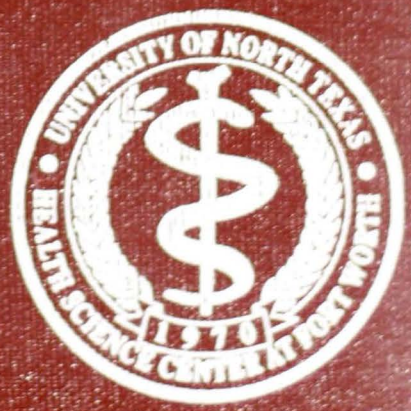

UNIVERSITY OF NORTH TEXAS
HEALTH SCIENCE CENTER AT FORT WORTH
1970

Ettinger, Eve Sari, The Role of Fibroblast Growth Factor-2 in the Expansion of Pancreatic Islets *In Vitro.* Doctor of Philosophy (Biomedical Sciences, Biochemistry and Molecular Biology), May 2004, 181pp., 7 Tables, 46 figures, 109 references

The focus of these studies was to identify a pool of progenitor cells within the adult islet, which can be expanded *in vitro* and aggregated into glucose responsive neoislets as a potential source for transplantation in diabetic patients. We hypothesize that normal adult islets contain an unspecialized proliferative progenitor cell population that can be expanded, aggregated by FGF-2 via PKC mechanism in 2-D culture or in a Rotating Wall Vessel Cell Culture System and matured into glucose-sensitive neoislets through the differential expression or activation of one or more PKC isoform(s). It was shown by immunofluorescence that normal adult islets from several species (rat, porcine, human and canine) contain proliferative endocrine precursor (endocrine epithelial) cells, which coexpress insulin, glucagon and somatostatin. These cells also express PDX-1, a marker of mature β cells and endocrine precursors as well as glut 2 and glucokinase, which are necessary components of glucose metabolism. Endocrine epithelial cells were shown to aggregate into neoislets either spontaneously in 2-D culture or via a rotating wall vessel cell culture system in the presence of fibroblast growth factor-2. FGF2 has been implicated in the aggregation of endocrine epithelial cells into neoislets via the FGFR-cadherin-catenin complex or through PKC signal transduction. Due to the variations in islet isolation, Ins-1 cells were used as a model of β cell aggregation. PKC profiles of endocrine epithelial cells, neoislets, native islets, Ins-1 cells and Ins-1 neoislets were

determined. PKC-α, βII, ε and ζ were the only isoforms expressed in these cells and are potential targets for modulation in the differentiation of endocrine epithelial cells. Insulin secretion in response to a glucose challenge was examined in normal islets, neoislets, endocrine epithelial cells, and Ins-1 cells. Although endocrine epithelial cells express insulin, show dense secretory granules and contain two critical proteins in the glucose-sensing cascade, they are immature with respect to glucose-responsive insulin secretion. Neoislets show insignificant insulin secretion in response to elevated glucose when compared to normal islets. Aggregation of endocrine epithelial cells may be the first step in differentiation into mature β cells, however glucose-responsive insulin secretion must be achieved *in vitro* for use as an alternate source of islets for transplantation in diabetic patients.

THE ROLE OF FIBROBLAST GROWTH FACTOR-2 IN THE EXPANSION OF PANCREATIC ISLETS *IN VITRO*

Eve Sari Ettinger, B.A.

APPROVED:

Major Professor

Committee Member

Committee Member

Committee Member

University Member

Chair, Department of Molecular Biology and Immunolgy

Dean, Graduate School of Biomedical Sciences

THE ROLE OF FIBROBLAST GROWTH FACTOR-2 IN THE EXPANSION OF PANCREATIC ISLETS *IN VITRO*

DISSERTATION

Presented to the Graduate Council of the

Graduate School of Biomedical Sciences

University of North Texas

Health Science Center at Fort Worth

In Partial Fulfillment of the Requirements

For the Degree of

Doctor of Philosophy

By

Eve Sari Ettinger, B.A.

Fort Worth, Texas

May 2004

ACKNOWLEDGEMENTS

I would like to take this opportunity to acknowledge the support I have received throughout my graduate studies. First, a special thanks is owed to my mentor, S. Dan Dimitrijevich, Ph.D. for his guidance and encouragement during my entire graduate career. To my dissertation committee, Julian Borejdo, Ph.D., Richard Easom, Ph.D., Andras Lacko, Ph.D., and Thomas Yorio, Ph.D., thank you for your support and direction. Thanks to Dr. Easom for introducing me to the world of research and for recognizing and supporting my aptitude for science.

I would like to thank my fellow labmates, past and present, for their assistance and friendship, particularly Jami Kern, Ph.D., Jwalitha Shankardas, M.S., and Tamara Reese. I would also like to express my graditude to Rahmi Heymann for his assistance in countless pancreatic isolations and Dr. Lou Tierce, DVM for enlightening me on the details of canine pancreas anatomy.

Lastly, I would like to express my deepest gratitude to my friends and family, especially my parents, Phyllis and Alan Ettinger for their unconditional love, support and patience throughout this experience and always.

TABLE OF CONTENTS

LIST OF TABLES

CHAPTER I

CHAPTER III

CHAPTER IV

LIST OF ILLUSTRATIONS

CHAPTER I

CHAPTER II

CHAPTER III

CHAPTER IV

CHAPTER I

STATEMENT OF PROBLEMS

During the past decade significant advances have been made, allowing islet transplantation to become a viable option in the treatment and/or cure of diabetes mellitus. However, several factors limit this procedure and these obstacles must be overcome before islet transplantation could become a practical treatment for diabetic patients. The leading problem facing islet transplantation is the gross disparity between the number of islets available for clinical transplantation and the number of patients who are eligible for the procedure. Due to the lack of availability of islets, other methods are being explored to obtain alternate sources of glucose-responsive, insulin secreting tissue.

During development it has been shown in rodents, that organogenesis of the pancreas is being fueled at the cellular level by the duct epithelium. Differentiation of the duct epithelial cells, orchestrated by the differential expression of an array of transcription factors and the developing mesenchyme result in formation of digestive enzyme secreting exocrine pancreas and glucose regulating islets of Langerhans. The vast majority of the research in this area has been based on the hypothesis that the differentiation of the duct epithelial cells is the strategy of choice for *in vitro* generation of pancreatic islets. One method of isolating these cells from pancreatic ducts is based on very delicate surgical technique. Because of the exposure to tissue digestive enzymes, isolating them as a bi-product of islets is also not optimal. Studies of islet homeostasis

confirm that there is a turnover of islet cells and clearly of the β-cells since they are the major cellular component [1]. Since in the adult organism the islets of Langerhans are no longer connected to the duct epithelium, it is unlikely that the cellular homeostasis of the islets is maintained solely by these cells. Similarly, because all the embryonic signals are no longer operative it is unlikely that islets are budding off the ductules. Instead in adult organisms, and in similarity of a number of adult tissues, there must be a proliferative compartment within the islets that supports the age or function related cell attrition.

Specific Aim 1: To obtain homogeneous cultures of endocrine epithelial cells from adult islets and characterize them as precursors of endocrine cells comprising the islets of Langerhans.

Hypothesis 1: A subset of cells referred to as endocrine epithelial cells, are present in, and can be derived from, normal adult islets due to their proliferative potential.

Islets from several species were initially obtained through a tissue distribution network, which eventually became inconsistent and unreliable. To combat this issue, an islet isolation technique was developed for canine pancreata that were available in-house. Endocrine epithelial cells from several species (rat, porcine, human and canine) were characterized by examining the expression of islet cells markers and other characteristic proteins using indirect immunofluorescence and western analysis. In addition, the optimal proliferative environment for endocrine epithelial cells was studied. During the studies with monolayer cultures of EECs, the observation was made that these cells had

the ability to spontaneously aggregate into islet-like structures (neoislets) when placed in an environment which supports differentiation.

Specific Aim 2: To effect the aggregation of endocrine epithelial cells into neoislets and determine the mechanism(s).

Hypothesis 2: Endocrine epithelial cells (EECs) can be aggregated *in vitro*, spontaneously or using a Rotating Wall Vessel Cell Culture System into multicellular structures (neoislets.) Aggregation of EECs into neoislets may take place by a differentiation mechanism that involves calcium, FGF2 (or serum), PMA and kinase signaling including protein kinase C (PKC) and/or receptor tyrosine kinase (RTK).

Endocrine epithelial cells must be aggregated into a three-dimensional neoislet prior to transplantation because islets are the basic endocrine unit that controls the glycemic index. Also, angiogenesis and innervation may then occur *in vivo* to complete organogenesis. Several phases are discernable in islet organogenesis: I) proliferation 2) differentiation 3) cell sorting 4) aggregation 5) glucose responsiveness or maturation 6) angiogenesis and 7) innervation. The order of these phases is still unknown and they may actually not be discreet events. There are several factors that promote and support differentiation and aggregation in epithelial cells. For example, in addition to being differentiation promoter that acts specifically via PKC signal transduction, PMA is also a known mutagen and may not be a player *in vivo* or useful *in vitro* [2]. On the other hand, epidermal growth factor (EGF), basic fibroblast growth factor (FGF2), retinoic acid and

high calcium are known differentiation promoters as well that could be involved *in vitro* and *in vivo* [3, 4] [5] [6].

Specific Aim 3: To determine which factors are involved in the maturation (glucose responsiveness) of neoislets *in vitro*.

Hypothesis 3: FGF also induces the complete differentiation (maturation) of glucose responsive neoislets through the differential expression or activation of one or more PKC isoform(s).

Although *in vitro* differentiation of the pancreatic β cells is being studied, phases in the production of mature glucose responsive neoislets have not been realized. Because proliferation and differentiation are mutually exclusive, cells must exit the cell cycle before differentiation begins. Aggregation of endocrine epithelial cell monolayer into neoislets may be considered as the first phase of differentiation but completion of the latter must include mature function in the form of native glucose responsiveness (i.e. upregulation of insulin secretion in response to elevated glucose concentrations). The signaling mechanisms involved in complete differentiation (proliferation arrest, aggregation and glucose responsiveness) are not known. Although the role of atypical PKCs in islet differentiation has been suggested, a systematic understanding of the controlling factors involved in β cell differentiation is not available. The understanding of such mechanism(s) would provide additional means for understanding the *in vivo* organogenesis (and perhaps decline) of the endocrine pancreas and provide the means of controlling more effectively the differentiation process *in vitro*. *In vitro* generated functionally mature neoislets, with long-term glucose responsiveness, will be suitable for

further evaluation in diabetic animal models. The organization of endocrine cells into islets during development is also a complex process and involves commitment, specialization, survival and apoptosis. *In vitro*, it is not clear if the appropriate process is induced differentiation of epithelial cells involving the abandonment of the proliferative phenotype and entry into a terminally differentiated functional phenotype.

Introduction to the Studies

Developmental Biology of the Pancreas:

The pancreas is a complex, vital organ that combines endocrine and exocrine function to regulate glucose homeostasis and digestion. The exocrine component represents 95-99% of the pancreas. This portion has a highly complex architecture composed of the acinar secretory cells that produce digestive enzymes as well as a network of ducts to discharge them into the digestive tract [7]. Embedded within the exocrine tissue, but not connected to the duct network, are the Islets of Langerhans, which consist of four distinct endocrine cell types. The islets are highly vascularized and innervated spherical cell clusters of endocrine cells organized so that the insulin-producing β cells reside in the core of the islet, whereas the glucagon-producing α cells, somatostatin-producing δ cells and the pancreatic polypeptide-producing PP cells occupy in the periphery (Fig 1) [8]. In addition to the major hormones produced by islets, insulin, glucagon, somatostatin, other peptide hormones such as amylin and polypeptide C are also products of pancreatic islet cells. Of the endocrine cells, 60-80% are β cells,

15-20% are α cells, 5-10% are δ cells and <2% are PP cells. The human pancreas has roughly one million islets, which are found to be most numerous in the tail of the pancreas. Each islet is composed of about 1-2000 cells (**Fig 1**).

The organogenesis and development of the pancreas is relatively well characterized in the chicken and rodents. Like the liver and gallbladder, the embryonic pancreas is formed from two buds that develop from the midgut endoderm (REF). The ventral bud is immediately adjacent to the hepatic diverticulum and the dorsal bud is on the opposite side of the gut tube. The rotation of the stomach and duodenum causes the ventral bud and hepatopancreatic orifice to come in contact and fuse with the dorsal bud (**Fig 2**) [9]. The ventral bud forms the posterior part of the head of the pancreas, while the dorsal bud forms the remainder of the organ (body and tail). The main pancreatic duct (duct of Wirsung) is formed by fusion of the ventral duct with the distal part of the dorsal duct. The proximal part of the dorsal duct becomes a small accessory duct (duct of Santorini) with its opening into the duodenum. Although endocrine cells have been detected very early in the developing pancreas, islets do not form until the end of the gestation period [10]. Neogenesis of islets continues throughout neonatal life but slows down considerably shortly after weaning [11]. Insulin gene expression in the β cell increases during birth and weaning and the ability to sense glucose and to regulate insulin secretion becomes established at this stage. Thus, like many other organs, the pancreas is not fully functional at birth and requires in vivo maturation. In normal development, after birth and through adulthood, the mass of β cells expands continuously [12]. With this expansion there is an increase in islet size due to the addition of new islet cells and

the increase in individual cell volume [1]. Throughout much of adult life there is continued addition of small islets by differentiation from ductal progenitors or neogenesis [13]. The endocrine pancreas is considered to be a slowly renewing tissue. The entire mass of β cells (in rats and mice) turns over slowly through apoptosis and regeneration from replication of differentiated β cells and from neogenesis [14]. For example, the β cell turnover is <3% new cells per day [12]. It has been shown that differentiated pancreatic cells (β cells, acinar, and duct) can replicate, but the capacity of mature β cells to replicate may be limited. Replication of the β cells has been shown by BrdU and tritiated thymidine incorporation to be localized in insulin positive cells. In addition, electron micrographs of mitotic figures in cells with insulin granules have been shown [13]. However, differences in function in replicative and senescent β cells may still exist. Perhaps there are different pools of replicative and quiescent β cells that replicate or, may dedifferentiate partially to proliferate at the expense of the loss or reduced function [13].

In the 1960's Golosow and Grobstein performed classical embryological experiments to study aspects of pancreatic specification, growth and morphogenesis. These experiments were the first to describe mesenchyme-to-epithelial signaling, which was found to be crucial for pancreatic growth, morphogenesis and differentiation during pancreas development [8]. Two families of signaling factors, epidermal growth factor (EGF) and fibroblast growth factor (FGF), have been linked to epitheliomesenchymal interactions as signals that stimulate growth and morphogenesis of the pancreas. Another important signaling mechanism that controls pancreatic cell growth and differentiation is the Notch pathway. Notch signaling controls the commitment of stem cells between

differentiated endocrine and progenitor cell fates in the developing pancreas. Therefore, activation of Notch signaling is an essential regulator that prevents premature differentiation of pancreatic progenitor cells, thereby allowing their proliferation and islet neogenesis [8].

Studies of pancreatic gene expression have identified a large number of markers, mainly transcription factors [10, 15-17] which have been used to monitor pancreatic cell differentiation (**Table 1**). The differentiation process in development is complex and includes commitment, specialization, survival and apoptosis. Cytodifferentiation within the pancreatic epithelium begins before the first signs of organogenesis become evident. Although it is known that all pancreatic cell types are derived from PDX-1 expressing progenitors, [18] it remains unclear how endocrine/exocrine cell fate decisions are made. It has been shown that mesenchymal proteins, such as growth factors, affect the proportion of endocrine and exocrine tissue, but whether these proteins directly influence cell fate decisions has not been established. The situation is further complicated since the Notch signaling pathway has been implicated in endocrine fate determination. Downregulation of this pathway at different stages of embryonic development in transgenic mice results in a depletion of pancreatic precursors due to accelerated endocrine differentiation [7, 19] [20]. This is caused by upregulation of the bHLH transcription factor, neurogenin 3 (NGN3), which has been shown to be necessary for the development of all endocrine cell lineages [21]. Although overexpression of NGN3 only induces the differentiation of α and δ cells, it has been shown that β cells and PP cells are derived from a common NGN3 expressing precursor [22]. NGN3 lies upstream of

several other transcription factors that seem to play an important role in endocrine cell differentiation. (Fig 3) Targeted disruption of the gene encoding ISL1, another early marker of endocrine cells, abolishes endocrine differentiation [23]. The paired homeodomain protein, PAX6, known to be downstream of ISL1, is initially expressed in all endocrine cells, and deletion of PAX6 causes disruption in the development of all endocrine cells [10]. Deletion of the gene encoding the bHLH factor NEUROD has the same effect [24]. Alternatively, the PAX4 protein is specifically necessary for the production of mature β and δ cells [25] and two members of the Nkx homeobox family (Nkx2.2 and its downstream target Nkx6.1) are also implicated in β cell differentiation. Nkx2.2 is expressed in the early pancreatic progenitor cells, the neurogenin3-expressing cells, and the differentiated islet cells [26]. On the other hand, Nkx6.1 is expressed in three populations of cells during pancreatic development: first in the undifferentiated epithelial cells of the early pancreatic buds; then, after the secondary transition, in a subset of proliferating islet cell progenitors; and finally in the differentiated β cells [27]. **Figure 4** shows a proposed simplified model for the role of transcription factors in endocrine differentiation in the developing pancreas. The proposed location in the lineage for each transcription factor is based on timing of expression, timing of predominant functional role, or both. Clearly, some factors function at several phases, but a single step is shown for simplicity [28]. Thus, progenitor pool derivation from ductal epithelium is a source of endocrine epithelial cells, specifically β cell precursors.

Physiology of the β cell:

The four endocrine cellular components of the islet, α, β, δ, and PP cells are responsible for secretion of glucagon, insulin, somatostatin and pancreatic polypeptide respectively. These hormones work in concert to regulate glucose homeostasis, and make the islet the basic glycemic sensing and control unit. For example, β cells release insulin in response to elevated glucose after food ingestion or during glucose release from glycogen stores. The other part of the equation is that target tissues participate in insulin controlled glucose uptake, such as liver, muscle and fat. In the liver, glucose production is inhibited in response to insulin. In contrast, the α cells respond to low blood-glucose by secreting glucagon, which stimulates glycogenolysis and gluconeogenesis. Somatostatin and pancreatic polypeptide exert inhibitory effects on both pancreatic endocrine and exocrine secretion [8].

β cells are electrically active and respond to glucose by generating action potentials. Over the past fifteen years, electrophysiological experiments have shown that insulin release is coupled to stimulation. Specifically, glucose enters the β cell via GLUT 2 transporters and is metabolized to produce ATP. The increase in the ATP/ADP ratio leads to the closure of ATP-sensitive K^+-channels (K_{ATP}-channels) in the plasma membrane, causing a gradual depolarization of the cell. Eventually, voltage-dependent Ca^{2+} channels (VDCC) become activated and initiate action potentials. Influx of Ca^{2+} through these channels and the resulting elevation of intracellular Ca^{2+} $[Ca^{2+}]_i$ then triggers exocytosis of insulin containing granules. In contrast, also upon membrane depolarization, voltage-dependent K^+ (K_v) channels open to repolarize the action

potential, limit Ca^{2+} entry through VDCCs and limit insulin secretion [29]. Other mechanisms that potentiate insulin secretion include activation of protein kinase C (PKC) and protein kinase A (PKA). Second messengers such as cAMP or diacylglycerol (DAG) increase insulin release through protein phosphorylation by cAMP-dependent PKA and Ca^{2+}-sensitive DAG-dependent PKC, respectively [30] (**Fig 5**).

Structure and Biosynthesis of Insulin:

Insulin is a peptide hormone composed of 51 amino acids arranged in two polypeptide chains, designated A and B which are linked together by two disulfide bonds (**Fig 6**). The A chain also contains a disulfide bond between amino acid residues 6 and 11. Although there is some variability in the amino acid sequence among species, many regions of the molecule are highly conserved. For example, beef insulin differs from human insulin at three amino acid positions, while porcine and canine insulin differ from human by only one amino acid [31].

Insulin is synthesized as a larger precursor, preproinsulin, which undergoes post-translational modifications. Proinsulin consists of three domains: an amino-terminal B chain, a carboxy-terminal A chain and a connecting peptide that links A and B chains known as the C-peptide. The amino-terminal signal peptide is removed cotranslationally by a signal peptidase located within the endoplasmic reticulum to generate proinsulin. Cleavage of this peptide by proteases occurs inside the insulin granules to form insulin, thus liberating C-peptide. C-peptide has no known biological function, but its presence in the blood is used as an indicator of insulin biosynthesis *in vivo* [24].

Mechanisms of Insulin Action:

The Insulin Receptor- The insulin receptor (IR) is synthesized as a single polypeptide that is glycosylated and cleaved into α– and β–subunits, which are then assembled into a tetramer linked by disulfide bonds. A hydrophobic domain in each β-subunit spans the plasma membrane, while the extracellular α-subunit contains the insulin binding site. The cytoplasmic domain of the β-subunit is a tyrosine kinase, which is activated by insulin. Binding of insulin to the α-subunit of the IR causes conformational changes that are transferred to the β-subunit, promoting autophosphorylation of a specific tyrosine residue of each β-subunit. It is known that receptor tyrosine kinase activity leads to phosphorylation of tyrosines of a peptide identified as insulin receptor substrate (IRS). Phosphorylated IRS appears to interact with a number of intracellular proteins such as SHP-2, PI3K, GRB2, and SHC. Therefore, insulin binding causes a complex cascade of phosphorylation and dephosphorylation reactions, terminating by dephosphorylation of the receptor (**Fig 7**).

Glucose Transport- Glucose can enter cells via two transport mechanisms. Facilitated transport is mediated by a family of at least five glucose transporters located in the plasma membrane which are designated GLUT-1 through GLUT-5 [26]. The glucose transporters share significant homology in their primary sequence but display a tissue-specific pattern of expression. For example, GLUT-1 is expressed in the placenta, brain, kidney and colon and is less abundant in muscle and adipose tissue [31]. GLUT-2 is present in the liver and pancreatic β cells, while GLUT-3 is found in multiple tissues, including brain, placenta and kidney [31]. GLUT-5 has been shown to predominantly

transport fructose and is mainly expressed in the small intestine epithelium and GLUT-4 is the only glucose transporter that is regulated by insulin [31]. It is expressed exclusively in insulin-sensitive tissues, which include skeletal and cardiac muscle and adipose tissue. In the absence of insulin, glucose transporters are present in cytoplasmic vesicles located close to the cell membrane where they are essentially inactive. The binding of insulin to receptors leads to the rapid fusion of transporter vesicles to the plasma membrane and insertion of the glucose transporters, allowing the cell to take up glucose. Cotransport is an energy-requiring process that facilitates entry of glucose into the cell, against a concentration gradient- from low glucose concentrations outside the cell to higher concentrations within the cell. Cotransport is a carrier-mediated process in which the entry of glucose into the cell is coupled to the concentration gradient of Na^+. This type of transport occurs in the intestinal epithelium, renal tubules and choroid plexus and is not regulated by insulin. It should be noted that some tissues such as the brain and the liver are unique and do not require insulin for efficient glucose uptake.

Metabolic Effects of Insulin:

Effects on carbohydrate metabolism- The effects of insulin on glucose metabolism are most prominent in liver, muscle and adipose tissue. In the liver, insulin decreases the production of glucose by inhibiting gluconeogenesis and the breakdown of glycogen. In muscle and liver, insulin increases glycogen synthesis. In muscle and adipose tissue, insulin increases glucose uptake by increasing the density of glucose transporters in the cell membrane.

Effects on lipid metabolism- Insulin decreases the level of circulating fatty acids by inhibiting the activity of hormone-sensitive lipase in adipose tissue. Insulin increases the transport and metabolism of glucose into adipocytes, providing the substrate glycerol-3-phosphate for triglyceride synthesis. Insulin also increases lipoprotein lipase activity of adipose tissue, providing fatty acids for esterification.

Effects on protein synthesis- Insulin stimulates the entry of amino acids into cells and protein synthesis, in most tissues.

The metabolic disorder of glucose metabolism is known as diabetes mellitus.

Diabetes Mellitus:

Diabetes mellitus afflicts approximately 18.2 million people in the United States and is the fifth leading cause of death by disease [32, #278, #278]. This metabolic disorder arises from the inability of the organism to regulate blood glucose levels, and can be broadly classified into two groups. Type I, or insulin dependent diabetes mellitus (IDDM) is proposed to be a multifactorial autoimmune disease resulting from a T-cell mediated destruction of pancreatic β cells, the body's only natural source of insulin [33]. The destructive process is gradual and ultimately leads to severe insulin depletion, which results in hyperglycemia due to decreased cellular uptake of glucose from the circulation (due to the lack of insulin), glycogenolysis and gluconeogenesis. In the absence of insulin, there is also an increase in fat breakdown via fatty acid oxidation, resulting in the excessive production of ketones [34]. High circulating glucose leads to imbalance of water homeostasis since glucose increases osmolarity in extracellular fluids. Historically, IDDM has been considered a predominantly childhood disease because occurrence

peaked between ages 10-14 years. However, the incidence of IDDM in adults may be more prevalent than initially believed and has been diagnosed in children younger than 10 years and in some adults [35]. IDDM patients manage the disease by insulin therapy, yet it is still difficult for them to maintain normoglycemia. They experience transient hyper- and hypoglycemic episodes, of which hypoglycemia is particularly dangerous and may be life threatening. This is because the brain cannot function properly without adequate supply of glucose. On the other hand, chronic hyperglycemia leads to complications such as end-stage renal failure, blindness, peripheral neuropathy, amputations and cardiovascular disease. It has been shown that "tight control" of blood glucose is clearly correlated with a dramatic reduction in the incidence of diabetic complications [36].

The early stages of the disease process leading to IDDM are characterized by insulitus, the infiltration of the pancreatic islets by mononuclear immune cells, including dendritic cells, macrophages and T cells [37]. This lymphocytic infiltrate is thought to contribute directly to β cell destruction. Autoreactive T cells specific for β cell proteins (including insulin, glutamic acid decarboxylase (GAD) and the protein phosphatase, IA-2) have been isolated from the peripheral blood in 85-90% of newly diagnosed individuals with diabetes [38]. Other studies have shown that some of these autoreactive T cells taken from diabetic patients are capable of destroying β cells *in vitro* [39]. Studies with the non-obese diabetic (NOD) mouse have shown that T cells play an important role in the disease pathogenesis. The NOD mouse spontaneously develops insulin deficient diabetes in a time course similar to the pathogenesis of IDDM. The

development of the disease in this animal model has been shown to be thymus dependent and require both CD4+ and CD8+ T cells [40].

The succeptibility to IDDM is influenced by both genetic and environmental factors. The environmental factors that have been suggested to contribute to the risk of developing IDDM, include viral infections, dietary components in early infancy, vaccination, climatic influences, toxins and stress [41]. The genetic determinants of susceptibility to IDDM are perhaps better understood than the environmental risk factors. The first diabetes susceptibility genes to be identified were the human leucocyte antigen (HLA) genes, located on chromosome 6p21 [42]. Subsequent studies demonstrated an association between the disease and the insulin gene region on chromosome 11p [43]. Several genome screens confirmed that the IDDM1 locus (the HLA gene region) is the major genetic determinant of disease risk, accounting for 42% of the familial inheritance. The IDDM2 locus (the insulin gene region) contributes a further 10% of genetic susceptibility [44].

Type II, or non-insulin dependent diabetes (NIDDM), the more prevalent form of diabetes, results from insensitivity of target tissues to insulin, rather than a lack of insulin secretion due to the destruction of β cells. Insulin resistance can be caused by mutations in the insulin receptor gene so that the function of insulin receptors is impaired. Alternatively, NIDDM may be caused by abnormalities in insulin secretion. NIDDM is characterized by the inability of muscle and adipose tissue to respond to normal levels of circulating insulin (insulin resistance). Consequently, due to continuous pressure to secrete increasing amounts of insulin, pancreatic β cells fail to maintain the increased

insulin secretion and overt diabetes occurs [26]. NIDDM is a heterogeneous syndrome that develops as a result of environmental as well as genetic factors. Risk factors for the development of NIDDM include sedentary lifestyle, nutritional imbalance and most importantly, obesity. For example, class 1 mutations include a decrease in the number of insulin receptors on the target cell surface caused by nonsense mutations that lead to truncated proteins, deletion mutations, or splicing defects. Class 2 mutations impair the transport of receptors through the RER and Golgi to the plasma membrane, while class 3 mutations impair receptor function by decreasing the binding affinity of insulin. The decrease or lack of receptor tyrosine kinase activity is due to a class 4 mutation. Finally, class 5 mutations accelerate receptor degradation by interfering with endocytosis, recycling, and degradation of insulin receptors, effectively decreasing receptor density [26].

In humans and lower mammals, insulin secretion is biphasic, with an early burst of insulin release within the first 10 minutes after feeding, followed by a progressively increasing phase of insulin secretion that persists as long as the hyperglycemic stimulus is present [45]. It has been suggested that loss of the first phase of insulin secretion is the earliest detectable abnormality in patients who are destined to develop NIDDM [46]. The loss of the first phase of insulin secretion has important pathogenetic consequences, particularly because this early burst of insulin release plays an important role in priming insulin target tissues (liver) that are also involved in maintenance of glucose homeostasis [17]. Several cases have been identified in which a point mutation in the insulin gene has led to the development of mild hyperinsulinemic diabetes mellitus. These patients often

have high circulating levels of preproinsulin and low circulating levels of C-peptide in the blood. Asymptomatic patients with insulin gene mutations have also been reported; overt diabetes is more likely to develop if these patients also demonstrate risk factors for insulin resistance. Maturity-onset diabetes of the young (MODY) is a monogenic diabetes subtype characterized by non-insulin dependence, autosomal dominant inheritance, an onset usually before 25 years of age, and an absence of ketosis [47]. Genetic linkage analysis studies have provided evidence for at least four unlinked loci on chromosomes 20, 7, 12, and 13 that can cause this form of diabetes, designated MODY1, MODY2, MODY3 and MODY4. The proteins encoded by the four MODY loci have been identified and shown to be hepatocyte nuclear factor 4 (HNF4a), glucokinase, hepatocyte nuclear factor 1 (HNF1a) and human homeodomain transcription factor IPF1 (PDX-1, STF-1, IDX-1), respectively [26].

Therapeutic Management of Diabetes:

All insulin-dependent and 40% of non-insulin-dependent diabetics must use daily insulin therapy to regulate their blood glucose level [48]. It has been shown that tight glycemic control requiring intensive insulin therapy with multiple daily injections or insulin pump delivery both coupled to frequent monitoring of blood glucose, can significantly reduce the chronic complications of diabetes. However, maintaining good glycemic control with insulin, by this type of titration strategy places an enormous burden on patients. Furthermore, there is a correlation between intensive insulin therapy and an increased incidence of severe hypoglycemia, which can be life threatening [49]. Some patients have wide swings in blood glucose with episodes of hyper- and hypoglycemia

despite strict adherence to insulin regimen prescribed [50]. Although significant advances in glucose monitoring and insulin therapy have continued, the only cure for diabetes resides in the presence of endogenous glucose sensitive insulin secretion. The two therapeutically essential events for the treatment of diabetes include the ability to continuously monitor blood glucose levels and the production of corresponding and sufficient levels of mature insulin to facilitate entry of glucose into muscle and adipose tissues [49]. This can be accomplished by either whole organ pancreas transplantation, or alternatively, islet or β cell implantation; however, significant obstacles to this strategy exist. A successful clinical islet transplant currently requires 2-4 donors per recipient to achieve independence from exogenous insulin. In addition, the shortage of cadaveric pancreata available for transplantation cannot fulfill the demand [51]. Post transplantation, continuous immunosuppressive therapy increases the risk for recurrent infections and certain cancers as well as have a serious impact on other vital organs specifically, the liver. Whole organ transplants have been shown to lead to sustained euglycemia and insulin-independence in the vast majority of recipients. Graft survival rates have been as high as 78% at 5 years [52], however, pancreatic transplants are generally considered in patients with end-stage renal failure and are still associated with peri-operative mortality and significant morbidity [53, 54]. Islet transplantation is an attractive alternative to whole organ transplantation. One of the advantages of islet implants, is that they could be achieved using a minimally invasive procedure. Islet transplantation involves isolation from cadaveric or autologous donor pancreata and intravascular infusion, typically through the portal vein by way of a percutaneous

catheter. The infused islets disseminate hematogenously to the liver where they lodge in the distal portal venules and become revascularized and reinnervated. Transplanted islets can remain viable and produce insulin in response to fluctuating glucose levels over extended periods of time [50]. However, islet transplantation is severely limited by the availability of donor pancreata. Of the 5,000 annual organ donors in the US, only a fraction of them provide a usable pancreas for transplantation [55].

Islet Isolation:

In addition to the importance of strict recipient selection, the quantity of high-quality islets transplanted, and the reduction of diabeteogenicity of the immunosuppressive regimen, several other issues require further study to optimize feasibility and outcome of the islet transplant endeavor. For example, donor characteristics significantly influence the yield and quality of islets isolated from cadaveric pancreata. Procurement factors (ischemia and time from tissue isolation to transplant) also play a role in efficient islet recovery. Advances in the techniques of islet isolation since the development of the automated Ricordi method [56] (**Fig 8**) have increased the purity, yield and viability of transplantable islets. Briefly, the Ricordi method is a continuous, controlled automated digestion approach during which the pancreas is placed in a dissociation chamber with steel spheres and is agitated in the presence of collagenase at 37°C. The dissociation solution is circulated while a fine mesh screen allows islets to be released and large, under-digested tissue pieces to remain in the chamber. Islets are then further purified by density gradient centrifugation using technology such as the COBE 2991 cell processor [57]. For islets to be suitable for

transplantation, they must conform to certain criteria. These include: 1) purity >30%, 2) product yield ≥ 4,000 IEQ/kg recipient body weight, 3) viability (flourescein diacetate dye inclusion and propidium iodide exclusion) ≥70%, 4) packed cell volume ≤10mL, 5) endotoxin content ≤5EU/mL final product volume/kg of recipient body weight, and 6) a negative gram stain [50]. Due to the difficulties in obtaining sufficient amounts of high-quality islets/insulin producing cells for transplantation, alternative sources of glucose responsive insulin producing (GRIP) cells are being studied. Two fundamental approaches involve either 1) *in vitro* expansion and differentiation of islets from stem/progenitor cells, or 2) introduction and guidance of stem/progenitor cells to achieve *in vivo* regeneration of islets. Potential sources of stem/progenitor cells include pancreatic ducts, bone marrow, mobilized peripheral blood, umbilical cord blood, fetal liver or embryonic stem cells.

In Vitro Production of Alternate Islet Cell Sources:

Throughout adult life, the pancreatic β cell population exists in a dynamic state of slow turnover, as cells undergo replication, neogenesis, apoptosis and changes in volume [58]. Although this tissue maintenance is slow, it has been shown in several animal models that there does appear to be some regenerative capacity of the endocrine pancreas. *In vitro*, terminally differentiated β cells are difficult to sustain due to their lack of mitotic activity presumably related to their functional role. Stem/progenitor cells offer a potential solution to the need for a proliferative, expanding cell population and yet full glucose responsive insulin secretion characteristics. It has been suggested that

intrapancreatic islet progenitor cells may be derived not only from the pancreatic duct epithelium [12, 59], but are also present within the islets themselves [60]. Soria, et al. demonstrated that mouse embryonic stem (ES) cells contained roughly 90% of the insulin present in a normal mouse islet and also responded to glucose by secreting insulin in a physiologically relevant manner [61]. However, when these cells were injected into streptozotocin-induced diabetic mice, they improved but could not normalize hyperglycemia. Another group has generated cells that produce insulin and other pancreatic endocrine markers from mouse ES cells [62] which contained roughly 50-fold less insulin content than pancreatic β cells, but these cells secreted insulin in a glucose responsive manner. When grafted subcutaneously into the shoulder of diabetic mice, hyperglycemia in the animals remained, but their body weight remained stable and survival improved [62]. *In vitro* studies of human ES cells demonstrated cytoplasmic insulin localization in cells of embryoid bodies (clusters of differentiated cells derived from ES cells) as well as the presence of insulin secreted into the medium. Furthermore, the expression of GLUT2 and glucokinase was confirmed by RT-PCR, but the cells did not secrete insulin in a glucose-responsive manner [63]. It has been realized that ES cells and other pluripotent stem cells will express the insulin gene. However, whether they can be induced *in vitro* to become glucose sensing β cells remains to be established.

It has been suggested that pancreatic endocrine development involves ductal progenitors, which differentiate into populations of specialized islet cells. Experiments involving partial pancreatectomy in young rats demonstrate that ductal epithelia are capable of regeneration [64]. Ramiya, et al. has shown that cells derived from pancreatic

duct epithelia have the ability to proliferate extensively and to form cell aggregates resembling islets *in vitro* [59]. These islets can 1) produce insulin, glucagon and somatostatin, 2) significantly upregulate insulin production upon the elevation of glucose in the presence of nicotinamide, and 3) decrease blood glucose levels following transplantation into NOD mice [59].

Nonpancreatic sources have been reported to produce cells with glucose responsive insulin production. For example, because of developmental relationship, hepatocytes seem to be an ideal target for studies of transdifferentiation to putative endocrine cells with glucose responsiveness. Transgene delivery using an adenoviral vector containing the PDX1 cDNA was used to infect hepatocytes *in situ.* While a large population of hepatocytes expressed PDX1, only a few cells expressed insulin. Streptozotocin-induced diabetic mice infected with this adenoviral vector had decreased blood glucose levels after a few days. [65] These studies suggest that in the context of a stem cell strategy, PDX1 alone might be capable of programming the genome of the stem cell to express the set of genes required to establish a fully functional β-cell. Whether this can be translated to an adult human organism is difficult to predict.

Stem Cell Stimulators and Differentiators:

Mulitpotential stem/progenitor cells can be induced to proliferate and differentiate when exposed *in vitro* to certain growth factors of cytokines. For example, AR42J cells (a rat pancreatic acinar cell line) can be converted to insulin-producing cells by exposure to the growth factors betacellulin and activin A or to hepatocyte growth factor [66]. Oxidative damage is proposed as a hurdle to *in vitro* expansion and nicotinamide is often

used to increase β cell survival as well as to increase insulin production [59]. Glucagon-like peptide (GLP-1), an insulinotropic hormone appears to be an effective agent for inducing differentiation of human pancreatic islet-derived progenitor cells [67] and duct cells into insulin producing cells. Notably, GLP-1 induces the expression of PDX1 in the progenitor cells [67] and duct cells [54] which appears to be essential for their differentiation or commitment into β cells. It is believed that the activation of the receptor by GLP-1 activates PDX1 both by phosphorylation via MAP kinase pathways, and by effecting its translocation into the nucleus [68, 69]. Cells derived from pancreatic islets from rat and human increase their rates of proliferation in the presence of basic fibroblast and epidermal growth factors (FGF-2 and EGF). [60] These cells can then be differentiated into islet-like clusters by the withdrawl of FGF and EGF and the addition of differentiating factors such as activin A, betacellulin, HGF [60] or GLP-1 [67].

Proliferation and Differentiation via FGF-2

The fibroblast growth factor (FGF) family consists of at least 18 homologous heparin-binding polypeptides that are potent regulators of cell proliferation, differentiation and function, depending on the target cell [70]. FGF signaling is mediated by high affinity tyrosine kinase receptors (FGFRs) and low affinity heparin-sulfate proteoglycan receptors that enhance ligand presentation to the FGFRs and stabilize FGF by protecting them from proteolysis. Acidic FGF (FGF-1) and basic FGF (FGF-2) are the prototypic FGF members and have been implicated in pancreatic islet embryogenesis [71]. FGFR1 and 2 are expressed during pancreatic development in the rat, and it has

been shown that FGF-2 induces proliferation of pancreatic epithelial cells during embryogenesis [72]. Also in the rat, FGF-1 and FGF-2 are expressed in the ductal epithelium on days 10-14 of intrauterine development, coinciding with increased cell replication [70]. Early development in chickens is tightly regulated by FGF-2 released from the notochord [73]. During embryogenesis in mice, FGFs are widely expressed in the region of the mesenchyme surrounding pancreatic precursor cells and are involved in the early stages of development of the endocrine pancreas [74]. Thus, in chicken and mice, mesenchyme-derived FGFs are thought to act via a paracrine mechanism to stimulate migration of pancreatic precursor cells into the mesenchyme and thereby initiate the process of pancreas development [75]. Other studies suggest that signals arising from endocrine pancreatic precursor cells themselves may be sufficient to promote cell clustering and differentiation during the formation of islets. For example, Hardikar, et al. showed that human pancreatic PANC-1 cells, as well as adult-derived precursor cells (hIPCs), form hormone expressing islet-like aggregates when exposed to serum-free media. Of several growth factors tested, FGF-2 was found to be the most effective chemoattractant in the aggregation of PANC-1 cells. Expression analysis of FGF-2 and its receptor suggests that FGF-2 signaling may also participate in the aggregation of hIPCs. This suggests that FGF-2 is not derived from the surrounding mesenchyme, but secreted by endocrine precursor cells, may be an important factor in the differentiation of pancreatic precursor cells to islets [75].

Cell-Cell Interactions

Cadherins constitute a superfamily of transmembrane proteins, which mediate Ca^{2+}-dependent cell-cell adhesions that are involved in many morphogenetic processes during tissue development. Cadherin isoforms are structurally highly homologous with a Ca^{2+}-binding extracellular domain consisting of five subdomains, a single transmembrane domain, and a highly conserved cytoplasmic domain [76] [77]. The cytoplasmic domain of cadherins interacts with cytoplasmic components of α-, β-, and γ-catenin, which mediate the linkage to the actin filaments. [78]. The amount of cadherin-catenin complexes varies depending on cell type and differentiation state of cells and is believed to be modulated during changes in the developmental stage of cells [79]. It has been shown that α- and β-catenin become phosphorylated at tyrosine residues and that this posttranslational modification correlates with changes of the epithelial cell phenotype [80]. In addition to a function in adherens junction formation, the cadherin-catenin complex is thought to transmit signals from neighboring cells through interactions with growth factor receptors [79]. For example, it has been shown that tyrosine phosphorylation of catenins was also observed in cells treated with EGF and HGF [81].

Studies with rat islets have shown that E-cadherin mediates most of the Ca^{2+}-dependent adhesion between all cell-types in the islets. The role of E-cadherin in the regulation of morphogenesis of the islets during pancreatic organogenesis was elucidated in studies by Dahl, et al. [76]. Transgenic mice carrying an E-cadherin cDNA lacking most of its extracellular domain, whose expression is driven by the rat insulin promoter 1 (Rip1), showed disrupted cadherin activity in β cells during pancreatic ontogeny. The

initial clustering of β cells, which begins at 13.5-14.5 days postcoitum in normal rats was disrupted. The aggregation of endocrine cells into islet-like structures which normally begins at 17.5-18 days postcoitum was inhibited in these transgenic animals [76].

Ins-1 Cell Line

Several β cell lines, such as INS-1 have been used as a model to study β cell physiology, due to several factors including: a limited availability of pancreatic endocrine tissue, the difficulty of preparing a sufficient quanitity of viable islets, cellular heterogeneity and the rapid decline of insulin production *in vitro* [82] [83]. The INS-1 cell line is a radiation-induced rat insulinoma, developed by Asfari et al. [84, 85]. INS-1 cells are well characterized and exhibit insulin gene transcription, preproinsulin biosynthesis, proinsulin processing and regulated insulin exocytosis in response to the metabolism of D-glucose. However, these cells have a diminished response to glucose, specifically at higher passages. In addition, INS-1 cells have been shown to synthesize small quantities of proglucagon, glucagon and GLP-1 [85].

Rotating Cell Culture System

Traditional two-dimensional cell culture conditions provide an environment which differentiated cells lose their specialized features and dedifferentiate [86]. Suspension culture is a popular solution that prevents dedifferentiation and maintains specialized features of cells. The rotating-wall vessel (RWV) is a suspension culture vessel optimized to produce laminar flow and eliminate the shear stress on cell aggregates in culture (**Fig 9**). Furthermore, the RWV allows three-dimensional spatial freedom, the delivery of nutrients and oxygenation by diffusion [86]. For example,

human hepatocytes cultured in the RWV remained viable for 60 days and aggregated into tissue-like structures [86, 87]. The RWV may be used as an alternative to aggregation in tissue culture flasks.

Figure 1. Diagram of the pancreas. The pancreas is an organ with combined exocrine and endocrine function. The mature pancreas is adjacent to the duodenum - the most anterior part of the small intestine (a). The function of the exocrine pancreas is to supply the gut with digestive enzymes, which are produced and secreted by acinar cells and subsequently transported to the intestine via the pancreatic ductal system (b). Endocrine pancreas consists of four hormone-producing cell types: α–, β–, δ– and pancreatic polypeptide (PP) cells. α–cells (red) secrete glucagon and make up 15–20% of the endocrine pancreas. β–cells (green) secrete insulin and make up 60–80% of the endocrine pancreas. δ–cells (yellow) secrete somatostatin and make up 5–10% of the endocrine pancreas, whereas PP cells (blue) secrete PP and make up less than 2% of the endocrine pancreas. [8]

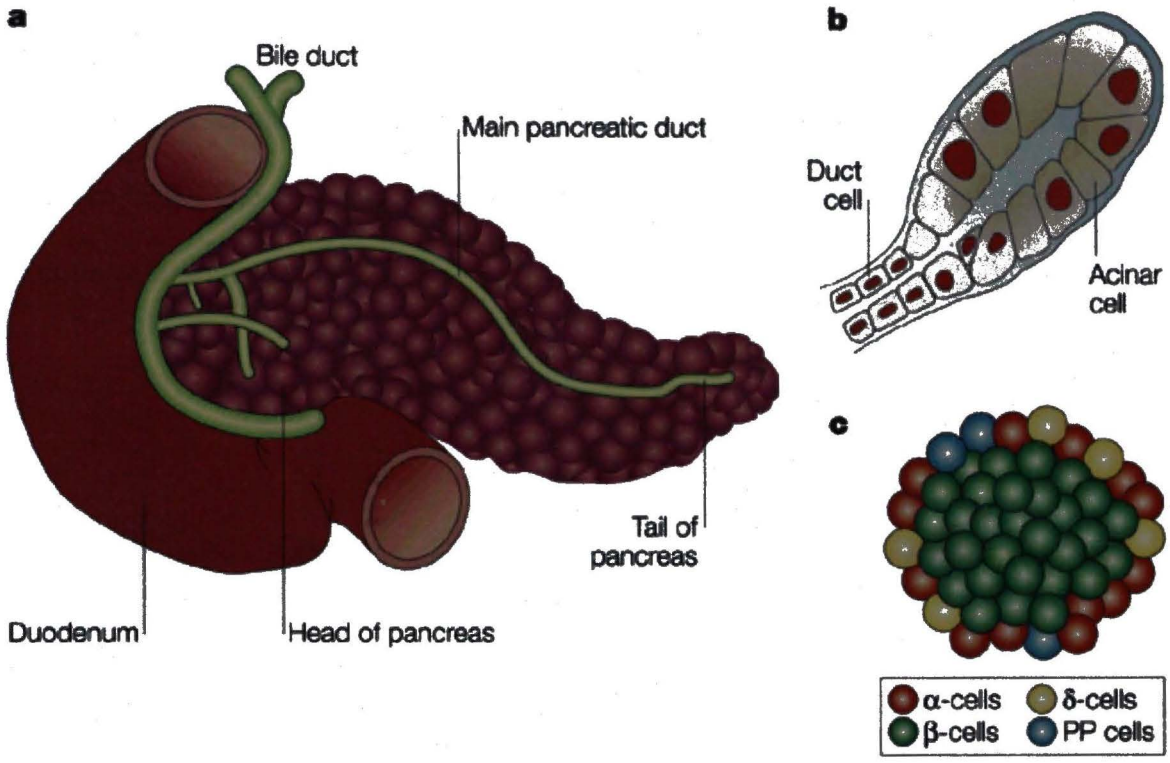

a
Bile duct
Main pancreatic duct
Tail of pancreas
Duodenum
Head of pancreas
b
Duct cell
Acinar cell
c
α-cells
δ-cells
β-cells
PP cells

Figure 2. The pancreas develops as evaginations of the primitive gut epithelium. Schematic representation of the pancreas at embryonic day (E) 9, E10 and E12 of a mouse embryo. The dorsal and ventral pancreatic buds rotate and fuse together to form the definitive pancreas. [8]

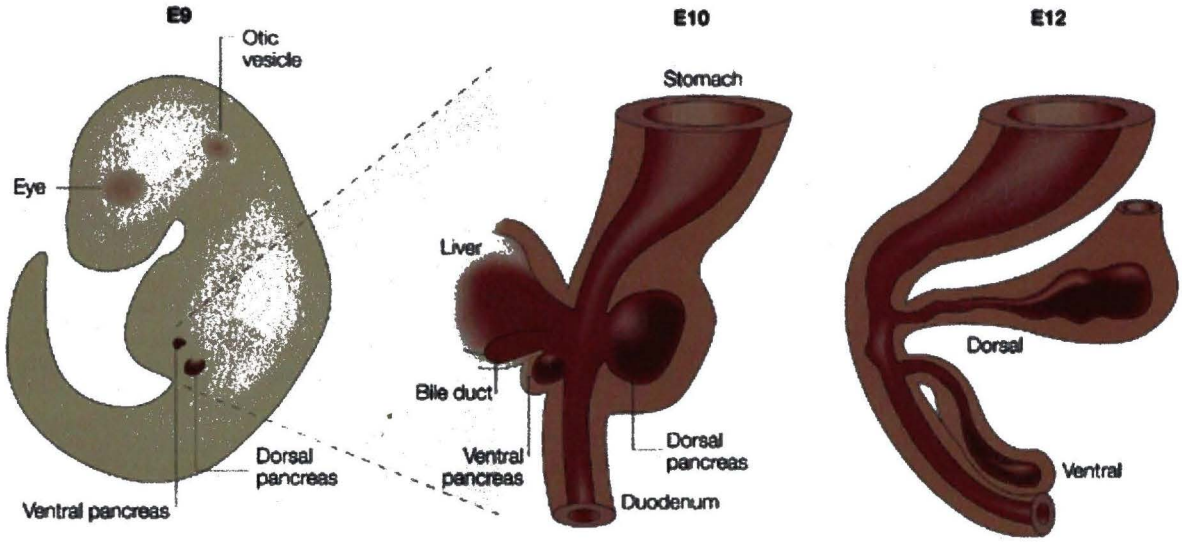

E9
Otic vesicle
Eye
Dorsal pancreas
Ventral pancreas
E10
Stomach
Liver
Bile duct
Ventral pancreas
Dorsal pancreas
Duodenum
E12
Dorsal
Ventral

Table 1. A partial list of transcription factors involved in pancreatic development including downstream target genes and mutations that occur. [28]

Transcription Factor	Family	Expression	Downstream Pancreatic Genes	Mouse mutations	Human mutations
Neurogenin 3	bHLH	Fetal pancreas (endocrine progenitor cells) and CNS	neuroD1/BETA2, pax4, nkx2.2	Diabetes, no islet cells	
NeuroD1/BETA2	bHLH	Islet, gut endocrine cells, CNS	Insulin	Diabetes, decreased islet cells	Late onset diabetes
PDX/IPF1	parahox homeodomain	β and δ cells, duodenum, stomach, CNS	Insulin, IAPP, glucokinase, glut2	Pancreatic agenesis	MODY4
Pax 2	Paired homeodomain	Islet, urogenital tract, CNS	glucagon	optic nerve, urogenital tract	Het:renal coloboma syndrome
Pax 4	Paired homeodomain	fetal pancreas, CNS	pax4 (autorepression)	decrease β and δ cells	Late onset diabetes;early diabetes
Pax 6	Paired homeodomain	Islet, gut endocrine cells, CNS	glucagon, insulin, somatostatin	decrease in all islet cells, decreased glucagon	Aniridia
Nkx2.2	NK-homeodomain	α, β, PP cells, CNS	nkx6.1, insulin, glut2, GK	diabetes, no insulin	
Nkx6.1	NK-homeodomain	β cells, CNS		decreased β cells, postnatal lethal	
Isl1	LIM-homeodomain	Islet, CNS	somatostatin, glucagon	No islet cells, embryonic lethal	late onset diabetes

Figure 3. Outline of pancreatic development: The pancreas is of endodermal origin. The notochord, the overlying mesoderm and ectoderm, as well as other tissues provide signals such as TGF-β, activin-B, and FGF2, which serve to induce changes such as subsequent upregulation of PDX-1. Other signals such as TGF-β and activin promote endocrine cell formation through a complex transcription factor cascade involving a number of transcription factors including ISL-1, Pax 6, Nkx 2.2, NGN3, and Beta2/NeuroD. Expression of these and other transcription factors including Pax 4, and Nkx 6.1 are further modulated by other less well-defined signals to promote specific fates among the endocrine precursor cells and drive them toward the mature cell types found in the islet including the β-cell, α-cell, δ-cell and PP cell, which produce insulin, glucagon, somatostatin and pancreatic polypeptide, respectively. [49]

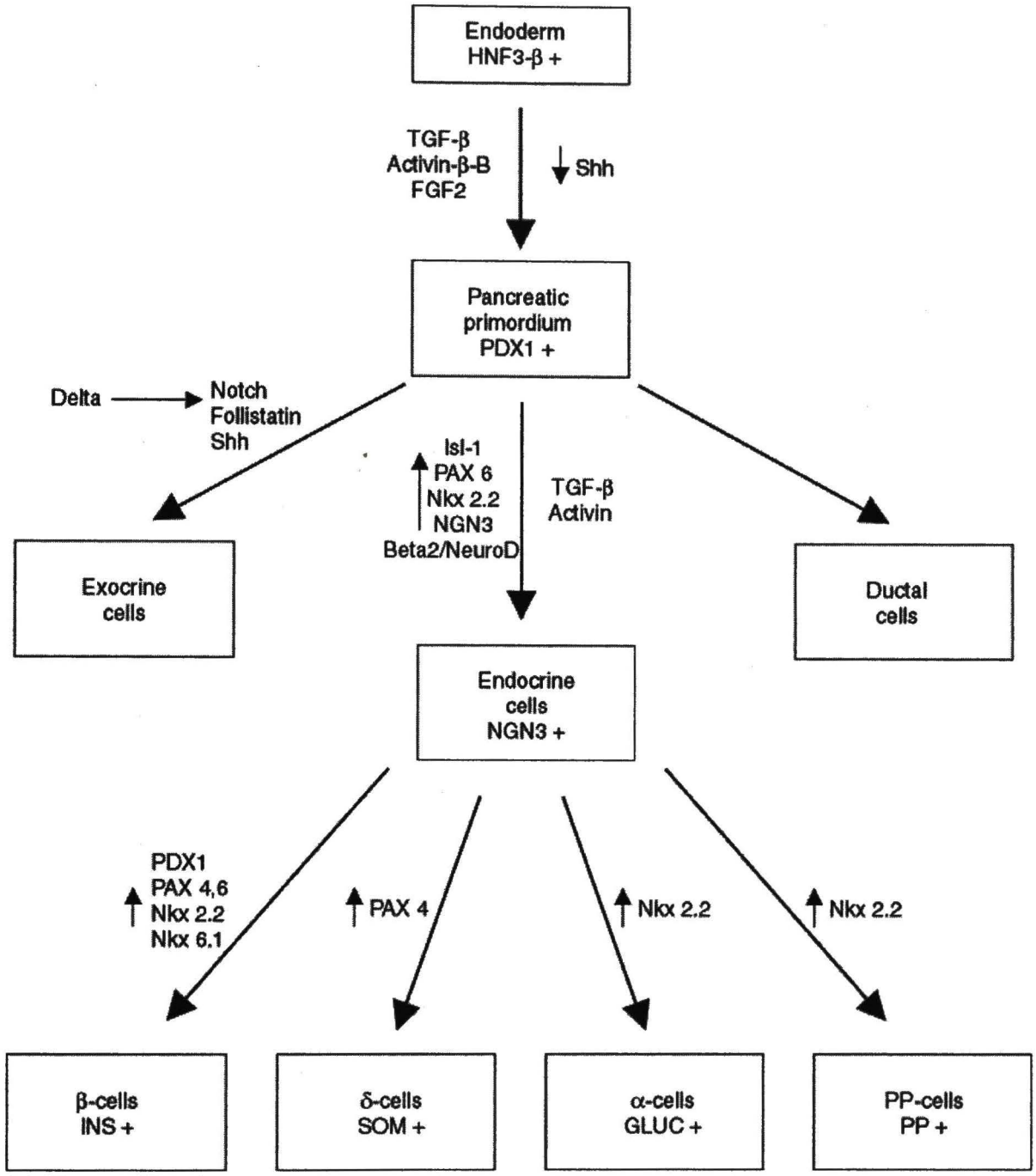

Endoderm
HNF3-β +
TGF-β
Activin-β-B
FGF2
Shh
Pancreatic
primordium
PDX1 +
Delta
Notch
Follistatin
Shh
Isl-1
PAX 6
Nkx 2.2
NGN3
Beta2/NeuroD
TGF-β
Activin
Exocrine
cells
Ductal
cells
Endocrine
cells
NGN3 +
PDX1
PAX 4,6
Nkx 2.2
Nkx 6.1
PAX 4
Nkx 2.2
Nkx 2.2
β-cells
INS +
δ-cells
SOM +
α-cells
GLUC +
PP-cells
PP +

Figure 4. A simplified model for the proposed role of islet transcription factors in endocrine differentiation in the developing pancreas. Endocrine and duct cells arise from PDX1 expressing progenitors whereas exocrine cells arise from p48 expressing progenitors. [28]

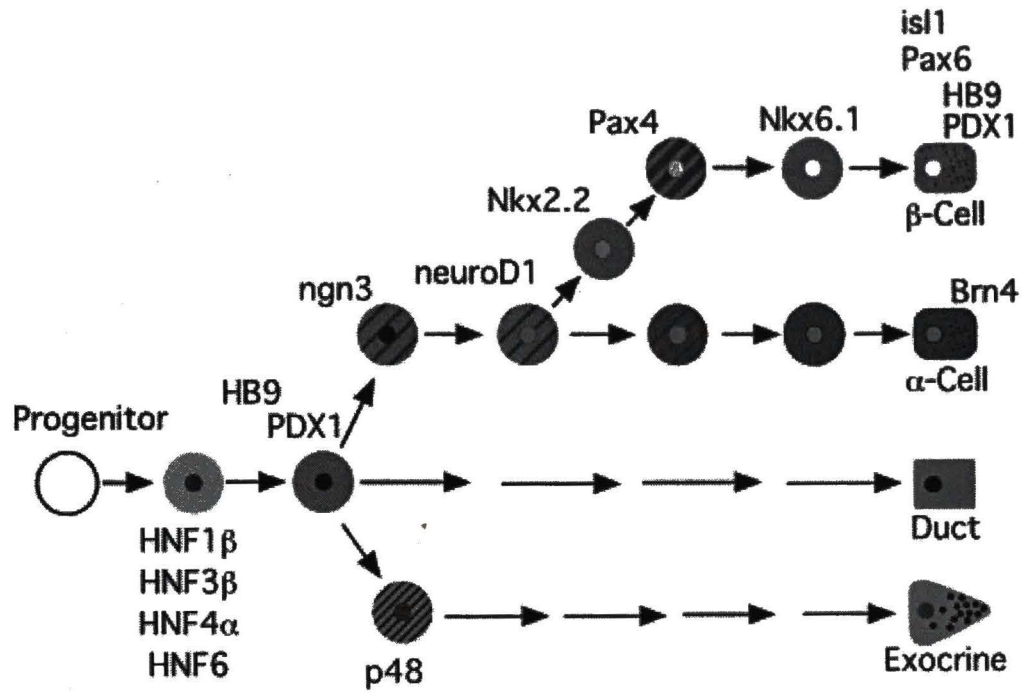

isl1
Pax6
HB9
PDX1
Pax4
Nkx6.1
Nkx2.2
β-Cell
ngn3
neuroD1
Brn4
α-Cell
HB9
PDX1
Progenitor
Duct
HNF1β
HNF3β
HNF4α
HNF6
p48
Exocrine

Figure 5. Mechanism of glucose induced insulin secretion. Glucose enters the β cell and metabolized which causes an increase in the ATP/ADP ratio. The ATP-dependent K^+ channels close, which causes an influx of Ca^{2+} through the voltage-gated Ca^{2+} channel. The increase in intracellular calcium promotes exocytosis of insulin granules (insulin secretion).

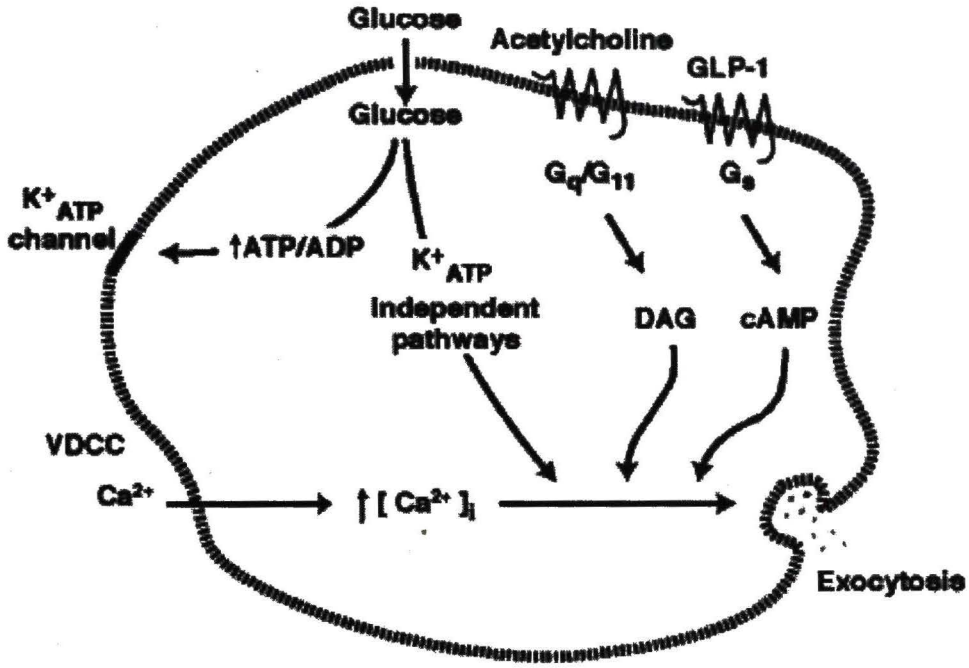

Glucose
Acetylcholine
GLP-1
Glucose
K^+_{ATP} channel
↑ATP/ADP
K^+_{ATP} Independent pathways
G_q/G_{11}
G_s
DAG
cAMP
VDCC
Ca^{2+}
↑ [Ca^{2+}]$_i$
Exocytosis

Figure 6. Structure of human insulin. Amino acid residues different in bovine insulin are shown in black and in porcine insulin in gray. [31]

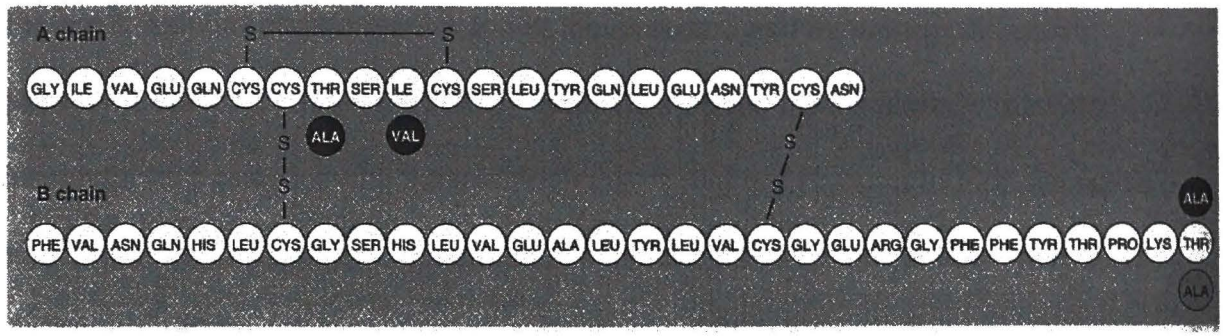

A chain
S
S
GLY ILE VAL GLU GLN CYS CYS THR SER ILE CYS SER LEU TYR GLN LEU GLU ASN TYR CYS ASN
ALA
VAL
S
S
S
S
B chain
ALA
PHE VAL ASN GLN HIS LEU CYS GLY SER HIS LEU VAL GLU ALA LEU TYR LEU VAL CYS GLY GLU ARG GLY PHE PHE TYR THR PRO LYS THR
ALA

Figure 7. Insulin receptor signaling. The appropriate signaling through the insulin pathway is critical for the regulation of glucose levels and the avoidance of diabetes. Insulin forms a complex with the Insulin Receptor (IR) and B chains to form the active signaling complex. Through recruitment of adaptor molecules and the activation of RAS, the activated IR can cause transcriptional activation [88].

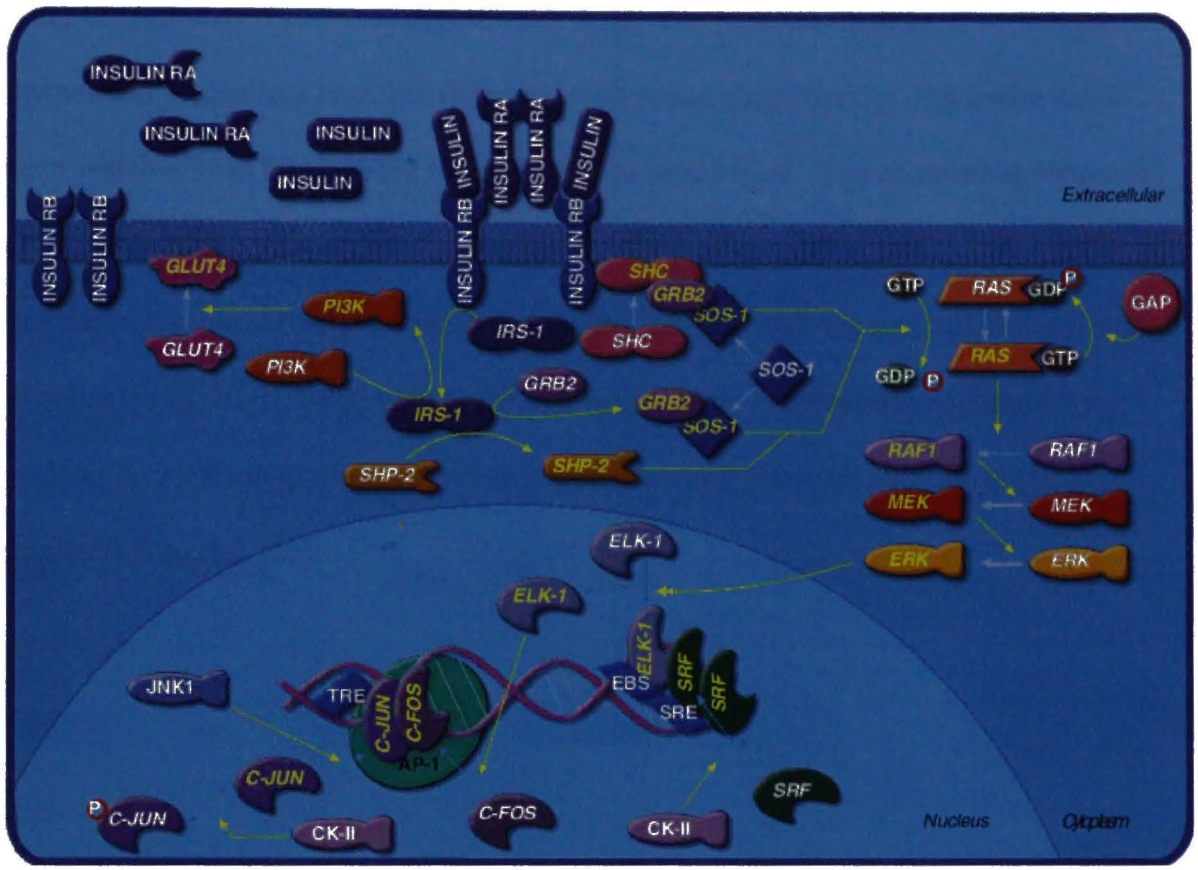

INSULIN RA
INSULIN RA
INSULIN
INSULIN
INSULIN RB
INSULIN RB
INSULIN RB INSULIN
INSULIN RA
INSULIN RA
INSULIN RB INSULIN
Extracellular
GLUT4
PI3K
GLUT4
PI3K
IRS-1
SHC
GRB2
SOS-1
GRB2
SOS-1
IRS-1
SHP-2
SHP-2
SHC
GRB2
SOS-1
GTP
RAS
GDP
P
GAP
GDP
P
RAS
GTP
RAF1
MEK
ERK
RAF1
MEK
ERK
ELK-1
ELK-1
ELK-1
SRF
SRF
EBS
SRE
JNK1
TRE
C-JUN
C-FOS
AP-1
C-JUN
P
C-JUN
CK-II
C-FOS
CK-II
SRF
Nucleus
Cytoplasm

Figure 8. Automated pancreas digestion system. The pancreas is infused with digestive enzymes and placed in a chamber (Ricordi) with seven glass marbles and a wire mesh screen which allows the digested tissue (islets) to be released. The chamber is connected to a shaker, which provides gentle agitation and the temperature is maintained at 37°C. Once the islets pass through the mesh, they are passed through the recirculation cylinder and collected in a flask filled with Hank's Solution (4°C). The system continuously circulates as islets are gradually freed from the digesting pancreas until the organ is completely digested [56].

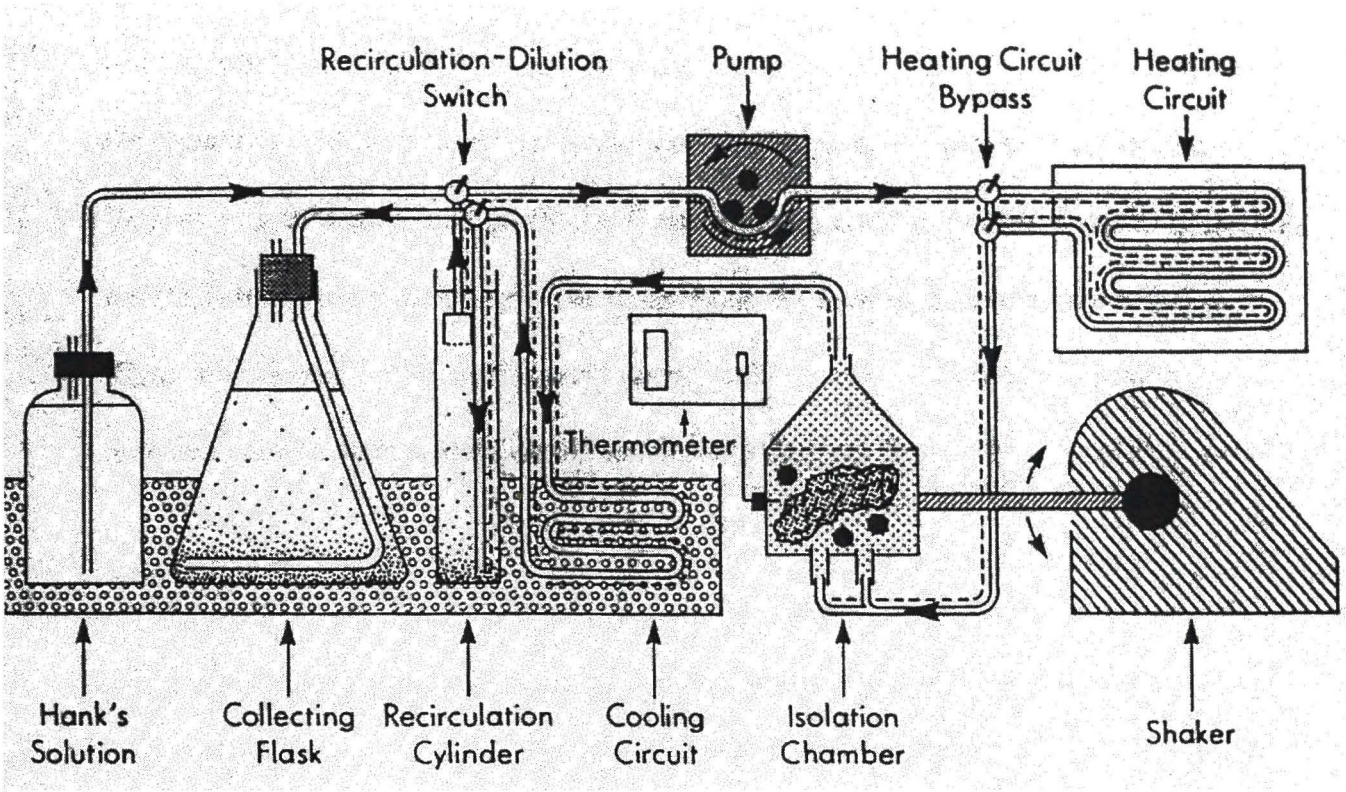
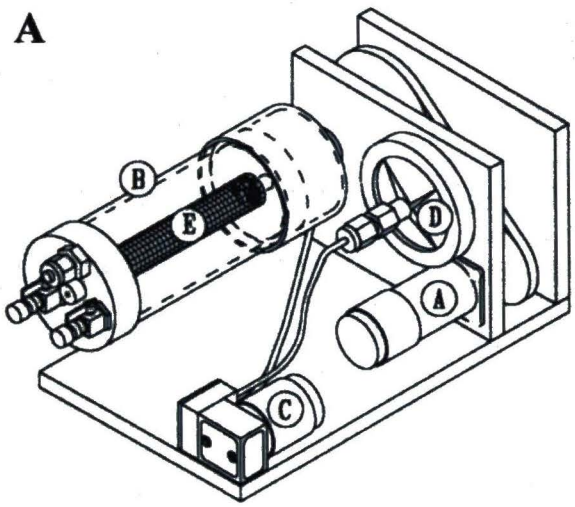

Recirculation-Dilution Switch
Pump
Heating Circuit Bypass
Heating Circuit
Thermometer
Hank's Solution
Collecting Flask
Recirculation Cylinder
Cooling Circuit
Isolation Chamber
Shaker

Figure 9. Rotating wall vessel cell culture system. *Left*: Schematic of the lateral turning rotating wall vessel (LTRWV). A 24-V direct current motor (A) drives a belt that rotates the cylindrical culture vessel (B) along its horozontal axis. An air pump (C) draws incubator air through a 0.22μm filter (D) and discharges it through a rotating coupling on the shaft that carriers the vessel. The oxygenator (E) is wrapped aroung the center post. *Right:* Photograph of the high aspect rotating wall vessel (HARWV) in operation. Selected vessel volumes and designs are shown. [86]

A



B

CHAPTER II

NORMAL ADULT ISLETS CONTAIN A POPULATION OF PROLIFERATIVE ENDOCRINE PRECURSOR CELLS THAT CAN BE EXPANDED *IN VITRO*

Introduction

One of the major limitations of successful islet transplant therapy for diabetic patients is the limited supply of pancreatic islets suitable for transplantation. Only about 3,000 donor pancreata are available per year in the United States, whereas about 35,000 new cases of IDDM are diagnosed each year [89]. Furthermore, a successful islet transplant requires 2-4 donors per recipient [51]. The rate of proliferation of all islet cells, including β cells, slows down by postnatal days 3 and 4 and continues to decline thereafter [14]. The rate of mitosis in adult pancreatic β cells is approximately 3% per day. β cells have a life span of approximately 50 days, after which they undergo apoptosis [90]. β-cell replacement is considered to occur through proliferation of islet β cells and also through neogenesis from precursor cells [91]. As a result of the limited capacity of β cells to proliferate *in vitro* and the gross tissue shortage, several alternative sources of pancreatic β cells for transplantation are currently the focus of many studies.

Embryonic stem (ES) cells have been considered as a source of β cells based on their proliferative capabilities and differentiation potential. Although ES cells rapidly

proliferate *in vitro*, it has been found that undifferentiated or partially differentiated ES cell grafts often formed tumors when implanted *in vivo* [92]. Adult duct cells also have been considered as a potential β cell source based on the hypothesis that new β cells arise from budding pancreatic duct cells [89, 93]. One method of isolating β cell progenitors from pancreatic ducts is based on very delicate surgical technique. Because of the exposure to tissue digestive enzymes, isolating them from whole pancreas digestion is not optimal. Since in adult organism the islets of Langerhans are no longer connected to the duct epithelium, it is unlikely that the islet cellular homeostasis is exclusively maintained by these ductal cells. Similarly, because all the embryonic signals are no longer operative it is unlikely that islets are budding off the ductules. Instead, in adult organisms, and in similarity to a number of adult tissues, there must be a proliferative compartment within the islets that supports the age or function related cell attrition. It has been shown that in adult mice when β cells are destroyed by treatment with streptozotocin, new β cells develop within the islets [94]. In addition, it has been shown that the presence of pancreatic progenitor cells within the islets is based on the pattern of expression of Neurogenin-3, a transcription factor expressed during prenatal life in pancreatic endocrine progenitor cells [95]. The presence of neurogenin-3-positive cells in the islets of the adult pancreas strongly suggests that endocrine precursors exist inside the islet. However, these intraislet progenitors have not been characterized extensively, which is the purpose of this study.

Islet isolation is a critical step in studying intraislet progenitor cells. Advances in the technique of islet isolation since the development of the automated Ricordi method

[56], has improved purity, yield and viability of isolated islets. Briefly, the Ricordi method is a continuous, controlled automated digestion approach during which the pancreas is placed in a dissociation chamber with steel spheres and is agitated in the presence of collagenase at 37°C. The dissociation solution is circulated while a fine mesh screen allows islets to be released and large tissue pieces to remain in the chamber. Islets can then be further purified by density gradient centrifugation. Based on the premise of the Ricordi method, a manual islet isolation procedure was designed to isolate islets from canine pancreata in the same way, without the use of the automated system.

Materials and Methods

Islet Acquisition: Isolated islets from porcine and human origin were acquired through a tissue procurement organization through Washington University, St.Louis, MO. Isolated rat islets were obtained in house from Dr. Richard Easom. Canine islets were isolated from pancreata that were donated by the laboratories of Dr. James Caffrey and Dr. Robert Mallet.

Islet Isolation: Canine pancreas is excised leaving a portion of the duodenum attached to the pancreas. The pancreas is cannulated via the main pancreatic duct through the papilla inside the duodenum with a 22G catheter and infused with 200 ml Liberase (0.20 WU/ml) (Roche) at 37°C. Depending on the anatomy of the animal, cannulation of the accessory duct is also cannulated and enzyme is infused. The openings of the ducts are tied off with suture material. The organ is manually, continuously rocked in a 500ml Erlenmeyer flask in a 37°C water bath throughout digestion. After 10 minutes, the digest

is passed through a 40μm sieve into a beaker for collection and transferred to a 50 ml centrifuge tube and washed with HBSS+10% FBS, while the undigested material continues to incubate. Every 10 minutes of incubation, the digest is passed through the sieve allowing islets that are freed from the tissue to be washed and collected. This process is repeated 4-5 times, until the entire pancreas is digested. After repeated washes and centrifugation, the pelleted material was suspended in 16ml 27% Ficoll (Sigma) in 50ml tubes (up to 2.5ml in each tube). The islets were separated by overlaying 23% (8ml), 20% (8ml), and 11% (8ml) Ficoll gradient and centrifugation. Islets were recovered from between 11% and 20% layers. Islets were washed free of Ficoll and transferred into RPMI medium. Isolated islets are identified using dithizone (DTZ) (Sigma) staining and counted. DTZ positive islets can also be handpicked from the digest, thereby eliminating the need for Ficoll.

Cell Culture Conditions: Isolated islets (1000-2000 islets) were placed in tissue culture flasks coated with FNC Coating Mix (BRFF) and allowed to attach. Confluent colonies epithelial cells were obtained within 7-10 days. Cells were passaged using Trypsin-EDTA (Gibco) and Trypsin activity was inactivated using Trypsin Inhibitor (Sigma). Culture medium used was KGM (a serum free defined medium containing low calcium (0.05mM), and the presence of bovine pituitary extract (BPE) (30μg/ml) for optimal attachment, epidermal growth factor (EGF) (5ng/ml), Insulin (5μg/ml), hydrocortisone (HC) (500ng/ml), Gentamycin (50μg/ml) and Amphoteracin (50ng/ml) or EpiLife (Clonetics). Medium was changed every other day. Canine islets required RPMI 1640 tissue culture medium (11.1mmol/L glucose, pH 7.4) supplemented with 10% fetal

bovine serum (Tissue Culture Biologicals), 0.1% pyruvate, 1.8 µl 2-mercaptoethanol, 1% Pen-Strep, 0.1% Gentamycin for optimal growth.

Indirect Immunofluorescence: Endocrine epithelial cells isolated from canine islets are cultured on glass coverslips. Once fixed with Methanol:Acetone (1:1) at 4°C, the cells are blocked with 1% BSA in PBS. Primary antibody (insulin, 1:100; glucagon, 1:100; somatostatin, 1:100; PDX-1, 1:500: cytokeratin 19, 1:100; β–tubulin, 1:200; glut2, 1:100; glucokinase, 1:100; FGFR, 1:100; EGFR, 1:200; VEGFR 1:100; and nestin 1:200) is diluted in 1%BSA in PBS and is incubated on the cells at 4°C overnight. Following multiple washes with 0.1% Tween 20 in 1X PBS, the cells are incubated with secondary antibody (AlexaFlour, Molecular Probes) diluted 1:1000 in 1% BSA in PBS for 1 hr at 37°C. The slides are washed thoroughly and incubated with DAPI (300nM) for 20 min at 37°C. Coverslips are mounted on slides using FluorSave (Calbiochem) and examined by fluorescence microscopy. Results are recorded photographically or digitally.

Western Blot Analysis: NeoIslets, endocrine epithelial cells and normal islets are lysed using a western extract buffer and protein concentration is determined using BCA assay. (Pierce) Proteins are run on 4-12% NuPage Novex gels (Invitrogen) at 200 V for 90 min, transferred at 30V for 60 min. Membranes are blocked at 4°C using PBS/5%Milk/1%BSA membranes were probed using insulin, 1:1000; glucagon, 1:1000; PDX-1, 1:5000; glut 2, 1:2000: glucokinase, 1:2000; FGFR, 1:100; EGFR, 1:200; VEGFR, 1:100 and β-tubulin, 1:500 antibodies. Membranes are incubated in primary antibodies at 4° C overnight and secondary antibody at room temperature for 60 min.

Blots are visualized by using an Extra-Sensitive Chemiluminescence (ECL) Kit (Amersham), exposed to film and developed.

Proliferation Studies: Proliferation is determined by the SRB (Sulforhodamine B) assay. INS-1 cells or endocrine epithelial cells are seeded in a 96 well plate at 5,000 cells per well. Cells are exposed to different conditions for 5-7 days (media changed every other day) and fixed with 10% TCA. Cells are then stained with SRB dissolved in 1% acetic acid and washed with 1% acetic acid. Plates are dried overnight. Prior to reading, 10mM Tris base was added per well to solubilize the dye. The plates were mixed for 5 minutes on a plate shaker and read at 564 nm on a microplate reader.

Results

Isolated islets were placed in a tissue culture flask coated with FNC to promote their attachment at a density of 1000-2000 islets/25cm^2 flask. When place in a specific environment which promotes epithelial cell mitosis, endocrine epithelial cells (EECs) were derived from adult islets. These conditions feature a serum free defined medium containing low calcium (0.05mM), and the presence of bovine pituitary extract (BPE) (30μg/ml) for optimal attachment, epidermal growth factor (EGF) (5ng/ml), Insulin (5μg/ml), hydrocortisone (HC) (500ng/ml), gentamycin (50μg/ml) and amphoteracin (50ng/ml). Several species including rat, porcine, human and canine islets all produced confluent monolayers of EECs in the same way (**Fig. 1**). However, for optimal growth, canine EECs required RPMI 1640+10%FBS, the classical proliferation conditions for

most β cell lines. Cultures of EECs can be propagated by subculture and can be sustained *in vitro* for several months.

Although EECs are derived from islets, their function is unclear. Extensive characterization of EECs is essential in establishing their role as putative islet progenitor cells. Immunofluorescence was used to determine the presence of proteins such as insulin, glucagon and somatostatin, which are characteristic of β, α and δ cells, respectively. EECs expressed insulin, glucagon and somatostatin, which are shown in **Fig 2**. Coexpression of insulin and glucagon is shown in **Fig 3**. Although EECs possess classical epithelial cell morphology, the presence of cytokeratin 19, an early epithelial marker, was confirmed. The expression of cytokeratin 19 was also shown in EECs (**Fig 3**).

Nestin is an intermediate filament expressed in neuroepithelial stem cells [96]. The expression of nestin in islet progenitor cells is somewhat controversial. It has been reported that *in vitro*, insulin-positive cells could be generated from nestin-positive cells located within rodent and human islets [60, 67, 93]. In contrast, it has recently been reported that during development, nestin is detected in mesenchymal cells and not in the epithelial cells that give rise to β cells [97]. In adult rat pancreas nestin expression was not detected in duct cells which are proposed islet cell progenitors, nor in a model of pancreas regeneration [98]. To clarify the controversy, we examined the presence of nestin by immunofluorescence and found its expression present in our endocrine epithelial cells (**Fig 4**).

Because the function of endocrine cells is primarily secretory, progenitor cells should have the necessary machinery to package and secrete endocrine hormones. For example, β-tubulin, the essential component of microtubules found in all eukaryotic cells, is required for vesicular transport of secretory cells. The lack of β-tubulin in EECs would compromise their prospective secretory function. Therefore, we examined the presence of β-tubulin in EECs and confirmed its presence via immunofluorescence and Western analysis (**Fig 5**).

The islet transcription factor, PDX-1 plays a key role in pancreatic development as well as in regulation of transcription of islet genes such as GLUT 2 and glucokinase. Lack of PDX-1 leads to pancreatic agenesis whereas mutations of PDX-1 lead to abnormalities in islet function and diabetes in humans and mice [16, 99, 100]. Adenovirus-mediated uptake of PDX-1 into liver cells *in vivo* can induce a β cell-like phenotype that produces sufficient insulin to restore normoglycemia in streptozotocin-induced diabetic rats [65]. β cell precursors must express PDX-1 to differentiate into functional β cells. The expression of PDX-1 is shown by western blot in porcine and canine EECs and immunofluorescence in canine EECs (**Fig 6**).

In the β cell, glucose enters the cell by facilitated transport via the glucose transporter, GLUT 2. Glucose can be further metabolized by the cell due to the phosphorylation of glucose (glucose 6-phosphate) by glucokinase. As β-cell progenitors mature, the issue of glucose-dependent insulin secretion becomes the critical function. These cells must be able to sense glucose and further respond by secreting insulin. GLUT 2 and glucokinase are two necessary components of the glucose-sensing insulin

secretion pathway. The presence of GLUT 2 and glucokinase was examined and shown to be present in canine EECs by immunofluorescence and western analysis (**Fig 7**).

FGF2 has been shown to have a mitogenic effect on pancreatic epithelial cells *in vivo* [72]. Therefore, the presence of the FGFR in EECs would be essential in responding to FGF-induced proliferation. The expression of FGFR was examined in EECs by immunofluorescence and western blot (**Fig 8**).

Discussion

The purpose of this study was to characterize the endocrine epithelial cells derived from adult islets of several species including rat, porcine, human and canine. Our preliminary studies began with rat islets. We continued our studies with porcine and human islets, which became available through a tissue distribution network. However, the tissue supply was unreliable and too sporadic to be of practical use. We turned our attention to canine islets for several reasons: 1) Canine tissue was available to us on a regular basis by donation from the investigators in the Integrative Physiology department 2) Canine islets have not been extensively studied as a source of islet progenitor cells 3) Dogs develop IDDM spontaneously as humans or diabetes can be induced chemically 4) Dr. Tune, an investigator in the department of Integrative Physiology used a diabetic dog model (alloxan induced) in his cardiovascular studies which could be particularly appropriate in the event *in vivo* studies.

In order to obtain intraislet progenitor cells, or endocrine epithelial cells we first had to optimize our islet isolation protocol. The foundation of our manual canine islet

isolation protocol was based on the premise of the automated digestion system designed by Ricordi [56]. Islets were recovered as they were gradually freed from the digesting organ so as to prevent further degradation of the fragile microorgans. Several factors in islet isolation must be optimized to yield good quality islets. These include temperature, enzyme concentration, time of digestion and inhibition of the enzyme. Enzyme concentration and the time of digestion are critical in establishing the fine line between over-digestion and under-digestion of the pancreas. In addition, complications such as anatomical differences within the species can lead to variations in the protocol. Most of these challenges were overcome and we were able to obtain thousands of good quality islets for further experimentation. However, islet yield continued to vary between islet preparations. Automated digestion systems are expensive and difficult to obtain. However, some degree of automation, would decrease variability of islet yields between preparations.

Most studies in the area of islet cell progenitors have been done on pancreatic duct cells, based on the hypothesis that islet cells are derived from duct cells during organogenesis. Duct cells are an unlikely source of islet progenitors in the adult animal due to the fact that although islets are no longer in contact with duct cells, there is still a turnover of islet cells. Therefore, there must be an internal compartment of progenitor cells within the islet that supports the replacement of islet cells. The use of intraislet progenitor cells may be a source of putative β cells that can address the issue of the shortage of transplantable islets. We have shown that we can obtain proliferative endocrine epithelial cells from normal adult islets in several species. The in-depth

characterization studies have defined these endocrine epithelial cells as β cell progenitors. Although EECs have been shown to express PDX-1, it would be useful to examine other transcription factors that are expressed during different stages of development. For example, Pax 6 and Nkx6.1 are present in progenitors later in the differentiation process of β cells. These factors are excellent markers in the identification of progenitor cells, however the external signals that control their expression must be identified in order to differentiate progenitor cells *in vitro*.

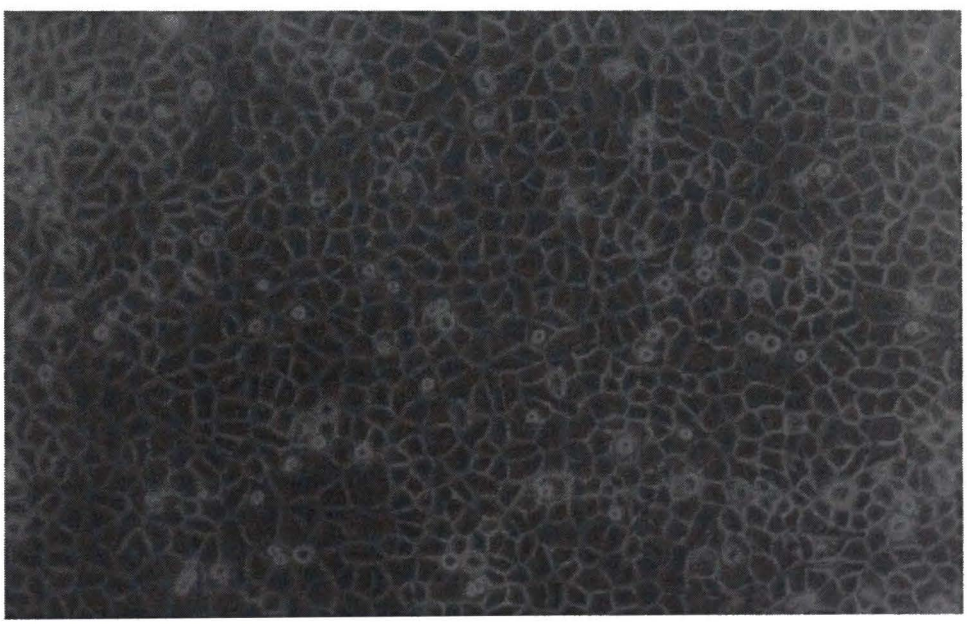
β cell progenitors should have the capability to express markers of differentiated β cells. These studies have shown that EECs express β cell markers such as insulin, Glut 2, and glucokinase, which are all essential in the proper function of β cells. However, the coexpression of islet hormones as well as the expression of nestin demonstrates their immaturity, or progenitor status. Although the expression of nestin in islet progenitor cells has been somewhat controversial, these studies definitively show nestin expression in EECs isolated from adult islets and is therefore identified as a marker for endocrine progenitor cells.

These findings are a positive beginning to what may be the solution to the lack of material suitable for transplantation in diabetic patients. If adult islets can provide progenitor cells, the culture techniques discussed here could give rise to an unlimited supply of β cell progenitors that can be further differentiated *in vitro* and used for transplantation *in vivo*.

Figure 1. Endocrine epithelial cell monolayer. Endocrine epithelial cells derived from porcine (A) and human (B) adult islets. Isolated islets (1000-2000 islets) were placed in tissue culture flasks coated with FNC Coating Mix (BRFF) and allowed to attach. Cells were cultured in KGM of EpiLife (human) and RPMI (canine). Confluent colonies of epithelial cells were obtained within 7-10 days.

A.

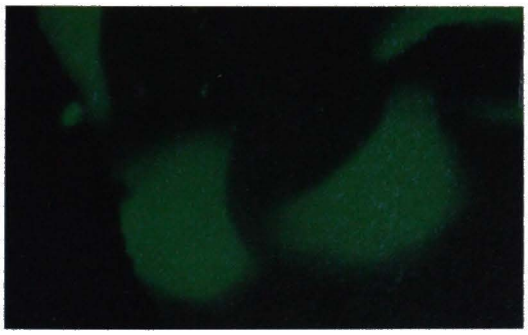
B.



A.

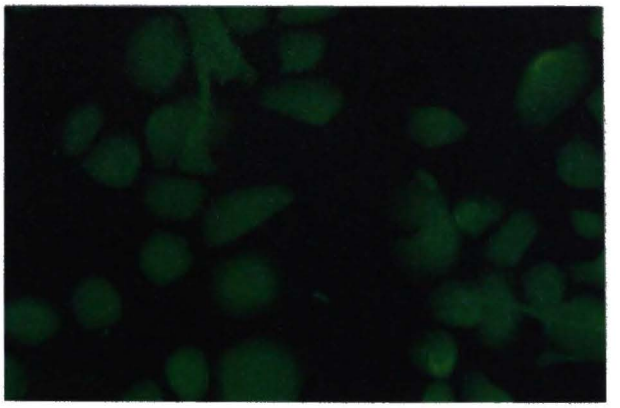
B.

C.



D.

E.



F.

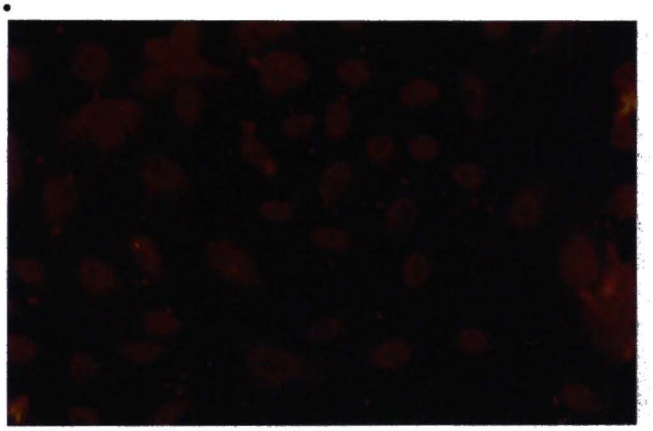
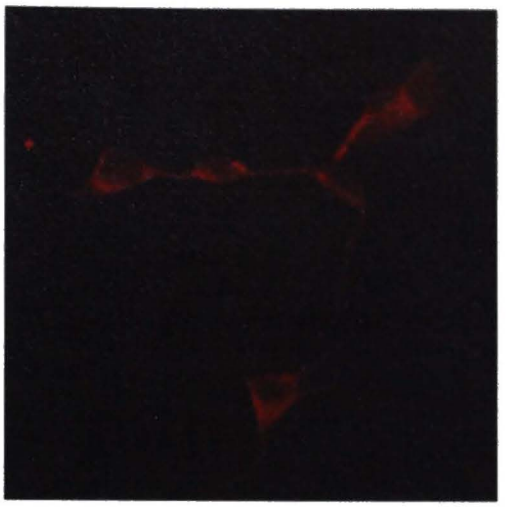
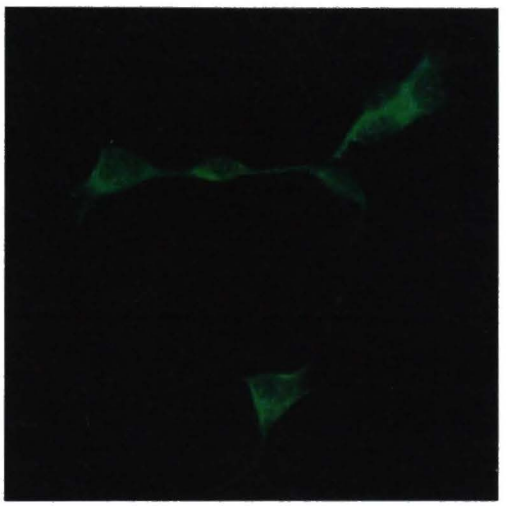


Figure 3. Coexpression of cytokeratin 19 (A) and insulin (B) in canine endocrine epithelial cells. Nuclei are stained with dapi (C). Canine EECs were cultured on coverslips, fixed and probed for cytokeratin 19 (mouse) and insulin (rabbit) antibodies. These proteins were labeled with anti-mouse (red) and anti-rabbit (green) fluorescent secondary antibodies.

A.



B.



C.

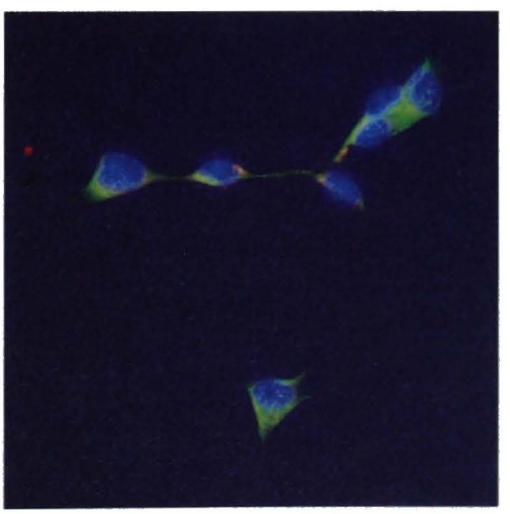


Figure 4. Nestin expression in canine endocrine epithelial cells shown by immunofluorescence. Canine EECs were cultured on coverslips, fixed and probed for nestin using an anti-nestin (rabbit) antibody. Nestin was labeled using a fluorescent anti-rabbit secondary antibody (red).

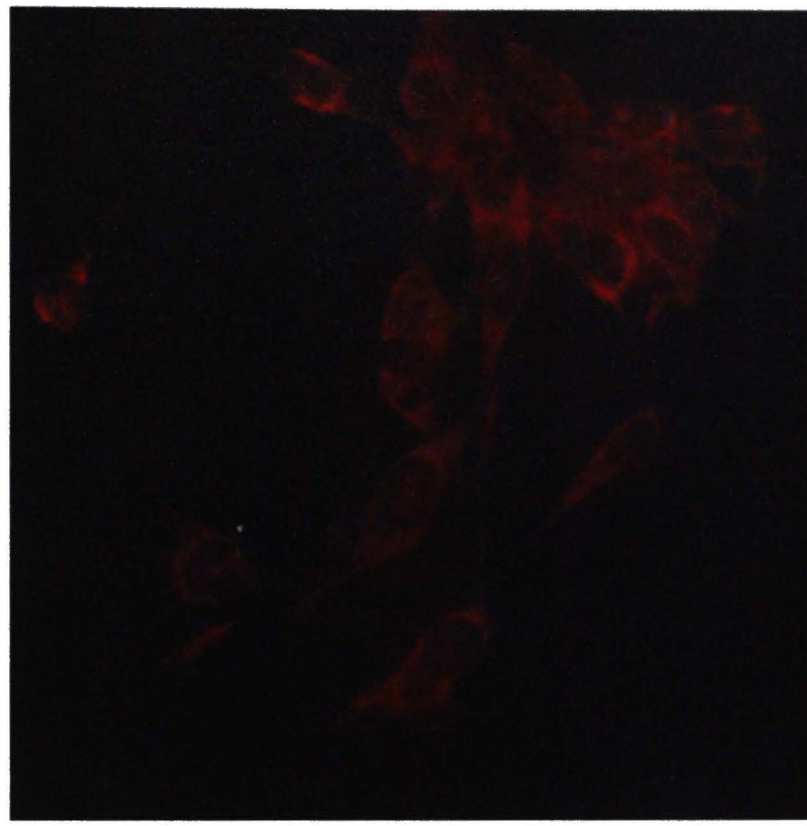
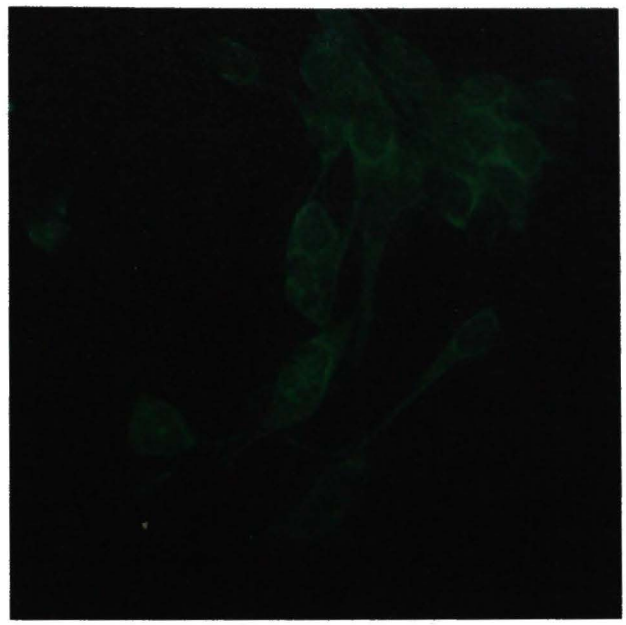


Figure 5. β-tubulin expression in EECs shown by immunofluorescence (canine) (A) and western analysis (porcine and canine) (B). EECs were cultured on coverslips, fixed and probed for β-tubulin using anti-β-tubulin antibody (mouse). β-tubulin was labeled using a fluorescent anti-mouse antibody (green) (A). Porcine and canine EECs were lysed and proteins were separated on a gel by electrophoresis, transferred to membrane and probed using anti-β-tubulin antibody (mouse). Membranes were incubated with anti-mouse secondary antibody and the presence of β-tubulin was visualized by chemiluminescence (B).

A.



β-tubulin

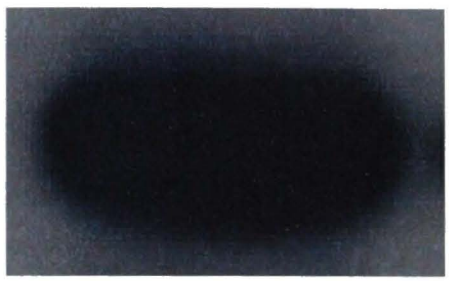
55kd →

porcine

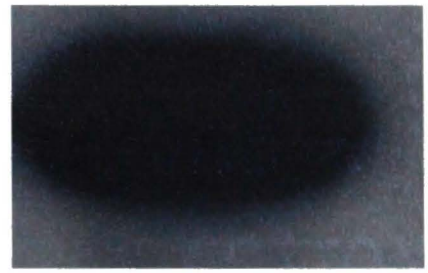
canine

Figure 6. Expression of PDX-1 in porcine (A) and canine (B) EECs shown by western blot and immunofluorescence (canine) (C). Canine EECs were cultured on coverslips, fixed and probed for PDX-1 using anti-PDX-1 antibody (rabbit). PDX-1 was labeled using a fluorescent anti-rabbit antibody (green) (C). Porcine and canine EECs were lysed and proteins were separated on a gel by electrophoresis, transferred to a membrane and probed for PDX-1 using anti-PDX-1 antibody (rabbit). Membranes were incubated with anti-rabbit secondary antibody and the presence of PDX-1 was visualized by chemiluminescence (A and B).

A. Porcine



B. Canine



←43kd

C. PDX-1

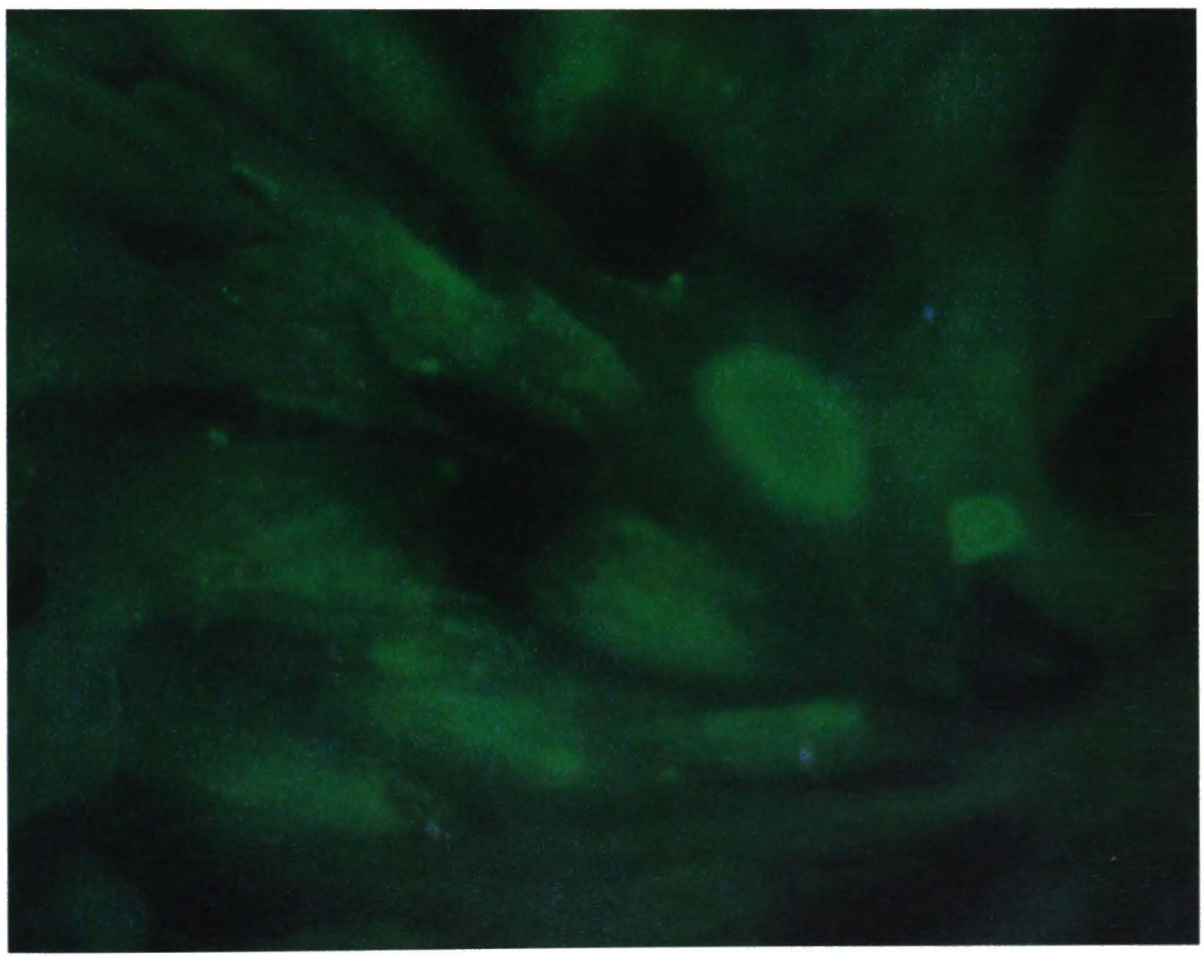
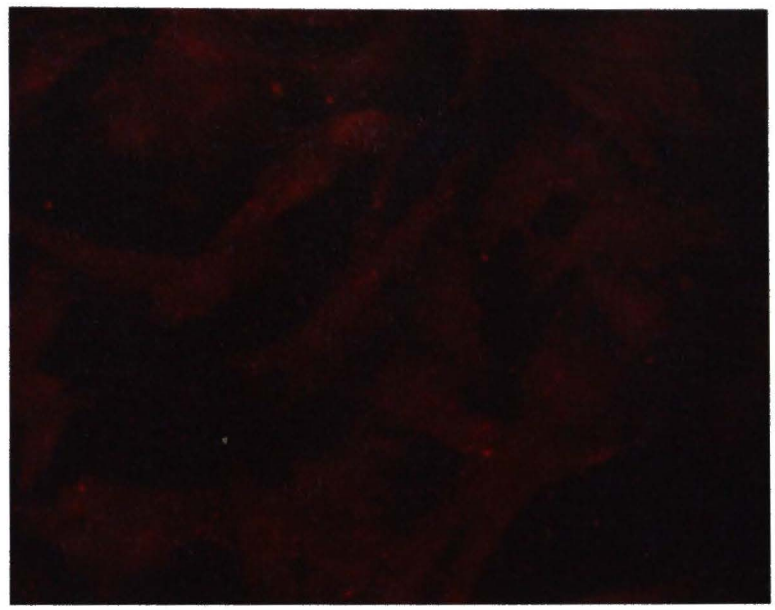


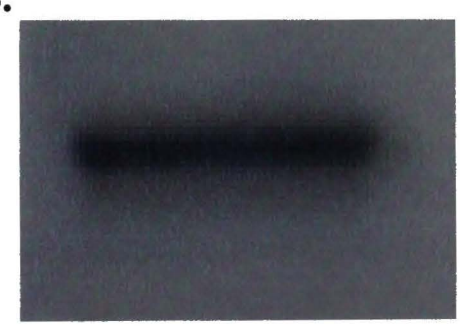
Figure 7. Expression of glucokinase by immunofluorescence (A) and GLUT 2 (B) by western in canine EECs. Canine EECs were cultured on coverslips, fixed and probed for glucokinase using anti-glucokinase antibody (mouse). Glucokinase was labeled using a fluorescent anti-mouse antibody (red) (A). Canine EECs were lysed and proteins were separated on a gel by electrophoresis, transferred to a membrane and probed for GLUT 2 using anti-GLUT 2 antibody (rabbit). Membranes were incubated with anti-rabbit secondary antibody and the presence of GLUT 2 was visualized by chemiluminescence (B).

A.

Glucokinase



B.

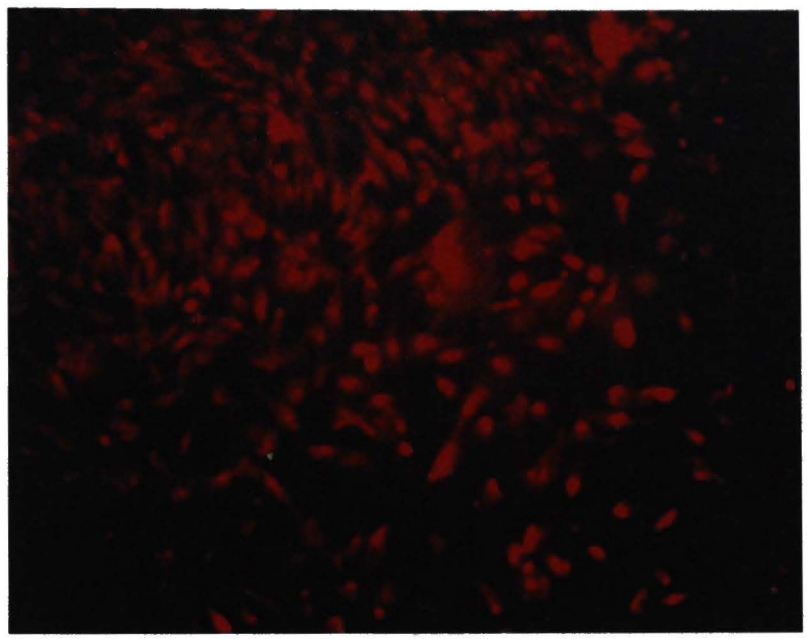


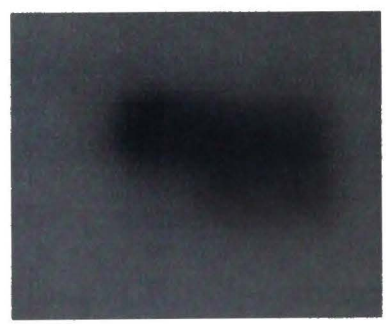
←60kd

GLUT 2

Figure 8. Expression of FGFR in canine endocrine epithelial cells by immunofluorescence (A) and western blot (B). Canine EECs were cultured on coverslips, fixed and probed for FGFR with anti-FGFR antibody (mouse). FGFR was labeled using a fluorescent anti-mouse antibody (red) (A). Canine EECs were lysed and proteins were separated using gel electrophoresis, transferred to a membrane and probed for FGFR using anti-FGFR antibody (mouse). Membranes were incubated with anti-mouse secondary antibody and FGFR was visualized by chemiluminescence (B).

A.





← 55kd
FGFR

CHAPTER III

THE *IN VITRO* MECHANISMS OF AGGREGATION OF ENDOCRINE EPITHELIAL CELLS INTO NEOISLETS

Introduction

The endocrine cells of the pancreas are highly organized into individual spherical structures with the β cells occupying the center of the sphere, while the α, δ, and pp cells reside in the periphery (Chapter I, Fig.1). This organization allows for the communication between different cell types thereby maintaining tight regulation of blood glucose. In the mouse, small clusters of endocrine cells are found forming around pancreatic ducts at embryonic day (E) 14.5. The definitive islets are formed late in gestation at E 18. Although some information has been derived about the mechanisms of aggregation *in vivo*, the mechanism of aggregation, of endocrine epithelial cells (EECs) *in vitro,* specifically the role of FGF, remains unclear.

Acidic FGF (FGF-1) and basic FGF (FGF-2) are the prototypic FGF members and have been implicated in pancreatic islet embryogenesis [71]. FGFR1 and 2 are expressed during pancreatic development in the rat, and it has been shown that FGF-2 induces proliferation of pancreatic epithelial cells during embryogenesis [72]. During embryogenesis in mice, FGFs are widely expressed in the region of the mesenchyme surrounding pancreatic precursor cells, and are proposed to be involved in the early

stages of development of the endocrine pancreas [74]. Some studies suggest that signals arising from endocrine pancreatic precursor cells themselves may be sufficient to promote cell clustering and differentiation during the formation of islets. For example, Hardikar, et al. [75] showed that human pancreatic PANC-1 cells, as well as adult-derived precursor cells (hIPCs), form hormone expressing islet-like cell aggregates when exposed to serum-free media [75]. Of several growth factors tested, FGF-2 was found to be the most effective chemoattractant in the aggregation of PANC-1 cells. Expression analysis of FGF-2 and its receptor suggests that FGF-2 signaling may also participate in the aggregation of hIPCs. This suggests that the source of FGF-2 is not only from the surrounding mesenchyme, but is also secreted by endocrine precursor cells. This autocrine influence of FGF2 may be an important factor in the differentiation of pancreatic precursor cells to islets [75].

Endocrine cell-cell contacts, specifically through the cadherin-catenin complex, have been shown to be critical in the development and the function of the well-organized islet. Cadherins constitute a superfamily of transmembrane proteins, which mediate Ca^{2+}-dependent cell-cell interactions that are involved in many morphogenetic processes during tissue development. Cadherins are structurally highly homologous with a Ca2+-binding extracellular domain consisting of five subdomains, a single transmembrane domain, and a highly conserved cytoplasmic domain [76] [77]. The cytoplasmic domain of cadherins interacts with cytoplasmic components of α-, β-, and γ-catenin which mediate the linkage to the actin filaments [78]. The density of cadherin-catenin complexes varies depending on cell type and differentiation state of cells and is believed

to be modulated during changes in the developmental stage of cells [79]. In addition to a function in adherens junction formation, the cadherin-catenin complex is thought to transmit signals from neighboring cells through interactions with growth factor receptors [79]. For example, it has been shown that tyrosine phosphorylation of catenins occurs in cells treated with EGF and HGF [81]. However, the role of FGF as an initiator of the aggregation of EECs has not yet been defined. Studies with rat islets have shown that E-cadherin mediates most of the Ca^{2+}-adhesion between all cell-types in the islets [76]. The role of E-cadherin in the regulation of morphogenesis of the islets during pancreatic organogenesis was elucidated in studies by Dahl, et al. [76]. Transgenic mice carrying an E-cadherin cDNA lacking most of its extracellular domain, whose expression is driven by the rat insulin promoter 1 (Rip1), showed disrupted cadherin activity in β cells during pancreatic ontogeny. The initial clustering of β cells, which begins at E 13.5-14.5 in normal rats, was disrupted. The aggregation of endocrine cells into islet-like structures which normally begins at E 17.5-18 was inhibited in these transgenic animals [76].

Aggregation studies have been performed *in vitro* under classical two-dimensional tissue culture conditions. Traditional two-dimensional cell culture conditions provide an environment in which differentiated cells lose their specialized features and dedifferentiate [86]. Suspension culture is a popular environment that prevents dedifferentiation and maintains specialized features of differentiated cells. The rotating-wall vessel (RWV) is a suspension culture system optimized to produce laminar flow and eliminate the shear stress on cell aggregates in culture (Chapter I, Fig 10.) because cells are completely immersed in liquid with no air space. A silicon membrane

allows gas exchange (oxygen and carbon dioxide) but prevents loss of liquid. Furthermore, the RWV allows for three-dimensional spatial freedom, the delivery of nutrients and oxygenation by diffusion [86]. For example, human hepatocytes cultured in the RWV remained viable for 60 days and aggregated into tissue-like structures [86, 87]. The RWV is a unique and efficient method in cell aggregation studies, particularly in the investigation of the aggregation of EECs into neoislets *in vitro*.

Several β cell lines, such as INS-1 have been used as a model to study β cell physiology, due to several factors including: a limited availability of pancreatic endocrine tissue, the difficulty of preparing a sufficient quanitity of viable islets, cellular heterogeneity and the rapid decline of insulin production *in vitro* [82, 83]. The INS-1 cell line is a radiation-induced rat insulinoma, developed by Asfari et al. [84, 85]. INS-1 cells are well characterized and exhibit insulin gene transcription, preproinsulin biosynthesis, proinsulin processing and regulated insulin exocytosis in response to the metabolism of D-glucose. However, these cells have a diminished response to glucose, specifically at higher passages. In addition, INS-1 cells have been shown to synthesize small quantities of proglucagon, glucagon and GLP-1 [85]. In our studies, Ins-1 cells will be used as a cellular model of β cell aggregation.

The aggregation of EECs into neoislets for transplantation must be accomplished *in vitro* for several reasons: 1) The islet is the optimal glycemic control unit, 2) complete maturation (angiogenesis and innervation) of the neoislet is facilitated when implanted *in vivo*, 3) single cells are more fragile and therefore, more vulnerable to immune

destruction than islets and 4) the number of neoislets (based on the amount of insulin secreted per islet) implanted can be applied to each patient, depending on body weight.

Materials and Methods

Cell Culture Conditions: Endocrine epithelial cells were cultured in MGM Clonetics) to promote aggregation of EECs into neoislets. The components of this medium are BPE (15μg/ml), PMA (50ng/ml), FGF2 (5ng/ml), FBS (0.5%), Insulin (5μg/ml), Calcium (1.5mM), Gentamycin (50μg/ml) Amphoteracin (50ng/ml). N2 Medium (DMEM:Hams F-12; 3:1) + PenStrep, Amph B (1:100), N2 Supplement (1:100) FGF2 (20ng/ml), EGF (20ng/ml) and Heparin (5mg/ml) was also used to promote aggregation. Neoislets formed under these aggregation conditions can be cultured in another flask and give rise to EECs under proliferative conditions. Ins-1 cells were cultured in RPMI 1640 (11.1mmol/L glucose, pH 7.4) supplemented with 10% fetal bovine serum, 0.1% pyruvate, 1.8 μl 2-mercaptoethanol, 1% Pen-Strep, 0.1% Gentamycin.

Proliferation Studies: Proliferation is determined by the SRB (Sulforhodamine B) assay. INS-1 cells or endocrine epithelial cells are seeded in a 96 well plate at 5,000 cells per well. Cells are exposed to different conditions for 5-7 days (media changed every other day) and fixed with 10% TCA. Cells are then stained with SRB dissolved in 1% acetic acid and washed with 1% acetic acid. Plates are dried overnight. Prior to reading, 10mM Tris base was added per well to solubilize the dye. The plates were mixed for 5 minutes on a plate shaker and read at 564 nm on a plate reader.

Indirect Immunofluorescence: Neoislets were fixed in a borosilicate glass culture tube with Methanol:Acetone (1:1) at 4°C. Neoislets are then cells are blocked with 1% BSA in PBS. Primary antibody (insulin, 1:100; glucagon, 1:100; somatostatin, 1:100; PDX-1, 1:500: cytokeratin 19, 1:100; glut2, 1:100; glucokinase, 1:100; FGFR, 1:100; EGFR, 1:200; VEGFR 1:100; and nestin 1:200) is diluted in 1%BSA in PBS and is incubated with the neoislets at 4°C overnight. Following multiple washes with 0.1% Tween in PBS, the neoislets are incubated with secondary antibody (AlexaFlour, Molecular Probes) diluted in 1% BSA in PBS for 1 hr at 37°C. The neoislets are washed thoroughly and incubated with DAPI (300nM) for 20 min at 37°C. Neoislets are mounted on depression slides so as not to alter their architecture using FluorSave (Molecular Probes) and examined by fluorescence microscopy.

Western blot analysis: NeoIslets and normal islets were lysed using a western extract buffer and protein concentration is determined using BCA assay (Pierce). Fifteen (15) μg of each sample was run on 4-12% NuPage Novex gels (Invitrogen) at 200 V for 90 min and transferred at 30V for 60 min. Membranes were blocked at 4°C using PBS/5%Milk/1%BSA overnight. Membranes were probed using PDX-1, 1:500; E-Cadherin, 1:2500; GLUT2, 1:200; glucokinase, 1:250; FGFR, 1:100; EGFR, 1:200; VEGFR 1:100; and β-tubulin 1:500; primary antibodies. Membranes are incubated in primary antibodies at 4° C overnight and secondary antibody at room temperature for 1 hr. Blots are visualized by using an Extra-Sensitive Chemiluminescence (ECL) Kit (Amersham), exposed to film and developed.

Aggregation of EECs into neoislets: EECs or INS-1 cells were seeded in a tissue culture flask at a cell density of $1x10^6$. Aggregates were counted manually under a dissecting microscope.

Aggregation of EECs into neoislets using a Rotating Wall Vessel (RWV) Cell Culture System: EECs or INS-1 cells were placed in the RWV and neoislets and single cells were counted after 24 hours in culture in the system.

Viability of Neoislets: Neoislets were tested for viability using Neutral Red staining. 1% Neutral Red was diluted 1:100 in a HBSS and incubated at 37°C for 10 minutes. Viable cells appear red under light microscopy do to the uptake of the dye.

Inhibition of Aggregation: EECs and INS-1 cells were cultured using the RWV in the presence of 1mM EGTA or 5uM Genistein (Sigma). Neoislets and single cells were counted after 24 hours, stained with neutral red and photographed under light microscopy. Ins-1 Cells ($5.0x10^6$) were incubated in 50μM and 100μM Bisindolymaleimide 1 (Calbiochem), or 10μM AG 957 (EMD Biosciences) in the presence of FGF2 (20ng/ml). Ins-1 cells were also treated with FGF2 (20ng/ml) + Chelerythrine chloride (1.0μM), FGF2 (20ng/ml) + U-73122 and FGF2 (20ng/ml)+ 5'-methylthioadenosine. After 24 hours neoislets and single cells were counted, assessed for viability with neutral red and some were stored at –20°C as pellets for immunoprecipitation.

Immunoprecipitation: INS-1 neoislets formed in the RWV were lysed and immunoprecipitated with anti-E-Cadherin antibody using Protein G beads (Sigma).

Samples were electrophoresed on a 12% Tris-Glycine gel and probed with anti-β-catenin and anti-FGFR antibodies.

Results

During the optimization of culture conditions for EECs derived from isolated islets, we examined the effect of MGM, a defined medium used for isolation and propagation of normal human melanocytes. The components of this medium are BPE (15μg/ml), PMA (50ng/ml), FGF2 (5ng/ml), FBS (0.5%), Insulin (5μg/ml), Calcium (1.5mM), Gentamycin (50μg/ml) and Amphoteracin (50ng/ml). This medium, when tested as a mitogenic environment for the ECCs was not very effective. However, when a near confluent culture of ECCs was incubated in this medium, spontaneous formation of spherical structures that eventually detached from the growth surface occurred (**Fig 1**). These neoislets could be generated in this environment even from nonconfluent cultures. Neoislets can be further expanded by using them to initiate a new culture of EECs, using a purely proliferative environment/medium such as KGM (Clonetics) or Epilife (Cascade). Using a 2-D culture method, EECs derived from normal adult islets can be aggregated into neoislets within 24 days (**Table 1**). We have shown, this cycle of native islets to EECs to neoislets to EECs can be maintained in culture for several years.

The spontaneous aggregation of EECs in the differentiating environment (MGM) could be attributed to the presence of high calcium (1.5mM), PMA, or FGF2. Due to the limited utility of PMA, based on its tumorigenicity, we examined the effects of FGF2 and

calcium on aggregation of EECs. A medium containing (DMEM:Hams F-12; 3:1) + PenStrep, Amph B (1:100), N2 Supplement (1:100) FGF2 (20ng/ml), EGF (20ng/ml) and Heparin (5mg/ml) was used as an alternative to MGM to promote aggregation of EECs.

Proliferation of canine EECs was examined using several concentrations of FGF2 (5, 10, 20ng/ml) with or without the presence of EGF (20ng/ml) using the SRB assay (**Fig 2**). The combination of FGF2 (20ng/ml) and EGF (20ng/ml) proved to be the most effective proliferative conditions. FGF2 (5ng/ml) in the presence of EGF showed an increased proliferative effect over FGF2 (10ng/ml) in the presence of EGF, yet not as high as FGF2 (20ng/ml) and EGF (20ng/ml). Among the concentrations of FGF2, without the presence of EGF, proliferation decreased as FGF2 concentrations increased. This is due to the differentiation effects of FGF2 at higher concentrations. The presence of EGF (20ng/ml) alone showed an equal amount of proliferation as when no growth factors were present.

Proliferation of Ins-1 cells and canine EECs was studied by treatment of the cells with different proliferative environments (growth factors) and evaluated with the SRB assay (**Fig 3**). The most proliferative environments for Ins-1 cells were complete RPMI + 10% FBS and Neural Progenitor Maintenance Medium (NPMM) which contains 20ng/ml EGF and 10ng/ml FGF2. Similarly, canine EECs were also most proliferative in NPMM, however, these cells showed no preference between the other media 1) complete N2 medium (20ng/ml FGF + 20ng/ml EGF), 2) with FGF2 (20ng/ml) only, 3) with EGF (20ng/ml) only or 4) without any growth factors added. Proliferation of canine EECs

significantly decreased in RPMI without serum and RPMI serum-free but with growth factors (EGF 20ng/ml and FGF 20ng/ml) added.

We have shown that using the RWV, EECs can aggregate into neoislets in less than 24 hours. Starting with 1.25 x 10^6 EECs, 310 neoislets are formed (Table 2). Assuming each neoislet is equivalent to a native islet, which contains 2000-3000 cells per islet, almost all of the EECs are aggregating to form neoislets. Similar to canine EECs, Ins-1 cells ($1x10^6$) aggregated into neoislets (283) in the RWV in less than 24 hours (**Fig 4**).

The characterization of EECs (discussed in chapter II), led to the question of whether these proteins found in EECs are also expressed in neoislets as well as Ins-1 cells. Although this was carried out in all species, **Figure 5** shows the coexpression of insulin and and glucagon in a porcine neoislet and the expression of insulin in a canine neoislet by indirect immunofluorescence. The expression of three growth factor receptors (FGFR, EGFR and VEGFR) (**Fig 6.**), were determined since FGF, EGF and VEGF are used in different culture conditions in the modulation of proliferation or differentiation. The FGFR was of particular interest due to the proposed role of FGF2 in the aggregation of neoislets. **Figure 7** shows the expression of FGFR in canine EECs, canine neoislets, Ins-1 cells and Ins-1 neoislets by western blot.

PDX-1 is a marker of endocrine progenitor cells, however it is present in mature β cells as well [10]. Due to the fact that aggregation is a form of differentiation, the expression of PDX-1 in Ins-1 cells and neoislets was shown by immunofluorescence to

determine if there was any change in expression from single cells to a further differentiated state of neoislets (**Fig 8**).

Cell adhesion molecules such as E-cadherin (present in islet cells) must be considered key players in the mechanism of aggregation of EECs into neoislets. The presence of E-cadherin was demonstrated through immunofluorescence and western blot (**Fig 9**) in Ins-1 cells and Ins-1 neoislets. The cytoplasmic domain of cadherins associates with catenins, which further interact with actin filaments of the cytoskeleton. The phosphorylation of catenins by receptor tyrosine kinases causes them to become activated and for the cytoplasmic complexes. The expression of β-catenin is shown by western blot in **Fig 10**. Since cadherins have an extracellular Ca^{2+} binding domain and are modulated by extracellular Ca^{2+}, Ins-1 cells were incubated in the RWV in the presence of 1mM EGTA for 24 hours and it was shown that aggregation of Ins-1 cells into neoislets did not occur. Only single cells and small clusters of 2-3 cells were formed (**Fig 11**). To further investigate the role of the cadherin-catenin complex, Ins-1 cells were incubated in the presence of genistein or AG 957, both tyrosine kinase inhibitors. **Figures 12 and 13** show the inhibition of the cadherin-catenin complex formation by these two receptor tyrosine kinase inhibitors. To investigate whether FGF2 is responsible for the activation of the cadherin-catenin complex in the aggregation of neoislets, Ins-1 cells were incubated in the presence of AG 957, FGF2 (20ng/ml) and in the absence of FGF2. Cell lysates were immunoprecipitated with E-cadherin antibody, electrophoresed, transferred and proteins were probed with FGFR and β-catenin antibodies (**Fig 15**). Cells treated with AG 957 or without FGF, did not show the FGFR in the immunoprecipitated

complex. However, cells treated with FGF (20ng/ml) did show the presence of FGFR in the cadherin-catenin complex. In contrast, β-catenin was shown in all cell lysates.

We have proposed that FGF2 mediates aggregation of endocrine precursor cells via a PKC-mediated mechanism (**Fig 16**). To investigate the role of PKC in FGF-induced aggregation, Ins-1 cells were incubated in the presence of FGF2+Bisindolymaleimide 1 (100μM), a global PKC inhibitor. Bisindolymaleimide was also used at a concentration of 50μM to ensure that PKA was not inhibited along with PKC. It was shown that aggregation was significantly decreased in the presence of Bisindolymaleimide 1 at both concentrations (**Fig 14**). These inhibition studies have been summarized in **Table 3**.

To further demonstrate that PKC is a key player in FGF2-induced aggregation of neoislets, Ins-1 cells were incubated in the presence of FGF2 + 1.0μM chelerthrine chloride, a selective inhibitor of PKC. It was shown (**Table 4**) that aggregation of Ins-1 cells into neoislets was significantly decreased. The PLC inhibitor, U-73122 was used to interfere with the activation of PKC by inhibition of PLC activation. Ins-1 cells incubated in the presence of FGF2 + U-73122 (2μM) showed a decrease in aggregation into neoislets. To further establish the role of FGF2 in the aggregation of Ins-1 cells into neoislets, the tyrosine kinase activity of the FGF2R was inhibited by the methyltransferase inhibitor, 5'-methylthioadenosine (1mM) shown by the decrease in the aggregation of Ins-1 cells. These studies have been summarized in **Table 4**.

Discussion

The purpose of these studies was to identify the mechanism(s) involved in aggregation of endocrine epithelial cells into neoislets. Because the islet exists as a spherical structure comprising the endocrine cells, it is imperative to reproduce that same architecture *in vitro,* to achieve proper function of endocrine cells. We have shown that endocrine epithelial cells can spontaneously aggregate or be induced to aggregate into islet-like structures, or neoislets. Cultures of EECs can be expanded by passaging via traditional culture methods or by neoislets initiating new cultures of EECs. We have sustained cultures of EECs for 2-3 years using this method, however the formation of neoislets by spontaneous aggregation is a slow but steady process.

Using our laboratory's expertise in dermal epithelial cell culture, we applied the knowledge of isolating pure cultures of melanocytes to endocrine epithelial cell culture. The specific environment that supports mitosis of melanocytes and gradually eliminates keratinocytes by terminal differentiation, also supports aggregation of EECs into neoislets. Factors present in the medium that could be responsible for driving differentiation/aggregation of epithelial cells include: high calcium (1.5mM), FGF2, and PMA. Although PMA is a known differentiaton promoter that acts specifically via PKC signal transduction, its tumorigenicity limits its use *in vitro,* particularly in the case of potentially transplantable tissue. The role of FGF2, due to its differentiation properties during development as well as *in vitro,* is a more likely driving force in the differentiation of EECs. We examined the proliferative influence of FGF2, as it is also a known mitogen. The basic understanding is that proliferation arrests before differentiaton

begins. Perhaps in the case of EECs, these two processes are less discrete and may overlap. The presence of high calcium in the aggregation medium is also noteworthy, as it was shown to be involved in the aggregation of EECs into neoislets by inhibition studies with EGTA. Due to the ongoing challenges that canine islet isolation continued to present, we established the similarity of Ins-1 cells and used them as a model in our aggregation studies particularly in those using the RWV.

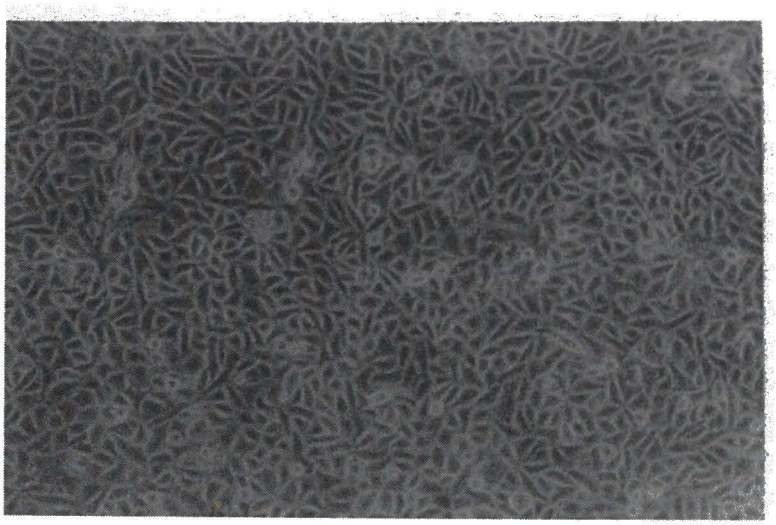
Traditional two-dimensional cell culture conditions provide attachment dependent cells with suitable proliferation signals in which differentiating cells lose their "specialized features". [86]. However, differentiation of epithelial cells in submerged conditions is not optimal and a number of epithelial cells differentiate more readily when exposed to air liquid interface. Furthermore, a number of cell types will form aggregates in submerged cultures including those of stratified epithelial lineage (i.e. keratinocytes). However, the process is slow and sporadic. Suspension culture is a popular solution to preventing dedifferentiation and maintaining specialized features of cells. The rotating-wall vessel (RWV) is a suspension culture vessel optimized to produce laminar flow and eliminate the shear stress on cells cultured within it. **(Fig 2)** However, because in this system the cells are brought in close proximity, this increases this increases the chances of cell-cell contact. Furthermore, the RWV allows for three-dimensional spatial freedom, the delivery of nutrients and oxygenation by diffusion [86]. This system is clearly suitable for forming stable aggregates with or without the help of the presence of culture beads. For example, human hepatocytes cultured in the RWV remained viable for 60 days and aggregated into tissue-like structures. [86] In addition, it has been shown that

wild type islets survive well in the RWV cell culture system [101]. We have shown the RWV to be a useful tool in the rapid aggregation of EECs into neoislets. This is advantageous to the formation of spontaneous aggregation due to the speed of aggregation (less than 24 hours), a more controlled environment, and the dynamics of the system that support aggregation.

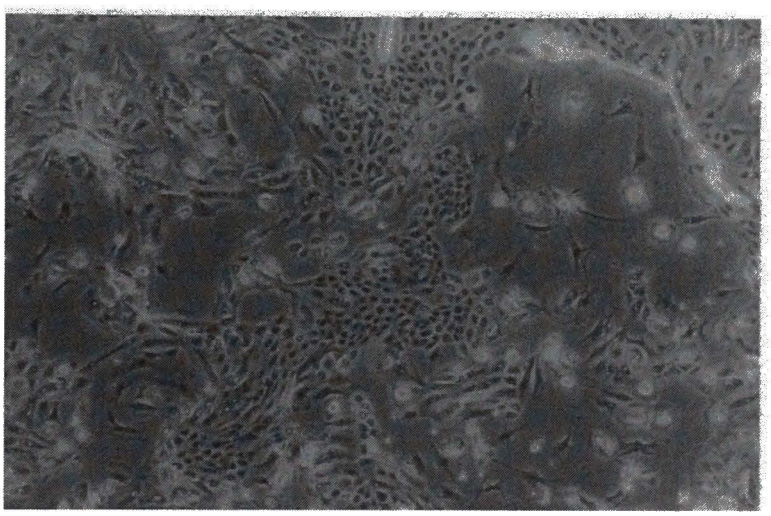
It has been shown in development that E-cadherin is critical in the aggregation of β cells into islets [76]. In addition, the cadherin-catenin complex not only functions in adherens junctions, but is thought to transmit signals from neighboring cells through interactions with growth factor receptors [79]. To further elucidate the role of FGF2 in the aggregation of EECs, we examined the cadherin-catenin complex and its association with the FGFR during aggregation. Immunoprecipitation studies identified the association of FGFR (in the presence of FGF) with the E-cadherin-catenin complex. However, the activation of β-catenin by tyrosine phosphorylation was not shown. The role of high calcium in the aggregation medium is particularly significant due to the calcium dependence of E-cadherin. Although the sequestering of calcium in the medium by EGTA inhibited aggregation, the direct effect on the cadherin-catenin complex was not shown. In all aggregation studies, the inhibition of aggregation by a specific inhibitor, was not complete but significantly reduced. It could be concluded that FGF induces aggregation of EECs by more than one mechanism. For example, global PKC inhibition by Bisindolylmaleimide 1 partially inhibited the aggregation of EECs.

Figure 1. Formation of neoislets via spontaneous aggregation. Human endocrine epithelial cells were cultured in a defined medium for epithelial cell proliferation (A). When placed under differentiation conditions (MGM), EECs presented a differentiated morphology (B) and began to aggregate into neoislets (C).

A.



B.



C.

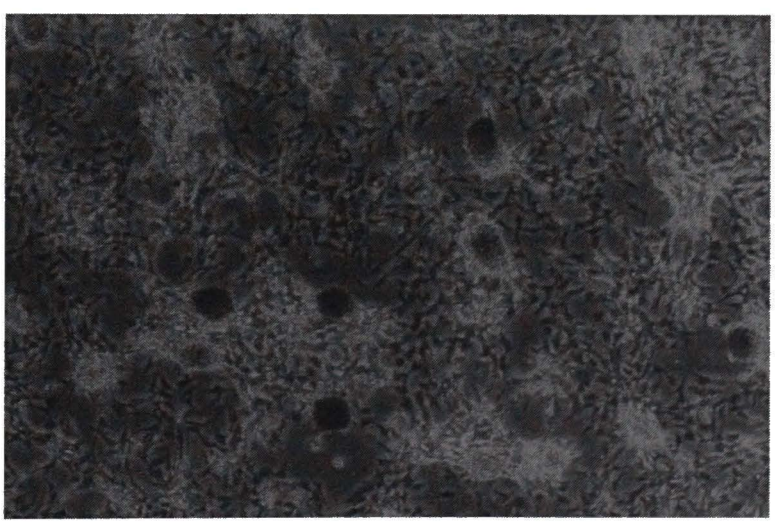


Table 1. Expansion of EECs derived from normal adult islets and their aggregation into neoislets. Canine Islets were seeded at a density of 2958±687, giving rise to $15.878x10^6 \pm 2.2x10^6$ EECs after 10 days. After 24 days in culture, the EECs aggregated to form $3.05x10^4 \pm 5.42x10^3$.

Canine Islets	Canine Islet cells (after 10 days)	Canine Neoislets (after 24 days)
2958±687	$15.878 \times 10^6 \pm 2.2 \times 10^6$	$3.05 \times 10^4 \pm 5.42 \times 10^3$

Figure 2. Canine endocrine epithelial cell proliferation determined by the SRB method. EECs were incubated in FGF2 (5, 10 and 20ngml) with or without the presence of EGF (20ng/ml). Proliferation of EECs in the presence of EGF (20ng/ml) alone and without either growth factor present was also measured.

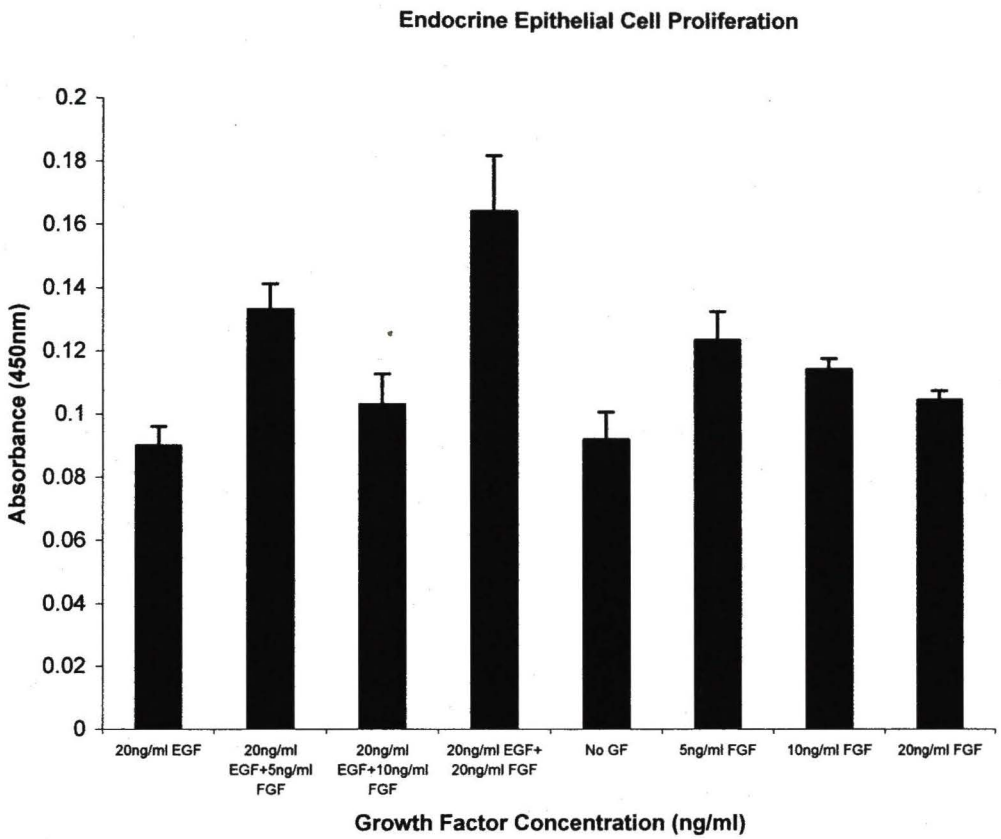
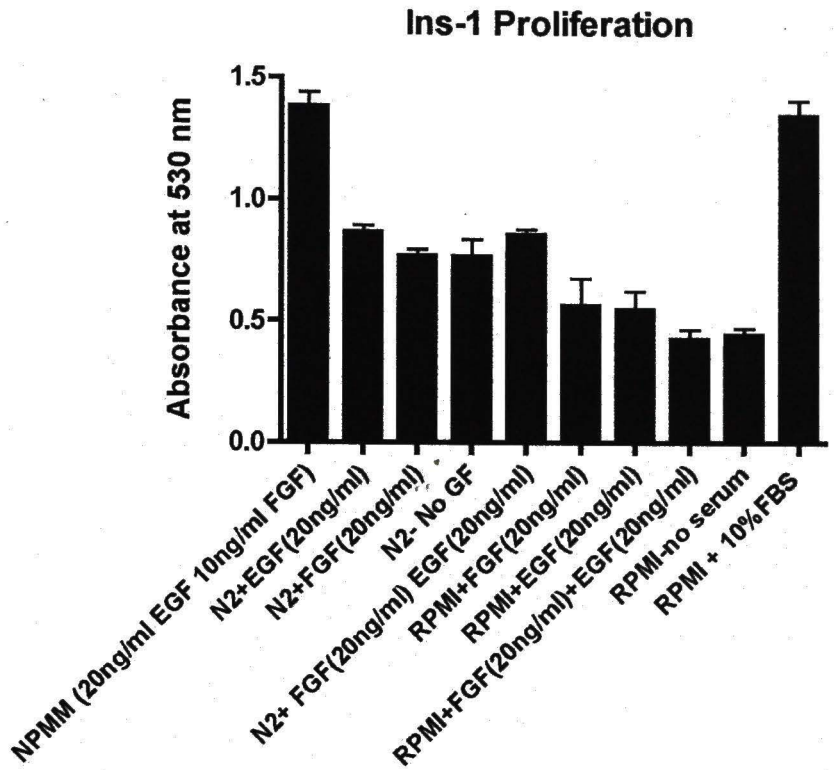

Endocrine Epithelial Cell Proliferation
Absorbance (450nm)
0.2
0.18
0.16
0.14
0.12
0.1
0.08
0.06
0.04
0.02
0
20ng/ml EGF
20ng/ml EGF+5ng/ml FGF
20ng/ml EGF+10ng/ml FGF
20ng/ml EGF+ 20ng/ml FGF
No GF
5ng/ml FGF
10ng/ml FGF
20ng/ml FGF
Growth Factor Concentration (ng/ml)

Table 2. Number of neoislets produced by aggregation of EECs and INS-1 cells in the RWV after 24 hours. Canine EECs ($1.25x10^6$) were cultured in the RWV and neoislets (310±16.68) were present after 24hr. Ins-1 cells ($1.0x10^6$) were cultured and neoislets (283.3±14.2) were present after 24 hr.

	Number of K9 EECs in RWV	Number of Ins-1 Cells in RWV
	$1.25x10^6$	$1.0x10^6$
Number of Neoislets formed after 24 hr	310±16.68	283.3±14.2

Figure 3. Proliferative conditions of Ins-1 (A) and canine EECs (B) determined by SRB assay. Ins-1 and Canine EECs were incubated in the presence of RPMI +10% FBS or without FBS (controls). EECs and Ins-1 cells were incubated in RPMI (no FBS) +EGF (20ng/ml), RPMI (no FBS) +FGF2 (20ng/ml) and RPMI (no FBS) +FGF2 (20ng/ml) +EGF (20ng/ml). Other conditions examined for proliferation of EECs included N2 basal medium (no growth factors) and N2 medium + FGF2(20ng/ml) + EGF(20ng/ml); N2 +FGF2(20ng/ml); N2+EGF(20ng/ml) and NPMM (20ng/ml EGF +10ng/mlFGF2).

A.


Ins-1 Proliferation
Absorbance at 530 nm
1.5
1.0
0.5
0.0
NPMM (20ng/ml EGF 10ng/ml FGF)
N2+EGF(20ng/ml)
N2+FGF(20ng/ml)
N2- No GF
N2+ FGF(20ng/ml) EGF(20ng/ml)
RPMI+FGF(20ng/ml)
RPMI+EGF(20ng/ml)
RPMI+FGF(20ng/ml)+EGF(20ng/ml)
RPMI-no serum
RPMI + 10%FBS

B.

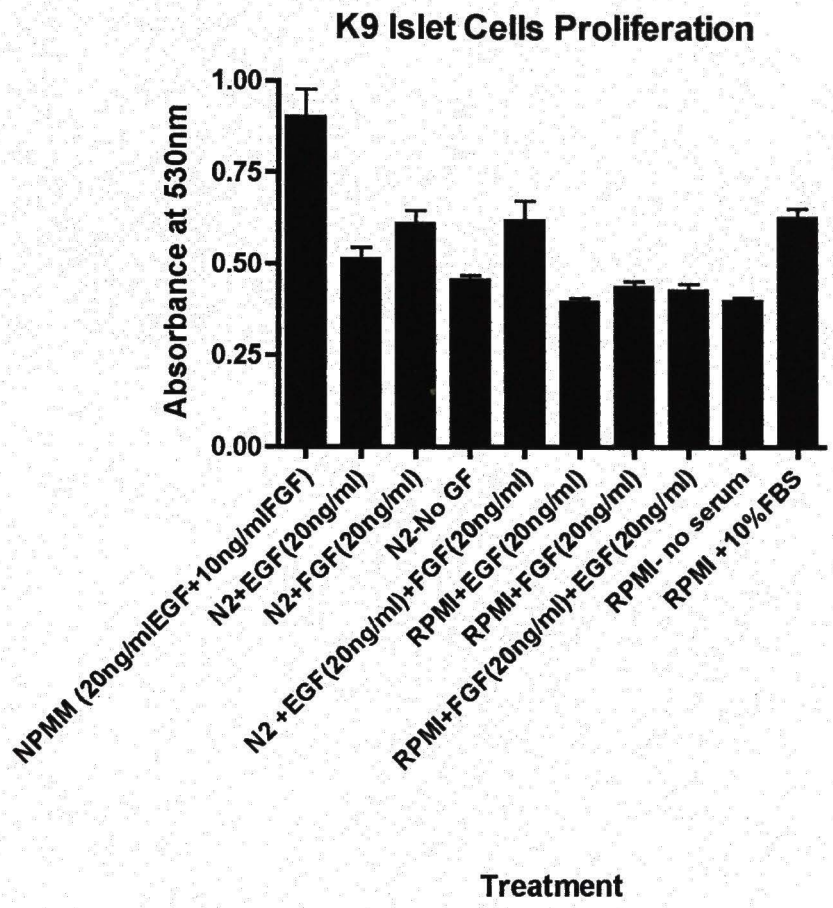
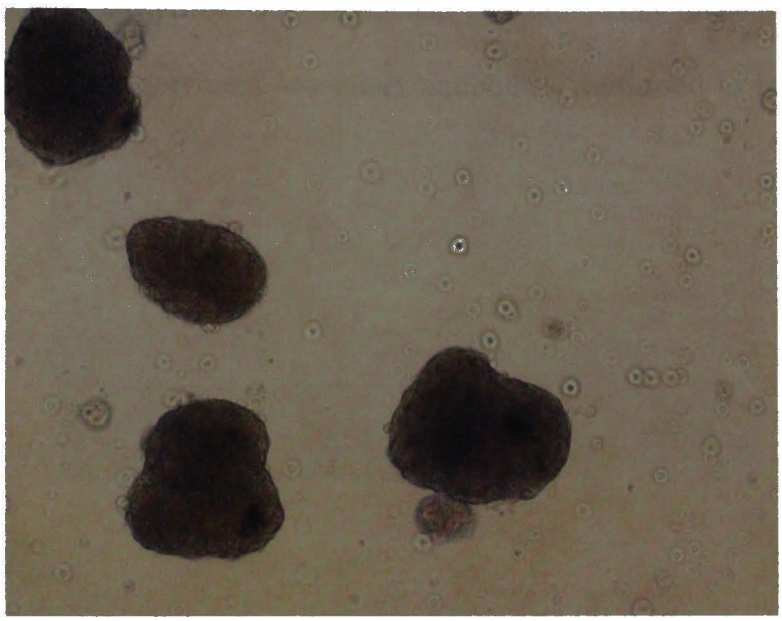

K9 Islet Cells Proliferation
Absorbance at 530nm
1.00
0.75
0.50
0.25
0.00
NPMM (20ng/mlEGF+10ng/mlFGF)
N2+EGF(20ng/ml)
N2+FGF(20ng/ml)
N2-No GF
N2 +EGF(20ng/ml)+FGF(20ng/ml)
RPMI+EGF(20ng/ml)
RPMI+FGF(20ng/ml)
RPMI+FGF(20ng/ml)+EGF(20ng/ml)
RPMI- no serum
RPMI +10%FBS
Treatment

Figure 4. Canine neoislets (A) and Ins-1 neoislets (B) formed from aggregation of EECs or Ins-1 cells in the RWV after 24 hours. EECs or Ins-1 cells were counted, placed in the RWV and incubated for 24hr. Magnification is 4X.

A. **Canine Neoislets**



Ins-1 Neoislets

B.

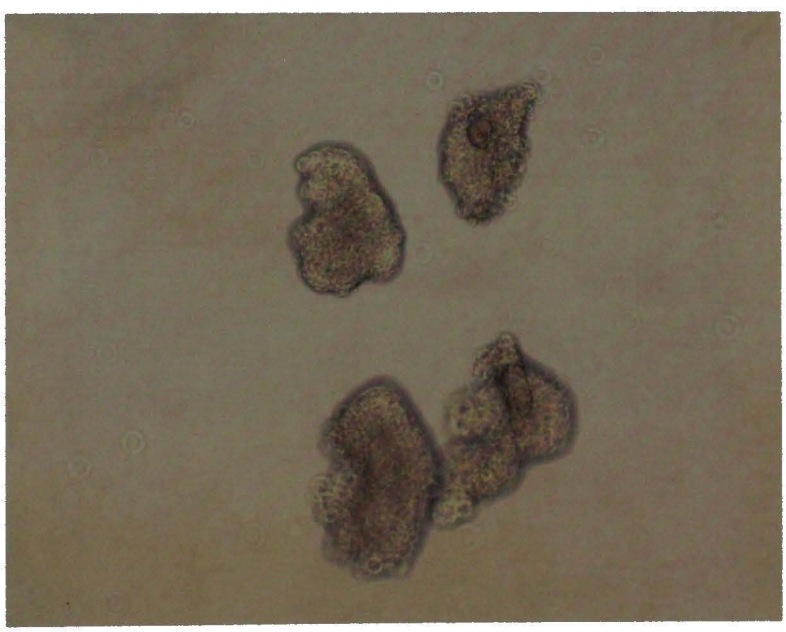
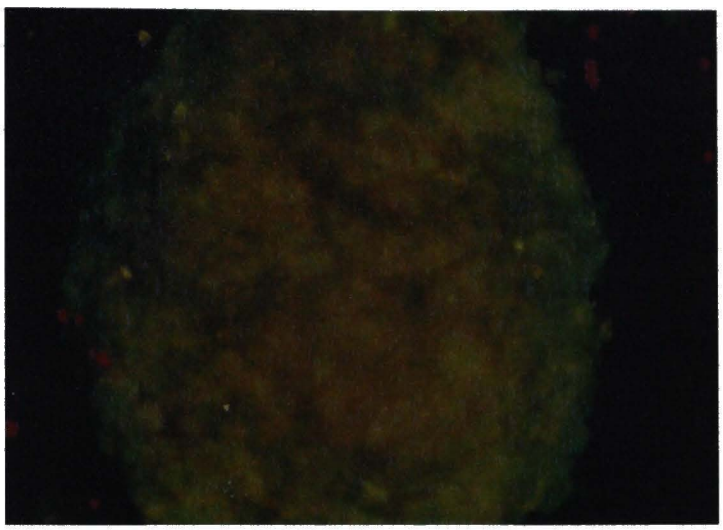


Figure 5. The coexpression of insulin and glucagon in a porcine neoislet (A) and the expression of insulin in a canine neoislet (nuclei labeled with dapi) (B). Neoislets were fixed in a glass tube and probed for insulin and glucagon using anti-insulin and/or anti-glucagon antibodies. Fluorescent secondary antibodies were used to label the proteins. Magnification is 40X.

A.



B.

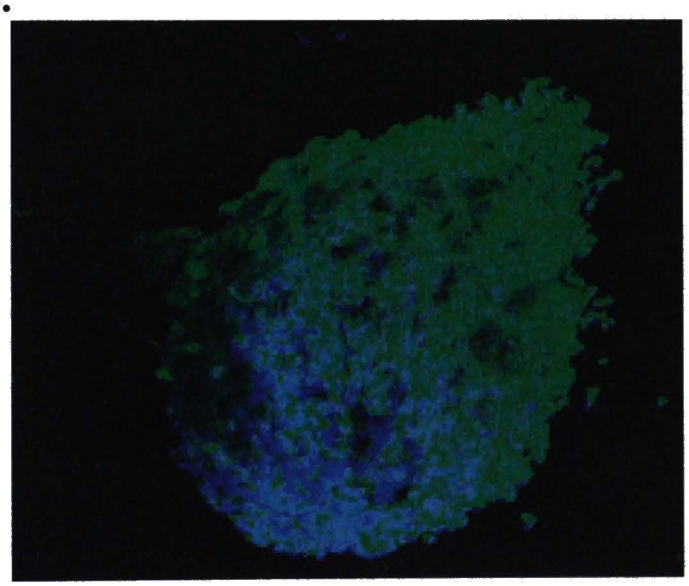
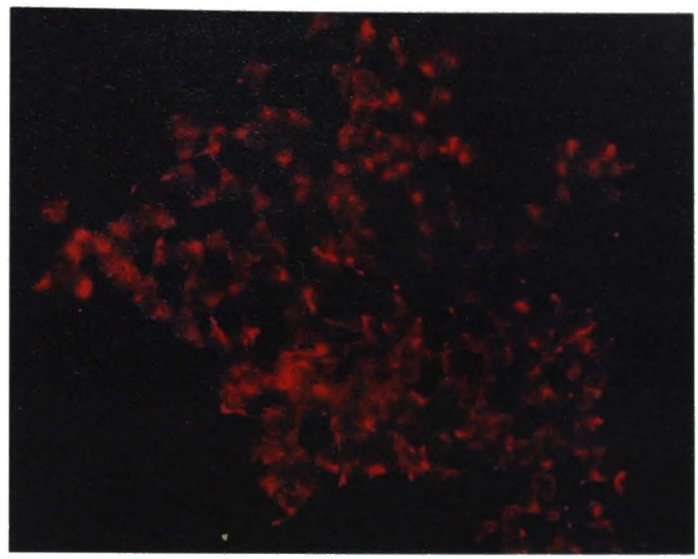


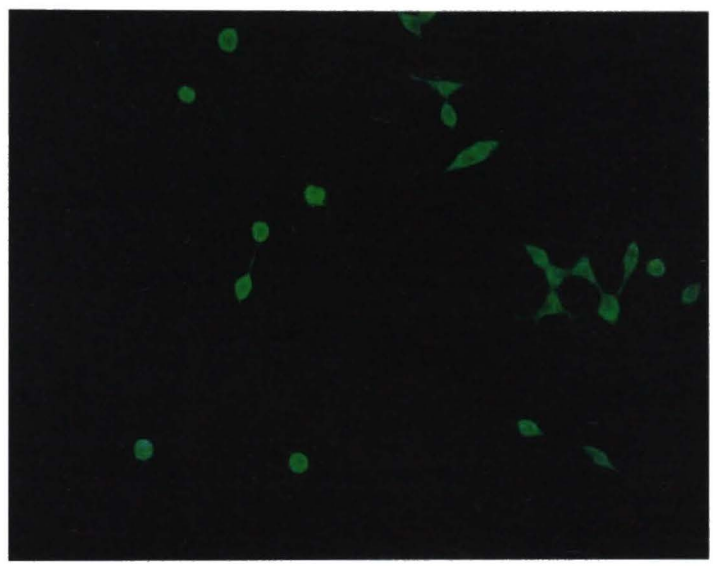
Figure 6. Expression of FGFR (A), EGFR (B), and VEGFR (C) in Ins-1 cells shown via immunofluorescence. Ins-1 cells were cultured on coverslips, fixed and probed with anti-FGFR, anti-EGFR and anti-VEGFR antibodies. Proteins were labeled using fluorescent secondary antibodies. Magnification is 4X.

A.



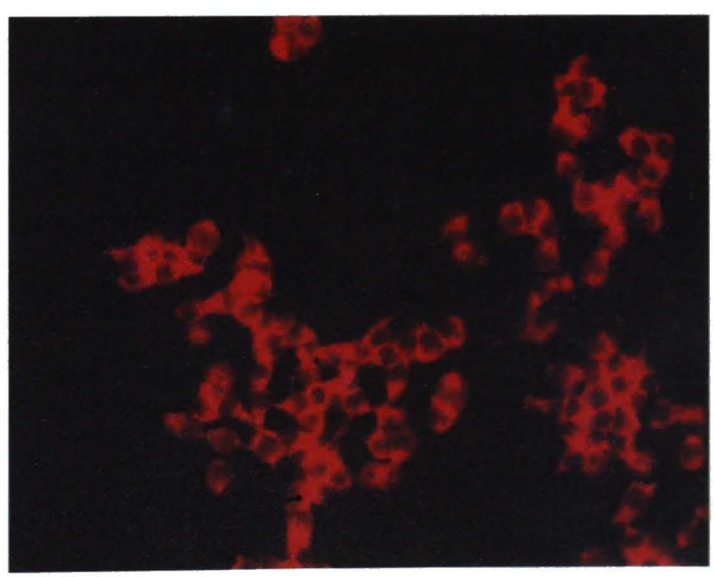
FGFR

B.



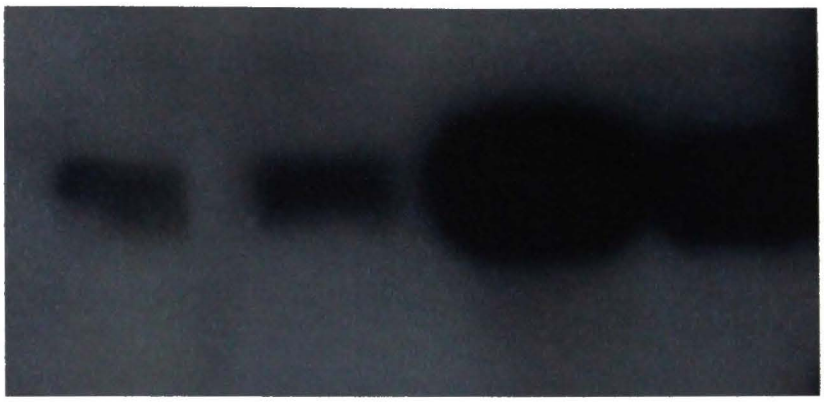
EGFR

C.



VEGFR

Figure 7. The expression of FGFR in canine EECs (A), canine neoislets (B), Ins-1 cells (C) and Ins-1 neoislets (D) shown by western blot. Samples (15 μg) were electrophoresed, transferred to a membrane and probed for FGFR with anti-FGFR antibody. Membranes were incubated with secondary antibody and visualized by chemiluminescence.

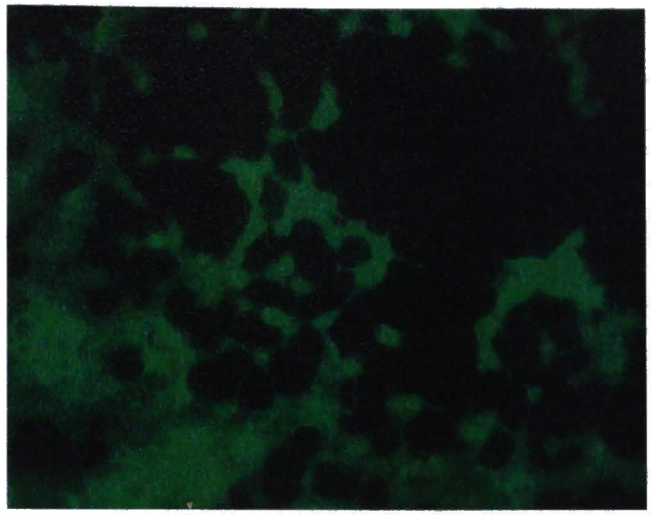


← 50kd

A B C D

Figure 8. Expression of PDX-1 in Ins-1 (A) cells and Ins-1 neoislets (B) is shown by immunofluorescence. Ins-1 cells were cultured on coverslips, fixed (neoislets fixed in a tube) and probed for PDX-1 using anti-PDX-1 antibody. PDX-1 was labeled using a fluorescent secondary antibody. Magnification is 4X.

A.



B.

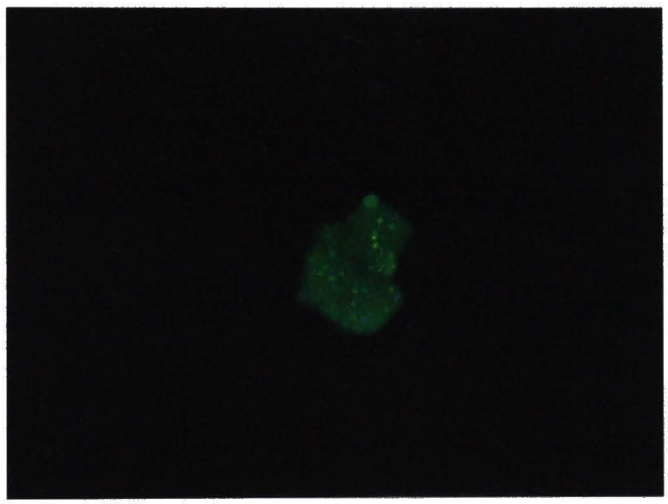
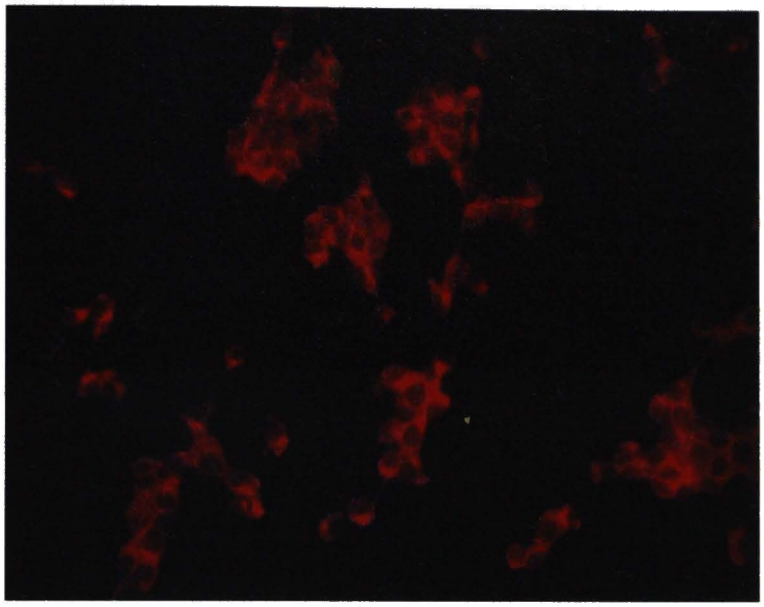


Figure 9. Expression of E-cadherin in Ins-1 cells and Ins-1 neoislets by immunofluorescence (A and B, respectively) and western blot (C and D, respectively). Ins-1 cells and neoislets were fixed and probed for E-cadherin using anti-E-cadherin antibody. E-cadherin was labeled using a fluorescent secondary antibody. Magnification is 4X. Samples (15μg) were electrophoresed, transferred to a membrane and probed for E-cadherin with anti-E-cadherin antibody. Membranes were incubated with secondary antibody and visualized by chemiluminescence.

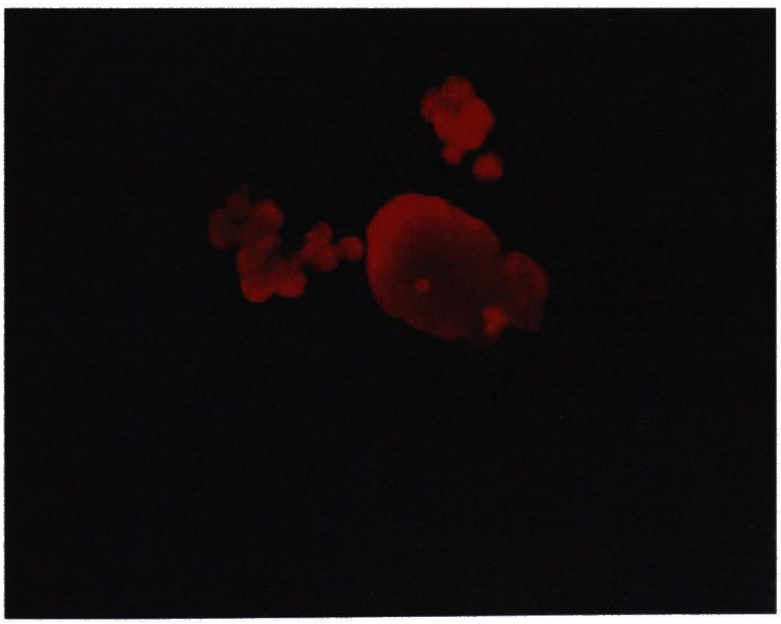
A. Ins-1 Cells



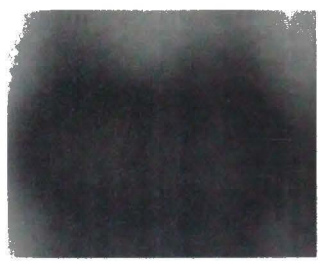
C.

Ins-1 Cells

B. Ins-1 Neoislets



D.



Ins-1 Neoislets

Figure 10. Expression of β-catenin in canine islets (A), canine EECs (B), canine neoislets (C), Ins-1 cells (D) and Ins-1 neoislets (E) shown by western blot. Samples (15μg) were electrophoresed, transferred to a membrane and probed for β-catenin using anti-β-catenin antibody. Membranes were incubated in secondary antibody and visualized by chemiluminescence.

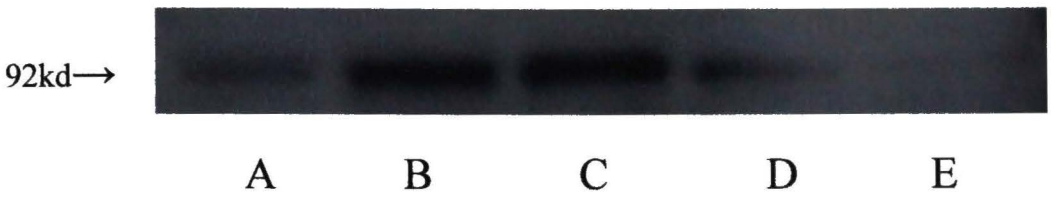
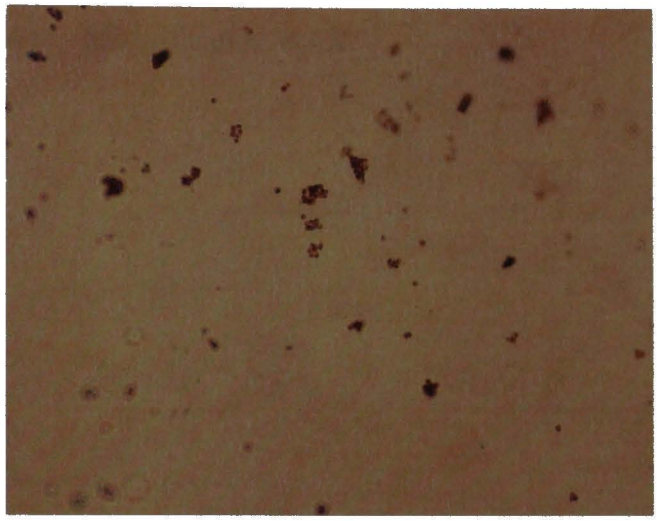

92kd⟶
A
B
C
D
E

Figure 11. Aggregation of Ins-1 cells with (A) and without (B) the presence of EGTA (1mM). Ins-1 cells were placed in the RWV in RPMI +10%FBS and RPMI +10%FBS + 1mM EGTA. Cells and neoislets were counted after 24 hours. Magnification is 4X (A, B).

A.



B.

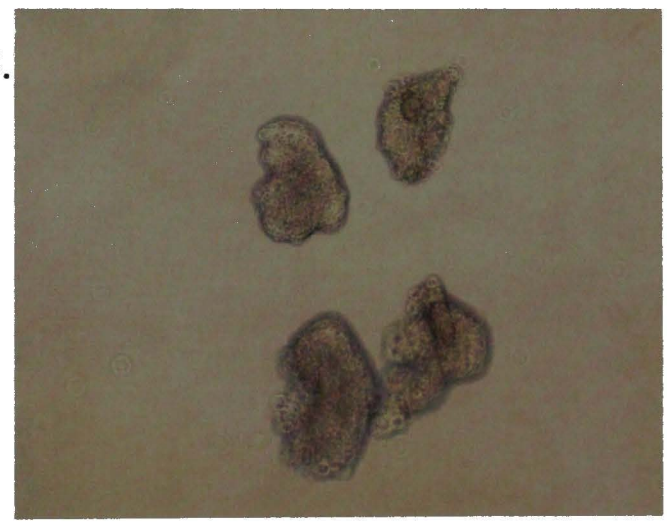
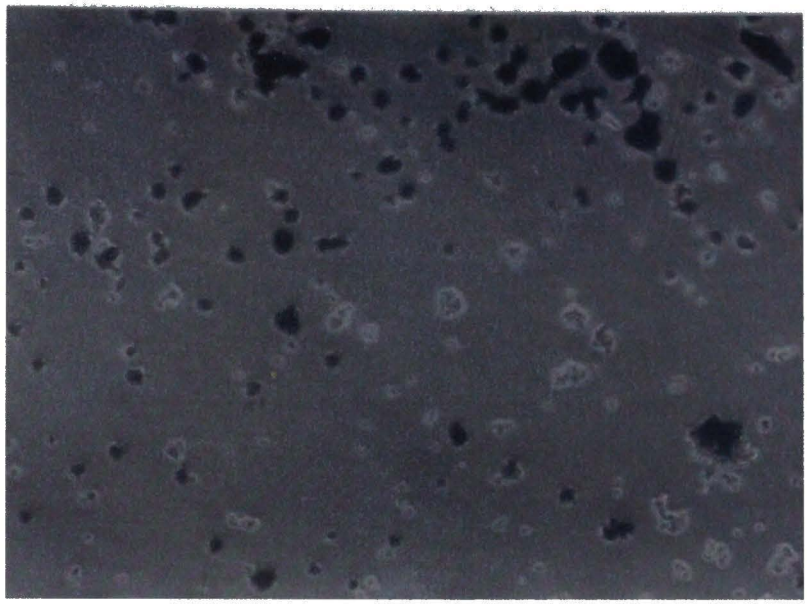


Figure 12. Inhibition of Ins-1 aggregation by Genistein (5μM). Ins-1 cells were placed in the RWV in the presence (B) or absence (A) of genistein (5μM). Cells and neoislets were counted after 24 hr. Magnification is 4X.

A.



B.

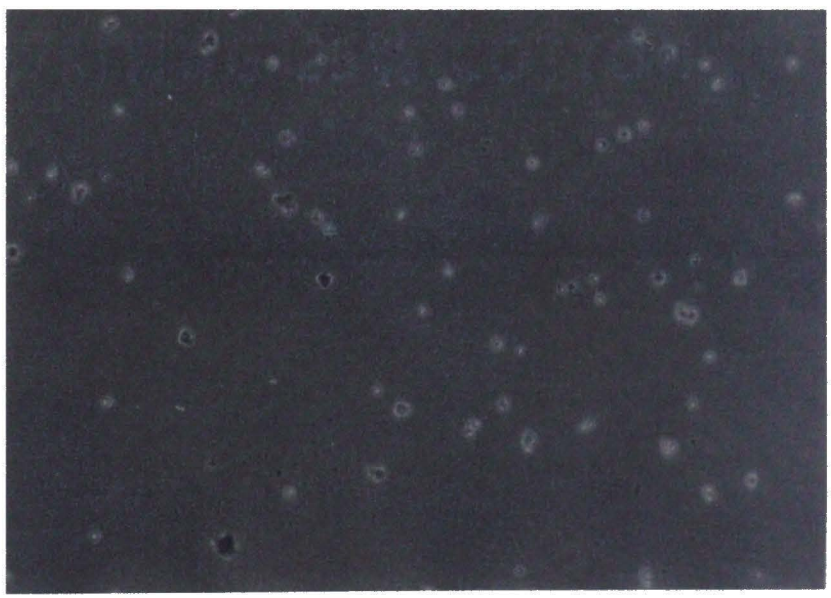
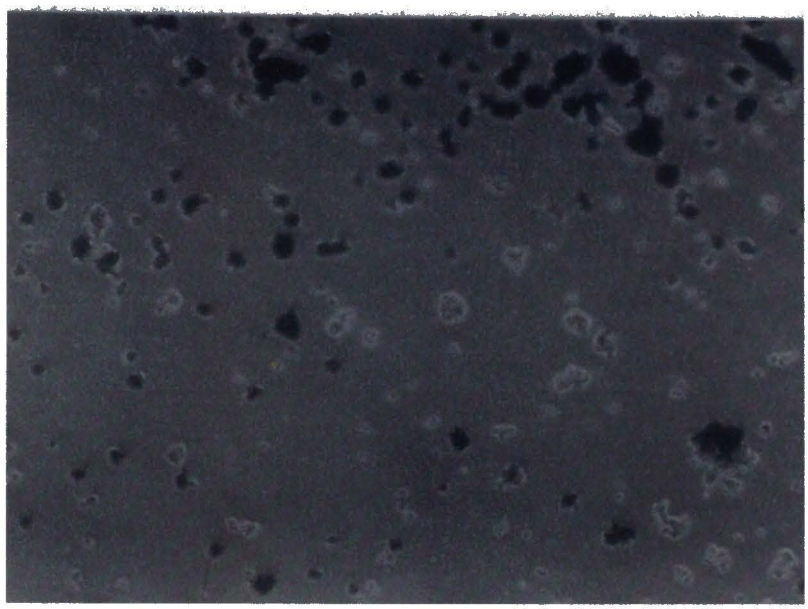


Figure 13. Inhibition of aggregation of Ins-1 cells by AG 957. Ins-1 cells were placed in the RWV in the presence (B) or absence (A) of AG 957. After 24hr, cells and neoislets were counted. Magnification is 4X.

A.



B.

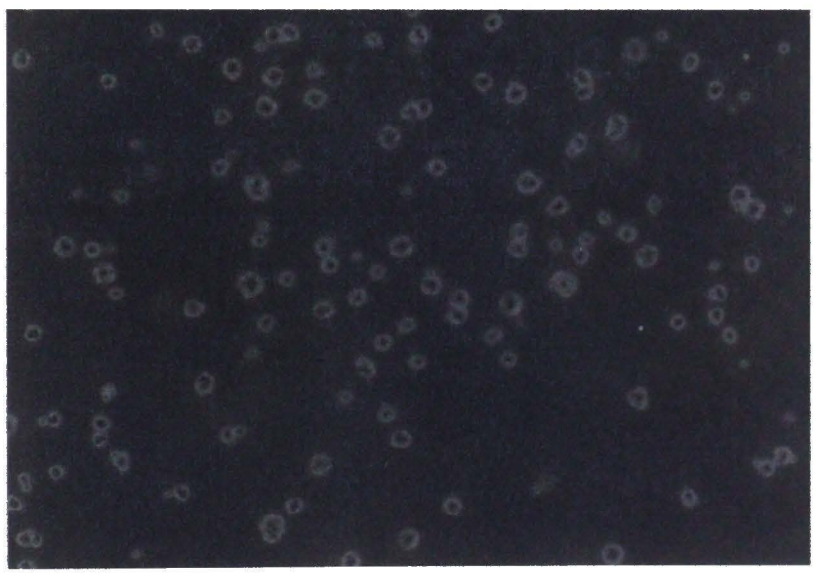
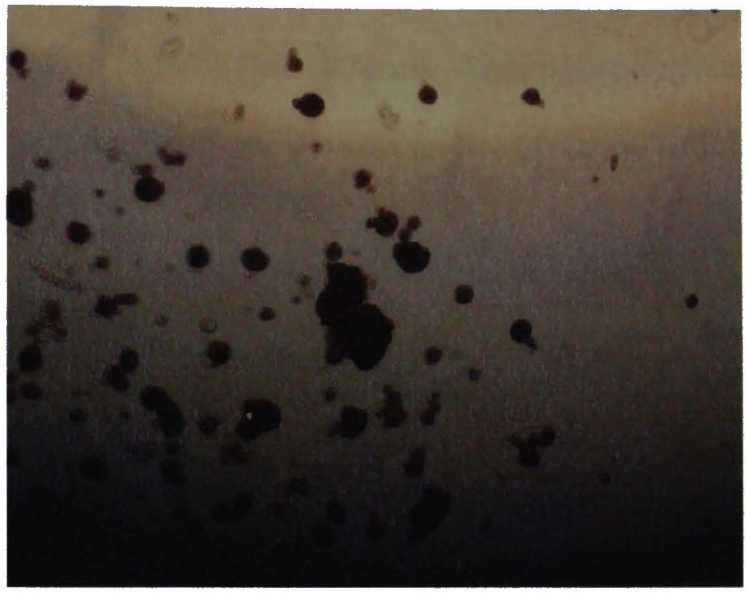


Figure 14. Inhibition of aggregation of Ins-1 cells by the global PKC inhibitior, Bisindolymaleimide 1 (100μM). Ins-1 cells were placed in the RWV in the presence (A) or absence (B) of Bisindolymaleimide 1 (100μM). Cells and neoislets were counted after 24 hr. Magnification is 4X.

A.



B.

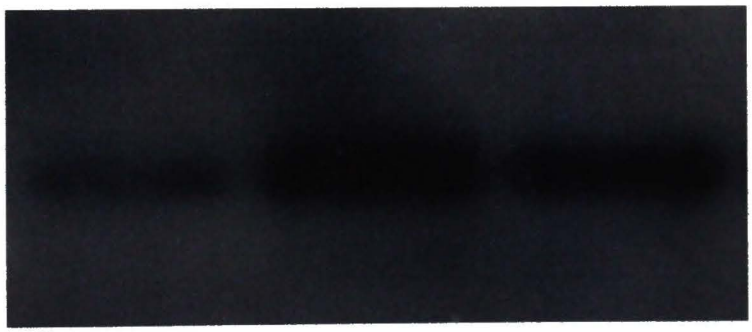
Figure 15. FGF induced aggregation of Ins-1 cells and association of FGFR with the E-cadherin-catenin complex. Proteins from lysates of Ins-1 cells aggregated in the presence of AG 957, without FGF and with FGF (20ng/ml) were immuoprecipitated with an E-cadherin-specific antibody. Immunoblots were probed with anti-FGFR (A) and anti-β-catenin (B) antibodies.

A.

← 50 kd

AG 957 **No FGF** **FGF (20ng/ml)**

B.



←92 kd

AG 957 **No FGF** **FGF (20ng/ml)**

Figure 16. Proposed mechanism of FGF2 signaling in endocrine epithelial cells. FGF2 binds to the FGF2R, causing the receptors to dimerize, which results in activation of protein kinases. The activation of the Ras protein initiates the MAPK cascade, which ultimately activates target proteins involved in proliferation. The second pathway involves the activation of PLC to split PIP_2 into IP_3 and DAG. PKC activation by DAG, Ca^{2+}, or PS (depending on the isoform) leads to phosphorylation of target proteins involved in differentiation.

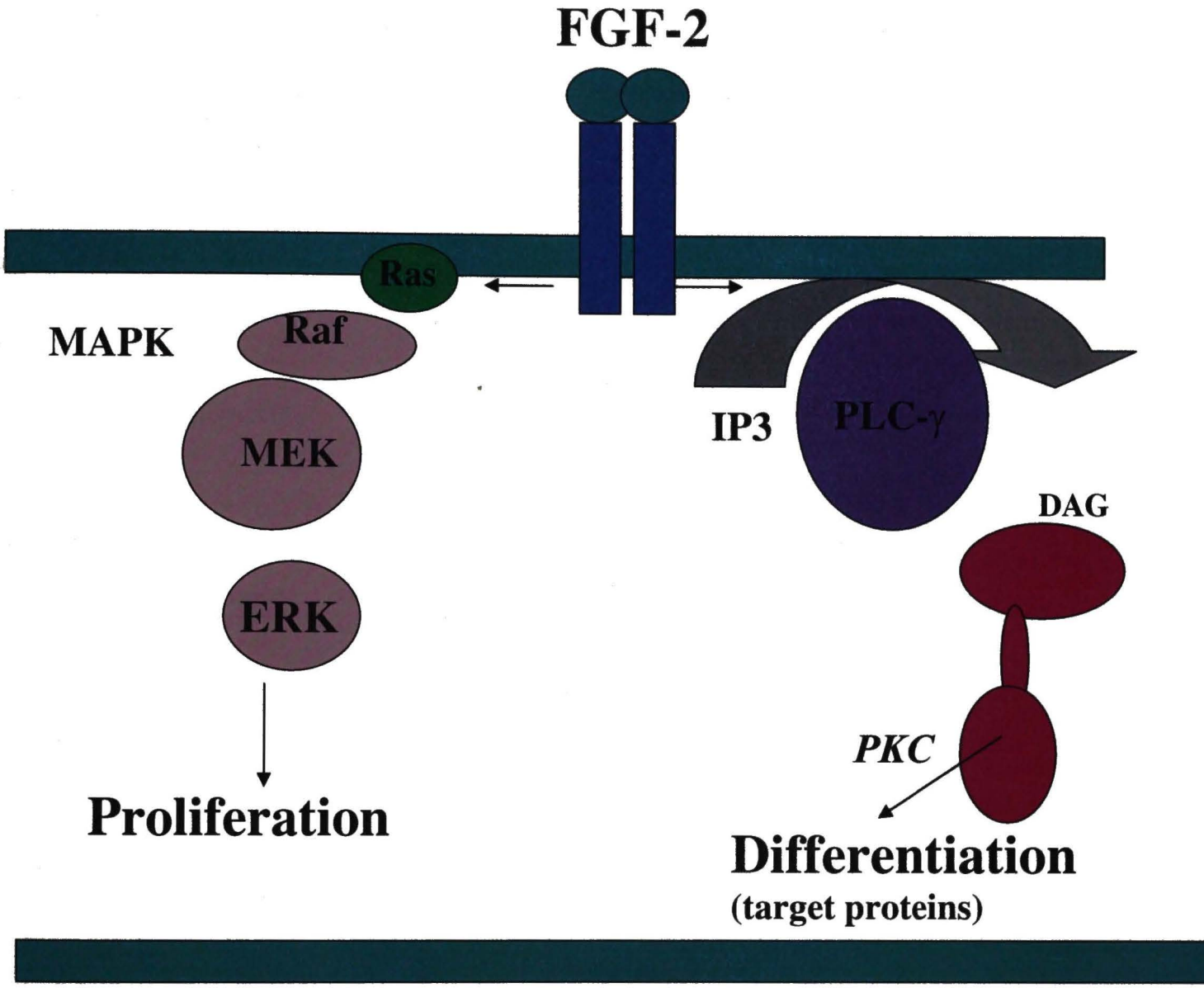

FGF-2
Ras
MAPK
Raf
MEK
ERK
Proliferation
IP3
PLC-γ
DAG
PKC
Differentiation
(target proteins)

Table 3. Summary of aggregation studies using Ins-1 (5.0×10^6) cells treated with FGF2 + Bisindolymaleimide 1(100μM and 50μM), AG 957, no FGF2 and FGF2 (20ng/ml) alone. Neoislets and single cells were counted and stained with Neutral Red for viability assessment.

Treatment	FGF2 + AG 957 (10μM)	FGF2 + Bisindolymaleimide (100μM)	FGF2 + Bisindolymaleimide (50μM)	No FGF2	FGF2 (20ng/ml)
# Neoislets	985	640	725	895	1435
# Single Cells	$2.1x10^6$	$3.6x10^6$	$3.45x10^6$	$3.4x10^6$	$8.0x10^5$

Table 4. Summary of aggregation studies using Ins-1 cells ($5.0x10^6$) cells treated with FGF2 (20ng/ml) + chelerythrine chloride (1.0μM), FGF2 (20ng/ml) + U-73122 (2μM) and FGF2 (20ng/ml) + 5'-methylthioadenosine (1mM). Neoislets and single cells were counted and stained with Neutral Red for viability assessment.

Treatment	FGF2 + U-73122 (2μM)	FGF2 + Chelerythrine Chloride (1μM)	FGF2 + 5'-methylthioadenosine (1mM)	No FGF2	FGF2 (20ng/ml)
# Neoislets	698	734	872	895	1435
# Single Cells	$3.56x10^6$	$3.3x10^6$	$3.4x10^6$	$3.4x10^6$	$8.0x10^5$

CHAPTER IV

THE ROLE OF FGF2 IN THE COMPLETE DIFFERENTIATION OF GLUCOSE RESPONSIVE NEOISLETS VIA PKC-MEDIATED PATHWAY

Introduction

Although *in vitro* differentiation of the pancreatic β cells is being studied extensively [102], the possibility that maturation of glucose responsive islets takes place in several phases, has not been considered. Because proliferation and differentiation are proposed to be mutually exclusive, cells must exit the cell cycle before differentiation begins. Aggregation of an endocrine epithelial cell monolayer into neoislets may be considered as the first phase of differentiation. Furthermore, completion of the process must include functional maturation in the form of native glucose sensing and responsiveness (i.e. upregulation of insulin secretion in response to fluctuating glucose levels) and takes place concurrently with endocrine cell sorting.

Signaling mechanisms involved in complete differentiation (proliferation arrest, aggregation and glucose responsiveness) of the β cells are not known. Various PKC isoforms have been identified in rat pancreatic islets including PKC α, βII, ε, and ζ [103]. However, the role of PKC and a systematic understanding of the controlling factors involved in signaling during β cell differentiation is not available. PKC is a known key component involved in many signal transduction pathways that regulate numerous

cellular functions [104]. The PKC family is comprised of 12 isoforms classified into three subfamilies: conventional, novel and atypical (**Table 1**). This family of serine-threonine kinases is typically activated by lipid second messengers, including diacylglycerol (DAG) or phosphatidylserine (PS), present in the plasma membrane. The phosphorylation of PKC molecules by upstream kinases contributes to the active state of PKCs [105, 106]. Several factors must be considered to elucidate the physiological functions of PKCs such as the individual features of each isoform, including expression, post-translational modification, substrate specificity, subcellular localization and cross-talk with other signaling pathways [106]. In particular, FGF2-induced PKC activation has not been elucidated and would constitute one of the likely mechanisms by which β cell differentiation occurs.

The understanding of such mechanism(s) would provide additional means for unraveling the *in vivo* organogenesis (and perhaps decline) of the endocrine pancreas and provide the means of controlling more effectively the differentiation process *in vitro*. For example, it has been shown that PKC-η is involved in the differentiation of keratinocytes. PKC-η overexpression induces G1 arrest and is accompanied by the transcriptional activation of transglutaminase I, a key enzyme and marker of squamous cell differentiation [106].

It has been suggested that FGF is a key player in the aggregation of endocrine precursor cells into neoislets in the mouse [107]. However, the role of FGF in the complete differentiation of neoislets remains unclear. *In vivo* organization of endocrine cells into islets during development is also a complex process and involves commitment,

specialization, survival and apoptosis. *In vitro*, it is not clear whether the appropriate process is chemokine-induced differentiation of epithelial cells in which a discreet change from the proliferative phenotype to a terminally differentiated functional phenotype takes place. I*n vitro* generation of functionally mature neoislets, with long-term glucose responsiveness, would be suitable for *in vivo* evaluation in diabetic animal models and furthermore, would be available for islet transplant in the long term treatment of diabetic patients, and perhaps a cure.

Unfortunately, studies with β cell progenitors have been so far very disappointing with respect to glucose-responsive insulin secretion. Although *in vivo* studies have shown promising results in the restoration of normoglycemia in diabetic mice by insulin producing cell clusters derived from mouse embryonic stem cells [108], i*n vitro* conditions which induce the terminal differentiation of the β cell progenitor (formation of insulin-containing granules and glucose responsiveness) have yet to be identified [109]. *In vivo* maturation of neoislets for the purposes of diabetes treatment would then require a surrogate organism. The surrogate's "foreign proteins" would then be generated and would contribute to the immunoincitive pool. For this strategy to be useful, a completely *in vitro* solution has to be found.

Materials and Methods

PKC profile: Western analysis was used to determine which PKC isoform(s) are present in endocrine epithelial cells, native islets and neoislets from canine pancreata as well as Ins-1 cells and Ins-1 neoislets. PKC profile in native islets from other species such as

mouse, rat, pig and human was also determined. Isoforms evaluated included: PKC-α, βI, βII, δ, γ, ε, ζ, and θ.

PKC inhibitor Studies: Aggregation of EECs was blocked by treating the endocrine epithelial cells with the global PKC isoform inhibitor, Bisindolymaleimide (50μM or 100μM) for 24 hours in the RWV.

Insulin Secretion and Content: Native islets and neoislets were preincubated at 37°C in KRB with 2mM glucose for 30 min and incubated in KRB with 5mM glucose for 1 hour, then in 20mM glucose for 1 hour. The supernatant was collected and secreted insulin was measured using Ultra Sensitive Human Insulin Radioimmunoassay (Linco Research) or ELISA (ALPCO). The non-secreted insulin within neoislet cells was extracted by incubation overnight at 4°C in acid/ethanol (1.5%HCL, 75% ethanol, 23.5% H20). Supernatants were stored at –20°C for subsequent measurement by RIA or ELISA. Islets cultured within a collagen matrix were preincubated at 37°C in 2mM glucose for 2 hours and incubated in 5mM glucose for 6-12 hours, then 20mM glucose for 6-12 hours. Media was collected at each stage and measured by RIA or ELISA.

Electron Microscopy: Native islets and neoislets were inoculated into a collagen gel to stabilize them for electron microscopy processing. The collagen gels containing the tissue were fixed in EM fixative, embedded in plastic and sectioned.

Results

A PKC profile of expression was determined for endocrine epithelial cells, neoislets, normal islets, Ins-1 cells and Ins-1 neoislets by western blot analysis of respective lysates. Isoforms examined included α, βI, βII, δ, γ, ε, ζ, η, and θ. The presence or absence of each isoform that was tested is illustrated in **Table 2**. Isoforms present in all samples were PKC-α, βII, ε and ζ. The expression of PKC-α was greatest in canine islets and neoislets and decreased in canine EECs (**Fig 1**). Ins-1 cells and neoislets expressed about the same amount of PKC-α. The expression of PKC-βII was significantly greater in Ins-1 cells and Ins-1 neoislets than in canine samples in which expression of this isoform significantly decreased (**Fig 2**). PKC-ε appears to be equally expressed in all samples (**Fig 3**), however, densitometric analysis was not performed. Highest expression of PKC-ζ was observed in canine islets and neoislets compared to canine EECs, Ins-1 cells and Ins-1 neoislets, which expressed the isoform at approximately the same level (**Fig 4**).

Normal islets and neoislets were examined by Electron Microscopy to compare the presence and organization of secretory granules in β cells or EECs. Dense, secretory granules are present and well organized in the normal β cell, however they seem to be fewer and less organized in the neoislet (**Fig 5**).

Glucose responsive insulin secretion was evaluated in endocrine epithelial cells, neoislets, normal islets, Ins-1 cells and Ins-1 neoislets. Islets or neoislets (25/tube) and 20,000 cells were incubated in 2mM glucose for 2 hours, followed by incubation in

20mM glucose for 2 hours and response of normal porcine islets is shown in **Fig 6.** The increase in insulin at 20mM glucose was even further increased in the presence of 10μM forskolin. The response to increased glucose by canine islets is shown in **Fig 7,** in which these islets increase their insulin secretion in response to an increase in glucose concentration. In contrast, canine EECs show little insulin secretion and no increase in response to elevated glucose (**Fig 8**). Similarly, canine neoislets (**Fig 9**) and porcine neoislets (**Fig 10**) had minimal insulin secretion and no response to changes in glucose concentration. Insulin secretion in response to glucose by Ins-1 cells compared to Ins-1 neoislets is shown in **Fig 11**. Although there is some increase in the insulin response to 20mM glucose of Ins-1 cells, this response is less pronounced in Ins-1 neoislets. **Figure 12** shows the glucose responsiveness of Ins-1 cells at 20, 40 and 60 minutes. The increase in insulin secretion at 20mM glucose is present at each time point. However, most insulin is secreted at the 60minute time point. Incubation with 20mM glucose + GLP-1 further increases insulin secretion.

Human islets were placed in a collagen type I gel matrix and insulin secretion was measured in response to a glucose challenge (**Fig 13**). The collagen matrix did not interfere with insulin secretion as there is an increased response to elevated glucose.

Discussion

All reports dealing with expansion of duct epithelial endocrine cells and our experience with islet derived endocrine epithelial cells and resulting neoislets converge

on the inability of these *in vitro* generated structures to exhibit wild type islet glucose responsiveness. The only strategy that has demonstrated such maturation of neoislets is implantation under the kidney capsule and ensuing *in vivo* maturation. However, such a procedure is of very little value as a source of transplant material for the treatment of IDDM and this goal has to be accomplished *in vitro*. The endocrine epithelial cells express insulin (as well as other endocrine hormones) and are clearly capable of synthesizing it. Neoislets from all species also express insulin and other endocrine hormones. In addition, EECs and neoislets express Glut 2 and glucokinase, two essential components in the glucose-sensing pathway. Neoislets embedded in a type I collagen gel and processed for EM were sectioned and showed the presence of insulin granules. However, these are not organized in the same fashion as in wild type islets. We have shown through various glucose challenge experiments, that neoislets are not glucose responsive and have very low insulin content. Forskolin and glucagon-like-peptide (GLP-1) have an added insulinotropic effect in the presence of glucose in normal islets or β cells. Although, there was some increase in insulin due to these factors, the amount of insulin secreted was insignificant. These results are in agreement with what has been shown in aggregates obtained from duct epithelial progenitors [89]. Thus, the aggregation process alone is only a part of functional maturation.

The PKC profile determined for normal islets, neoislets, EECs, Ins-1 cells and Ins-1 neoislets established the expression of PKC-α, βII, ε and ζ in all samples. However, expression of each isoform varied depending on the state of differentiation

(cells or aggregates). Perhaps modulation of a specific isoform may give rise to a terminally differentiated state.

Clearly, the lack of glucose responsiveness in neoislets is the biggest obstacle in this specific aim, which seems to be a common problem in this area of research. When compared to organogenesis *in vivo*, the cues that are missing are: 1) the contribution of the mesenchyme (source of FGF) and other growth factors and cytokines 2) the lack of vascular connection and presumably circulating serum-borne signaling molecules and 3) the lack of neurogenic contribution for the sensory systemic connection. Whereas we have shown that the presence of a three-dimensional environment (collagen matrix) has a beneficial effect on the survival of both native and neoislets, these matrices did not contain any other cellular components or extracellular components other than collagen. This format may be utilized to stabilize islets while in transit or storage (a losing proposition at this time) and even as a vehicle in subcutaneous implantation.

Future experiments may involve the combination of fibroblasts and neoislets in a collagen matrix, which may provide some of the necessary signals for neoislet maturation *in vitro*. At the very least, the presence of fibroblasts would be a source of FGF2. We have expertise in obtaining fibroblasts from tissue and have no reason to suspect that the fibroblasts and neoislets would be incompatible.

Table 1. PKC subfamilies, isoforms and their requirements for activation.

Subfamily	Isoforms	Requirements for Activation
Conventional	α, βI, βII, γ	**DAG, PS, Ca2+**
Novel	δ, ε, η, θ	**DAG, PS**
Atypical	λ, ζ, μ	**PS**

Table 2. PKC profile of endocrine epithelial cells, neoislets, native islets, Ins-1 cells and Ins-1 neoislets. EECs, neoislets, native islets, Ins-1 cells and Ins-1 neoislets were lysed and 15μg protein/sample were electrophoresed, transferred to a membrane and probed for each PKC isoform using anti-PKC antibodies. Membranes were incubated in secondary antibodies and proteins were visualized by chemiluminescence.

	PKC-α	PKC-βI	PKC-βII	PKC-δ	PKC-ε	PKC-γ	PKC-θ	PKC-ζ	PKC-η
Ins-1	+	-	+	-	+	-	-	+	-
Ins-1 Neoislets	+	-	+	-	+	-	-	+	-
Canine Islets	+	-	+	-	+	-	-	+	-
Canine Neoislets	+	-	+	-	+	-	-	+	-
Canine EECs	+	-	+	-	+	-	-	+	-
Porcine EECs	+	-	+	-	+	-	-	+	-

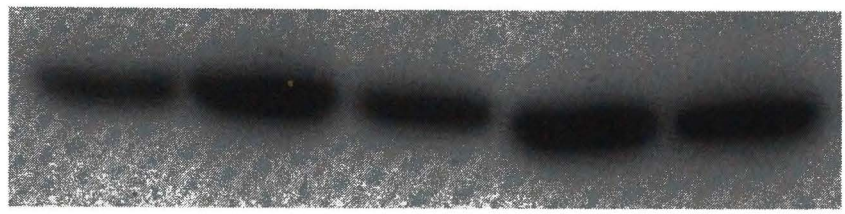
Figure 1. Expression of PKC-α determined by western blot (A). Proteins were run on a gel, transferred to a membrane and probed for PKC-α. Membranes were stripped and reprobed for GAPDH (B). PKC-α expression was normalized to GAPDH and analyzed by densitometry (C).

A.

← 80kd

Canine islets | Canine EECs | Canine Neoislets | Ins-1 Cells | Ins-1 Neoislets

B.



← 40kd

C.

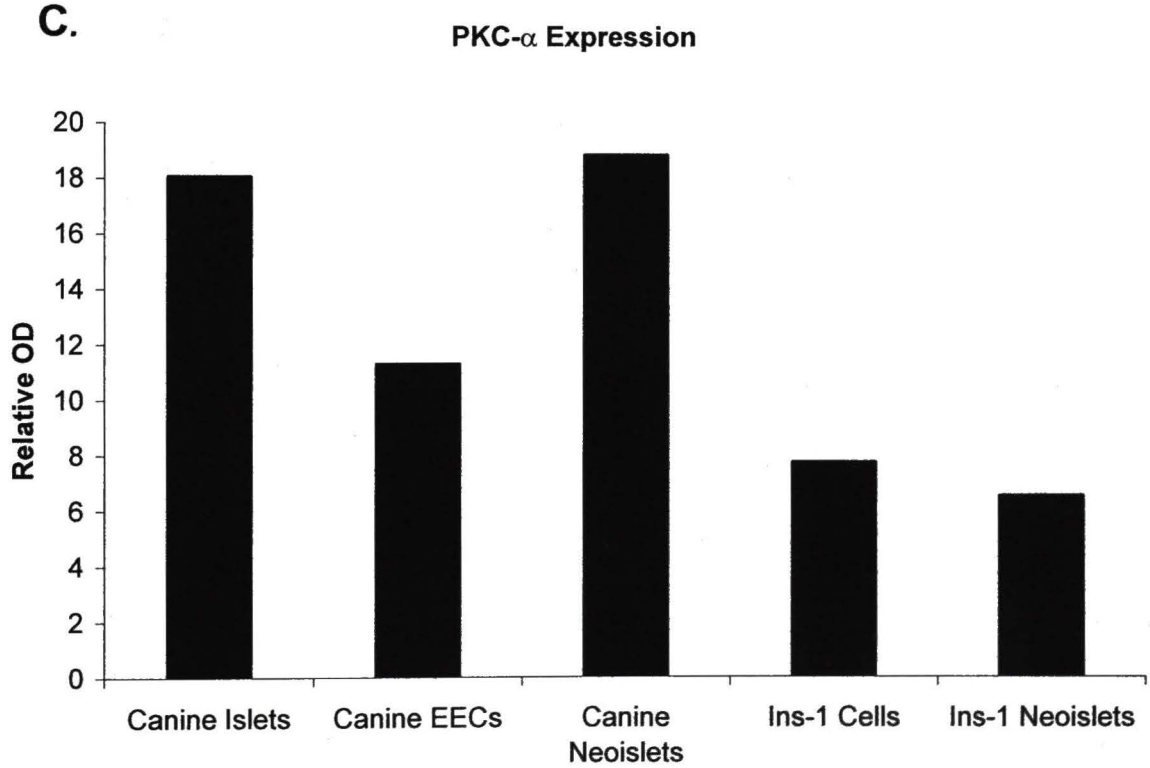
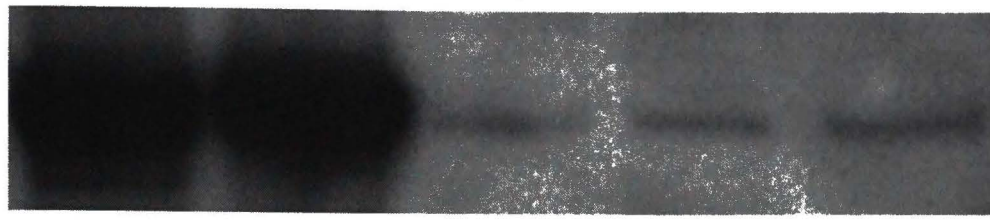


Figure 2. Expression of PKC-βII determined by western blot (A). Proteins were run on a gel, transferred to a membrane and probed for PKC-βII. Membranes were stripped and reprobed for GAPDH (B). PKC-βII expression was normalized to GAPDH and analyzed by densitometry (C).

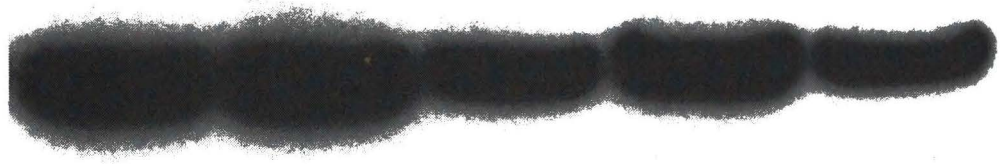
A.



←80kd

Ins-1 Neoislets | Ins-1 Cells | Canine Neoislets | Canine EECs | Canine Islets

B.



← 40kd

C.

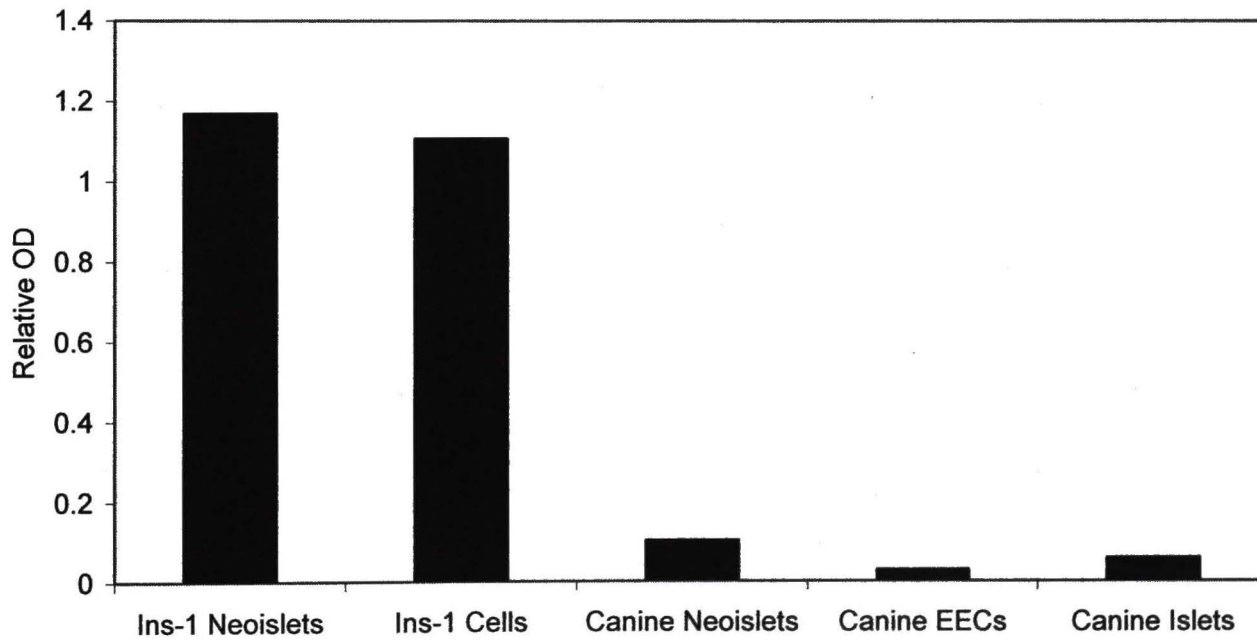


Figure 3. Expression of PKC-ε determined by western blot (A). Proteins were run on a gel, transferred to a membrane and probed for PKC-ε. Membranes were stripped and reprobed for GAPDH (B).

A.

← 80kd

B.

K9 Islets | K9 EECs | K9 Neoislets | Ins-1 Cells | Ins-1 Neoislets

←40kd

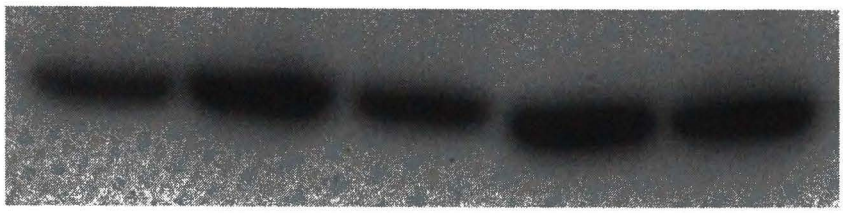
Figure 4. Expression of PKC-ζ determined by western blot (A). Proteins were run on a gel, transferred to a membrane and probed for PKC-ζ. Membranes were stripped and reprobed for GAPDH (B). PKC-ζ expression was normalized to GAPDH and analyzed by densitometry (C).

A.

← 80kd

Canine Islets | Canine EECs | Canine Neoislets | Ins-1 Cells | Ins-1 Neoislets

B.



← 40kd

C.

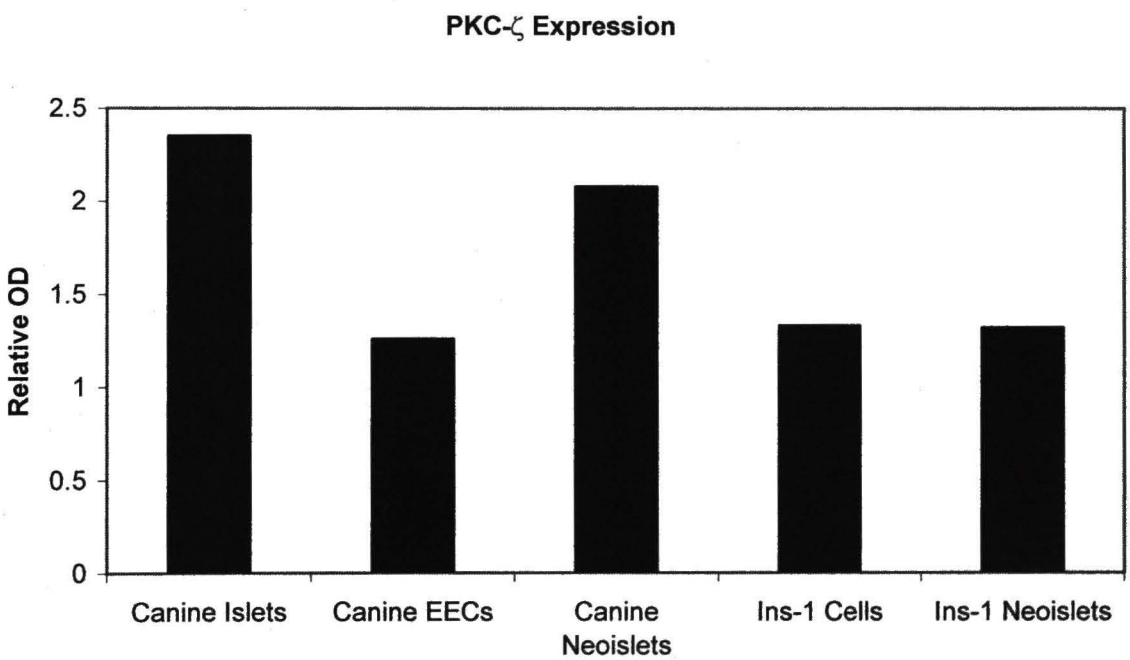
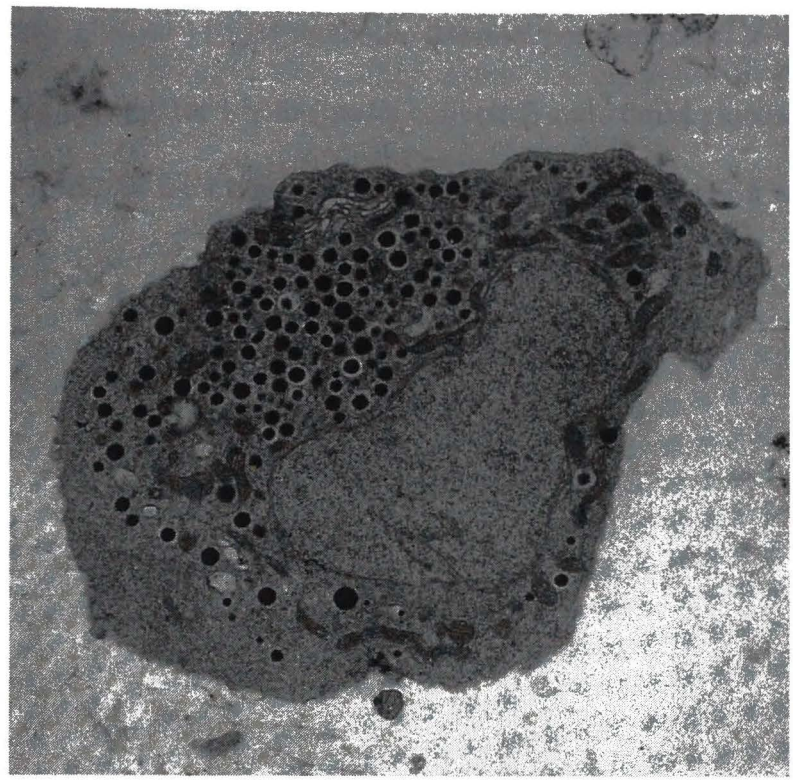


Figure 5. Electron microscopy of a β cell (A) and neoislet (B), showing dense secretory granules.

A.



B.

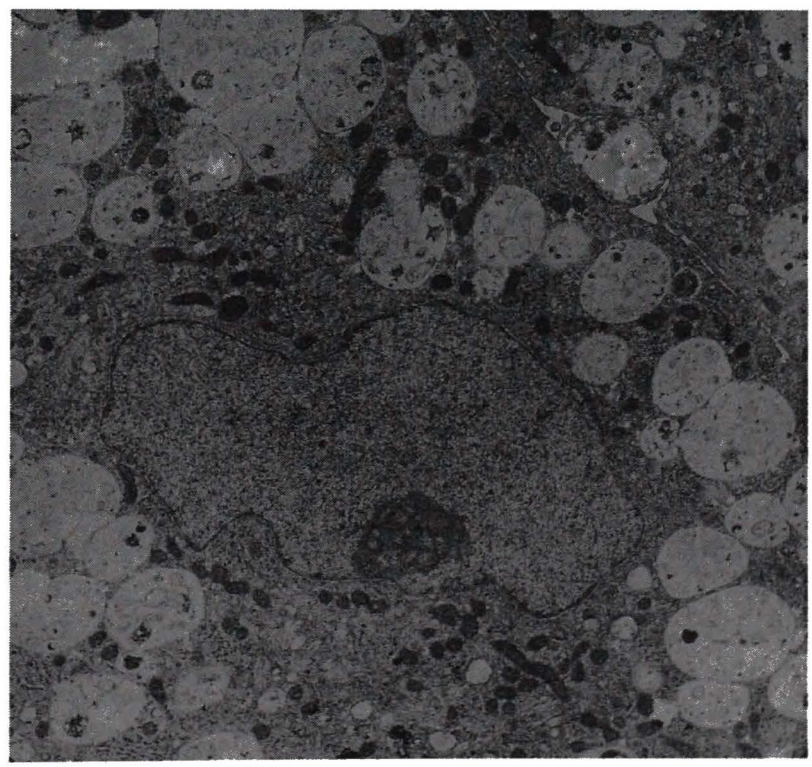


Figure 6. Normal porcine islet insulin secretion in response to 3mM glucose, 20mM glucose and 20mM glucose +10μM forskolin. Normal porcine islets (25/tube) were incubated in 3mM glucose for 1 hour (media collected) and 20mM glucose or 20mM glucose +10μM forskolin for 1 hour (media collected). Insulin secretion was measured by RIA.

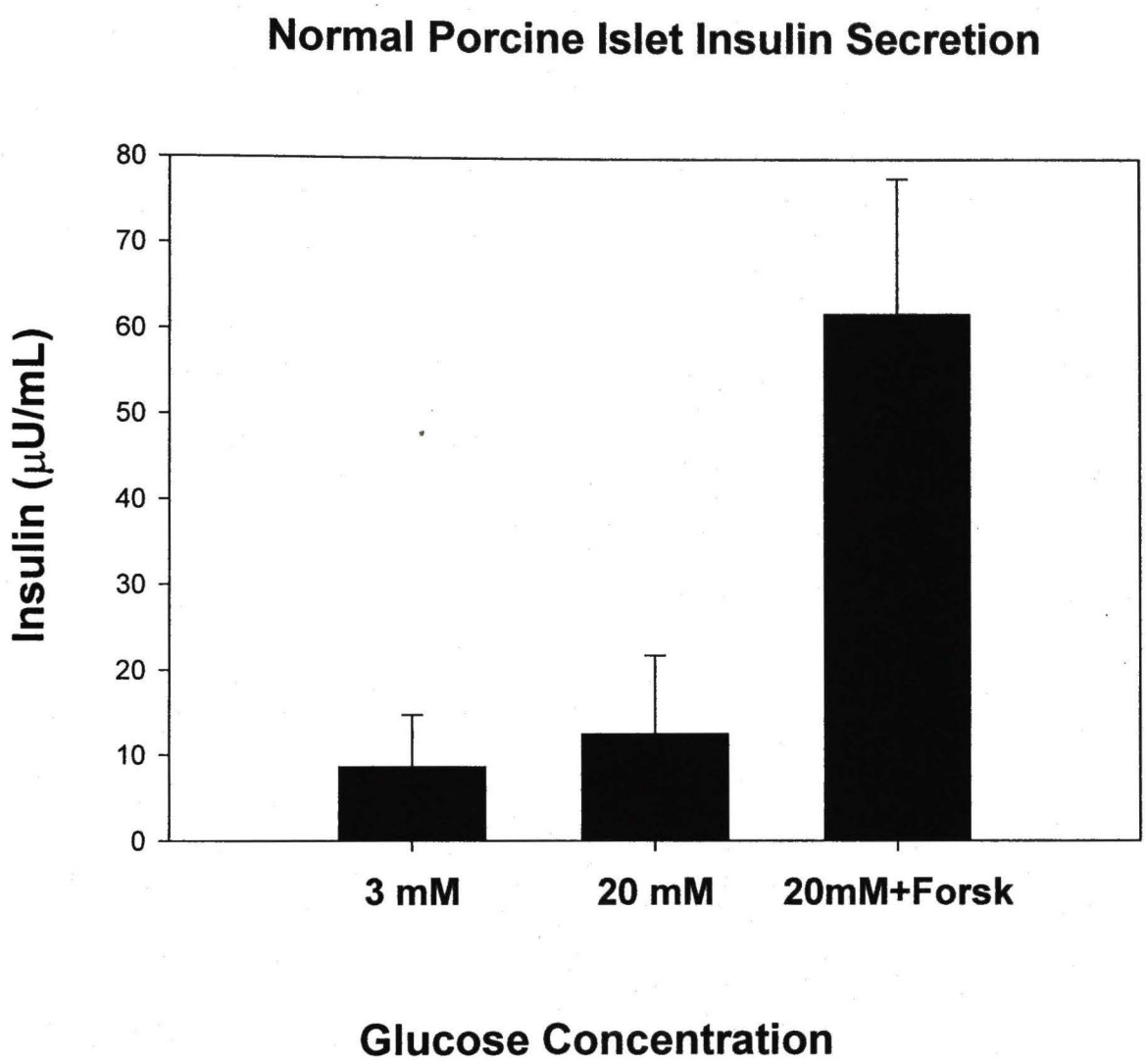
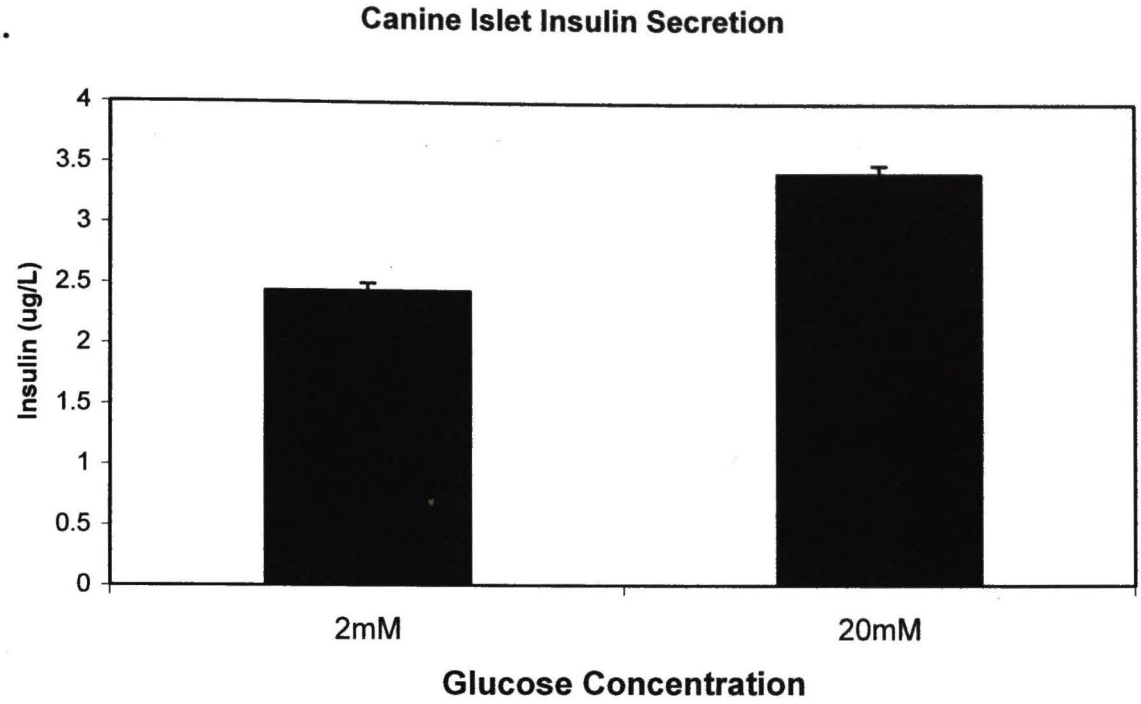

Normal Porcine Islet Insulin Secretion
Insulin (μU/mL)
80
70
60
50
40
30
20
10
0
3 mM
20 mM
20mM+Forsk
Glucose Concentration

Figure 7. Canine islet insulin secretion in response to 2mM and 20mM glucose. Canine islets (25/tube) were incubated in 2mM glucose for 1hr (media collected) and 20mM glucose for 1 hr (media collected). Insulin secretion was measured by ELISA and is presented as insulin (μg/L) (A) and % change (B).

A.


Canine Islet Insulin Secretion
Insulin (ug/L)
4
3.5
3
2.5
2
1.5
1
0.5
0
2mM
20mM
Glucose Concentration

B.

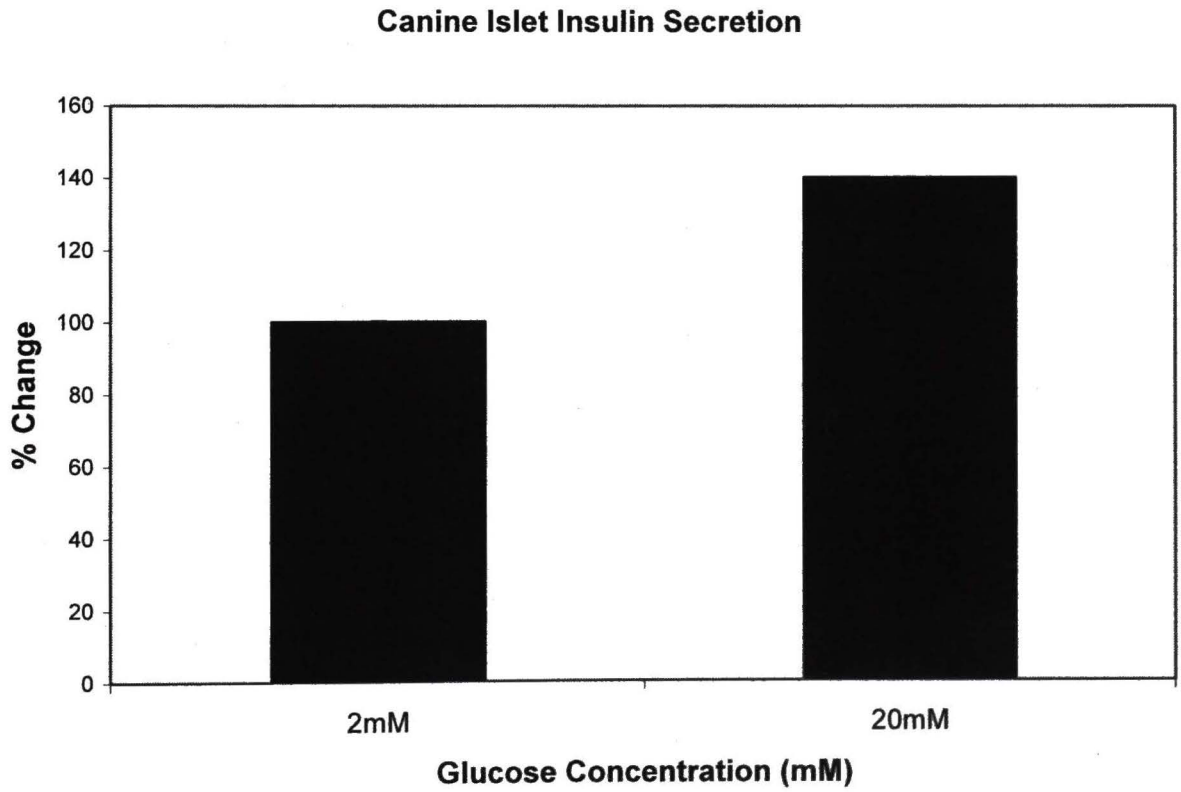
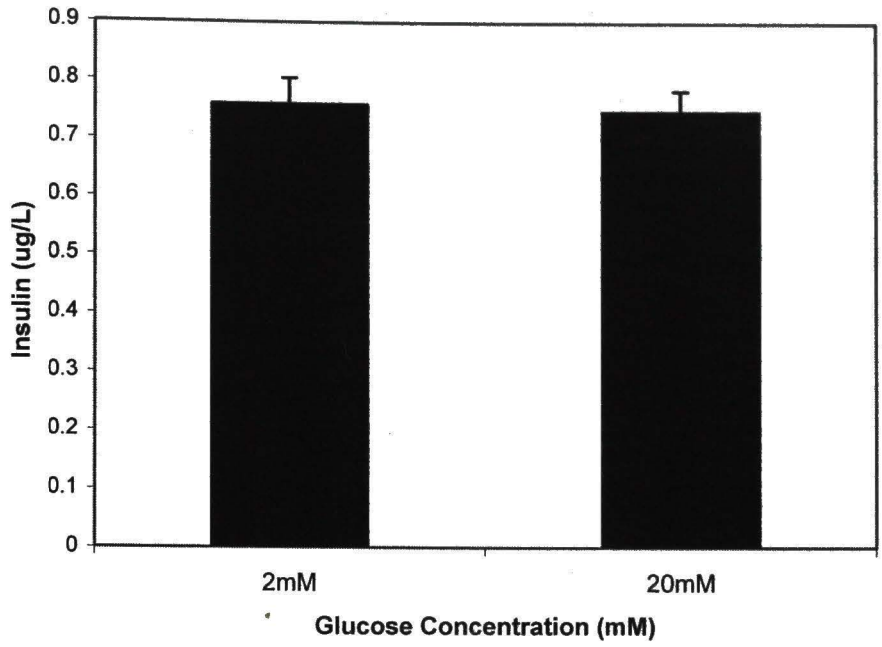

Canine Islet Insulin Secretion
% Change
160
140
120
100
80
60
40
20
0
2mM
20mM
Glucose Concentration (mM)

Figure 8. Canine EECs insulin secretion (20,000 cells) in response to 2mM glucose and 20mM glucose. EECs were incubated in 2mM glucose for 1 hr (media collected) and 20mM glucose for 1 hr (media collected). Insulin secretion was measured by ELISA and is presented as insulin (μg/L) (A) and % change (B).


Canine EECs Insulin Secretion
0.9
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Insulin (ug/L)
2mM
20mM
Glucose Concentration (mM)

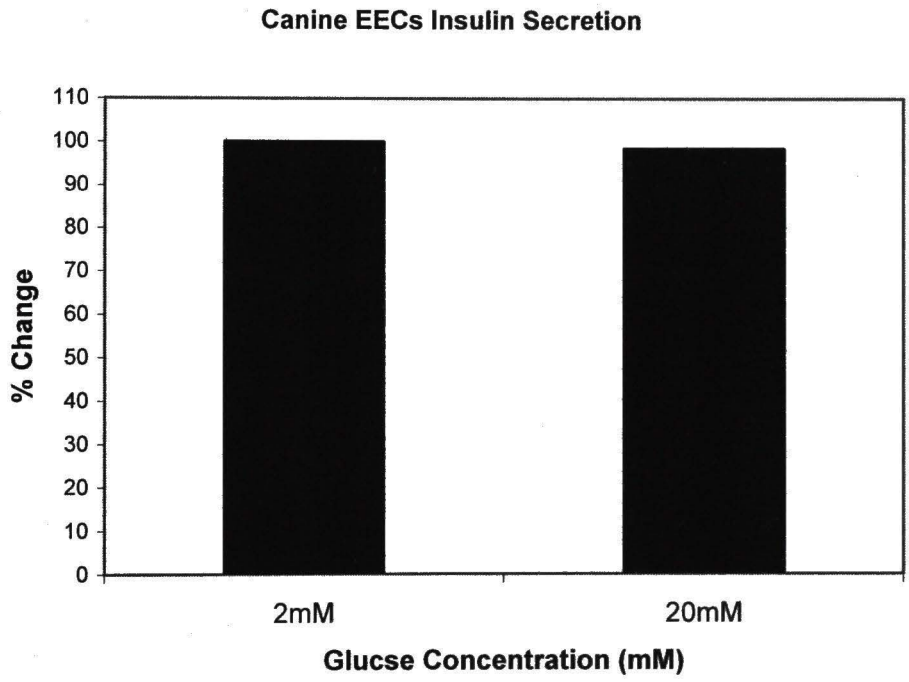
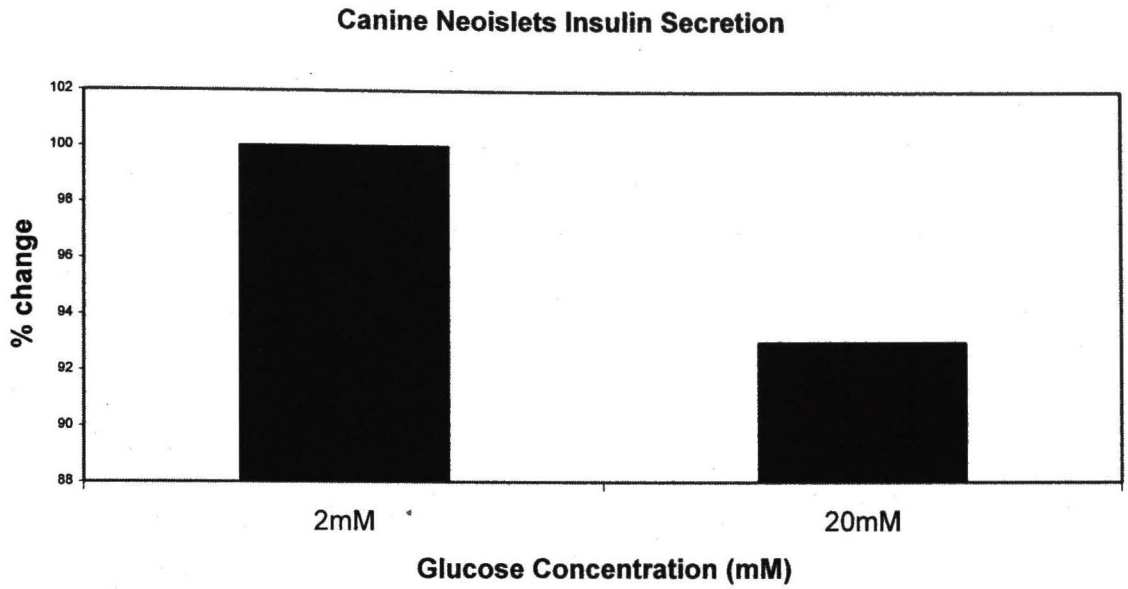

Canine EECs Insulin Secretion
110
100
90
80
70
60
50
40
30
20
10
0
% Change
2mM
20mM
Glucse Concentration (mM)

Figure 9. Canine neoislet insulin secretion in response to 2mM glucose and 20mM glucose. Canine neoislets (25/tube) were incubated in 2mM glucose for 1hr (media collected) and 20mM glucose for 1 hr (media collected). Insulin secretion was measured by ELISA and is presented as insulin (μg/L) (A) and % change (B).


Canine Neoislets Insulin Secretion
% change
102
100
98
96
94
92
90
88
2mM
20mM
Glucose Concentration (mM)

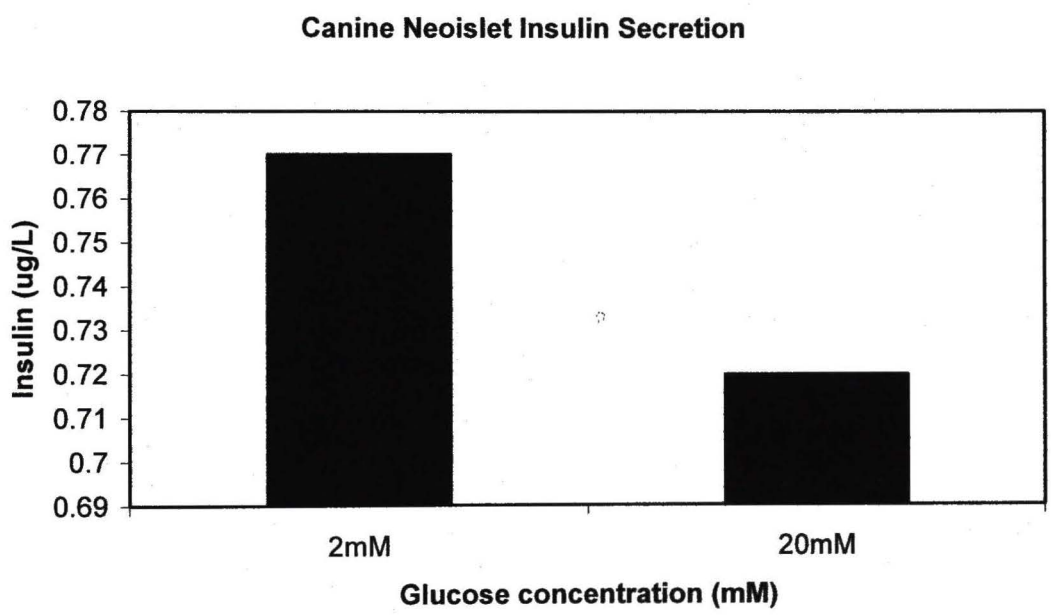

Canine Neoislet Insulin Secretion
Insulin (ug/L)
0.78
0.77
0.76
0.75
0.74
0.73
0.72
0.71
0.7
0.69
2mM
20mM
Glucose concentration (mM)

Figure 10. Porcine neoislet insulin secretion in response to 3mM glucose, 20mM glucose and 20mM glucose +10μM forskolin. Porcine neoislets were incubated in 3mM for 1 hr (media collected) and 20mM glucose or 20mM glucose +10μM forskolin for 1hr (media collected). Insulin secretion was measured by ELISA presented as insulin (μg/L) (A) and % change (B).

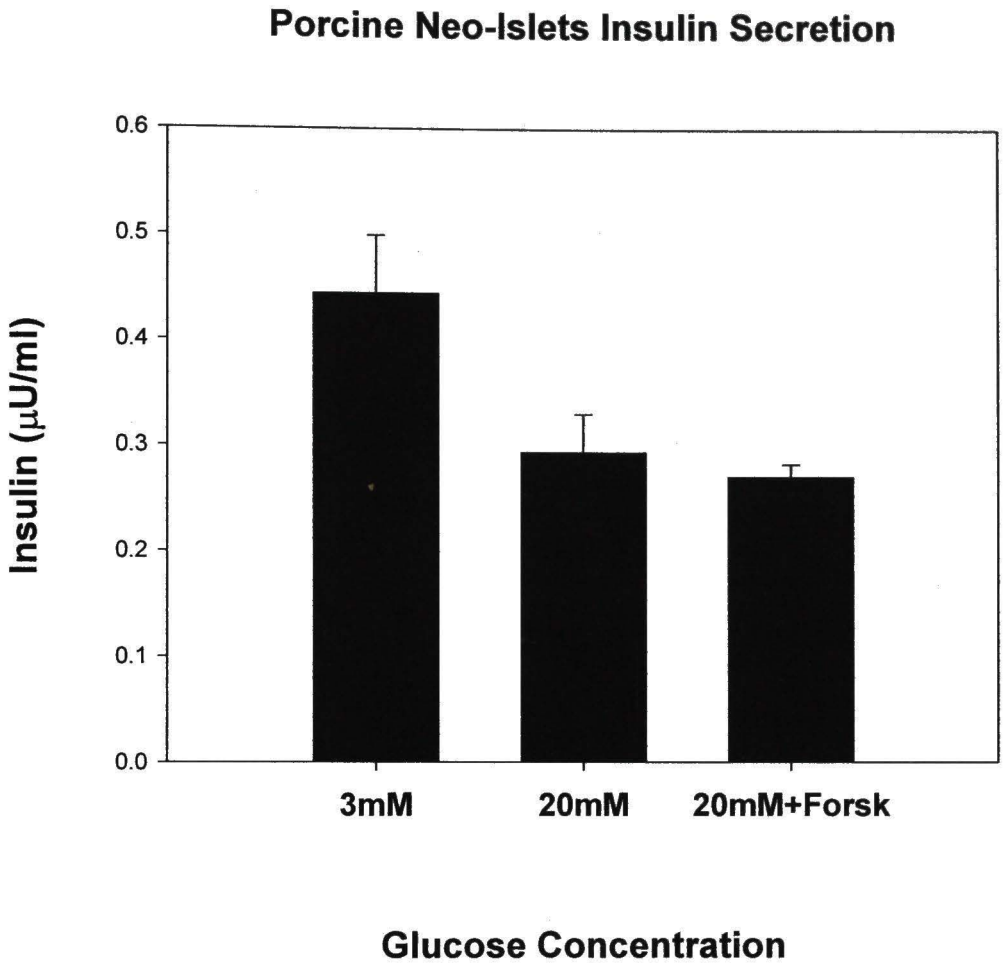

Porcine Neo-Islets Insulin Secretion
Insulin (μU/ml)
0.6
0.5
0.4
0.3
0.2
0.1
0.0
3mM
20mM
20mM+Forsk
Glucose Concentration

Figure 11. Ins-1 cells and Ins-1 neoislet insulin secretion in response to a glucose challenge. Ins-1 cells (20,000 cells/well) and Ins-1 neoislets (25 neoislets/tube) were incubated at 2mM glucose for 1 hr (media collected) and 20mM glucose for 1 hr (media collected). Insulin secretion was measured by ELISA.

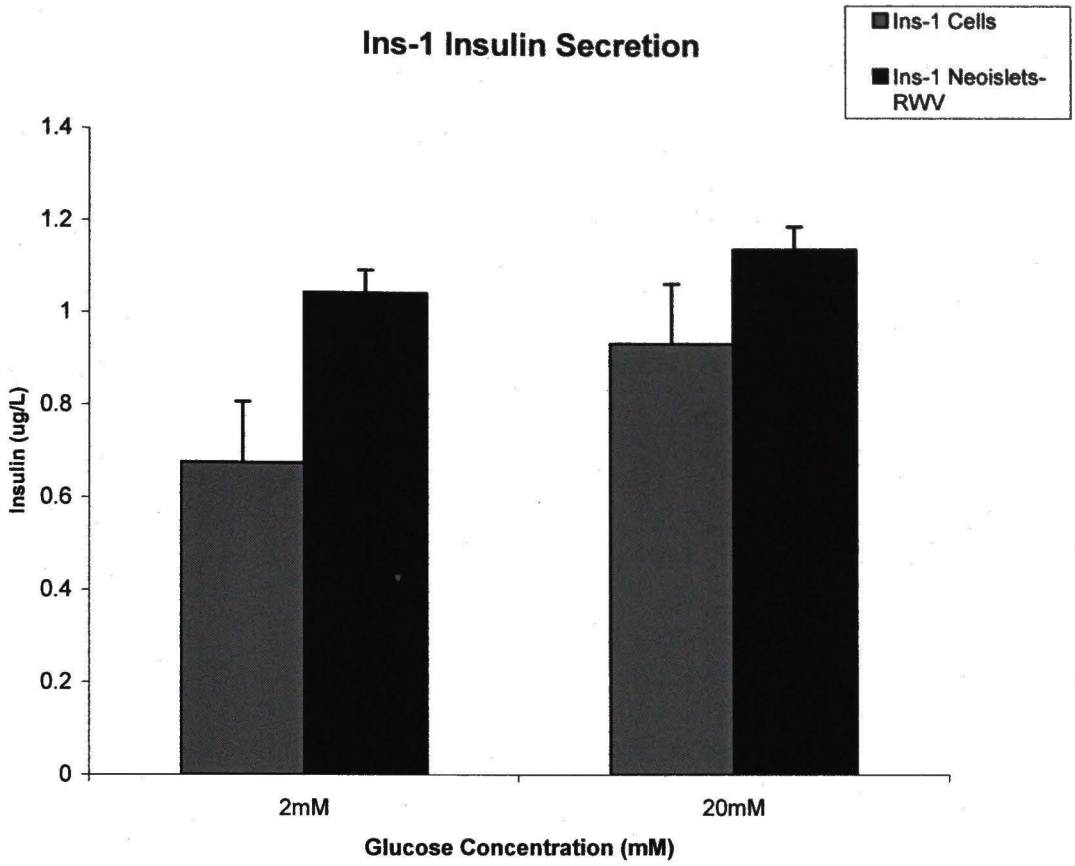

Ins-1 Insulin Secretion
Ins-1 Cells
Ins-1 Neoislets-RWV
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Insulin (ug/L)
2mM
20mM
Glucose Concentration (mM)

Figure 12. Ins-1 cells insulin secretion in response to 2mM glucose and 20mM glucose or 20mM glucose +10nM GLP-1 at 20, 40 and 60 min. Ins-1 cells (20,000 cells/well) were incubated at 2mM glucose and 20mM glucose or 20mM glucose +10nM GLP-1 for 20, 40, and 60 min. Insulin secretion was measured by ELISA.

Ins-1 Insulin Secretion

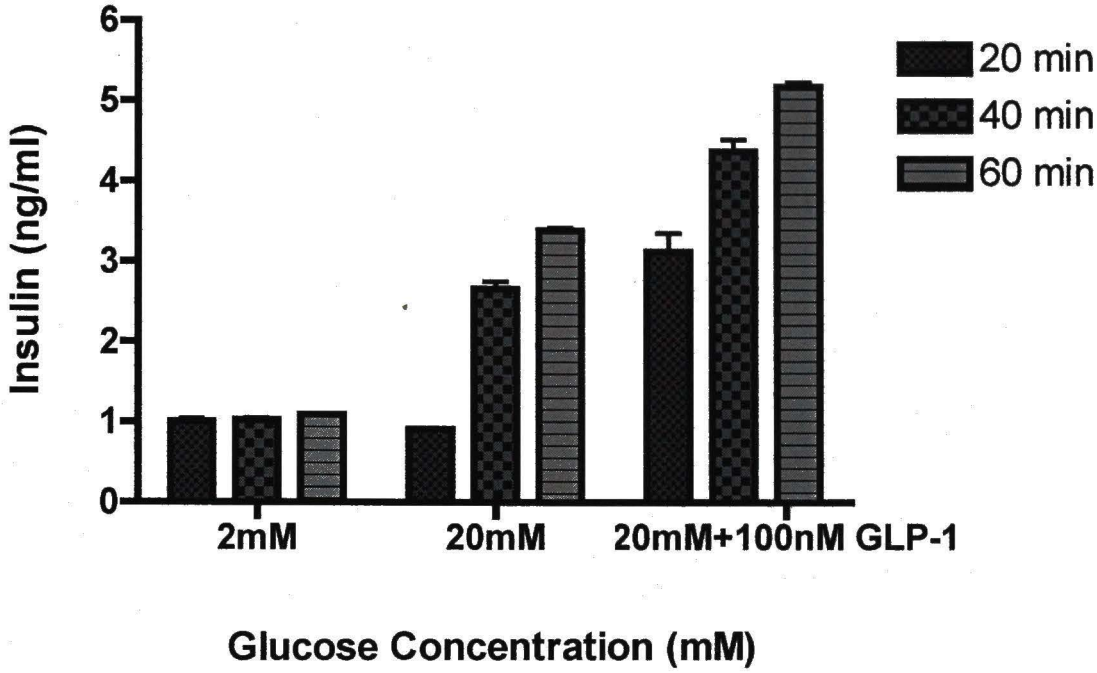
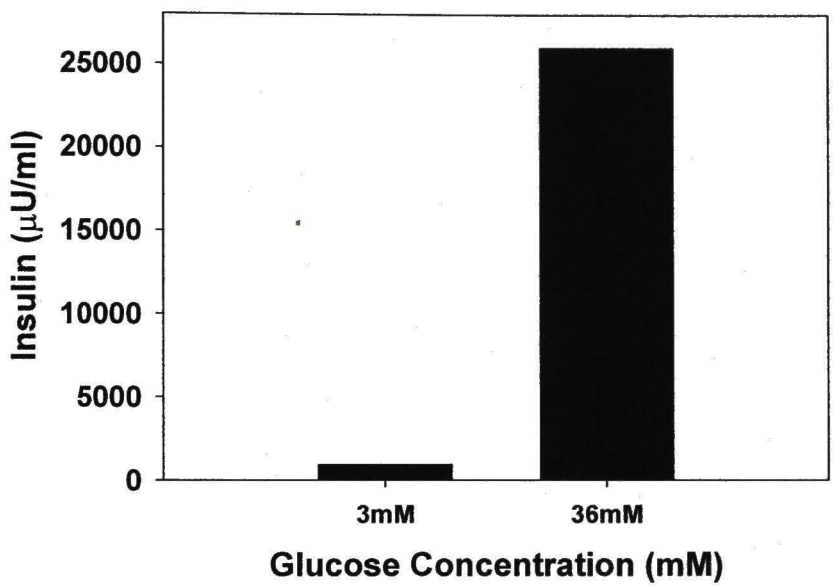


Figure 13. Glucose challenge on human islets embedded in a collagen matrix. Human islets were embedded in a type 1 collagen gel and incubated in 3mM glucose for 12 hr (media collected) and 36mM for 12 hr (media collected). Insulin secretion measured by RIA.


25000
20000
15000
10000
5000
0
Insulin (μU/ml)
3mM
36mM
Glucose Concentration (mM)

CHAPTER V

CONCLUSIONS AND FUTURE DIRECTIONS

The incidence of diabetes mellitus currently afflicts over 16 million people in the United States. Due to the recent epidemic of obesity in the western world, the number of new cases of diabetes diagnosed each year is consistently on the rise. Several debilitating side effects including blindness, peripheral neuropathy, amputations, end-stage renal failure, heart attack and stroke can occur due to chronic disregulation of glucose homeostasis. Currently, the treatment for diabetes includes regulation of blood glucose with exogenous insulin therapy. Even the tightest regulation of glucose may lead to dramatic fluctuations in circulating glucose levels which can lead to the aforementioned side effects. Pancreatic transplants are limited by the number of available organs, and the procedure is associated with high mortality and morbidity. Recently, islet transplantation has become a viable alternative due to extensive study in this research area. The procedure is relatively low-risk and can restore glucose homeostasis, however there are several limitations to this approach. The major obstacle includes the shortage of islets available for transplantation. Because the procedure requires 2-4 donors per recipient, the number of islets available cannot fulfill the demand. Therefore, the generation of alternate sources of glucose-responsive, insulin-secreting tissue is being pursued.

The purpose of these studies was to identify and characterize an intraislet progenitor pool of cells and effect their aggregation into a glucose-responsive neoislet. We have shown that we can isolate, culture and expand endocrine epithelial cells derived from adult islets of several species (rat, human, porcine and canine). The study of canine islet cells became an alternative resort due to poor availability sporadic delivery of porcine and human tissue. However, canine tissue is particularly pertinent in this study because the canine species develops IDDM in the same way as humans. As a result, a protocol for canine islet isolation was established based on Ricordi's automated method. Although islet isolations were variable, large numbers of quality islets were obtained. The RWV may be used as a tool in islet isolation, as it can be custom designed and can serve as an automated step, thereby eliminating some of the mechanical demands. For example, a RWV with two separate chambers (one for the pancreas, the other to collect freed islets), separated by a mesh screen, with directed flow, would allow the passage of islets released from the digesting pancreas. Furthermore, islets could be easily removed from the chamber and washed, to prevent further digestion.

Endocrine epithelial cells were well characterized by immunofluorescence and western blot analysis. Coexpression of endocrine hormones (insulin, glucagon and somatostatin) was shown in EECs. The expression of PDX-1 was of particular interest because it is a marker of β cell progenitors and is found in differentiated β cells. However, other transcription factors involved during islet cell development need to be investigated to make sure that EECs do not lack a critical transcription factor that is involved in the terminal differentiation of β cell progenitors. Perhaps establishing a

profile of transcription factors in EECs as well as neoislets would lead to potential targets for differentiation.

In these studies, differentiation is a complex process of organogenesis and aggregation of the EECs into neoislets, organization of α, β, δ and pp cells, as well as maturation of the neoislets into glucose-responsive insulin secreting structures. If in fact, differentiation of EECs begins with aggregation, the best practical method and a detailed mechanism that modulates this process must be defined. Having shown that the RWV cell culture system is the best method for aggregation, our goal was to determine how EECs aggregate into neoislets. Future studies to further optimize the use of the RWV to generate neoislets may include the determination of the relationship between rotation speed and neoislet size and number. We identified FGF2 as a key player in EEC aggregation mechanism due to its differentiation role in development of the pancreas *in vivo,* and its contribution to spontaneous aggregation of EECs *in vitro* in 2-D cultures. We have shown that FGF2 induced aggregation of EECs works via the activation of the calcium dependent cadherin-catenin complex. PKC signaling was also shown to play a role in aggregation/differentiation. However, the relationship between PKC signaling and cadherin-catenin assembly has not been established. Perhaps β-catenin is activated by PKC, allowing for more interactions with cadherin to occur. Other cell-cell interactions need to also be examined, for example, gap junctional proteins such as connexins. Future studies may include the identification of downstream targets of PKC. The definition of a PKC profile in native islets, neoislets and EECs was a necessary step in identifying the isoform profiles that would allow a specific isoform involved in

differentiation to be identified. Of the ten isoforms, only PKC-α, BII, ε and ζ were expressed in the above cells and aggregates. The expression of these PKC isoforms is a necessary starting point from which to explore the exact pathway involved in EEC differentiation. Studies defining the determination of PKC isoform activity and/or translocation to the plasma membrane would yield more definitive conclusions Identification of downstream targets of these PKC isoforms, such as MAPK, would provide a more detailed mechanism involved in the differentiation of neoislets as well as provide potential targets for inducing maturation.

The final goal of this dissertation was to achieve glucose responsiveness of neoislets. EECs have been shown to express insulin, contain dense secretory granules and express two essential components of a functional β cell, yet glucose responsive insulin secretion and insulin content of EECs and neoislets is insignificant. FGF2 may contribute to, but is not sufficient enough to establish functional maturity of neoislets. Although the aggregation studies using the RWV implicate PKC as a major player in FGF2-induced differentiation, the mechanism remains unclear. The lack of glucose responsiveness in endocrine progenitor cells is a common hurdle encountered by everyone working in this field. The most promising results have come from *in vivo* studies in which islet progenitors can restore normoglycemia in a diabetic animal. However, maturation must be achieved *in vitro* in order for this strategy to be useful in islet transplantation. Perhaps the complete differentiation of neoislets involves signals from the mesenchyme or factors that come in contact with the islets via angiogenesis or innervation. A three-dimensional collagen matrix would provide an environment that

could be manipulated (coculture with fibroblasts or neuronal cells) to provide some of the necessary components found *in vivo* that contribute to islet cell differentiation. The RWV would be a useful tool in sustaining these tissue equivalents as well as providing an environment, which allows for glucose challenge experiments to be performed with ease. In addition, the RWV may be used to transport islets for transplants, as it has been shown to protect the fragile islets as well as maintain their insulin secretion capability for a longer period of time. Although, advances have been made in maturation process of neoislets in vivo, the ultimate goal is to generate a functionally mature neoislet *in vitro,* which would provide a solution to the lack of available islets for transplantation in diabetic patients.

REFERENCES

1. Bonner-Weir, S., *Life and death of the pancreatic beta cells.* Trends in Endocrinology & Metabolism, 2000. **11**(9): p. 375-8.
2. Geiger, M., et al., *Defining the human targets of phorbol ester and diacylglycerol.* Curr Opin Mol Ther, 2003. **5**(6): p. 631-41.
3. Tulachan, S.S., et al., *All-trans retinoic acid induces differentiation of ducts and endocrine cells by mesenchymal/epithelial interactions in embryonic pancreas.* Diabetes, 2003. **52**(1): p. 76-84.
4. Bikle, D.D., et al., *Calcium- and vitamin D-regulated keratinocyte differentiation.* Mol Cell Endocrinol, 2001. **177**(1-2): p. 161-71.
5. Cras-Meneur, C., et al., *Epidermal growth factor increases undifferentiated pancreatic embryonic cells in vitro: a balance between proliferation and differentiation.* Diabetes, 2001. **50**(7): p. 1571-9.
6. Kim, S.K. and R.J. MacDonald, *Signaling and transcriptional control of pancreatic organogenesis.* Curr Opin Genet Dev, 2002. **12**(5): p. 540-7.
7. Johansson, K.A. and A. Grapin-Botton, *Development and diseases of the pancreas.* Clin Genet, 2002. **62**(1): p. 14-23.
8. Edlund, H., *Pancreatic organogenesis--developmental mechanisms and implications for therapy.* Nat Rev Genet, 2002. **3**(7): p. 524-32.
9. Slack, J.M., *Developmental biology of the pancreas.* Development, 1995. **121**(6): p. 1569-80.
10. Sander, M. and M.S. German, *The beta cell transcription factors and development of the pancreas.* J Mol Med, 1997. **75**(5): p. 327-40.
11. Githens, S., *The pancreatic duct cell: proliferative capabilities, specific characteristics, metaplasia, isolation, and culture.* J Pediatr Gastroenterol Nutr, 1988. **7**(4): p. 486-506.
12. Bonner-Weir, S., *Islet growth and development in the adult.* Journal of Molecular Endocrinology, 2000. **24**(3): p. 297-302.
13. Bonner-Weir, S. and A. Sharma, *Pancreatic stem cells.* J Pathol, 2002. **197**(4): p. 519-26.
14. Ryan, E.A., et al., *Long-term consequences in offspring of diabetes in pregnancy: studies with syngeneic islet-transplanted streptozotocin-diabetic rats.* Endocrinology, 1995. **136**(12): p. 5587-92.
15. Edlund, H., *Transcribing pancreas.* Diabetes, 1998. **47**(12): p. 1817-23.
16. Edlund, H., *Pancreas: how to get there from the gut?* Curr Opin Cell Biol, 1999. **11**(6): p. 663-8.
17. St-Onge, L., et al., *Pax6 is required for differentiation of glucagon-producing alpha-cells in mouse pancreas.* Nature, 1997. **387**(6631): p. 406-9.
18. Song, Z. and K. Gu, *[Transdifferentiating of adult human pancreatic stem cells into islet cells].* Zhonghua Wai Ke Za Zhi, 2002. **40**(11): p. 807-10.

19. Jensen, J., et al., *Independent development of pancreatic alpha- and beta-cells from neurogenin3-expressing precursors: a role for the notch pathway in repression of premature differentiation.* Diabetes, 2000. **49**(2): p. 163-76.
20. Apelqvist, A., et al., *Notch signalling controls pancreatic cell differentiation.* Nature, 1999. **400**(6747): p. 877-81.
21. Gradwohl, G., et al., *neurogenin3 is required for the development of the four endocrine cell lineages of the pancreas.* Proc Natl Acad Sci U S A, 2000. **97**(4): p. 1607-11.
22. Schwitzgebel, V.M., et al., *Expression of neurogenin3 reveals an islet cell precursor population in the pancreas.* Development, 2000. **127**(16): p. 3533-42.
23. Ahlgren, U., et al., *Independent requirement for ISL1 in formation of pancreatic mesenchyme and islet cells.* Nature, 1997. **385**(6613): p. 257-60.
24. Naya, F.J., et al., *Diabetes, defective pancreatic morphogenesis, and abnormal enteroendocrine differentiation in BETA2/neuroD-deficient mice.* Genes Dev, 1997. **11**(18): p. 2323-34.
25. Sosa-Pineda, B., et al., *The Pax4 gene is essential for differentiation of insulin-producing beta cells in the mammalian pancreas.* Nature, 1997. **386**(6623): p. 399-402.
26. Wang, H., et al., *Activation of c-Jun amino-terminal kinase is required for retinoic acid-induced neural differentiation of P19 embryonal carcinoma cells.* FEBS Lett, 2001. **503**(1): p. 91-6.
27. Sander, M., et al., *Homeobox gene Nkx6.1 lies downstream of Nkx2.2 in the major pathway of beta-cell formation in the pancreas.* Development, 2000. **127**(24): p. 5533-40.
28. Wilson, M.E., D. Scheel, and M.S. German, *Gene expression cascades in pancreatic development.* Mech Dev, 2003. **120**(1): p. 65-80.
29. Macdonald, M.J., *Export of metabolites from pancreatic islet mitochondria as a means to study anaplerosis in insulin secretion.* Metabolism, 2003. **52**(8): p. 993-8.
30. Yu, W., et al., *Synergism of protein kinase A, protein kinase C, and myosin light-chain kinase in the secretory cascade of the pancreatic beta-cell.* Diabetes, 2000. **49**(6): p. 945-52.
31. Champe, P.C.a.H., Richard A., *Biochemistry 2nd Edition.* 2nd ed, ed. J. Schott. 1994, Philadelphia: Lippincott-Raven Publishers. 443.
32. http://diabetes.niddk.nih.gov/dm/pubs/statistics/index.htm#7.
33. Halvorsen, T. and F. Levine, *Diabetes mellitus-cell transplantation and gene therapy approaches.* Curr Mol Med, 2001. **1**(2): p. 273-86.
34. Kelly, M.A., et al., *Molecular aspects of type 1 diabetes.* Mol Pathol, 2003. **56**(1): p. 1-10.
35. Atkinson, M.A. and G.S. Eisenbarth, *Type 1 diabetes: new perspectives on disease pathogenesis and treatment.* Lancet, 2001. **358**(9277): p. 221-9.
36. Eastman, R.C., *Is intensive glycemic control worth the expense?* Cleve Clin J Med, 1997. **64**(8): p. 410-3.

37. Gepts, W., *Pathologic anatomy of the pancreas in juvenile diabetes mellitus.* Diabetes, 1965. **14**(10): p. 619-33.
38. Leslie, C.A., *New insulin replacement technologies: overcoming barriers to tight glycemic control.* Cleve Clin J Med, 1999. **66**(5): p. 293-302.
39. Boitard, C., et al., *Lymphocytes from diabetics suppress insulin release in vitro.* Diabetologia, 1981. **21**(1): p. 41-6.
40. Todd, J.A., et al., *Genetic analysis of autoimmune type 1 diabetes mellitus in mice.* Nature, 1991. **351**(6327): p. 542-7.
41. Knip, M. and H.K. Akerblom, *Environmental factors in the pathogenesis of type 1 diabetes mellitus.* Exp Clin Endocrinol Diabetes, 1999. **107 Suppl 3**: p. S93-100.
42. Nerup, J., et al., *HL-A antigens and diabetes mellitus.* Lancet, 1974. **2**(7885): p. 864-6.
43. Bell, G.I. and R. Sanchez-Pescador, *Sequence of a cDNA encoding Syrian hamster preproinsulin.* Diabetes, 1984. **33**(3): p. 297-300.
44. Davies, M.J., et al., *Loss of the first phase insulin response to intravenous glucose in subjects with persistent impaired glucose tolerance.* Diabet Med, 1994. **11**(5): p. 432-6.
45. Curry, D.L., L.L. Bennett, and G.M. Grodsky, *Dynamics of insulin secretion by the perfused rat pancreas.* Endocrinology, 1968. **83**(3): p. 572-84.
46. Efendic, S., *Pathogenesis of NIDDM.* Diabetes Res Clin Pract, 1988. **4 Suppl 1**: p. 8-10.
47. Fajans, S.S., *Maturity-onset diabetes of the young (MODY).* Diabetes Metab Rev, 1989. **5**(7): p. 579-606.
48. Ogawa, A., et al., *Roles of insulin resistance and beta-cell dysfunction in dexamethasone-induced diabetes.* J Clin Invest, 1992. **90**(2): p. 497-504.
49. Kaczorowski, D.J., et al., *Glucose-responsive insulin-producing cells from stem cells.* Diabetes Metab Res Rev, 2002. **18**(6): p. 442-50.
50. Logdberg, L., et al., *Islet transplantation, stem cells, and transfusion medicine.* Transfus Med Rev, 2003. **17**(2): p. 95-109.
51. Shapiro, A.M. and J.R. Lakey, *Future trends in islet cell transplantation.* Diabetes Technol Ther, 2000. **2**(3): p. 449-52.
52. Sollinger, H.W., et al., *Experience with 500 simultaneous pancreas-kidney transplants.* Ann Surg, 1998. **228**(3): p. 284-96.
53. Manske, C.L., *Risks and benefits of kidney and pancreas transplantation for diabetic patients.* Diabetes Care, 1999. **22 Suppl 2**: p. B114-20.
54. Hui, H., C. Wright, and R. Perfetti, *Glucagon-like peptide 1 induces differentiation of islet duodenal homeobox-1-positive pancreatic ductal cells into insulin-secreting cells.* Diabetes, 2001. **50**(4): p. 785-96.
55. Hauptman, P.J. and K.J. O'Connor, *Procurement and allocation of solid organs for transplantation.* N Engl J Med, 1997. **336**(6): p. 422-31.
56. Ricordi, C., et al., *Automated method for isolation of human pancreatic islets.* Diabetes, 1988. **37**(4): p. 413-20.

57. Lake, S.P., et al., *Large-scale purification of human islets utilizing discontinuous albumin gradient on IBM 2991 cell separator.* Diabetes, 1989. **38 Suppl 1**: p. 143-5.
58. Bonner-Weir, S., *beta-cell turnover: its assessment and implications.* Diabetes, 2001. **50 Suppl 1**: p. S20-4.
59. Ramiya, V.K., et al., *Reversal of insulin-dependent diabetes using islets generated in vitro from pancreatic stem cells.* Nat Med, 2000. **6**(3): p. 278-82.
60. Zulewski, H., et al., *Multipotential nestin-positive stem cells isolated from adult pancreatic islets differentiate ex vivo into pancreatic endocrine, exocrine, and hepatic phenotypes.* Diabetes, 2001. **50**(3): p. 521-33.
61. Soria, B., et al., *Insulin-secreting cells derived from embryonic stem cells normalize glycemia in streptozotocin-induced diabetic mice.* Diabetes, 2000. **49**(2): p. 157-62.
62. Lumelsky, N., et al., *Differentiation of embryonic stem cells to insulin-secreting structures similar to pancreatic islets.* Science, 2001. **292**(5520): p. 1389-94.
63. Assady, S., et al., *Insulin production by human embryonic stem cells.* Diabetes, 2001. **50**(8): p. 1691-7.
64. Bonner-Weir, S., et al., *A second pathway for regeneration of adult exocrine and endocrine pancreas. A possible recapitulation of embryonic development.* Diabetes, 1993. **42**(12): p. 1715-20.
65. Ferber, S., et al., *Pancreatic and duodenal homeobox gene 1 induces expression of insulin genes in liver and ameliorates streptozotocin-induced hyperglycemia.* Nat Med, 2000. **6**(5): p. 568-72.
66. Mashima, H., et al., *Betacellulin and activin A coordinately convert amylase-secreting pancreatic AR42J cells into insulin-secreting cells.* J Clin Invest, 1996. **97**(7): p. 1647-54.
67. Abraham, E.J., et al., *Insulinotropic hormone glucagon-like peptide-1 differentiation of human pancreatic islet-derived progenitor cells into insulin-producing cells.* Endocrinology, 2002. **143**(8): p. 3152-61.
68. Buteau, J., et al., *Protein kinase Czeta activation mediates glucagon-like peptide-1-induced pancreatic beta-cell proliferation.* Diabetes, 2001. **50**(10): p. 2237-43.
69. Hussain, M.A. and J.F. Habener, *Glucagon-like peptide 1 increases glucose-dependent activity of the homeoprotein IDX-1 transactivating domain in pancreatic beta-cells.* Biochem Biophys Res Commun, 2000. **274**(3): p. 616-9.
70. Arany, E. and D.J. Hill, *Ontogeny of fibroblast growth factors in the early development of the rat endocrine pancreas.* Pediatr Res, 2000. **48**(3): p. 389-403.
71. Otonkoski, T., et al., *Hepatocyte growth factor/scatter factor has insulinotropic activity in human fetal pancreatic cells.* Diabetes, 1994. **43**(7): p. 947-53.
72. Le Bras, S., et al., *Fibroblast growth factor 2 promotes pancreatic epithelial cell proliferation via functional fibroblast growth factor receptors during embryonic life.* Diabetes, 1998. **47**(8): p. 1236-42.
73. Hebrok, M., S.K. Kim, and D.A. Melton, *Notochord repression of endodermal Sonic hedgehog permits pancreas development.* Genes Dev, 1998. **12**(11): p. 1705-13.

74. Powers, C.J., S.W. McLeskey, and A. Wellstein, *Fibroblast growth factors, their receptors and signaling.* Endocr Relat Cancer, 2000. **7**(3): p. 165-97.
75. Hardikar, A.A., et al., *Human pancreatic precursor cells secrete FGF2 to stimulate clustering into hormone-expressing islet-like cell aggregates.* Proc Natl Acad Sci U S A, 2003. **100**(12): p. 7117-22.
76. Dahl, U., A. Sjodin, and H. Semb, *Cadherins regulate aggregation of pancreatic beta-cells in vivo.* Development, 1996. **122**(9): p. 2895-902.
77. Geiger, B.a.A., O., *Cadherins.* Annu. Rev. Cell Biol., 1992. **8**: p. 307-332.
78. Ozawa, M., H. Baribault, and R. Kemler, *The cytoplasmic domain f the cell adhesion molecule uvomorulin associates with tree independent proteins structurally related in different species.* Embo J, 1989. **8**: p. 1711-1717.
79. Hoschuetzky, H., Aberle, H. and Kemler, R, *B-catenin Mediates the Interaction of the Cadherin-Catenin Complex with Epidermal Growth Factor Receptor.* J Cell Biol, 1994. **127**(5): p. 1375-1380.
80. Matsuyoshi, N., et al., *Cadherin-mediated cell-cell adhesion is perturbed by v-src tyrosine phosphorylation in metastatic fibroblasts.* J Cell Biol, 1992. **118**(3): p. 703-14.
81. Shibamoto, S., et al., *Tyrosine phosphorylation of beta-catenin and plakoglobin enhanced by hepatocyte growth factor and epidermal growth factor in human carcinoma cells.* Cell Adhes Commun, 1994. **1**(4): p. 295-305.
82. Hamid, M., et al., *Comparison of the secretory properties of four insulin-secreting cell lines.* Endocr Res, 2002. **28**(1-2): p. 35-47.
83. Hohmeier, H.E., et al., *Regulation of insulin secretion from novel engineered insulinoma cell lines.* Diabetes, 1997. **46**(6): p. 968-77.
84. Asfari, M., et al., *Establishment of 2-mercaptoethanol-dependent differentiated insulin-secreting cell lines.* Endocrinology, 1992. **130**(1): p. 167-78.
85. Chepurny, O.G. and G.G. Holz, *Over-expression of the glucagon-like peptide-1 receptor on INS-1 cells confers autocrine stimulation of insulin gene promoter activity: a strategy for production of pancreatic beta-cell lines for use in transplantation.* Cell Tissue Res, 2002. **307**(2): p. 191-201.
86. Hammond, T.G. and J.M. Hammond, *Optimized suspension culture: the rotating-wall vessel.* Am J Physiol Renal Physiol, 2001. **281**(1): p. F12-25.
87. Khaoustov, V.I., et al., *Induction of three-dimensional assembly of human liver cells by simulated microgravity.* In Vitro Cell Dev Biol Anim, 1999. **35**(9): p. 501-9.
88. www.biocarta.com.
89. Bonner-Weir, S., et al., *In vitro cultivation of human islets from expanded ductal tissue.* Proceedings of the National Academy of Sciences of the United States of America, 2000. **97**(14): p. 7999-8004.
90. Finegood, D.T., *Delineation of an apparent threshold for glycemic normalization after intraportal islet transplantation in streptozotocin-diabetic rats.* Transplant Proc, 1994. **26**(2): p. 663.
91. Ryan, F., et al., *Hyperinsulinism: molecular aetiology of focal disease.* Arch Dis Child, 1998. **79**(5): p. 445-7.

92. Thomson JA, I.-E.J., Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS et al., *Embryonic stem cell lines derived from human blastocysts.* Science, 1998. **282**: p. 1145-7.
93. Scharfmann, R., *Alternative sources of beta cells for cell therapy of diabetes.* European Journal of Clinical Investigation, 2003. **33**: p. 595-600.
94. Fernandes, A., et al., *Differentiation of new insulin-producing cells is induced by injury in adult pancreatic islets.* Endocrinology, 1997. **138**(4): p. 1750-62.
95. Gu G, D.J., Melton DA., *Direct evidence for the pancreatic lineage: NGN3+ cells are islet progenitors and are disctinct from duct progenitors.* Development, 2002. **129**: p. 2447-57.
96. Lendahl U, Z.L., McKay RD., *CNS stem cells express a new class of intermediate filament protein.* Cell, 1990. **60**: p. 585-95.
97. Selander L., E.H., *Nestin is expressed in mesenchymal and not epithelial cells of the developing mouse pancreas.* Mech Dev, 2002. **113**: p. 189-92.
98. Lardon J, R.I., Bouwens L., *Nestin expression in pancreatic stellate cells and angiogenic endothelial ells.* Histochem Cell Biol, 2002. **117**: p. 535-40.
99. Watada, H., et al., *Distinct gene expression programs function in progenitor and mature islet cells.* J Biol Chem, 2003. **278**(19): p. 17130-40.
100. Brissova M, M.S., Nicholson W, Gannon M, Knobel S, Piston D, Wright C, Powers, A., *Reduction in Pancreatic Transcription Factor PDX-1 Impairs Glucose-stimulated Insulin Secretion.* J Biol Chem, 2002. **277**(13): p. 11225-11232.
101. Tobin B, L.-W.S., Hashemi B, Smith S, Sams C., *Altered TNF-a, glucose, insulin and amino acids in islets of Langerhans cultured in a microgravity model system.* Am J Physiol Endocrinol Metab, 2001. **280**: p. E92-E102.
102. Soria, B., *In-vitro differentiation of pancreatic beta-cells.* Differentiation, 2001. **68**(4-5): p. 205-19.
103. Kaneto, H., et al., *Involvement of protein kinase C beta 2 in c-myc induction by high glucose in pancreatic beta-cells.* J Biol Chem, 2002. **277**(5): p. 3680-5.
104. Nishizuka, Y., *The molecular heterogeneity of protein kinase C and its implications for cellular regulation.* Nature, 1988. **334**: p. 661-665.
105. Parekh, D.B., W. Ziegler, and P.J. Parker, *Multiple pathways control protein kinase C phosphorylation.* Embo J, 2000. **19**(4): p. 496-503.
106. Kahshiwagi M, O.M., Chida K, Kuroki, T, *PKCn: Its Involvement in Keratinocyte Differentiation.* J Biochem, 2002. **192**: p. 853-857.
107. Elghazi, L., et al., *Role for FGFR2IIIb-mediated signals in controlling pancreatic endocrine progenitor cell proliferation.* Proc Natl Acad Sci U S A, 2002. **99**(6): p. 3884-9.
108. Peck, A.B., et al., *Generation of islets of Langerhans from adult pancreatic stem cells.* J Hepatobiliary Pancreat Surg, 2002. **9**(6): p. 704-9.
109. Cornelius, J.G., et al., *In vitro-generation of islets in long-term cultures of pluripotent stem cells from adult mouse pancreas.* Horm Metab Res, 1997. **29**(6): p. 271-7.