University of Alberta

Optimizing Cultural Conditions for Duct Cells

by

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This is dedicated to my parents; who are always supporting me no matter how far I am. I would not have achieved anything if it was not for you.

To my first friend my brother.

To my lovely wife you mean the world to me.

To my sweet Jasmine keep shining with your smile.

ABSTRACT

In-vitro expansion of the duct cells of the pancreas was associated with loss of the epithelial phenotype of these cells and acquisition of mesenchymal phenotype. These changes have been explained by epithelial mesenchymal transition.

We have tested the effect of several growth factors that have been used in epithelial cell cultures with a number of different base media. We used geneticin that has been used to limit the growth of mesenchymal cells. Flow cytometry was employed to determine the percentages of EpCAM and Vimentin positive cells in the NEPT.

The use of geneticin was helpful in preserving epithelial cells (~70% EpCAM positive cells) compared to the control (56% EpCAM positive cells) after a week in culture. Moreover we observed that a pancreatic media yielded the highest percentage of EpCAM positive cells (27%) and the lowest percentage of Vimentin positive cells (55%) compared to conventional media after 20 days *in vitro*.

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APCs	Antigen-presenting cells
bFGF	Basic fibroblast growth factor
BSA	Bovine serum albumin
CA 19-9	Carbohydrate antigen 19-9
CD	Cluster of differentiation
CK 19	Cytokeratin 19
DCs	Dendritic cells
DMEM	Dulbecco’s modified Eagle's medium
E-cadherin	Epithelial cadherin
EGF	Epidermal growth factor
EMT	Epithelial mesenchymal transition
EpCAM	Epithelial cell adhesion molecule
ESC	Embryonic stem cell
FBS	Fetal Bovine Serum
GLP-1	Glucagon-like peptide-1
GLUT-2	Glucose transporter 2
HbA1c	Glycated hemoglobin
HBSS	Hanks balanced salt solution
HDL	High density lipoproteins
HESC	Human embryonic stem cell
HLA	Human leukocyte antigen

Il-2	Interlukin 2
IMT	Intima media thickness
ITS	Insulin-transferrin-selenium
KGF	Keratinocyte growth factor
LDL	Low density lipoproteins
MHC	Major histocompitability complex
NCV	Nerve conduction velocity
NEPT	Non-endocrine pancreatic tissues
NeuroD1	Neurogenic differentiation 1
Ngn-3	Neurogenin 3
NOD	Non obese diabetic
Oct-4	Octamer-binding transcription factor 4
Pax-4	Paired box gene 4
Pax-6	Paired box gene 6
Pdx-1	Pancreatic and duodenal homeobox 1
RT-PCR	Reverse transcription polymerase chain reaction
SOX-2	Sex determining region Y-box 2
SPK	Pancreas transplant simultaneous with a kidney

CHAPTER ONE

General Introduction

1.1 Diabetes Mellitus

1.1.1 Definition

Diabetes mellitus describes a metabolic disorder of many causes characterized by disturbances of carbohydrate metabolism leading to chronic hyperglycemia, as well as disturbed fat and protein metabolism. It occurs when the pancreas does not produce enough insulin, or when the body cannot effectively use the produced insulin. The effects of diabetes mellitus include long–term damage, dysfunction and failure of various organs.

There are two main types of diabetes mellitus, namely type 1 diabetes mellitus (T1 DM) and type 2 diabetes mellitus (T2 DM) (1).

1.1.2 Epidemiology

Diabetes is a very common disease, according to the Canadian Diabetes Association, diabetes mellitus affects 246 million people around the world. Its incidence is increasing rapidly, and it is estimated that by the year 2030, this number will almost double. Diabetes mellitus occurs throughout the world, but is more common (especially type 2) in the more developed countries. However, the

increase in incidence might take place in developing countries. This increase may be caused by changes in lifestyle that follows the developed countries (2). For at least 20 years, the incidence of diabetes in North America have been increasing substantially, the rate of diabetes mellitus increases with age, it is expected that the numbers of older persons with diabetes might grow as the elderly population increases in number.

In 2005, an estimated 1.1 million people died from Diabetes. The World Health Organization (WHO) expects that deaths due to diabetes will be increased in the next 10 years by more than 50% (3).

1.1.3 Classification

The classification of diabetes mellitus can be summarized as: Clinical diabetes may be divided into four general subclasses, including: a. type 1 (caused by beta cell destruction and characterized by absolute insulin deficiency), b. type 2 (characterized by insulin resistance and relative insulin deficiency), c. other specific types of diabetes (associated with various identifiable clinical conditions or syndromes), d. gestational diabetes mellitus (4).

1.1.4 Pathophysiology of Type 1 Diabetes Mellitus

Type 1 DM is characterized by minimal amounts or even absent circulating insulin. Exogenous insulin is required to reverse this catabolic condition, prevent ketosis, and for normal lipid and protein metabolism.

Type 1 DM is an autoimmune disease. Almost 85% of patients have circulating islet cell antibodies, and most type 1 DM patients have detectable anti-insulin antibodies before starting insulin therapy, also there is the lymphocytic infiltration of the pancreas leading to destruction of insulin-secreting cells of the islets of Langerhans. Type 1 DM can be associated with other autoimmune diseases, such as Addison disease, Graves' disease, and Hashimoto thyroiditis. The presence of HLA-DQs can be considered as a specific marker of type 1 DM susceptibility. Approximately 95% of patients with type 1 DM have either human leukocyte antigen (HLA)-DR3 or HLA-DR4.

It is believed that damage of β-cells could be precipitated by an infectious (e.g., mumps, rubella, coxsackie B4) or environmental agent chemical factors as toxins and physical factors as irradiation. These factors might triggers the immune system in persons with genetic susceptibility to develop an autoimmune response against altered pancreatic beta cell antigens (5).

1.1.5 Pathophysiology of Type 2 Diabetes Mellitus

Patients of Type 2 DM usually have insulin resistance which means that the body cells -importantly muscle, fat and liver cells- do not respond appropriately to insulin, and later on declining beta β-cell function, eventually leading to possible β-cell failure. These patients are usually obese, and their obesity might be a part of metabolic syndrome (also known as syndrome X or insulin resistance syndrome).

Family history is also an important risk factor, hereditary influence is demonstrated in monozygotic twins, offspring and siblings of diabetic patients who are at high risk for the disease. No HLA markers have been found to be related to type 2 diabetes mellitus (4).

1.1.6 Diagnosis of Diabetes

The classical symptoms are polyuria and polydipsia. These symptoms may develop rapidly - weeks - in type 1 DM, particularly in children. In type 2 diabetes symptoms usually develop slowly and may be absent. Significant weight loss might occur in patients with type 1 DM. Blurred vision is a common complaint that may lead to a diabetes diagnosis, with rapid deterioration in type 1 DM in comparison to the more gradual deterioration in type 2 DM.

Once glucose concentration in the blood is higher than the renal threshold (about 10 mmol/L), the reabsorption of glucose from the proximal kidney tubules becomes incomplete leading to glucose loss in urine (glycosuria). Urine osmolality is increased with more glucose, thus reabsorption of water by the kidney is inhibeted, resulting in increased excretion of urine (polyuria) and increased fluid loss, resulting in dehydration and thirst (6).

The diagnosis of diabetes is confirmed when fasting glucose is equal to or greater than 126 mg/dL on at least two separate occasions. Fasting glucose levels less than 110 mg/dL is considered a normal glucose level, while values between 110 and 126 mg/dL, although not diagnostic, should be examined further, results

in this range are classified as impaired fasting glucose. Oral glucose tolerance test (OGTT) is done for patients of this category, to help detecting diabetes at its earliest stage. Glycated hemoglobin (HbA1c) of 6.0% or higher is considered abnormal by most labs; HbA1c is primarily used to follow up potency of treatment and estimates the average blood glucose levels over the past 3 months (4).

1.1.7 Acute Complications

Diabetic ketoacidosis (DKA) is an acute and dangerous complication that is considered as a medical emergency. It is characterized by disturbed level of consciousness that may progress to coma. Ketoacidosis can cause hypotension, shock, and death. The incidence of ketoacidosis is much more common in type 1 DM than type 2 DM.

Another form of acute complications is hyperosmolar nonketotic state (HNS), it is similar to DKA, but has a different origin and a different treatment. Also hypoglycemia -low blood glucose- is an acute complication that can be caused by several factors, such as inappropriate insulin dose or time, too much or incorrectly timed exercise or poor food intake (7).

Diabetes mellitus also weaken the immune response, leading to increased susceptibility to infections especially skin and respiratory infections such as pneumonia and influenza (8).

1.1.8 Chronic Complications

Chronic elevation of blood glucose level leads to microvascular disease and macrovascular disease. Microangiopathy of the cardiac microcirculation leads to development of diabetic cardiomyopathy, and eventually heart failure (52).

Diabetic nephropathy can lead to chronic renal failure, finally those patients require dialysis. Diabetes mellitus is the most common cause of adult kidney failure worldwide in the developed world.

Neuropathy in the form of glove and stocking together with damaged blood vessels can lead to diabetic foot (55). Diabetes can lead to development of, growth of friable and poor-quality new blood vessels in the retina known as diabetic retinopathy. Also it can cause macular edema that can lead to severe vision loss or blindness (54).

Macrovascular disease leads to cardiovascular disease; by accelerating atherosclerosis coronary arteries leading to ischemic cardiac disease, diabetic myonecrosis, peripheral vascular disease, stroke and diabetic encephalopathy (9).

1.1.9 Treatment

Type 1 DM patients require insulin therapy to control hyperglycemia and maintain serum electrolytes and hydration. Some changes in the life style should be done as eating healthy food and regular exercise (4).

The treatment of type 2 DM often consists of monotherapy with diet, metformin, sulfonylureas etc. However, with progressive β-cell failure, the combination of more than one oral hypoglycemic drug might be needed; eventually insulin may be required to achieve normoglycemia (4).

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1.1.10 Insulin

In 1869 Paul Langerhans, noticed tissue clumps scattered throughout the bulk of the pancreas, these clumps were named after him. The first commercially available biosynthetic human insulin was called Humulin appeared in 1982. Most of the current available insulin is biosynthetic recombinant "human" insulin. Insulin can be given as shots or using insulin pump. Other forms of insulin are also available as inhaled powder form of recombinant human insulin which is not used clinically (10, 11).

The use of insulin for type 1 DM patients helped to improve the symptoms of diabetes mellitus and to decrease the morbidity and the mortality in these patients; however these patients were still exposed to development of secondary complications of diabetes. The development of micro and macrovascular complications despite daily injections of insulin is thought to be due to the lack of physiological variations in exogenous insulin compared to the dynamic changes of endogenous insulin in relation to changes of serum glucose levels.

Thus the development of methods of production of endogenous insulin in diabetic patients is thought to decrease the incidence of chronic complications in

diabetics. Pancreas and islet transplantations have become the only therapies that achieve normal glucose levels by reestablishing endogenous insulin secretion responsive to normal feedback regulation (12).

1.2. Pancreatic Transplantation

1.2.1 History of Pancreatic Transplantation

Pancreatic transplantation was first tried as a treatment of type 1 diabetes in humans in 1966 (13). Within more than 40 years, over 10,000 pancreatic transplantations had been recorded in the International Pancreas Transplant Registry. The success rates in different centers differ depending on operative experience of the surgical team and patient selection.

The introduction of improved immunosuppressive regimens, new surgical techniques, and the selection of healthier recipients helped in obtaining higher survival rate for the grafts and the patients (14).

1.2.2 Techniques of Pancreatic Isolation

The main source for pancreata for transplantation was the dead bodies. To a lesser extent, a segment of pancreas is obtained from a living related donor after hemipancreatectomy.

Patients receiving pancreatic transplantation receive intense immunosuppression and antibody induction therapy using either a monoclonal or polyclonal agent.

Pancreas transplant can be performed coincident with a kidney (SPK; 90%), or after a kidney transplant (4%) and also pancreas transplant alone (6%) (14).

1.2.3 Metabolic Results

Normal blood glucose concentrations, and normal HbA1c values, were achieved. The patients responded properly to responses to oral and intravenous glucose stimulation tests demonstrated by changes in insulin levels (15).

The lipid profile of the recipients showed some beneficial changes. Triglyceride and LDL cholesterol concentrations decrease and serum HDL cholesterol concentrations increase in pancreatic transplant recipients (16, 17). Also pancreatic transplantation improved glucose counter regulation after hypoglycemia (18), due to improvement of both glucagons and epinephrine counter regulatory response (19).

1.2.4 Effects on the Chronic Complications of Diabetes

Examining the renal structure in patients receiving pancreatic transplantation showed diminished mesangial mass in patients receiving SPK. Those receiving a kidney alone did not have a similar effect (20).

Improvement of nerve conduction velocities in both motor and sensory nerves has been demonstrated in recipients. Partial reversal of neuropathy was observed 10 years after transplantation (21). However, no beneficial effects have been demonstrated for established retinopathy (22) or for abnormalities of gastric motility (23). SPK minimizes atherosclerotic vascular risk, since SPK was shown to improve fasting lipid profiles and blood pressure (24), also SPK decreased the carotid intima thickness.

1.2.5 Acute and Chronic Rejection

Rejection of a transplanted graft may occur within days (acute) or after years (chronic) of successful transplantation. In patients receiving simultaneous pancreas kidney transplantation, an increase in serum creatinine can be used as a signal that both organs are undergoing a rejection (15).

1.2.6 Morbidity and Mortality

Risks associated with pancreas transplantation include clinical complications caused by the surgery, intra-abdominal infections and abscess, vascular graft thrombosis, anastomotic leak, and duodenal stump leak and other complications caused by the usage of chronic immunosupprssive agents (25).

1.3 Islet Cell Transplantation

1.3.1 Rationale for Islet Transplantation in Type 1 Diabetes

As mentioned above transplantation of the whole pancreas is associated with major surgical complications (26). In contrast, islet cell transplantation has fewer incidences of complications since it is performed as a minimally invasive procedure (27).

1.3.2 Definition of Islets

Islets were described as separate islands that surrounded by pancreatic exocrine tissue (28), fig. (1.1). Islets represent approximately 2 to 3 % of the total pancreatic volume.

The islets are supplied by the portal circulation, with blood flowing from beta to alpha to delta cells. The central nervous system; regulate secretion of different cells of the islets. β-cells secrete insulin in response to hyperglycemia, while α-cells produce glucagons in case of hypoglycemia (29).

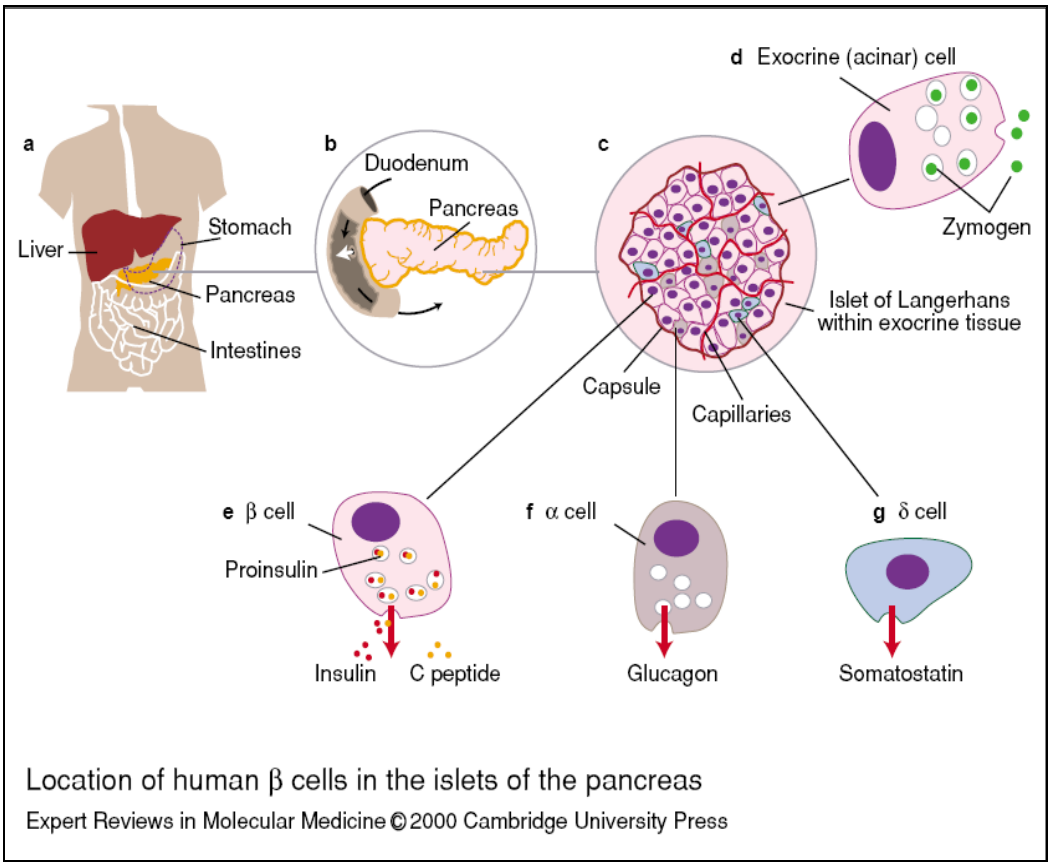

Figure 1.1: Human β-cells are located in the islets of the pancreas and secreate insulin and c peptide, the pancreas also secretes glucagon and somatostatin from α cells and δ cells respectively (Thomas Titus et al., 2000) (116).

1.3.3 Previous Trials

In 1894 Williams used minced sheep's pancreas and extracts of pancreas in glycerine for oral and subcutaneous therapy (30). He used sheep xenografts without immunosuppression and this trial of course did not succeed. In the early seventies of last century Ballinger and Lacy reported reversal of streptozocin-induced diabetes in rats after receiving islet isografts from normal rats (31).

Few years later successful transplantations of islet autografts in humans were reported. The transplanted islets can not go through the sinusoids so they are kept in the liver (32, 33).

In 1992, Pyzdrowski et al. (34) reported that transplantation of 265,000 islets achieved insulin independence. In 1995, Wahoff et al. reported an insulin-independence rate in 14 patients after autologous islet transplantation of more than one third of a million islets, the rate of insulin independence reached 74 % two years after transplantation (35).

The success of allogeneic islet transplantation in patients with type 1 DM with the use of immunosuppressive agents and purified human islets from cadaveric donors was reported, but, the overall rates of success were reported as less than 10 percent (36).

In 2000, Shapiro et al. reported 80% insulin independence in seven patients for one year and 50% of patients maintain insulin independence 5 years after islet transplantation. This high rate may have been due to the many differences in their approach as compared with previous techniques, the recipients' body weights had to be monitored carefully, the corticosteroids were

avoided because of its diabetigenic effect, sirolimus and low-dose tacrolimus together were added to the immunosuppressive regimin, also the patients received daclizumab (anticytokine drug), and finally multiple infusions of islets from different human donors were used to collect a transplanted islet mass that would be sufficient to achieve insulin independence after transplantation (37).

1.3.4 Islet Donation and Purification

The process of islets isolation is complex, many methods have been developed. The automated method of islet isolation figure (1.2) was able to recover a sufficient number of islets from a single donor for successful transplantation in a diabetic recipient (37).

The good care of the donor pancreas is very important for the success of islet isolation also. Young donors are preferred (20–50 years) with minimal warm and cold ischemia because both are damaging to the pancreas (38).

Purification of islets helps to make the transplantation safer as it decreases the volume of unwanted acinar tissue, improve islet revascularization and decrease the post operative complications. Large volumes of unpurified pancreatic digest infused into the portal vein or spleen have led to portal hypertension, hepatic or splenic infarction, disseminated intravascular coagulation, and deaths (39, 40).

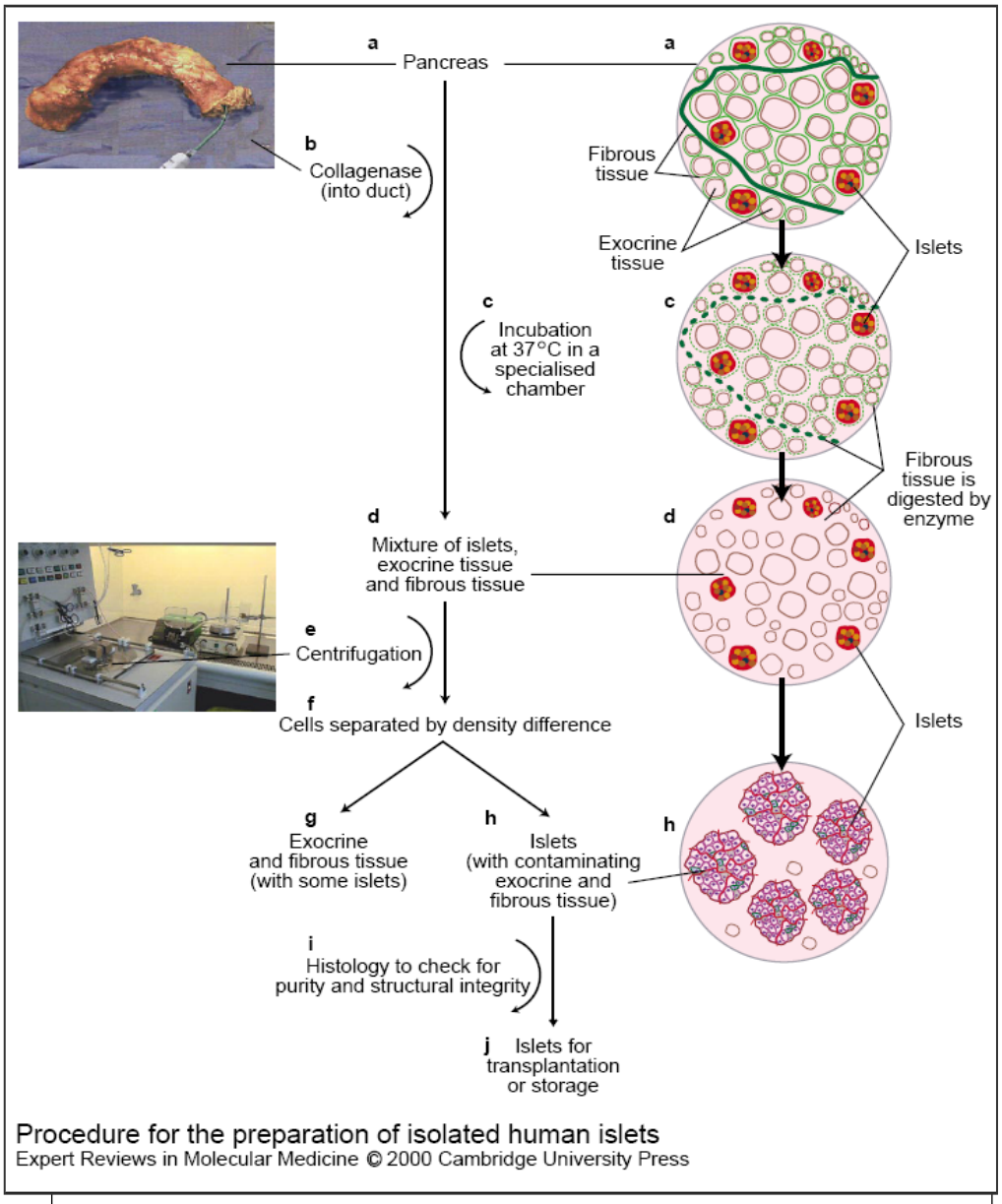

Figure 1.2: Procedure for the preparation of isolated human islets. (a) surgical removal of the pancreas; (b) Collagenase is injected into the duct, (c) break down of the collagen structure. (d) Liberation of islets. (e) Islets separated are from the surrounding, (f) exocrine tissue using differential density centrifugation. (g–i) Before use, islets can be identified by dithazone staining, and their structural

integrity and purity are confirmed by histological examination then they are ready for transplantation (Thomas Titus et al., 2000) (116).

1.3.5 Transplantation Site

Many trials on different sites have been tested such as the renal sub-capsular space, the spleen, intraperitoneal space, and the intraportal site is the most common. Islets are infused intra-operatively directly into the hepatic portal venous circulation under direct view or percutaneously.

Potential complications of islet infusion into the liver include hemorrhage, portal venous thrombosis, and portal hypertension. The presence of transplanted islets in the liver expose islets to environmental toxins and potentially medications that would be absorbed from the gastrointestinal tract and delivered into the portal vein (41).

1.3.6 Metabolic Effects of Islet Transplantation

Many reports showed achieving insulin independence and normalization of glucose blood levels following islet transplantation. Unfortunately, insulin independence in the long term is achieved in a small percent of patients who received islet transplantation (37). In recipients, insulin is secreted in the liver and, by insulin cleavage in the liver peripheral hyperinsulinemia is avoided (42).

A number of studies support that transplantation of islets in the liver respond suitably to hypoglycemia. Luzi et al. showed that functioning islet grafts

lead to normalizing basal hepatic glucose output, improve insulin action and normalize plasma concentrations of amino acids (43).

Several studies showed improvement of abnormal lipid profile, in the form of decreased triglycerides level and LDL (44).

1.3.7 Effect of Islet Transplantation on Patient Survival

Islet transplantation on type 1 diabetic patients' decreased the morbidity and mortality. One study compared two populations of kidney-islet-transplanted patients, before transplantation the two groups shared the same general characteristics, metabolic status. After transplantation they had the same immunosuppressive regimens and similar kidney graft function. After 7 years of follow up, the survival among patients in the group with successful islet transplantation and restoration of β-cell function was significantly higher (90%) than among patients in the unsuccessful transplantation group (51%) (45).

1.3.8 Effects of Islet Transplantation on Long-Term Diabetic Complications

a. Islet Transplantation and The kidney

One of the most common complications of type 1 DM is nephropathy. The success of islet transplantation helps to increase graft survival and prevent reduction in vascular function of the kidney graft. The potential positive effect of

islet transplantation on kidney function was confirmed by improvement of the kidney function tests in the form of decreases in the urinary excretion of albumin, the urinary fractional excretion of sodium and the creatinine clearance (46).

b. Islet Transplantation and the Heart

Some animal studies showed that Islet transplantation can improve diabetic cardiomyopathy, islet transplantation can correct the changes in protein tyrosine phosphorylation in the myocardium in diabetic mice receiving islet transplant. Islet transplantation has been associated with an improvement in diastolic function and in the width of QT interval in Electrocardiogram. Also, the reduction of atrial / ventricular natriuretic peptide was evident during the follow-up period (47, 48).

c. Islet Transplantation and the Blood Vessels

Patients with type 1 DM are at high risk for macro-microangiopathy. A study of over 34 patients with type 1 DM who received kidney transplants and islet transplantation at a single institution observed a reduction in carotid intima media thickness (IMT), showing the positive effect of successful islet transplantation on micro and macro vascular complications (48).

d. Islet Transplantation and the Eyes

Diabetic retinopathy, the main cause of blindness in developed countries, is a potentially serious complication of all types of diabetes mellitus and is characterized by retinal neovascularization. Retinal blood flow velocity was increased significantly at 1 year in patients with type 1 DM who received islet transplants. Proper glucose control after islet transplantation can stop alterations in retinal microcirculations (49).

e. Islet Transplantation and the Nervous System

Lee et al. examined peripheral nerve function with a nerve conduction velocity (NCV) index in islet-transplanted patients; that improved in both sensory and motor nerve fibers. Also kidney-transplanted diabetic patients who received a functioning islet transplant showed improvement of polyneuropathy (50).

1.4. Problems Facing Islet Transplantation

1.4.1 Availability of Islets

Lack of human tissue for clinical transplantation is a problem that all organ transplant programs face. This is even more serious with regard to islet transplantation, since successful islet transplantation needs more than 300,000 islets and a single patient might need multiple transfusions (51).

1.4.2 Adverse Effects of Current Immunosuppressive Protocols

Immunosuppressive drugs are needed to prevent rejection in transplantation. Current immunosuppressive regimes based on corticosteroids, cyclosporin and tacrolimus have been shown to increase the risk of infection and malignancy is another concern (52, 53).

Corticosteroids have a diabetogenic effect as they lead to reduction of the number and affinity of insulin receptors. Also corticosteroids inhibit insulin secretion in humans (54).

The use of tacrolimus and cyclosporin can induce structural damage of the islets as they led to cytoplasmic swelling, apoptosis and abnormal immunostaining for insulin, with reduced secretory granules in the β-cells (55).

It is important to mention that the corner stones of the Edmonton protocol are the avoidance of corticosteroids with combined sirolimus, tacrolimus and anti-Il-2-receptor antibody, the use of more than two fresh islet preparations, as well as the careful monitoring of the patients preoperatively (56).

1.4.3 Islet Cell Allograft Rejection

Graft rejection is one of the major problems that reduce the success rate of islet cell transplantation. The introduction of newer nondiabetogenic immunosuppressive regimens is believed to decrease islet cell allograft loss (57).

Immunological tolerance can be used to decrease the need of immunosuppressive agents for islet cell transplantation; one of the techniques that has been extensively investigated for this purpose is immunomodulation (58).

Encapsulation of islets into aliginate-polylysinealginate capsules that have the ability to prevent immune reaction and in the same time they allow nutrients and oxygen to reach the islets and also allow insulin to be released into the bloodstream, but these capsules. However most encapsulated islet grafts still fail because of capsule fibrosis and bio-incompatibility (59).

1.4.4 Islet Cell Autoimmunity

Diabetes Mellitus is an autoimmune disease, so the transplanted islets may be exposed to the immune defect that originally caused the disease. Some cases of autoimmune recurrence have been described after islet transplantation. Even therapeutic immunosuppression has not been able to prevent autoimmune recurrence (60).

1.4.5 Primary Nonfunction and Ischaemia

Primary non-function of transplanted islets commonly occurs in human islet transplantation. This could be the result of several factors: (a) transplanting an inadequate number of islets (the number of transplanted islets could be quantified but not all the transplanted islets are viable (61). (b) Processing the pancreas during islet isolation can reduce the viability and function of islets (62). (c) Nonspecific inflammation damaging the transplanted islets by Kupffer cells

and hepatic macrophages (63). (d) Immune-mediated destruction may involve macrophages and dendretic cells either from the donor or from the host leading to failure of the graft.

Trials to decrease this rejection process included means like using low-temperature culture, and ultraviolet irradiation. Anti-MHC class II antibodies and anti-DC antibodies can also be used to prevent immunerejection (63).

The rate by which glucose levels decreases in serial glucose tolerance tests can be used as a marker of rejection (64).

1.4.6 Inadequate Knowledge Regarding Islet Function after Transplantation

Normalization of blood glucose levels after islet transplantation is the main goal of the islet transplantation. However, the normal islet function includes other hormones, such as glucagon, somatostain and pancreatic polypeptide; more investigations have to be made to study the effect of these hormones on carbohydrate, protein and lipid metabolism. It is unclear as to how important these other hormones would be in the maintenance of normal glucose homeostasis following transplantation (65).

1.4.7 Alternative Approaches to Islet Transplantation

If researchers managed to reach higher success rates in islet transplantation, the availability of human pancreatic tissue would rapidly become

insufficient compared to the large number of diabetic patients and also the number of islets required for each patient, now some research is directed towards the use of other sources of islets. Islet xenotransplantation, and stem cell technology, are the main areas of focus to solve the problem of limited donor supply of islets for transplantation into type 1 diabetic patients (66).

1.4.8 Xenotransplantation

It is the transplantation of living cells, tissues or organs from one species to another such as from pigs to humans. The have been trials using a number of animal models models in an attempt to find suitable sources of xenogenic islets for transplantation. Porcine islets represent the most promising source for limitless supplies of islets, because the pigs share many anatomical and physiological similarities to human pancreas. Other advantages of pigs over other animal models are the large supply and short gestation period (67).

Moreover, porcine insulin was the standard therapy for diabetes for many years before introduction of recombinant insulin, there is only one amino acid difference between human and porcine insulin (68).

There are two major obstacles facing the success of porcine islet xenotransplantation into humans, first are safety issues related to xenosis and the second is xenorejection of the transplants (68).

1.5. Stem cells

1.5.1 Introduction

Stem cells are special cells that have the ability to renew themselves through mitotic cell division and to differentiate into many specialized cell types, so “offspring” cells of stem cells can be either stem cells (and hence, self-renewing) or specialized cells (i.e., differentiated cells) (69, 70).

In a developing embryo, stem cells have the ability to differentiate into all of the specialized embryonic tissues. In adult organisms, stem cells maintain the normal turnover of regenerative organs, such as blood and skin (71).

1.5.2 Potency Definitions

Potency is the potential of the stem cell to differentiate into various cell types. Totipotent stem cells are able to differentiate into embryonic and extraembryonic cell types e.g. placenta. Pluripotent stem cells of the morula can differentiate into nearly all cells derived from endoderm, ectoderm or mesoderm. The ability of stem cells to differentiate into cells of a closely related family is called multipotency, while oligopotent stem cells can differentiate into a small number of cells. Unipotent cells can produce only one cell type, their own (72).

1.5.3 Different Types of Stem Cells

There are several types of stem cells: embryonic stem cells, fetal stem cells, adult stem cells, embryonic germ cells, and amniotic and umbilical cord stem cells (71).

1.5.4 Potential Uses of Stem Cells

Adult stem cells derived from bone marrow have been used for long time for successful treatment of multiple blood-based disorders (73).

In the future, medical researchers wish to use technologies derived from stem cell research to treat various diseases such as cancer, diabetes, multiple neurological diseases as Parkinson's disease, multiple sclerosis, and others (74).

The ability of bone marrow stem cells to produce liver cells has been used as a treatment for patients with hepatic malignancies (75). Human embryonic stem cells have been made to differentiate into endothelial precursor cells; that were used to form blood vessels in mice (76). Stem cells have also been used for the treatment of stroke and heart ischemia animal models by human umbilical cord blood transplants in rats (77, 78), and the ability of embryonic stem cells to differentiate into cardiac myocytes (79).

The benefit of stem cell research is not only regenerative medicine, but also it is very useful in studying cells' proliferation and differentiation. This is of particular importance in the fight against cancers and in general research directed towards the development and normal life cycle of cells (80). Another benefit for

stem cells research is that they could be used to model organs for the testing of drugs or new surgical techniques (81).

1.5.5 Controversy Surrounding Stem Cell Research

There is a widespread controversy surrounding human embryonic stem cell research. The focus of this first controversy is on when life begins and whether any individual has the right to terminate a life. Many countries either ban embryonic stem cell research or severely restrict it (82). The second issue is about the stem cells' potential to produce malignancies, once implanted due to their theoretically immortal nature given their ability to proliferate continuously, these cells carry an increased likelihood of mutations, which in turn increases the probability that they will grow out of control and become cancerous (83). The third main controversy concerns whether adult stem cells are as beneficial as embryonic stem cells.

In this study our focus would be on the stem cells possibly present in adult human pancreas.

1.6 β-cell regeneration from Stem Cells

Different stem cells have been investigated to be source for β-cells progenitor such as embryonic stem cells, bone marrow-derived mesenchymal

stem cells, hepatic oval cells, splenocytes, umbilical cord blood cells and adult pancreatic-derived multipotent progenitor, pancreatic duct cells, (figure 1.3).

Evaluation of the ability of these progenitors to produce mature β-cells could includes: insulin staining, presence of activated specific cell genes such as PDX1, nGn3, neuroD1 or PaX4, *in vitro* response to higher glucose levels, and the ability of progenitor cell to reverse hyperglycemia in animal models of type 1 DM (84).

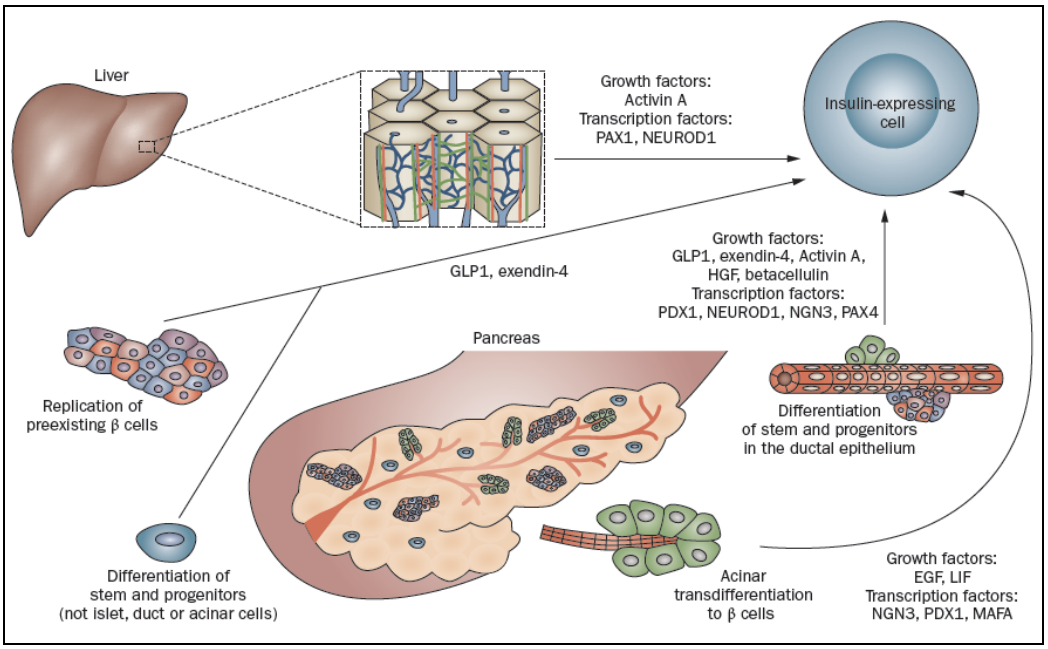

Figure 1.3: Various strategies to obtain β-cells from organ specific stem cells (Bonner-Weir, S. et al.2010) (117).

1.6.1 Embryonic Stem Cells as Renewable Source of Functional β-Cells

a. Early Attempts for *in vitro* β-cell Differentiation

Various approaches have been tried to generate insulin-producing β-cells from ESCs. Many studies reported the generation of insulin producing cells from mouse (85), monkey (86), and hESCs (87). However, none of these studies have produced cells that would secrete physiologically sufficient amounts of insulin in response to glucose.

Trials to generate β-cells from nestin positive ESCs led to the generation of neuronal cell types. This goes with the belief that nestin does not mark endocrine progenitor cells but it is rather a marker for neural progenitors and pancreatic exocrine progenitors (88).

Lineage-specific gene markers can be used to follow differentiation of ESCs into definitive endoderm (DE) and later into pancreatic cells (89).

b. Directed Differentiation of HESCs

Assady et al. reported that β-cells can be produced by spontaneous differentiation using hESCs (90). Consequent studies used the signals that regulate embryonic endoderm and pancreas formation, which might mimic the *in vivo* sequence of events through a multi-step protocol that promotes differentiation of ESCs through the normal developmental stages. Reports from

D'Amour et al. (91) and Kroon et al. (92) are considered the most successful attempts. When given the appropriate stimuli, final differentiation of β-cells and other hormone-secreting cells was achieved (93).

c. Generation of Insulin-Secreting Cells through Nuclear Reprogramming.

Nuclear reprogramming has been examined by expressing certain transcription factors in ESCs. The use of ESCs expressing high levels of exogenous Pdx1, Ngn3, and Pax4 has produced more obvious pancreatic differentiation; however no functional β-cells were generated from these protocols (94).

1.6.2 New β-Cell Formation from Adult Pancreatic Stem Cells

β-cell mass is regulated by the balance between new cells generation by replication of existing β-cells or production of new islet from adult stem cells as well as by the rate of apoptosis of β-cells. The endocrine pancreatic cells undergo dynamic changes in response to growth, development, and other physiological conditions such as pregnancy or obesity. The decreased β-cell mass diabetic patients may be restored by either increasing the rate of new β-cells formation or by blocking β-cells apoptosis pathway (95, 96).

a. β-Cell Adaptation

Chronic high-dose glucose infusion in normal animals or in rat diabetic models, led to an increase in β-cell mass and function. Also chronic hyperglycemia caused increased neogenesis and higher cell replication indices (97). "In another study; young near-totally-pancreatectomized rats presented a spontaneous eight-week-regeneration of 27% of pancreas weight and 42% of the endocrine pancreas" (98). In humans, 39-year-old type 1 DM Japanese patient received SPK that was complicated by an abdominal incisional hernia 2 years after SPK. The patient received Immunosuppressive drugs since transplantation and his serum glucose levels were regulated without exogenous insulin. At the time of surgery for the hernia, the original pancreas biopsy showed a 4 fold increase in the percent of β-cells compared to that seen in type 1 DM patients treated with insulin (99).

b. Transdifferentiation of Exocrine Tissue

Transdifferentiation of acinar cells into β-cells has been suggested based on the presence of single β-cells scattered throughout the exocrine parenchyma in diabetic patients after immunosuppressive treatment (100).

Initial studies showed that acinar cells obtained from human pancreas have been able to transdifferentiate into amylase-negative and cytokeratin 19-positive positive duct cells (101). Another study was able to produce functional β-cells from rat exocrine cells after *in-vitro*, exposure to a combination of EGF and

leukemia inhibitory factor. *In vivo*, these exocrine-derived β-cells were able to restore normoglycemia in diabetic mice (102).

c. Generation of New Islets from Pancreatic Duct Cells

Pancreatic duct cells are believed to be the main source for β-cell growth and regeneration after injury. Many methods have been described to drive pancreatic duct cells to express insulin including hepatocyte growth factor, β-cellulin, GLP1 and exendin-4 (103).

Partial duct ligation of the pancreatic duct in adult mice resulted in new β-cell formation and it is thought to be due to activation of Ngn3 expressing cells near or within the ducts (104).

In vitro culture of the duct cells of the pancreas was associated with some changes in the phenotype in the form of loss of the epithelial markers and acquisition of mesenchymal markers (105).

1.6.3 Generation of New Islets from Splenic Mesenchymal Cells

Transplantation of a combination of splenic mesenchymal cells with complete Freund's adjuvant reversed diabetes by regeneration of insulin producing islets. The mesenchymal cells originated from spleen and transplanted under certain conditions are helpful both against immune destruction of islets in

check, and as a source of progenitors that transdifferentiate into pancreatic β-cells (106).

1.6.4 Generation of New Islets from Hepatic Stem Cells

Rat oval hepatic stem cells were transdifferentiated to islet like structure. This may be due to the common endodermal origin, of liver and pancreas. The differentiated hepatic stem cells lose the expression of hepatocyte markers. Acquisition of β-cell markers was proved by RT_PCR and immunocytochemistry showed expression of pdx-1, pax-4, pax-6, nkx2.2, nkx6.1, insulin 1 and 2, glucagon, pancreatic poly peptide, and GLUT-2. Implantation of these cells under the renal capsule of streptozotocin-diabetic NOD-SCID mice led to restoration of normal glycemia (107).

1.6.5 Bone Marrow Mononuclear Cells

Bone marrow (BM) has been shown to contain multipotent stem cells that can differentiate towards ectodermal or endodermal directions (108). Several studies showed that BM-derived stem cells can differentiate *in vitro* into insulin-producing cells with many characteristics of true β-cells (109, 110). Using a diabetic patient's own bone marrow as a source of autologous insulin-producing β-cells is an interesting idea that would minimize the use of immunosuppressive agents.

A study done in Argentina and Peru were they used bone marrow mononuclear cells that were introduced via splenic artery to treat diabetic patients of both type 1 and 2. After one year follow up type 1 DM patients, showed no metabolic improvement as observed by high HbA1c levels, doses of daily exogenous insulin given for the patients to control hyperglycemia and by no increase in C-peptide levels (111).

1.6.6 Autologous Umbilical Cord Blood Transfusion

Haller and colleagues followed up 7 diabetic children that were recently diagnosed with type 1 DM for 6 months. Patients who received umbilical cord blood transfusion had lower insulin requirements and lower HbA1c levels when compared to patients who received insulin therapy alone. However, C-peptide levels have decreased and none of the patients became insulin-free (112).

1.6.7 Challenges Facing the Use of Stem Cells in Treatment of Diabetes Mellitus

a. Risk of Cancer Formation up on Transplantation of HESC-derived Cells

One of the main problems facing the use of undifferentiated ESCs is the risk of causing tumors after cell transplantation. This can be prevented by transplantation of a purified, homogenous population of partially or fully differentiated cells generated from hESCs. Fluorescence-activated cell sorting and

magnetic-activated cell sorting could be used to obtain pure cell populations for transplantation (113).

b. Transplantation-Related Immune Rejection

Grafted tissues in patients of type 1 DM are not only exposed to the usual immune reaction being foreign entities that initiate a host vs. graft alloimmune reaction, but also they face the risk of being destroyed by autoimmune response as other type 1 diabetes patients, where β-cells are destroyed by an autoimmune response (60).

The use of some autologus adult stem cells or umbilical cord stem cells could be beneficial to prevent alloimmune response of the immune system (112).

c. Full Maturation of β Cells in Vitro

One of the things hindering the development of fully differentiated β-cells under *in vitro* is the absence of cell-cell interactions between mesenchymal and epithelial cells that is normally present between cells during embryonic pancreas development *in vivo* (114). In the most recent *in vitro* differentiation protocol, cells are grown in two-dimensional cultures as monolayers. The use of a co-culture system with pancreatic mesenchyme or endothelial cells and three dimensional cultures may improve the efficiency of β-cell differentiation (115).

1.7 Summary

Diabetes mellitus is a very common disease and its' incidence is increasing allover the world. Two major types of diabetes are known type 1 DM and type 2 DM. Clinical diagnosis of both types is similar, the serum glucose level has to be higher than normal on two separate occasions. Diabetes can lead to serious complications that might lead to death sometimes.

Since the production of commercial insulin it has been the main treatment for type 1 DM, despite the improvement in these patients' survival rates these patients are still at the risk of developing secondary complications. That was explained by the lack of physiological variations in insulin level corresponding to fluctuations in serum glucose level. In order to solve this problem researchers have been looking to find source of more physiologic insulin. Pancreatic transplantation was the first proposed method; however it was modified later to the smaller version which is islets transplantation to avoid the major surgical risk associated with transplantation o the whole pancreas.

Islet transplantation successfully was able to improve type 1 DM style of life, decreasing the risk of developing secondary complications of diabetes.

Similar to other forms of human organ transplantation, the major hurdle facing islet transplantation is the availability of donor tissue. Xenotransplantation and stem cell therapy have the potential to offer limitless amounts of tissues to be used as an alternative to islets transplantation.

Many stem cells have been tried to obtain functional β-cells that could reverse hyperglycemia, among them are cells within the pancreas itself such as duct cells.

1.8 Thesis Objectives

Duct cells of the pancreas are one of the candidates that are thought to represent β-cell progenitor, however *in vitro* culture of these cells was associated with some phenotypic changes. These cells are thought to lose there epithelial phenotype in two dimensional culture. The objective of this study is to monitor changes occurring on the phenotype of the human non-endocrine pancreatic tissues (NEPT) remaining after islets isolation, composed basically of epithelial acinar and duct tissues. In monolayer culture we are testing various protocols that are thought to help preserving the epithelial phenotype of these cells and prevent the acquisition of mesenchymal markers. We are testing the use of geneticin, effect of bFGF, EGF, KGF and other growth factors over the phenotype of the NEPT.

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CHAPTER TWO

Towards Optimizing Culture Condition of Duct Cells of the Pancreas

2.1 Introduction

Type 1 diabetes mellitus (T1DM) is a metabolic disorder caused by autoimmune destruction of the insulin-producing pancreatic β-cells, leading to increase in plasma glucose levels; as a result, patients with T1DM are dependent on exogenous insulin for their blood glucose control (1).

Although the treatment with insulin has largely improved survival of these patients for the long term, patients with T1DM are still not exempt from the development of diabetic complications. Patients with diabetes mellitus can suffer from acute complications such as acute hypoglycemia and diabetic ketoacidosis and chronic complications in the form of nephropathy, neuropathy, retinopathy and cardiovascular problems (2).

The development of these complications may be due to the absence of physiological variations in insulin secretion in response to changes in plasma glucose levels; thus, it is very important to search for effective ways to re-establish a functional β-cell mass in patients with T1DM. For this purpose, first was the transplantation of a whole human pancreas, in which recipients undergo major surgery and are at risk of serious complications. These complications can be avoided by the less invasive procedure of cadaveric human islet transplantation

(3, 4) which enabled patients with T1DM to become insulin-independent. However, the single islet transplantation procedure requires at least 10,000 islet equivalents/kg to achieve insulin independence, which necessitates the use of 2-3 donor pancreata for each recipient (5).

To solve the problem of limited supplies of islets, researchers proposed alternative sources of insulin-producing tissue such as porcine tissue (6), engineered beta-cell lines (7), and differentiation of stem cells into functional β-cells.

Among stem cells, studies have focused on human embryonic stem cells (8), induced pluripotent stem cells (9), umbilical cord blood (10), bone marrow-derived mesenchymal stromal cells (11), and organ-specific stem cells, such as cells from liver (12), and the pancreas itself.

Within the pancreas four types of cells are suspected sources of β-cell progenitors, duct tissue (13, 14), acinar tissue (15, 16), mesenshymal cells (17, 18), and islets of langerhans (19, 20).

Pancreatic duct cells have been proposed as the main source of progenitors for growth and regeneration within the pancreas (21). Genetic lineage tracing has of cells that express carbonic anhydrase II within the ductal structures showed that they contribute to the β-cell population both after birth and after injury (22). It is important to mention that Jorge Ferrer and colleagues used HNF1b expression to oppose this theory and showed that duct cells do not contribute to endocrine pancreatic cells after birth (29).

Another study showed that partial ligation of the pancreatic duct in adult mice resulted in new β-cell formation and it is thought to be due to activation of Ngn 3 expressing cells near or within the ducts (23).

In vitro, various methods have been reported to drive pancreatic ductal cells to express insulin, including certain growth factors such as hepatocyte growth factor, β-cellulin (24, 25), GLP1 and exendin-4 (26, 27). Additionally, viral infection to introduce transcription factors required for islet development for example (Pdx1, Ngn3, NeuroD1 or Pax4) has been shown to induce the β-cell phenotype (28). Expansion followed by transplantation of human ductal cells, purified by carbohydrate antigen 19-9 (CA19-9) expression, into immunocompromised NOD/SCID mice, confirmed the ductal origin of these insulin-positive cells (30).

However, ductal cells within the NEPT showed a decrease in cell number with time in cell culture. During *in vitro* expansion the percent of single-positive epithelial cells expressing (amylase, CK19, EpCAM and E-Cadherin) decreases, whereas the percent of single-positive mesenchymal cells expressing (Vimentin, CD105, CD90, CD13, CD29, and CD44) increases, possibly from epithelial cells that dedifferentiated intomesenchymal cells, Epithelial Mesenchymal Transition (EMT) (31).

In this study we are trying to preserve the epithelial phenotype of the duct cells *in-vitro* through treating cells with geneticin (G418) which is an aminoglycoside antibiotic that is known for its differential cytotoxic effects on eukaryotic cells with different growth rates as it decreases polypeptide synthesis

in dividing cells with more effect on more rapidly dividing fibroblasts compared to epithelial cells (32).

Several factors that have been used in primary epithelial cell culture were tested by adding them to the different base media, in order to maintain a robust duct cell population that could be differentiated to functional β-cell later on.

In this study we hypothesize that G418 exposure followed by culturing cells in pancreatic media with no serum could help preserving the epithelial phenotype of duct cells within human NEPT.

2.2 Materials and Methods

2.2.1 Tissue Samples

Non-endocrine pancreatic digests from independent human donors ranging from 27 to 65 years of age were obtained following islet purification in the human islet lab at the University of Alberta. The predominately epithelial cell fraction - composed of acinar and duct cells - was collected from the Cobe bag and washed three times with Hanks balanced salt solution (HBSS; Sigma-Aldrich, Oakville, Canada) supplemented with 0.5% bovine serum albumin (BSA; fraction V, Sigma). Dithizone staining of representative samples was performed to assess islet content. NEPT were re-suspended in RPMI 1640 (Gibco/Invitrogen, Burlington, Canada) supplemented with 1% insulin-transferrin-selenium (ITS), and 0.5% BSA (RPMI/BSA/ITS) in a ratio of 1: 10 of the packed tissue volume. Protocols used in this study were approved by the Research Ethics Board of the University of Alberta.

2.2.2 Culturing of Non-Endocrine Pancreatic Tissues (NEPT)

Approximately 5 ml of the NEPT suspension were cultured per non-treated 150 mm plate (Fisher Scientific, Edmonton, Canada). The media used, was RPMI/BSA/ITS. The islet-depleted tissues were cultured in the humidified incubator at 37°C and 5% CO_2, for 24-48 hours.

After 24-48 hours, NEPT were collected in 50 ml conical tubes, re-suspended in 10 ml of RPMI with 10% Fetal Bovine Serum (FBS, Gibco).

A fraction of the tissue suspension was collected, then cells of this tissue fraction were dissociated into single cells using 0.1% trypsin (Sigma- Aldrich, Oakville, Canada) and cells were counted on light microscope 10x (Leica). Of these single cells, $5x10^6$ cells - representing passage 0 - were fixed in 1% formaldehyde for antibody staining, and then flow cytometry analysis was done.

Passage 1 started with culturing 0.5 ml of the suspension in 150 mm tissue culture treated plates (Fisher Scientific) for control plates. Medium used was RPMI 1640 supplemented with 10% FBS, 10 mM HEPES, 1 mM sodium pyruvate, 71.5 μM β-mercaptoethanol, 20 ng/ml of epidermal growth factor (EGF, Invitrogen), 20 ng/ml basic fibroblast growth factor (bFGF, Invitrogen), 100 U penicillin and 1000 U streptomycin (Lonza), this media is referred to as (RPMI-FL). Medium was changed every 2-3 days.

For the experimental plates 1-1.5 ml of the suspension was cultured in the same type of plates using RPMI-FL same as the control. Media change was done every 2-3 days.

2.2.3 Exposure to G418

Geneticin (G418) was previously used to selectively eliminate fibroblasts contaminating human melanocytes cell cultures (33). We treated our NEPT to G418 to eradicate the fibroblasts that prevail in NEPT cultures.

After 4-5 days, cellular aggregates, composed primarily of ductal epithelium were 60-80% confluent. Then tissues in experimental plates were treated with RPMI-FL containing (G418) 50 mg/ml (Gibco) with a concentration of 100 μg/ml for 24-48-hours, controls were not exposed to G418.

After 24-48 hours, ductal aggregates of both experimental and control plates were harvested and dissociated with 0.1% trypsin, and cells were counted. Of these single cells $5x10^6$ cells - representing passage 1 - were fixed in 1% formaldehyde for antibody staining. The phenotype of the stained and fixed samples was tested by flow cytometry analysis. The non fixed cells of the ductal aggregates were further passaged to test the effect of culturing them in various media types.

2.2.4 Testing Different Types of Media

After dissociation of the NEPT aggregate of passage 1, passage 2 started by culturing cells into different types of media on 150 mm tissue culture treated plates. In the following density, for the control $0.5\text{-}1x10^6$ cells were cultured in (RPMI-FL). The cells previously exposed to G418 were cultured at higher density of $2\text{-}3x10^6$ into various types of media.

We used RPMI-FL, and RPMI-EGF, which is similar to RPMI-FL but lacked bFGF. We also used pancreatic media described by Abunang et al. (34), that had DMEM F12 1:1(Gibco) as a medium base, supplemented with EGF 0.1 μg/ml (Invitrogen), Dexamethasone 0.4 μg/ml (Sigma-Aldrich), Bovine pituitary extract 0.025 mg/ml (US Biological), Tri-iodo-thyronine 0.05 μM (Sigma-Aldrich), ITS 100x (Sigma-Aldrich), Soy bean trypsin inhibitor 0.1 mg/ml (US Biological). Finally we used Keratinocyte serum-free medium described by Angelica et al. (35) that had DMEM F12 1:1(Gibco) as a medium base, supplemented with EGF 5 ng/ml (Invitrogen), Bovine pituitary extract 0.05 mg/ml (US Biological) and Keratinocyte growth factor (KGF) 25 ng/ml. The effect of serum on cell growth in these types of media was tested by changing the concentration of FBS from 10 to 1%.

Media change was made every 2-3 days; cells were left to grow till 80-100% confluent. For cells exposed to G418 they take around 18-24 days. The control plates of passage 2 became 100% confluent quickly (7-10 days), so they were collected and passaged into new plates passage 3, to be collected with other cells at the end of the experiment (Figure 2.8a).

NEPT aggregates were dissociated with 0.1% trypsin into single cells; of these cells $5x10^6$ were fixed with 1% formaldehyde representing passage 2 for experimental plates and passage 3 of the control. The single fixed cells were stained using EpCAM FITC and Vimentin FITC to characterize the epithelial and mesenchymal phenotypes of the cells respectively.

2.2.5 Immunophenotyping by Flow Cytometry

To determine the phenotype of the NEPT cultured on monolayer culture, cell surface antigen expression of single cells obtained from each passage were analyzed by flow cytometry. Briefly, cells were fixed with cold 1% formaldehyde (BDH Laboratory Supplies), washed once with PBS and stained with the primary antibodies EpCAM FITC (Stem Cell Technologies Inc) 1:5 and Vimentin FITC (Progen) 1:10 after permeabilization with 3% saponin. The isotype control used was IgG1 FITC (Cedarlane) 0.6:10. Cell populations were analyzed on the FACS Calibur (BD Biosciences, Mississauga, Canada) using Cell Quest Pro software and compared to the isotype control and the unstained sample.

2.2.6 Imaging

Images of culture plates in different media were taken using a Leica microscope with 10x magnification power.

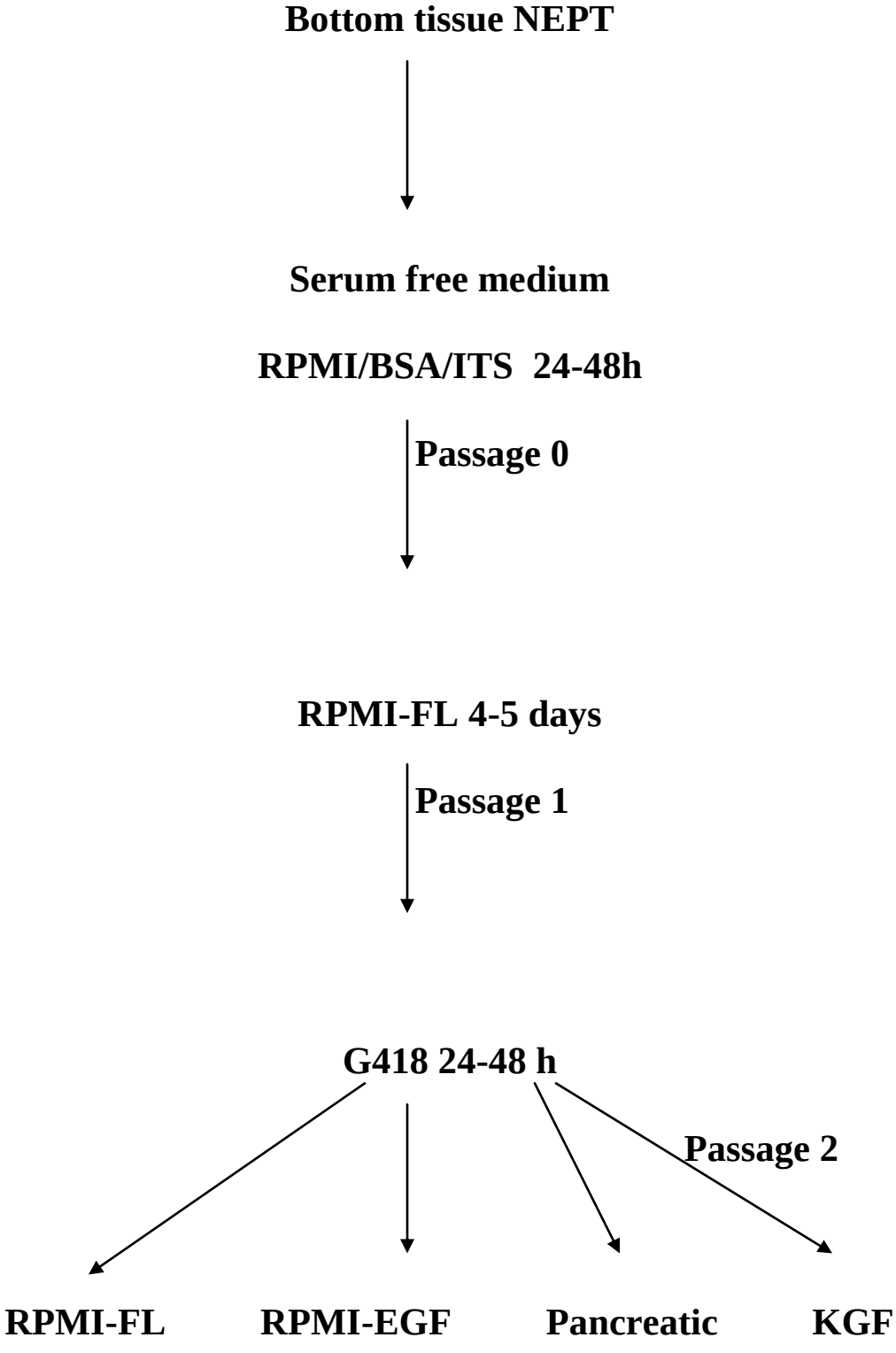
2.2.7 Statistical Analysis

Values are expressed as mean ± standard error of the mean (S.E.M.). Statistical significance of differences was calculated by a one-way analysis of variance (ANOVA) and Tuekey's test. Tests were performed on Graph Pad Prism 4. A P value < 0.05 was considered significant.

Table 2.1: Summary of media used in this study

Media	composition
RPMI/BSA/ITS	RPMI 1640,1% insulin-transferrin-selenium (ITS), 0.5% BSA
RPMI-FL	RPMI 1640 supplemented with 10% FBS, 10 mM HEPES, 1 mM sodium pyruvate, 71.5 μM β-mercaptoethanol, 20 ng/ml of epidermal growth factor (EGF) 20 ng/ml basic fibroblast growth factor (bFGF)100 U penicillin and 1000 U streptomycin .
RPMI-EGF	Same as RPMI-FL with no (bFGF)
Pancreatic	DMEM F12 1:1 as a medium base, supplemented with EGF 0.1 μg/ml, Dexamethasone 0.4 μg/ml, Bovine pituitary extract 0.025 mg/ml, Tri-iodo-thyronine 0.05 μM, ITS 100x, Soy bean trypsin inhibitor 0.1 mg/ml.
KGF	DMEM F12 1:1as a medium base, supplemented with EGF 5 ng/ml, Bovine pituitary extract 0.05 mg/ml and Keratinocyte growth factor (KGF) 25 ng/ml.

Figure 2.1 Summary of design of the experiment



2.3 Results

2.3.1 Loss of EpCAM Positive Cells over Time

Stained single cells of passage 0 i.e before culturing them in tissue culture treated plates were compared to passage1 of the control plates, and passage 3 of the control plates at the end of the experiment.

The average of EpCAM positive cells in passage 0 n = 4, was 72.6 ± 4.3 %, and for the Vimentin positive cells the average was 5.2 ± 2.3%.

Analysis of cells from (passage 1) n = 7, showed that, the average of EpCAM positive cells was 56.4 ± 8.7% and for the Vimentin positive cells the average was 84.3 ± 3.2%. The cells collected at the end of passage 3 n = 7, revealed that, the average of EpCAM positive cells was 0.5 ± 0.3% and for the Vimentin positive cells, the average was 96.8 ± 1.3%. Data summarized in figure 2.2.

Statistical analysis showed a significant decrease in EpCAM positive cells over time. Also a significant increase of Vimentin positive cells over time, when comparing passage 0 and passage 3 results.

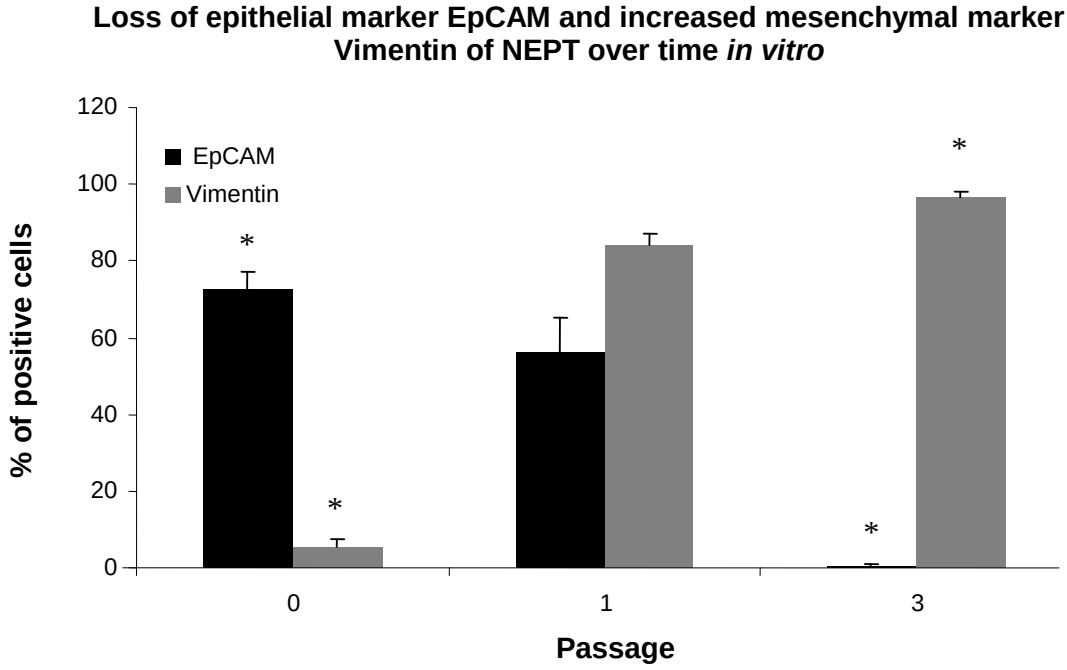


Figure 2.2: Loss of epithelial marker EpCAM of NEPT *in-vitro* and acquisition of mesenchymal marker Vimentin over time. Values are means ± SEM from different human donors showing the phenotype of NEPT cells cultured in RPMI-FL harvested at different time points, before the beginning of culture (passage 0) n = 4, after 5-6 days (passage 1) n = 7, and at the end of the experiment 18-24 days (passage 3) n = 7. Statistical significance of difference between the three groups was calculated by one-way ANOVA; has shown that there is consecutive decrease of average of EpCAM positive cells and increase of average of Vimentin positive cells over time; * denotes $p < 0.05$.

2.3.2 Effect of Exposure to G418 for 24 and 48 Hours

Hao et al. used G418 to eradicate mesenchymal cells from NEPT culture (36). To evaluate the ability of G418 to preserve the epithelial phenotype of the duct cells within the NEPT and eliminating the mesenchymal cells. After 4-5 days in RPMI-FL the NEPT was treated with G418 at a concentration of 100 μg/ ml for 24 and 48 hours. Then cells were harvested using 0.1% trypsin and fixed in 1% formaldehyde. Fixed single cells were then stained with antibodies (Vimentin and EpCAM). The phenotype of the cells was tested using flow cytometry.

The average of EpCAM positive cells in the control n = 7 at this time point, was 56.4 ± 8.7% and the average of Vimentin positive cells was 84.3 ± 3.2%. Those treated with G418 for 24 hours n = 7 had an average of was 70.6 ± 8.8% EpCAM positive cells, and an average of 69.4 ± 5.5% Vimentin positive cells. For the group of cells treated with G418 for 48 hours n = 7, the mean of EpCAM positive cells was 64.4 ± 10.6%, and the mean of Vimentin positive cells was 74.8 ± 8.4%. This data is summarized in figure 2.3.

Statistical analysis showed non significant differences; however there was a trend showing that cells treated with G418 have more EpCAM positive and less Vimentin positive cells compared to the control. Those treated with G418 for 24 hours had more EpCAM positive cells and less Vimentin positive cells than those treated with the same concentration of G418 for 48 hours.

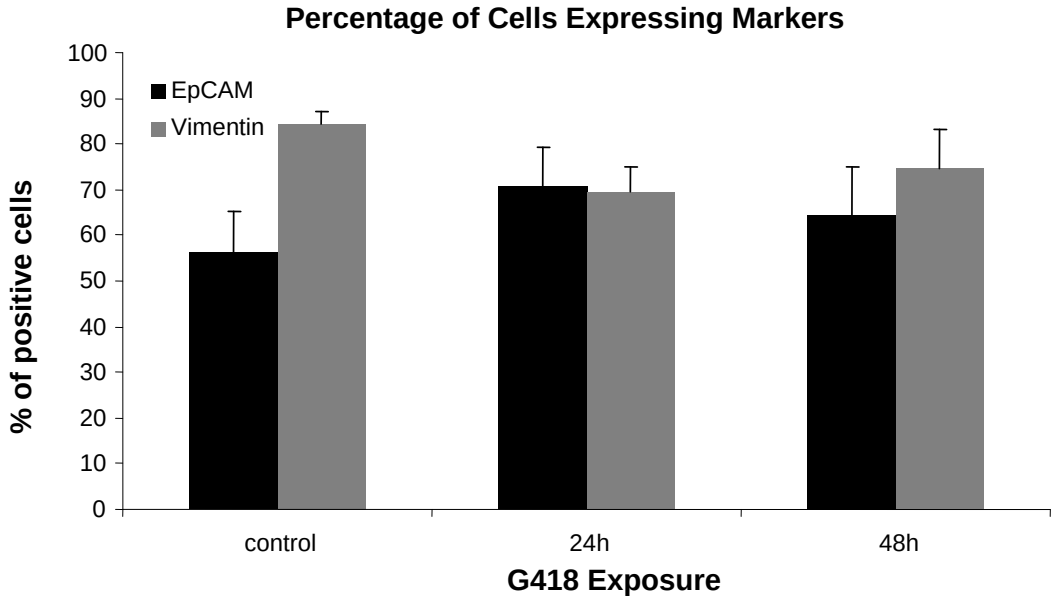


Figure 2.3: Exposure to 100 μg/ml of G418 for 24 and 48 hours after 4-5 days in culture helped in preserving the epithelial phenotype of duct cells in NEPT with higher EpCAM positive cells and lower Vimentin positive cells in G418 treated cells compared to the control. Results are expressed as means ± S.E.M. of percentage of EpCAM positive and Vimentin positive cells. NEPT from different human donors were cultured in RPMI-FL for 4-5 days, then exposed to 100 μg/ml of G418 for 24 hours n = 7 and 48 hours n = 7 vs. control not exposed to G418 n = 7.

2.3.3 Culturing NEPT in RPMI-FL and RPMI-EGF after G418 Treatment

To determine the effect of bFGF on the composition of cells. Cells were cultured in RPMI-FL that contains bFGF and RPMI-EGF without bFGF. After treatment of the cells with G418 the cells were cultured in RPMI-FL and RPMI-EGF. The growth rate and expansion of cells previously exposed to G418 was slow. It took the cells in RPMI-Fl 18-22 days to become 80-100% confluent, while those in the media with RPMI-EGF took more time 20-24 days to reach 80-100% confluence.

Given this long culture time, cells started to appear as elongated spindle cells rather than rounded cells (Figure 2.8b). The control group were cultured in RPMI-FL. The control plates became 100% confluent usually after 7-10 days at the end of passage 2. They were harvested and passaged to passage 3 to be collected at the end of the experiment with cells of experimental plates. Flow cytometry was used to analyze the phenotype of the harvested, fixed and stained cells.

Cells cultured in RPMI-FL following exposure to G418 for 24 hours n = 7 the average of EpCAM positive cells 2.9 ± 0.9 % and the average of Vimentin positive cells was 97.9 ± 0.8 %. The cells cultured in the RPMI-FL after 48 hours treatment with G418 n = 6, showed an average of 2.4 ± 1.2% EpCAM positive cells and 97.3 ± 0.8% Vimentin positive cells. On the other hand cells cultured in RPMI-EGF media after 24 hours of treatment with G418 n = 4 showed an average of 5.2 ± 1.9% EpCAM positive cells and 95.4 ± 2.3% Vimentin positive. Those

cultured in RPMI-EGF media after 48 hours exposure to G418 n = 4, showed a mean of 3.2 ± 1.2% EpCAM positive cells and 96.7 ± 1.5% Vimentin positive cells. Control group n = 7 showed an average of 1.8 ± 0.6% EpCAM positive cells and 98.5 ± 0.3% Vimentin positive cells. Data summarized in figure 2.4.

Statistical analysis showed non significant difference between control cells cultured in RPMI-FL with no G418 exposure to those exposed to G418 for 24 and 48 hours and cultured in RPMI-FL and RPMI-EGF.

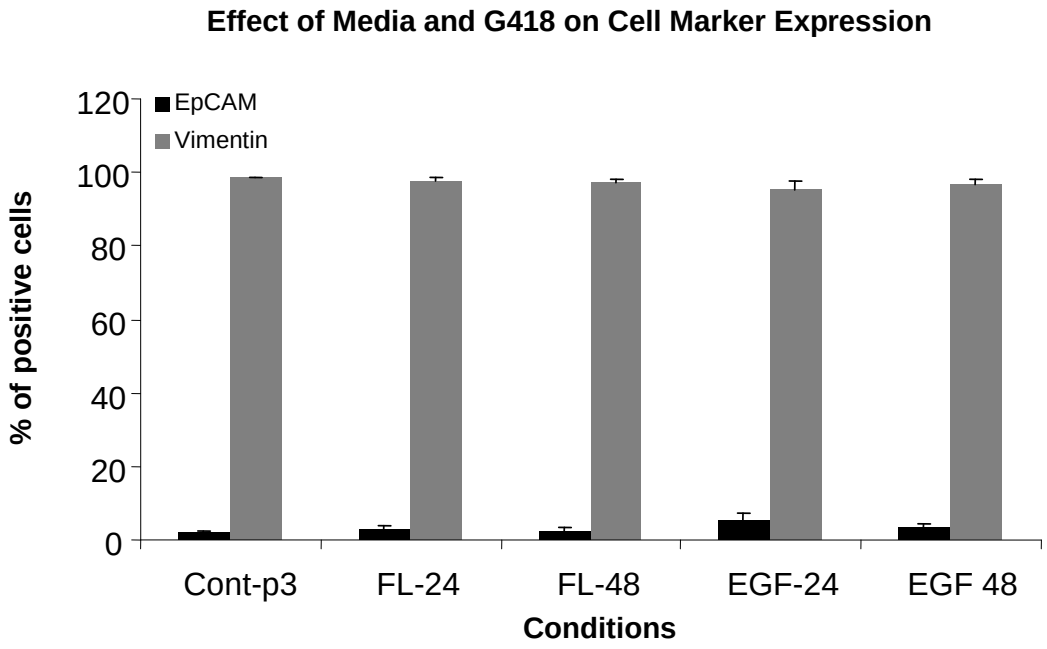


Figure 2.4: The phenotype of NEPT cells from different human donors cultured in RPMI-FL and RPMI-EGF after 24 and 48 hours exposure to 100 μg/ml of G418 vs. control cultured in RPMI-FL. Statistical analysis using one-way ANOVA showed no significant difference of EpCAM positive cells and Vimentin positive cells between cells cultured in RPMI-FL after G418 for 24 hours n = 7 and for 48 hours n = 6 and cells cultured in RPMI-EGF after G418 for 24 hours n = 4 and for 48 hours n = 4 vs. control cultured in RPMI-FL without G418 n = 7.

2.3.4 Changing the FBS Concentrations in RPMI-FL and RPMI-EGF Media after Exposure to G418

To observe the effect of changes in FBS concentration on the phenotype of the cells cultured in RPMI-FL and RPMI-EGF Media after exposure to G418, cells were cultured in these media supplemented with 10 and 1% FBS.

Cells of experimental plates were exposed to 100 μg/ml of G418 after 4-5 days in culture in RPMI-FL. After 24 hours, cells were harvested, and then they were dissociated into single cells using 0.1% trypsin. Approximately 2-3x10^6 cells were cultured in RPMI-FL with 1 and 10% FBS, and RPMI-EGF with 1 and 10% FBS. Cells of the control group were cultured in RPMI-FL.

Cell growth in the media with 1% FBS was slower than those cultured in media with 10% FBS. The cells were left to become 80-100% confluent, and then they were harvested using 0.1% trypsin. Single cells were fixed in 1% formaldehyde and stained with EpCAM FITC and Vimentin FITC antibodies.

Analysis of the phenotype of cells using flow cytometry showed that cells exposed to 100 μg/ml G418 for 24 hours, then cultured in RPMI-FL 10% FBS n = 7 had an average of 4.1 ± 1.3 % EpCAM positive cells and 93.5 ± 3.2% Vimentin positive cells. While those cultured in RPMI-FL 1% FBS n = 5 showed a mean of 1.1 ± 0.4% EpCAM positive cells and 96.1 ± 2.4% Vimentin positive cells.

For cells cultured in RPMI-EGF 10% FBS n = 7 the average EpCAM positive cells were 4.5 ± 1.1% and 97.8 ± 0.4% Vimentin positive cells. The cells cultured in RPMI-EGF 1% FBS n = 6 showed an average of 2.3 ± 1.4% EpCAM positive cells and 98.4 ± 0.1% Vimentin positive cells. Control group n = 7 showed 0.5 ±

0.4% EpCAM positive cells and 96.8 ± 1.4% Vimentin positive cells. Data summarized in figure 2.5.

Statistical analysis of these data, showed non significant effect of changing the concentration of FBS in RPMI-FL and in RPMI-EGF media.

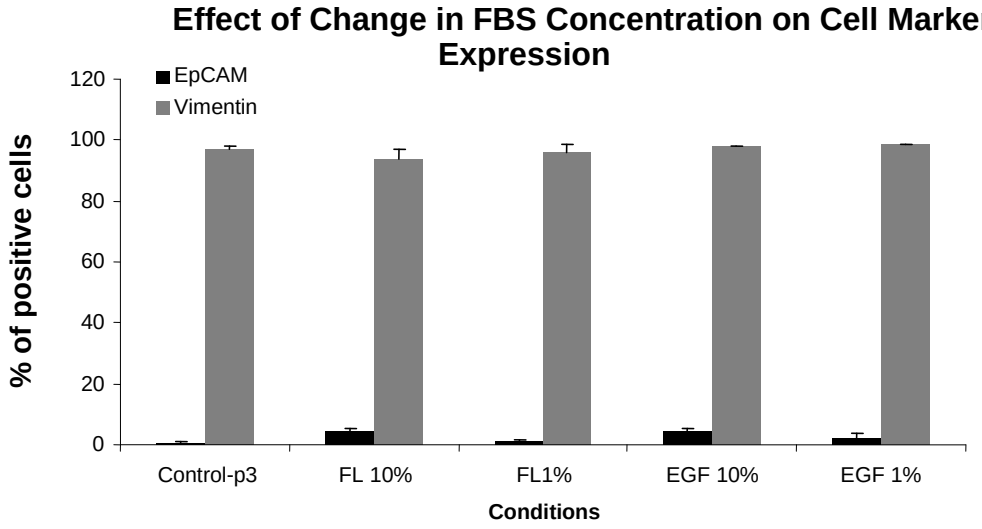


Figure 2.5: Percentage of EpCAM positive cells and Vimentin positive cells expressed as means ± S.E.M. of NEPT from different human donors cultured in RPMI-FL 1% FBS n = 5 and 10% FBS n = 7 and RPMI-EGF 1% n = 6 and 10% FBS n = 7 after 24 hours exposure to 100 μg/ml of G418 vs. control n = 7 cultured in RPMI-FL. Statistical analysis using one-way ANOVA showed no significant effect of changing the FBS concentration in RPMI-FL and RPMI-EGF post G418 over the phenotype of NEPT *in vitro.*

2.3.5 Culturing NEPT in Pancreatic Media after G418 Treatment

Pancreatic media described by Abunang et al. (34), was tested to determine if it can affect the phenotype epithelial cells. 2-3x10^6 single cells of the NEPT were cultured in pancreatic medium after treatment with G418 for 24 hours and 48 hours. Growth rate in this medium was slower than the rate of cells cultured in RPMI-FL experimental and control plates (Figure2.8a, b and c) with much slower rate for those previously treated with G418 for 48 hours. The cells cultured in pancreatic medium were collected when 60-70% confluent at the same time those in RPMI-FL are 80-100% confluent after 18-22 days. Flow cytometry analysis of the phenotype of cells cultured cells in pancreatic media after 24 hours exposure to 100 μg/ml G418 n = 6, showed an average of 27.1 ± 2.8% EpCAM positive cells and an average of 54.4 ± 11.1% for Vimentin positive cells. Those cultured in pancreatic media after 48 hours treatment with 100 μg/ml G418 n = 3 had an average of 20.7 ± 9.3% EpCAM positive cells and 59.3 ± 11.3% Vimentin positive cells. The cells cultured in RPMI-FL 10% FBS showed an average of 2.8 ± 0.9 % and 1.2 ± 0.2 % EpCAM positive cells, and 98.1 ± 0.4 % and 97.9 ± 0.4% Vimentin positive cells after 24 hours n = 6 and 48 hours n = 6 G418 respectively.

Control plates cultured in RPMI-FL, n = 6 showed 1.9 ± 0.5% EpCAM positive cells and 98.6 ± 0.5% Vimentin positive cells. Data summarized in figure 2.6.

Statistical analysis showed significantly higher percentage of EpCAM positive cells and lower percentage of Vimentin positive cells in tissues cultured

in pancreatic media after being treated with G418 for 24 hours compared to the experimental plates cultured in RPMI-FL and to the control.

The cells cultured in pancreatic media after 48 hours of exposure to G418 had a significantly lower percentage of Vimentin positive cells compared to the experimental plates cultured in RPMI-FL and to the control plates. The percentage of EpCAM positive cells was non-significantly higher than both the experimental plates cultured in RPMI-FL and to the control.

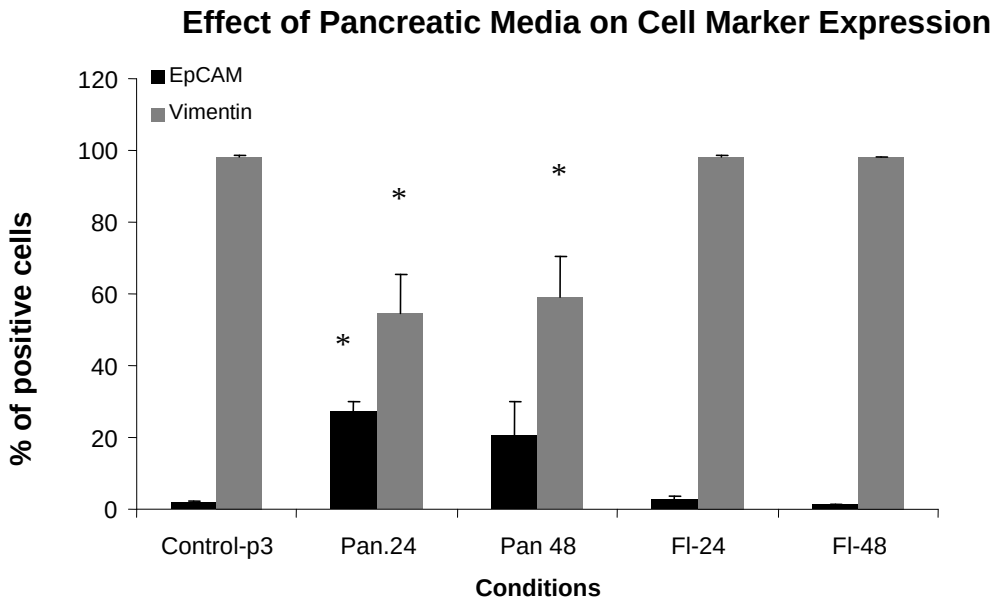


Figure 2.6: Values are percentage of EpCAM positive cells and Vimentin positive cells in NEPT from different human donors presented as an average ± S.E.M. Cells cultured in pancreatic media after exposure to 100 μg/ml of G418 for 24 hours n = 6 and 48 hours n = 3 compared to those in RPMI-FL, after exposure to 100 μg/ml of G418 for 24 hours n = 6 and 48 hours n = 6 vs. control n = 6 cultured in RPMI-FL without G418 exposure. Statistical analysis using one-way ANOVA showed significantly higher percentage of EpCAM positive cells and lower percentage of Vimentin positive cells for cells cultured in pancreatic media compared to RPMI-FL and to the control; * denotes $p < 0.05$.

2.3.6 Changing FBS Concentration in Pancreatic Media

To observe the effect of adding different concentrations of FBS in pancreatic media over the phenotype of cells. NEPT cultured in pancreatic media with no serum showed highest EpCAM positive cells and lowest Vimentin positive cells; however those cells were slowly growing. Adding 1 and 10% FBS to pancreatic media increased the growth rate, cells cultured in pancreatic media with 10% FBS were collected earlier 14-17 days when 100% confluent, while those in pancreatic media with no FBS and 1% FBS were collected 18-22 days when 60-80% confluent.

As shown previously the mean of EpCAM positive was 27.1 ± 2.8% and the mean of vimentin positive cells was 54.4 ± 11.1% in pancreatic media with no serum after G418 n = 6. The mean of EpCAM positive was 7.9 ± 2.2% and the mean of 79.9 ± 5.7% Vimentin positive cells was in pancreatic media with 1% FBS n = 6. While the mean of EpCAM positive was 7.1 ± 4.2% and the mean of Vimentin positive cells was 87.2 ± 2.6% in pancreatic media with 10% FBS n = 5. Data summarized in figure 2.7.

Statistical analysis showed significantly higher EpCAM positive cells and lower Vimentin positive in pancreatic media without FBS compared to the same media with 1 and 10% FBS.

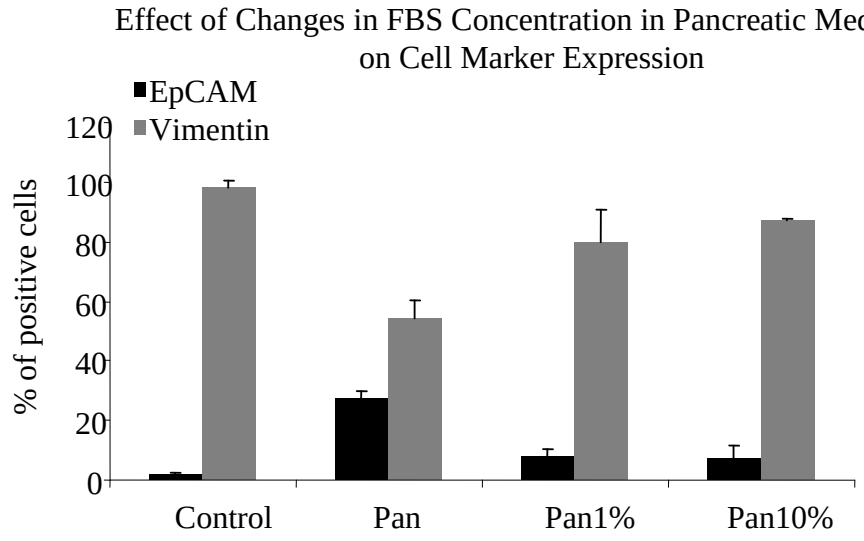
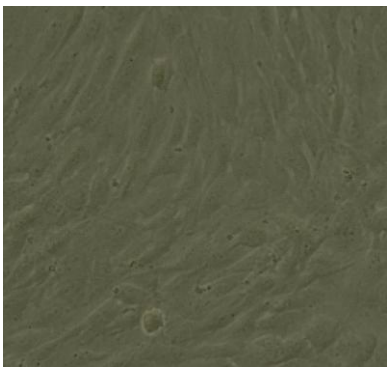


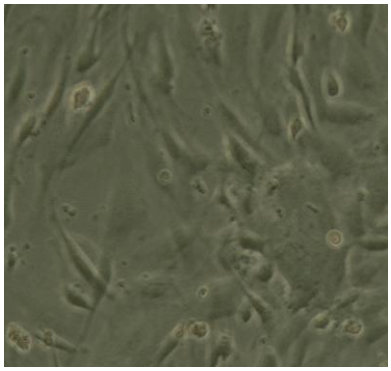
Figure 2.7: Results are expressed as means ± S.E.M. of percentage of EpCAM positive and Vimentin positive cells. NEPT of different human donors were cultured in pancreatic media with no serum n = 6, pancreatic media with 1% FBS n = 6 and pancreatic media with 10% FBS n = 5, post 100 μg/ml G418 exposure vs. control cultured in RPMI-FL with no G418 n = 6. Statistical analysis using one-way ANOVA showed significantly higher percent of EpCAM positive cells and lower percent of Vimentin positive cells in pancreatic media with no serum compared to pancreatic media with 1 and 10% FBS and to the control; * denotes $p < 0.05$.

2.3.7 Culturing NEPT in KGF Media after G418 Treatment

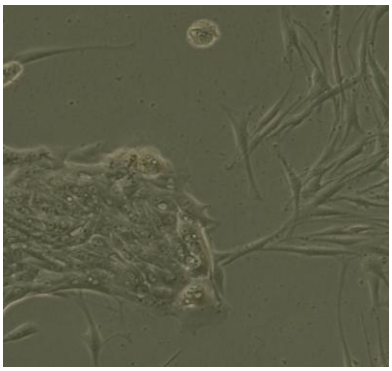
KGF has been used in establishing three dimensional cultures for human pancreatic duct epithelial cells (35). After exposure to 100 μg/ml of G418 for 24 hours, NEPT were cultured in KGF serum free medium. The cells did not stick to the plates, and their growth rate was slow (fig. 2.8d). We could not collect enough cells for staining to do flow cytometry.



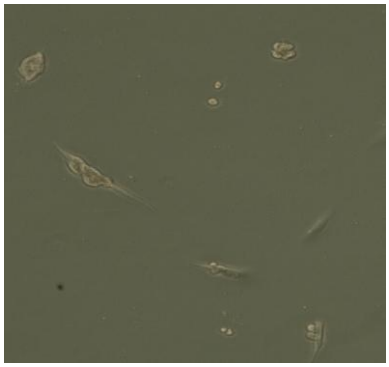
a. Control



b. RPMI-FL



c. Pancreatic media



d. KGF media

Figure 2.8: The photomicrographs depict changes in epithelial cell morphology from monolayer culture of NEPT after 15 days in different media. (a) Cells in control plates cultured in RPMI-FL are elongated fibroblast like cells and 80-100% confluent. (b) NEPT cultured in RPMI-FL after exposure to 100 μg/ml G418 hours, cells were 70-80% confluent with very few rounded cells and mostly elongated cells. (c) NEPT cultured in pancreatic medium with no serum after exposure to 100 μg/ml of G418 hours showed less growth rate compared to the control, 50-60% confluent, and aggregates of duct cells are present with less elongated cells compared to control. (d) NEPT cultured in KGF medium after exposure to 100 μg/ml of G418 for 24 hours, cells grew slowly compared to cells from other types of media.

2.4 Discussion

Exogenous insulin improves the life style of type 1 DM patients, and decreased the morbidity and the mortality these diabetic patients. However, type 1 DM patients are still at risk of developing secondary microvascular and macrovascular complications (2). Islet transplantation is a promising treatment for type 1 DM, because transplanted islets produce endogenous insulin in a more physiological way compared to the current medical therapy (3). Unfortunately the application of islet transplantation as a treatment for type 1 DM is hindered by the limited supply of donor tissues. To overcome this shortage of islets, there have been a number of proposed alternatives such as xenotransplantation (6) and stem cell therapy.

Many studies have pursued the use of stem cells to produced β-cells. Various types of stem cells have been explored such as umbilical cord (10), bone marrow (11), as well as hepatic (12) and pancreatic stem cells have been tried. Duct cells of the pancreas are of particular importance because of their large population. They are believed to play a major role in β-cell formation and maturation (21). Islets of langerhans-like structures were present within the ductal epithelium; however, the absence of polyploidy suggested young islets (37). *In vivo* studies on animal models showed that pancreatic duct ligation, could produce some sort of inflammation that induced cell differentiation. Linage tracing provided evidence that the source of increased β-cells is duct cells (23). *In vitro* human duct cells have been manipulated with certain transcription factors to generate insulin producing β-cells. *In vitro*, various methods have been reported to

drive pancreatic ductal cells to express insulin, including certain growth factors such as hepatocyte growth factor, β-cellulin, GLP1 and exendin-4 (24-30).

However, exposure of the pancreatic ductal tissue to processing and digestion by collagenase might introduce some changes in the expression of epithelial and mesenchymal antigens by epithelial cells (31).

Monolayer culture of NEPT - composed basically of epithelial cells acinar and duct tissues - was associated with some morphological changes in the form of loss of the rounded cobble-stoned pattern of epithelial cells and acquisition of the elongated spindle shape of fibroblast-like cells. These changes can be explained by Epithelial Mesenchymal Transition (EMT) (31).

EMT has been observed to occur both during normal development (38) and in cancer (39). During EMT, cell-cell adhesion is affected by loss of E-cadherin and increase in cell mobility (38). Vimentin is an intermediate filament protein normally expressed in cells of mesenchymal origin and absent from epithelial cells (40). The rapid up-regulation of Vimentin expression has been described in epithelial cells involved in physiological and pathological processes that require cell migration and has been interpreted as a marker of EMT (41).

In our conventional culture condition, cells stained double positive for EpCAM and Vimentin decreased with prevalence of single Vimentin positive cells and disappearance of single EpCAM positive cells (31).

In this study, our goal was to develop a method to preserve the epithelial phenotype of the ductal cells of the human NEPT, and to limit the growth of mesenchymal cells *in-vitro* through enhancing the culture conditions of the duct cells of the pancreas.

First, the NEPT were cultured for 24-48 hours in RPMI-BSA-ITS to eliminate the remaining acinar cells (42). Comparison of samples taken from this tissue, passage 0 i.e before the beginning of culture, and further passages (passage 1 after 6 days in culture and passage 3 at the end of experiment 18-24 days) confirmed the loss of the epithelial phenotype and acquisition of mesenchymal phenotype over time. Immunophenotyping demonstrates that there is a statistically significant decrease in the percentage of EpCAM positive cells and a concomitant increase in the percentage of Vimentin positive cells between passages 0 and 3 for cells cultured in RPMI-FL.

Hao et al. reported that the use of Geneticin (G418), at a concentration of 400 μg/ml for 4 days helped to eliminate mesenchymal and remaining β-cells from culture of NEPT (36). We previously tried between 50-400 μg/ml of G418 for shorter periods of time. Because of the toxicity of G418, cultured tissues did not survive at >200 μg/mL but 50μg/mL did not affect the growth of fibroblast-like cells.

Thus, NEPT were cultured in RPMI-FL media for 4-5 days, and then treated with 100 μg/ml of G418 for 24 and 48 hours. After 24h treatment with G418, EpCAM expression was not significantly higher and Vimentin positive cells expression was not lower compared to control; also there was no significant difference in cell phenotype between 24 and 48 hours treatment with G418, however there was a trend toward higher percentage of EpCAM positive and a lower percentage of Vimentin positive cells in those treated with G418 compared to the control.

Subsequently, we were interested in the effect of culture media on NEPT after exposure to G418. RPMI-FL is the conventional media used in our lab to culture NEPT. To limit fibroblast growth, we excluded bFGF from our RPMI-FL and called this modified media RPMI-EGF. The rate of expansion from the experimental plates was slow, which is likely due to toxicity of G418 as a much slower growth rate was observed after 48 hours compared to 24 hours treatment. On the other hand, the control plates showed quicker morphological changes with the appearance of spindle shaped fibroblast like cells and decreased proportions of the rounded cells, as well as a faster overall cell growth rate. These control plates become 100% confluent at the end of passage 2 earlier - 7-10 days - than other cells, so they were passaged and cultured till confluence for three passages compared to the slower growing experimental plates which could only be cultured to the end passage 2. Statistical analysis of EpCAM positive and Vimentin positive cells showed no significant difference between cells cultured in RPMI-FL and RPMI-EGF post exposure to G418 compared to control.

We further wanted to see whether decreasing the concentrations of serum growth factors such as transforming growth factor beta (TGFβ) present in FBS within RPMI-FL and RPMI-EGF media that contain 10% FBS, would help us achieving our goal of preserving the epithelial phenotype and eliminating the mesenchymal phenotype of duct cells within the NEPT *in-vitro*. After 24 hours treatment with G418, harvested cells were cultured in RPMI-FL and RPMI–EGF media with 1 and 10% FBS. The rate of cell growth in media with 10% FBS was higher than that of media with 1% FBS. Flow cytometry analysis showed very

low percentage of EpCAM positive cells and very high percentage of Vimentin positive cells in RPMI-FL and RPMI-EGF with 1 and 10% FBS. Statistical analysis of results of these media showed non-significant difference had been found between RPMI-FL and RPMI-EGF media with 1 and 10% FBS compared to the control.

Pancreatic media that was described by Agbunag et al had 10% FBS for three dimensional culture of duct cells of rat pancreas (36); however we tried the same media with no serum, using human pancreatic tissues. NEPT cultured in RPMI-FL as usual were treated with G418 100 μg/ml for 24 and 48 hours and harvested cells were cultured in pancreatic media with no serum and in RPMI-FL. Despite that the cells were slowly growing in this media this medium was able to preserve relatively high concentration of EpCAM positive cells 27% and low concentration of Vimentin positive cells 54% compared to previous media used. Analysis of the results revealed statistically significant higher percentage of EpCAM positive cells and statistically significantly lower percentage of Vimentin positive cells in pancreatic media after 24 hours G418 compared to control and RPMI-FL after 24 and 48 hours of exposure to G418. However, those cells with 48 hours of exposure to G418 then cultured in pancreatic media showed only statistically significant lower Vimentin positive cells compared to other conditions but no significant difference concerning EpCAM positive cells. It is important to mention that the pancreatic media was used in three dimensional culture of NEPT but we used it on a monolayer culture because of the difficulty of retrieving enough cells from the matrix to perform flow cytometry.

No significant effect was shown by changing FBS concentration in RPMI-FL and RPMI-EGF, but we wanted to see whether there is an effect of changing FBS concentration on pancreatic medium.

After 24 hours exposure to 100 μg/ml G418, NEPT were cultured in pancreatic media with 1, and10% FBS. Despite of the faster growth rate in pancreatic media with FBS compared to the same media with no FBS, results showed significantly higher EpCAM positive and lower Vimentin positive cells with pancreatic media compared to other conditions; while pancreatic with 1 and 10% FBS showed no significant difference in both EpCAM and Vimentin positive cells in compared to control.

Daily keratinocyte growth factor administration to adult rats led to increased proliferation of pancreatic duct epithelial cells. That was proved by proliferating cell nuclear antigen expression within the intercalated, interalobular, and interlobular duct cells (43). *In vitro* KGF has been used in establishing three dimensional cultures for human pancreatic duct epithelial cells (35).

To observe the effect of KGF on cells post treatment with the G418, we cultured NEPT after exposure to G418 for 24 hours in KGF serum free medium. The cells did not grow well in this medium; they did not stick to the culture dishes. The collected cells were not enough to be stained for phenotype analysis with flow cytometry.

It worth noting that starting our cultures in a media with no serum did not help cells to settle in the plate and their number was not enough to perform analysis using Flow cytometry.

2.5 Conclusion

The previous data confirmed the *in-vitro* loss of the epithelial marker EpCAM and acquisition of mesenchymal marker Vimentin of the NEPT mainly duct cells - after loss of the remaining acinor cells by culture in serum free medium - with the usual types of media even with lower concentrations of serum. The use of G418 was helpful in achieving our goal but its effect was lost with time in RPMI-FL and RPMI-EGF. Despite changes made in FBS concentrations in RPMI-FL and RPMI-EGF the epithelial phenotype of duct cells was lost in these media. On the other hand, culturing cells in pancreatic media with no serum on a monolayer culture after G418 exposure was best at preserving the epithelial phenotype and likely decreases the EMT. The use of KGF media with monolayer culture has not been evaluated because of the low ability of cells to grow in this media in monolayer cultures.

In the future isolation of duct cells that are CA 19-9 positive from NEPT using magnetic beads, and then treating cells with G418 could preserve the phenotype of the duct cells. Elimination of fibroblasts by picking or by using antifibrotic agents could be helpful in purification of epithelial cell cultures. Culturing duct cells in the pancreatic media with no serum on three dimensional cultures could allow us to test differentiation protocols of epithelial cells to obtain β-cells.

The produced β-cells could be tested for the production of insulin by immunohistochemistry and by gene analysis using RT-PCR.

The response of these cells to changes in glucose level could be tested by insulin assay. *In vivo* experiments could be performed to test the effect of the differentiated cells in reversing hypoglycemia in diabetic animals.

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CHAPTER THREE

General Discussion and Conclusion

3.1. Discussion

Diabetes mellitus is a common metabolic disorder characterized by abnormal carbohydrate metabolism leading to high glucose serum level. Two major types of diabetes exist, type 1 diabetes mellitus, that appears due to complete loss of insulin production due to destruction β-cells in islets of Langerhans of the pancreas and type 2 diabetes mellitus that appears due to decreased insulin production and/or defective use of the produced insulin caused by abnormal insulin receptors or post receptor defect. There is an emerging global epidemic of diabetes especially type 2 DM due to life style changes that led to increased obesity and decreased physical activity. According to world Health Organization, around 200 million people worldwide suffer from diabetes and the number of deaths by diabetes is expected to increase by 50% in the coming 10 years.

Diabetic patients are susceptible to develop acute or chronic complications such as retinopathy which is the leading cause of blindness in developed countries, and nephropathy that is also the main cause of renal failure in these countries.

Insulin is the current treatment for type 1 diabetes mellitus, despite the improvement of these patients' conditions they are still not exempt from

developing hypoglycemic attacks which can lead to coma and death and secondary complications such as cardiocvascular diseases (1).

Production of endogenous insulin in type 1 diabetic patients would be secreted in response to changes in glucose levels is the ultimate goal for researchers in the filed of type 1 DM. Transplantation of the whole pancreas was effective but patients developed many surgical complications (2). Then the islets transplantation was tried and it proved to be very effective especially after modification of immunosuppressive regimens by Edmonton protocol in 2000, which used steroid free immunosuppression, leading to 100% success rate where seven out seven patients achieved insulin independence for one year (3).

The major problem affecting the predominance of islet transplantation as the main therapy for type 1 DM patients is the limited numbers of donor supplies compared to the number of patients that need islets transplantation. Alternative sources of islets could be genetically engineered cells, xenotransplantation and stem cells (4, 5).

Since the discovery of stem cells with their special properties of repeated divisions and differentiation into different types of cells, researcher believed in the ability of stem cells to play a major role in regenerative medicine. They are currently used in treatment of some blood cancers and studies are trying to use them in treatment of cancers, neurological disorders such as Parkinson's, Multiple sclerosis and Amyotrophic sclerosis, also many investigations have been done to use stem cells in treatment of type 1 DM (6).

Adult stem cells in the liver (7), bone marrow (8) and pancreas have been tried. The endocrine cell population in the pancreas may also undergo dynamic changes in response to growth, development, and conditions such as pregnancy or obesity. Plasticity and regeneration capacity of β-cell mass was demonstrated in animal models as well as in humans (9). In the pancreas four main types of cells have been investigated as a possible source for β-cells progenitors, acinar cells (10), mesenchymal cells (11), β-cells and duct cells (12) and duct cells.

Duct cells have been proposed as a possible source for β-cells progenitors because they contribute to β-cell population after birth and after injury (13). Moreover partial ligation of the duct of the pancreas has been associated with increased β-cell mass in the ligated part in adult mice and it is thought to be due to activation of neurogenin 3 expressing cells near or within the ducts (14).

In vitro studies showed the ability of the duct cells to be differentiated into β-cells after exposure to certain growth factors such as hepatocyte growth factor (15) and extendin - 4 (16), however developed β-cells are not fully responsive to changes in glucose level. A possible explanation as to why fully differentiated β-cells have so far not been generated under cell culture conditions might come from the absence of cell-cell interactions between mesenchymal and epithelial cells that occur during embryonic pancreas development *in vivo* (17).

In vitro expansion of duct cells was associated with phenotepic changes in the form of loss of the epithelial phenotype and acquisition of mesenchymal phenotype and that was explained by occurrence of epithelial mesenchymal transition (EMT) (18).

We have tried to test different growth factors and different media that were described for epithelial cell growth *in vitro.* The objective was to hinder the development of the EMT to be able to apply various differentiation protocols on duct cells to generate β-cells.

3.2. Conclusion

Non-endocrine pancreatic tissues (NEPT) are the pancreatic tissue remaining after islets isolation it is mainly formed of epithelial tissues; acinar cells are lost with apoptosis after culturing the cells for two days in RBMI/BSA/ITS (19).

Culturing cells in the conventional media (RPMI-FL) in monolayer culture was associated loss of the epithelial phenotype and acquisition of mesenchymal phenotype over time as seen in the high percentage of EpCAM positive and low percentage of Vimentin positive cells in NEPT at the beginning of the experiment and the low percentage of EpCAM positive and high percentage of Vimentin positive cells at the end of the experiment.

Geneticin (G418) that was previously used in a concentration of 400 μg/ml for 4 days to eliminate the mesenchymal cells from NEPT culture (20). We have tried between 50-400 μg/ml of G418 for shorter periods of time. Because of the toxicity of G418, cultured tissues did not survive at >200 μg/mL but 50μg/mL did not affect the growth of fibroblast-like cells. We used 100 μg/mL of G418 for 24 and 48 hours and it helped to preserve the epithelial phenotype of NEPT as seen with higher percentage of EpCAM positive cells and lower percentage of

Vimentin positive cells compared to the non- G418 treatred NEPT -control-. It was obvious that cells treated with G418 are growing much slowly compared to those not treated with G418 and this may be due to the toxic effect of this antibiotic on growing cells, proved by longer culture time to reach confluence.

Removal of b-FGF from the conventional media RPMI-FL did not help in preventing EMT. Neither lower concentration of FBS in the same media was helpful to preserve the epithelial phenotype of the NEPT.

Pancreatic media was previously used for culturing rat duct cells on three-dimensional culture contained 10% FBS (21). We used the same media in 2 dimensional cultures on human NEPT with no FBS, after 24 hours treatment with G418, this media yielded the highest percentage of EpCAM positive cells and the lowest percentage of Vimentin positive cells compared to cells cultured in conventional media after G418 and the control. We used 2 dimensional cultures to be able to retrieve enough number of cells to do flow-cytometry since 3 dimensional cultures do not allow that.

Adding FBS to the pancreatic media as the originally described was associated with loss of the pattern seen in the same media with no serum as the percentage of EpCAM positive cells decreased and the percentage of Vimentin positive cells increased in pancreatic media with 1 and 10% FBS post 100 μg/mL G418 treatment for 24 hours.

We also tried to use KGF serum free media that was previously used in 3 dimensional cultures of human duct cells (22); however the rate of cell growth in

this media after G418 exposure was so slow, not enough cells could be collected to stain them for doing flow-cytometry analysis.

3.3. Future Directions

Islets transplantation is an optimal treatment for type 1 DM patients to prevent development of secondary complications in these patients currently treated with insulin. The limited supplies of islets could be overcome by the use of stem cells to produce islets. Duct cells of the pancreas are proposed to be progenitors for β-cells, however in vitro cultures of NEPT containing primarily duct cells was associated with phenotypic changes. In order to test the ability of duct cells to generate β-cells, we tried to hinder this phenotypic changes explained by EMT.

The use of antifibrotic agents e.g. interferon (IFN)-γ could be helpful to limit the growth of fibroblasts that contaminate the *in vitro* cultures of NEPT.
In the future to be the use of G418 followed by culturing cells in pancreatic media with no serum could be used to culture selected duct cells using magnetic beads that pick CA-19/9 positive cells only.

These conditions could be also tested on three dimensional cultures since they offer three-dimensional interactions between epithelial cells as they occur during islet formation *in vivo*. Also performing single cell clones of duct cells in the pancreatic media could allow us to isolate the potent stem cells lie within duct cells, the isolated potent stem cells could be exposed to multi-step differentiation

protocols including dexamethasone, extendin -4, hepatocyte growth factor and other growth factors.

The produced β-cells could be further tested for insulin production by immunohistochemistry and by RT-PCR to look for insulin gene expression and other genes expressed by ordinary β-cells such as PDX-1, ISL-1, NeuroD, Nkx 2.2, Ngn3 and PAX-4. The response of these cells to changes in glucose level *in vitro* could be tested using insulin assay.

Further differentiated duct cells could be labeled with lentiviruses to detect them when transplanted *in vivo* in diabetic immunocompromised mice, to test the ability of differentiated duct cells to reverse hyperglycemia in diabetic animals.

Another interesting idea for using duct cells to generate β-cells in diabetics could be the use of autologus duct cells of diabetic patients and differentiate them into β-cells then re-infuse them into the patients to decrease the use of immunosuppressive agents that have many deleterious side effects.

Finally it worth noting that differentiation of duct cells into functional β-cells will not be sufficient since the functioning islets composed of different type of cells that work in harmony to regulate serum glucose levels in healthy individuals, so trials to differentiate duct cells to α-cells and δ-cells and PP cells would be very beneficial.

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