancreatic ductal tissue and islet tissue. Although existing in very low frequency (~0.02%), these pancreatic progenitor cells have the ability to differentiate into a variety of pancreatic cell lineages as well as neural lineages. In addition, they exist in both ductal tissue as well as islet tissue at similar frequency, showing that such progenitors are not localized to a specific tissue and therefore are not likely to be terminally differentiated beta cells or ductal cells. This study also looked into the distribution of nestin positive cells and the association of nestin expression and PMP population, and found that there is no correlation between the generation of PMP colonies and the expression of nestin marker. This is by far the first study to confirm the presence of a precursor cell type in adult mouse pancreas. However, because of the nature of progenitor cells, it was not possible to analyze the property of these precursor cells which give rise to multiple lineages retroactively, and thus their characteristics remain a mystery.

Recently, Ngn3-expressing islet cell progenitors were identified and isolated from pancreata of adult mice (Xu *et al*, 2008). These non-insulin secreting, Ngn3-expressing cells were found to arise after pancreatic duct ligation, a known model of pancreatic damage that induces regeneration. Interestingly, their presence coincided with the increased expression of pancreatic ductal marker, suggesting that their presence might be associated with ductal cells (or

might come directly from ductal cells). Furthermore, these cells could be purified using fluorescent-activated cell sorting and induced to express islet cell markers *in vitro*. This is indeed a further step toward the identification and purification of adult pancreatic progenitors. It would be interesting to determine the exact signal that induces the increase of Ngn3 expressing cells and evaluate its possible use in diabetic treatments.

1.4.6 Other Sources of Islet Cells

Because of its ability to differentiate into various cell types and to self-renew, embryonic stem cells (ES cells) are considered a valuable candidate to generate insulin secreting beta cells. Even though various protocols have been established in an attempt to generate beta cell from embryonic stem cells, it has proven to be an extremely difficult task (Santana *et al*, 2006; Kania *et al*, 2004). First of all, the embryogenesis of pancreatic islets is extremely complicated, and it is difficult to take all factors involved into consideration. Secondly, one must ensure the maturation of beta cells generated from ES cells and prevent them from further differentiation or de-differentiation. This includes preventing ES cells from generating cancerous tumors. Last but not least, it was found previously that ES cells have the ability to uptake exogenous insulin in their culture media, giving a false sense of them differentiating into insulin positive cells (Hansson *et al*, 2004). This shows that stringent standards must be set for functional criteria to confirm the ability of differentiated ES cells to secrete insulin. Novocell Inc. recently generated insulin positive cells using human ES cells by introducing extracellular factors on a schedule mimicking normal embryogenesis of human pancreas (D'Amour *et al*, 2006). Differentiated cells were found to express common islet markers and are positive of islet hormones including insulin and glucagon. They also secreted C-peptide, a by-product of *de novo* insulin secretion. Although their insulin content is relatively low compared with human islets, these differentiated ES cells are the first step in large-scale generation of islet cells using ES cells.

Other types of stem cells, including bone marrow, mesenchymal, liver, and gut stem cells, have also been suggested to generate insulin positive beta cells (Gangaram-Panday *et al*, 2007; Hussain and Theise, 2004). More studies are required to clarify the role of cell fusion and to ensure beta cell phenotypes can be maintained after transplantation.

Other than stimulating ES cells to express beta cell phenotypes using extracellular factors, genetic manipulation has also been used to allow the expression of essential beta cell proteins in other cell types. The difficulty is in generating cells with two essential beta cell qualities simultaneously: glucose sensing and insulin secreting. Liver and intestinal stem cells have been used mostly because they arise from the same endoderm lineage as the pancreas. The general approach is to deliver genes essential in pancreatic development, such as *pdx-1*, *neuroD*, or *ngn-3*, either individually or combined into cells of interest *in vitro* and look for expression of insulin or other beta-cell phenotypes, including expression of Glut-2, SUR1, Kir6.2, or other development related proteins and their corresponding genes (Sapir *et al*, 2005; Kojima *et al*, 2002). The issues in these studies are that the cells usually assume partial beta cell phenotypes and therefore secrete a relative small amount of insulin in comparison to true beta cells. Another approach is to deliver the insulin gene into cells with similar secretory machinery. For example, by introducing insulin gene into glucose sensitive intestinal K cells that usually secrete gastric inhibitory peptide, one can generate cells that are responsive to glucose and secrete insulin accordingly (Cheung *et al*, 2000). These genetic modification methods have also been used to study the possibility of future gene therapy for type 1 diabetes (Samson and Chan, 2006). Such techniques should also be applicable to *in vitro* generation of islet like tissue. One thing to note is that an efficient and specific gene delivery method must be established before gene therapy can be available to patients. Because of this, the possibility of generating surrogate beta cells *in vitro* and introducing these cells by transplantation seems to be a more immediate goal than using gene therapy to treat type 1 diabetes. Another related approach is to generate human beta cell

lines that can secrete insulin consistently. In the past, almost all human beta cell lines failed to generate insulin on the long-term because cells tend to de-differentiate and lose their ability to secrete insulin upon glucose stimulation (Baroni *et al*, 1999; Hohmeier and Newgard, 2004). Moreover, cell lines are usually not suitable for transplantation since they will divide and progress rapidly into a tumour. Recently, Narushima *et al* successfully generated a reversible immortalized human beta cell line that is able to maintain insulin secretion for thirty weeks after being transplanted into streptozotocin-induced diabetic mice (Narushima *et al*, 2005). This is strikingly different from other beta cell lines previously established. In addition, it was recently shown that transplantation of pure beta cells, although in a larger volume, can still correct high glucose levels in diabetic SCID mice (King *et al*, 2007). These two studies demonstrated the possibility of transplanting pure beta cell lines to treat type 1 diabetes.

Another alternative source of islet tissue is xenogeneic tissue harvested from the pancreata of other species. The advantage is that xenografts are readily available and can be procured easily. Normally, transplantation of xenografts procured from porcine results in hyper acute rejection by primate recipients due to the expression of galactose alpha-1, 3-galactose (Gal) epitope (Lin *et al*, 2000) or other downstream immunopathologic events (Kirchhof *et al*, 2004). Several methods have been studied to prevent rejection resulted from transplantation of porcine xenografts. First, the use of microencapsulation has been studied extensive for the use of preventing autoimmune rejection found in type 1 diabetic patients, hyper acute rejection in xenograft transplantation, as well as other rejections found in allotransplantations. A study by Kobayashi *et al* first demonstrated that agarose-microencapsulated syngeneic mouse islets can be transplanted into immune competent diabetic NOD mice without the use of immunosuppression and maintain function for more than 100 days after transplantation (Kobayashi *et al*, 2003). It was further demonstrated by Schneider *et al* that long-term function could be preserved in alginate-encapsulated adult and human islets after transplantation in immunocompetent diabetic

mice (Schneider *et al*, 2005). These studies demonstrated the potential of using microencapsulation as a method to prevent all types of rejections, including hyper acute rejections. Another method is to use antibody regimen to prevent hyper acute rejection. Regimen such as antibody mediated CD154 or CD28-CD154 costimulatory blockade have been shown to successfully prevent hyper acute rejection when porcine islets were transplanted into macaques (Hering *et al*, 2006; Cardona *et al*, 2006). Last but not least, many attempts have been made in generating genetically modified pigs deficient in galactose alpha-1, 3-galactose (alpha 1, 3 Gal) or of alpha1, 3-galactosyltransferase (alpha 1, 3 GT), the enzyme that generates alpha 1, 3 Gal (Lai *et al*, 2002; Dai *et al*, 2002; Phelps *et al*, 2003). However, it was found that baboons transplanted with kidneys from alpha 1,3 Gal knockout pigs still suffer from rejections caused possibly by T-cell responses (Chen *et al*, 2005; Yang and Sykes, 2007).

Other issues encountered by xenotransplantation include the ethical use of organs from animals for transplantation purposes, the rights of the patients who are to receive xenografts, and the possibility of transmitting infectious agents between pigs and humans (MacKenzie *et al*, 2003; Anderson, 2006, Quante and Wiedebusch, 2006). Several clinical trials are now underway despite the controversial nature of this subject (Rood and Cooper, 2006); many studies of non-human primate should still be necessary for the use of xenograft as an alternative source of islet tissue.

Chapter 2 Thesis Investigation

2.1 Objectives

Although various groups have proposed ways to generate islet tissue *in vitro*, there has been no consensus on which method is more efficient. Beta cell specific gene expression, immunohistochemical staining, and insulin to DNA ratio are three common methods used to demonstrate achievement or maintenance of beta cell phenotype. Nevertheless, there are several reasons why gene expression and immunohistochemical staining alone are not sufficient to fully support successful generation of islet tissue. First of all, genetic expression of insulin or other beta cell specific transcription factors often does not guarantee actual synthesis and secretion of insulin. An example is that beta cell lines which fail to secrete insulin could still have insulin and Glut-2 mRNA expression detected (Baroni *et al*, 1999). Results from PCR might not be reliable if contamination by other cell types is to occur (Sipione *et al*, 2004). Second, previous stem cell studies confirmed that intracellular insulin might be absorbed from exogenous insulin in the culture media (Hansson *et al*, 2004; Sipione *et al*, 2004). In these cases, even though immunohistochemical staining demonstrated the presence of insulin in the differentiated cells, no *de novo* insulin synthesis and secretion have occurred. In the Bonner-Weir protocol, increase in insulin to DNA ratio at different stages of culture was considered to be an indication of increased insulin positive cells. Tissue samples were sonicated in distilled water directly. The same sample was used to determine DNA and insulin level. This however presents several problems. First of all, in order to assay DNA and insulin from the same sample, acid extraction was used to ensure all the insulin is dissolved in the sample. It is not possible to precisely determine total DNA content from acid treated samples. The lack of insulin extraction might result in underestimation of insulin content in the sample. Secondly, with a sample of mixed cell type, increase in insulin to DNA ratio can be explained by other possibilities including the death of a large amount of

non-insulin containing cells; this phenomenon is normally observed in isolated exocrine tissue. It can also be explained by beta cell replication. Last but not least, it does not reflect the increase in the total number of insulin positive cells. Samples in which there is a mixture of few mature insulin positive cells as well as many non-insulin positive cells might have the same total insulin to DNA ratio compared to samples in which there are numerous immature insulin positive cells (with low levels of insulin). Therefore, although these well-developed methods are extremely helpful in providing information about the islet-like tissue generated, more stringent criteria are required to confirm, for example, progenitor differentiation into insulin secreting beta cells.

Glucose-regulated insulin secretion is the hallmark of human beta cells. Therefore, such a characteristic should be considered essential in generating cells with the ability to replace beta cells. A perifusion system is commonly employed to test insulin secretion by islets or cell lines. In a perifusion system, media containing glucose is pumped through a chamber filled with insulin-secreting islets. In the chamber, islets are subjected to media with various glucose concentrations that mimic their physiological environment. The media flow past the islets and can then be collected and assayed for insulin concentration. The physiological behaviour of islets in insulin secretion can be monitored and studied. This is the most unbiased method to detect insulin secretion by islets and to ensure the islets do maintain proper glucose responsive insulin secretion. This method is commonly used in combination with immunohistochemical staining and gene expression detection to study islet physiology. Nevertheless, the use of perifusion also has its challenges. It usually takes a long time to set up the system and the set up requires specialized technicians. In addition, since each chamber allows only one condition to be tested, it is extremely difficult to check multiple conditions simultaneously using the perifusion system.

Another method to test insulin secretion is the static incubation method. In this method, islets are maintained in media containing low glucose. The low glucose media is then replaced with low glucose media (as a control) or the media containing the condition to be tested (such as

media containing high glucose or soluble factors to stimulate insulin secretion). In the end, the testing media is collected and assayed for insulin content. This allows testing of various conditions simultaneously. In addition, the protocol for static incubation is extremely easy and does not require specially trained personnel. Static incubation is therefore a convenient way to test insulin secretion by newly generated islet-like clusters.

2.2 Hypothesis and Specific Aims

In this study, I hypothesize that insulin secreting human islet-like clusters could be generated from pancreatic ductal cells, a potential pancreatic progenitor cell type. The specific aims are as follows:

1. To generate human islet-like clusters *in vitro*. Previously studied protocols will be used to find the suitable conditions for generating islet-like clusters *in vitro*.

2. To explore methods to purify ductal cells (potential progenitors) and beta cells (as a positive control). Fluorescent activated cell sorting (FACS) and magnetic activated cell sorting (MACS) will be tested to determine which method might be suitable for the purification of potential pancreatic progenitors.

3. To develop a static incubation protocol for testing insulin secretion by islet-like clusters. MIN-6 (mouse insulinoma cells) will be used to develop a reliable and convenient static incubation system.

4. To evaluate insulin secretion of human islet-like clusters generated from impure ductal tissue and purified ductal cells. Using the established static incubation protocol, this study hopes to evaluate insulin secretion by islet-like clusters generated from impure ductal tissue or purified ductal cells.

Chapter 3 Methods and Materials

3.1 Human Tissue

Pancreatic exocrine tissues (with research consent) were obtained from cadaveric adult human organ donors from the Ike Barber Clinical Human Islet Transplant Laboratory and University of Alberta Clinical Islet Transplant Program. Donor information is summarized below in Table 1. Human pancreata were processed as previously described (Warnock *et al*, 2005; Ryan *et al*, 2005). The exocrine portion containing acinar and ductal cell clusters was collected from discarded non-islet tissue after islet purification using Ficoll density gradient for the purpose of this study. The exocrine portion had approximately 1-5% islet contaminants as assessed by dithizone staining. Exocrine tissue was cultured at 22°C until use to maintain maximum tissue viability.

This study was approved by the Clinical Research Ethics Board of UBC (Appendix 1).

Table 1 General Information of Donors Whose Tissue Was Used in Experiments

Used in	Donor ID	Age (years)	Weight (kg) or BMI	Cold Preservation Time (hours:minutes)	Culture Time* (hours:minutes)
Sequential Static Incubation	BC054	55	65	8:15	
	BC055	60	91	3:24	
	BC057	60	65	2:21	
	AB1077	52	BMI = 25.0	N/A	40:00
Parallel Static Incubation	BC086	47	77	3:40	
	BC090	17	80	3:00	
	BC091	50	72	4:22	
Cell Sorting (FACS or MACS)	BC068	61	109	10:31 (Tissue procured in Ontario)	
	BC069	67	80	2:00	
	BC079	42	70	4:17	
	BC089	56	70	2:29	
	AB1032	50	BMI = 30.8	9:00	17:00
	AB1037	47	BMI = 24.4	3:00	74:00

* Only applicable to Alberta Tissue

3.2 Generation of Islet-like Clusters

Exocrine tissue was cultured according to modified Bonner-Weir method (Bonner-Weir *et al*, 2000) and Todorov method (Todorov *et al*, 2006) to generate islet-like clusters. Throughout the tissue culture period, cells were maintained at 37°C and 5% CO_2 unless otherwise indicated. In the Bonner-Weir method (Bonner-Weir *et al*, 2000), exocrine tissue was diluted 5 fold (v/v) and 20 μL was seeded into each well in a 24 well plate. Both tissue culture untreated (used in the Bonner-Weir study to remove islet tissue) and treated plates were used for comparison in the elimination of islets and the ability to induce the generation of islet-like clusters. Cells were cultured in CMRL-1066 media (Invitrogen) containing 10% fetal bovine serum (FBS, Invitrogen) and 5% L-Glutamine (Sigma) for the first 5-7 days with frequent media change until tissue clusters flattened to form a monolayer. This first week of culture in serum-containing media was considered "**stage one**." Then the media was changed to serum-free media containing DMEM/F12 (1:1, Invitrogen, 1400 mg/L glucose), 1 g/L Insulin-Transferrin-Selenite supplement (ITS, Sigma), 2 g/L bovine serum albumin fraction V (BSA, Sigma), and 10 mM nicotinamide (Sigma). In the original Bonner-Weir protocol, 10 ng/mL keratinocyte growth factor (KGF) was included in the serum-free culture media. However, this was found to rapidly increase cell proliferation, making it difficult to maintain tissue culture. In addition, in a previous study done evaluating critical factors for differentiation in the Bonner-Weir protocol, it was found that KGF was not essential in the generation of islet-like clusters (Gao et al, 2003). Therefore, the use of KGF was eliminated in crude exocrine tissue culture. This second week of culture in serum-free media was considered "**stage two**." After a one-week culture in serum-free media, an extracellular matrix Matrigel (BD Biosciences) was diluted 2 to 3 fold and layered on top of the cells (100 μL/well of a 24 well plate). At this stage, monolayer cells were induced to migrate into three-dimensional structures. The cells were cultured in this condition for three

weeks further to ensure the formation and maturation of the islet-like clusters. The last phase of culture with matrigel was considered "**stage three**."

The Todorov method (Todorov *et al*, 2006) was modified as follows. Cells were again cultured into a monolayer in CMRL-1066 as described in the Bonner-Weir method. After the first week of culture, cells were lifted using trypsin (0.25 % Trypsin-EDTA solution, Invitrogen) and maintained in serum free media in a non-tissue culture treated dish on a shaker at 37°C and 5% CO_2.

3.3 Dissociation of Exocrine Tissue into Single Cells

Exocrine cell clusters were washed in dissociation media containing Ca^+ free, Mg^+ free Hanks Balanced Salt Solution (HBSS, invitrogen), 1 mM ethylenediamine tetraacetic acid (EDTA, Gibco/Invitrogen), 10 mM Hepes (MediaTech), and 0.5% BSA fraction V. The wash solution was discarded and the total volume was brought to 20 mL with the dissociation media. The cell clusters together with the dissociation media were transferred to a 10 cm suspension dish (Corning) and incubated at 37°C for 7 minutes. Then, 200 µL 0.25% trypsin (final concentration = 25 µg/mL) and 200 µL DNase stock solution (final concentration = 4 µg/mL) were added to the cell clusters. The plate was returned to incubator for another 10 minutes. To avoid over-digestion, plate content was monitored every two minutes until dissociated single cells could be observed. The clusters were further broken into single cells by pipetting up and down using a P-1000 pipet tip. Trypsin was inactivated by the addition of 20 mL serum containing media. Single cells were filtered through a 40 µm cell strainer and collected into a new 50 mL tube. The dissociated cells were washed twice and the total volume was brought to 10 mL. Cell concentration and viability were then determined through Trypan Blue staining (Sigma).

3.4 Dithizone Staining

Dithizone solution was prepared as previously described (Shiroi *et al*, 2002). Dithizone stock was prepared by dissolving 50 mg of Dithizone (Diphenylthiocarbazone, Sigma) in 5 mL of dimethyl sulfoxide (DMSO, Sigma) and stored at -20°C. Before staining, 100 µl was added into 10 mL of PBS or phenol red-free DMEM and filtered through a 0.2 µm syringe filter. Islet-like clusters were incubated for 5 minutes in dithizone-containing media and examined under an inverted microscope.

3.5 Immunofluorescence (sections and single cells)

Antibody reagents and dilutions: All antibodies were diluted in DAKO antibody dilution solution.

Primary antibodies:

Antibody	**Source**
Monoclonal mouse anti-human carbohydrate antigen 19-9 (CA19-9) – 1:200 (0.25 µg/mL)	Vision Biosystems
Polyclonal sheep anti-human carbonic anhydrase II (CAII) – 1:500	R&D Systems
Polyclonal guinea pig anti-swine insulin – 1:250	DAKO
Polyclonal goat anti-human Pdx-1 – 10 µg/mL	R&D Systems
Polyclonal rabbit anti-human GLP-1 receptor – 1:150	From Dr. Timothy Kieffer, UBC

Secondary antibodies: Unless otherwise indicated, all secondary antibodies were diluted 1:200

Antibody	**Source**
Polyclonal donkey anti-sheep (AF488)	Invitrogen
Polyclonal donkey anti-guinea pig (Texas Red)	Jackson ImmunoResearch
Polyclonal donkey anti-guinea pig (AF488)	Invitrogen
Polyclonal goat anti-rabbit (AF568)	Invitrogen
Polyclonal donkey anti-mouse (AF488)	Invitrogen
Polyclonal anti-goat (AF488)	Invitrogen

Pancreatic tissue was fixed in 10% formalin, paraffin-blocked, and sectioned at 5µm thickness. Pancreatic sections were deparaffinized and hydrated by passing the sections through the following solutions: Histoclear II (Diamed, 3 × 10 minutes), 100% ethanol (2 × 10 minutes),

95% ethanol (1 × 10 minutes), and 70% ethanol (1 × 10 minutes). Hydrated slides were washed in PBS before proceeding to staining. To prepare single cell slides, cells were washed and resuspended in PBS. Cell suspension was left on Histobond slides (Marienfeld) to allow attachment of cells to hydrophilic slides. Cells were then fixed in formalin-acetic acid fixative solution (10 mL 37% formaldehyde, 90 mL dH_2O, 5 mL acetic acid) for 15 minutes, washed with PBS, and then stored in 70% ethanol until use.

Slides were transferred from ethanol or PBS to 0.25% Triton X 100 (in PBS) and incubated for 10 minutes. If antigen recovery was required, the slides were microwaved 3 × 5 minutes in 10 mM sodium citrate solution, pH 6.0. The slides were cooled down until room temperature and washed in PBS for 5 minutes. Tissue to be stained was circled using DAKO hydrophobic pen. Slides were incubated in universal protein block (DAKO) for 15 minutes and then incubated with primary antibody with appropriate dilution overnight at 4°C. After overnight incubation, slides were washed in PBS and incubated with appropriate secondary antibodies at room temperature in dark for 1 hour. After the slides were washed in PBS and then dH2O, slides were incubated with DAPI (1 μg/mL) diluted in water. After several washes with dH2O and PBS, slides were mounted with Vectashield aqueous mounting media (Vector Laboratories) or Prolong Gold (Invitrogen). Slides were sealed using nail polish for permanent mounting.

3.6 FACS Sorting and Analysis

3.6.1 Experimental Design and Rationale

It was speculated that contaminating islets might contribute greatly to the insulin secretion detected by static incubation. An attempt was therefore made to purify islet beta cells and pancreatic ductal cells. By evaluating insulin secretion by islet-like clusters generated with purified cell populations, one might clarify which cell type contributed to the generation of the beta cells within the islet-like clusters.

CA19-9 is a marker commonly used to identify pancreatic carcinoma. In normal pancreas, CA19-9 is a pan-ductal surface antigen that is normally expressed by pancreatic ductal, ductular, and centroacinar cells (Itzkowitz *et al*, 1988; Takasaki *et al*, 1988; Gmyr *et al*, 2004; Githens S, 1988). It is a cell surface antigen that is not expressed by islet cells (Githens S, 1988), making it a suitable marker for FACS sorting. Antibodies against CA19-9 have been used to purify CA19-9 positive ductal cells (Gmyr *et al*, 2004).

Since it is possible that the insulin-secreting cells in islet-like clusters arise from beta cell replication, a protocol was tested to purify human islet beta cells. Using purified beta cells from the same donor as a control, one can demonstrate whether islet-like clusters could indeed be generated from purified beta cells. Additionally, by double staining cells with ductal and beta cell markers, one can ensure elimination of beta cells in the purified ductal cell population through FACS sorting.

3.6.2 Experimental Procedure

Preparation

Cells were dissociated as previously described and incubated at 37°C and 5% CO_2 for at least 30 minutes for recovery. After the recovery period, cells were washed in the sorting solution containing PBS, 2% FBS, and 40 µg/mL of DNase. All of the following staining procedures were done in the sorting solution.

Newport Green

Insulin positive beta cells were sorted according to a previously established protocol using Newport Green (Lukowiak *et al*, 2001). Cells were incubated in sorting solution containing 10 µM Newport Green (Invitrogen) for 60 minutes. Negative control (cells incubated without Newport Green) was prepared simultaneously. After incubation, cells were washed in

sorting solution and filtered through a 40 μm cell strainer and kept on ice until FACS sorting. Propidium iodine (Invitrogen) or 7-AAD (Amino-actinomycin, BD Biosciences) was added to the cell suspension to allow elimination of non-viable cells.

CA19-9

Pancreatic ductal cells were sorted according to a previously established protocol (Gmyr *et al*, 2004). Cells were washed in the sorting solution and incubated with mouse anti-CA19-9 antibody (Vision Biosystems) at 4°C for 30 minutes at 40 μL/mL of cell suspension at the concentration of 1×10^7 cells/mL. Isotype control was prepared by incubating cells with mouse IgG1 negative control (DAKO). After incubation, cells were washed in PBS and then incubated with 5% Alexa Flour 647 anti-mouse antibody (Invitrogen) for 30 minutes at 4°C. After the second incubation, cells were washed in sorting solution and filtered through a 40 μm cell strainer and kept on ice until FACS sorting. Propidium iodine (Invitrogen) or 7-AAD (Amino-actinomycin, BD Biosciences) was added to the cell suspension to allow elimination of non-viable cells.

Sorting

FACS sorting was done at three FACS core facilities: Terry Fox Laboratory Flow Cytometry Core Facility, UBC Flow Cytometry Facility, and Child & Family Research Institute Flow Cytometry Facility. FACS analysis was done at the Terry Fox Laboratory Flow Cytometry Core Facility. Sorted cells were collected into media containing 50% CMRL and 50% FBS to increase cell viability. After sorting, cells were spun down and resuspended in appropriate media and prepared for further analysis or cell culture.

3.7 MACS Sorting

3.7.1 Experimental Design and Rationale

Two MACS systems were tested. The Stem Cell Technologies (SCT) system involves the use of an "easy sep" magnet that allows quick separation by marking the cells of interest with magnetic beads, immobilizing these cells on the wall of a tube placed in a magnet, and separation of these cells from the negative population by decanting. In the Miltenyi Biotech (MB) system, target cell population was marked by magnetic beads and retained in the purification column with an external magnet. Their abilities to purify pancreatic ductal cells were compared.

Carbonic anhydrase II (CAII) is an intracellular enzyme that can be found mostly in ductal cells and occasionally in acinar cells (Kumpulainen and Jalovaara, 1981; Githens S, 1988; Inada *et al*, 2006). It is used as a marker to identify ductal cells in the purified cell population. CA19-9, as previously mentioned, continued to be used as the marker for sorting the cells.

3.7.2 Experimental Procedure

Preparation

Cells were dissociated as previously described and incubated at 37°C and 5% CO_2 for at least 30 minutes for recovery. After the recovery period, cells were washed in the sorting solution containing PBS, 2% FBS, and 40 µg/mL of DNase. All of the following staining procedures were done using the sorting solution.

Stem Cell Technologies EasySep (One step labeling)

Magnetic cocktails were prepared according to manufacturer's instructions. Mouse anti-CA19-9 antibody was mixed with cocktail components provided in this kit to prepare EasySep positive selection cocktail. Dissociated cells were incubated with the positive selection cocktail in a round bottom 5mL polystyrene tube at room temperature for 15 minutes. Magnetic

nanoparticles were then added into the cell suspension and incubated for another 10 minutes. The cell suspension was then placed in the EasySep magnet for 8 minutes. The supernatant was decanted into another polystyrene tube. This tube contains the CA19-9 negative population (with the tube still in the magnet). The original tube was then removed from the EasySep magnet and the cells were resuspended and placed back into the magnet. This was repeated three times. The purified cell population (CA19-9 positive) in the original tube as well as the rest of the cells in the second tube (CA19-9 negative) were resuspended in appropriate media and prepared for further analysis or culture. Cell viability was determined by trypan blue staining.

Miltenyi Biotech (Two step labeling)

Cells were first incubated with mouse anti-CA19-9 antibody at 4°C for 10 minutes. Cells were washed and incubated with goat anti-mouse IgG magnetic microbeads for 15 minutes at 4°C. Cells were washed and resuspended in sorting buffer before proceeding to sorting. Cell suspension was run through the MS separation column that was placed in a magnetic field. CA19-9 positive cells remained in the column while the negative cells were eluted. Cells in the column were washed three more times and the total effluent was collected and marked as CA19-9 negative. The column was then removed from the magnetic field and the cells in the column were eluted using the sorting solution. The purified cell population (CA19-9 positive) as well as the rest of the cells (CA19-9 negative) were resuspended in appropriate media and prepared for further analysis or culture. Cell viability was determined by trypan blue staining.

3.8 Culture Dissociated and Purified Single Cells

Purified cells were cultured in the same conditions as outlined by the original Bonner-Weir protocol (with the use of KGF) as previously described, except with the use of plates with tissue culture treated surface. The purpose of using tissue culture treated surface was to promote

cell adhesion to the culture surface after cell sorting. KGF was used here because it was found to promote ductal cell proliferation (Yi et al, 1994), and would hopefully enhance the culture condition for sorted ductal cells.

3.9 Glucose Stimulated Insulin Release (Static Incubation)

3.9.1 Experimental Design and Rationale

A sequential static incubation method was first considered rather than a parallel static incubation method to eliminate well-to-well variation caused by pipetting errors and difference in the number of insulin-secreting beta-like cells generated. In a sequential static incubation, islet-like clusters were incubated in solution containing low level of glucose (2.8mM). After the first incubation period, samples were collected and the same islet-like clusters were subsequently incubated in solution containing high level of glucose (20mM). It was later found that this method created several technical difficulties, including problems in washing and handling the clusters, and therefore a parallel static incubation system was tested and used instead for detecting insulin secretion by human islet-like clusters. In a parallel static incubation system, islet-like clusters were generated in multi-well plates. Clusters in each well were subjected to a specific condition, such as different glucose levels and insulin secretagogues. Insulin secretion in different wells were measured and compared to evaluate insulin secretion upon stimulation.

A mouse inulinoma cell line (Min6) was first used to confirm that the parallel static incubation system set up could reliably demonstrate glucose stimulated and GLP-1 stimulated insulin secretion. Insulin-secreting Min6 cells were exposed to various conditions including low glucose level (3 mM), mid glucose level (11.5 mM), high glucose level (20 mM), low glucose level with potassium chloride, high glucose level with 10 nM GLP-1, and high glucose level with 50 nM GLP-1. GLP-1 is a known insulin secretagogue that plays important physiological roles as one of the two incretins. In addition, GLP-1 receptors were found to be expressed by mature

beta cells (Huypens et al, 2000). Therefore, its effect on differentiated islet-like clusters is an area of interest. The effect of exposure to potassium chloride was also studied. Potassium chloride has often been used in perifusion and static incubation studies. Exposure to potassium chloride results in an increase in potassium level in beta cells and leads to the release of insulin granules without involving the glucose sensing part of the glucose stimulated insulin secretion pathway. Static incubation with potassium chloride can therefore be used to evaluate the functionality of the islet-like clusters generated. If one sees no insulin secretion except with the exposure of potassium chloride, the islet-like clusters generated might then have deficiency in glucose sensing but not the synthesis of insulin.

During sample collection, samples were spun and the debris was removed by transferring supernatant into new collection tubes. Islet-like clusters were washed repetitively before static incubation by a gentle procedure to ensure the removal of culture media without disturbing islet-like clusters. Insulin secretion was normalized by the amount of DNA in each well to ensure that variation in cell number between wells does not affect insulin secretion results.

3.9.2 Sequential Static Incubation

The steps of sequential static incubation are outlined in Figure 1 below. Pancreatic exocrine tissue was cultured in 6 well plates to generate islet-like clusters as previously described. Viability media (Media Tech) containing 2.8 mM and 40 mM of glucose was prepared and filter sterilized. Islet-like clusters were washed twice in low glucose viability media and then incubated in 1500μL of the same viability media for 2 hours. After incubation, 700 μL of the low glucose media was removed and stored in –20°C for further analysis. 700 μL of 40 mM viability media was then added into the well so that the total volume in each well was 1500 μL and the glucose concentration of the viability media in the well is 20.16 mM. The islet-like clusters were

incubated for another 2 hours and the viability media was stored at –20°C until radioimmunoassay.

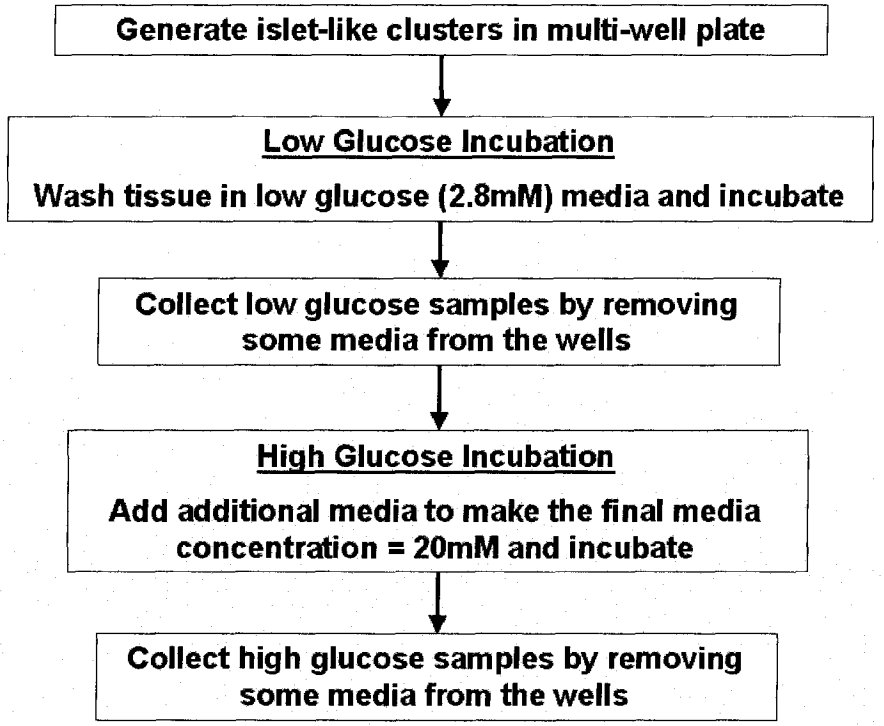

Figure 1 General Procedure for Sequential Static Incubation of Islet-like Clusters.

3.9.3 Parallel Static Incubation

The steps of parallel static incubation are outlined in Figure 2 below. Parallel static incubations were done using Krebs–Ringer bicarbonate buffer (KRB buffer) containing 129 mM NaCl, 4.8 mM KCl, 1.2 mM $MgSO_4.7H_2O$, 1.2 mM KH_2PO_4, 2.5 mM $CaCl_2.2H_2O$, 5 mM $NaHCO_3$, 10 mM HEPES, 3 mM Glucose, and 0.5% BSA (all chemicals from Sigma). This solution is termed "3G KRB" solution. KRB containing 11.5 mM (11G KRB) and 20 mM (20G KRB) of glucose were also prepared. KCl-KRB buffer, used to stimulate the release of insulin granules, contained 30 mM KCl (Sigma). To test whether GLP-1 has any stimulatory effect in insulin secretion by the islet-like tissue generated, KRB buffer solutions containing 10 nM and

50 nM of GLP-1 (From Dr. David Thompson, UBC) were also prepared. ITS free serum free DMEM/F12 media (as described in the Bonner-Weir method without the addition of ITS supplement) was prepared for washing.

Sufficient amount of various KRB solutions and serum free ITS free DMEM/F12 were incubated at 37°C in 5% CO_2 for at least 30 minutes to ensure the temperature and CO_2 content of the prepared solutions equilibrated with the incubator environment. Cells in 24 well plates were washed three times with serum free ITS free media. Each wash involves decanting the media by inverting the plate over an alcohol sanitized catching plate to prevent direct contact with the cells, adding new wash solution, and incubating at 37°C in 5% CO_2 for 20 minutes. The cells were then washed twice with 3G KRB solution, with the second wash left in the wells and incubated for 30 minutes before removal. Again the media was removed by flipping the plate over. In each well, 500µL of various KRB solutions containing 3 mM glucose, 11 mM glucose, 20 mM glucose, KCl, 10 nM GLP-1, and 50 nM GlP-1 were added to respective wells. Islet-like clusters were incubated for 90 minutes. After 90 minutes, 400 µL of the incubated solutions were slowly collected from each well into microtubes. Samples were spun at 2000 rpm for 10 minutes to remove any cell debris. 350 µL of the supernatant was transferred into new microtubes and samples were stored at –20°C or lower until radioimmunoassay. Leftover media in the wells was removed by inverting the plate over the catching plate. The 24 well plate was stored at -80°C until DNA quantification.

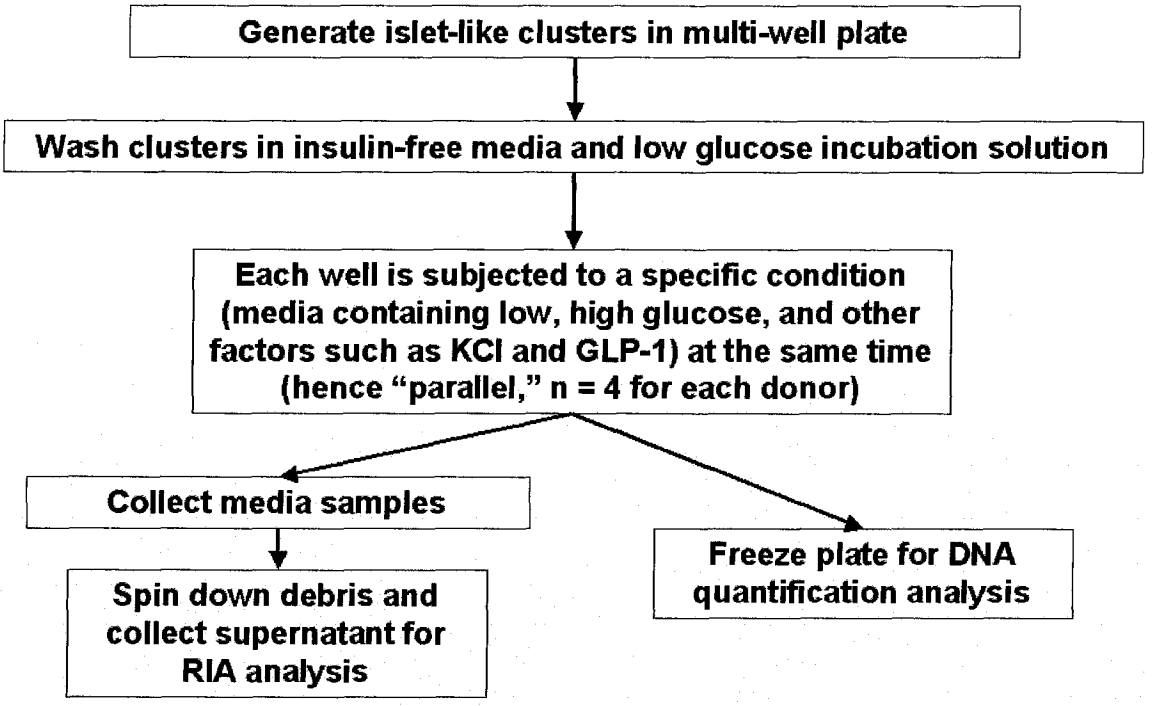

Figure 2 General Procedure for Parallel Static Incubation of Islet-like Clusters

3.9.4 DNA Quantification

CyQuant Cell Proliferation Assay Kit (Invitrogen) was use to quantify the amount of DNA in each well of the 24 well plate. Cells in each well were lysed with 250 μL of cell lysis buffer. The cell lysis buffer was prepared using the 20X stock provided with the kit with distilled water supplemented with 180 mM NaCl and 1 mM EDTA. The partially lysed cells were transferred into 1.5 mL microtube and further broken down by 3 × 5 seconds sonication with a dismemberator (Sonic Dismemberator, Fisher Scientific). The final lysed cell samples were diluted to appropriate concentration and assayed according to the manufacturer's instruction.

3.9.5 Radioimmunoassay

Insulin and C-peptide concentrations in the static incubation samples were determined using normal human insulin RIA kits (HI-14K), ultra sensitive human insulin RIA kits (HI-11K),

and human C-peptide RIA kits (HCP-20K) according to manufacturer's instructions (Linco/Millipore). According to previously experience, half of the reagent amount suggested by the manufacturer is sufficient in detecting the presence of insulin and C-peptide. Therefore, the amounts of the sample and reagents used were reduced to half. Samples with insulin concentration outside of standard curve were further diluted with PBS and re-assayed. Insulin concentrations in samples were normalized by their respective quantity of DNA.

3.10 Data Analysis and Statistical Test

Figures in this thesis were plotted using GraphPad Prism 4.0 computer program and shown with means ± standard error of mean unless otherwise indicated. Data were tested using Bartlett's test to determine whether data log transformation was necessary before statistical analysis. If log transformation was necessary, data were then expressed as geometric means and confidence intervals. Student's t-test, one-way ANOVA, and two-way ANOVA were carried out using GraphPad Prism 4.0.

Chapter 4 Results

4.1 Generation of Islet-like Clusters

Pdx-1 protein expression is an important indicator of potential putative progenitors to differentiate into islet cells. Through immunofluorescent staining, Pdx-1 protein was found to be expressed by both insulin positive beta cells as well as CA19-9 positive pancreatic ductal cells (Figure 3). This demonstrated the potential of using pancreatic ductal tissue to generate islet-like clusters *in vitro*.

Dithizone-positive islet-like clusters could be generated using both the Bonner-Weir and the Todorov protocols (Figure 4) (Bonner-Weir *et al*, 2000; Todorov *et al*, 2006). Islet-like clusters generated using the Bonner-Weir method were approximately 150-250 μm in diameter. It was common to observe islet-like clusters that were partially but not completely dithizone positive. These islet-like clusters remained attached to the plate until being flushed with a strong stream of media using a pipetter. In the original Bonner-Weir protocol, non-tissue culture treated surface was used to prevent islet attachment to the culture plates. Here, tissue culture treated and untreated plates were compared in their ability to generate islet-like clusters (Figure 5). It was found that cells cultured on treated plates tended to stay in the monolayer after Matrigel was added to the culture. On the other hand, when non-treated plates were used, cells quickly migrated into clusters once Matrigel was added. Islet-like clusters generated using the Todorov method were slightly smaller in size than those generated with the Bonner-Weir method, but were also found to be Dithizone positive. A large amount of cell debris and small clusters were also found. The Bonner-Weir protocol was selected as the method to generate islet-like clusters for the following static incubation experiments. This is because the Bonner-Weir protocol was based on the same hypothesis of this thesis study, and that adherence of the islet-like clusters to their culture surface allowed better handling of the clusters in a static incubation protocol.

Islet-like clusters generated using the Bonner-Weir protocol were found to consist mainly of CA19-9 and Carbonic anhydrase II positive pancreatic ductal cells (Figure 6). Some insulin positive cells, although few, were visible. It was however difficult to distinguish the insulin positive cells from non-specific staining. This further emphasizes the importance of using insulin-secretion as a criterion to evaluate the generation of islet-like clusters *in vitro*.

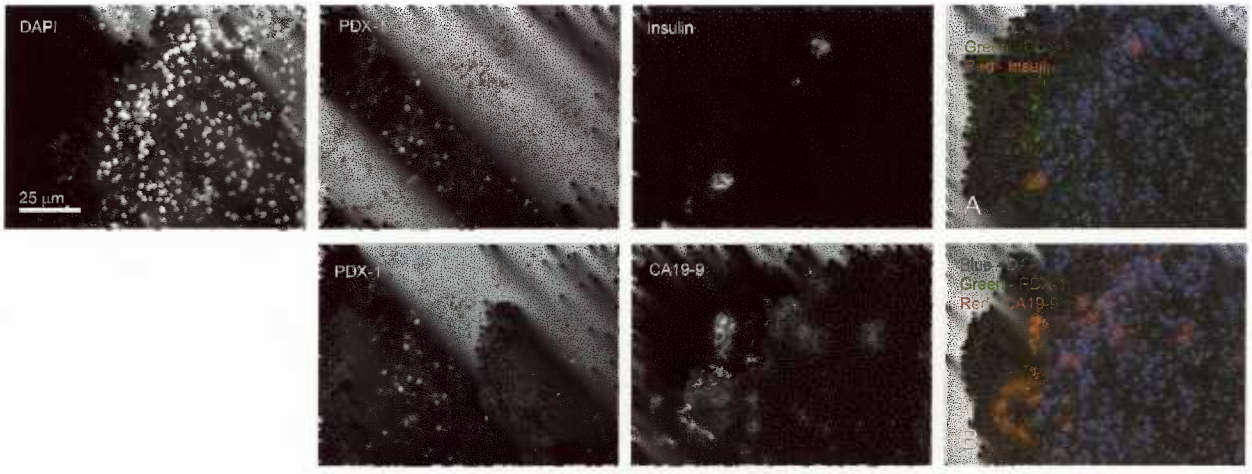

Figure 3 Pdx-1 Expression by Human Pancreatic Ductal Cells and Beta Cells. Pdx-1 protein was expressed by both insulin positive beta cells (A) and CA19-9 positive ductal cells (B).

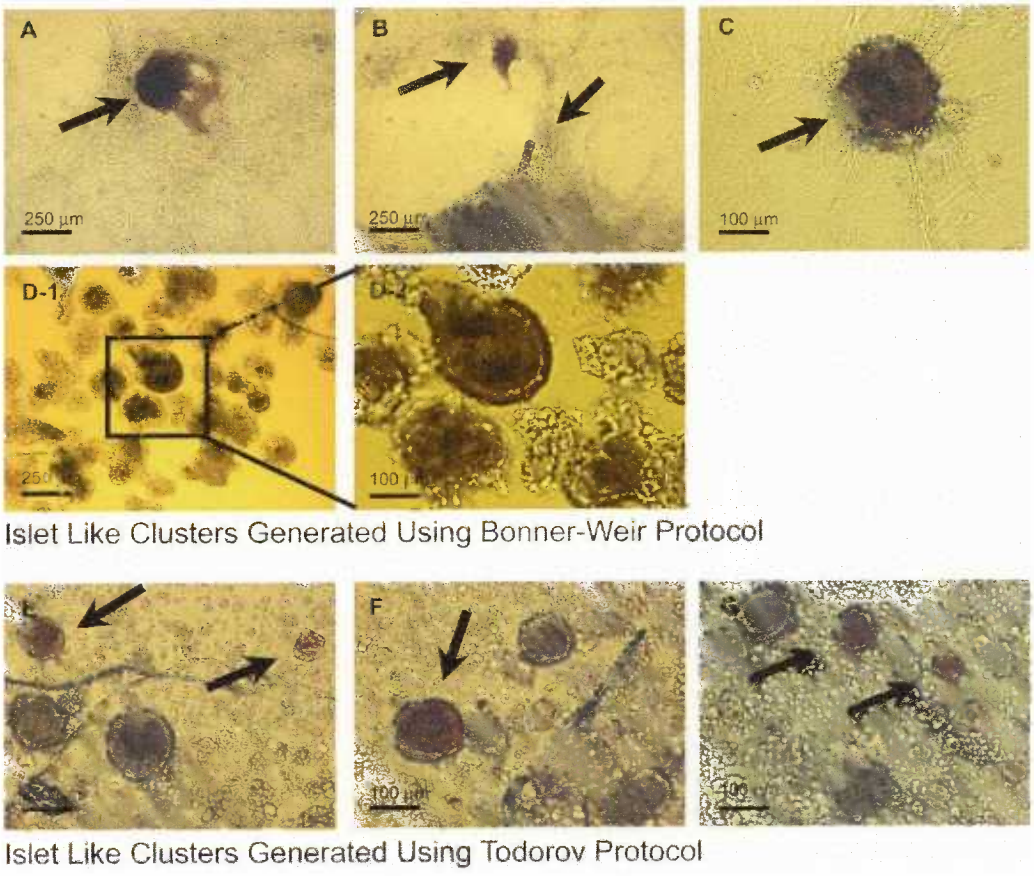

Figure 4 Generation of Dithizone-positive Islet-like Clusters (Arrows) Using Crude Human Exocrine Tissue. A-D: Islet-like clusters generated using the Bonner-Weir protocol. D: Islet-like clusters after they were flushed off from tissue culture surface. E-G: Islet-like clusters generated using the Todorov protocol.

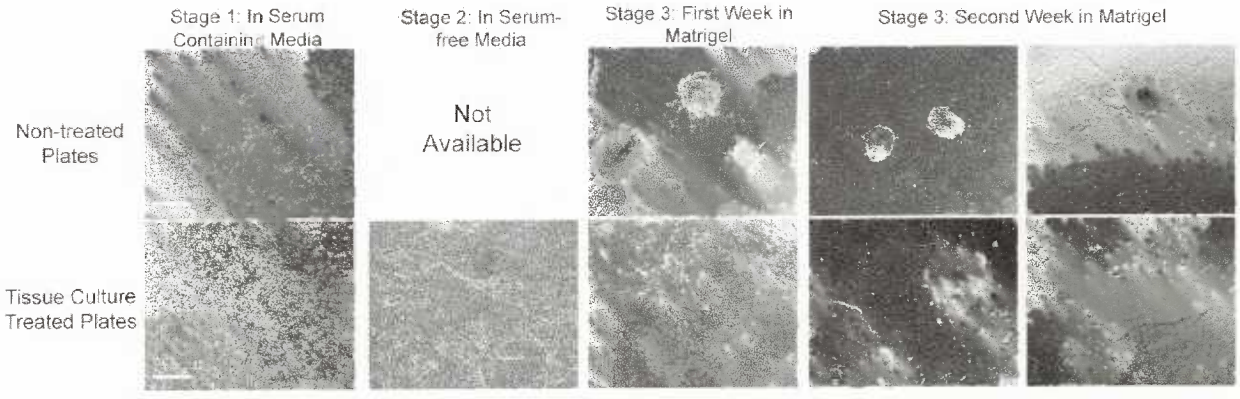

Figure 5 Human Islet-like Clusters Generated Using Tissue Culture Treated and Non Treated Plates. Islet-like clusters were generated more readily when non-treated plates were used, while cells in the treated plates tended to remain in a monolayer after Matrigel was layered on top.

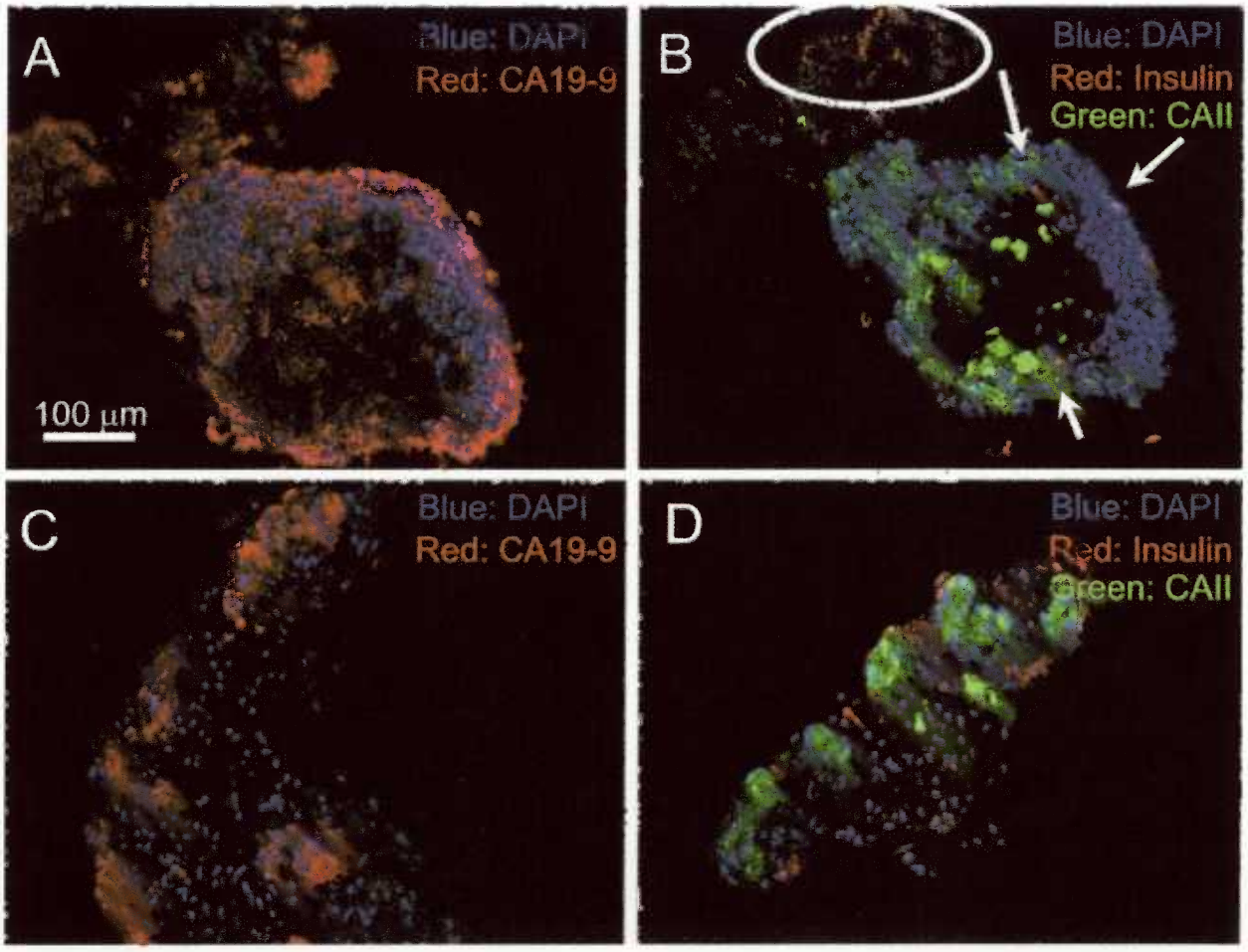

Figure 6 Beta Cell and Ductal Cell Marker Expression by Human Islet-like Clusters Generated Using The Bonner-Weir Protocol. The islet-like clusters consisted mostly of ductal cells (positive for CA19-9 and CAII). Some insulin positive cells (arrows in B, D) were observed in these islet-like clusters. Cell nuclei were labeled by DAPI. A and B are consecutive sections.

4.2 Static Incubation

4.2.1 Sequential Static Incubation

Islet-like clusters were generated and tested for their ability to secrete insulin into the static incubation media using a sequential static incubation system. Percentage of basal insulin secretion was calculated to demonstrate whether glucose-stimulated insulin release was observed in the islet-like clusters generated. It was found that out of 9 experiments using tissue from four donors, only two demonstrated glucose-stimulated insulin secretion (Table 2 and Figure 7). Overall, average insulin secretion upon glucose stimulation was 87% of basal (n = 4 donors). Two-way ANOVA analysis demonstrated that there was no significant insulin secretion compared with basal when the islet-like clusters were exposed to high glucose level. In fact, a significant decrease was found to be associated with increase glucose level ($p = 0.0098$, $F = 10.11$).

Table 2 Sequential Static Incubation of Islet-like Clusters Generated Using Exocrine Tissue from Various Donors (n = 9). Statistical analysis using two-way ANOVA demonstrated that incubation of islet-like clusters in high glucose level ([glucose] = 20 mM) resulted in a significant decrease in insulin secretion ($p = 0.0098$).

Sample #	Donor ID	Insulin concentration at 2.8mM glucose (μU/mL)	Insulin concentration at 20mM glucose (μU/mL)	% of basal insulin secretion
1	BC54	46.3	12.7	27.46
2	BC54	4.40	19.7	445.97
3	BC54	21.8	25.3	115.82
4	BC55	25.7	21.7	84.60
5	BC55	30.5	27.4	89.84
6	BC57	62.9	16.4	26.00
7	BC57	100.9	29.0	28.76
8	AB1077	90.2	50.9	56.46
9	AB1077	200.0	28.8	14.38

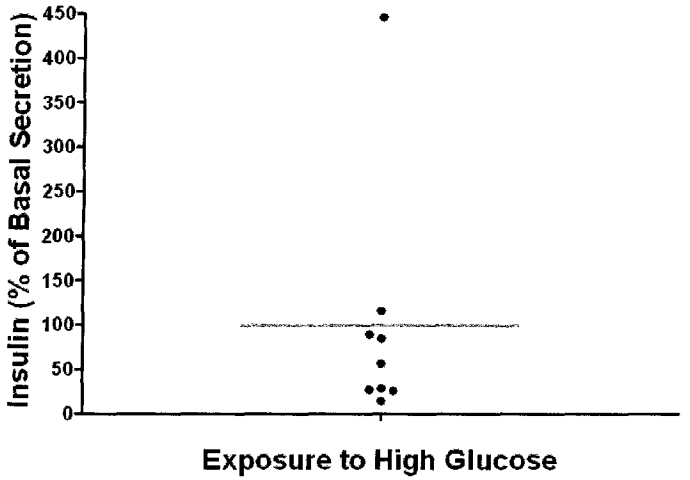

Figure 7 Insulin Secretion (As Percentage of Basal) by Islet-like Clusters in Sequential Static Incubation. Raw data (n = 9 experiments). The gray line represents 100% of basal secretion. Any data point that is above the gray line demonstrates an increase in insulin secretion upon glucose stimulation.

4.2.2 Parallel Static Incubation with Min6 Cells and DNA Normalization

Results of Min6 parallel static incubation are shown in Figure 8. Significant increases in insulin secretion (unit: 10^{-5}mL) compared with basal (geometric mean, 20.51; C.I., 14.27-29.47) were observed when Min6 cells were exposed to mid glucose level (49.89, 43.28 - 57.51, vs. basal; $p<0.001$), high glucose level (57.02, 49.06 - 66.27, vs. basal; $p<0.001$), and potassium chloride (251.19, 201.54 - 313.07, vs. basal; $p<0.001$). Insulin secretion by cells incubated in high glucose was further enhanced by the introduction of 10 nM GLP-1 (230.67, 192.85 - 275.91, vs. basal; $p<0.001$) and 50 nM GLP-1 (234.96, 186.02 - 296.79, vs. basal; $p<0.001$) into the incubation solution.

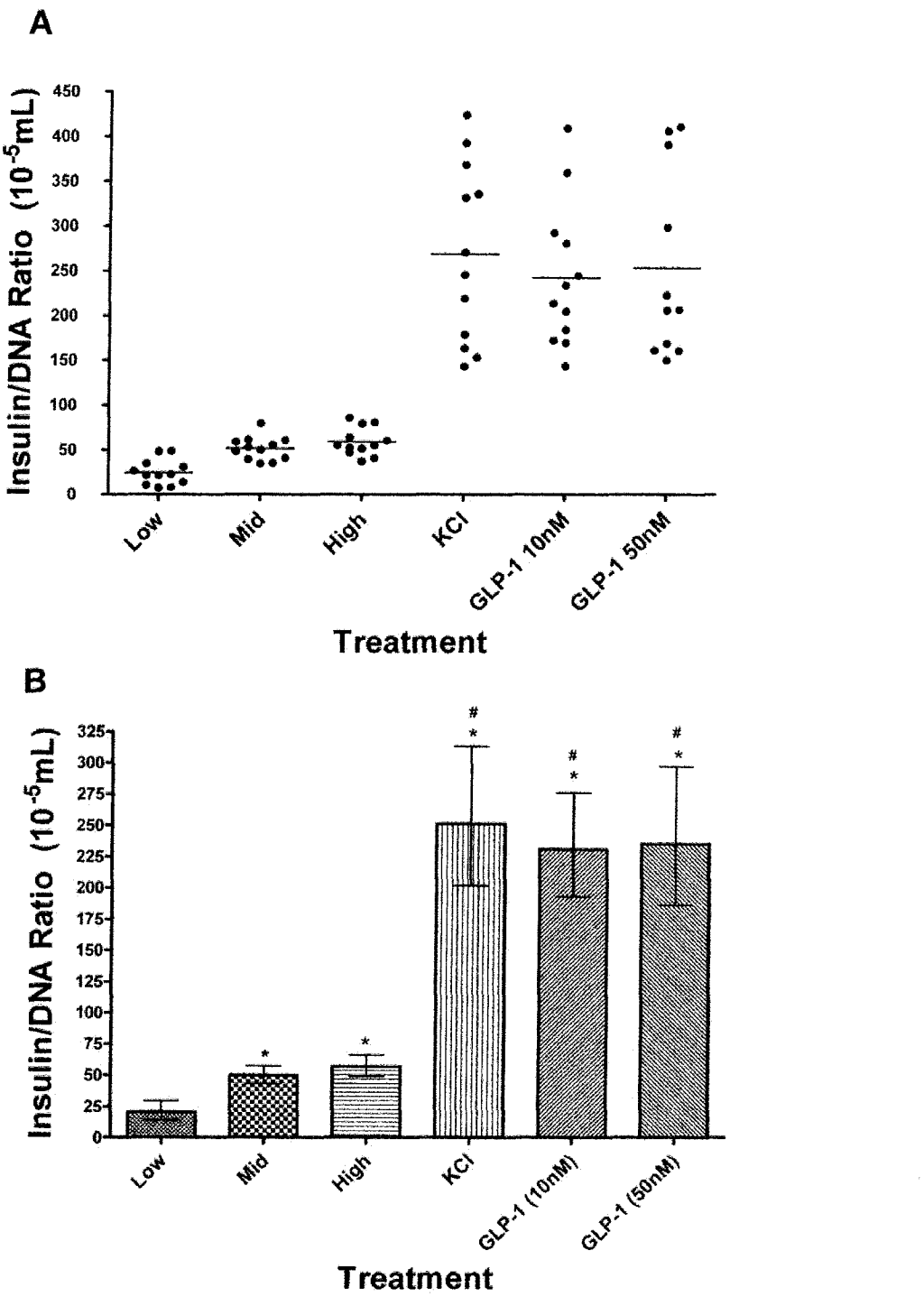

Figure 8 Parallel Static Incubation with Min6 Cells after DNA Normalization (n=12). A: Raw data (•) and mean (–). B: Geometric mean (column bar) and confidence interval (error bar). Significant increases in insulin secretion compared with basal were observed when Min6 cells were exposed to higher glucose levels and insulin secretagogues (*, vs. low glucose, $p<0001$). Insulin secretion at high glucose level was significantly increased upon exposure to GLP-1 at 10nM and 50nm ($^{\#}$, vs. high glucose, $p<0.001$). Original data were log-transformed, analyzed with one-way ANOVA, and back-transformed.

4.2.3 Parallel Static Incubation with Human Islet-like Clusters

Based on the parallel static incubation system established using Min6 cells, insulin secretion by islet-like clusters generated using pancreatic tissue from three different donors was evaluated. Exocrine tissue culture at three stages of differentiation was studied.

Insulin Secretion Upon Stimulation by Glucose and Other Factors

Results of parallel static incubation were summarized and plotted as percentage of basal insulin secretion in Figure 9 below. At stage one, significant increases in insulin secretion were found with the exposure of mid glucose level (mean ± SEM, 465.0 ± 166.8 % of basal, $p<0.05$) and GLP-1 both at 10 nM (483.5 ± 169.2 % of basal, $p<0.05$) and 50 nM (794.4 ± 169.2 % of basal, $p<0.01$). However, at stage two, significant increases in insulin secretion were only observed with the exposure of potassium chloride (224.3 ± 169.2 % of basal, $p<0.01$) and 50 nM GLP-1 (295.1 ± 88.6 % of basal, $p<0.05$). At stage three, similarly to stage one, increases in insulin secretion were found with exposure of mid glucose level (194.2 ± 27.7 % of basal, $p<0.05$) and GLP-1 at both 10 nM (212.6 ± 22.7 % of basal, $p<0.05$) and 50 nM (415.2 ± 83.3 % of basal, $p<0.01$). At stage one, significant increases in insulin secretion confirmed the presence of insulin-secreting beta cells in crude exocrine tissue. Crude exocrine tissue at stage one and islet-like clusters at stage three exhibited similar responses to glucose levels and GLP-1, while tissue at stage two did not respond as strongly to glucose levels but significantly to the exposure of potassium chloride.

Interestingly, none of the tissue at stage one, two, and three responded significantly to high glucose level at 20 mM (stage 1, 252.4 ± 97.7 % of basal, $p = 0.097$; stage 2, 291.4 ± 118.2 % of basal, $p = 0.090$; stage 3, 96.6 ± 34.4 % of basal, $p = 0.46$), contrary to what was commonly observed with human islet tissue in culture (Harrison *et al*, 1985; Ritzel *et al*, 2003). However,

exposure to GLP-1 at high glucose level allowed tissue to respond to incubation in high glucose media, resulting in significant increases in insulin secretion at stage 1 and stage 3.

To further analyze the effect of culture stages to insulin secretion by tissue from individual donors, data were plotted (Figure 10) and summarized in table form (Appendix 2). Furthermore, raw insulin data were transformed and analyzed using two-way ANOVA (Table 3). It was found that for islet-like clusters generated with tissue from BC086 and BC091, culture stages had a significantly positive effect on insulin secretion in some cases. However, this effect was not found in the culture of tissue from BC090. In fact, insulin secretion in response to 50 nM GLP-1 stimulation decreased significantly with the progression of culture stages. It was noted that BC090 tissue was from a donor younger (17 years old) than donors BC086 and BC091 (47 and 50 years old).

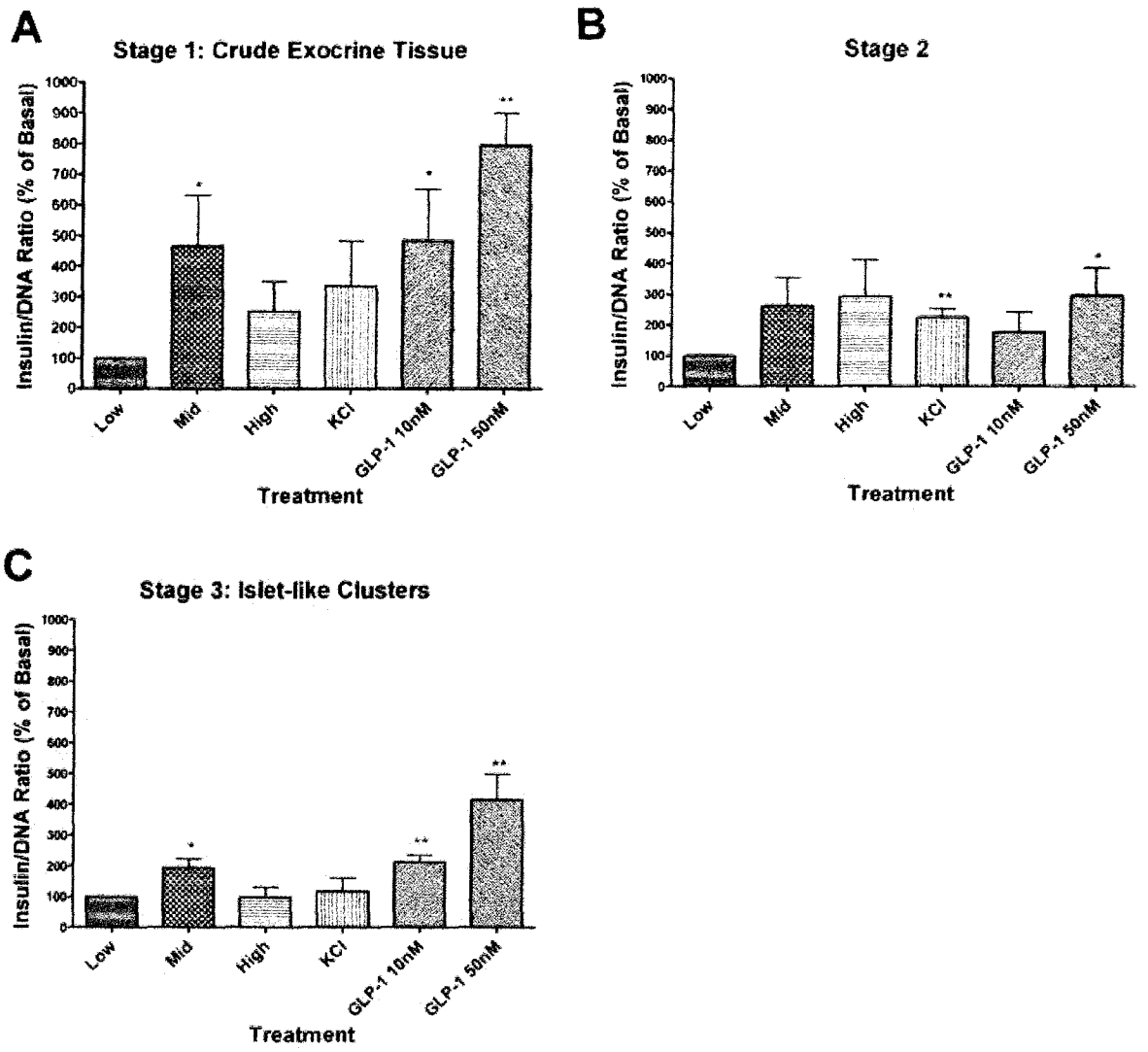

Figure 9 Insulin Secretion by Human Islet-like Clusters with Response to Stimulation by Glucose and Other Factors (n = 3 donors). Results are shown as mean (column bar) and SEM (error bar). At stage one, significant increases in insulin secretion were observed with exposure to mid glucose level and GLP-1. At stage two, exposure to potassium chloride and GLP-1 resulted in significant increases in insulin secretion. At stage three, significant increases in insulin secretion were found when islet-like clusters were incubated with mid glucose level and GLP-1, similar to that at stage one. Significance was determined by Student's t-test and denoted by * (*, $p<0.05$; **, $p<0.01$).

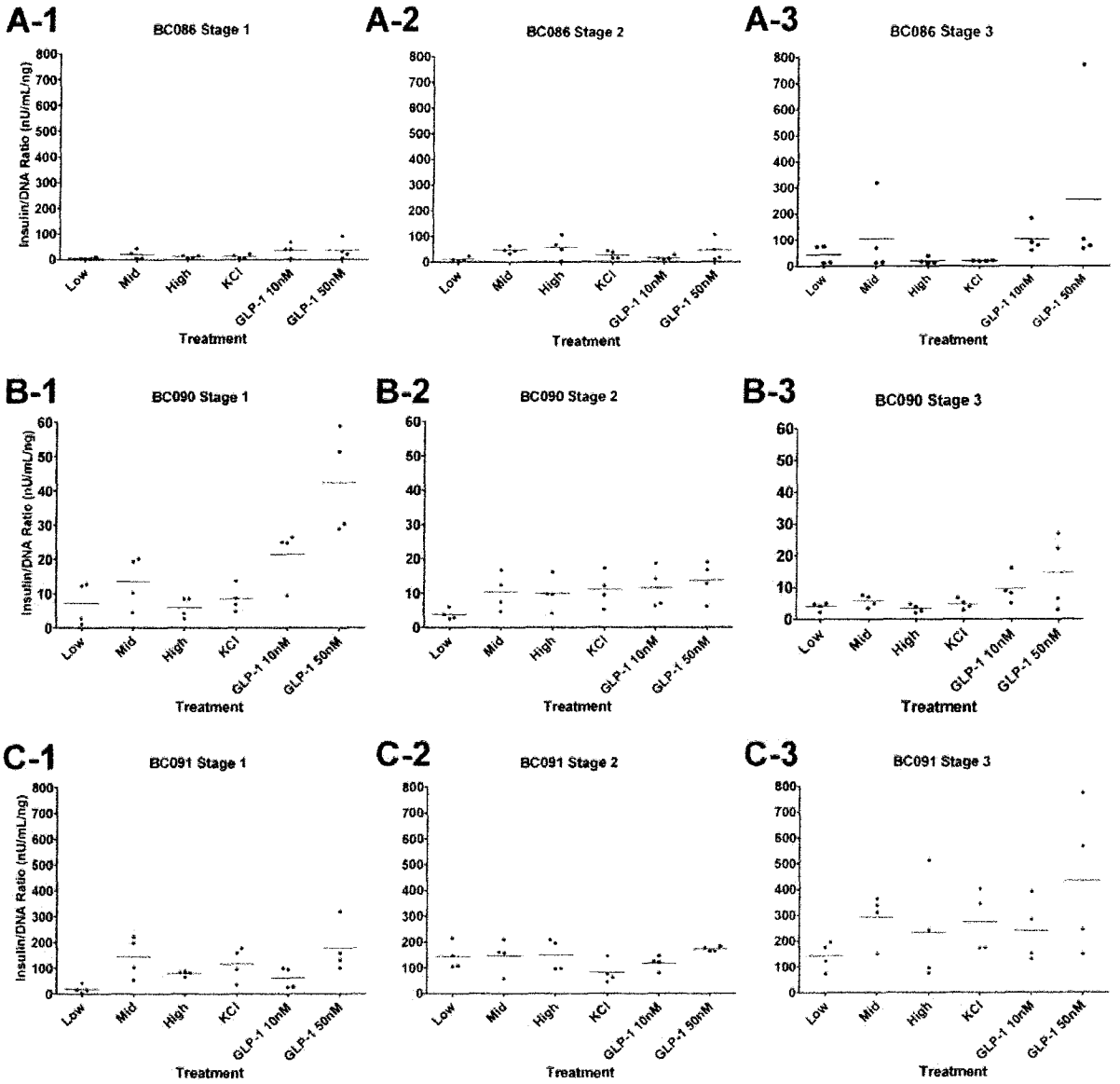

Figure 10 Insulin Secretion by Human Islet-like Clusters Generated Using Tissue from Individual Donors (n = 4 for each donor). A1-3: Tissue from donor BC086; B1-3: Tissue from donor BC090; C1-3: Tissue from donor BC091. Raw data (·) and mean (-) were shown in each graph. All data were normalized by DNA quantity.

Table 3 Two-way ANOVA Analysis of The Effect of Culture Stages on Insulin Secretion Results.

Donor ID	Donor Age	Overall*	Stage 1 vs. Stage 2	Stage 1 vs. Stage 3**	Stage 2 vs. Stage 3**
BC086	47	$P < 0.0001$, $F = 12.50$	NS	+, $p < 0.01$ (Low glucose)	+, $p < 0.05$ (GLP-1 10nM)
BC090	17	$P < 0.0001$, $F = 14.06$	NS	-, $p < 0.001$ (GLP-1 50nM)	NS
BC091	50	$P < 0.0001$, $F = 17.13$	NS	+, $p < 0.01$ (GLP-1 50nM)	+, $p < 0.01$ (GLP-1 50nM)

* Overall analysis demonstrated statistical significance in all donors.

** Data were transformed prior to two-way ANOVA analysis: + denotes a significant increase in insulin secretion; - denotes a significant decrease in insulin secretion; NS denotes non-significance.

Increase in Basal Insulin Secretion

Using the same set of data, analysis was done on the amount of basal insulin secreted by tissue from different donors at different stages (Figure 11). At stage three of islet-like cluster generation, significant increases in basal insulin secretion (at 3mM glucose) were observed in two out of three donors. In BC086, there was a significant increase in basal insulin secretion from 3.329 μU/mL/ng at stage one (geometric mean, CI: 1.477-7.504) to 31.842 (CI: 12.127-83.607) at stage three. In BC091, there was an increase of basal insulin secretion from 12.853 μU/mL/ng at stage one (CI: 4.312-38.311) to 137.088 (CI: 98.534-190.727) at stage two and to 133.660 (CI: 87.041-205.248) at stage three. However, no significant increase in basal insulin secretion was observed in islet-like clusters generated using exocrine tissue from donor BC090 (stage one, 4.534 with CI: 1.340-15.342; stage two, 3.628 with CI: 2.526-5.211; stage three, 3.873 with CI 2.730-5.493)

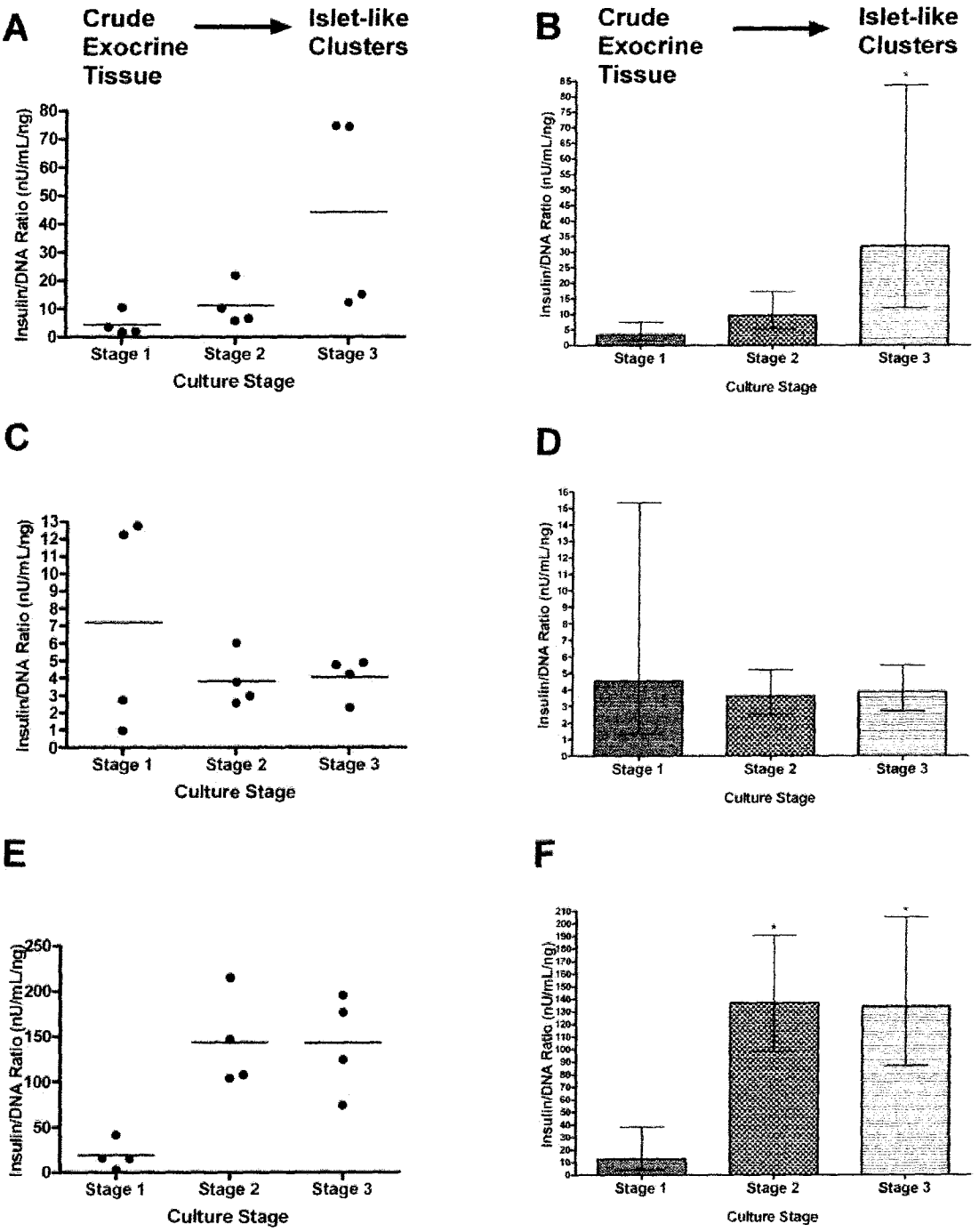

Figure 11 Basal Insulin Secretion by Islet-like Clusters at Three Stages of Cell Differentiation. A, B: from donor BC086; C, D: from donor BC090; E, F: from donor BC091. A, C, and E: Raw data (•) and mean (–). B, D, and F: Geometric mean (column bar) and confidence interval (error bar). Significant increases in basal insulin secretion were observed at stage three of BC086 tissue and at stage two and three of BC091 tissue. No significant increase in basal insulin secretion was found at stage two and three of BC090 tissue. Original data were log-transformed, analyzed with one-way ANOVA, and back-transformed. Significances were determined by one-way ANOVA and denoted with * ($p<0.05$).

C-peptide Secretion by Islet-like Clusters

To ensure that insulin secretion detected was indeed *de novo* secretion and not an artifactual, C-peptide secretion was measured from the same sample where insulin secretion was measured. Since only a regular C-peptide assay was available (ultra sensitive insulin RIA assay kit was used in the measurement of parallel static incubation samples), many samples were found to contain no detectable level of C-peptide. An arbitrary C-peptide concentration at half of the detectable level was assigned to these samples.

Data (normalized by DNA quantity) of C-peptide secretion were plotted (Figure 12) and compared with raw data of insulin secretion (Figure 10). Raw C-peptide secretion data were summarized in a table format in Appendix 3. Similar to what was observed with insulin secretion, C-peptide secretion was detected from tissue of all donors at stage 1 upon exposure to glucose and other factors, demonstrating the presence of insulin-secreting beta cells within crude exocrine tissue. What was also clear was that C-peptide secretion data appeared to correspond closely to insulin secretion data, demonstrating the presence of *de novo* insulin secretion.

To study whether culture stage is relevant to C-peptide secretion, a two-way ANOVA analysis was done for tissue from each donor at three stages (Table 4). It was found that culture stages significantly affected C-peptide secretion results (BC086, $p = 0.0062$, $F = 5.60$; BC090, $p = 0.0006$, $F = 10.30$; BC091, $p < 0.0001$, $F = 17.38$). C-peptide secretion by BC086 tissue at 50 nM GLP-1 was found to increase significantly from stage one to stage three ($p<0.001$) and from stage two to stage three ($p<0.001$) with the progression of culture stages. Similarly, C-peptide secretions by BC091 tissue in high glucose and in 10 nM GLP-1 were found to significantly increased from stage one to stage three (high glucose, $p<0.05$; 10nM GLP-1, $p<0.01$). On the other hand, responses to 10 nM GLP-1 and 50 nM GLP-1 appeared to decrease significantly with the progression of culture stages for tissue from BC090 (10nM GLP-1, $p<0.01$; 50nM GLP-1,

p<0.001). As mentioned previously, BC090 tissue was from a donor younger (17 years old) than donors BC086 and BC091 (47 and 50 years old).

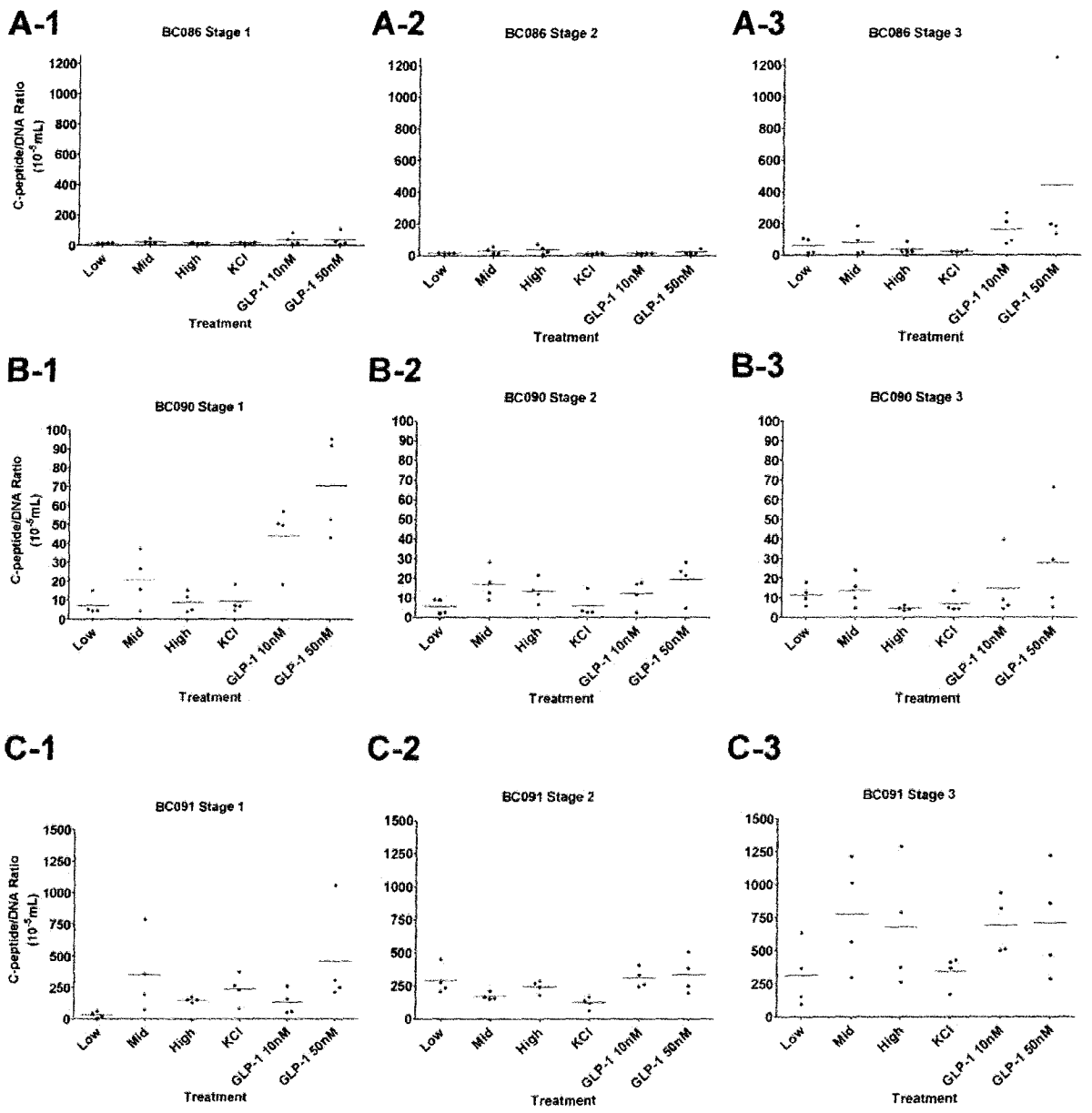

Figure 12 C-peptide Secretion by Human Islet-like Clusters Generated Using Tissue from Individual Donors (n = 4 for each donor). A1-3: Tissue from donor BC086; B1-3: Tissue from donor BC090; C1-3: Tissue from donor BC091. Raw data (•) and mean (–) were shown in each graph. All data were normalized by DNA quantity.

Table 4 Two-way ANOVA Analysis of The Effect of Culture Stages on C-peptide Secretion Results.

Donor ID	Donor Age	Overall	Stage 1 vs. Stage 2	Stage 1 vs. Stage 3	Stage 2 vs. Stage 3
BC086	47	P = 0.0062 F = 5.60	NS	+, P < 0.001 (GLP-1 50nM)	+, P < 0.001 (GLP-1 50nM)
BC090	17	P = 0.0002 F = 10.30	-, P < 0.01 (GLP-1 10nM) -, P < 0.001 (GLP-1 50nM)	-, P < 0.01 (GLP-1 10nM) -, P < 0.001 (GLP-1 50nM)	NS
BC091	50	P < 0.0001 F = 17.38	NS	+, P < 0.05 (High glucose) +, P < 0.01 (GLP-1 10nM)	+, P < 0.01 (Mid glucose)

* Overall analysis demonstrated statistical significance.

** **+** denotes a significant increase in insulin secretion; **-** denotes a significant decrease in insulin secretion; NS denotes non-significance.

Comparison between Treated and Non-treated Culture Surfaces

It was suggested by the Bonner-Weir group (Bonner-Weir *et al*, 2000) that the use of non-treated tissue culture surface prevents attachment of islet-tissue from culture surface. Therefore, basal insulin secretion by crude exocrine tissue cultured in treated and non-treated culture surfaces was compared for tissue from donors BC090 and BC091 (Figure 13). It was found that while a significant difference in basal insulin secretion was found for BC091 (untreated, 18.74 ± 8.180 vs. treated, 53.34 ± 11.15), no significant increase in basal insulin secretion was observed for BC090 (untreated, 7.184 ± 3.093 vs. treated, 11.03 ± 5.192). Hence, the use of non-treated culture surface could not eliminate unwanted islet tissue from crude exocrine tissue.

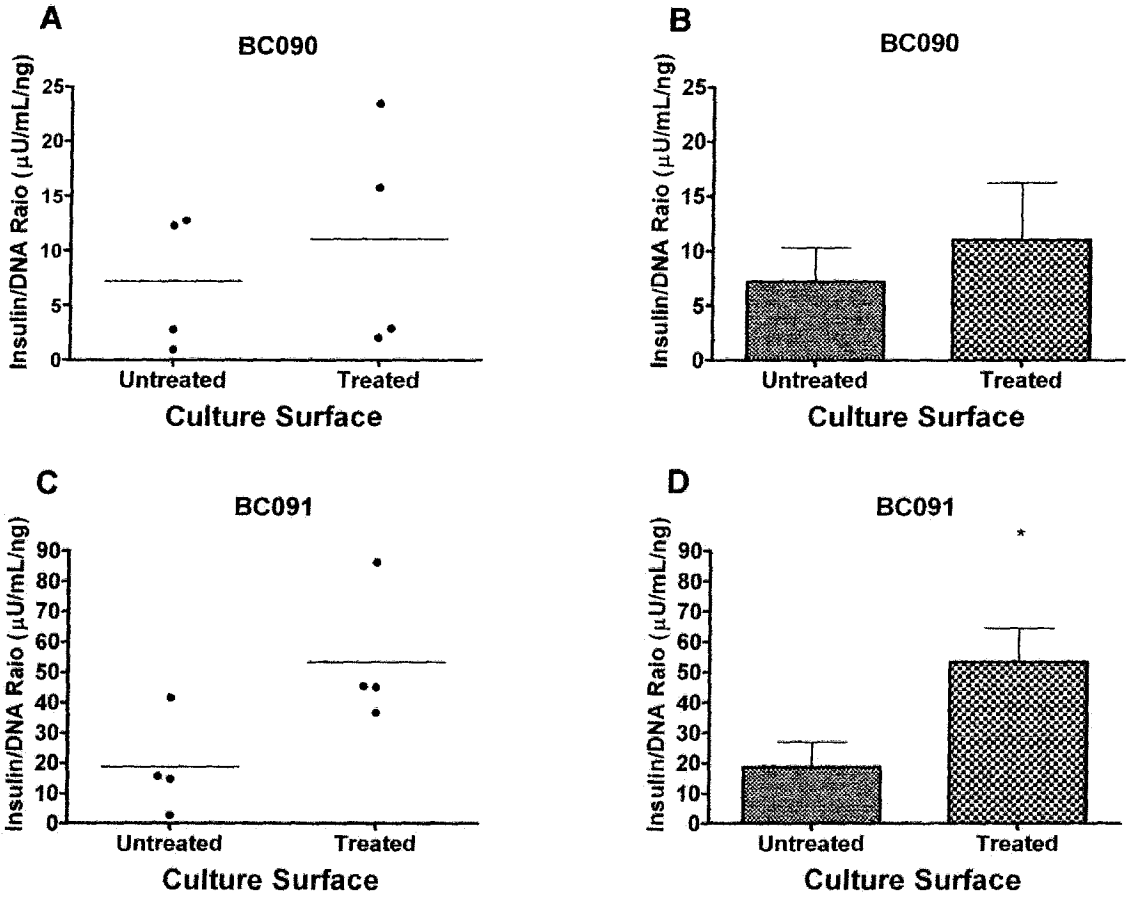

Figure 13 Comparison between Basal Insulin Secretion of Crude Exocrine Tissue Cultured on Non-treated and Treated Culture Surfaces. In BC091, significant increase in insulin secretion was found in crude exocrine tissues cultured on treated tissue culture surface.

4.3 Cell Purification and Culture

4.3.1 Purification of Ductal Cells

Carbonic anhydrase II (CAII) and carbohydrate antigen 19-9 (CA19-9) were used in the identification and purification of human pancreatic ductal cells (Figure 14). Neither of these two markers was found to be expressed by islet cells (Figure 15).

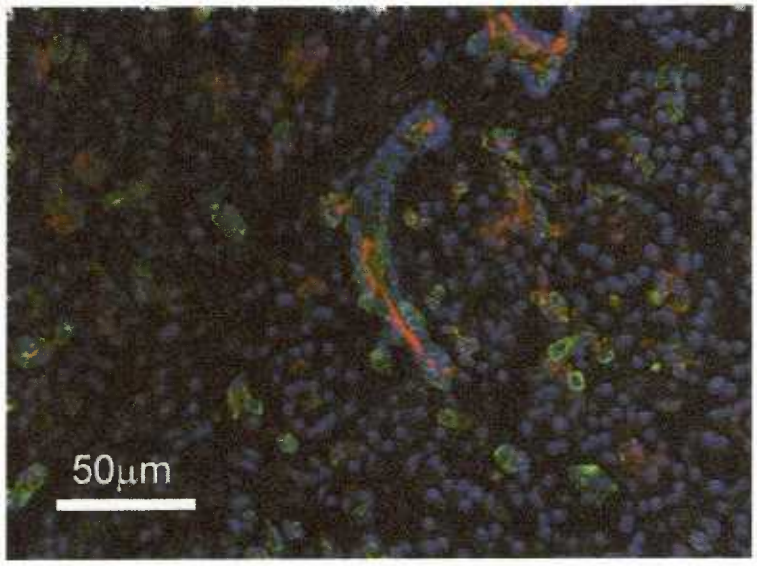

Figure 14 CA19-9 (Red) and CAII (Green) Co-localized in Human Pancreatic Ductal Cells. CA19-9 and CAII were expressed by human pancreatic ductal cells. Cell nuclei were labeled by DAPI (Blue).

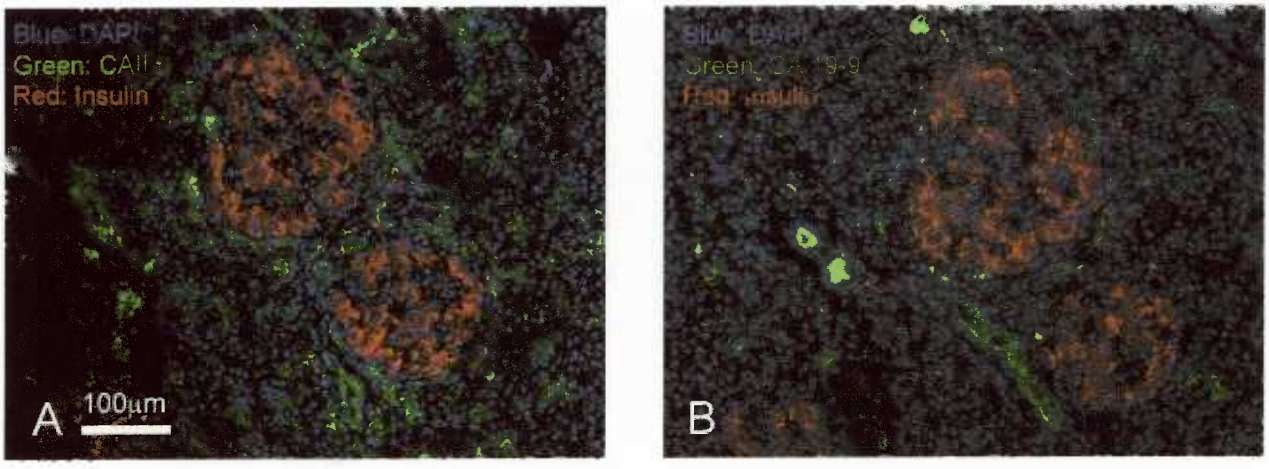

Figure 15 Ductal Cells Are Closely Associated with Islets in Human Pancreas. Markers of ductal cells (A: CAII, B: CA19-9) were not co-expressed by islet cells. A and B are consecutive sections. Cell nuclei were labeled by DAPI (Blue).

FACS Purification

CA19-9 positive pancreatic ductal cells were purified from 5 different donors (including two procured in Alberta and three locally procured) through FACS. A clear separation between CA19-9 positive and negative populations could be found in all cases (Figure 16). The percentages of CA19-9 positive cells from Alberta exocrine tissue were approximately 50% in both cases (Table 5). On the other hand, the percentages of CA19-9 positive cells from BC exocrine tissue were in the range of 20-40%.

Purified human CA19-9 positive cells were cultured according to the Bonner-Weir protocol except with the use of tissue culture treated plates and the addition of keratinocyte growth factor. As mentioned previously, this is to provide the FACS sorted cells the opportunity to adhere to the culture surface and hence provide a better chance for them to proliferate after sorting. Ductal cells purified from all five donors were cultured, with only one (BC068) demonstrating cell survival with ductal cell morphology (Figure 17). Cell cultures from other donors were either non-viable in culture or overgrown by fibroblast.

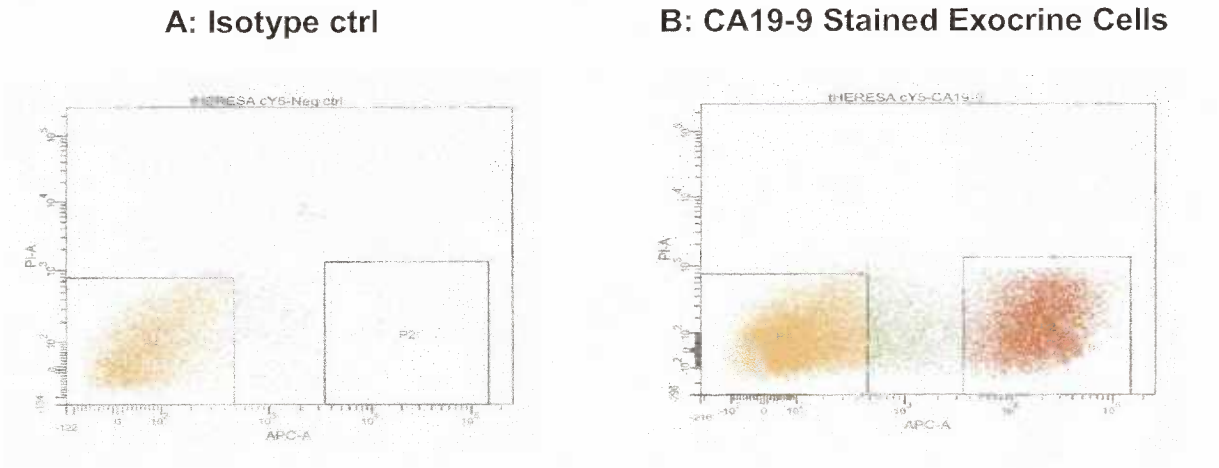

Figure 16 FACS Separation of CA19-9 Positive and Negative Human Pancreatic Cells. Human pancreatic cells were stained with (A) Isotype control anti-mouse IgG antibody, or (B) anti-human CA19-9 antibody to separate human pancreatic ductal cells from non-ductal cells.

Table 5 Comparison of Viability* and Percentage of CA19-9 Positive Cells in FACS Sorted Cells.

Donor ID	Age	Pre-sort Viability**	Control Viability**	Sample Viability**	% of Positive (of Viable)	Cultured?	Proliferated Ductal Cells?
AB1032	**50**	73.48	20.90	29.44	54.91	Yes	No
AB1037	**47**	55.86	75.00	72.80	50.20	Yes	No
BC068	**61**	52.36	45.20	45.90	27.60	Yes	Yes
BC069	**67**	53.04	50.94	56.61	36.23	Yes	No
BC079	**42**	65.25	56.58	55.89	21.92	Yes	No

* Viability was determined by propidium iodine (PI) and 7-Amino-actinomycin D (7-AAD) staining.
** Expressed in percentage

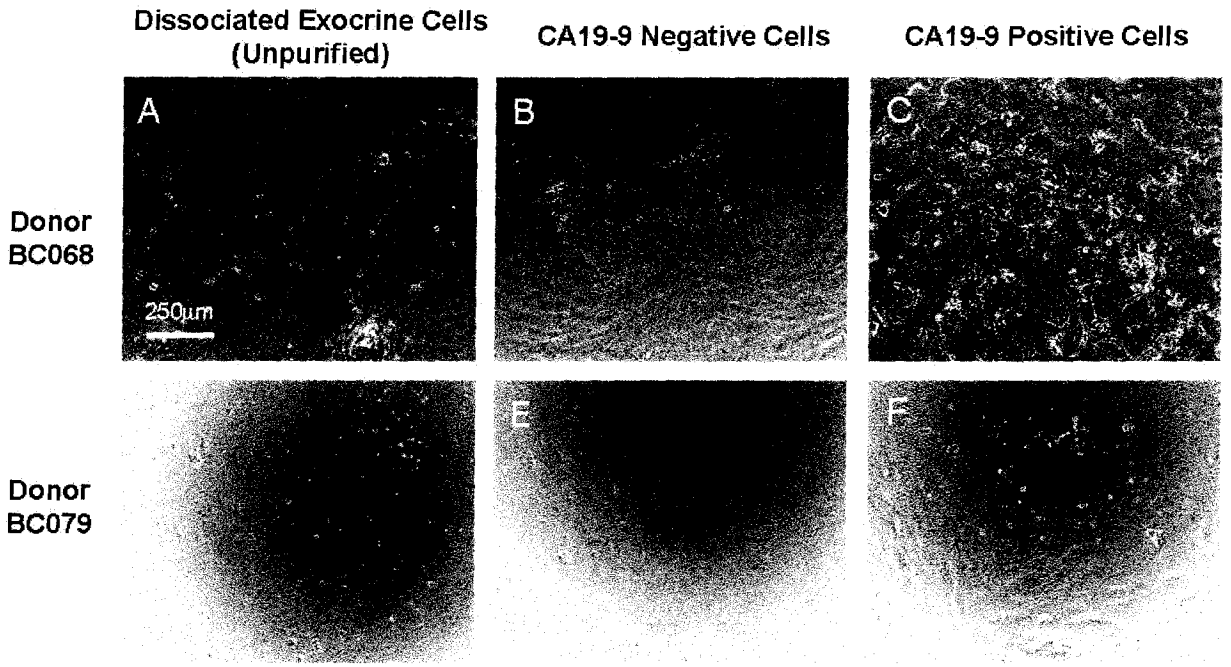

Figure 17 Cultures of FACS Sorted Human Ductal Cells from Two Donors. Cells were cultured in the same condition as outlined in the Bonner-Weir Protocol. A-C: Donor BC068. D-F: Donor BC079. Unsorted (A, D), CA19-9 negative (B, E) and CA19-9 positive pancreatic ductal cells (C, F) were cultured and studied by morphological observation. Out of cells from 5 different donors, only one (BC068) appeared to demonstrate maintenance of ductal cell culture.

MACS Purification

Ductal cells from two donors were purified through magnetic-activated cells sorting (MACS) (Table 6 and 7). Preliminary results showed that the Stem Cell Technologies (SCT) system was capable of isolating human ductal cells from the exocrine cell population (purity

changed from 27.30 to 72.78%, and from 15.51 to 38.10%) However, purities varied greatly. The Miltenyi Biotech (MB) system did not seem to be able to purify human ductal cells. This might be due to user's inexperience with the MACS system. MACS purified human ductal cells were cultured according to the Bonner-Weir protocol. It was found that cultures of MACS purified cells all resulted in the lack of ductal cell proliferation and fibroblast overgrowth (Figure 18).

Table 6 Percent Viability and Recovery Rate of MACS Sorted Cells.

Donor ID	Age	Sorting Method*	Pre-sort Viability	CA19-9+ Viability	CA19-9 – Viability	Recovery Rate	Cultured?	Proliferated Ductal Cells?
BC079	42	SCT	65.25	78.5	66.9	52.89	Yes	No
		MB	65.25	97.89	92.50	64.77	Yes	No
BC089	56	SCT	67.82	70.83	50.90	30.00	Yes	No

* SCT: Stem Cell Technologies; MB: Miltenyi Biotech

Table 7 CA19-9 Purity of MACS Sorted Cells* Determined by Immunofluorescent Staining of Carbonic Anhydrase II.

For BC079	Pre-sorting (control)	SCT CA19-9 +	SCT CA19-9 -	MB CA19-9 +	MB CA19-9 -
CAII Expression	27.30	72.78	21.46	39.90	51.93
Insulin Expression	1.89	3.80	0.81	7.21	0.60

For BC089	Pre-sorting (control)	SCT CA19-9 +	SCT CA19-9 -		
CAII Expression	15.51	38.10	25.36		
Insulin Expression	0.81	0	0.01		

* All numbers are expressed in percentage

** SCT: Stem Cell Technologies; MB: Miltenyi Biotech

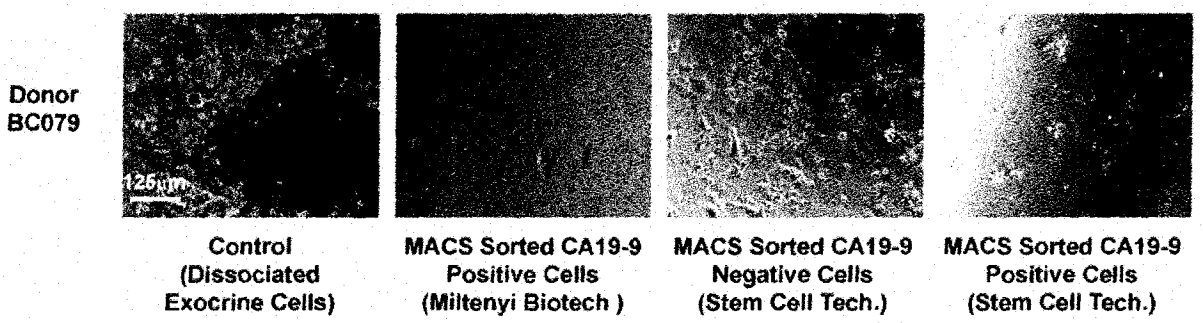

Figure 18 MACS Sorted CA19-9 Cells after Culture. Cultures of MACS sorted cells all resulted in fibroblast overgrowth.

4.3.2 Purification of Islet Beta Cells

Purification of human islet beta cells was attempted using a previously known protocol to isolate beta cells from human islets (Lukowiak *et al*, 2001). It was found that the Newport Green dye used resulted in a general shift in fluorescence for all exocrine cells. No cell separation was observed at all for residual pancreatic beta cells although their presence in the crude exocrine tissue was confirmed by dithizone staining and static incubation (Figure 19).

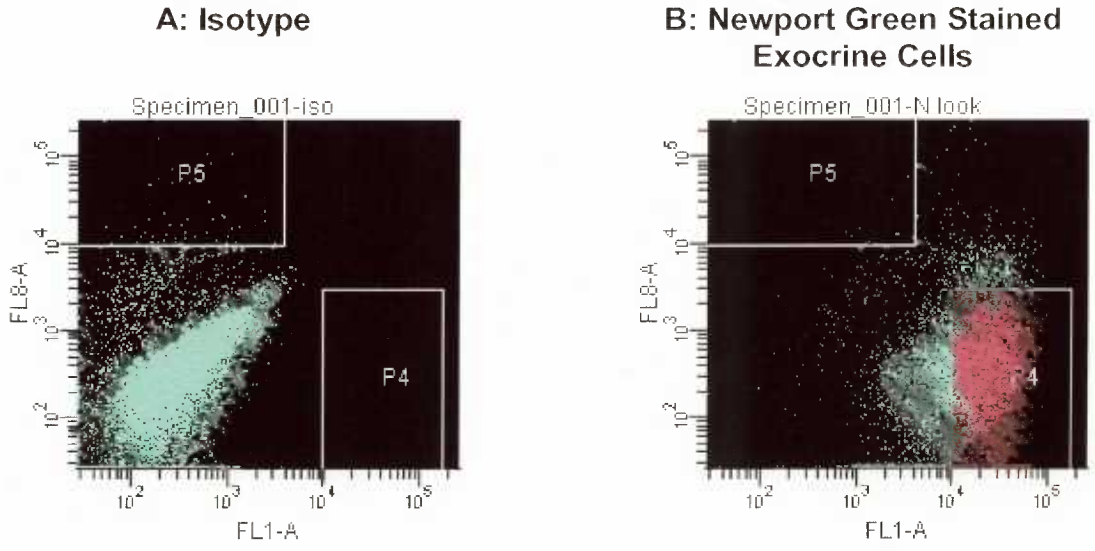

Figure 19 FACS Sorting of Pancreatic Beta Cells Stained with Newport Green. A. Green fluorescence FACS dot plot of Newport Green stained cells. It was found that Newport Green stain results in a significant shift in fluorescence in all cells when compared with B.

Chapter 5 Discussion

5.1 Generation of Islet-like Clusters

Both the Bonner-Weir protocol and the Todorov protocol were considered in the generation of islet-like clusters. The generation of islet-like clusters with the Bonner-Weir method allowed the use of static incubation without having to transport and wash clusters in suspension. This prevented the introduction of mechanical factors that would likely result in non-glucose stimulated insulin secretion. In addition, adherence allowed convenient manipulation without the loss of sample tissue. Therefore, the Bonner-Weir protocol was selected as the method to generate islet-like clusters for the purpose of evaluating insulin secretion in static incubation.

In the original Bonner-Weir protocol, non-treated tissue culture surface was used to eliminate islets from culture. In this study, it was found that the use of non-treated surface had an additional benefit. Compared with crude exocrine tissue cultured on treated surface, cells cultured on non-treated surface did not adhere to the surface as tightly and were able to migrate faster into three-dimensional structures that resembled islets. This is extremely interesting since it was speculated that a three-dimensional structure might promote islet cell differentiation. However, this is beyond the scope of this thesis and was not studied further. As originally suggested by the Bonner-Weir protocol, the use of non-treated surface did sometimes prevent islet tissue from attaching. Nevertheless, insulin secretion in response to glucose level was still found at the crude exocrine tissue stage, demonstrating that the use of non-treated surface could not effectively eliminate islet tissue. To eliminate islet tissue or beta cells from cell culture, one must consider other purification methods such as fluorescent-activated cell sorting and magnetic-activated cell sorting, or utilize an *in vitro* lineage-tracing system.

Immunofluorescent staining was also used to determine whether insulin was expressed by cells in islet-like clusters. However, this was found to be extremely difficult due to strong non-specific staining. This showed that a functional assay is necessary to determine whether insulin-secreting cells were generated.

5.2 Static Incubation to Detect Insulin Secretion

5.2.1 Sequential Static Incubation

The major advantage of sequential static incubation was that there would be no between-group errors. Therefore, the potential of using this static incubation method was first evaluated. It was found that no significant increase in insulin secretion was observed in sequential static incubation when islet-like clusters were exposed to media with increased glucose content. In fact, a significant decrease in insulin secretion was observed according to two-way ANOVA analysis. This was possibly contributed by the following factors. First of all, intensive pipetting during the course of static incubation could disturb islet-like clusters. This could result in an increase in non-glucose stimulated insulin secretion during basal condition and reduce the observable effect of glucose-stimulated insulin secretion. Furthermore, intensive pipetting could also cause the detachment of islet-like clusters from their tissue culture surface. Suspending cells and clusters were collected with media samples and eventually contributed to an increase in insulin secretion by means of additional secretion after incubation period or cell death, which released intracellular insulin into media samples. Last but likely the most important factor was that the high basal insulin level could be caused by the lack of proper washing before static incubation. Islet-like clusters were cultured in serum-free media containing bovine insulin at a level of 5 mg/L. According to manufacturer's instructions, the anti-insulin antibody in the insulin RIA kit used to measure insulin secretion has a 62% specificity toward bovine insulin. This was confirmed by the large amount of insulin detected in the serum-free media using the insulin RIA

kit (data not shown). Even after a two-night incubation at 37°C, 88.25 μU/mL of insulin was still detected in the media. Any remaining culture media could make the basal insulin secretion appear higher than it was. A drop in insulin secretion after the islet-like clusters were exposed to incubation media with a high level of glucose was simply the result of further removal of contaminating serum-free media in the first sample collection.

Because of these factors, sequential static incubation with the protocol established was not suitable for evaluating the ability of islet-like clusters to secrete insulin. In addition, improvements were made in techniques such as washing when the parallel static incubation was developed.

5.2.2 Parallel Static Incubation

Significant increases in insulin secretion by Min6 cells with response to elevated glucose levels and other known secretagogues were demonstrated using the parallel static incubation protocol. The data agreed with what was expected of the normal responses to glucose and GLP-1 by Min6 cells (Park *et al*, 2006; O'Driscoll *et al*, 2004). This demonstrated that the established static incubation system was reliable and could be used to evaluate the effects of glucose concentration and other insulin secretagogues to insulin secretion by insulin-secreting cells.

C-peptide secretion data, although not as sensitive as the corresponding insulin secretion data, did support *de novo* insulin secretion by relating closely to the insulin secretion data. Therefore, the insulin secretion found through static incubation was not an artifact caused by absorption of bovine insulin present in the serum-free media. As mentioned previously, insulin secretion was observed at stage one, showing the presence of insulin-secreting cells in crude exocrine tissue used to generate islet-like clusters. Crude exocrine tissue at stage one and islet-like clusters at stage three appeared to respond similarly to glucose levels and GLP-1, despite reduced relative levels of responses at stage three. This demonstrated that the insulin-secreting

cells at stage one and those at stage three behaved similarly. At stage two, tissue did not seem to have significant responses to glucose or low level of GLP-1. There are two possible yet very different interpretation based on these evidences. First of all, the similarity between the secretion pattern by tissue at stage one and at stage three might support that the insulin-secreting cells originated from beta cell replication. Lack of insulin secretion at stage two suggests that islet beta cells might have gone through a period of proliferation, which could result in de-differentiation (Beattie *et al*, 1999). On the other hand, it is also possible that the lack of insulin secretion suggests the death of beta cells in the crude tissue, and the islet-like clusters generated originated from the differentiation of ductal cells or other progenitor cell type.

Two-way ANOVA showed that as culture stages progressed, increases in insulin secretion and C-peptide secretion could be observed in some cases from islet-like clusters generated using tissue from BC091 and BC086. This was strikingly different from the generation of islet-like clusters using tissue from BC090, which actually had a reduced response to GLP-1 (50 nM) at stage three. Furthermore, there did not seem to be an increase in basal insulin secretion by islet-like clusters generated using BC090 tissue. Interestingly, donor BC091 and BC086 are a lot older than BC090. Could the difference be caused by age difference? Or was this caused by the pancreas digestion process, in which pancreas from younger donors usually requires longer digestion time (Ziliang Ao, personal communicatin) and might endure more tissue damage? With only data from three donors, it is not possible to confirm the origin of islet-like clusters generated in stage three based solely on this static incubation data. Increased number of experiments will be necessary in order to determine whether these three culture stages do promote the generation of islet-like clusters, and whether donor age affects the ability of exocrine tissue to generate insulin-secreting islet-like clusters. Further cell purification and supplemental protein and genetic characterization will also be necessary to determine whether any individual cell type has the ability to differentiate into insulin-secreting islet-like clusters.

It was very interesting to see that the insulin-secreting cells in the crude exocrine tissue at stage one responded a lot better to mid glucose level (11.5 mM) than to high glucose level (20 mM). This was very different than what was suggested by the literature. In the past, it was observed that insulin secretion by human islet tissue increased with respect to increased glucose levels (Harrison *et al*, 1985; Ritzel *et al*, 2003). Such an increase plateaued at approximately 15~20 mM glucose. It is possible that there exists heterogeneity in islets, and the islet tissue that remained in the crude exocrine tissue simply responded better in mid glucose. It is also likely that these attached islets were actually not as healthy as the suspended islets, resulting in their inability to tolerate stimulation by glucose at a higher level. This lack of response with the exposure to high glucose level seemed to be corrected by GLP-1, which agrees with protective effect that GLP-1 can exert on human islets.

An overall increase in basal insulin secretion suggests that there might be an increased number of insulin positive cells. Decreased fold increases in insulin responses to external secretagogues (hence reduced relative levels of responses) implies that these cells, although with the ability to secrete insulin at basal conditions, might be immature so that they did not respond well to increases in glucose and GLP-1 levels. It would be interesting to see whether it is possible to further their maturity by *in vitro* culture with other external factors known to stimulate islet growth and maturation.

This thesis study was done based on the protocol of islet-generation published by the Bonner-Weir group in 2000. In the Bonner-Weir study, it was suggested that insulin-secreting islet-like clusters could be generated from ductal tissue. This ductal tissue fraction contained approximately 1-15% of islets, similar to what was used in this thesis study. Their only means of eliminating islets from the ductal tissue fraction was using non tissue culture treated flasks. In this thesis study, however, it was found that the use of non-treated flask could not effectively eliminate all insulin-secreting cells. In addition, the method used by the Bonner-Weir group to

confirm the elimination of islets was by determining the insulin content to DNA ratio. It was to our knowledge and experience that without the use of insulin extraction with acid, it is likely that the insulin content of crude exocrine tissue at stage one was underestimated in the Bonner-Weir study. Since no insulin-secretion analysis was done at early stages of culture in the Bonner-Weir study, it is not possible to know whether the insulin content to DNA ratio correctly reflects the number of insulin-secreting cells in the original tissue cultured. This is not to say that ductal cells cannot differentiate into insulin-secreting cells. Nevertheless, more reliable functional assays should be utilized to confirm the presence of newly generated insulin-secreting cells.

5.2.3 Cell Purification

Through FACS sorting, it was found that crude exocrine tissue from Alberta contained more ductal cells than observed in exocrine tissue from BC. This might be caused by further differentiation of exocrine tissue after prolonged culture as previously observed (Gmyr *et al*, 2004), or the fact that non-ductal cells were dying during the process of transportation. To ensure that only ductal cells are purified, one should use locally procured tissue to prevent the use of ductal cells that originate from acinar cell differentiation. Alternatively, *in vitro* lineage tracing can be utilized to track ductal cells.

Magnetic cell sorting is a recently popularized method to purify cell populations. The benefit of MACS is that MACS sorting does not require an expensive FACS machine and specially trained personnel. MACS also provides a less harsh sorting process and allows for better viability and recovery (Bonner-Weir, personal communication, October 5, 2006). The disadvantage of MACS, however, is that it usually does not provide the same purity FACS sorting does, as observed in this study. In addition, the sorting process could not be visualized through a computer. More debris was also observed with the MACS sorted cell suspension in this study. Further experiments should be carried out to test and confirm whether different

MACS systems will result in better purification. Purified cells could then be further analyzed through FACS analysis.

Regardless of the method used to purify pancreatic ductal cells, fibroblast overgrowth seemed to be a major issue. Based on morphological observations, most of the cells found at stage three were fibroblast. Purified ductal cells could have died after FACS or MACS sorting processes. Another possibility was that fibroblasts proliferated at a rate higher than ductal cells, thus preventing ductal cells from proliferation. Further studies using lineage tracing and proliferation assays, although difficult, should be done to evaluate ductal cell proliferation after purification. Therefore, it order to generate islet-like clusters using purified ductal cells and to demonstrate that they can different into islet-like clusters, it is necessary to develop methods to eliminate fibroblast contamination. One method is to eliminate fibroblasts by adding factors, such as G418, which can selectively eliminate fibroblast. This has been demonstrated in previous studies (Hao *et al*, 2006). However, this might still affect the viability of ductal cells and result in increased cell death (data not shown). Another method is to consider extracellular agents that promote ductal cell growth after purification. Factorial analysis can be done for this purpose.

5.3 Significance and Future Perspectives

Many studies have tried to pinpoint the ability of adult pancreatic cells to regenerate islet tissue *in vitro*. For most of them, very little functional analysis was done to address the ability of islet-like clusters, in particular human islet-like clusters, to secrete insulin under different conditions. Instead, they relied on immunohistochemical characterization as well as genetic expression analysis such as PCR. In this study, a static incubation system was established to evaluate the ability of human islet-like clusters to secrete insulin upon stimulation by glucose and other known secretagogues. In addition, an attempt was made to generate islet-like clusters using purified human ductal cell populations. This system was found to be a convenient and effective

way to demonstrate glucose and secretagogues stimulated insulin secretion by insulin-secreting cells such as Min6. This system can easily be incorporated into other studies as a way to provide additional functional analysis data for *in vitro* generated islet-like tissue. Although GLP-1 was used here, other factors, such as amino acids, lipids, and known secretagogues, could also be included in the system to provide a comprehensive overview of insulin secretion responses by islet-like clusters. This system also provides more flexibility than a perifusion system, which is restricted by the number of chambers and the requirement of specially trained technicians to operate it. Furthermore, with the ability of multiplex systems nowadays, it is possible to evaluate the secretion of multiple hormones by these islet-like clusters in the future. It should also be noted that this system should be used in conjunction with other methods of analysis to provide a complete picture for islet-like clusters, instead of being a stand-alone system.

Although the attempt to purify and culture ductal cells was not very successful, this experience identified the important issues one must address in order to generate islet-like clusters from purified cell populations. A recent study attempted to generate islet-like clusters using MACS purified ductal cells (Yatoh *et al*, 2007). Although very few insulin positive cells were found initially, more cells were observed to be insulin positive after the cells were cultured on stromal cells and subsequently transplanted into NOD/SCID mice. This suggests that maybe ductal proliferation and differentiation could be stimulated by the addition of extracellular factors. Using the static incubation system, one can easily subject purified ductal cells to different culture conditions and then assess their ability to differentiate. It is my hope that one day, this static incubation system can be utilized to confirm the ability of purified adult progenitor populations to generate islet-like clusters.

The goal of generating human islet-like clusters was to use these clusters in human islet transplantation. This study successfully demonstrated the ability of these human islet-like clusters to secrete insulin. In addition, this study provided information of their responses when

exposed to GLP-1, a factor known to stimulate insulin secretion, to protect islet tissue, and to promote differentiation. Since GLP-1 was found to stimulate further insulin secretion by islet-like clusters, it is conceivable that future transplantation treatment might include transplantation of islet-like clusters with the administration of GLP-1. Indeed, further studies, especially animal studies, should be completed to confirm this possibility.

Chapter 6 Conclusion

A static incubation system was established to evaluate insulin secretion by human islet-like clusters in response to glucose and other known insulin secretagogues. A parallel static incubation system was selected because it did not have the technical difficulties found in a sequential static incubation system. Indeed, glucose and secretagogue stimulated insulin secretion by Min6 cells was reliably portrayed. With the difficulty in determining the functionality of islet-like clusters through immunofluorescent staining and genetic expression, functional assay is still the more reliable way to ensure insulin-secreting cells were generated. This study also provided important information on the ability of these *in vitro* generated human islet-like clusters to secrete insulin upon stimulation. With donor variances, more experiments will be necessary to confirm the results observed. An attempt was made to generate islet-like clusters from purified ductal cells. However, difficulties in the culture of purified ductal cells suggest that further studies are required to identify factors that can eliminate fibroblast in culture or promote ductal cell proliferation and differentiation.

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Appendix 1: Ethical Approval

The University of British Columbia
Office of Research Services
Clinical Research Ethics Board – Room 210, 828 West 10th Avenue, Vancouver, BC V5Z 1L8

ETHICS CERTIFICATE OF EXPEDITED APPROVAL: RENEWAL

PRINCIPAL INVESTIGATOR:	DEPARTMENT:	UBC CREB NUMBER:
Garth Warnock	UBC/Medicine, Faculty of/Surgery/General Surgery	H03-70453

INSTITUTION(S) WHERE RESEARCH WILL BE CARRIED OUT:

Institution	Site
Vancouver Coastal Health (VCHRI/VCHA)	Vancouver General Hospital
UBC	Point Grey Site

Other locations where the research will be conducted:
N/A

CO-INVESTIGATOR(S):
James M. Piret
Ziliang A. Ao
Dawei Ou
James D. Johnson
Zehua He
Robert Mark Meloche
Theresa Liao
Timothy J. Kieffer
T. Michael Underhill

SPONSORING AGENCIES:
Juvenile Diabetes Research Foundation International - "Autocrine Survival Factors in Human Islets" - "Prolonging Diabetes Reversal in Islet Xenotransplantation by B7-H4" - "Cryopreservation of Human Islets for Clinical Transplant in Type I Diabetes"
Michael Smith Foundation for Health Research - "The Centre of Human Islet Transplant and Beta Cell Regeneration"
Taplow Ventures Ltd. - "High content, high throughput screening for molecules that promote beta-cell formation"
VGH and UBC Hospitals Special Opportunities Research Fund - "Autocrine Survival Factors in Human Islets" - "Cryopreservation of Human Islets for Clinical Transplant in Type I Diabetes"

PROJECT TITLE:
Cryopreservation of Human Islets for Clinical Transplant in Type I Diabetes

To add a New title "The Centre of Human Islet Transplant and Beta Cell Regeneration"

High content, high throughput screening for molecules that promote beta-cell formation

EXPIRY DATE OF THIS APPROVAL: June 26, 2008

APPROVAL DATE: June 26, 2007

CERTIFICATION:
In respect of clinical trials:
1. The membership of this Research Ethics Board complies with the membership requirements for Research Ethics Boards defined in Division 5 of the Food and Drug Regulations.
2. The Research Ethics Board carries out its functions in a manner consistent with Good Clinical Practices.
3. This Research Ethics Board has reviewed and approved the clinical trial protocol and informed consent form for the trial which is to be conducted by the qualified investigator named above at the specified clinical trial site. This approval and the views of this Research Ethics Board have been documented in writing.

The Chair of the UBC Clinical Research Ethics Board has reviewed the documentation for the above named project. The research study, as presented in the documentation, was found to be acceptable on ethical grounds for research involving human subjects and was approved for renewal by the UBC Clinical Research Ethics Board.

Approval of the Clinical Research Ethics Board by one of:

[redacted]

Dr. Bonita Sawatzky, Associate Chair

Appendix 2 Insulin Secretion Data from Parallel Static incubation

Table 8 Insulin Secretion by Islet-like Clusters from Three Donors with Response to Stimulation by Glucose and Other Factors (n = 4 for each treatment at each culture stage). Mean ± SEM is shown in the table. All numbers are expressed as insulin/DNA ratio (unit: nU/mL/ng). This data was plotted in Figure 12 and compared with C-peptide secretion data.

		Low Glucose	Mid Glucose	High Glucose	KCl	GLP-1 (10nM)	GLP-1 (50nM)
BC086	Stage1	4.42 ± 2.08	19.54 ± 8.83	11.21 ± 2.77	11.98 ± 4.09	36.34 ± 13.20	37.51 ± 5.21
	Stage 2	11.09 ± 3.70	46.05 ± 6.41	56.54 ± 21.08	27.91 ± 7.60	16.14 ± 4.29	45.30 ± 2.190
	Stage 3	44.10 ± 17.57	104.24 ± 72.95	20.10 ± 6.41	20.15 ± 0.99	103.25 ± 27.20	254.86 ± 172.59
BC090	Stage 1	7.18 ± 3.09	13.54 ± 3.75	5.93 ± 1.47	8.50 ± 1.90	21.45 ± 4.01	42.42 ± 2.09
	Stage 2	3.83 ± 0.76	10.31 ± 2.70	9.96 ± 2.45	10.99 ± 2.49	11.52 ± 2.95	13.64 ± 2.81
	Stage 3	4.04 ± 0.60	5.74 ± 0.96	3.33 ± 0.61	4.67 ± 0.81	9.55 ± 2.33	14.72 ± 5.84
BC091	Stage 1	18.75 ± 8.18	143.30 ± 39.55	78.91 ± 4.84	115.96 ± 32.34	61.95 ± 20.59	177.09 ± 13.58
	Stage 2	173.42 ± 25.72	145.96 ± 32.25	149.21 ± 30.27	82.02 ± 22.10	117.54 ± 13.92	172.90 ± 4.32
	Stage 3	142.82 ± 27.36	291.61 ± 48.10	231.44 ± 100.88	273.48 ± 58.94	238.91 ± 61.28	433.17 ± 144.29

Table 9 Insulin Secretion by Islet-like Clusters from Three Donors with Response to Stimulation by Glucose and Other Factors (n = 4 for each treatment at each culture stage). Geometric means and confidence interval (in brackets) are shown in the table. All numbers are expressed as insulin/DNA ratio (unit: nU/mL/ng). This data was used in two-way ANOVA analysis.

		Low Glucose	Mid Glucose	High Glucose	KCl	GLP-1 (10nM)	GLP-1 (50nM)
BC086	Stage1	3.33 (1.48-7.50)	13.34 (4.74-37.52)	10.47 (6.80-16.12)	9.85 (4.77-20.34)	22.03 (5.10-95.24)	24.60 (8.22-73.67)
	Stage 2	9.58 (5.31-17.28)	44.77 (34.12-58.76)	35.65 (9.13-139.10)	24.66 (13.86-43.88)	14.83 (9.52-23.07)	30.55 (10.85-85.99)
	Stage 3	31.84 (12.13-83.61)	45.50 (35.11-153.80)	17.74 (10.40-30.26)	20.09 (18.25-22.12)	94.41 (59.44-149.93)	142.56 (46.61-436.01)
BC090	Stage 1	4.53 (1.34-15.34)	11.59 (5.84-22.99)	5.31 (3.05-9.24)	7.90 (5.13-12.16)	19.86 (12.22-32.29)	40.36 (28.32-57.54)
	Stage 2	3.63 (2.53-5.21)	9.19 (5.25-16.08)	8.96 (5.19-15.49)	10.07 (6.19-16.37)	10.40 (6.20-17.45)	12.53 (7.62-20.60)
	Stage 3	3.87 (2.73-5.49)	5.48 (3.84-7.82)	3.15 (2.17-4.58)	4.45 (3.12-6.35)	8.76 (5.47-14.03)	10.47 (3.81-28.78)
BC091	Stage 1	12.85 (4.31-38.31)	123.88 (64.85-236.63)	78.34 (68.97-88.99)	97.72 (46.94-203.47)	50.70 (24.14-106.47)	160.32 (98.65-260.55)
	Stage 2	137.09 (98.53-190.72)	131.22 (74.24-231.93)	139.64 (92.28-211.30)	74.30 (45.51-121.30)	114.82 (88.86-148.36)	172.58 (164.36-181.22)
	Stage 3	133.66 (87.04-205.25)	276.06 (185.14-411.62)	172.58 (72.56-410.51)	254.10 (164.22-393.16)	215.77 (129.40-359.81)	355.63 (169.65-745.49)

Appendix 3 C-peptide Secretion Data from Parallel Static incubation

Table 10 Raw Data of C-peptide Secretion by Parallel Static Incubation (n = 4 for each donor, unit = $1/10^5$ mL). Mean ± SEM was shown. All data was normalized by DNA quantity. This data was plotted in Figure 13 and compared with raw data of insulin secretion.

		Low Glucose	Mid Glucose	High Glucose	KCl	GLP-1 (10nM)	GLP-1 (50nM)
BC086	Stage1	15.28 ± 1.447	22.29 ± 7.828	16.42 ± 2.031	17.62 ± 2.077	37.87 ± 16.32	40.19 ± 23.13
	Stage 2	15.18 ± 0.4822	30.51 ± 9.694	38.86 ± 13.13	14.01 ± 1.008	15.27 ± 0.4642	24.43 ± 7.859
	Stage 3	60.64 ± 24.08	80.32 ± 39.54	38.89 ± 16.38	24.14 ± 2.656	161.5 ± 45.72	441.1 ± 269.7
BC090	Stage 1	7.174 ± 2.580	20.86 ± 7.031	8.979 ± 2.657	9.369 ± 3.104	43.95 ± 8.660	70.69 ± 13.24
	Stage 2	5.744 ± 1.879	16.91 ± 4.163	13.44 ± 3.088	5.991 ± 2.971	12.29 ± 3.493	19.59 ± 5.080
	Stage 3	11.61 ± 25.62	13.70 ± 4.123	4.877 ± 0.5781	6.877 ± 2.235	14.84 ± 8.258	27.74 ± 13.90
BC091	Stage 1	32.83 ± 12.74	351.0 ± 156.1	152.6 ± 9.291	237.5 ± 60.24	132.4 ± 49.03	459.1 ± 200.9
	Stage 2	292.8 ± 54.15	172.4 ± 13.25	245.4 ± 22.99	124.5 ± 37.10	312.0 ± 37.10	335.0 ± 70.65
	Stage 3	314.2 ± 122.3	776.0 ± 208.8	680.0 ± 232.	343.3 ± 59.74	692.7 ± 110.2	709.1 ± 208.5