University of Alberta

Characterization of Human Pancreatic Beta-cell Progenitors as a Means to Alleviate the Shortage of Donor Tissue for Islet Transplantation

by

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Abstract

The current shortage of islet tissue from cadaveric human donors is not sustainable and will preclude islet transplantation from becoming a widespread therapeutic treatment for those with type 1 diabetes. A potential alternative source of tissue may be through isolating, expanding, and differentiating beta-cell progenitors within the adult human pancreas. We sought to characterize the population of origin of beta-cell progenitors.

Our human studies use pancreatic beta-cell progenitor marker, Pdx-1 to study the development and origin of beta-cells. *In vivo* immunohistochemical study of fetal development demonstrates morphologic evolution of islets throughout maturation and expression and interaction of several factors important to beta-cell development. *In vitro* study utilizes a novel cell culture media permitting long-term study of the epithelial population within the adult pancreas as well as lentiviral reporters and immunostaining to observe the differentiation of potential progenitor cells. Together our data suggests that Pdx-1 expressing progenitor cells are epithelial cells.

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List of Abbreviations

α	glucagon producing
β	insulin producing
δ	somatostatin producing
ε	ghrelin producing
ARX	aristaless related homeobox
BMP	bone morphogenic protein
CAII	carbonic anhydrase II
CFU-F	colony-forming unit fibroblastic
CK19	cytokeratin 19
Cpa-1	carboxypeptidase A
DD	double diabetes
EGF	epidermal growth factor
EGFP	enhanced green fluorescent protein
EMT	epithelial-to-mesenchymal transition
EpCAM	epithelial cell adhesion molecule
ESCs	embryonic stem cells
FACS	fluorescence activated cell sorting
FBS	fetal bovine serum
FGF	fibroblast growth factor
Hes1	hairy enhancer of split 1
HGF	hepatocyte growth factor
HLA	human leukocyte antigen
IDPT	islet depleted pancreatic tissue
IF	immunofluorescence

INF	interferon
INGAP	islet neogenesis-associated protein
Ins1	*Insulin 1*
ISCs	islet survivor cells
LADA	latent autoimmune diabetes in adults
LIF	leukaemia inhibitory factor
MIAMI	multilineage inducible
MODY	mature onset diabetes of the young
mRFP	monomeric red fluorescent protein
MSCs	mesenchymal stem cells
NGS	normal goat serum
OHSU	Oregon Health and Science University
PBS	phosphate buffered saline
PCNA	proliferating cell nuclear antigen
PDX-1	pancreatic and duodenal homeobox factor 1
PP	pancreatic polypeptide producing
Ptf1a	pancreas transcription factor 1a
RA	retinoic acid
RPMI	Roswell Park Memorial Institute
Shh	sonic hedgehog
SOX17	sex-determining region on Y box 17
STZ	streptozotocin
TGF-β	transforming growth factor β
VEGF	vascular endothelial growth factor
wpc	weeks post conception
wpn	weeks post natal

CHAPTER ONE

INTRODUCTION

1-1. GENERAL INTRODUCTION

Currently, over 346 million people worldwide are suffering from diabetes [1]. The fact that every hour of every day more than 20 people are diagnosed with diabetes suggests that the incidence of diabetes within the population is escalating to pandemic proportions [2]. It is anticipated that by the year 2030 this disease will affect 552 million people unless drastic measures are pursued [3]. In 2010, it was estimated that 7.3% of Canadians had diabetes; this number is expected to grow to 9.9% by 2020 [2]. Each year, diabetes is a contributing factor in about 41,500 deaths in Canada [4].

This global pandemic has and will increasingly become a significant burden on healthcare expenditures. Currently, 9-15% of total healthcare expenditure in developed countries supports diabetes care [5]. In Canada, the economic burden of diabetes has almost doubled since 2000, rising to an estimated $12.2 billion in 2010. As the prevalence of diabetes increases, this number is likely to rise to nearly $17 billion by the year 2020 [2]. It is important to note that the true economic cost of diabetes is likely higher than the numbers reported. Not included in these cost projections are such things as the costs associated with long-term care, public health and capital spending by the healthcare sector, the direct costs associated with third-party support, and the cost of care provided by family caregivers and voluntary healthcare spending [2]. On a personal level, an individual diagnosed with diabetes can face a $1,000-$15,000 bill per year related to direct costs for medication and diabetes supplies [4]. This high cost of care and the lack of access to medications, devices, supplies, and health care providers has

contributed to the 57% of diabetic Canadians not adhering to their prescribed therapy [2]. As a consequence, increased hospitalization rates, higher healthcare system costs, and more serious co-morbidities and complications result [2]. Improved access to treatment, new treatment measures, and ultimately progress towards a cure for diabetes is necessary.

Since the monumental discovery of insulin in 1921 by Frederick Banting and his colleagues, diabetes has become a treatable disease. However, in Banting's words: insulin is not a cure for diabetes; it is a treatment [6]. Almost a century later, we are still working towards a cure. Advances such as the procedure known as the Edmonton Protocol have brought islet transplantation to the forefront of possible cures. Using this protocol, a group of Edmonton researchers was the first to successfully transplant islets of Langerhans from cadaveric donors into seven diabetic patients to produce insulin independence [7]. However, this procedure is limited by the shortage of cadaveric pancreas available for transplant, as well as the complications that result from the need for chronic immune suppression therapy to prevent graft rejection in the patient. In order for islet transplantation to become a more applicable therapy to treat type I diabetic patients two main obstacles must be overcome: first, the development of an unlimited source of β cells; second, optimization of anti-rejection strategies such that graft tolerance is induced and sustained, but the detrimental side effects of that therapy are minimized [8,9,10].

1-2. DIABETES

1-2.1 Definition

Diabetes mellitus is a chronic metabolic disease characterized by abnormally high levels of glucose in the bloodstream resulting from the insulin deficiency or impaired effectiveness of insulin action, or a combination of the above [11]. The high levels of glucose in the blood, termed hyperglycemia, can damage organs, blood vessels, and nerves by inducing endothelial cell damage from pathways that enhance oxidative stress via excess presence of reactive oxygen species [12]. These secondary complications are what contribute to the high morbidity and mortality for the disease [2]. Worldwide, 50% of people with diabetes die of cardiovascular disease; 10-20% of patients die of kidney failure, and 42% of new kidney dialysis patients in Canada in 2004 were also diabetic patients [1,2]. Nerve damage from diabetic neuropathy affects 50% of people with diabetes yielding tingling, pain, numbness, or weakness in the extremities, combined with reduced blood flow. Diabetic neuropathy encompasses seven out of ten non-traumatic limb amputations [2]. Diabetic retinopathy is another complication that damages the microvasculature of the retina, and is the single leading cause of blindness in Canada [2]. Summed, these complications result in a significantly reduced life span and quality of life as the overall risk of dying is doubled for patients with diabetes compared to those without [1].

Diabetes mellitus is classified into a number of different subtypes. Type 1 diabetes mellitus and type 2 diabetes mellitus are the most prevalent and there are a number of other types that are less common including, but not limited to,

gestational diabetes, prediabetes, maturity onset diabetes of the young (MODY), latent autoimmune diabetes in adults (LADA), and double diabetes (DD).

1-2.1.1 Type 2 Diabetes

Type 2 diabetes mellitus has previously been known as non-insulin-dependent diabetes mellitus or maturity-onset diabetes [1,11]. Type 2 diabetes is a progressive disease where upon patients are diagnosed as type 2 diabetic when their fasting blood glucose levels are greater than 7mmol/L [11,13]. Type 2 diabetes represents about 90% of diabetes cases [1,2]. The hormone insulin is normally produced and released by β-cells, residing within the islets of Langerhan in the pancreas, as a physiological response to glucose sensed in the blood. Insulin promotes the storage of glucose in the form of glycogen in the liver and muscle cells [11]. These patients do produce insulin, however the efficiency in action of the insulin produced is hampered since the cells of the liver which are to respond to the insulin have become insensitive. The failure of these cells to sense and respond to insulin disrupts a delicate feedback loop to the secreting β-cells and the accumulation of glucose in the blood has a glucotoxic effect on the β-cells [13], thereby leading to β-cell failure and reduction in β-cell mass [14]. Genetic causative factors play a limited role in type 2 diabetes overpowered by primarily causal factors of environment namely over nutrition, sedentary lifestyle, and obesity [15].

1-2.1.2 Type 1 Diabetes

Type 1 diabetes mellitus, was formerly known as insulin-dependent diabetes mellitus or juvenile-onset diabetes [1,11]. Type 1 diabetes represents 10% of

diabetes cases [1,2]. These patients are deficient in insulin production and thus require daily insulin injection therapy for survival [1,2]. Type 1 diabetes is the result of an autoimmune destruction of β-cells due to a defect of peripheral tolerance and/or central tolerance involving the thymus which instigates a T-cell mediated response against β-cell antigens [16,17]. Consequently there is a reduced capacity to produce insulin production that leads to hyperglycemia. The exact trigger of this autoimmune reaction against β-cells is not completely understood at this time. Type 1 diabetic risk is not fully explained by genetic factors since monozygotic twin studies show disease concordance ranges from 30-50% [15]. In particular, the human leukocyte antigen (HLA) region on chromosome 6, at staining region 6p21 may be responsible for the histocompatibility disorder where β-cells display improper antigens to T cells [18]. This may account for 40% of genetic risk [15]. Environmental factors, potentially including drugs, toxins, infant dietary exposure, low vitamin D status, and the factors associated with hygiene hypothesis may also play a role [17]. Enteroviral infection, namely from the coxsackievirus B, has also been postulated. This is evidenced in part by researchers finding anti-coxsackievirus antibodies in greater frequency in type 1 diabetics compared to control subjects [19] as well as a temporal relationship between diabetic onset and peak enteroviral infection; interferon (IFN)-alpha produced by enterovirus-infected cells could initiate the faulty autoimmunity towards β-cells [17,19]. Though the exact cause of type 1 diabetes is not understood, it is likely a complex interaction of both genetic and environmental factors.

1-2.1.3 Other Types of Diabetes

Gestational Diabetes is diagnosed during pregnancy affecting 2-4% of all pregnancies [2]. A diabetic state is incurred in some females because their β-cells are unable adapt to an increase in metabolic load and progressive insulin resistance brought on by pregnancy [20]. Following pregnancy, diabetic state resolves [2]. Since gestational diabetes is an indicator of having poor β-cell adaptive capabilities, it is not unforeseen that long term follow up studies indicate that of those women who became diabetic during pregnancy, up to 70% developed type 2 diabetes later in life [20].

Prediabetes is a state in which a patient's blood glucose levels are elevated but have not yet reached a level to be classified as type 2 diabetes (greater than 7mmol/L) [11,13]. With proper management type 2 diabetes can be avoided, unfortunately in Canada 50% of prediabetes cases will advance to type 2 diabetes [2].

Maturity Onset Diabetes of Youth, or MODY, is a group of disorders caused by a mutation in one of at least six identified genes, which leads to β-cell dysfunction and diabetes mellitus [21]. MODY has an autosomal dominant mode of inheritance, thus often presents clinically in non-obese patients during adolescence and early adulthood with a prominent family history of diabetes in successive generations. In developed countries, MODY may account for 1-5% of the diabetic population [21].

Finally, underscoring a continuum between type 1 and 2 diabetes are the cases of latent autoimmune diabetes in adults (LADA), and double diabetes (DD) in

children and adolescents [15]. A mixture of both obesity induced insulin resistance as well as autoimmune destruction of β-cells occurs in these cases. Accurately diagnosing double diabetes from type 2 diabetes is quite important since double diabetic children and adolescents must be treated for the possible increased risk of complications associated with type 1 diabetes in addition to macrovascular complications of type 2 diabetes. [15].

1-2.2 Treatment of Diabetes

The first defense against type 2 diabetes is modification of diet and exercise routines. Oral anti-diabetic medications may also be used. Currently Metformin is the drug of choice, and acts by reducing the amount of glucose released to the blood by the liver from glycogen stores as well as increasing the cellular uptake of glucose in skeletal muscle tissue. This alleviates some of the stress placed on β-cells for insulin production, and has an added benefit of promoting weight loss in patients. If lifestyle modification and anti-diabetic medications fail, insulin must be administered to control hyperglycemia. [22].

Type 1 diabetes has a complex pathophysiology, and our limited understanding of the precise etiological triggers and autoimmune mechanisms that generate the disease make treatment challenging. An intervention strategy that consistently and safely prevents or reverses type 1 diabetes has yet to be developed [23]. Present therapy for these patients consists of daily administration of insulin [1].

The historic discovery and purification of insulin in 1921 by Frederick Banting, Charles Best, James Collip, and John McCleod changed the face of diabetes care and radically reduced the mortality of diabetic patients [5]. Reduced

mortality revealed the secondary long-term complications and side-effects from blood glucose variation in type 1 diabetics [5,24].

Intensive exogenous insulin therapy treatment studies which strive to maintain plasma glucose levels within the range of non-diabetics through careful monitoring and administration of insulin show a protection against excessive hypo- and hyperglycemic events [23]. As a consequence long-term complications of diabetes are delayed [23]. For instance, retinopathy risk decreased by 53%, and retinopathy progression decreased by 70% [25]. Though an adverse reaction to intensive therapy to maintain blood glucose levels within a narrow margin includes a three fold increase in risk of severe hypoglycemic events, this did not outweigh the alternative long-term morbidity of secondary complications [25].

Work towards a technologically advanced therapeutic approach for type 1 diabetes includes a man-made closed-loop system consisting of a glucose-sensing arm and an insulin delivery arm [26]. Continuous glucose sensors increase awareness of blood glucose levels, but benefits are limited by the user's capability to respond to the data provided [27]. Insulin pumps using a subcutaneous cannula allow for a gradual insulin release and additional bolus doses, which provides greater freedom for users to control their disease treatment and improve quality of life [27]. Work is underway to combine the sensory and delivery arms by creating software that would automatically translate measured glucose levels into appropriate insulin doses [26]. Before widespread clinical use of these devices occurs the computer algorithms controlling them must be thoroughly tested *in silico* with computer simulation [27]. Despite a drastic quality of life

improvement, ultimately man-made closed-loop systems will not cure type 1 diabetes.

A more physiological approach to deliver insulin is through tissue transplantation [28]. Due to favorable results in animal models, in 1966 the first vascularized whole pancreas transplant was attempted by at the University of Minnesota by Drs. Kelly, Lillehei, Merkel, Idezuki, and Goetz [29]. Though the patient demonstrated improved blood glucose levels, pulmonary embolism causing death occurred three months later [29]. Further investigation into this procedure has improved initial outcomes to reliably restore normoglycemia and glucose homeostasis [5]. However, like all major organ transplantations, pancreatic transplantation requires patients to continuously take immunosuppressive drugs to prevent rejection of the foreign tissue. Side effects and risks associated with these drugs may mask the benefits provided by the transplant's natural insulin production. Furthermore, this complex surgical procedure has a significant morbidity and mortality rate associated with it due to the technical surgical difficulties in re-establishing proper exocrine drainage and vasculature in the new pancreas [30]. Thus whole pancreatic transplantation is often reserved for patients suffering from severe secondary complications or for those who are already or will be administered immunosuppressive drugs due to previously or simultaneously transplanted tissue [5].

1-3. ISLET TRANSPLANTATION TO TREAT TYPE 1 DIABETES

A promising alternative to daily insulin injections or whole pancreas transplantation is the transplantation of β-cell containing islets of Langerhan to establish a more physiological system of restoring glucose homeostasis. This could potentially reverse the progression of the metabolic and neurovascular complications associated with type 1 diabetes [28]. Data confirms that transplantation of islets can yield stabilization of glucose metabolism, sustained decrease in number of hypoglycemic episodes, restoration of symptom awareness, and finally a reduction in HbA1C levels [30].

1-3.1 History of Islet Transplantation

In 1889, researchers Joseph Baron von Mering and Oscar Minkowski of the University of Strasbourg definitively connected blood glucose regulation to the pancreas by demonstrating that pancreactomized dogs became hyperglycemic. With this understanding, significant research towards treatment and ultimately a cure for type 1 diabetes was underway [31]. To date, substantial progress in the realm of islet transplantation as a means to cure diabetes has been made.

The first reported instance of islet transplantation for diabetes treatment is credited to Dr. Watson-Williams and Harshant in 1894. These researchers subcutaneously transplanted small fragments of a sheep pancreas into a young boy experiencing diabetic ketoacidosis. Though an improvement in blood glucose levels was seen, unfortunately the transplant was rejected and the boy died shortly thereafter [28].

Coinciding with previously mentioned advances in whole pancreas transplantation [29], Dr. Paul E. Lacy, who is considered the true "Father of Islet Transplantation" [31], had turned to a rat animal model to study islet transplantation. In particular, he explored islet isolation procedures to increase the transplantable islet mass. Building on previous advancements made regarding isolation and purification procedures, in 1972 Ballinger and Lacy successfully demonstrated the first sustained reversal of chemically induced diabetes in a rat model through the transplantation of 400-600 isolated rat islets. Furthermore, when this islet graft was removed, the rats returned to a hyperglycemic state [32].

In order to extend success of the rat model to humans, rat islet isolation and purification procedures had to be altered and refined. Technologies such as the Ricordi digestion chamber and the COBE continuous purification system, use of the digestive enzyme collagenase to enact controlled pancreatic distension, and a purified enzyme blend that had low endotoxin levels enhanced the procurement and purity of human islets [5]. These advances facilitated the transition of human islet transplantation into the realm of clinical applicability.

From 1974 through 1999, islet allotransplants were received by over 450 type 1 diabetic patents; one year post surgery, less than 10% of these patients achieved insulin independence. Failure of these grafts has been attributed to insufficient islet transplant mass, inadequate measures taken against allograft rejection, and the use of toxic immunosuppressive drug treatments [5].

The face of diabetes research drastically changed in the year 1999 with a landmark study conducted by a group of Edmonton researchers that would later

become known as the Edmonton Protocol [33]. The Edmonton group's new transplant protocol resulted in all seven type 1 diabetic recipients achieving and maintaining insulin independence beyond one year [7]. This protocol was successful first, because the patients received islets from two to three donors amounting to a mean (±SD) islet mass of 11 547 ± 1604 islet equivalents per kilogram of body weight, and second, the glucocorticoid-free immunosuppressive regimen consisting of sirolimus, lowdose tacrolimus, and a monoclonal antibody against the interleukin-2 receptor to protect against rejection provided a more potent therapy with less diabetogenic side effects [7]. This protocol has set the standard worldwide and now many other groups have achieved similar success [28]. At the University of Alberta, as of 2006, 66 patients had received islet transplantations in accordance to the Edmonton Protocol. Of these 66 patients, 82% remain insulin free after one year, 70% after two years, and 50% after three. Also, 88% of patients demonstrate sufficient enough islet function five years following transplantation to avoid severe hypoglycemic reactions, as evidenced by endogenous secreted insulin and C-peptide presence [5]. Nonetheless, only 10% of patients remain totally insulin independent five years post-transplant [34].

1-3.2 Islet isolation and transplantation

A number of steps are involved in islet transplantation, and success of islet transplantation depends on a number of factors associated with each step of the procedure. The basic steps involved in preparing and transplanting islets are outlined below (*Figure 1-1*).

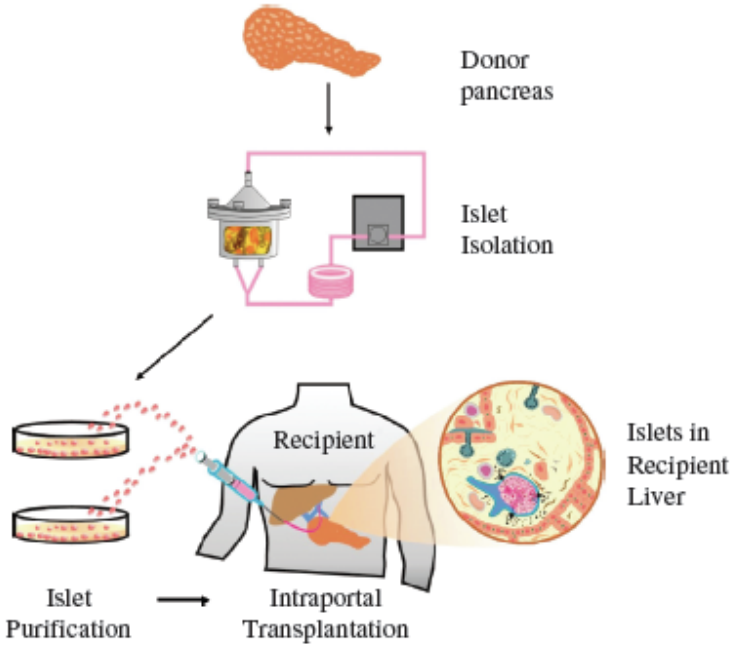

Figure 1-1. Islet transplantation to treat type 1 diabetics- the basic steps involved in preparing islets from donor pancreatic tissue for infusion into the portal vein of patients [5].

Obtaining, isolating, and purifying high quality viable islets from a harvested pancreas is crucial in the islet transplantation procedure. Preparation of suitable tissue for transplant depends on donor factors, surgical technique, and technologies and protocols involved in the islet isolation and purification [5,31].

Donor factors seem to affect quality of the islets. Factors such as age, body mass index, serum glucose levels, and haemodynamic stability play a role. Also brain death of the donor may release brain-derived inflammatory peptide that can have a toxic affect upon the islets [5]. Pancreatic lipomatosis, characterized by the replacement of exocrine pancreatic parenchymal tissue with adipose tissues, is a condition associated with obesity, ageing, obstruction of the pancreatic duct and congenital disorders. Interestingly, a pancreas from a donor with this condition is

not suitable for whole organ transplant, however these organs tend to yield a higher islet count from isolation procedures [31].

A critical first step of islet procurement is an intraductal infusion of the digestive enzyme collagenase into the whole pancreas to aid in pancreatic digestion [5]. Of paramount importance during initial procurement of the pancreatic tissue from a donor is an informed knowledge of pancreas anatomy since it is vital that the infusing catheters be correctly inserted into the ductal system [31]. It is especially important to be able to recognize and adjust procedures to account for anatomical variation present with this organ such that a uniform distention and digestion of the pancreatic tissue occurs [31].

Technological advancements such as the aforementioned Ricordi digestion chamber and the COBE continuous purification system were key to increasing islet yield and quality [5]. Unfortunately, the labour intensive, expensive isolation process is also frequently inconsistent. Current islet isolation techniques at best are only able to recover about 20-50% of the potential islet mass from a donor pancreas [5]. Culturing islet tissue prior to transplantation can also enhance the purity of the islet tissue, and does not appear to change the viability and function of the islets. This extra time can allow for the best recipient candidate match, and pre-transplant conditioning of the recipient's immune system is also possible [5].

Following isolation and purification, islets from multiple donors are infused on separate occasions through a catheter inserted through a percutaneous trans-hepatic cannula [26] into the portal vein [5]. The islets travel along the portal

vein and become embedded in the liver mass where they will reside secreting insulin [5]. A potential negative implication for islet survival with the portal infusion site is that the islets are exposed to environmental toxins. In particular, medications that are absorbed in the gastrointestinal tract travel to the portal system having not yet had a neutralizing first pass effect in the liver [26]. In addition, oxygen supply to the islets at this site is relatively reduced, since the blood of the portal vein is a collection of the venous blood already deoxygenated by the gastrointestinal system [26]. As a result of these factors, most of the islets are lost immediately through apoptosis and other nonimmune-mediated inflammatory pathways leaving only 25-50% of the transplanted mass is left to engraft [5, 35]. Alternative sites for transplantation are under investigation to reduce islet damage incurred at the portal site [28]. Potential sites include the omentum, pancreas, gastrointestinal submucosa, muscular tissue, and subcutaneously, however initial research is showing negligible improvements to islet survival [26].

Due to inefficiencies in islet isolation and poor initial graft survival, a significantly large initial islet mass is essential for islet transplantation. Usually this amounts to using islets from two to three pancreas' in multiple procedures in order for the recipient to become insulin independent [7]. Further optimization to isolation and purification techniques and transplant site may allow for increased number of β-cells available to achieve sufficient engraftment in islet transplant recipients [7].

1-3.3 Barriers to islet transplantation

Despite the landmark success of the Edmonton Protocol, currently, islet transplantation as a curative treatment for type 1 diabetics is limited in its long-term success. Barriers to clinical islet transplantation include challenges associated with immune system and the limited supply of cadaveric transplantable tissue.

Following transplantation, graft tissue is challenged by the recipient immune system. Progressive loss in β-cell mass and function due to chronic allograft rejection results, as well β-cell toxicity from immunosuppressive drugs, reoccurrence of autoimmunity, and immunosuppressive-related loss of β-cell regenerative capacity [28]. Eventually, these factors result in a return to insulin dependence in the majority of cases [14].

Development of novel immunosuppressive therapies that are less toxic to β-cells and other tissues are presently being researched [5]. Ultimately, inducing transplant tolerance by "reprogramming" the immune system to see the foreign tissue as "self" would be a ground-breaking advancement [5]. Development of monoclonal antibodies against T-lymphocytes is also a promising approach to minimize the damaging immune response towards the graft [36]. Some bodily tissue sites are naturally immunologically privileged including the brain, cornea, placenta and fetus, and the testis; studying the immune tolerance of these tissues may shed light on inducing tolerance of transplanted islet tissue [37]. Another potential strategy to avoid immune recognition may be microencapsulation of

islets prior to transplantation. Microencapsulation places islets into immunoprotective devices that would allow small molecules like nutrients, oxygen, and water to permeate while the membrane would be impermeable to larger molecules like immune cells and antibodies [38]. Following transplantation, normal islets rely on diffusion of oxygen and nutrient diffusion from surrounding tissue until revascularization occurs via angiogenesis- this process takes 7-10 days. Unfortunately, microencapsulated islets do not revascularize, thus hypoxia and eventual islet death occurs [38]. This problem is currently being addressed through nanotechnology and tissue engineering fields of research [38].

Following transplantation, long-term survival of islet tissue will remain contingent upon optimizing anti-rejection therapies to allow graft tolerance while minimizing detrimental side effects of that therapy.

Currently, each patient usually requires islets from two to three cadaveric pancreas' transplanted through multiple procedures in order for the recipient to become insulin independent [7]. Before islet transplantation can become a more widespread therapy to treat type 1 diabetic patients, identifying and establishing an unlimited source of β-cells suitable for transplant will also be imperative [8,9,10,39].

1-4. ALTERNATE SOURCES OF DONOR TISSUE FOR ISLET TRANSPLANTATION

The tremendous shortfall of human cadaveric islets for transplantation will preclude islet transplantation as a widespread therapeutic treatment of type 1 diabetics [14]. To address the insufficient islet supply, many research groups are exploring ways to create alternative cell sources from both human and non-human origin.

1-4.1 Xenotransplantation

Xenotransplantation (or cross-species transplantation) using pig islets is a source of islets showing tremendous potential as a nearly unlimited source of islets [5,26,33]. Pig tissue is at the forefront of candidate animal donors for several reasons: first, pig insulin is structurally similar to human insulin differing by only one amino acid; second, pig islets have a lower sensitivity compared to human islets to potential recurrent autoimmune destruction of β-cells; finally, the potential to genetically modify pig donors is being researched such that human recipient immune response to the foreign tissue could be moderated [40]. Microencapsulation to immuno isolate the pig islets may also be a potential way to prolong islet graft survival [26]. Despite these advantages, immune rejection as well as the risks of zoonotic viral transmission still have to be dealt with to bring this therapy to fruition [40].

The generation of β-cells from alternate human sources is a reasonable means to investigate in addressing the shortage of donor islets [14]. Such alternate sources may include: differentiation of human embryonic stem cells,

transdifferentiation of non-pancreatic cells, or the differentiation of potential progenitor cell populations within the adult pancreas [41]. Since this research is relatively recent, there is extensive debate and disagreement regarding the potential of these various populations to generate β-cells.

1-4.2 Differentiation of human embryonic stem cells

Embryonic stem cells (ESCs) are undifferentiated cells derived from the inner cell mass of blastocysts with ability to self-renew indefinitely and capability of differentiating into all embryonic cell types [42]. The combined capacity of self-renewal and pluripotency in ESCs offers potential as an almost unlimited variety of self-renewing therapeutic cells for a wide array of degenerative diseases [42]. One such disease being diabetes mellitus.

Under the appropriate *in vitro* culture conditions ESCs can respond to extracellular signals and differentiate into various cell types, while maintaining their proliferative capacity allowing an almost unlimited expansion without hindering their differentiation capacity [43]. Proving challenging however has been the directing of ESCs towards a specific desired lineage [43]. Early studies were able to generate cells that produced some insulin in mouse [44,45] and rhesus monkey [46] models. In studies with human ESCs, through the use of sequential culturing protocols, researchers have been able to bring these ESCs to an immature β-cell form [47, 48,49]. These cells do produce insulin, however the level of insulin produced was low, cells had a polyhormonal phenotype, and functional response to glucose stimulation was poor. However in a study by Kroon and colleagues, when these immature β-cells are transplanted into diabetic

mice, they resulted in insulin positive cells capable of restoring normoglycemia. Removal of the transplant resulted in return to hyperglycemia [50]. Thus it is inferred that a yet unidentified signal or cell-to-cell interaction *in vivo* is necessary to bring the ESC derived β-cells to a mature form [43]. The study to date underscores the continued importance of studying human embryonic and fetal development especially to understand the intricacies of the cell-to-cell interactions and the inductive signals cast onto immature β-cells from surrounding tissues which guides these cells to a functionally mature form [51].

A safety concern associated with the use of ESCs is that the persistence of undifferentiated ESCs in a transplant has a high risk of forming teratomas [35, 52, 53]. Thus to avoid the chance of tumor formation, transplants would have to be purified to only contain fully differentiated β-cells [43]. This would necessitate identifying a cell surface marker to sort the preparations that would discriminate the fully mature compared to the immature β-cells [43,35]. However, this would not fully alleviate concern. Use of a mouse model to prove the success of this technique is problematic. The length of time that would be used to assess risk of teratoma formation in the mouse model is too short to be clinically sufficient to disprove tumor risk in human patients [42].

As is the case with current islet transplantation, another concern is that the transplant recipient's immune system must be circumvented to allow for long term islet transplant success. Both the autoimmune response specifically targeting β-cells as well as the alloimmune response to foreign tissue must be overcome [42].

Human ESCs are obtained by first, procuring either fresh or frozen embryos in the cleavage stage of development, then culturing them to bring them to the blastocyst stage, the inner cell mass is isolated, and finally from this an ESC line is established [54]. As research progresses into the potential use of human ESCs as a source of therapeutic cells, a continued careful balance between the ethical dilemmas associated with the isolation of human ESCs and related embryonic germ cells as opposed to the potential clinical benefit must be continually examined [55].

1-4.3 Transdifferentiation of non-pancreatic cell sources

Cell sources outside of the pancreas have been explored as potential pools of cells to transdifferentiate into insulin producing cells [41,43,56,57]. One approach is to use cells from tissues such as the liver and intestines which share the common embryonic gut origin to the pancreas [56,43]. A number of pathways have been proposed for the mechanism of generation of insulin producing cells from non-pancreatic sources (see *Figure 1-2*): first, true transdifferentiation of a differentiated cell type into a new differentiated cell type; second, dedifferentiation of one cell type into a common progenitor cell followed by differentiation into a new cell type; third, *de novo* differentiation of pluripotent cells which have persisted in adult tissue; fourth, fusion of a pluripotent cell with a cell that has already differentiated in order to give a new cell type [56,41].

Mesenchymal stem cells (MSCs) are adult stem cells that are pluripotent (capable of progressing into ectoderm, mesoderm, and endoderm fates) [58]. From these germ layers further differentiation into a variety of cell types

including bone, muscle, adipose tissue, cartilage, cardiomyocytes, astrocytes, and hepatic cells has been demonstrated [59]. Since there is significant controversy in defining these cells, they may also be termed: multipotent mesenchymal stromal cells, mesenchymal stromal cells, marrow stromal cells, mesodermal progenitor cells, marrow isolated adult multilineage inducible (MIAMI) cells, and colony-forming unit fibroblastic (CFU-F) [58,60].

Insulin producing cells have also been successfully generated by the transdifferentiation of MSCs originating from bone marrow [61-64], umbilical cord blood [65], and adipose tissue [66].

Compared to ESCs, MSCs are advantageous since they are easily obtained and expanded in culture, are free of the ethical issues associated with ESCs, and usually do not form teratomas [41]. Furthermore, MSCs are thought to have immunomodualtory and anti-inflammatory properties, as such these cells may be able to evade destruction by the immune system [26]. Unfortunately, like ESC sourced insulin producing cells, cells differentiated from non-pancreatic sources have yet to attain sufficient levels of insulin production to be physiologically applicable. To advance the functional efficacy of insulin producing cells from non-pancreatic sources it will be necessary to further explore transdifferentiation pathways to understand what mechanisms are necessary to differentiate into a fully functional (mature) β-cell [61,62].

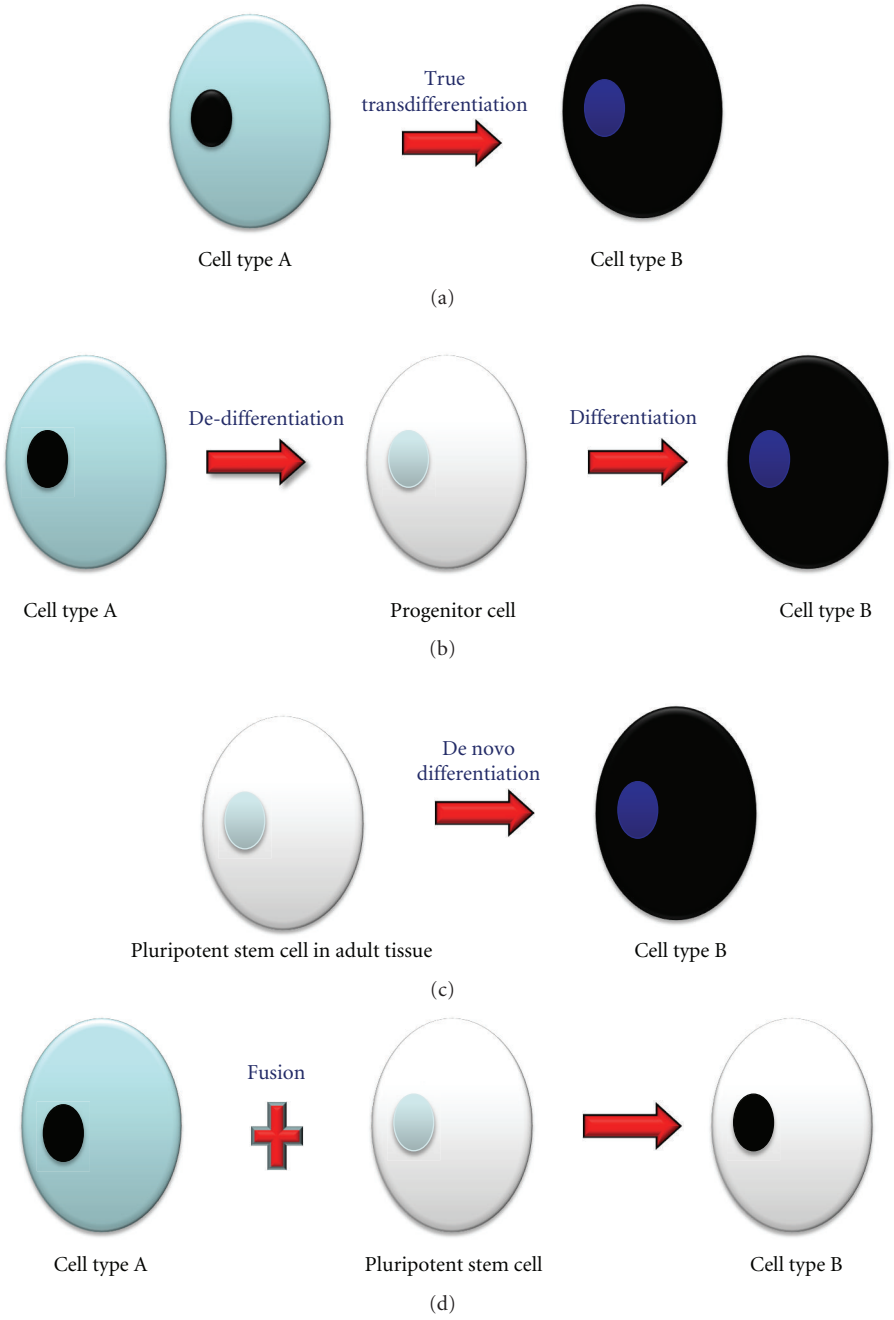

Figure 1-2. Proposed pathways for the generation of insulin producing cells from a non-pancreatic origin. A. True transdifferentiation of a differentiatied cell type into a new differentiated cell type. B. Dedifferentiation of one cell type into a common progenitor cell proceeded by differentiation into a new cell type. C. De novo differentiation of pluripotent cells which have persisted in adult tissue. D. Fusion of a pluripotent cell with a cell that has already differentiated to give a new cell type. [41].

1-4.4 The adult pancreas as a source of β-cells

Under normal adult physiological conditions β-cell mass is a slowly renewed tissue mass [67,68]. However, under certain physiological and pathophysiological conditions β-cell mass becomes dynamic responding to increased insulin demand brought on by pregnancy, obesity, or partial pancreactomy [68]. For instance, during pregnancy there is a prolonged increase in metabolic demand and progressive insulin resistance. In response, the endocrine pancreas can compensate by increasing β-cell mass. This is achieved through: increasing β-cell size as well as increasing cell number. This adaptive condition demonstrated during pregnancy and other conditions is being explored as a potential means to generate β-cells for islet transplantation [20].

What remains unclear, and the topic of frequent controversy, is the origin of newly formed β-cells [68]. Some believe that these cells are generated only by replication of pre-existing β-cells [69,67] while others propose a role in increasing β-cell number is performed by the differentiation of pancreatic progenitor cells [70,71].

A paper published in 2004 by Dor et al. claims adult pancreatic β-cells are the product of replication of pre-existing β-cells rather than the differentiation of progenitor populations [69]. This conclusion was drawn from transgenic mouse studies in which the insulin promoter drove the expression of a tamoxifin-dependent Cre-recombinase (RIP-CreER). According to this mouse model β-cell replication is the main source of new β-cells during normal adult life as well as in the replacement of β-cell mass following partial pancreactomy. Furthermore,

their findings maintain the idea that the number of islets during adult life is fixed. These findings were supported in a follow up study by Teta et al. in which β-cell mass expansion by self-duplication was confirmed using a novel DNA based lineage tracing technique that used serial thymidine analog labeling in mice [67].

However, several limitations with the Dor et al. study exist. First, these reports cannot prove the absence of progenitor cells [69,71]. Since there are limitations associated with the leakiness of the Cre-lox system over time, only a proportion of cells were counted, and there was only a 30% labeling of β-cells [71]. It is also possible that facultative progenitor cells residing in the ducts, acini, or islets remain unmarked by the Cre-lox system, but could acquire the progenitor phenotype following stimulation [69]. Second, insulin gene expression marked by the RIP-expression in cells does not necessarily exclusively mark fully functional β-cells, since insulin gene expression has been detected in immature cell types leading to mature β-cells. As such other mature β-cell specific markers like MafA should be used to confirm self-duplication [72]. Third, both the Dor et al. and Teta et al. studies were performed in mice, thus it must be considered that a species difference in mode of β-cell regeneration exists [69]. For instance according to a human study by Butler et al. during pregnancy, the β-cell mass increase is accompanied by an increase in small new islets, no increase in β-cell replication, and duct cells also positive for insulin were noted [73]. These results support β-cell neogenesis theories rather than self-duplication as a means to increase β-cell mass during pregnancy. Conversely, a study by Meier et al. sought to determine the extent and timing of expansion of β-cell mass in humans

from early childhood to adolescence as well as the predominant source of the newly formed β-cells by observing pancreatic samples from autopsies ranging in age from two weeks to 21 years old [74]. This study determined that the several fold expansion of β cell mass was accomplished by increasing β-cell numbers within islets rather than an increase in islet number. Thus indicating that replication of existing β-cells is the primary mechanism responsible for the postnatal expansion of β-cell mass [74]. On the other hand, 50% pancreatectomy in adult humans does not trigger any β-cell regeneration, instead those patients who have undergone a pancreatic resection have a high incidence of diabetes [75]. Clearly, there are inconsistencies between findings in rodent models compared to humans as well as in mode of β-cell mass expansion under varying conditions amongst species, caution must be applied when translating lessons of the rodent model to the clinical setting, and further work using human tissue must be pursued.

Specialized progenitor cells are essential to tissue development and maintenance, and show a powerful capacity for regeneration in the human body [72,67]. Cell populations such as bone marrow, gastrointestinal and skin epithelia have demonstrated progenitor cell activity [72]. It is thus hypothesized that similar specialized progenitors could already exist within the adult pancreas or be induced by dedifferentiation of other pancreatic cells [51,72]. Once identified, the regenerative capacity of these cells could be harnessed, and expanded source of β-cell mass for transplantation [51]. A number of approaches are being explored to this end, potential progenitor cell sources within the adult pancreas including

within islets, acinar, or pancreatic ductal epithelial tissue are all being considered in order to create functional β-cells.

In a 2004 study, Gershengorn and colleagues found that they could culture human islets such that existing β-cells dedifferentiated *in vitro* into proliferative mesenchymal-type cells, these cells could be expanded, then with intervention of serum withdrawal, could be directed to redifferentiate back into cells with a low level of insulin expression. It was postulated that the β-cells underwent a reversible epithelial-to-mesenchymal transition (EMT). It was believed that this process could be exploited to generate cells for β-cell replacement therapy [76]. One problem with the 2004 study was that the original human β-cells could not be identified and followed in culture [77]. Thus follow-up studies by the Gershengorn group made use of transgenic mice to attempt to confirm the origin of the expanding cell population, also assuming conservation across species in their study of these cells [77]. These subsequent findings by Morton et al. were able to view the initial change of epithelial β-cells to mesenchymal-type cells, however it also determined that the proliferating progenitor cells were likely not derived from β-cells, rather may be cells from contaminating non-islet tissues [77]. Another follow-up study by Davani et al. identified the proliferating progenitor cells as a specialized type of MSC normally present within the islets [78]. Unlike bone marrow derived MSCs, these MSCs possessed chromatin modifications of the insulin gene characteristic of active genes [79], which may reflect a unique endocrine commitment [78].

Without directly tracing the original β-cells, it cannot be determined if the loss of epithelial phenotype in expanded cells was the result of β-cell dedifferentiation or β-cell death accompanied by expansion of a preexisting MSC population [80]. In a separate research group, the initial hypothesis of the 2004 Gershengorn et al. study was confirmed. In 2008, Russ et al. used a lentiviral lineage tracing tool to provide the first direct evidence that adult human β-cells could survive *in vitro*, dedifferentiate through EMT, and significantly proliferate [80]. Furthermore, the study illustrated a species difference, since mouse β-cell derived MSCs showed significantly less proliferation *in vitro* compared to human β-cell derived MSCs. Though this study supports EMT theory in human β-cell expansion, it does not exclude the possible presence of an additional expanding population of cells such as MSCs. Interestingly, it was determined that 40% of expanding cell mass was derived from a β-cell origin, while the unmarked population consisting of either unmarked β-cells, MSCs, or other cell types, proliferated at a similar rate [80]. A further study by Russ et al. in 2009 determined that neither β-cell derived MSCs or potential preexisting islet MSCs were able to significantly differentiate into mesodermal cell types, thus could not be classified as *bona fide* MSCs. Likely, these cells retain a restricted redifferentiation potential towards β-cells [81].

Overall, the data suggests that the MSC-like cell population from expansion of islet tissue originates from at least two sources: proliferating dedifferentiated β-cells, and proliferation of islet specific MSCs. Since these MSC-like cells retain some endocrine characteristics they could more easily be differentiated to a

functional β-cell source and become a potential source of β cells for transplantation [68].

Acinar pancreatic tissue has also been proposed as an ideal source of tissue to differentiate into β-cells since it is the most abundant cell type in the pancreas [82]. From a developmental point of view, acinar cells may be easier to redirect towards β-cell characteristics than ductal cells since acinar tissue has a common progenitor with endocrine cells following the partition of the ductal lineage [83]. An *in vitro* study by Baeyens et al. in 2005 was able to transdifferentiate rat exocrine cells to glucose responsive β-cells using the growth factors epidermal growth factor (EGF) and leukaemia inhibitory factor (LIF) following an initial dedifferentiation of the acinar cells to progenitor like cells [84]. These cells were able to induce normoglycemia in alloxan-diabetic mice [84]. However, only 10% of the dedifferentiated acinar cells went on to transdifferentiate into insulin expressing cells, thus this process may be enhanced by optimizing cell culture conditions [82]. In a human study by Lipsett et al., 90% of acinar tissue transformed into duct-like-structures by the 8th day of *in vitro* culture, if these duct-like structures were then exposed to gastrin, hepatocyte growth factor (HGF), and islet neogenesis-associated protein (INGAP) for 6 days, then β-cell mass expansion was induced [85]. Finally, an *in vivo* mouse study by Zhou et al. was able to induce direct conversion of acinar cells to β-cells through the adenoviral vector delivery of transcription factors *Ngn3*, *Pdx1*, and *MafA*. Mice were then tested to see if the acinar induced β-cells were functional by administering streptozotocin (STZ) which specifically ablates islet β-cells.

Increased glucose tolerance was observed in the mice containing the acinar induced β-cells, however since the induced acinar β-cell mass was less than that of β-cell mass in normal animals, effectiveness was limited. Also, since the new β-cells did not organize into islet structures remaining as single cells or small clusters, the lack of organization likely impairs functioning due to lack of signaling amongst β-cells for insulin release [86]. Further studies must be performed to determine if transdifferentiating and expanding human acinar tissue is a possible means in increase the available tissue for transplantation.

Significant evidence suggests that the pancreatic ductal epithelium could be a source of progenitor cells. Theories favoring a ductal epithelial progenitor for β-cell generation bear a resemblance to β-cell development in utero where it is thought that β-cells arise from epithelial progenitor cells residing close to or within the pancreatic ductal epithelium [87]. One of the first groups to report the expansion and differentiation into glucose responsive islet-like structures *in vitro* from human ductal tissue left over from islet isolation procedures was Bonner-Weir et al. in the year 2000 [88]. Next the same group used immunomagnetic sorting to purify a CA19-9 positive ductal population from the tissue immediately following islet isolation [89]. Compared to crude ductal preparations, purified preparations were slow to expand, had poor aggregation, and poor engraftment when transplanted into NOD/SCID mice. Interestingly, improvements were observed if mesenchymal cells were added to constitute 0.1% of the preparation. It is thought that the mesenchymal cells secrete growth factors or extracellular

matrix to support the growth in other cells. Control mesenchymal cells did not contain insulin, thus ductal cells were likely the source of the islet structures [89].

Pancreatic duct ligation studies in mice show that β-cell progenitors can be activated within the ductal epithelia. In a human study, Hereremans et al. through adenoviral mediated delivery of Ngn3 forced expression of Ngn3, a marker of islet cell progenitors, in human pancreatic duct cells. Subsequently these cells expressed insulin [90]. A follow up study from the same group by Xu et al. showed pancreatic duct ligation in BALB/C mice significantly increases density of duct structures and leads to activation of expression of Ngn3 [91]. These Ngn3 cells could be differentiated into functional β-cells. Ngn3-specific RNA interference confirmed the contributive role of the Ngn3 positive progenitor cells following duct ligation, as this significantly hampered the previously observed increase in β-cell mass [91]. Thus increase in β-cell mass following ductal ligation is due to both β-cell replication as suggested by Dor et al. and differentiation of progenitor cells [69,91]. The study by Xu et al. provided the first direct evidence for endogenous progenitor cells within the adult mouse pancreas, however could not directly source these progenitors to the ductal epithelial cells [91]. This evidence was provided by Inada et al. through lineage tracing in transgenic mice such that Cre expression was directed by the promoter of carbonic anhydrase II (CAII), a marker of mature ductal cells [92]. This study showed that CAII expressing ductal cells could be stimulated to dedifferentiate into progenitor cells, then redifferentiate into islet cells after birth and ductal ligation [92].

On the contrary, a study by Solar et al. [93] suggests that the pancreatic ductal epithelium does not make a significant contribution to either acinar or endocrine cells postnatally even in a regenerative post injury setting. These findings were based on transgenic mouse studies which replaced the *Hnf1β* exon (marks pancreatic ductal cells) with a Cre-containing transgene with 65% transduction efficiency. These marked *Hnf1β* cells did not generate islet cells in this model. However as noted in a recent review by Jiang et al., since only one copy of the *Hnf1β* was rendered nonfunctional, haploinsuficiency of *Hnf1β* may have blocked the differentiation of marked ductal cells [72]. Previous studies by Haumaitre et al. in mice [94] and humans [95] observe pancreatic agenesis in heterozygous *Hnf1β* mutants. Despite the contradictory indications amongst the findings of mouse studies, cells from the human pancreatic ductal epithelium should continue to be explored as a source of β-cells.

Seeberger et al. hypothesized that MSCs derived from the pancreatic ductal epithelial enriched population remaining following islet isolation could be directed towards a β-cell fate, thus creating a plentiful source of insulin-producing cells for transplantation [39]. The pancreatic MSCs isolated in this study expressed similar cell surface antigens as bone marrow derived MSCs, and furthermore these MSCs were multipotent since they could be differentiated into osteocytes, adipocytes, and chondrocytes. Following *in vitro* differentiation these pancreatic MSCs also expressed mRNA for Pdx1, NeuroD, Ngn3, and Pax4 which are necessary for islet and β-cell development, however, the protein products of these genes were not detected [39].

In a follow-up study, Seeberger et al. attempted to identify the mechanism by which the MSCs of the previous study were derived [96]. It was thought that MSCs derived from the islet depleted pancreatic tissue (IDPT) remaining following islet isolation may undergo a similar reversible EMT as previously described in a β-cell model by Gershengorn et al. [96,76]. Initial pancreatic sections of IDPT showed, as expected, no evidence of epithelial cells coexpressing MSC antigens. Following a collagenase digestion and initial two day culture, cell populations present included single positive MSC, single positive epithelial cells, and epithelial cells coexpressing MSC antigens. During expansion, epithelial populations as well as coexpressing cells decreased, while MSC expressing cells increased. It is possible that the increase in MSC population was due to EMT, however it is also plausible that the initial single positive MSC population expanded while culture conditions hampered the growth of the epithelial populations. Also, differentiation of the MSCs to revert to an epithelial phenotype as the reversible EMT model outlines was unsuccessful in the *in vitro* culture conditions of the study. It was further hypothesized that the few remaining epithelial cells observed following differentiation were what contributed to detection of developmental genes in the RT-PCR results [96]. In order to understand if and how the mesenchymal and epithelial populations within the adult human pancreas contribute to β-cell regeneration, specific study of these populations will be necessary.

Assessing the clinical feasibility of this source will also be a consideration when transitioning from the bench to bedside. For instance, if exploring

endogenous treatment strategies for type 1 diabetic patients, the likelihood of expanding β-cells from a pre-existing pool through β-cell replication is limited since the necessary preexisting β-cells are likely defective or absent [51]. However, this may be an ideal therapy for type 2 diabetic patients since the prerequisite β-cells to replicate are usually intact [97]. On the other hand, a therapy promoting β-cell development from a progenitor population is more favorable in the case of type 1 diabetes since non-β cell populations are present, and newly developed β-cells may retain some of their source characteristics, thus concealing them from detection and destruction by the immune system [74].

Despite the tremendous wide spread exploration in this field of research, controversy remains [14]. The precise cell or cell populations that could be isolated to expand into a clinically applicable pool of transplantable cells has yet to be conclusively identified.

1-5. PANCREATIC AND β-CELL DEVELOPMENT

A thorough understanding of pancreas development and β-cell formation, especially specific to humans, will be key to ongoing research to identify, isolate, and differentiate a progenitor cell population into functional β-cells. Pancreas and subsequent β-cell formation is coordinated by a complex network of signaling pathways and transcription factors that establish early pancreatic specification and differentiation of exocrine and endocrine lineages.

In order to appreciate pancreatic development, first, the mature pancreatic physiologic components must be grasped.

1-5.1 Physiologic components of the adult human pancreas

The pancreas is an accessory digestive organ with three main classes of pancreatic cell types: acinar, exocrine cells which produce and secrete digestive enzymes to the duodenum through the pancreatic ducts; ductal, which connect acinar cells to ducts and secrete acid-neutralizing bicarbonate; and endocrine, which are hormone producing cells of the islets of Langerhan. [98,99,100]. Each islet is composed of several endocrine cell types: α (glucagon-producing), β (insulin-producing), δ (somatostatin-producing), PP (pancreatic polypeptide-producing), and ε (ghrelin-producing) [99]. The vast majority (more than 90-95%) of pancreatic tissue is acinar tissue secreting digestive enzymes including lipases, carbohydrases, and amylases [43]. A mere 1-2% of the pancreas organ by mass is comprised of islets [24]. Within the endocrine compartment, insulin-producing cells make up 65-90% of endocrine cells, glucagon-producing 15-20%, somatostatin-producing 3-10%, and pancreatic polypeptide and ghrelin-producing cells are about 1% each [100]. The insulin producing β-cells play an essential role in the regulation of blood glucose levels, and an absolute or relative deficiency of β-cells may lead to type 1 or type 2 diabetes mellitus respectively [24].

1-5.2 Steps of β-cell development

Due to the dual functionality of the pancreas, both as an exocrine and endocrine organ, the development of the pancreas is quite complex. Understanding how the endocrine and exocrine cells develop through interconnected pathways may aid our efforts to generate an alternative source of cells for islet transplantation. The steps of β-cell development can be divided into four approximate phases: endoderm formation, pancreas specification, endocrine specification, and finally β-cell maturation [35]. A great deal of our understanding of these phases of pancreas development and the molecular and transcription factors involved has been the result of study using a mouse mammalian model [14,43].

1-5.2.1 Endoderm formation

"It is not birth, marriage, or death, but gastrulation which is truly the most important time in your life." –Lewis Wolpert

Gastrulation is a formative process during the third week of human embryological development by which the three germ layers, the precursors of all embryonic tissue are established. The axial orientation of the body is also established at this time [101]. The three germ layers include: the ectoderm, mesoderm, and endoderm. The ectoderm will give rise to the epidermis, central and peripheral nervous systems, the eye and inner ear, and various connective tissues of the head. The mesoderm will give rise to all skeletal muscles; connective tissues like cartilage, bones, tendons, ligaments, and dermis; blood cells and the lining of blood vessels; all visceral smooth muscle; linings of all

body cavities; the reproductive and excretory system ducts; and the majority of the cardiovascular system. Finally, the endoderm will give rise to the epithelial linings of the respiratory and digestive tracts including the glands opening into the digestive tract such as the pancreas, and the glandular cells associated with these glands [101].

1-5.2.2 Pancreas specification

During pancreas specification, a particular subset of endodermal cells residing in the wall of the embryonic foregut is directed to become pancreatic progenitors [35]. Specification of endodermal cells into pancreatic progenitors is directed by patterning signals provided by mesodermal tissues in proximity to the endodermal tissue [43,102]. As such, initiation and early development of the dorsal and ventral pancreatic primordial occur by separate mechanisms related to the proximal mesodermal tissues [103]. Signaling pathways including retinoic acid (RA), fibroblast growth factors (FGF), transforming growth factor β (TGF-β) superfamily members, Wnt members, and sonic hedgehog (Shh) have been implicated in regionalization of the foregut by regulating expression of key transcription factors [35,102,104]. RA signaling is thought to be important in the overall patterning of the foregut endoderm, since alterations in signaling results in defects in a number of foregut derivatives including the pancreas [102]. Shh signaling is expressed throughout the endoderm of the developing gut tube [102]. Since Shh signaling is inhibitory to pancreatic formation and early pancreatic and duodenal homeobox factor 1 (PDX-1) expression, suppression of this pathway is crucial for pancreatic specification and development [14,35].

Initially, the dorsal pancreatic primordia is in close proximity to the notochord. It is the FGF2 and activin (TGF-β member) signals from the notochord which act to repress the Shh expression, thereby permitting the dorsal bud to envaginate the overlying mesenchyme [102]. In humans, this occurs on the 26th day of development [104]. Following this, Pdx-1 expression is detected in the dorsal pancreas [103].

In contrast, the ventral bud is controlled by signals from the cardiac and septum transversum mesoderm- namely pro-hepatic FGF signals, which favor liver formation over ventral pancreatic formation, as well as bone morphogenic protein (BMP) (TGF-β member) signals [103,104]. These signals repress ventral pancreatic formation by upregulating the Shh signaling pathway [14]. It is only later- in the case of humans, 6 days following dorsal bud evagination [104]- with anatomical gut rotation that the ventral pancreas moves away from the cardiac mesoderm, thereby downregulating the repressive signals such that the ventral pancreas may initiate development [14].

Pdx-1 expression is crucial for pancreatic growth, specification, and maturation of β-cells [43,105]. During pancreatic development, Pdx-1 expression is maintained in progenitor cells, but later becomes restricted to β-cells in the mature pancreas [105].

1-5.2.3 Endocrine specification

Following evagination of the dorsal and ventral pancreas, many signals and subsequent transcription factors are at play in the proliferation of pancreatic progenitors and determining the endocrine and exocrine lineages [104]. Some of

these key transcription factors are outlined in a figure published in 2008 by Kaneto et al. (see *Figure 1-3*) [105]. Molecular regulation of the endocrine specification stage is mostly controlled by the Notch signaling pathway [35].

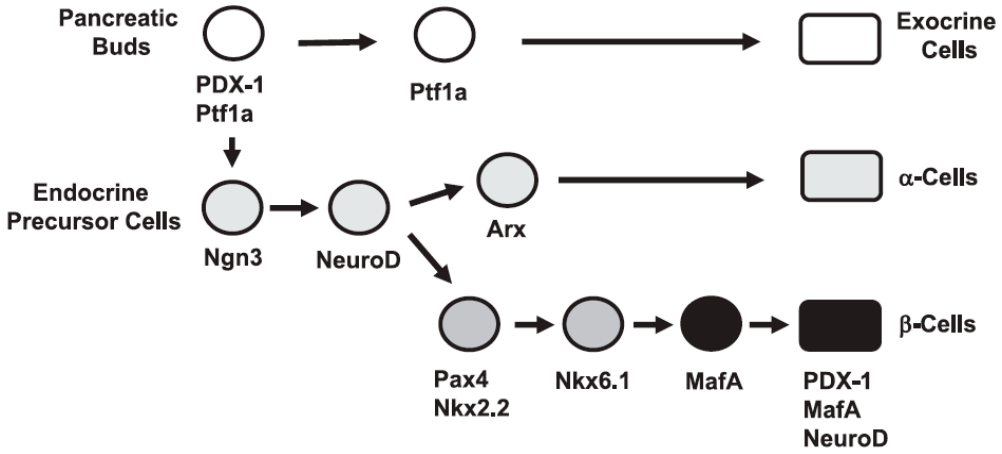

Figure 1-3. Heirarchy of key transcription factors involved in endocrine specification and endocrine cell maturation. PDX-1 expression is observed in both pancreatic progenitors as well as mature β-cells since it is crucial for pancreatic growth, specification, and maturation. Ptf1a is also expressed by pancreatic progenitors, and is later important in exocrine differentiation. Ngn3 expression which marks all endocrine progenitor cells activates the NeuroD, from which the marture α- and β-cell cells originate via differentiation. [105]

In the previous stage, the dorsal pancreas was in close proximity to the notochord, however eventually the dorsal pancreas looses contact with the notochord due to fusion of the paired dorsal aorta [104]. This fusion brings the endothelial cells of the dorsal aorta adjacent to the dorsal pancreas, thereby inducing pancreas transcription factor 1a (Ptf1a) expression in the dorsal pancreas [14]. Ptf1a is known to be expressed in pancreatic progenitor cells; the transcription factor can bind to Area III of the Pdx-1 promoter, which enhances Pdx-1 expression in progenitor cells thereby instructing these cells towards a

pancreatic fate rather than a duodenal, hepatic, or bile duct fate [104]. As well, Ptf1a has complex interactions with members of the Notch signaling pathway during endocrine/exocrine lineage specification, and subsequently is involved in exocrine cell differentiation [105].

Active Notch signaling maintains PDX-1 positive progenitor cells in an undifferentiated state through upregulation of hairy enhancer of split 1 (Hes1), a neurogenin 3 (Ngn3) repressor [104]. In addition, Notch signaling activates the FGF10 signaling, which along with EGF, and TGF-β members is important in expanding the PDX-1 positive pancreatic progenitor pool as well as the branching and differentiation of epithelium to a sufficient extent prior to endocrine differentiation [47,102,104]. RBP-J is a transcription factor that mediates signaling of all Notch receptors depending upon which RBP-J paralog is bound to Ptf1a [104]. In the RBP-Jκ form, Notch signaling is active. However likely as a result of PTF1a expression, acinar cells begin to form, which amplifies expression of the RBP-Jl form such that Notch signaling is repressed [104]. With Notch signaling repressed, Hes1 signaling no longer represses Ngn3 expression. Coexpressing Pdx-1 and Ptf1a pancreatic progenitor cells located at the tips of the branching pancreatic epithelia are also marked with carboxypeptidase A (Cpa-1). It is from this pool of cells that future endocrine cells will begin to express Ngn3 [106]. Ngn3 expressing cells are considered multipotent progenitor cells able to give rise to all endocrine cells of the pancreas [102]. Ngn3 initiates a cascade of transcription factors that controls the subsequent endocrine cell differentiation [47].

As a side note, from an anatomical standpoint, at day 42 of gestation, gut tube growth and rotation brings the ventral bud in proximity to the dorsal bud and fusion of the tissue and ductal system occurs (see *Figure 1-4*) [101]. This is important with respect to the resulting molecular factors acting on the pancreas due to spatial proximity [104].

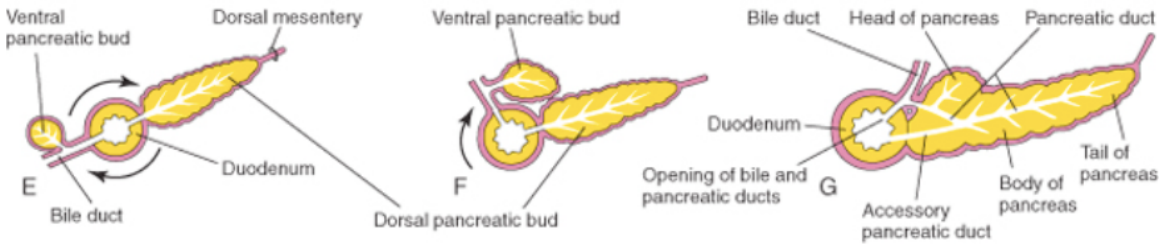

Figure 1-4. Schematic transverse sections through the duodenum and pancreas from the fifth through eighth week of human development. Growth and rotation of the duodenum brings the ventral bud of the pancreas towards the posterior side of the dorsal bud, and subsequent fusion occurs on gestational day 42. Note that it is the ventral duct that is in contact with the bile duct, hence it is the ventral pancreatic duct that becomes the main pancreatic duct. The proximal portion of the dorsal pancreatic duct usually obliterates, though it may persist as an accessory pancreatic duct. [101]

1-5.2.4 β-cell maturation

The final stage of endocrine pancreatic development is the maturation of Ngn3 progenitor cells into the various mature hormone-producing cells [35]. This process is mediated by many signaling pathways and numerous transcription factors which are not completely understood at this time [35,102]. Factors thought to be necessary for specification into the distinct endocrine lineages are listed, but not limited to those noted in *Table 1-1*. These factors interact through both synergistic and antagonistic action with each other.

Table 1-1. Transcription factors for differentiation of distinct endocrine lineages. *[43,102,103,105,107]*

Endocrine Cell Type	Hormone Produced	Transcription factors for Differentiation
α	glucagon	Pax6, Nkx2.2, Foxa2, Arx
δ	somatostatin	Pax4, Pax6
PP	pancreatic polypeptide	Nkx2.2
ε	ghrelin	Nkx2.2
β	insulin	Pax4, Pax6, Nkx2.2, Nkx6.1, Hlxb9, MafB/A, and Pdx-1

MafB functions in the formation of insulin and glucagon positive cells, however is later turned-off in the insulin positive cells with the transition from immature to mature β-cells which express MafA. This transition depends on MafB function since MafB activates the MafA gene [104]. MafA is thought to be a specific marker for mature β-cells, and acts as an activator of the insulin gene [103]. Ectopic MafA expression is capable of inducing some endogenous insulin expression in a variety of non-β-cell lines. However, MafA in combination with Pdx-1 and NeuroD drastically enhances insulin gene promoter activity to yield greater amounts of insulin mRNA and protein. Enhanced expression is understood to be through synergistic interaction effects of MafA, Pdx-1, and NeuroD [108].

Also crucial to the final maturation and effectiveness of islets is the process whereby the islets become vascularized through islet angiogenesis [43]. It is

thought that vascular endothelial growth factor (VEGF) expression by islet cells is necessary for both recruitment of endothelial cells as well as for the proper formation of endothelial fenestrations in intra islet vasculature, which is important for proper glucose sensing [14,104]. Recruited endothelial cells then synthesize basement membrane laminin chains α4 and α5. Interaction between these laminin chains and the β1-integrin containing laminin receptors on β-cells is also thought to contribute to enhancing insulin gene expression and β-cell proliferation [14].

Transgenic rodent studies have provided a vast insight into the workings of the numerous signaling pathways and the transcription factors involved. Continued research will be required in order to fully understand the signaling pathways and transcription factors involved in pancreatic and β-cell development. In particular, studies examining β-cell maturation will be crucial, since these may translate to improvements of human *in vitro* culturing protocols attempting to differentiate cells into insulin-producing cells by recapitulating pathways observed during development.

1-6. SUMMARY

Despite advances made through transgenic rodent studies, our present understanding of the signaling pathways and complex tissue interactions that are essential to pancreas and β-cell development is incomplete. Though the same type of studies cannot be performed on human subjects, other methods are being applied to understand pancreatic and β-cell development in the human model,

such as lineage tracing of *in vitro* cultured human pancreatic tissue. Knowledge gained from the rodent studies has informed the selection of growth factors used in human studies to create differentiation protocols that recapitulate developmental pathways *in vitro*. Unfortunately, current protocols have been unable to generate β-cells that produce enough insulin to be clinically applicable [109]. Moreover, controversy remains over the precise cell population that might be a clinically relevant starting source of cells to isolate for this differentiation. In order to move closer to the goal of differentiating functional β-cells in therapeutic numbers for islet transplantation, two crucial steps must be taken: first, gaining a better understanding of human pancreatic and β-cell development *in vivo*, and second, characterizing and isolating an *in vitro* human β-cell progenitor population upon which differentiation protocols could be applied and refined.

Contained in this thesis are two studies which examine *in vivo* and *in vitro* islet and β-cell development in order to characterize the β-cell progenitor population. Pdx-1 is a transcription factor essential for pancreatic development, differentiation of β-cell progenitors, transactivation of the insulin gene, and maintenance of mature β-cell function [43,105]. Since Pdx-1 expression is a key marker of pancreatic progenitor cells [105], observation of cells expressing Pdx-1 were central to our studies of pancreatic progenitors. In chapter two, immunohistochemical analysis of human fetal pancreatic tissue was performed examining expression of insulin and glucagon as well as transcription factors PDX-1 and SOX17. The goal of this study was to observe expression patterns of key pancreatic hormones and transcription factors, relative amounts and timing of

that expression, coexpression of these factors, and the changing islet morphology throughout fetal human development. In chapter three, we sought to study the origin of β-cell progenitors *in vitro* within the adult human pancreas with particular attention to the pancreatic epithelial population of cells. Previously, specific study of the pancreatic epithelial population has been difficult since mesenchymal stem cell (MSC) populations usually overtake the culture [39,96]. However, in chapter three, we describe a novel pancreatic epithelial culture media that preserves the epithelial population by preventing dedifferentiation and minimizing MSC overgrowth of the culture. This allowed for long-term study and differentiation of the epithelial population *in vitro*. Utilizing lentiviral vectors which mark Pdx-1 and insulin expressing cells combined with immunohistochemical characterization, we were able to characterize Pdx-1 expressing progenitor cells as members of the pancreatic epithelial population.

Taken together, the *in vivo* and *in vitro* studies contained in this thesis indicate that a β-cell progenitor population is likely contained within the pancreatic epithelial population. Further characterization and isolation of Pdx-1 expressing pancreatic progenitor cells within the pancreatic epithelial population as well as continued research to fully understand the signaling pathways and transcription factors involved in endocrine ontogeny in the human fetal pancreas will aid in improving *in vitro* culture and differentiation protocols towards the ultimate goal of differentiating functional β-cells in therapeutic numbers for use in islet transplantation.

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CHAPTER TWO

IMMUNOHISTOCHEMICAL ANALYSIS OF INSULIN, GLUCAGON, PDX-1, AND SOX17 EXPRESSION IN HUMAN FETAL PANCREATIC DEVELOPMENT

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A version of this chapter is in preparation for submission.

2-1. INTRODUCTION

In order for islet transplantation to become a more applicable therapy for the treatment of type I diabetes, there is a need to identify an unlimited source of β-cells for transplantation [1]. Potential progenitor populations, which could be isolated from the adult human pancreas and differentiated into a clinically applicable β-cell source is an appealing option to address the shortfall in cadaveric donor tissue. However in order for this to come to fruition, current protocols to generate insulin-producing cells *in vitro* must be improved since current protocols have yet to generate cells that secrete physiologically sufficient levels of insulin [2-4].

Understanding the normal developmental biology associated with pancreatic endocrine cell ontogeny is likely key to identifying β-cell progenitors and refining *in vitro* differentiation protocols. Extensive research has been conducted in transgenic mouse models [summarized in 5,6,7], however, significant differences exist between human and rodent islet biology thereby cautioning the direct extrapolation of lessons learned in the mouse model to human biology [8]. Some of the discrepancies noted include the timing of developmental stages [9], the sequence of key developmental events [10,11], composition and organization of islet cell clusters [12-14] including a lower proportion of β-cells in human islets compared to mouse islets [14], there are two insulin genes in mice compared to only one in humans [15], and innervation patterns of islets differ [16]. Examination of human fetal pancreatic endocrine cell development is likely to provide further insight in defining the developmental patterns of endocrine cells.

Recent human pancreatic studies affirm the importance of continued study of pancreatic development in the human model. Studies examining human fetal pancreatic tissue have used both immunohistochemistry and gene expression profiling to examine pancreatic ontogeny. Findings of this work have observed an evolving islet morphology throughout development [8,17,18]. Unlike mouse development this does not include a late gestation secondary transition islet formation [11,19], rather islets are observed by the end of the first trimester [9,19]. A major finding of the Bouwens et al. [20], Polak et al. [21], and Meier et al. [22] studies were that the endocrine hormone expressing cells first appeared within or adjacent to primitive ductal epithelium suggesting that islet cells differentiate from progenitor cells located in this region. Piper et al. [9] and Lyttle et al. [23] found that PDX-1 expression is initially located in the primitive ductal epithelia and later transitions to be more prominently expressed in islets. Pancreatic and duodenal homeobox factor-1 (Pdx-1) is a key transcription factor necessary in pancreatic development, β-cell progenitor differentiation, and maintenance of mature β-cell function through transactivation of the insulin gene and other genes in mature β-cells [3]. PDX-1 is initially expressed in pancreatic progenitor cells and is later restricted to mature β-cells [3]. Another aspect of pancreatic ontogeny in the human model observed by Polak et al. [21] and Piper et al. [9] is the coexpression of insulin and glucagon in early fetal development. They hypothesized that these were primitive endocrine cells that would later mature into monohormonal cells. Recently Riedel et al. [24] suggested that these coexpressing insulin and glucagon cells give rise to mature α-cells, since these

cells also produce the α-cell transcription factor, aristaless related homeobox (ARX) and lack specific β-cell transcription factors. SOX17 is a transcription factor with a functional role in lineage specification of the pancreatic endoderm [25]. In a human study by McDonald et al. [25], the fetal expression of transcription factor sex-determining region on Y box 17 (SOX17) was significantly higher than in adult islets by qRT-PCR, however by microarray no change in expression from fetal pancreata age 8 to 21 wpc compared to adult islets was detected. Their findings remain inconclusive, and further studies of expression patterns of SOX17 are required to determine if this transcription factor has a role in islet formation and β-cell maturation. These studies provide a solid base and illustrate the value of future developmental studies using human fetal tissue.

In this present study we performed immunohistochemical analysis of human fetal pancreatic sections aged from 7.7 to 38 weeks post conception (wpc), as well as 10 weeks post natal (wpn), and adult sections. We examined expression of the hormones insulin and glucagon as well as the transcription factors Pdx-1 and SOX17. The purpose of this study was to observe expression patterns of key pancreatic hormones and transcription factors, to examine the expression and coexpression relationships of these factors, and to describe the changing islet morphology throughout fetal human development. Our study contributes to the overall knowledge of human fetal development by confirming previous findings of others, expanding observation of key factors to the entire fetal development period, and presenting new observations of the transcription factor SOX17. A

detailed knowledge of developing islet morphology, maturation of β-cells, and expression of key hormones and transcription factors may aid in differentiating functional β-cells in therapeutic numbers for islet transplantation.

2-2. MATERIALS AND METHODS

2-2.1 Human Sections

Immunofluorescent (IF) analysis was performed on paraffin-embedded fetal and post natal human pancreatic tissue sections of various developmental time points (Fig. 2-1). Human fetal pancreatic tissue sections ranging in age from 7.7 to 14.4 weeks post conception (wpc) were obtained from the Birth Defects Research Laboratory at the University of Washington in compliance with US State and Federal regulations. Specimens were collected within 12 h post-mortem, and fixed with formalin (Richard-Allan Scientific, Kalamazoo, MI, USA. Human fetal pancreatic tissue sections ranging in age from 15 weeks to 38 wpc were also obtained from Alberta Health Services. The 10 weeks post natal (wpn) sample was obtained from the Alberta Diabetes Institute (ADI) IsletCore. The adult pancreatic samples were obtained from the Edmonton Clinical Islet Transplant Program. All samples were embedded in paraffin, cut into 5um sections, and mounted on histobond slides (Fischer Scientific, Ontario, Canada).

2-2.2 Double and Triple Immunofluorescent Staining

Paraffin sections were rehydrated, and immersed in Tris EDTA for antigen retrieval in a Sanyo Model-F301 1260W microwave for 15 minutes at 80%, and let cool for 20-25 minutes. Sections were rinsed with phosphate buffered saline

(PBS), and blocked with 20% normal goat serum (NGS) in PBS for 1 h. If biotinylated primary antibodies were used, and avidin/biotin (Vector Laboratories Inc., Burlingame, CA, USA) blocking step was performed prior to the first primary antibody, and sections were rinsed two times with 5% NGS. Primary antibodies were diluted in 5% NGS at the following concentrations: 1:1000 guinea pig anti-porcine insulin (DAKO, Carpinteria, CA, USA), 1:5000 mouse monoclonal anti-glucagon (Sigma, Saint Louis, MO, USA), 1:1000 rabbit polyclonal to PDX-1 (Abcam, Cambridge, MA, USA), and 1:50 biotinylated anti-human SOX17 antibody (R&D Systems, Minneapolis, MN, USA), and incubated for 1h, then washed two times with 5% NGS. The appropriate species-specific secondary antibody of goat anti guinea pig IgG Alexa Fluor 488, goat anti mouse IgG Alexa Fluor 488, goat anti rabbit IgG Alexa Fluor 594, or Streptavidin Alexa Fluor 594 were diluted 1:200 in 5% NGS, and goat anti mouse IgG Alexa Fluor 350 was diluted 1:100 in 5% NGS (Invitrogen Molecular Probes, Eugene, OR, USA) and applied for 30 minutes in the dark. Prior to application of second and following third rounds of primary antibody application, sections were washed twice in 5% NGS. Negative controls were sections from each age point incubated without primary antibodies, all control slides yielded no labeling. Slides were coversliped with ProLong Gold anti-fade reagent with DAPI to counter stain nuclei or ProLong Gold antifade reagent without DAPI in the case of an AlexaFluor 350 secondary (Invitrogen) to preserve fluorescence. All slides were visualized with a Zeiss Axioscope II equipped with AxioCam MRc camera and

analyzed with Axiovision 4.6 (Carl Zeiss MicroImaging GmbH, Göttingen, Germany).

2-2.3 Image Analysis

Images collected from the fetal human pancreatic sections were separated into the three trimesters of human development. The following groupings were made: first trimester (1-12 wpc), second trimester (13-24 wpc), and third trimester (24 wpc to birth (generally 38 wpc)) [26]. Furthermore a 10 wpn and an adult sample were grouped with the third trimester results to providing a representation of a mature pancreas. To quantify the number of cells producing insulin, glucagon, or both insulin and glucagon, representative images (n=2-3 from each pancreatic section) were manually counted using ImageJ software [27]. Data is expressed as the percentage of insulin and/or glucagon expressing cells as a percentage of total insulin and/or glucagon hormone expressing cells.

2-2.4 Statistical Analysis

Quantification data is expressed as mean ± SEM. Statistical comparisons were performed using the Mann-Whitney test, assuming representative sampling and independence. Given the small sample size, this test is used since it does not assume a normal distribution. The acceptable level of significance was considered $p \leq 0.05$.

2-3. RESULTS

2-3.1 Analysis of insulin and glucagon expression

To characterize endocrine hormone expression and islet morphology during human pancreatic development we performed a qualitative immunohistochemical analysis of insulin and glucagon expression in fetal pancreas between 7.7 and 38 wpc, 10 wpn, and in the adult pancreas (Figs. 2-2 A,B,C) as well as a quantitative analysis of the proportion of insulin and/or glucagon expression as a percent of total insulin and/or glucagon expressing cells (Figs. 2-3 A,B,C). During the fetal period of human development, evolving islet morphology is observed from the initiation of endocrine hormone expression in scattered cells, to the clustering of those cells into islet-like structures, and further remodeling of those structures to acquire the adult islet phenotype.

Insulin and glucagon expression was first detected late in the first trimester (Fig. 2-2 A) at the beginning of the fetal period at 8.4wpc in the form of isolated single cells. At 7.7 wpc, no insulin or glucagon expression was observed. Insulin and glucagon expression significantly increases between 8.4 and 10.6 wpc, and the first islet like clusters develop in this time. These clusters consist mostly of insulin positive cells with a few of those cells also coexpressing glucagon at the periphery of the positive structures. Islet size continues to increase between 10.6 and 12.1 wpc. In the first trimester, the majority of insulin and glucagon expressing cells are distributed amongst the tissue as isolated cells. Throughout the first trimester the proportion of insulin expression was higher than glucagon expression ($p \leq 0.025$). Significant coexpression of insulin and glucagon was

observed from 8.4wpc up to and including 23 wpc (23 wpc data not shown). As a proportion of total endocrine cells, the greatest amount of coexpression of insulin and glucagon is observed during the first trimester compared to second ($p \leq 0.025$) and third ($p \leq 0.025$) trimesters, and post natally ($p \leq 0.025$). Interestingly, in the first trimester, most glucagon cells coexpress insulin (82.21±15.79%), while cells only positive for insulin were most often located at the center of the islet-like clusters.

During the second trimester (Fig. 2-2 B), glucagon expression was proportionately greater than insulin expression ($p \leq 0.025$). By 13 wpc, the size of islet clusters increased, and unlike the first trimester a greater proportion of expressing cells are localized to islet structures rather than as isolated cells. Furthermore, the islets of the second trimester are reminiscent of a rodent model [28] as within the clusters the insulin and glucagon expressing cells self-aggregate. During the second trimester, a few coexpressing insulin and glucagon cells (14.71±3.97 of hormone expressing cells) are usually located in the margin between the self-aggregated endocrine cells, in addition, amongst these islets there are smaller islet clusters similar to those of the first trimester with higher levels of coexpressing insulin and glucagon (Fig. 2-2 B). The heterogeneous nature of islet morphology in the second trimester is likely indicative of a transitioning morphology into a more mature form.

In the third trimester (Fig. 2-2 C), the proportion of glucagon expression becomes equivalent to insulin hormone expression ($p \geq 0.05$). During the third trimester, islet morphology takes on a phenotype increasingly similar to the adult

human phenotype. Insulin and glucagon expressing cells become intermixed within the islet structures, and islet size becomes more homogeneous across the tissue sample. The 10 wpn islet morphology is similar to other samples of the third trimester as well as that of the adult human islet suggesting that mature islet morphology is acquired during the third trimester. Finally, negligible coexpression of insulin and glucagon is observed amongst hormone positive cells in the third trimester (1.88±0.68%) and post natal (1.21±0.12%) samples.

2-3.2 Expression of PDX-1 and its colocalization with insulin expression

In the first trimester (Fig. 2-4 A), PDX-1 expression is located in numerous duct-like structures, and is also coexpressed in all insulin cells. At 8.4 wpc scattered insulin expressing cells were located amongst the PDX-1 positive cells. It was interesting to visualize the close association of newly forming insulin positive clusters in close association with the PDX-1 positive duct-like structures. It appeared as though the initial isolated insulin positive cells were a part of the duct like structures in the 8.4 wpc sample, and as more insulin positive cells aggregate in the 10.6 wpc sample the islet like structures seemed to bud off from the duct structure.

In the second trimester (Fig. 2-4 B) a transition of PDX-1 expression can be distinctly observed. Between 14.4 and 15 wpc (14.4 wpc data not shown), as islets increase in size the association with the ductal structures is diminished, PDX-1 expression within the duct-like structures decreased and increased in the insulin expressing cell clusters. In addition to PDX-1 expression in all insulin

positive cells, PDX-1 is most pronounced in non-insulin expressing cell clusters surrounding the insulin cell clusters.

As maturation proceeds into the third trimester (Fig. 2-4 C), PDX-1 is minimally detected in the duct-like structures and decreased in number in those cells bordering the insulin positive clusters of cells. PDX-1 expression also is weaker in the insulin expressing cells. The first non-PDX-1 staining insulin cells are observed in the 37wpc sample. The late third trimester expression pattern resembled that of the adult pattern where PDX-1 is weakly coexpressed in the insulin positive cells and there are a few more strongly expressing PDX-1 cells at the periphery of the insulin expressing cells of the islets. As such it is assumed that islets are approaching maturity near the end of the fetal period in the third trimester.

2-3.3 Expression of SOX17 and its colocalization with insulin and glucagon expression

In the first trimester (Fig. 2-5 A) SOX17 only expressing cells are initially scattered non-specifically amongst the insulin and glucagon expressing cells at 8.4 and 10.6 wpc. Following, from 13 through 29 wpc (Figs. 2-5 B and C), the SOX17 expressing cells are spatially associated with the glucagon expressing cells. This spatial association is particularly emphasized in the second trimester samples (Fig. 2-5 B) where the insulin and glucagon cells have self-aggregated, and the SOX17 expressing cells are found in the glucagon cell clusters. Between 29 and 38 wpc in the third trimester (Fig. 2-5 C), the insulin, glucagon, and

SOX17 expressing cells disperse amongst the entire islet to acquire the adult phenotype.

Occasional SOX17 and insulin coexpressing cells were observed in the 8.4 through 14.4 wpc samples. Due to technical constraints associated with immunofluorescent microscopy, in Figure 2-5 no nuclear marker was used. Though this did allow for more direct observation of the interaction of both insulin and glucagon positive cells with SOX17 expressing cells throughout pancreatic development, in order to confirm suspected SOX17 and insulin coexpression, samples from selected time points were restained with insulin, SOX17, and nuclear marker DAPI (Fig. 2-6). These results affirmed that coexpression of insulin and SOX17 is detected in a small number of cells of the 8.4 through 14.4 wpc samples.

2-4. DISCUSSION

To date a significant portion of our understanding of pancreas and β-cell development comes from research that has been conducted in rodent animal models [5-7]. It must be acknowledged that there are significant differences between human and rodent biology, islet composition, and development [8-16]. For instance, the timing of corresponding initial insulin expression is not equivalent; mice begin expressing insulin at E11, projected to a human model, expression would be expected at 33 days post conception (4.7wpc) [9] rather than the observed 8.4wpc (Fig. 2-2 A). Morphological studies show mouse islets are properly formed within a few days of birth following a secondary wave of β-cell

differentiation [11] where as human islets initially appear in the first trimester (Fig. 2-2 A) and attain a mature form during the third trimester (Fig. 2-2 C). Further research using human tissue is needed to correlate the observations made in the rodent model and further refine the developmental model to the human condition.

As studies using human fetal pancreatic tissue are relatively new in the field of developmental research, the timing and developmental staging of the human fetal pancreas amongst studies is not always consistent [18]. For instance, several studies observe endocrine hormone expression as early as 9wpc [8,18,24], while others have noted a few insulin positive cells at 6wpc [8]. In our studies we conclude that insulin and glucagon expression initiates between 7.4 and 8.4wpc. Due to the limited availability of human fetal pancreatic tissue for study, conclusions are usually based on a very limited number of samples per fetal age. Furthermore, usually research is only conducted on a smaller time frame of fetal development rather than across the entire period. However, the advantage of our studies was that we were able to observe the entire period of fetal development thus we were able to visualize islet development from the initiation of endocrine expression through to the formation of phenotypically mature islets. As additional studies are conducted it is expected that the collective sample size of fetal data will be enhanced and impact of individual sample variation will decrease, thereby allowing for a more precise staging of islet development.

Differences between rodent and human islets are observed particularly when examining islet cytoarchitecture. In adult mice, 71% of insulin cells had

exclusively homeotypic associations contrary to only 29% in adult humans [13]. Using serial optical sectioning through both mouse and human islets, Brisova et al. was able to compare the three-dimensional structures of the islets [13]. Findings indicate adult mouse islets have a β-cell core with a non-β-cell mantle while in human islets this architecture was not observed as the β-cells were intermingle with the α and δ cell types [12]. According to Bocian-Sobkowska et al. the four stages of human islet development: first, scattered polyhormonal (9-10 wpc), second, immature polyhormonal islet stage (11-15 wpc), third, insulin monohormonal core islet stage (16-29 wpc) and finally polymorphic islet stage (30wpc onward) [18]. Our data shows a good correlation with the predictions made and perhaps shows a more explicit picture of these stages than previously presented. It was interesting to observe the insulin and glucagon expressing cells appear to self-aggregate during the second trimester such that an architecture pattern reminiscent to that of mouse islets rather than mature human islets is observed (Fig. 2-2 B).

Jeon et al. analyzed pancreatic samples between 6 and 20wpc to demonstrate some of the stages of islet development, and presented a schematic drawing predicting that after 19 to 20 wpc a mature human islet morphology with an intermingling of cell types would occur [8]. Our results confirm and build upon these results as they cover the duration of fetal development, and thus are able to show the transition between the third monohormonal core islet stage and the final polymorphic mature islet stage [18,19]. As far as the specific timing of the intermingling of islet cell types again there are inconsistencies. Our observations

are consistent with Bocian-Sobkowska et al. observing the polyhormonal islet after 30wpc, while Meier notes polymorphic mature islets from 26 wpc through to birth [18,22]. Specifically isolating the time frame for transition into the mature polyhormonal islet stage will likely require observation of additional fetal human pancreatic samples of the third trimester.

Endocrine cells which coexpress insulin and glucagon during early fetal development are thought to signify cells that are multipotent progenitor cells for islet cells [22,29]. According to a recent study by Riedel et al., coexpressing insulin and glucagon cells may represent an alpha-cell progenitor population [24]. In our studies insulin and glucagon coexpression was observed from 8.4 through 23 wpc, with the greatest proportion of coexpression in the first trimester ($p \leq 0.025$). From the first to second trimester, a decrease in coexpression by 37.59±8.60% and an increase in glucagon only expressing cells by 48.40±3.34% was observed. This observation may illustrate alpha-cell maturation. According to Riedel et al. during this transition there is little PCNA expression in endocrine cells and this shift is not accompanied by apoptosis [24]. Perhaps lending further support to this hypothesis is our observations of heterogeneous islet morphology in the second trimester. Presumably less mature islets with a high proportion of coexpression of insulin and glucagon were observed in the second trimester which upon visual inspection appear similar to the islets of the first trimester where coexpression was 52.29±7.63%. In these islets, it was noted that most glucagon expressing cells coexpress insulin, similar to those islets of the first trimester where 82.21±15.79% of glucagon expressing cells also coexpress insulin. A

population of insulin only expressing cells were also present in these islets. The coexpressing cells may be alpha-cell progenitors, while the insulin only expressing cells may be destined for a β-cell fate.

In transcription factor studies of the 8 to 21 wpc fetal pancreatic samples Lyttle et al. noted initial PDX-1 expression in CK19 positive cells, following this the PDX-1 and CK19 coexpression decreased with a simultaneous increase in PDX-1 and insulin coexpressing cells [23]. According to Piper et al. in early fetal development, CK19 marks pancreatic epithelial cells [9]. In our study, we also note PDX-1 expression initially localized to duct-like structures, and later in development it shifts to be islet associated. Taken together, these observations may indicate an epithelial origin of β-cell progenitors.

Though our observations of SOX17 expression are not necessarily conclusive, perhaps our data will contribute to determining if SOX17 has a functional role in pancreas development beyond from initial endoderm specification.

The main strength of this study lies in that observations of developmental patterns was conducted on human fetal pancreatic tissue rather than using a rodent model. Since we were able to obtain a reasonable number of samples across the entire fetal development period, we were able to demonstrate morphological and hormone expression changes throughout pancreatic development rather than within a select period of weeks [8]. This provided a better overview of the entire process of endocrine cell clustering to form mature islets. Procurement of human fetal tissue is an inherent difficulty associated with human developmental studies. As such the number of samples per age point are limited. Furthermore, the effect

of donor and maternal health status as well as cause of pregnancy termination in relation to normal fetal development is unaccounted for in the findings of this study since this background information was unavailable. However, despite a limited pool of samples, and the potential of unaccounted for deviation from normal development amongst donor samples, the results attained do illustrate a progressive picture of human islet development.

Future advancement of this study should include correlating the qualitative observations made in this study with quantitative data such as RT-PCR. Should more samples be obtained, particularly from the third trimester, more specific event timings could be determined, for instance the transition from the insulin monohormonal islet core to the polyhormonal mature islet. Other developmentally relevant hormones and transcription factors could be explored across the entire fetal development to add to the current picture of human fetal development. The MafA and MafB transcription factors would be interesting to observe since MafB is a marker of immature β-cells while MafA is expressed in mature β-cells [30]. Finally, expression patterns in fetal human islet development must be correlated to *in vitro* culture studies of islet differentiation to better understand β-cell development.

Overall, this study was able to present a unique qualitative assessment of insulin, glucagon, PDX-1, and SOX17 expression and coexpression patterns, across the entire human fetal development. Comprehensive morphologic changes during the formation and development of human fetal islets were observed. These results contribute to a better understanding of human islet development, and may

support an epithelial origin of β-cell progenitors. Further characterization of these β-cell progenitors during fetal development *in vivo*, may allow for specific isolation of these cells in an *in vitro* study attempting to isolate, expand, and differentiate progenitor cells as a viable source of β-cells for transplantation.

2-5 ACKNOWLEDGEMENTS

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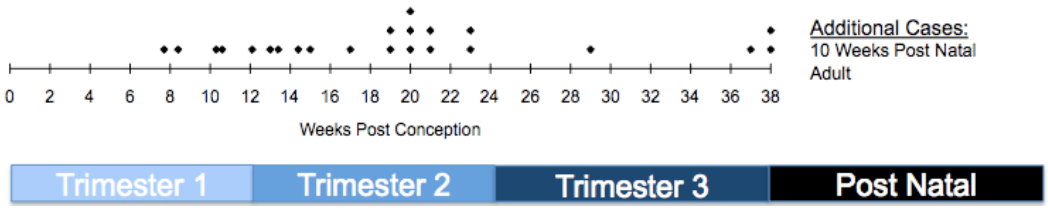

Figure 2-1 Summary of cases for immunohistochemistry.

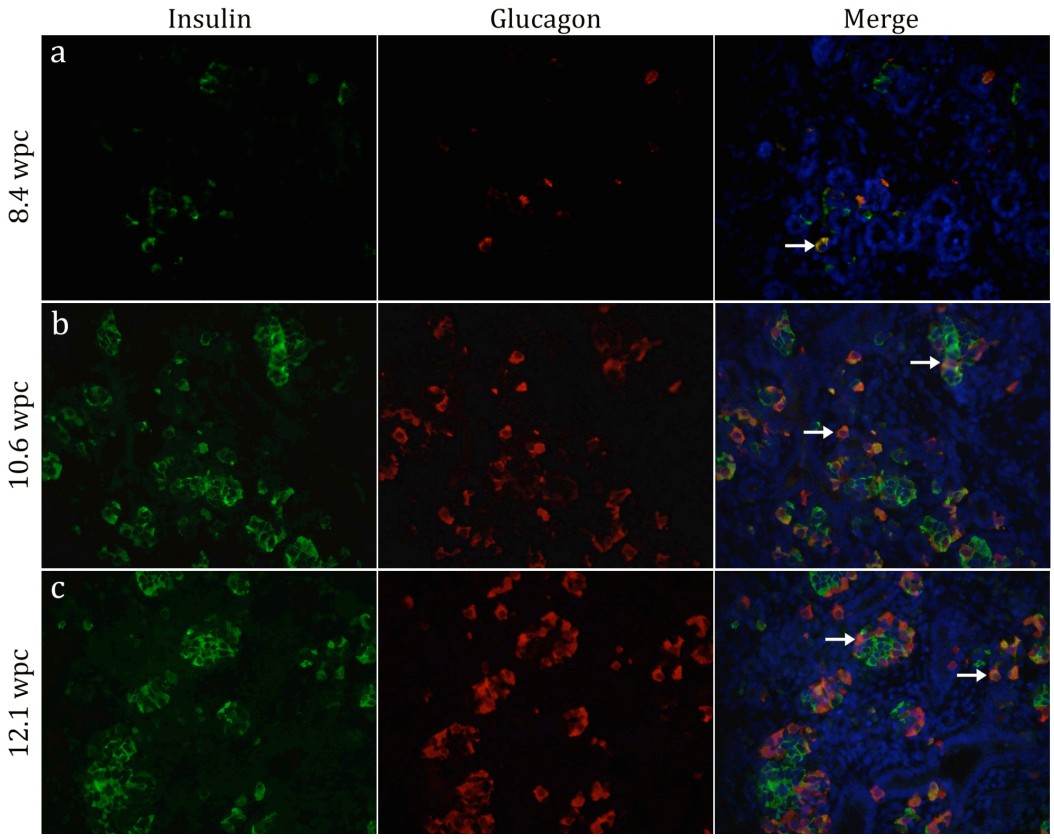

Figure 2-2 A. Insulin (green) and glucagon (red) expression during the first trimester of human fetal development (1- 12 wpc), as demonstrated by samples 8.4 (a), 10.6 (b), and 12.1 (c) wpc. DAPI nuclear counterstain (blue). Arrows demonstrate examples of coexpressing insulin and glucagon cells. Prior to 8.4 weeks there is no positive insulin or glucagon staining. At 8.4 wpc insulin and glucagon expression is observed in scattered cells. By 10.6 wpc insulin and glucagon expression has significantly increased, and the first islet-like clusters are visible, consisting mostly of insulin positive cells, while a few insulin and glucagon coexpressing cells are observed on the periphery of the clusters. Islet size continues to increase between 10.6 and 12.1 wpc. Overall, in the first trimester, the majority of insulin and glucagon expressing cells are found as isolated cells. During the first trimester, there is a greater proportion of insulin expressing cells compared to glucagon expressing and most glucagon positive cells are coexpressing insulin. Magnification is 400x.

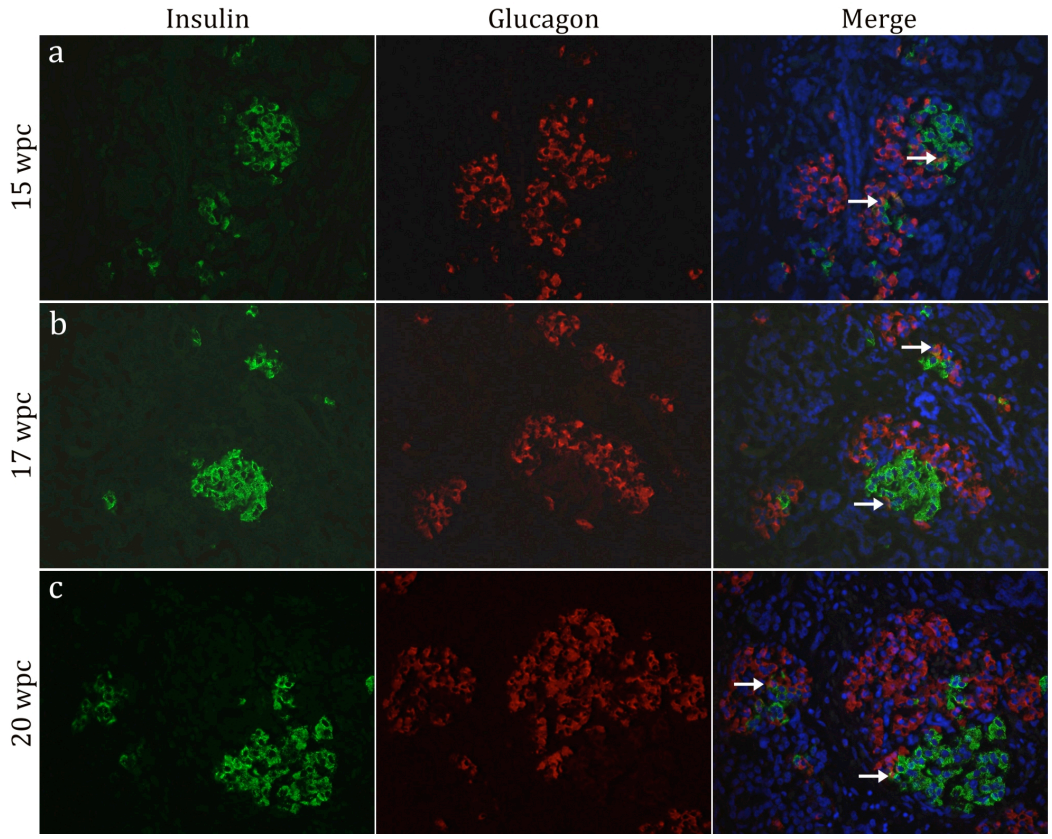

Figure 2-2 B. Insulin (green) and glucagon (red) expression during the second trimester of human fetal development (13- 24 wpc), as demonstrated by samples 15 (a), 17 (b), and 20 (c) wpc. DAPI nuclear counterstain (blue). Arrows demonstrate examples of coexpressing insulin and glucagon cells. During the second trimester, islet size continues to increase and glucagon expression is proportionally equivalent to insulin expression. Unlike the first trimester, in the second trimester, the majority of insulin and glucagon expressing cells are localized to islet structures rather than as isolated cells. Within islet structures, the insulin and glucagon expressing cells self-aggregate, and a few coexpressing insulin and glucagon cells are located in the margin between the self-aggregated cell types. In addition, smaller islets-like clusters of insulin and glucagon expressing cells, similar to those of the first trimester, with a greter proportion of coexpressing cells are also observed. Magnification is 400x.

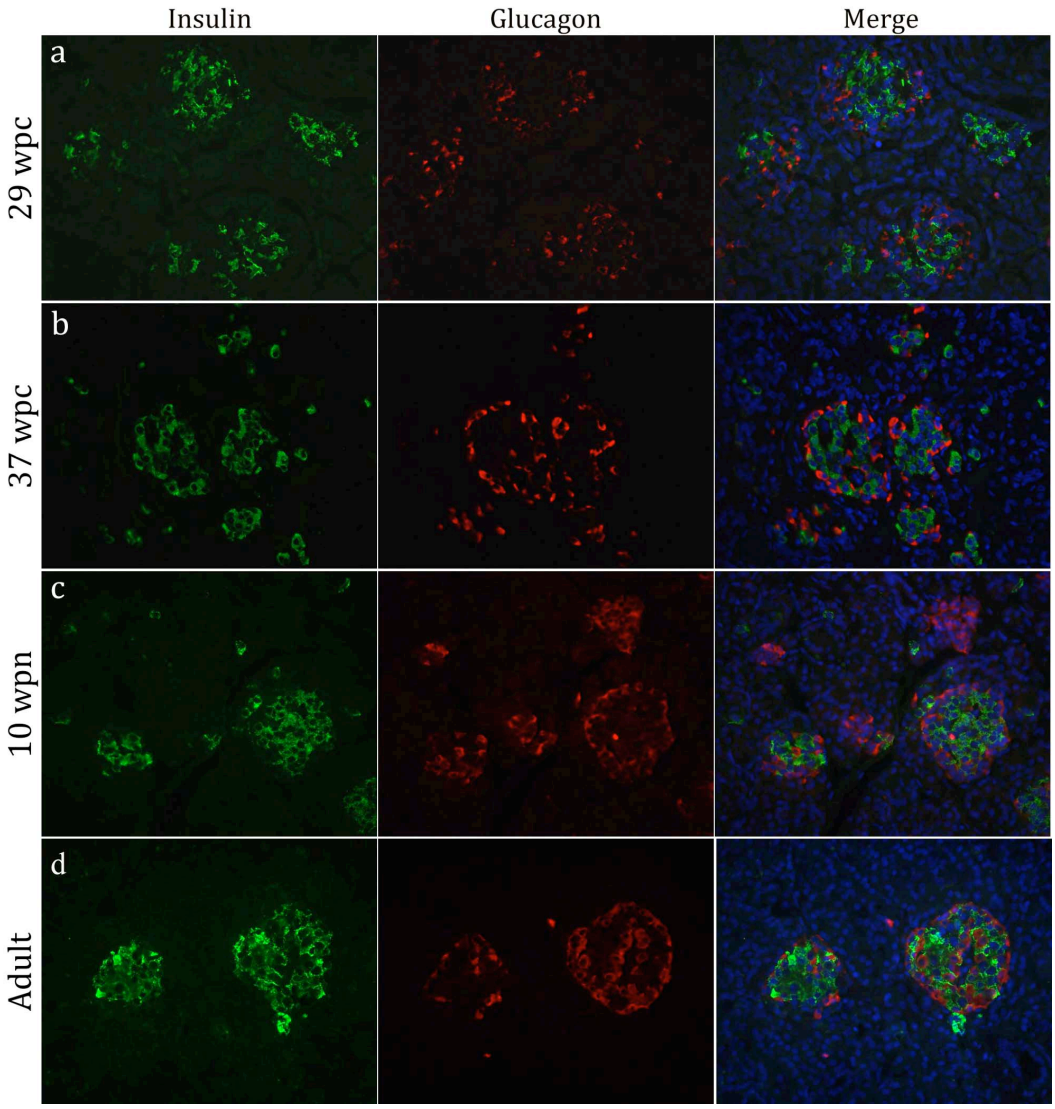

Figure 2-2 C. Insulin (green) and glucagon (red) expression during the third trimester of human fetal development (24 wpc-birth(38 wpc)) as demonstrated by samples 29 (a) and 37 (b) wpc, as well as 10 (c) wpn, and adult (d). DAPI nuclear counterstain (blue). In the third trimester, the overall proportion of insulin and glucagon expressing cells decreases compared to the second trimester, and insulin expressing cells are the predominant cell type in the islets. Similar to the post natal samples, insulin and glucagon expressing cells are intermixed within the islets. In the third trimester and post natal samples, islet size is more homogeneous across the tissue section. No Coexpression of insulin and glucagon was observed. Magnification is 400x.

A

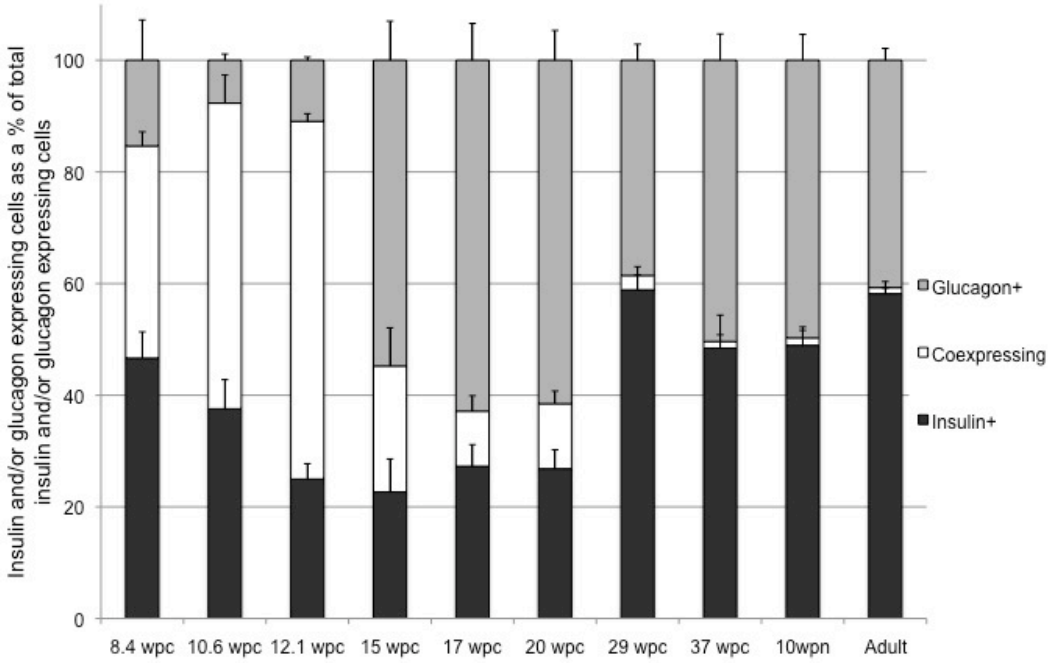

Figure 2-2 A. Quantification of insulin and glucagon expressing cells in the fetal and post natal pancreas. Percentage of insulin only expressing (dark gray), coexpressing insulin and glucagon (white), and glucagon only expressing (light gray) cells as a proportion of total population of insulin and/or glucagon expressing cells. Data is expressed mean ± SEM, n= 2-3 sections per group.

B

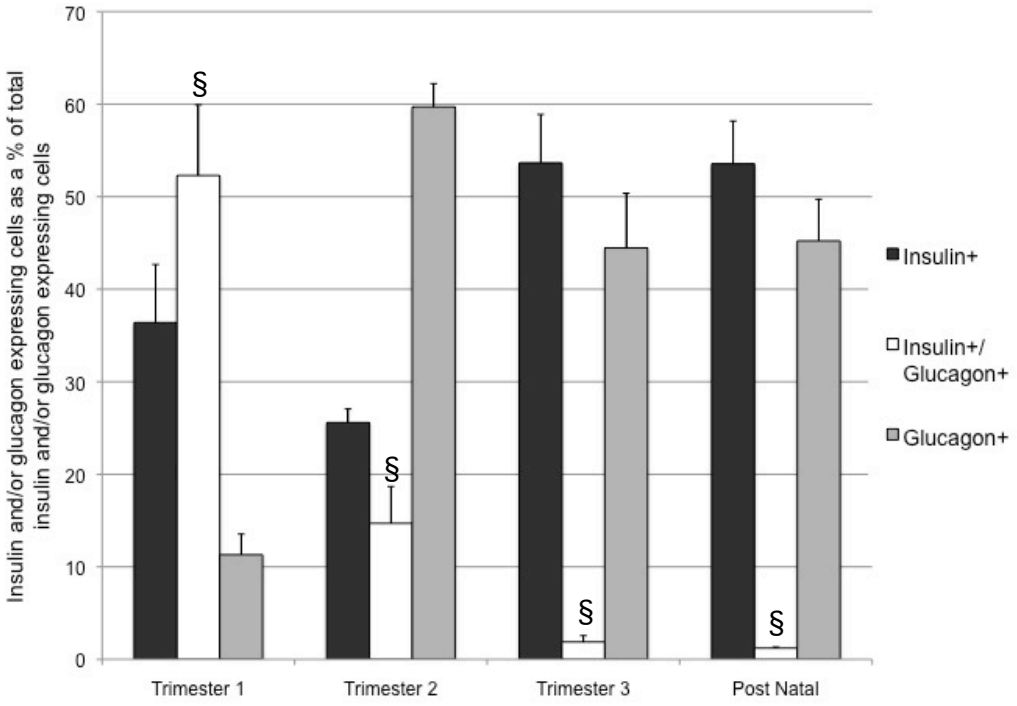

Figure 2-2 B. Quantification of insulin and glucagon expressing cells in the fetal and post natal pancreas. Percentage of insulin only expressing (dark gray), coexpressing insulin and glucagon (white), and glucagon only expressing (light gray) cells as a proportion of total population of insulin and/or glucagon expressing cells. Data is expressed mean ± SEM, n= 4-9 sections per group.

§$p \leq 0.025$ for coexpression of insulin and glucagon in the first trimester greater than in the second and third trimesters and post natal

C

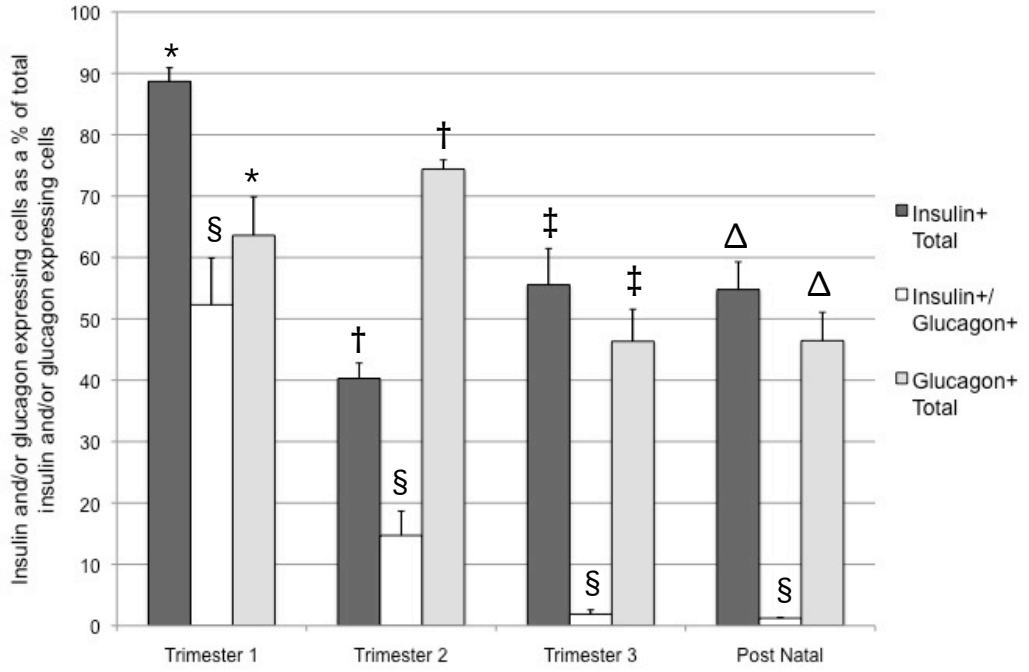

Figure 2-2 C. Quantification of insulin and glucagon expressing cells in the fetal and post natal pancreas. Percentage of total insulin expressing (dark gray), coexpressing insulin and glucagon (white), and total glucagon expressing (light gray) cells as a proportion of total population of insulin and/or glucagon expressing cells. Data is expressed mean ± SEM, n= 4-9 sections per group.

*$p \leq 0.025$ for insulin greater than glucagon expression in first trimester

†$p \leq 0.025$ for glucagon greater than insulin expression in second trimester

‡$p \geq 0.05$ for insulin expression equivalent to glucagon expression in third trimester

Δ$p \geq 0.05$ for insulin expression equivalent to glucagon expression post natal

§$p \leq 0.025$ for coexpression of insulin and glucagon in the first trimester greater than in the second and third trimesters and post natal

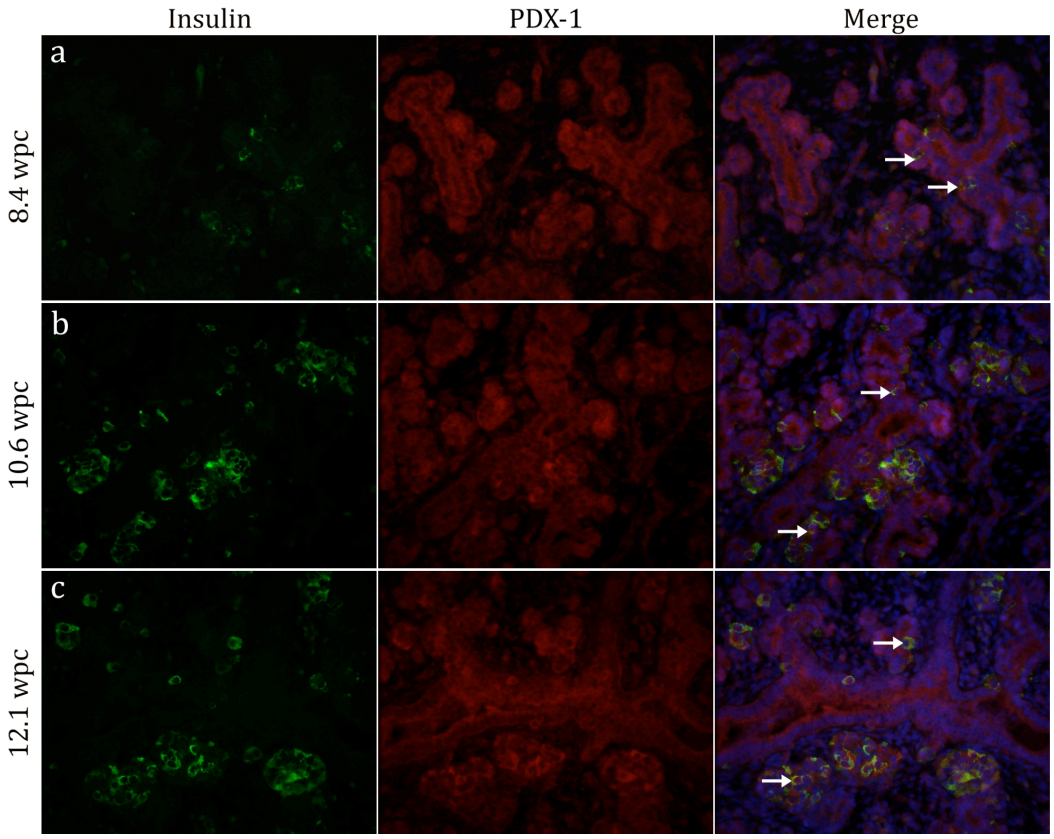

Figure 2-4 A. Insulin (green) and PDX-1 (red) expression during the first trimester of human fetal development (1- 12 wpc), as demonstrated by samples 8.4 (a), 10.6 (b), and 12.1 (c) wpc. DAPI nuclear counterstain (blue). Arrows demonstrate examples of coexpressing insulin and PDX-1 cells. In the first trimester, PDX-1 expressing cells are located in numerous duct-like structures, and insulin expressing cells always coexpress PDX-1. At 8.4 wpc, scattered insulin expressing cells are located amongst the duct-like PDX-1 expressing cells. As more insulin expressing cells aggregate at 10.6 and 12.1 wpc, the islet-like structures seem to bud off from the PDX-1 expressing duct-like structures. Magnification is 400x.

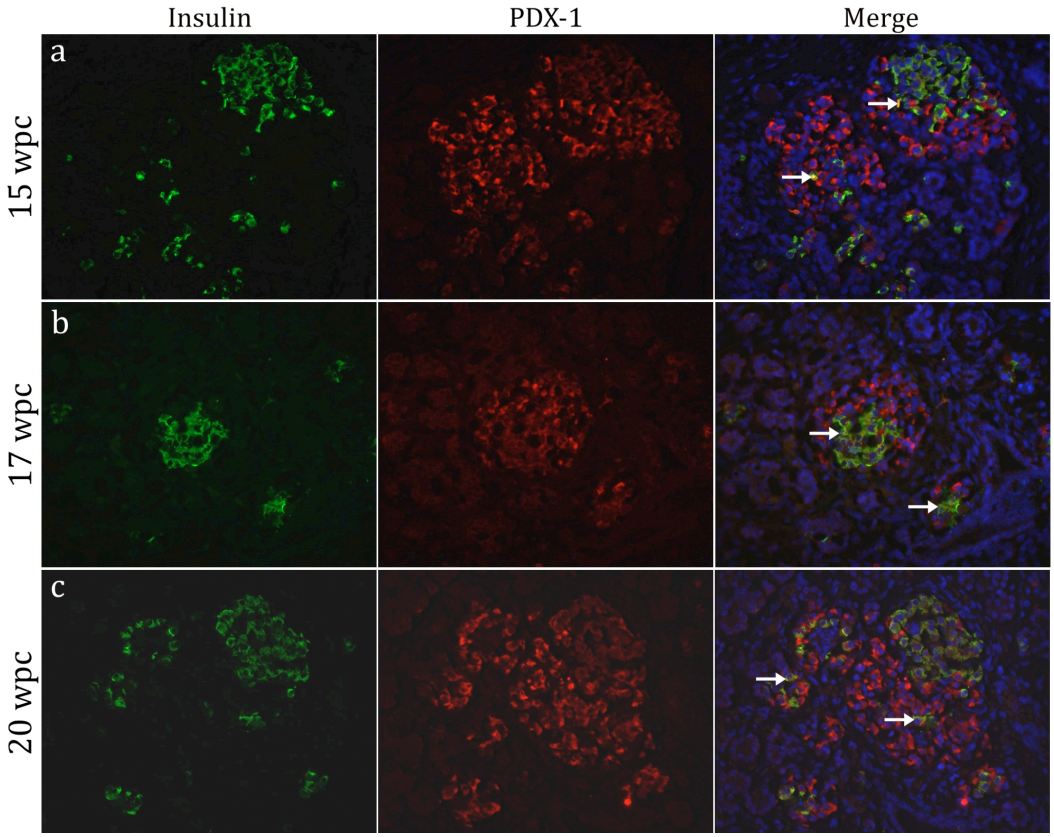

Figure 2-4 B. Insulin (green) and PDX-1 (red) expression during the second trimester of human fetal development (13- 24 wpc), as demonstrated by samples 15 (a), 17 (b), and 20 (c) wpc. DAPI nuclear counterstain (blue). Arrows demonstrate examples of coexpressing insulin and PDX-1 cells. In the second trimester, PDX-1 expression is localized mainly to islet structures, and PDX-1 expression is most pronounced in cells surrounding the insulin expressing cells. All insulin expressing cells coexpress PDX-1. The islet structures have less spatial association to the duct-like PDX-1 expressing structures. Magnification is 400x.

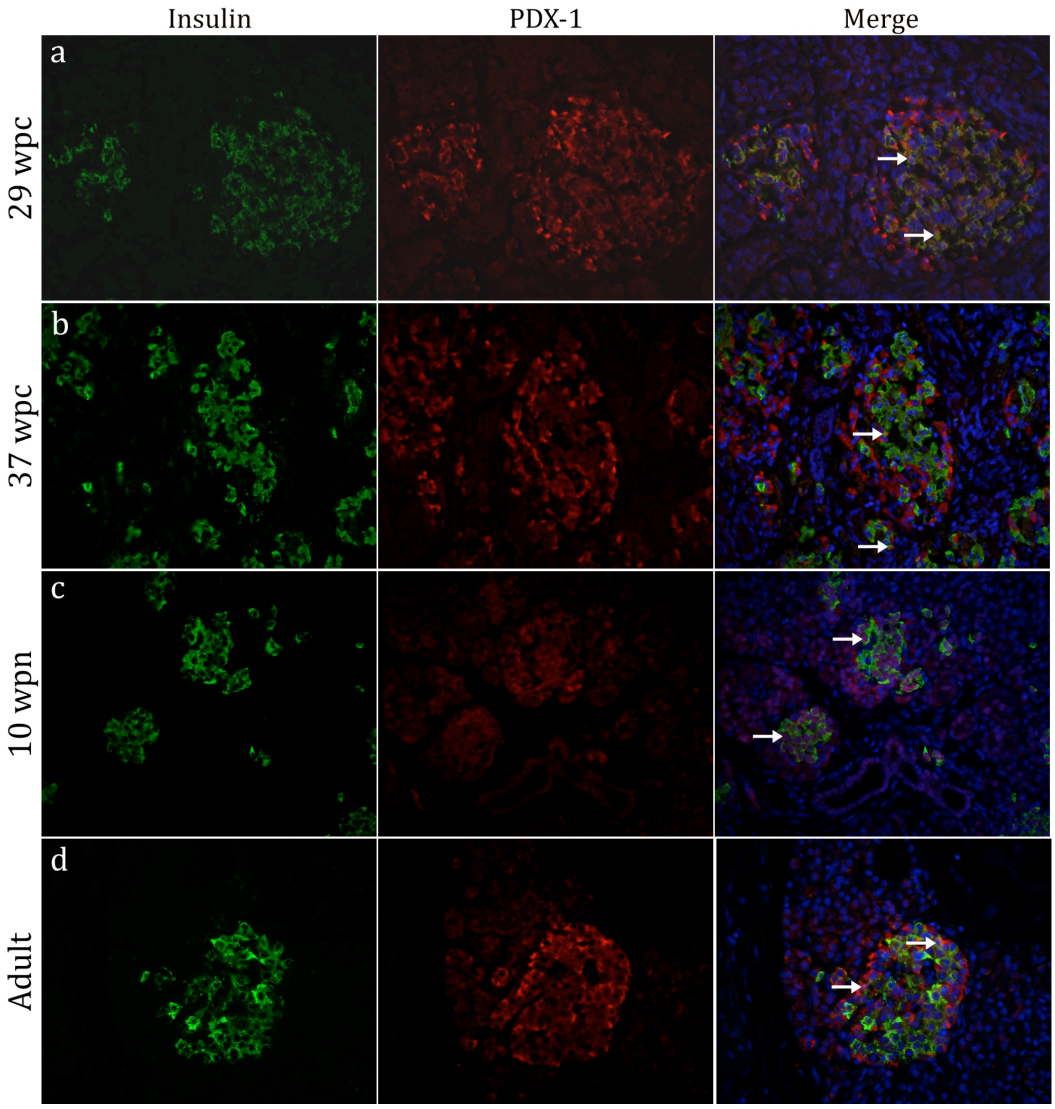

Figure 2-4 C. Insulin (green) and PDX-1 (red) expression during the third trimester of human fetal development (24wpc-birth(38wpc)) as demonstrated by samples 29 (a) and 37 (b) wpc, as well as 10 (c) wpn, and adult (d). DAPI nuclear counterstain (blue). Arrows demonstrate examples of coexpressing insulin and PDX-1 cells. In the third trimester, PDX-1 expression is minimally detected in duct-like structures, and expression is also proportionatally less compared to the second trimester in the islet structures. The third trimester expression pattern is similar to the post natal and adult, where PDX-1 is weakly coexpressed in insulin expressing cells, and a few strongly expressing PDX-1 alone cells are located at the periphery of the the insulin expressing clusters of cells. The first insulin alone expressing cell is observed at 37 wpc. Magnification is 400x.

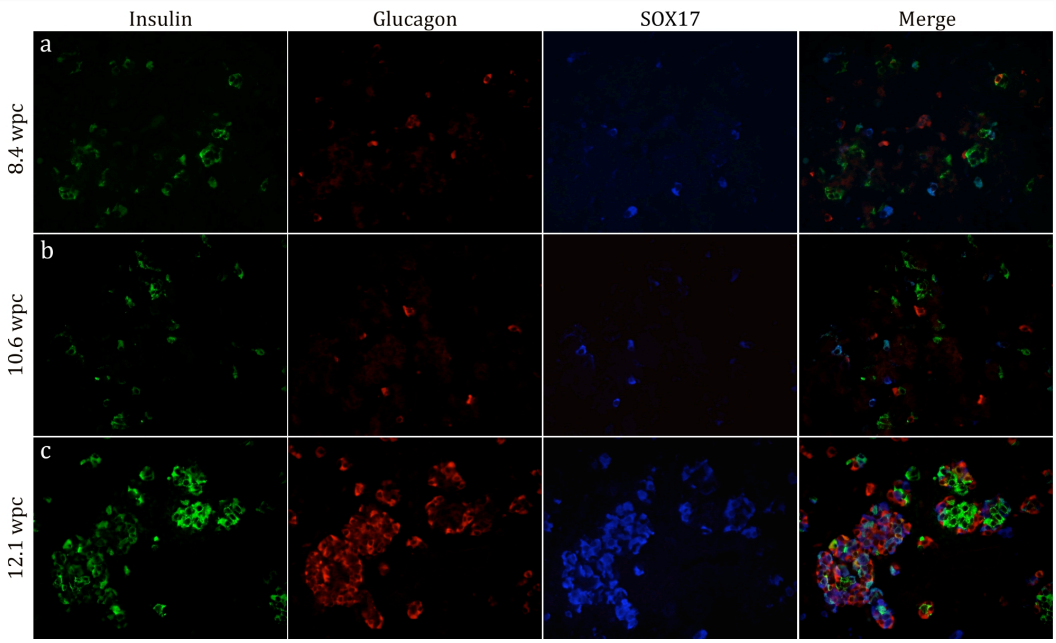

Figure 2-5 A. Insulin (green), SOX17 (red) and glucagon (blue) expression during the first trimester of human fetal development (1- 12 wpc), as demonstrated by samples 8.4 (a), 10.6 (b), and 12.1 (c) wpc. In the first trimester, SOX17 expressing cells are scattered non-specifically amongst the insulin and glucagon expressing cells. Upon islet-like cluster formation at 10.6 wpc SOX17 expressing cells are also found in the islet-like clusters. SOX17 is not coexpressed in glucagon expressing cells, however select insulin expressing cells appear to coexpress SOX17. Magnification is 400x.

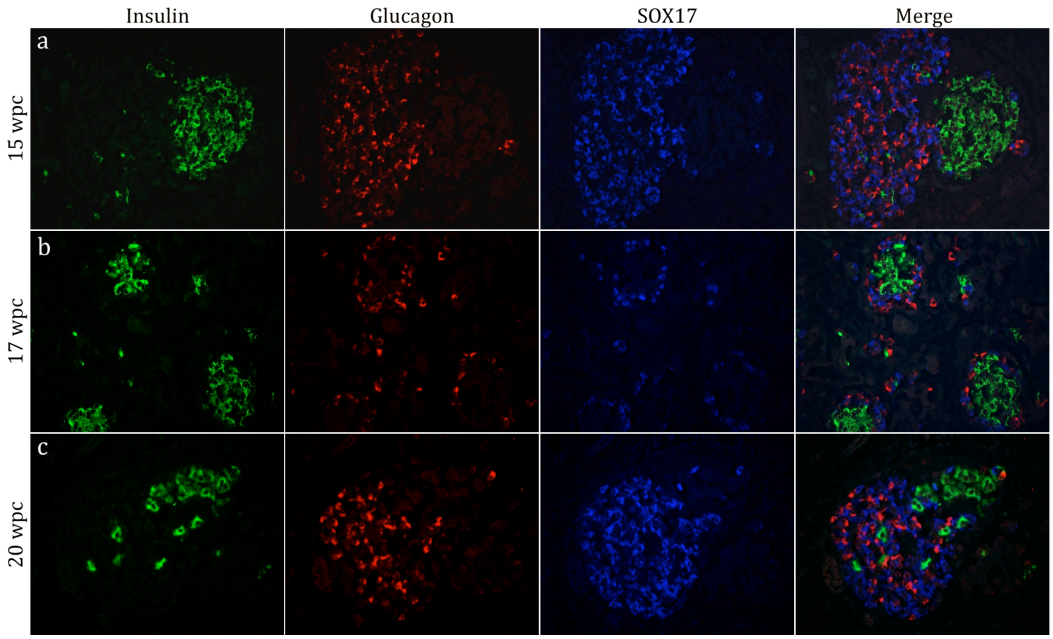

Figure 2-5 B. Insulin (green), SOX17 (red) and glucagon (blue) expression during the second trimester of human fetal development (13- 24 wpc), as demonstrated by samples 15 (a), 17 (b), and 20 (c) wpc. In the second trimester, SOX17 expressing cells are spatially associated with glucagon expressing cells within the islet structures. At 15, 17, and 20 wpc SOX17 is not coexpressed in insulin or glucagon expressing cells. Magnification is 400x.

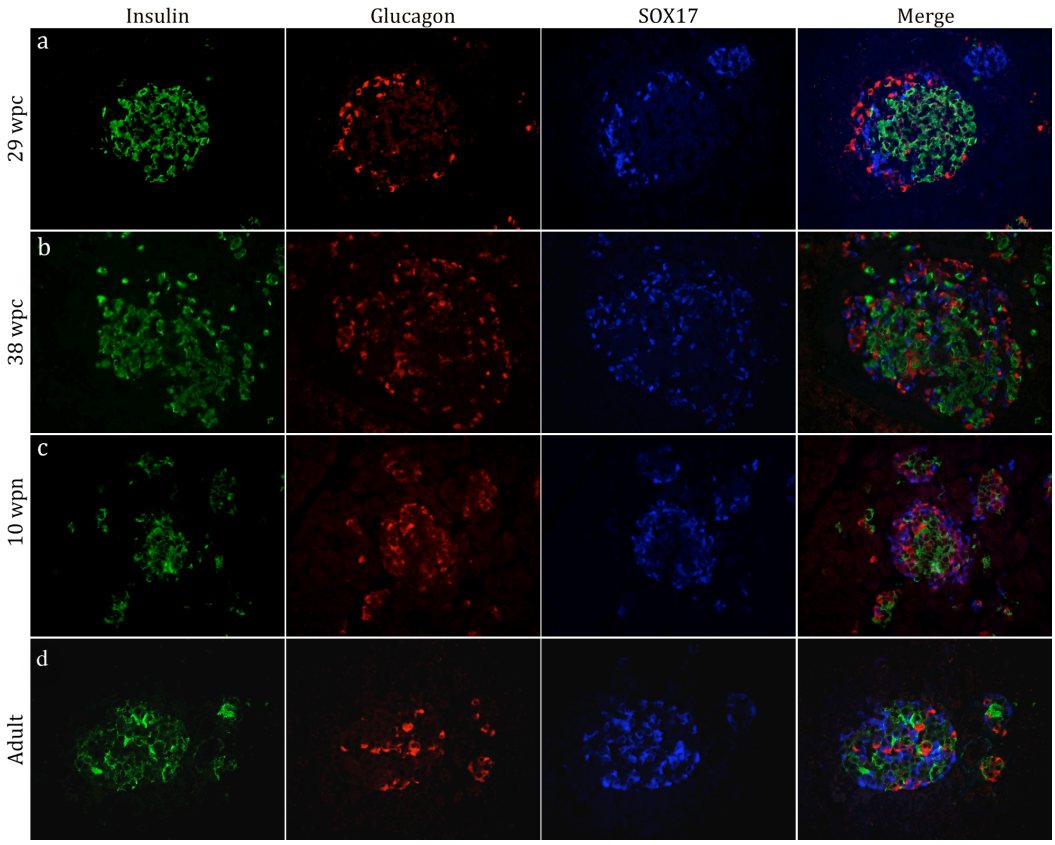

Figure 2-5 C. Insulin (green), SOX17 (red) and glucagon (blue) expression during the third trimester of human fetal development (24wpc-birth(38wpc)) as demonstrated by samples 29 (a) and 38 (b) wpc, as well as 10 (c) wpn, and adult (d). In the third trimester, SOX17 expressing cells are initially associated with glucagon expressing cells at 29 wpc, however as the insulin and glucagon expressing cells become intermixed by 37 wpc and post natally, the SOX17 expressing cells also are intermixed with other cell types within the islets. SOX17 is not coexpressed in insulin or glucagon expressing cells in the third trimester or post natally. Magnification is 400x.

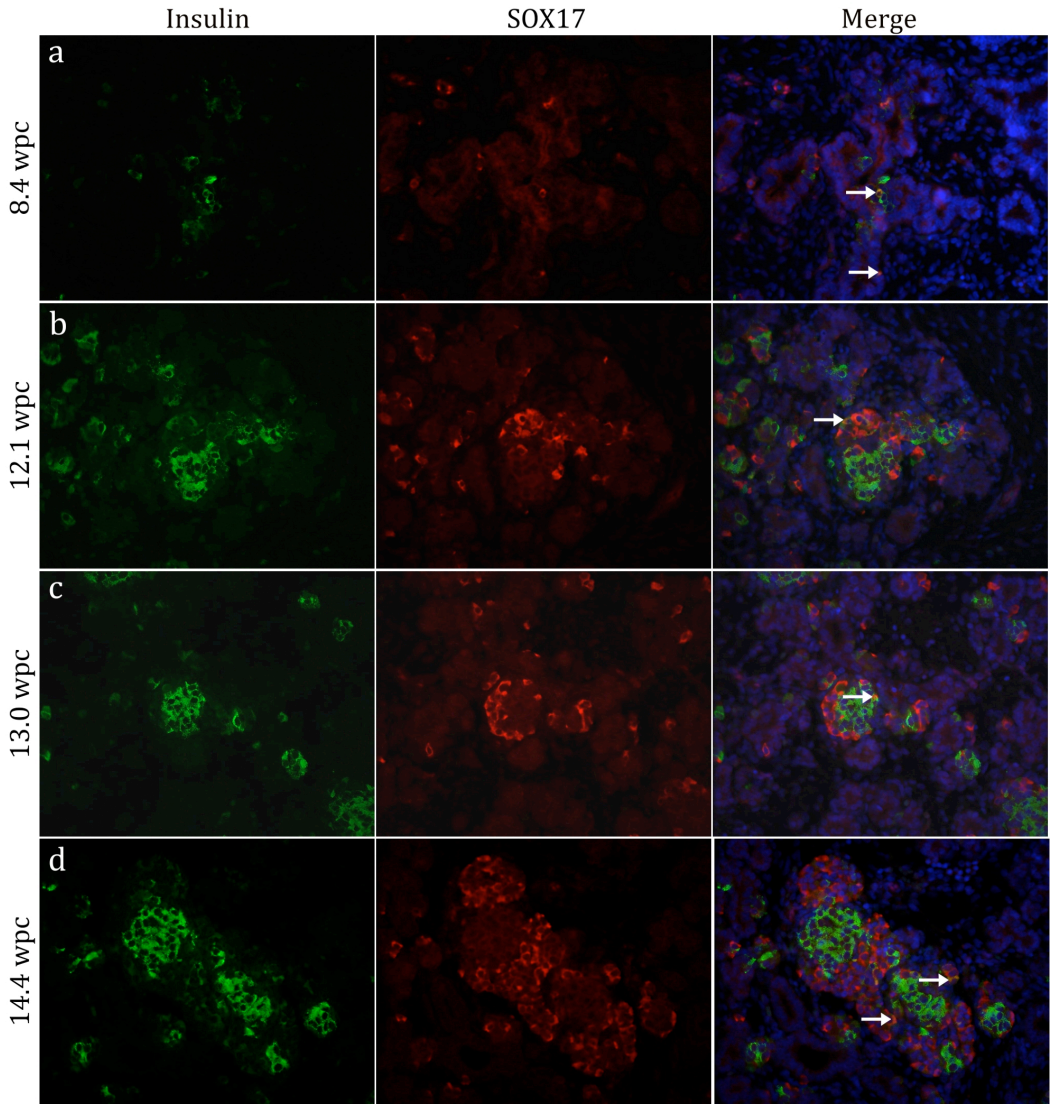

Figure 2-6. Confirmation of insulin and SOX17 coexpression in occasional cells at 8.4 (a), 12.1 (b), 13.0 (c), and 14.4 (d) wpc using insulin (green), SOX17 (red), and a nuclear counterstain, DAPI, (blue). Arrows demonstrate examples of coexpressing insulin and SOX17 cells. Coexpression of insulin and SOX17 is observed in occasional cells in the first trimester and early second trimester at 14.4 wpc. Magnification is 400x.

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CHAPTER THREE

IDENTIFICATION AND DIFFERENTIATION OF PDX1 EXPRESSING β-CELL PROGENITORS WITHIN THE HUMAN PANCREATIC EPITHELIUM

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3-1. INTRODUCTION

Islet transplantation is an attractive alternative to daily insulin injections to achieve a more physiological means for restoring glucose homeostasis [1-3]. Identification and understanding the origin of a potential human β-cell progenitor could alleviate the current shortage of donor islets and contribute to the overall knowledge of β-cell regeneration. However, the study of β-cell progenitors is fraught with controversy, as several models and possible mechanisms describing the origin and existence of these progenitor cells have been proposed. Despite lineage tracing experiments utilizing transgenic mouse models [4-6] the exact origin of β-cell progenitors residing within the pancreas is yet to be elucidated. For example β-cell progenitors have been proposed to originate from: β-cell replication [4], acinar cell transdifferentiation [7,8], ductal cell transdifferentiation [9-12], pancreas derived multipotent precursors [13], pluripotent islet survivor cells [14] and β-cell dedifferentiation with expansion of mesenchymal stem cell intermediates [15-20].

Previously we reported [21] that mesenchymal stem cells (MSCs) also referred to as multi-potent stromal cells [22] could be expanded from human islet depleted pancreatic tissue (IDPT) that remains following islet isolation and demonstrated that these pancreatic MSCs could be partially differentiated into islet-like cells. However, in a follow up study [23] we could not restore an epithelial phenotype during tissue culture or generate glucose responsive islet cells. Unlike the reversible epithelial-mesenchymal transition (EMT) model first described by Gershengorn et al. [15] and the dedifferentiation of β-cells then replication of β-

cell –derived cells described by Russ et al. [20] we propose that epithelial β-cell progenitors reside within the human pancreatic epithelium and that these cells have the potential to respond favorably to *in vitro* differentiation.

The purpose of this study was to determine if a β-cell progenitor population resides within the pancreatic epithelium by utilizing a novel pancreatic epithelial cell culture medium (PEM) that preserves the pancreatic epithelial population *in vitro,* as well as lentiviral lineage tracing to study the origin of β-cell progenitors within the adult human pancreas, and determine if these cells respond favorably to differentiation protocols. In this study we report that during *in vitro* human pancreatic MSC expansion epithelial cells also proliferate. In addition, if epithelial cells are selected, following differentiation this epithelial cell enriched population expresses developmental transcription factors indicative of a β-cell progenitor. We describe a novel culture medium, (PEM), that minimizes epithelial cell dedifferentiation and MSC overgrowth and maintains epithelial cell phenotype thereby allowing long-term *in vitro* study of this cell population. Furthermore, by utilizing dual and single lentiviral reporters developed by Szabat et al. where enhanced green fluorescent protein (EGFP) is controlled by the rat *Insulin 1* promoter and monomeric red fluorescent protein (mRFP) under the control of the mouse *Pdx1* promoter [24] we determined that $Pdx1^{+}$cells after 25 days post-differentiation are indeed epithelial cells. Overall we report a novel media that promotes the survival of pancreatic epithelial cells and minimizes MSC overgrowth that post-differentiation $Pdx1^{+}$ cells are indeed epithelial cells and not MSCs.

3-2. MATERIALS AND METHODS

3-2.1 Cell expansion and differentiation

Human islets (n=9) and islet depleted pancreatic tissue (IDPT; n=13) were obtained from the Edmonton Clinical Islet Transplant Program. Average donor age was 54 (30-71 years) and islet purity assessed by dithizone staining ranged between 10-40%. IDPT (<5% insulin positive cells) was obtained following removal of islets by density gradient purification [21,23,25]. Upon receipt, IDPT was cultured in Roswell Park Memorial Institute (RPMI) 1640 medium (Invitrogen, Burlington, ON Canada) supplemented with 0.5% w/v fraction V bovine serum albumin (Sigma-Aldrich, Oakville, ON Canada), 1.0% insulin-transferrin-selenium (Sigma-Aldrich) and 100 U penicillin/1000 U streptomycin (Invitrogen). Islets were cultured in CMRL-1066 medium (Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 2 mM L-glutamine (Invitrogen), 10 mM HEPES and 100 U penicillin/1000 U streptomycin (Invitrogen). Both IDPT and islets were cultured in 150 mm non-tissue culture treated plates (Corning, NY USA) and maintained for 24-48 hours at 37°C in 5% CO_2 95% air. Following culture, single cell suspensions were derived by dissociating islets or cellular aggregates derived from the cultured IDPT with 0.05% trypsin, 0.5 mM EDTA (Invitrogen) in 1% PBS.

These single cell preparations were further cultured and expanded in pancreatic MSC medium composed of RPMI 1640 supplemented with 10% FBS, 10 mM HEPES, 1 mM sodium pyruvate (Invitrogen), 71.5 μM β-mercaptoethanol (Sigma-Aldrich), 20 ng/mL epithelial growth factor (EGF, R&D, Minneapolis,

MN USA), 20 ng/mL fibroblast growth factor (Invitrogen) and 100 U pencillin/1000 U streptomycin [21,23,25]. Cells expanded in this medium achieved confluence within 10-14 days and required passaging every 7 days after that. From both islets and IDPT at the 2nd and 3rd passage we routinely generated a cell population with MSC characteristics as previously described [21,23,25]. These expanded cell populations were subsequently differentiated using a multi-step protocol and characterized by flow cytometry, immunohistochemistry and RT-PCR [21,23,25]. For differentiation the cell monolayer is treated with 20 ng/mL OncostatinM (R&D) for 72 hours. In steps 2 and 3 the medium is supplemented with 10 mM nicotinamide (Sigma-Aldrich) for 72 hours followed by 10 mM nicotinamide and 10 nM exendin4 (Sigma-Aldrich) for another 72 hours. In step 4, 10 ng/mL of transforming growth factor-β1 (TGFβ-1; EMD Millipore, Billerica, MA USA) is included with nicotinamide and exendin4 for 3-7 days with media changes every 72 hours. Cell monolayers are detached with trypsin and aggregated by reconstituting cells at 125, 000 cells/mL in medium supplemented with nicotinamide, exendin4, TGFβ-1, 0.5X ITS^{+}premix (BD Biosciences, Beford, MA USA) and transferred to ultra-low attachment non-tissue culture treated plates (Corning).

In another experiment to preserve epithelial cell phenotype, single cells derived from islets or the IDPT were also cultured and expanded in a pancreatic epithelial expansion medium either on 150 mm tissue culture treated plates (Corning) or 12 mm poly-l-lysine coated cover slips (BD Biosciences) placed in 24 well tissue culture treated plates (Corning). Pancreatic epithelial expansion

medium is composed of D-MEM/F12 (Invitrogen) supplemented with 0.5% FBS, 0.1 μg/mL EGF, 0.4 μg/mL dexamethasone (Sigma-Aldrich), 14 mg/mL bovine brain extract (Lonza, Walkersville, MD USA), 0.05 μM tri iodo-l-thryonine sodium salt (Sigma-Aldrich), 0.1 mg/mL soybean trypsin inhibitor (US Biological, Swampscott, MA USA), 0.5X ITS^{+}premix (BD Biosciences) and 100 U penicillin/1000 U streptomycin. These cell preparation were subsequently differentiated using the multi-step protocol as previously described but without transferring to ultra-low attachment plates.

3-2.2 Immunohistochemistry

The following antibodies [26] that recognize several endocrine and non-endocrine cell surface epitopes: HPi1, HPi2, HPi3, HPi4 (endocrine cells); HPa1, HPa2 (alpha cells); HPx1, HPx2 (exocrine cells); and HPd1 and HPd2 (duct cells) were purchased from the Oregon Health and Science University (OHSU), Portland, Oregon USA under the appropriate Material Transfer Agreement between institutions.

Double immunofluorescence (IF) analysis was performed on paraffin sections of single cells that had first been fixed with 1% formalin (Fisher Scientific, Nepean, ON Canada) and embedded in 2% low melting point agarose (Sigma-Aldrich) or cells which had been differentiated on 12 mm poly-l-lysine cover slips (BD Biosciences). Paraffin sections were processed and immunostained as previously described [23]. Cover slips were fixed in 1% formalin for 30 minutes in the dark at 4°C, and then washed twice with 5% normal goat serum (NGS) in PBS. For antibodies requiring permeabilization, 0.3% saponin (Sigma) in PBS

was applied for one minute, and another two washes of 5% NGS followed. All cover slips were then blocked with 20% NGS for 1 hour in the dark. Primary antibodies were diluted in 5% NGS at the following concentrations: 1/200 anti-epithelial cell adhesion molecule (EpCAM, Stem Cell Technologies, Vancouver, BC Canada), 1/100 anti-vimentin (Dako, Mississauga, ON Canada), 1/25 anti-human proliferating cell nuclear antigen (PCNA, Invitrogen), 1/25 anti-CA19-9 (AbD, Serotec, Oxford, UK), 1/50 anti-CK19 (Dako), 1/5000 anti-glucagon (Sigma-Aldrich), 1/1000 anti-insulin (Dako), 1/1000 anti-pancreatic polypeptide (Dako), 1/1000 anti-somatostatin (Dako), 1/1000 anti-Pdx1 (Abcam, Cambridge, MA USA), 1/20 HPi1 (OHSU), 1/20 HPi2 (OHSU), 1/20 HPi3 (OHSU), 1/10 HPi4 (OHSU), 1/10 HPd1 (OHSU) and 1/10 HPd2 (OHSU). All appropriate species-specific secondary antibodies were AlexaFluor 488 or 594 conjugates (Molecular Probes, Eugene, OR USA) and diluted 1/200 in 5% NGS. Slides and or cover slips were cover-slipped with ProLong Gold anti-fade reagent with DAPI (Invitrogen) to counter stain nuclei and preserve fluorescence. Negative controls were incubated without primary antibodies. All slides were visualized with an Axioscope II equipped with AxioCam MRC and analyzed with Axiovision 4.6 (Carl Zeiss, Gottingen, Germany).

3-2.3 Flow cytometry

Single cells from 24-48 hour cultured IDPT were stained and sorted using the BD Influx cell sorter (BD Biosciences). Cells were co-stained with 3/50 anti-CD29-PE-Cy5 (Invitrogen) and 1/5 anti-EpCAM-FITC (Stem Cell Technologies). $CD29^{+}EpCAM^{+}$ and $CD29^{+}EpCAM^{-}$ cell populations were sorted and purity was

95-97%. These cell populations were expanded and differentiated as outlined above. Fluorescence activated cell sorting (FACS) analysis of cells expanded *in vitro* and single cells from islets and IDPT stained with the OHSU antibodies was performed using the FACS Calibur (BD Biosciences) and Cell Quest Pro software and compared to matched isotype controls. Secondary antibodies were goat anti-mouse IgG (H+L) conjugated to FITC or PE (SouthernBiotech, Birmingham, AL USA). Values are expressed as mean percent ± SEM.

3-2.4 RNA isolation and RT-PCR

Islets and IDPT cells prior to cell culture, during expansion and post differentiation were preserved in Trizol reagent (Invitrogen) and stored at -80°C. RNA was extracted in combination with the RNeasy Mini Kit (Qiagen, Mississauga, ON Canada) as per the manufacture's protocol. cDNA was synthesized as described [21] and 1 uL of cDNA was amplified for 35 cycles using Platinum Taq polymerase (Invitrogen) as described [21]. Primer pairs to PDX1, NeuroD, insulin, glucagon, Neurogenin3 and β-2-microglobulin (house keeping gene) are described elsewhere [21] and EGFP and mRFP primers were described by Szabat et al. [24]. All RT-PCR products were resolved on 2% agarose gels stained with eithidium bromide. cDNA negative controls contained water in place of RNA and RT-PCR negative controls contained water in place of cDNA, β2-microglobulin (beta-2) ensured cDNA integrity.

3-2.5 Lentivirus infection

Lentiviral vectors were kindly provided by Dr. James D Johnson (University of British Columbia, Vancouver, BC, Canada). Cloning of these vectors is

described in detail by Szabat et al. [24]. We received the following vectors: dual reporter mouse *Pdx1* promoter-monomeric red fluorescent protein (mRFP) /rat *Insulin 1* promoter-enhanced green fluorescent protein (EGFP), single reporter mouse *Pdx1* promoter-mRFP, single reporter rat *Insulin 1* (Ins1)-EGFP as well as the structural and envelope vectors. Virus was produced by transfection of 293T cells that were a gift from Dr. Patrick MacDonald (University of Alberta, Edmonton, AB, Canada) utilizing FuGENE6 Transfection Reagent (Roche Diagnostics, IN, USA) and the protocol first described by Dr. Garry Nolan Lab (http://www.stanford.edu/group/nolan/index.html). Virus was titred using the rat INS1 cell line (a gift from Dr. Patrick MacDonald) and titres were between 2-4 X 10^6 TU/mL with an infection efficiency of 40-70%. Single cells from human islets or IDPT were plated at a density of 0.3 X 10^6cells/well onto a 24 well plate that contained 12 mm poly-l-lysine cover slips and cultured in pancreatic epithelial expansion medium. Cells were allowed to adhere over a 48-72 hour period and infected at a MOI of <1. Protein expression (fluorescence) was monitored daily and peak fluorescence of human primary cells was routinely detected between 7-10 days post infection. Differentiation of infected primary cells was started at 7 days post-infection and 10 days after plating. Absolute counts of positive mRFP and EGFP cells were counted using ImageJ software [27].

3-2.6 Statistical analysis

Data is expressed as mean ± SEM. Statistical comparisons were performed with STATA 11 (StataCorp LP, College Station, TX) using one-way analysis of variance and Bonferroni post-hoc tests, since data collected is assumed to be

independent, representative, and normally distributed. The acceptable level of significance was considered $p < 0.05$.

3-3. RESULTS

3-3.1 Epithelial cells from IDPT proliferate in MSC cell culture conditions and respond favorably to in vitro differentiation

Previously, we demonstrated that during *in vitro* cell expansion of cells from IDPT, the proportion of epithelial cells decreased while vimentin positive cells (MSCs) significantly increased [21,23]. We hypothesized that this observation was either due to simple over growth of preexisting MSCs (vimentin$^+$ cells) or possible dedifferentiation and EMT of pancreatic epithelial cells due to our cell culture conditions. Therefore, to determine if epithelial cells were still capable of proliferation, in this study we assessed cell proliferation after 14 days in culture via dual immunofluorescence (IF) staining. Cells that were formalin fixed and embedded in agarose were stained with antibodies against epithelial cell adhesion molecule (EpCAM) or vimentin then co-stained for proliferating cell nuclear antigen (PCNA). After 14 days in culture we confirmed that vimentin$^+$ cells were the predominant cell population and that these cells were also PCNA$^+$ and proliferating (Figs. 3-1A-C). A small proportion of epithelial cells (EpCAM$^+$) were still present and were also PCNA$^+$ thus still proliferating (Figs. 3-1D-F).

To further verify that epithelial cells can still proliferate in our MSC expansion medium we sorted CD29$^+$ EpCAM$^+$ epithelial cells (n=3), expanded these cells for 14 days and determined their epithelial cell composition. The CD29$^+$ cell

surface antigen was chosen as we have previously determined that at the time of isolation [23] within the IDPT the $CD29^+$ cell population co-expresses the most epithelial cell antigens. This population was compared to $CD29^+EpCAM^-$ (n=3) and non-sorted IDPT cells (control) from which MSCs ($vimentin^+$ cells) rapidly expand from. After 14 days in culture $CD29^+EpCAM^+$ cells cultured in MSC expansion medium still expressed EpCAM and were PCNA positive as determined by double IF staining, confirming that the epithelial cell population was still proliferating (Figs. 3-1G-I). The proportion of epithelial cells was compared between sorted $CD29^+EpCAM^+$ and $CD29^+EpCAM^-$ cell populations after 14 days in culture (Table 3-1). $CD29^+EpCAM^+$ cells expressed more pan-epithelial (EpCAM) antigens and ductal epithelial cytokeratin 19 (CK19) cell antigens than the $CD29^+EpCAM^-$ cell population (p=0.014). The $vimentin^+$ population in $CD29^+EpCAM^+$, $CD29^+EpCAM^-$ and control were similar (p=0.442, Table 3-1) indicating that these culture conditions do indeed favor MSC expansion over epithelial cells. However, compared to the control cells that had less than 17.42±6.88% epithelial cells, the sorted $CD29^+EpCAM^+$ cell population still contained 47.33±6.36% $epithelial^+$ cells (p=0.048, Table 3-1).

To determine which cell population responds favorably to our differentiation protocol, $CD29^+EpCAM^+$ cells and MSCs derived from non-sorted IDPT were expanded then differentiated utilizing our multi-step differentiation protocol [21,23] and mRNA expression was compared (n=3). Comparison of mRNA expression demonstrated that differentiated MSCs and $CD29^+EpCAM^+$ cells expressed Pdx1, NeuroD, insulin and glucagon but only the $CD29^+$ $EpCAM^+$ cells

upon aggregation into cell clusters post differentiation expressed Ngn3 (Fig. 3-1J) a transcription factor expressed in endocrine progenitor cells [5] thus we determined that a starting cell population which contains more epithelial cells has a better outcome when differentiated *in vitro*.

3-3.2 Novel pancreatic expansion medium preserves epithelial cell phenotype

Our current MSC expansion medium limits pancreatic epithelial cell growth *in vitro* [21,23]. We determined that sorted epithelial cells could expand and divide in MSC expansion medium but vimentin$^+$ MSCs were still the predominating cell population in this medium (Table 3-1). Therefore we sought to modify our cell culture conditions in order to prevent epithelial cell dedifferentiation and MSC overgrowth. By reducing the serum content from 10% to 0.5% and modifying the growth factor content [28-30] we were able to preserve the epithelial cell population and phenotype thus minimizing MSC overgrowth. Compared to control cells (MSCs expanded from unsorted IDPT) and sorted epithelial cells expanded in MSC expansion medium, IDPT cells cultured in pancreatic epithelial expansion medium contained significantly less vimentin$^+$ cells (85, 80 and 44% respectively, $p=0.007$, Table 3-1). Therefore, pancreatic epithelial expansion medium was used for the remainder of this study to trace cell fate and was supplemented for cell differentiation.

3-3.3 Characterization of islet and IDPT cells with OHSU antibodies

Novel antibodies obtained from the Oregon Health and Science University (OHSU) that recognize several endocrine and non-endocrine cell surface epitopes [26] were used to determine cell composition of dissociated IDPT and islets prior

to expansion (Table 3-2). The IDPT is composed mainly of epithelial cells [21,23], which include ductal epithelial cells (CA19-9) and exocrine cells (HPx1 and HPx2). Within the epithelial cell fraction of the IDPT there exists sub-sets of ductal epithelial (HPd1 and HPd2) and contaminating endocrine cells [21,23] as indicated by HPi2 staining (Table 3-2). Single cells from dissociated islets were also analyzed via OHSU antibodies (Table 3-2). The percentage of single cells isolated from islets that were positive for the OHSU pan-endocrine (Hpi1-Hpi4) antibodies was less than 12%. These results are comparable to Dorrell et al., who reported that in islets that were between 30-40% dithizone positive, both staining for HPi1 and HPi2 were less than 10% positive [26]. These antibodies were also used to characterize cells following expansion and differentiation by immunofluorescent staining.

3-3.4 Lineage tracing and characterization of dissociated islet and IDPT epithelial cells expanded in pancreatic epithelial expansion medium and differentiated in vitro

To determine the progenitor cell content within the pancreatic epithelium, islets and IDPT were dissociated and cultured in pancreatic epithelial expansion medium, infected with either the single or dual reporter lentivirus, differentiated and characterized via IF staining and RT-PCR.

Single cells from dissociated islets and IDPT were seeded onto poly-l-lysine coated cover slips placed in the wells of 24 well plates. Cells were expanded in pancreatic epithelial medium and infected with, the *Pdx1*-mRFP-*Insulin 1*-EGFP dual reporter, *Pdx1*-mRFP or *Insulin 1*-EGFP single reporter lentivirus prior to

differentiation as a monolayer. Peak fluorescence was detected between 7-10 days post infection in both islet and IDPT preparations at which time differentiation commenced. Controls were infected but not differentiated. During differentiation of islet cells, cell aggregates formed throughout the cell monolayer. Within these adherent aggregates $Pdx1^+$ and Insulin 1^+ (Ins1) expressing cells were observed (Figs. 3-2 A-C and 3-3 A-B). In undifferentiated conditions fewer cell aggregates were observed and $Pdx1^+$ and $Ins1^+$ cells were scattered throughout the monolayer and not localized within cell aggregates (Figs. 3-2 D-F and 3-3 C-D). Overall less $Pdx1^+$ and $Ins1^+$ cells were observed in undifferentiated cell preparations. When analyzing image fields, in 4/4 cell preparations approximately twice as many $Pdx1^+$ and $Ins1^+$ cells were observed in differentiated conditions (51.25±17.24 mRFP and 28.13±9.34 EGFP) versus undifferentiated conditions (31.75±10.83 mRFP and 14.50±4.03 EGFP) as determined by absolute counts. The majority of fluorescent cells were single positive for either $Pdx1^+$ or $Ins1^+$, double positive $Pdx1^+Ins1^+$ were infrequent but present. In addition in both the differentiated and undifferentiated cell conditions approximately twice as many $Pdx1^+$ cells than $Ins1^+$ cells were observed. A similar pattern was observed in differentiated (Figs. 3-3 E-F) and undifferentiated (Figs. 3-3 G-H) wells of cultured IDPT infected with *Pdx1*-mRFP or *Ins1*-EGFP although much less *Pdx1* and *Insulin 1* (not shown) expression was observed compared to islet cell cultures. Overall IDPT did not respond as well to cell differentiation conditions as islet cell preparations.

3-3.5 Characterization of lentiviral infected differentiated cells by immunofluorescence staining

To determine which cells within the differentiated cell aggregates were $Pdx1^+$, cells that were infected with the *Pdx1*mRFP lentivirus were characterized by IF staining (Fig. 3-4) utilizing the following antibodies: OHSU pan-endocrine (HPi1-i4) and ductal epithelial (HPd1 and HPd2), EpCAM, vimentin, CK19, glucagon, insulin, pancreatic polypeptide and somatostatin. Remarkably cells positive for glucagon, pancreatic polypeptide, somatostatin, HPi1 and HPi4 still remained post 25 days in culture although these cells were infrequent and did not express $Pdx1^+$ (not shown). $Pdx1^+$ cells did not stain positive for vimentin (Figs. 3-4 A-C) or CK19, HPd1 and HPd2 (not shown). In addition, we can also conclude that after 25 days in culture $vimentin^+$ cells have not overgrown the wells. $Pdx1^+$ cells did co-stain with EpCAM (Figs. 3-4 D-F), HPi2 (Figs. 3-4 G-I) and HPi3 (Figs. 3-4 J-L) thus $Pdx1^+$ cells are localized to the epithelial cell population. Pdx1 antibody and insulin antibody staining (Fig. 3-5) verified expression results. Nuclear and cytoplasm Pdx1 staining (Figs. 3-5 A-C) was observed while insulin staining was confined to the cytoplasm (Figs. 3-5 D-F).

RT-PCR was used to confirm lentivirus infection as well as Pdx1 and insulin expression (Fig. 3-5 G). Message for EGFP and mRFP or both was detected in single and or dual infected cells. Correspondingly message for Pdx1 in differentiated islet cells was detected while message for Pdx1 was not detected in IDPT or undifferentiated islet cells. Message for insulin and glucagon was detected in both differentiated and undifferentiated islet and IDPT cells.

3-4. DISCUSSION

Although, several recent human studies that involve lineage tracing [13,14,20,24] have been conducted the debate continues as to the origin of human β-cell progenitors. For example, Russ et al. has provided convincing evidence that human β-cells can indeed be expanded *in vitro* and can be successfully redifferentiated into insulin producing cells [20] thereby confirming EMT first suggested by Gershengorn et al. [15]. However, Russ et al. has also determined that although EMT is possible, EMT does not induce multipotent cells [19]. In contrast, White et al. studying a similar adherent cell population derived from dissociated islets termed islet survivor cells (ISCs) determined that these cells expressed the pluripotency-associated stem cell marker complex OCT4/SOX2/NANOG [14]. In addition, Smukler et al. has determined that similar to the mouse pancreas where they first identified a precursor population termed pancreas derived multipotent precursors [31] the human pancreas also contains a similar population of multipotent cells [13]. In their study they determined that although these pancreas-derived multipotent precursors derived from human pancreas can contribute to both pancreatic and neural lineages these cells do not express OCT4.

In our previous studies we were able to expand MSCs from the IDPT [21,23] and partially differentiate these cells into islet-like clusters but we were unable to restore epithelial cell phenotype or detect insulin protein by IF staining. We have since hypothesized that in our differentiation conditions it was the low percentage of epithelial cells, which still remained after cell expansion that responded

favorably in our differentiation protocol [21,23] and not differentiated MSCs or an MSC intermediate. Thus, in our previous *in vitro* studies [21,23] one single limitation exists: the de-differentiation of islet or IDPT cells during long-term culture results in the loss of epithelial cells thus making this population difficult to study *in vitro*.

Since our MSC expansion medium either selected vimentin$^+$ cells or encouraged dedifferentiation of pancreatic epithelial cells, in this study we sought to prolong epithelial cell phenotype by utilizing a novel pancreatic epithelial cell culture medium in combination with our differentiation protocol. We also included the OHSU antibodies [26] that recognize cell surface epitopes on human endocrine cells to help identify a potential progenitor with the long-term goal of being able to isolate this population. In addition we used lentiviral vectors [24] where the fluorescent protein was under the expression of the *Pdx1* or *Insulin 1* promoter to observe morphological changes during differentiation and allow characterization of Pdx1$^+$ cells we observed *in vitro*.

We determined that although the predominating cell population during cell expansion was vimentin$^+$ cells, epithelial cells were also proliferating and were PCNA$^+$. By sorting and differentiating CD29$^+$EpCAM$^+$ cells we determined that compared to CD29$^+$EpCAM$^-$ cell population, EpCAM$^+$ cells expressed the transcription factor Ngn3 post expansion and differentiation. Routinely in our differentiation model, Pdx1, Ngn3 and Pax4 expression is almost always up regulated [21,23]. Ngn3 may indicate some similarity to Xu et al. where during

tissue regeneration they demonstrated that $Ngn3^+$ cells generate several islet cell types [5].

In order to study epithelial cells expanded from islets or IDPT we required a culture medium that would preserve the epithelial cell phenotype. Unlike our previous data, in this study we were able to maintain epithelial phenotype for greater than 25 days and prevent $vimentin^+$ cell overgrowth. In this model by preventing dedifferentiation and MSC overgrowth, progenitors that pre-exist can be studied. If in fact EMT [15,20] is a necessary process where progenitors or β-cells must dedifferentiate to replicate and then be redifferentiated into insulin producing cells then an alternate cell culture model must be employed. However, it is unclear if dedifferentiation, expansion then redifferentiation is a preferential model for increasing β-cells since β-cells generated in this model do not secrete physiologic levels of insulin compared to normal β-cells [15,20]. In our previous studies by this EMT mechanism we have failed to detect insulin hormone protein post differentiation and have not been able to truly restore epithelial cell phenotype [21,23].

By utilizing lentiviral tracing [24] in combination with OHSU antibodies [26], for the first time we were able to observe morphological changes during *in vitro* differentiation without multiple passages or MSC overgrowth. Compared to lentiviral infected controls (undifferentiated) we have concluded that it is within the adhered differentiated cellular aggregates where $Pdx1^+$ and $Ins1^+$ cells reside. In addition, it is within these cellular aggregates where epithelial cells ($EpCAM^+$, $HPi2^+$ and $HPi3^+$) that are $Pdx1^+$ are located. More importantly we did not

observe vimentin^{+} or ductal epithelial cells (CK19^{+}) that were Pdx1^{+} or Ins1^{+} in this study. Double positive cells (Pdx1^{+}Ins1^{+}) cells were also infrequent during the culture period and post differentiation. In addition, the infrequent cells we observed that were pancreatic polypeptide, somatostatin, glucagon, HPi1 and HPi4 positive did not express either *Pdx1* or *Insulin 1*. RT-PCR results verified lentiviral infection and expression of Pdx1 and insulin in islets. However, due to the very low expression of *Pdx1*-mRFP in undifferentiated islets or differentiated and undifferentiated IDPT, mRNA message for Pdx1 was not detected though message for mRFP was detected. Szabat et al. demonstrated by quantitative RT-PCR that in their Pdx1^{+}Ins^{+} cell population gene expression for mRFP was 2 fold higher than Pdx1 gene expression [24]. Therefore, as there are fewer Pdx1+ cells in our undifferentiated cell preparations the transcript for Pdx1 could be below our detection level.

The Pdx1 transcription factor is crucial for pancreas development, differentiation of pancreatic stem cells, and regulates insulin expression and maintains function of mature β-cells [32,33]. Its expression is also crucial in islet progenitor studies. For example in partial pancreatectomized rodents Pdx1 is up regulated in proliferating islets [34]. In human β-cell studies utilizing their *Pdx1*/*Insulin 1* lentiviral dual reporter Szabat et al. determined that human β-cells exist in distinct states *in vitro* [24]. Immature β-cells were distinguished as *Pdx1* positive and *Insulin 1* negative or low expressing (Pdx1^{+}Ins1^{-}) and mature β-cells were distinguished as Pdx1 positive and insulin positive (Pdx1^{+}Ins1^{+}). In a time-lapse study they determined that Pdx1^{+}Ins^{-} cells could mature *in vitro* without cell

division and convert to $Pdx1^{+}Ins1^{+}$ cells [24]. They concluded that a proportion of adult β-cells share similar characteristics to immature embryonic β-cell progenitors. In a follow up study [35] they determined by genomic profiling that the immature $Pdx1^{+}Ins1^{-}$ cells shared multiple genes associated with β-cell development and progenitor cells.

Using the same lentiviral vectors the largest proportion of $Pdx1^{+}$cells we observed through out our study and post differentiation were $Pdx1^{+}Ins1^{-}$. Double positive cells were infrequent during our study period and $Pdx1^{-}Ins1^{+}$ cells were also observed. It is important to note that unlike Szabat et al. [24] the length of our study was quite different. Our cell culture medium allowed us to exceed 25 days and still detect $Ins1^{+}$ and $Pdx1^{+}$ cells within differentiated cell aggregates without MSC overgrowth or cell dedifferentiation and EMT. However, the proportion of $Pdx1^{+}Ins1^{-}$ cells found in our adhered aggregates may indicate that we have not yet completed differentiation as these cells may be immature [24,35,36] and have not transitioned to $Pdx1^{+}Ins1^{+}$ cells. Another explanation is that the growth factor content of our pancreatic epithelial medium may have selected for immature β-cells [36]. The $Pdx1^{-}Ins1^{+}$ cells we observed could be preexisting β-cells that may be reaching senescence in culture and are losing Pdx1 expression due to the high glucose content of the medium [37]. In addition as with all *in vitro* studies when not using cells from transgenic animals, this study is also limited by our transduction efficiency (40-70%).

In summary we describe a unique cell culture condition for long-term study of pancreatic epithelial progenitor cells that minimizes overgrowth of MSCs (vimentin^{+}) and dedifferentiation of epithelial cells through EMT. We confirmed that during differentiation via lentiviral reporting that Pdx1^{+} cells were confined to epithelial cell aggregates that form during differentiation and not vimentin+ cells suggesting that EMT is not a mechanism for generating pancreatic progenitor cells.

3-5. ACKNOWLEDGEMENTS

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Table 3-1. Flow cytometry analysis of single cells from IDPT expanded for 14 days in MSC expansion and pancreatic epithelial medium. Values are expressed as mean ± SEM.

Antibody	MSC Expansion Medium			Epithelial Medium
	Control	$CD29^{+}EpCAM^{+}$	$CD29^{+}EpCAM^{-}$	
	% Positive (n=7)	% Positive (n=3)	% Positive (n=3)	% Positive (n=3)
Vimentin	85.28 ± 8.02[§†]	80.00 ± 9.54[†]	98.70 ± 0.33[†]	44.87 ± 2.49[§]
EpCAM	17.43 ± 6.88[‡]	47.33 ± 6.36[*‡]	3.00 ±1.00[*]	24.10 ± 8.60
CK19	11.57 ± 4.85	14.00 ± 1.73	4.00 ± 1.00	

The mesenchymal (vimentin) and epithelial cell (EpCAM and CK19) cell surface antigen expression was compared between MSCs (control) and sorted cell populations (CD29+EpCAM+ and CD29+EpCAM-) that were expanded in MSC expansion medium for 14 days. Epithelial and mesenchymal cell antigen expression was compared between MSCs (control) expanded in MSC expansion medium and IDPT cells expanded in pancreatic epithelial expansion medium for 14 days.

[*]p=0.014 for CD29+EpCAM+ vs. CD29+EpCAM- in MSC Expansion Medium
[†]p=0.442 for vimentin+ in MSC Expansion Medium
[‡]p=0.048 for CD29+EpCAM+ vs. Control in MSC Expansion Medium
[§]p=0.007 for vimentin+ in MSC Expansion Medium vs. Epithelial Medium

Table 3-2. Flow cytometry analysis of single cells from human IDPT and islets stained with OHSU and commercial antibodies expressed as mean ± SEM.

	% Positive IDPT (n=4)	% Positive Islets (n=3)
OHSU Antibody		
HPi1	0.84 ± 0.19	10.64 ± 1.25
HPi2	5.78 ± 1.42	12.26 ± 2.92
HPi3	3.84 ± 1.00	10.89 ± 2.21
HPi4	0.24 ± 0.03	1.56 ± 0.20
HPa1	0.32 ± 0.03	1.74 ± 1.43
HPa2	0.12 ± 0.03	0.81 ± 0.37
HPd1	11.33 ± 1.08	
HPd2	7.68 ± 0.87	
HPx1	45.04 ± 5.35	
HPx2	35.02 ± 5.36	
Commercial Antibody		
CA19-9	62.17 ± 5.14	
EpCAM	74.92 ± 0.81	79.33 ± 3.89
CD29	76.78 ± 5.66	

Survey of cell composition of cell aggregates from IDPT and islets and cultured for 24-48 hours was determined by staining with OHSU antibodies and subject to FACS analysis.

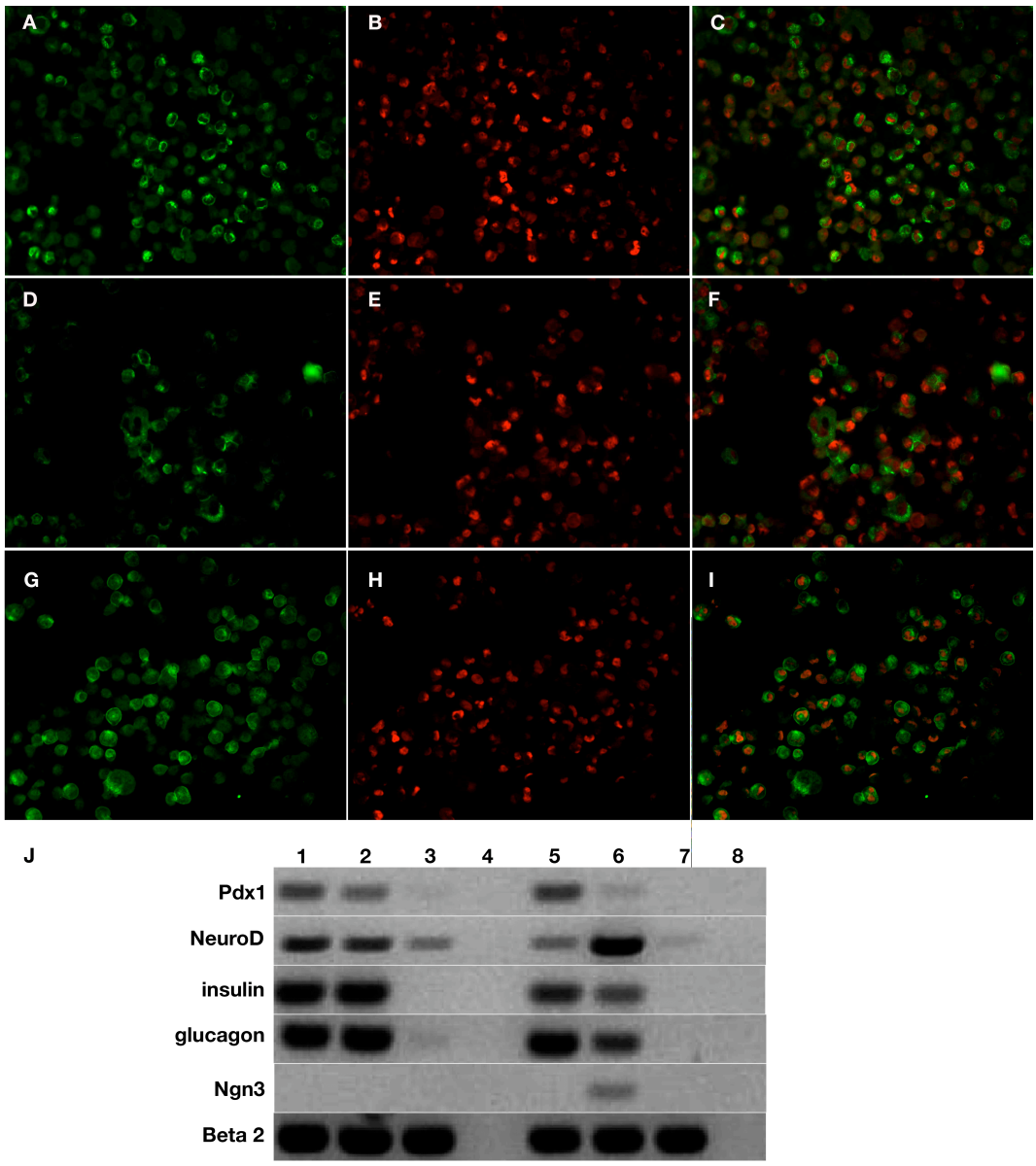

Figure 3-1.

Double IF staining of IDPT derived MSCs and $CD29^{+}EpCAM^{+}$ cells after 14 days in culture (A-I). MSCs stain positive for vimentin (green, A), EpCAM (green, D) and PCNA (red, B and E). Both vimentin and EpCAM positive cells are positive for PCNA staining (C and F merged). After 14 days in culture $CD29^{+}EpCAM^{+}$ selected cells (bottom row) are still positive for EpCAM (green, G). $EpCAM^{+}$ cells proliferate in culture, and are positive for PCNA after 14 days in culture (red H and I merged). Magnification is 400x.

RT-PCR of differentiated, IDPT derived MSCs and $CD29^{+}EpCAM^{+}$ cells (J). MSCs differentiated as a monolayer (lane 1) and aggregated into clusters (lane 2) express Pdx1, NeuroD, insulin and glucagon. $CD29^{+}EpCAM^{+}$ cells differentiated as a monolayer (lane 5) express Pdx1, NeuroD, insulin, and glucagon. $CD29^{+}$

$EpCAM^+$ cells aggregated into cell clusters (lane 6) express less Pdx1 but these clusters express Neurogenin3. Lane 3 and lane 7 are undifferentiated MSCs and undifferentiated $CD29^+EpCAM^+$ cells cultured for the same length of time as differentiated cells. Lane(s) 4 and 8 are water blanks. β2 microglobulin denotes RNA and cDNA integrity. Genomic DNA controls were negative (not shown).

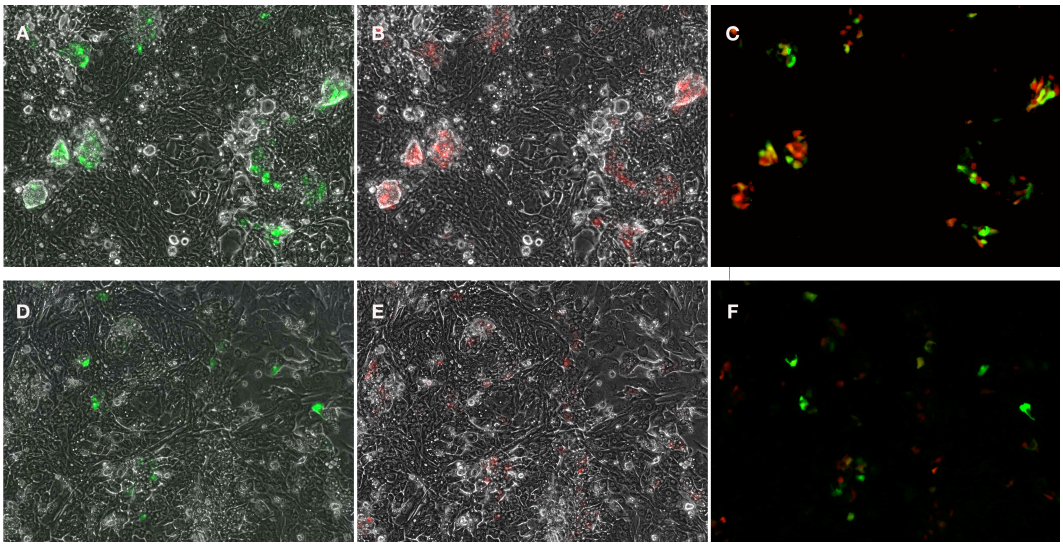

Figure 3-2.

Comparison of differentiated and undifferentiated islet cells infected with *Pdx1*-mRFP/*Insulin 1*-EGFP. Islet cells cultured in differentiation medium form adherent cell aggregates within the cell monolayer and $Ins1^+$ (green, A) and $Pdx1^+$ (red, B) expressing cells are localized within these cell aggregates (C merged). Islet cells cultured in control medium (undifferentiated) have fewer aggregates and $Ins1^+$ (D) and $Pdx1^+$ (E) cells (F merged) are found throughout the cell monolayer. In this representative image absolute counts determined that differentiated conditions had 57 $Pdx1^+$and 28 Ins^+ cells versus undifferentiated conditions that contained 25 $Pdx1^+$ and 14 Ins^+ cells. Magnification is 100x.

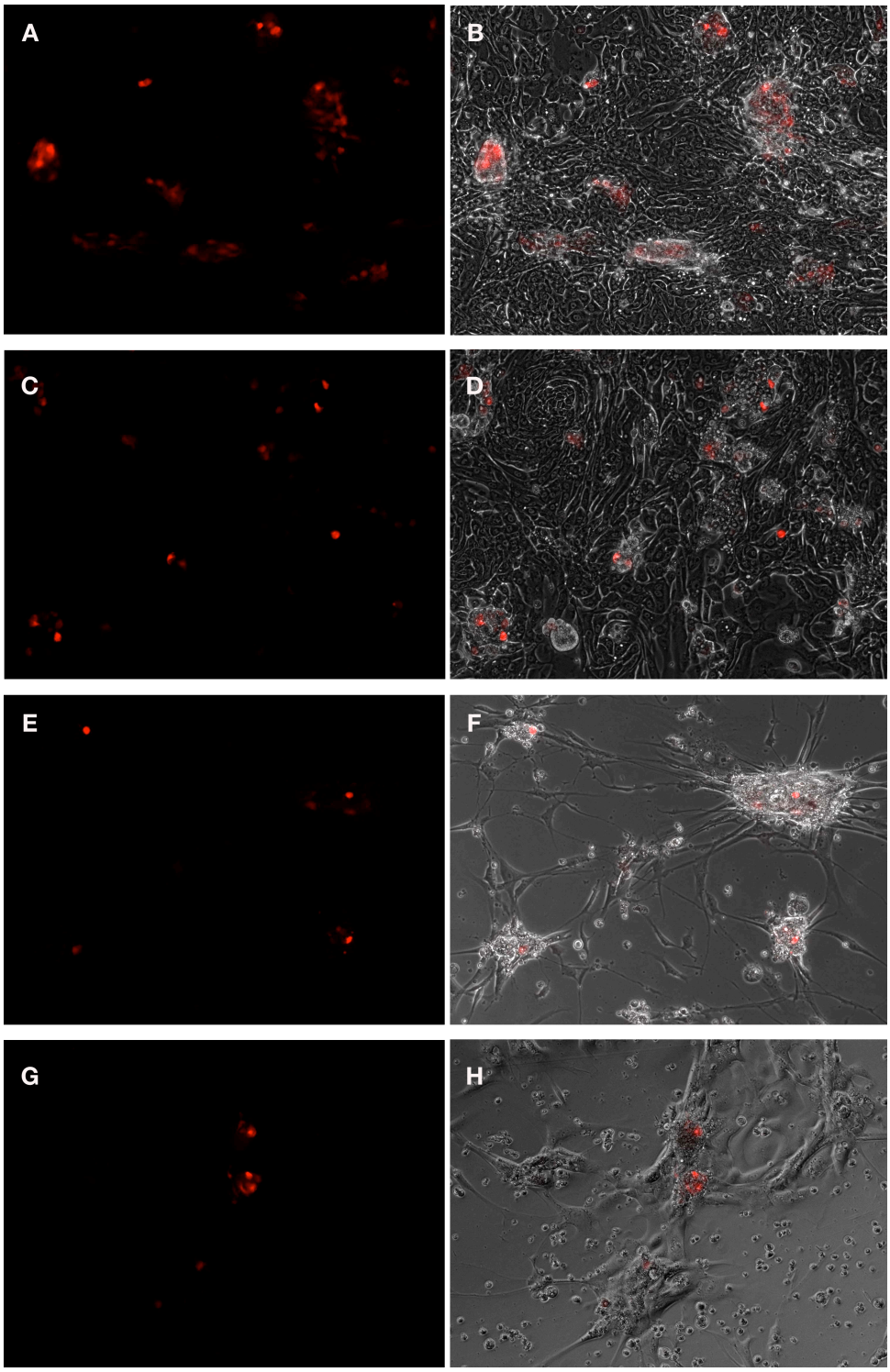

Figure 3-3.

Comparison of differentiated and undifferentiated dissociated islet cells (A-D) infected with *Pdx1*-mRFP. $Pdx1^{+}$ cells are localized within adhered cell aggregates when cultured in differentiation medium (A and B) while $Pdx1^{+}$ cells

are scattered throughout the monolayer when cultured in control medium (C and D). Magnification is 100x.

Comparison of differentiated and undifferentiated cells from the IDPT (E-H) infected with *Pdx1*-mRFP. A few $Pdx1^{+}$ cells are seen within adhered aggregates in the differentiated condition (E and F). No cell aggregates are apparent in the undifferentiated cell conditions and $Pdx1^{+}$ cells are within the monolayer (G and H). Magnification is 100x.

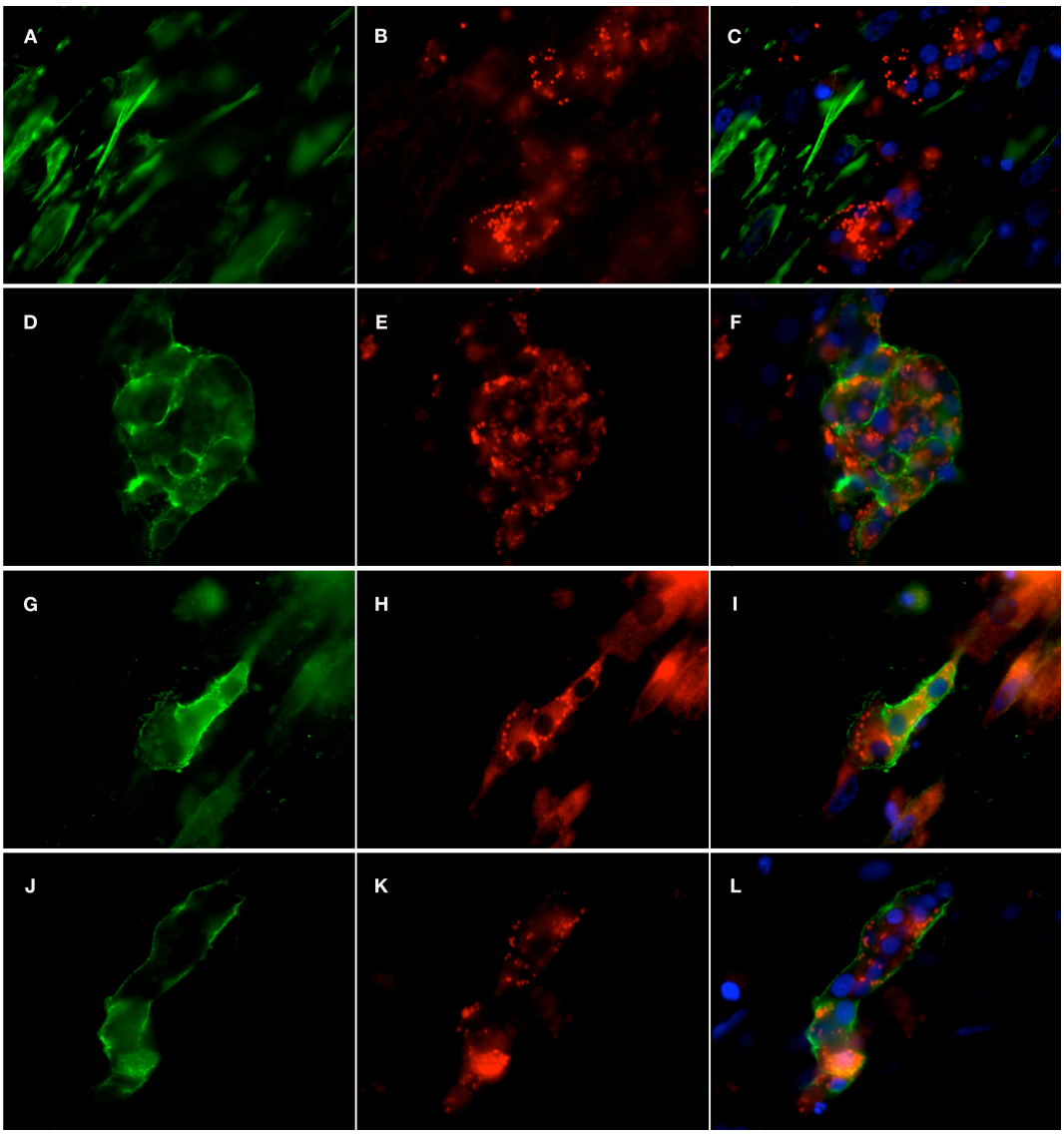

Figure 3-4.

IF staining of differentiated *Pdx1*-mRFP infected islet cells with primary antibodies to: vimentin (A), EpCAM (D), Hpi2 (G) and Hpi3 (J) and secondary antibodies conjugated to Alexa-488 (green). $Pdx1^{+}$ positive cells (B, E, H, and K red) are not co-localized with vimentin (C merged), but are co-localized with EpCAM (F merged), Hpi2 (I merged) and Hpi3 (L merged). Nuclei are stained blue with DAPI. Magnification is 1000x.

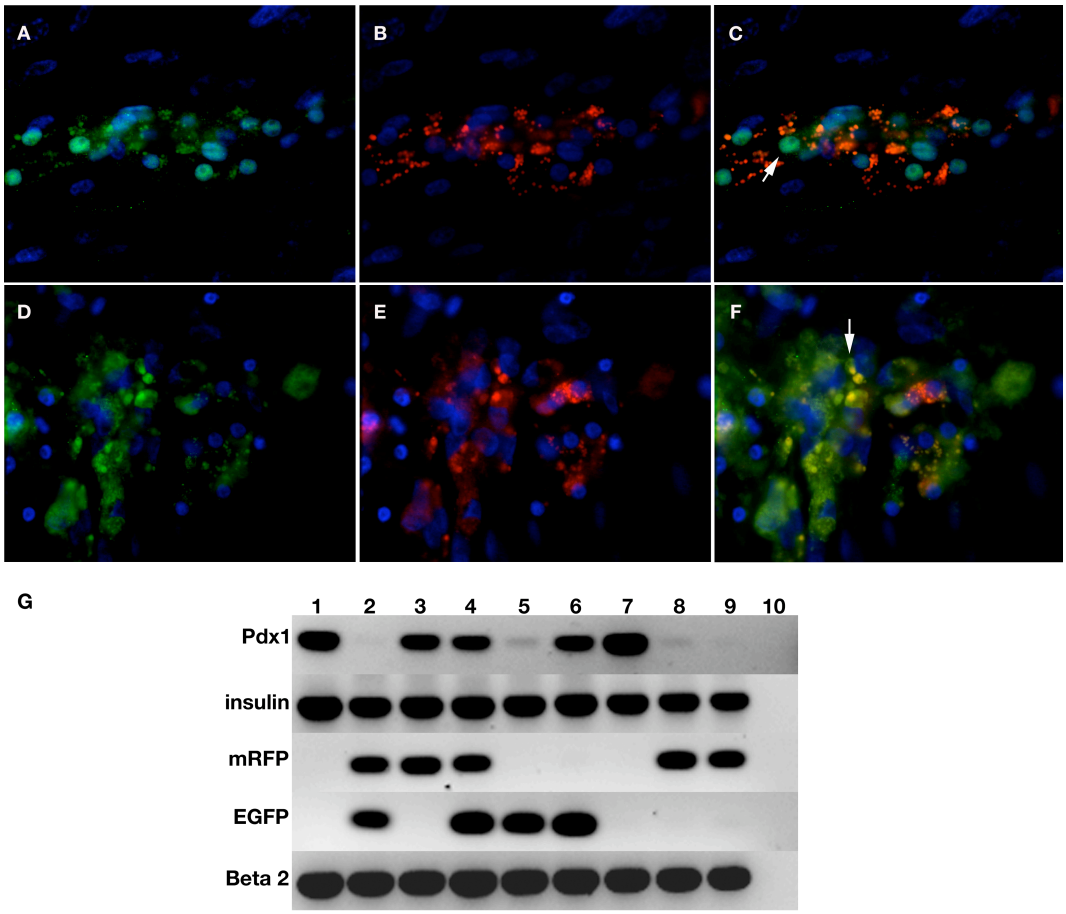

Figure 3-5.

IF staining of differentiated *Pdx1-mRFP* infected islets with primary Pdx1 and insulin antibodies with secondary antibody conjugated to Alexa-Fluor488 (green) (A-F). $Pdx1^{+}$ cells are red (B, E and merged C, F). Pdx1/Alexa-488 antibody (A, and merged C) stains nuclear Pdx1 denoted by arrow (C). Insulin/Alexa-Fluor488 (D, and merged F) stains insulin within $Pdx1^{+}Ins^{+}$ cells. Nuclei are stained blue with DAPI. Magnification is 1000x.

RT-PCR comparing Pdx1, insulin, mRFP and EGFP expression of differentiated and undifferentiated islets and IDPT after lentiviral infection (G). Starting tissue, islets (lane 1) and IDPT cells (lane 7) express Pdx1 and insulin and no mRFP or EGFP. Undifferentiated *Pdx1*-mRFP/*Insulin 1*-EGFP infected islets (lane 2) are negative for Pdx1, positive for mRFP and EGFP compared to differentiated *Pdx1*-mRFP infected islets (lane 3) and *Pdx1*-mRFP/*Insulin 1*-EGFP infected islets (lane 4) that are positive for Pdx1 and mRFP and EGFP respectively. Undifferentiated *Insulin 1*-EGFP infected islets (lane 5) express very little Pdx1 (faint band) compared to differentiated *Insulin 1*-EGFP infected islets (lane 6) that express Pdx1. Neither undifferentiated *Pdx1*-mRFP infected IDPT (lane 8) nor differentiated *Pdx1*-mRFP infected IDPT (lane 9) express Pdx1 but express mRFP. All preparations express insulin. Beta2 microglobulin (Beta 2) denotes RNA and cDNA integrity and genomic DNA control (lane 10) is blank.

3-6. REFERENCES

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CHAPTER FOUR

GENERAL DISCUSSION AND CONCLUSION

4-1. GENERAL DISCUSSION

Type 1 diabetes is a chronic disease which results from an improper autoimmune response against the β-cells of the pancreas [1]. As a consequence β-cells are destroyed, yielding a deficit in insulin production, and ultimately hyperglycemia results [1]. With the discovery and purification of insulin by Banting and colleagues type 1 diabetes became a treatable disease [2]. Exogenous insulin therapy is able to reduce mortality associated with type 1 diabetes thereby revealing morbidity associated with secondary complications [3]. Secondary complications including diabetic nephropathy, neuropathy, and retinopathy as well as increased risk for cardiovascular disease result from imprecise regulation of blood glucose over time [4,5]. Intensive insulin therapy which strives to maintain plasma blood glucose within a normal range through careful monitoring and administration of insulin serves to delay long-term complications [4,5]. Never the less, this is not a cure. Ultimately, a more physiological approach to insulin delivery in patients with type 1 diabetes is essential to avoid complications and improve quality of life for those suffering from this disease [6].

Islet transplantation is a promising alternative to daily insulin injections as a treatment option for patients with type 1 diabetes [6]. The landmark success of the Edmonton protocol study in 1999 resulting in seven out of seven type 1 diabetic patients achieving and maintaining insulin independence following islet transplantations provides support for islet transplantation as an approach to treat and possibly cure patients with type 1 diabetes [7]. However, presently there are

a number of barriers which must be overcome in order for islet transplantation to become a viable treatment option. There are two main barriers which must be overcome. First, immune rejection of graft tissue must be controlled, currently this is through the continuous use of immune suppressing medications which have detrimental side effects [6]. In the future, this may be overcome by creating less toxic immunosuppressive therapies or manipulating graft tissue to avoid immune recognition [3]. Second, the current source of islet tissue from cadaveric human donors is not sustainable and will preclude islet transplantation from becoming a widespread therapeutic treatment for those with type 1 diabetes [8]. It is essential to realizing the potential of islet transplantation that an alternative source of insulin-producing cells be created.

A number of options are being explored in order to create an alternative source of cells for islet transplantation. These options include using xenogeneic pig tissue [9], live human donor islets [10], or generating β-cells from alternative human sources [8]. Methods being explored to generate insulin-producing cells from human cells include differentiating human embryonic stem cells, transdifferentiating non-pancreatic cell sources, and manipulating cell populations within the adult pancreas to a new fate [11]. Perhaps the most applicable and ethically sound source of islet-tissue for transplant remains the adult human pancreas. Under certain physiological conditions, β-cell mass within the pancreas can increase to accommodate increased insulin demand [12,13]. The origin of the newly formed β-cells contributing to the increase in β-cell mass is controversial [12]. Some research claims this expansion is through β-cell replication alone

[14,15], however substantial research has also shown that differentiation of progenitor populations within the pancreas also contributes [16,17]. Numerous groups are doing research in hopes of identifying and differentiating progenitor cells that may pre-exist or be induced by dedifferentiation of other pancreatic cell populations within the adult pancreas. Thus far, a clear consensus on potential sources of progenitor cells has not been reached [18]. Once identified, the regenerative potential of these cells could be harnessed, such that the cell population was expanded and differentiated into β-cells to be used for transplantation [18].

Since it has been frequently hypothesized that maturation of progenitor cells *in vitro* would mimic *in vivo* β-cell development it is logical to examine both *in vivo* and *in vitro* β-cell development and maturation in order to characterize β-cell progenitors and understand their development. Much research in this field at this point has been conducted in rodent models- while these models have provided valuable insight into processes otherwise inaccessible to study in humans, with increasing knowledge in the field discrepancies have emerged between the rodent and human models. Therefore, for continued progress in understanding islet and β-cell development, focus must be shifted toward a human model such that lessons learned will have increased clinical applicability. As such, in this thesis, in order to characterize β-cell progenitors we examined both *in vivo* fetal human development as well as *in vitro* differentiation of adult human pancreatic tissue.

Pdx-1 is a key transcription factor necessary for pancreatic development, differentiation of β-cell progenitors, transactivation of the insulin gene, and

maintenance of mature β-cell function [19-21]. During pancreatic development, Pdx-1 expression is localized to pancreatic progenitor cells, however throughout development, expression becomes increasingly specific to β-cells in the mature pancreas [20]. *In vitro* β-cell maturation studies by Szabat et al. using lentiviral vectors demonstrated that *Pdx1* positive and *Insulin* negative or low expressing ($Pdx1^{+}Ins1^{-}$) cells expressed β-cell developmental genes including MafB, Nkx2.2, and Pax4, and that the same cells could transition, without cell division, to a more mature state denoted by *Pdx1* positive and *Insulin1* positive ($Pdx1^{+}Ins1^{+}$) expression accompanied by increased expression of mature β-cell transcription actors including MafA and Nkx6.1 [22,23]. As such, in studies of β-cell progenitors observation of the Pdx1 transcription factor is essential.

In chapter two, immunohistochemical analysis was performed on human fetal pancreatic tissue using antibodies towards insulin, glucagon, and PDX-1, and SOX17. The goal of this qualitative study was to observe the timing of expression of key pancreatic hormones and transcription factors, relative amounts of that expression, presence of coexpression, and finally the changing islet morphology through fetal human development. Age range of specimens observed is from 7.7 to 38 weeks post conception (wpc), 10 week post natal, as well as observed in adult donors. Our results are able to demonstrate the four stages of islet development previously described by Bocian-Sobkowska et al. including: scattered polyhormonal, immature polyhormonal, insulin monohormonal core islet stage, and finally polymorphic or adult islet stage [24]. A recent study by Riedel et al. in 2011, indicates that cells coexpressing insulin and glucagon may

be an alpha-cell progenitor population [25]. This hypothesis may be supported by our observation that in the first trimester almost all glucagon positive cells also expressed insulin, and that in the transition into the second trimester an inverse relation is observed between the decrease in insulin and glucagon coexpressing cells and the increase in glucagon alone expressing cells. In addition, the transition of PDX-1 localization from duct-like structures in early fetal development to islet structures in late development may support an epithelial origin of β-cell progenitors. This hypothesis is supported by Lyttle et al. noting in a 2008 study that Pdx1 positive cells coexpress CK19, which according to Piper et al. in early fetal pancreas CK19 marks pancreatic epithelial cells [26,27].

The steps of pancreatic and β-cell development can be divided into four approximate phases: endoderm formation, pancreas specification, endocrine specification, and β-cell maturation [28]. Lineage tracing studies by Spence et al. suggest that the ventral pancreas and biliary system develop from a common progenitor distinct from the liver [29]. Pancreaticobilliary progenitor cells express both Sox17 and Pdx-1 while biliary structures are $Sox17^{+}Pdx\text{-}1^{-}$ and pancreatic progenitor cells are $Sox17^{-}Pdx\text{-}1^{+}$. McDonald et al. observed Sox17 expression by qRT-PCR, and noted higher expression in the fetal human pancreas compared to isolated adult islets [30]. Our data contained in chapter two demonstrates an interesting spatial association of Sox17 expressing cells with glucagon expressing cells throughout fetal development. Furthermore, in other preliminary data (unpublished), we show that these Sox17 expressing cells also express somatostatin. At this point, the functional role Sox17 may have following

pancreatic specification in the fetal and postnatal pancreas has yet to be elucidated. Given these observations, we have adapted a figure by Kaneto et al. to also include Sox17 expression data (see *Figure 4-1*). Continued study of Sox17 expressing cells is required to determine if these observations are of any significance.

An inherent limitation associated with human fetal developmental studies is that conclusions are drawn from post mortem samples. As researchers, we do not usually receive background information regarding donor and maternal health status, cause of pregnancy termination, and resultant pathologies that may have altered the normal developmental course of the fetus. As such, one must be aware of this limitation when conclusions are drawn from post mortem samples and applied to normal developmental circumstance. The observations made in this study add to the relatively recent and small pool of data available specific to human fetal pancreatic development, and will hopefully contribute to refining our understanding of pancreatic and islet development to the human model.

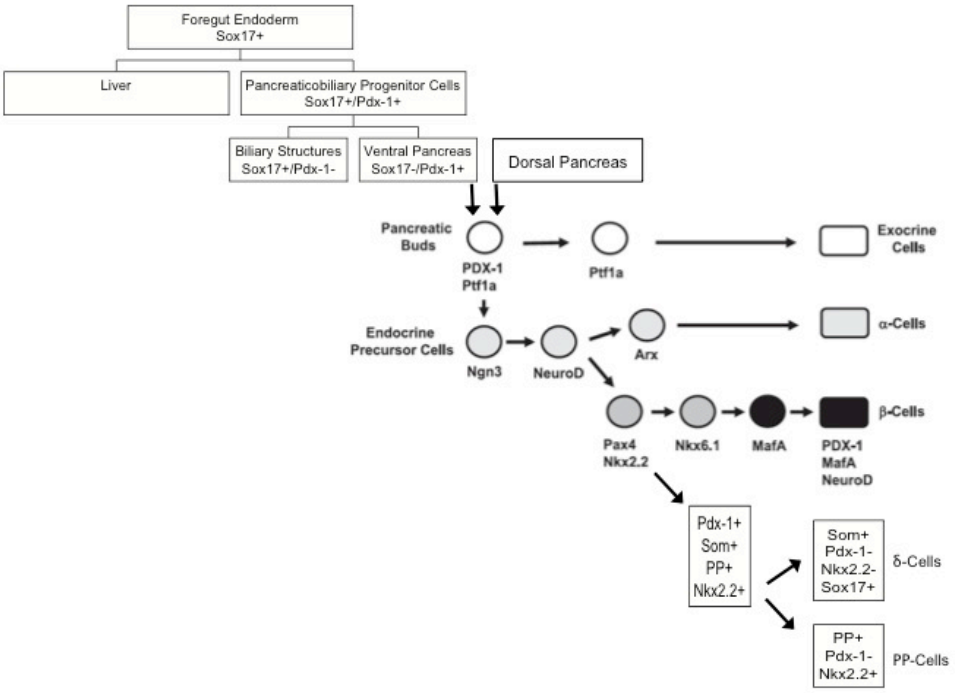

Figure 4-1. Heirarchy of factors involved in pancreatic specification, endocrine specification, and endocrine cell maturation. Sox17 is expressed in the definitive foregut endoderm from which the pancreas, liver, and biliary structures arise. Sox17 expression is involved in determining biliary vs. pancreatic specification from pancreaticobiliary progenitor cell population. Sox17 is later expressed in δ-cells as marked by somatostatin (Som) expression. [20, 29, 30]

The in vitro study presented in chapter three follows two previous studies out of the Korbutt lab in 2006 and 2009 [31,32]. In the 2006 study, Seeberger et al. hypothesized that MSCs could be derived from epithelial cells in IDPT from the adult human pancreas undergoing a reversible EMT, and that upon expansion and differentiation, these MSCs could represent a potential alternative source of cells for islet transplantation [31]. This model of reversible EMT was similar to a β-cell dedifferentiation to a mesenchymal state described by Gershengorn et al. [33]. The population of cells isolated in this study expressed similar cell surface markers to bone marrow MSCs, and could be differentiated into osteocytes, adipocytes, and chondrocytes [31]. These cells could also be differentiated to express mRNA for some genes necessary in islet and β-cell development, however the protein products were not observed [31]. In the following 2009 study, an epithelial phenotype could not be restored to the MSC cells nor were the cells glucose responsive [32]. It was hypothesized that the increase in MSC population observed in both studies was due to culture conditions favoring expansion of a preexisting MSC population, and the observed expression of developmental genes may have been due to a surviving epithelial population responding favorably to the differentiation conditions [32].

In the study presented in chapter three, we sought to study the origin β-cell progenitors with particular attention to the pancreatic epithelial population of cells. First we determined that during the *in vitro* MSC expansion, epithelial cells were in fact, still proliferating, and prior to expansion if enriched, this epithelial population expressed developmental transcription factors indicative of a β-cell

progenitor when subjected to differentiation protocols. Following, we describe a novel pancreatic epithelial cell culture media that preserves the epithelial population *in vitro* by preventing dedifferentation, and minimizing MSC overgrowth of the culture. This allowed for long-term study and differentiation of the epithelial population *in vitro*. Utilizing the same lentiviral reporter vectors developed by Szabat et al. of the Johnson group where monomeric red fluorescent protein (mRFP) is under control of the mouse *Pdx1* promoter and enhanced green fluorescent protein is under control of the rat *Insulin 1* promoter [22], we show that epithelial cells following differentiation form cell-aggregates where Pdx1 positive and insulin positive cells are localized. Furthermore, by immunohistochemical characterization, we determined that Pdx1 expressing β-cell progenitors following differentiation were in fact, epithelial cells rather than mesenchymal cells. These results suggest that EMT is not a mechanism for the generation of pancreatic progenitor cells.

In the future, further characterization of the Pdx-1 expressing progenitor cells will be necessary to determine the specific origin of these cells within the subpopulations of pancreatic epithelial cells. An excellent tool in this work will be the novel antibodies developed by Dorrell et al. which recognize cell surface markers for pancreatic subpopulations panendocrine, alpha, exocrine, and ductal cells [34]. Initial study indicates that Pdx-1 expressing epithelial cells are not positive for HPd1, HPd2, or commercial antibody for CK19, thus denoting that these cells are not of the pancreatic ductal epithelium [Chapter 3]. Continued study will be necessary to isolate the specific subpopulation of pancreatic cells

from which the Pdx1 expressing progenitor cells originate. Since the antibodies developed by Dorrell et al. recognize cell surface antigens, once a specific population is identified to contain the Pdx-1 expressing progenitor cells this population may be purified via flow cytometry remaining viable for *in vitro* culture [34].

A potential caveat to establishing a purified population of the cells of interest prior to *in vitro* culture and differentiation is that growth factors secreted by other cell populations that are necessary for the growth of the cell population of interest may not be present in the purified culture [35,36]. For instance, a study by Yatoh et al. used immunomagnetic sorting to isolate a CA19-9 positive ductal population, and found that purified populations had slow expansion, poor aggregation, and when transplanted had poor engraftment [35]. On the other hand, significant improvement was observed if mesenchymal cells were added to constitute just 0.1% of the preparation [35]. Should purified cell populations require the presence of other factors to create ideal culture conditions for growth use of either conditioned medium or a transwell culturing approach may be necessary [37]. Should these methods fail, direct cell-to-cell contact may be needed and direct co-culture may be necessary [37]. In this case, purification of the target population for transplantation may be necessary following *in vitro* culture and differentiation to reduce the total cell mass for transplant.

In our *in vitro* study, a high proportion of Pdx-1 positive but insulin negative cells were noted [Chapter 3]. This may indicate that the majority of the Pdx-1 positive cells in our culture conditions have not yet completed differentiation into

mature Pdx-1 positive insulin positive cells, and thus remain as immature β-cells [22,23]. As such improvements upon our differentiation protocol may be necessary in order to generate functionally mature β-cells. At this point, an *in vitro* protocol that successfully differentiates β-cell progenitors into fully functional β-cells has not been identified [38]. Currently, the most effective differentiation of β-cell progenitors has been by providing a final *in vivo* maturation step, indicating that certain factors present *in vivo* must be added to *in vitro* protocols to enhance efficiency [39]. It would be interesting to observe if transplantation of the $Pdx1^{+}Insulin^{-}$ cells generated in our study are able to mature *in vivo*. Following this, functional assays to assess if and how effectively these cells respond to glucose stimulation would provide insight as to the potential of these cells as a source of insulin producing cells for transplantation.

The safety implications associated with transplantation of undifferentiated tissue with proliferative capacity includes the potential for teratoma formation [39]. For clinical applicability, the necessary factors for β-cell maturation should ideally be administered *in vitro*, and graft tissue should be purified to only contain fully differentiated β-cells [19]. Also associated with proliferative capacity, candidate cells for replacement therapy must possess mechanisms to prevent unregulated expansion of β-cell mass, which would lead to severe hypoglycemia from excess insulin production [36]. Finally, above all, candidate cells for β-cell replacement must be able to efficiently synthesize, process and store insulin, and release that insulin in physiologically sufficient amounts to maintain plasma glucose in a normal range [36].

4-2. CONCLUSION

Islet transplantation is an exciting therapy which has potential replace daily insulin injections for type 1 diabetic patients, in favor of a more physiological system of restoring glucose homeostasis, there by reducing secondary complications for patients [4-6]. In order to realize the potential of this therapy option, a sustainable source of insulin producing cells must be generated. One potential source of these cells is the expansion and differentiation of β-cell progenitor populations within the adult human pancreas [16,17]. At this point, the precise cell or cell populations that could be isolated to expand into a clinically applicable pool of transplantable cells has yet to be conclusively identified.

The objective of our studies is to study the origin of β-cell progenitors within the fetal and adult human pancreas. In this thesis, we have shown a qualitative observation of several factors important in β-cell development in the human fetal pancreas. Pdx-1 positive progenitor cells in this study appeared originate from the pancreatic epithelium. Following this, an *in vitro* study was conducted using adult human pancreatic tissue. We described a novel media that is able to prevent MSC overgrowth of the culture thereby allowing long term study of the epithelial population. Utilizing lentiviral vectors which mark Pdx-1 and insulin positive cells allowed us to observe Pdx-1 and insulin positive cells localize within cell aggregates during differentiation. Immunohistochemical characterization of the Pdx-1 expressing progenitor cells indicates that these cells are pancreatic epithelial cells. Taken together, the *in vivo* and *in vitro* studies of this thesis indicate that continued study of the pancreatic epithelial population is necessary

to isolate a source of cells applicable for islet transplantation. Furthermore, understanding normal pancreatic development remains key to successful manipulation of *in vitro* differentiation protocols.

It is my hope that the research presented in this thesis will provide insight into islet and β-cell development such that isolation and differentiation of a β-cell progenitor residing within the pancreatic epithelia of the adult human pancreas may eventually serve as a viable source of cells for islet transplantation. Ultimately, I wish for this contribution to one day improve the quality of life for those suffering from type 1 diabetes.

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