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Islet Regenerative Properties of Ex Vivo Expanded Hematopoietic Progenitor Cells

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A thesis submitted in partial fulfillment of the requirements for the Master of Science degree in Physiology

ISLET REGENRATIVE PROPERTIES OF *EX VIVO* EXPANDED HEMATOPOIETIC PROGENITOR CELLS

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by

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Abstract

Human umbilical cord blood (UCB) progenitor cells with high aldehyde dehydrogenase activity ($ALDH^{hi}$), can stimulate endogenous islet regeneration after transplantation into mice with steptozotocin (STZ)-induced diabetes. However, UCB $ALDH^{hi}$ cell are extremely rare, and expansion will be required to develop cell-mediated strategies to treat patients with diabetes. To increase the number of progenitor cells available for clinical application, we expanded $ALDH^{hi}$ UCB cells under clinically applicable, serum-free hematopoietic-restricted conditions. Six day expansion resulted in a 15-fold increase in total cell number, and a 3-fold increase in the number of HPC retaining high ALDH ($ALDH^{hi}$ HPC) activity. $ALDH^{hi}$ HPC highly expressed primitive hematopoietic cell surface markers, and demonstrated hematopoietic colony forming capacity *in vitro*. Culture-expanded $ALDH^{hi}$ HPC transplanted STZ-induced mice demonstrated improved islet function, increased islet size, and vascularization. Therefore, culture-expanded $ALDH^{hi}$ HPC represent a novel population for the development of cellular therapies to promote islet regeneration.

Keywords: Aldehyde Dehydrogenase, Hematopoietic Progenitor Cells, Stem Cell Expansion, Stem Cell Transplantation, Islet Regeneration, Islet Angiogenesis, Diabetes, Streptozotocin-treated NOD/SCID mice.

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Co-Authorship Statement

The following contains material from a manuscript in preparation co-authored by Ayesh Seneviratne, Gillian Bell, Tyler Cooper, Stephen Sherman, David Putman, and David Hess. Ayesh Seneviratne performed all the experimental work presented in this thesis. Gillian Bell, Hess lab technician helped with cell transplantation into hyperglycemic mice, and animal care. Tyler Cooper, Hess lab summer student helped phenotype culture-expanded hematopoietic progenitor cells. Stephen Sherman, Hess lab MSc student helped with animal care. David Putman, Hess lab PhD student helped phenotype culture-expanded hematopoietic progenitor cells.

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List of Abbreviations

ALDH ─ Aldehyde Dehydrogenase

ANGPT2 ─ Angiopoietin 2

ANOVA ─ Analysis of Variance

AREG ─ Amphiregulin

AUC ─ Area under the curve

β2M ─ β2 microglobulin

β-gal ─ β-galactosidase

BM ─ Bone marrow

BODIPY ─ 4,4-difluoro-5,7-dimethyl-4-bora-3a,4adiaza-5-proprionic acid

CAM ─ Chorioallantonic membrane

CBP ─ Common B Cell Progenitor

CD ─ Cluster of differentiation

CA/TD ─ Centoracinar and Terminal Duct

cGY ─ Centigray

CK 19 ─ Cytokeratin 19

CLP ─ Common lymphoid progenitor

CMP ─Common myeloid prognitor

CTP ─ Common T cell progenitor

DEAB ─ diethylaminobenzaldehyde

EdU ─ 5-ethynyl-2'-deoxyuridine

EGF ─ Epidermal growth factor

EFGR ─ Epidermal growth factor receptor

EREG ─ Epiregulin

ETS ─ E26 transformation-specific

FACS ─ Fluorescence activated cell sorting

FBN2 ─ Fibrilin 2

FC ─ Fold change

FLT-3L─ Fms-related tyrosine kinase 3

G-CSF ─ Granolucyte colony stimulating factor

GMP ─ Granulocyte/macrophage progenitor

GUSB ─ Glucouronidase

HAP ─ Human alkaline phosphatase

hESC ─ Human embryonic stem cells

HGF ─ Hepatocyte Growth Factor

HLA ─ Human leukocyte antigen

HPC ─ Hematopoietic progenitor cells

HSC ─ Hematopoietic stem cells

IAPP─ Islet associated polypeptide

IL-3 ─ Interleukin-3

iPan ─ Intra-pancreatic

iPSC ─Induced pluripotent stem cells

IV ─ Intravenous

Lin ─ Lineage

MAPK ─ Mitogen-activated protein kinase

M-CSF ─ Macrophage colony – stimulating factor

MEF ─ Mouse fibroblast

MEP ─ Megakaryocytic/erythroid progenitor

mESC ─ Mouse embryonic stem cells MFG-E8 ─ Milk Fat Globule-EGF factor 8

MFG-E8 ─ Milk Fat Globule - EGF factor 8

MPS VII ─ Mucopolysaccharidosis VII

Ngn3 ─ Neurogenin 3

NK ─ Natural Killer

NKP ─ NK cell progenitor

NOD ─ Non-obese diabetic

PBS ─ Phosphate buffered saline

PMP ─ Pancreas-derived multipotent progenitor

PP-Pancreatic Polypeptide

PROM1 ─ Prominin 1

RA ─ Retinoic acid

RAR ─ Retinoic acid receptor

RIP-CreER ─ tamoxifen-inducible Cre transgene rat insulin promoter

ROS ─ Reactive oxygen species

SCF ─ Stem Cell Factor

SCID ─ Severe combined immunodefficient

SDF-1 ─ Stromal derived Factor-1

SEM ─ Standard error of the mean

SEMA4A ─ Semaphorine 4A

SFRP1 ─ Secreted frizzled-related protein 1

SR1 ─ StemRegenin1

SRC ─ SCID repopulating cell

STZ ─ Streptozotocin

TGF β ─ Transforming Growth Factor β

TIMP ─ Tissue inhibitors of metalloaproteinases

TPO ─ Thrombopietin

UCB ─ Umbilical cord blood

UKPDS ─ United Kingdom Prospective Diabetes Study

VEGFA ─ Vascular endothelial growth factor

WISP1 ─ WNT1 inducible signaling pathway protein 1

1.0 Introduction

1.1 Human Pancreas

The human pancreas, located in the abdominal cavity behind the stomach, is composed of exocrine cells (98%), and endocrine cells (1-2%). The exocrine acinar cells synthesize enzymes such as trypsin, chymotrypsin, lipase, and amylase. The synthesized acinar cell enzymes as well as bicarbonate secreted by pancreatic ductal epithelial cells, enter the duodenum of the small intestine *via* pancreatic ducts, where these enzymes play an important role in digestion.[1] In contrast, endocrine cells are clustered throughout the exocrine tissue in islets of Langerhans, and synthesize hormones that play an important role in glucose homeostasis.

There are approximately 1 million islets in an adult human pancreas. The islets receive a rich vascular supply from the splenic and mesenteric arteries, while the hormones synthesized by the islets are secreted into capillaries collected by the portal vein. Pancreatic islets consist of four major endocrine cell types: α-cells (35-40%), β-cells (50%), δ-cells (10-15%), and pancreatic polypeptide (PP) cells, as well as more recently described Epsilon cells.[2] α-cells secrete glucagon, a 29 amino acid peptide hormone, when blood glucose levels are low. Glucagon then binds to glucagon receptors on hepatocytes to increase blood glucose levels, by stimulating the breakdown of glycogen into glucose, and the synthesis of new glucose molecules from non-glucose fuel sources. Human β-cells of the pancreas sense blood glucose levels via the GLUT1 and GLUT3 glucose transporters, and secrete insulin, a 51 amino acid hormone, when blood glucose levels are high.[2] Once released insulin stimulates insulin responsive cells to uptake, store, and supress the production of glucose. δ-cells of the pancreas secretes somatostatin that has potent inhibitory effects on insulin, and glucagon release. PP-cells secrete the hormone PP, which regulates both endocrine and exocrine secretions.[3] Finally, Epsilon cells of the human pancreas secrete Ghrelin, which has been shown to stimulate insulin secretion in the rat pancreas, however its exact physiological role in the human islet is not known.[4]

1.2 Diabetes Mellitus

The ancient Egyptian text, the *Ebers Papryus* written in 552 BC, described a disease that caused patients to frequently urinate.[5] In 234 BC the Greek physician Aretaeus, named this disease diabetes. Diabetes mellitus, as it is now known, is a metabolic disease associated with high blood glucose levels. According to the international diabetes federation 382 million people worldwide currently have diabetes. It is most prevalent in individuals above 65 years of age, and in people who live in urban areas. With a continued increase in the aging population, and increased urbanization, it is predicted that 592 million people will have diabetes in the year 2035. There are two main forms of diabetes, type 1 diabetes also known as insulin-dependent diabetes mellitus or juvenile onset diabetes, and type 2 diabetes also known as insulin-independent diabetes mellitus or adult onset diabetes (Figure 1.1).

1.2.1 Type 1 Diabetes

Type 1 diabetes is an auto-immune disorder where the body's immune system mediates the destructions of insulin secreting β cells of the pancreas.[6] Currently, Type 1 diabetes accounts for only 5-10% of total diabetic patients, and is the most predominant type of diabetes in children. Recent epidemiological data suggest that, the incidence of type 1 diabetes is increasing worldwide at a rate of 3% a year.[6]

Histological analysis has shown that the pancreata of patients with type 1 diabetes have lymphocytic infiltration of the pancreatic islets with destruction of insulin producing β cells, resulting in an overall decrease in β cell mass. However, α cells, δ cells, and PP cells of the islet are intact.[7] The inflammatory infiltrate consists of $CD8^+$ and $CD4^+$ T cells, B lymphocytes, macrophages, and natural killer (NK) cells.[8]

The non-obese diabetic (NOD) mouse model provides insight into the pathophysiology of type 1 diabetes. NOD mice spontaneously develop autoimmune diabetes at around 10 weeks of age.[9] Similar to human patients with type 1 diabetes, autoreactive $CD4^+$ and $CD8^+$ cells, macrophages, and lymphocytes are detected in the pancreatic infiltrate of NOD mice.[10] Studies using NOD mice have found that the process of β cell destruction begins

when macrophages and dendritic cells present β cell antigens to naïve $CD4^+$ T-cells, using the major histocompatibility complex. $CD4^+$ T-cells are then activated.[11] In turn, activated $CD4^+$ T-cells activate $CD8^+$ T-cells, which are directly responsible for mediating β cell death.[6] As a result of β cell death additional antigens are released, which permits antigen presenting cells further access to self-antigens. Ongoing sampling of these autoantigens, leads to the activation of additional auto-reactive T-cells, which results in the amplification of the initial autoimmune response.[6] Also, the inability of regulatory T-cells to inactivate β cell specific autroreactive T-cells contributes to the pathogenesis of type 1 diabetes.[12]

Genetic and environmental factors both play a role in the etiology of type 1 diabetes. The gene most associated with the risk for type 1 diabetes is the human leukocyte antigen (HLA)-DR loci on chromosome 6.[13] One in 40 individuals with a HLA-DR3 or HLA-DR4 at the HLA-DR loci will develop type 1 diabetes. Environmental factors such as diet, cow's milk, protein exposure, vitamin D deficiency, viral infections, drug toxins, and oxidative stress are also thought to trigger type 1 diabetes in genetically predisposed individuals.[14]

Patients with type 1 diabetes present with weight loss, polyuria, and polydipsia. Hyperglycemia, as a result of insulin deficiency, caused by a significant loss of β cell mass explains these symptoms.[6] Similar to patients with type 1 diabetes, patients with type 2 diabetes also demonstrate a loss of β cell function, and β cell mass, at the late stages of the disease.

1.2.2 Type 2 Diabetes

Type 2 diabetes mellitus is a complex metabolic disorder characterized by hyperglycemia, insulin resistance-a condition in which cells of the body fail to respond to insulin properly-and relative impairment in insulin secretion. Type 2 diabetes is the most common type of diabetes, as it accounts for 90%-95% of diabetic patients.[15] In the past decade, the prevalence of type 2 diabetes has risen alarmingly primarily due to an increase in obesity and a sedentary life style. Furthermore, it is worrisome that this disease which was predominantly found in adults, is now increasingly seen in children and adolescents.[15]

The development of insulin radioimmunoassays in the 1960's led to the finding that plasma insulin levels were often increased in type 2 diabetic patients, and therefore lead scientist to believe that the failure to respond to insulin alone could explain the pathogenesis of type 2 diabetes.[16] However, these studies did not measure insulin in the context of insulin resistance. As a result, the role of β cell dysfunction in the pathogenesis of type 2 diabetes was greatly overlooked.[17] Research conducted in the past 15 years has shown that β cell inadequacy is a fundamental part of type 2 diabetes. A longitudinal study on 48 Pima Indians, showed that β cell dysfunction was prominent in those destined to progress to type 2 diabetes.[18] Multiple measures of insulin sensitivity, and insulin secretion were carried out on this very high risk population group. After an average of 5 years, 35% of these subjects progressed from normal glucose tolerance, to impaired glucose tolerance, and then to diabetes.[15] Interestingly, the β cells of the subjects who eventually developed type 2 diabetes did not secrete enough insulin to overcome insulin resistance, indicating diminished β cell function.[19] Indeed, the insulin secretion of the subjects that developed diabetes declined by 78%, while their insulin sensitivity only decreased by 14%.[19] In a matched group of subjects who did not develop diabetes, insulin sensitivity declined by 11%, but insulin secretion increased by 30%.[19] In addition, data from the United Kingdom Prospective Diabetes Study (UKPDS) that followed 3867 type 2 diabetes patients suggest that β cell dysfunction commenced years before overt hyperglycemia developed.[20]

The diabetic milieu contributes to both β cell dysfunction, and insulin resistance in type 2 diabetic patients. Hyperglycemia impairs β cell function by increasing the rate of β cell death, via the production of reactive oxygen species.[21] An increase in circulating Non-esterfied fatty acids, is another characteristic of type 2 diabetic patients. Non-esterfied fatty acids have been shown to reduce insulin mediated glucose uptake, and impair β cell function both *in vitro*, and *in vivo*.[16,22] The consumption of increased dietary fat triggers the deposition of amyloid in pancreas.[23] Amyloid plaques in islets are formed by the aggregation of islet associated polypeptide (IAPP), and analysis of pancreas sections of type 2 diabetic patients revealed that islets with large amyloid deposits often have fewer β cells.[24,25] When human IAPP is overexpressed in mice or rats, the result is worsening diabetes associated with more amyloid deposition.[25] It appears that the toxicity is exerted

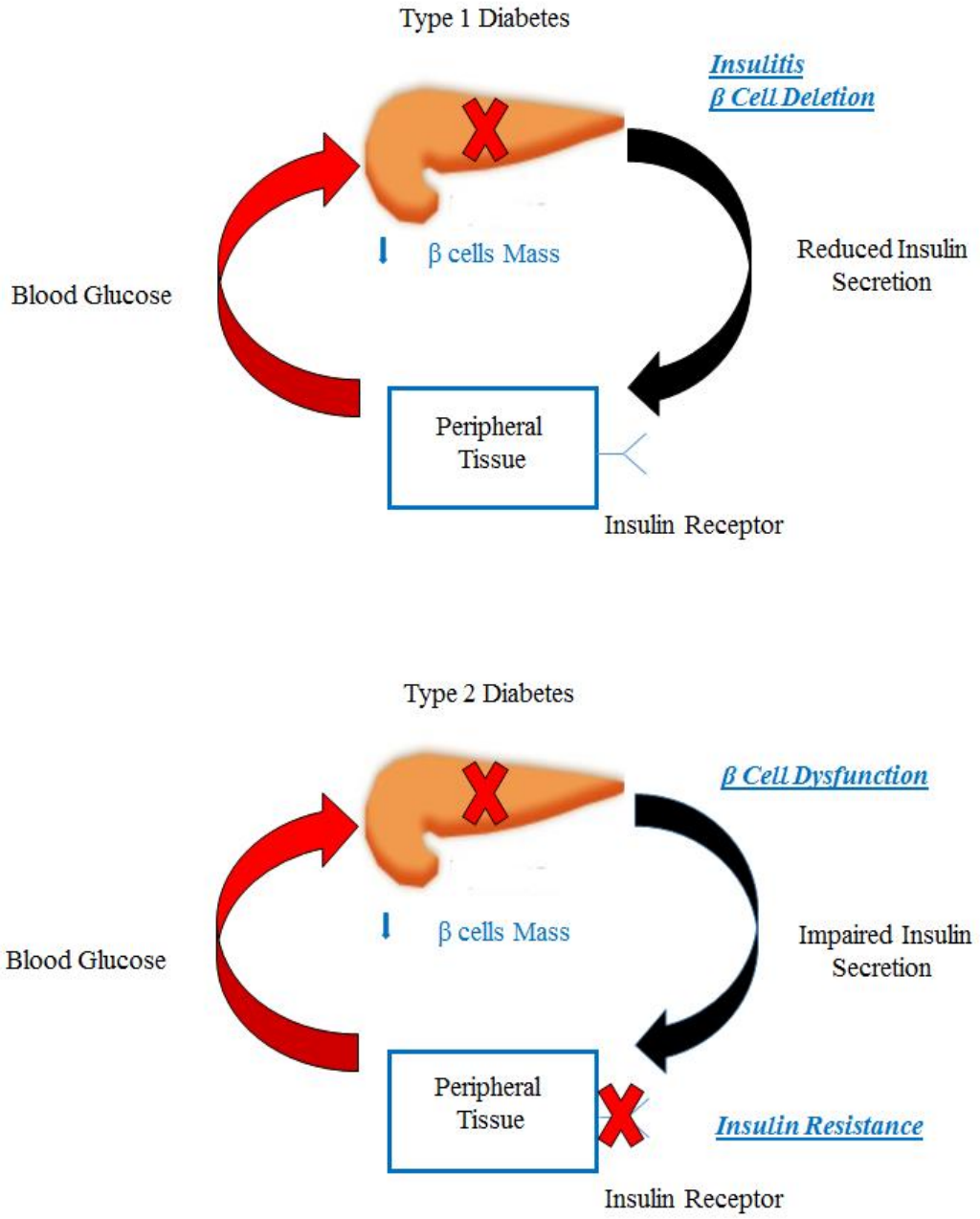

Figure 1.1 The mechanisms of Type 1 and Type 2 diabetes. Schematic representation of the mechanisms underlying the pathogenesis of Type 1 and Type 2 diabetes. Type 1 diabetes is an autoimmune disorder where β cells are destroyed, leading to a reduced β cell mass and reduced insulin secretion. Type 2 diabetes is a metabolic disease associated with peripheral insulin resistance which reduces the uptake of glucose in tissues, and β cell dysfunction which leads to impaired insulin secretion. Although glucose homeostasis is impaired by varying mechanisms, both types of diabetes are associated with high blood glucose levels.

by a specific oligomeric components of the amyloid deposit that can damage cell membranes, and increased lipid peroxidation.[16]

Genetic predispositions, and environmental factors such as diet, physical activity, and age play a large role in the development of the disease.[16] Furthermore, certain insults during intrauterine fetal growth, such as placental insufficiency, have been associated with the fetus developing type 2 diabetes later on in life.[26] Since, the exact cause of type 2 diabetes is not known, current therapeutic strategies aim to treat the symptoms of the disease.

1.3 Management of Diabetes

The symptoms associated with β cell dysfunction in both type 1 and type 2 diabetes are treated using exogenous insulin. Furthermore, oral hypoglycemic agents are used to treat the peripheral insulin resistance observed in type 2 diabetic patients.

1.3.1 Insulin Therapy

Fedrick Banting, Charles Best and John McLeod, demonstrated that intra-venous (i.v.) injection of a pancreatic extract from dogs, could reduce blood glucose levels in diabetic dogs.[5] They called this extract "isletin", which is now known as insulin.[5] After this seminal discovery, Banting, Best together with their new team member Collip attempted to increase the supply of insulin for human use, by purifying insulin from the pancreas of cattle.[5] The pancreas extracts from cattle were first administered into Leonard Thompson, a 14 year old boy with diabetes, on the 11th of January 1922.[5] After the administration of insulin injections Leonard's condition dramatically improved. His blood glucose, and glucosuria decreased, and his ketouria disappeared.[27] Similar outcomes were archived when insulin was administered to six other diabetic patients.[5]

Many advances to insulin have been made since its initial discovery in 1922. Eli Lily and August Krogh worked independently to improve the extraction and purification of insulin from animal sources. In the 1930's protamine and zinc were added to animal insulin to prolong its action.[28] Then in 1978 the first human synthetic insulin was prepared by David Goeddel and his colleagues at Genentech.[5] The rapid and the short acting forms of synthetic

insulin were marketed under the brand names Humulin R and Humulin NPH respectively.[5]

The discovery of insulin dramatically increased the life expectancy of diabetic patients. Before the discovery of insulin the average life expectancy in diabetic patients was 6.1 years from diagnosis. Soon after insulin therapy became available to the general public the average life expectancy of a diabetic patient increased to 18.2 years after diagnosis.

Now with advances such insulin pumps, and continuous blood glucose monitoring systems both type 1 and type 2 diabetic patients can be managed more effectively.[15] However, since type 2 diabetic patients also demonstrate insulin resistance, additional pharmacological agents are required to manage this disease. Even with modern insulin therapies and pharmacological agents, the life expectancy of diabetic patients is reduced by 15 years.

1.3.2 Pharmacological Agents

Currently, patients presenting with type 2 diabetes symptoms are initially managed using oral antidiabetic agents. These agents act through a variety of mechanisms to achieve euglycemia. The American Diabetes Association, and the European Association for the study of diabetes recommends a patient-centered approach with respect to medication choices. When initiating pharmacological treatments, Metformin seems to be the drug of choice, unless it is not tolerated by the patient.[29] Metformin reduces blood glucose levels in type 2 diabetic patients by reducing gluconeogenesis in the liver.[30] Similar to Metformin, Thiazolidinediones also reduce gluconeogenesis in the liver.[31] Specifically, Thiazolidinediones re-direct fat from the liver to subcutaneous adipose depots, thereby improving insulin sensitivity in the liver. In contrast, Sulfonylureas lowers blood glucose levels by stimulating insulin secretion from β cells.[32] Type 2 diabetic patients also have elevated triglyceride levels, which is a risk factor for cardiovascular disease. In addition to lowering plasma blood glucose levels, the D2-dopamine agonist Bromocriptine reduces plasma triglyceride and free fatty acid levels without increasing plasma insulin levels.[33] However, it has been documented that approximately 25% of type 2 diabetic patients do not respond sufficiently to the available therapies.[29]

1.4 Diabetes Complications

Even with current insulin and drug therapy more than 90% of diabetic patients develop long-term vascular complications.[34] Diabetic vascular complications are grouped under either microvascular or macrovascular disease. Microvascular complications arise due to damages in small blood vessels, and includes: eye disease or retinopathy, kidney damage or nephropathy, neural damage or neuropathy.[35] The major macrovascular complications include: accelerated cardiovascular disease resulting in myocardial infarction, and cerebrovascular disease manifesting as strokes.[35] The chronic elevation in blood glucose levels is suggested to give rise to vascular complications by either damaging the cells of the affected organs, or damaging the blood vessels that supply the affected organs. The progression of these complications can be slowed but not stopped with aggressive management of glycemia and blood pressure, laser therapy for advanced retinopathy, and the administration of an angiotensin converting enzyme inhibitor or angiotensin receptor II blocker for nephropathy.[35] Thus, to prevent the morbidities associated with these complications curative therapies that re-establish a functional β cell mass are currently under intense investigation.[36]

1.5 Whole Pancreas Transplantation

Transplantation of cadaveric pancreata is performed in patients with type 1 diabetes, type 2 diabetes, and those who have undergone a total pancreatectomy.[37] The first pancreas transplant was performed in 1966 by William Kelly and Richard Lihelli at the University of Minesota, USA.[38] Since then, >27,000 pancreas transplants have been performed in the United States of America.[37] Over the years pancreas transplants have evolved from experimental procedures to the standard of care for patients with severe diabetes mellitus and uremia.[37] Pancreas transplants are performed on three major categories of patients: 1) patients with uremia who undergo simultaneous pancreas and kidney transplants, 2) patients who are posturemic who undergo a pancreas transplant after a kidney transplant, 3) patients with brittle diabetes without uremia who undergo a pancreas transplant alone.[37]

Overall the average graft survival, defined by insulin independence, was >96% 1 year post transplant, and > 80% at 5 years.[39] The time it takes to lose 50% of graft function ranges from 7-14 years. Furthermore, a successful pancreas transplant seem to be a lot more effective than intensive insulin therapy at lowering levels of HBA1c.[40,41] Even 10 years after transplantation, a successful pancreas transplant can preserve insulin secretion and provide good glycemic control.

1.6 Islet Cell Transplantation

Islet cell transplantation is an alternate strategy that can be used to re-establish functional β cell mass in severely diabetic patients. This procedure is gaining popularity, because of the relatively low perioperative risks associated with islet cell transplantation when compared to pancreas transplantation.[42] The first attempt at islet transplantation was made by Watson-Williams and Harsant in 1893, when they transplanted minced sheep's pancreas into the subcutaneous tissue of young boy with ketoacidosis.[43] After the discovery of insulin the interest in islet transplantation dwindled, but was renewed in 1972 when Drs. Ballinger and Lacey demonstrated a method for isolating intact islets of Langerhans from rodents that retained function after transplantation into a rodent model of diabetes.[44] Twenty years later Scharp *et al*.[45] was able use the islet transplantation methodology to achieve insulin independence in a human diabetic patient for nearly 1 month. Further advances and experimentation contributed to the development of the Edmonton protocol in the year 2000. In this procedure, islets were procured from multiple (2-4) deceased donors, purified, and then infused percutaneously into the portal vein of the recipient. Furthermore, this is the first protocol that did not use steroid-based immunosuppressant therapy due to its toxic effect on β cells. One-hundred percent of the recipients achieved insulin independence after one year of transplantation, and none experienced severe hypoglycemic episodes.[46] However, continued autoimmune destruction of donor islets, the need for life-long immunosuppressant therapy, and the critical shortage of donor pancreata limits the use of this procedures.[36]

1.7 Stem Cells

Due to the limitation of current β cell replacement therapies, methods of utilizing stem cells to establish functional β cells for transplantation are being studied. Stem cells play an important role in the development and regeneration of tissue and organ systems.[47] These cells have two distinguishing features, the ability to self-renew through cell division, and the ability to differentiate into multiple cell types with specialized function.[48] There are three main types of stem cells: (1) Embryonic stem cells (ESC), (2) induced pluripotent stem cells (iPSC), (3) adult stem cells (Figure 1.2).

1.7.1 Embryonic Stem Cells

ESC are derived from the inner-cell mass of 4-5 day old mouse or human blastocyst. These cells are termed pluripotent, thereby have the ability to give rise to any of the 3 germ layers, ectoderm, mesoderm, and endoderm.[49] Martin Evans was the first to isolate and culture mouse embryonic stem cells (mESC).[50] To maintain pluripotency *in vitro,* Evans cultured mESC on a feeder layer of division incompetent mouse fibroblast (MEF). Seventeen years later the first human embryonic stem cells (hESC) was established, by Jamie Thompson.[51] Similar to mESC, hESC were maintained *in vitro* by embryonic fibroblast feeder cells. The establishment of hESC provide exciting prospects for cellular therapies. Currently, researchers are attempting to differentiate human hESC into cell types of a wide range cell lineages, to treat diseases associated will cellular deficiency.[49]

1.7.2 Induced Pluripotent Stem Cells

Induced pluripotent stem cell (iPSC) has flourished, as a result of the controversies surrounding hESC research. iPSC were first generated by Shinya Yamanaka in 2007, by expressing the transcription factors Oct4, Sox2, KLF4, and c-myc through retroviral transduction in human skin fibroblasts.[52] Similar to hESC, iPSC are pluripotent, and thereby have the ability to differentiate into cells of all three germ layers of the body. iPSC are currently used as tools for drug development, and modeling diseases. However,

transduction using retroviruses may cause insertion mutagenesis, and subsequent adverse effects such as cancer.[53] Therefore, researchers are currently attempting to generate retroviral free induction methods in order to generate iPSC that can be used in transplantation medicine.[54–58]

1.7.3 Adult Stem Cells

Currently, there are few therapies using hESC or iPSC despite the promise that these cells offer. This is due to difficulties of directing the production of a functional effector cells, and their potential for teratoma forming capacity. In contrast, adult stem cells are safely used in the clinic for a number of medical procedures including bone marrow (BM) transplantation to treat hematological malignancies.

Adult stem cells, also known as somatic stem cells, are undifferentiated cells found among differentiated cells within an organ. These cells are usually considered multipotent, since they have the ability to differentiate into mature cells of a particular lineage, or germ layer. The physiological role of adult stem cells is organ maintenance and repair, by replenishing damaged or dying cells, so that organ function is maintained. Adult stem cells have been identified in almost every organ in the human body including: the brain, BM, umbilical cord blood (UCB), blood vessels, skeletal muscle, skin, gastro intestinal tract, liver, ovarian epithelium, and testes. Of these many types of adult stem cells, hematopoietic stem cells (HSC) which are derived from adult BM or UCB are extremely well characterized.

1.7.3.1 Hematopoietic Stem Cells

HSC demonstrate the ability to self-renew, and differentiate into specialized blood cells of both the myeloid and lymphoid lineages. The initial evidence for the existence of HSC came in the atomic era. It was found that the lethal consequences of radiation was due to BM failure, and that this condition could be rescued following injection of spleen or BM cells from unirradiated donors.[59] The study of HSC moved from observational to functional when landmark by Till and McCulloch showed that the regenerative potential of HSC could be assayed using clonal *in vivo* repopulation assays, thus establishing the existence of a multipotential HSC.[60] Xenotransplantation assays were used to functionally characterize

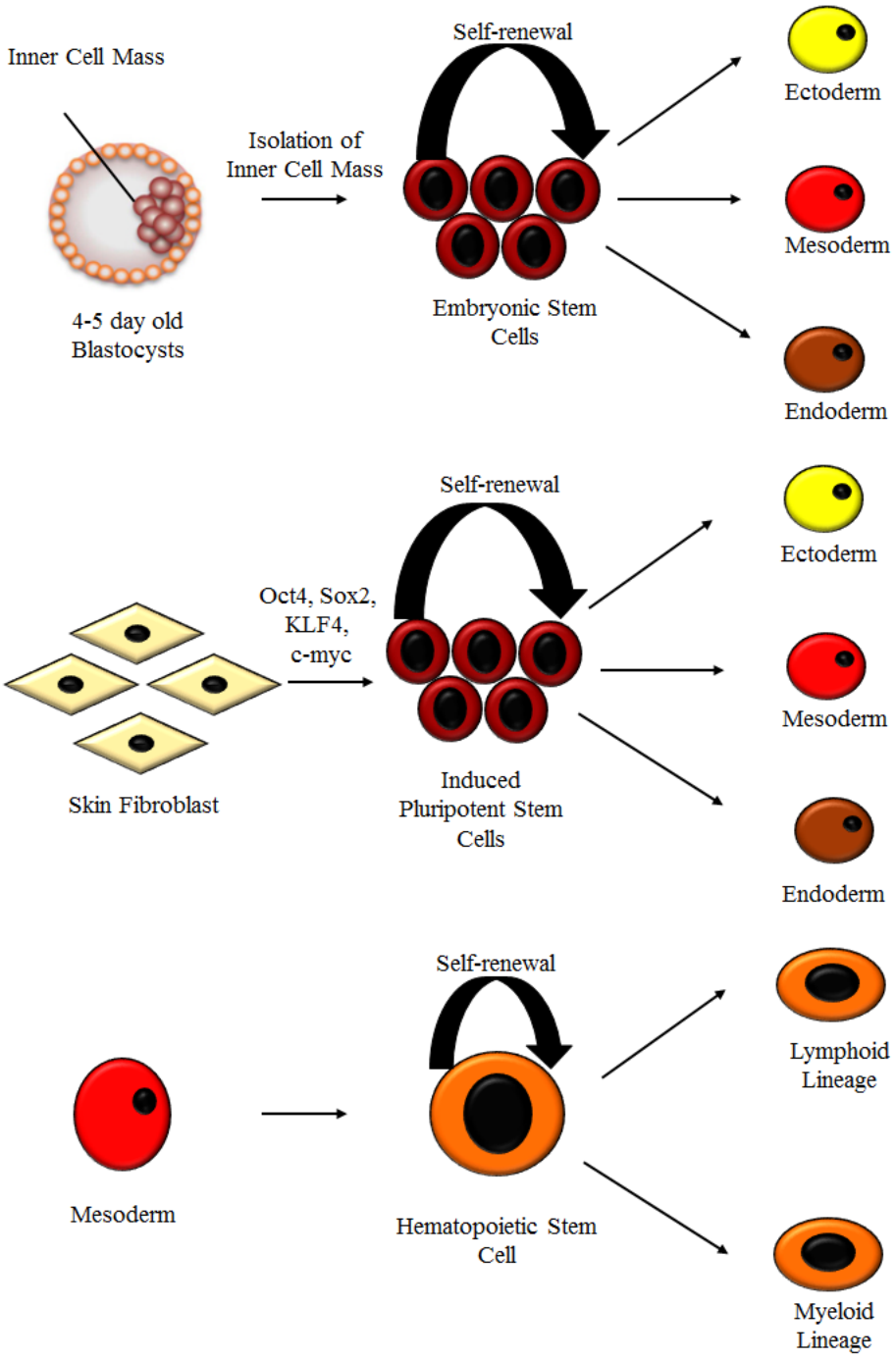

Figure 1.2 Differentiation Potential of Stem Cells. Schematic of the differentiation potential of embryonic stem cells stem (ES) cells, induced pluripotent stem (iPS) cells, and adult stem cells. ES cells, iPS cells, and adult stem cells all possess the ability to self-renew, and differentiate. However, ES and iPS cells are pluripotent thereby can differentiate into cells of all three germ layers. Whereas, adult stem cell such as hematopoietic stem cells (HSC) are germ-layer specific in their differentiative ability.

human HSC, and still remain the gold standard. The multipotency of HSC was demonstrated by the ability of human HSC to repopulate the myeloid and lymphoid compartment of the BM of an irradiated immunodeficient mouse.[47,61] The self-renewal potential of human HSC has been demonstrated by the ability of a single HSC to repopulate the BM of a primary host, and give rise to other HSC that can reconstitute all blood lineages in secondary hosts.[47] In contrast, the most immediate progeny of HSC, hematopoietic progenitor cells (HPC), loose their capacity for self-renewal, and therefore were unable to reconstitute the BM of a secondary host.[47,61]

Cell surface molecules expression as HSC mature during hematopoiesis is a highly regulated, and conserved process (Figure 1.3).[62] For instance, early HSC and HPC express the cell surface antigens CD34 CD133 and do not express CD38.[63] Whereas, the more lineage committed cells such as: myeloid progenitors, early thymic progenitors, and B/NK cell progenitors do not express CD133, and express both CD34 and CD38. Finally, the mature effector cells of the lymphoid, and myeloid lineage express lineage specific markers, but do not express CD34 or CD38.[61] Fluorescent monoclonal antibodies (CD34, CD38, and CD133), and fluorescence activated cell sorting (FACs) can be used to separate HSC and HPC populations (HSC/HPC) from more mature lineage committed cells (Lin^+).

However, considerable controversy exists regarding the expression of CD34 on true HSC. Bhatia *et al*.[64] demonstrated that $CD34^-/CD38^-$ cells isolated from UCB depleted of mature lineage committed cells (Lin^-) were functionally distinct from UCB $Lin^-/CD34^+$ HSC/HPC, and these cells had the ability to initiate multilineage hematopoiesis in non-obese diabetic severe combined immunodeficient (NOD/SCID) mice. It has also been reported that, UCB $Lin^-/CD34^-$ cells can generate $CD34^+$ cells once transplanted into NOD/SCID mice.[65] A recent study showed that, $Lin^-/CD34^-/CD38^-/CD93^+$ UCB cells initiated multilineage hematopoietic engraftment in both primary, and secondary hosts.[66] $Lin^-/CD34^-/CD38^-/CD93^+$ UCB cells were highly quiescent, compared to $Lin^-/CD34^+/CD38^-$ cells. Furthermore, the progeny of $Lin^-/CD34^-/CD38^-/CD93^+$ cells, were more efficient at engrafting to a secondary host, compared to the progeny of $Lin^-/CD34^+/CD38^-$ progenitor cells. Suggesting that, $Lin^-/CD34^-/CD38^-/CD93^+$ cells are more primitive than $Lin^-/CD34^+/CD38^-$ cells.

Alternatively, HSC/HPC can be isolated according to stem cell function, rather than phenotype. In order to facilitate the daily production of approximately one trillion (10^{12}) blood cells in the human body, the HSC/HPC pool must be maintained throughout the life of the organism.[61] Thus, enzymes such as aldehyde dehydrogenase (ALDH) that protects against reactive aldehydes, and reactive oxygen species (ROS) are highly expressed in HSC/HPC.[67] In contrast, more mature blood effector cells have low ALDH activity (Figure 1.3). There are 19 structurally related ALDH isoforms in humans, and 20 ALDH isoforms have been described in mice.[68] Out of these isoforms ALDH1A1 is the most abundantly expressed in HSC/HPC, but ALDHA1 knockout did not adversely affect HSC/HPC function.[69] Interestingly, ALDH1A1 knockout was associated with an increase in ALDH3A1 in the HSC/HPC pool, suggesting this enzyme compensates for the loss of ALDH1A1. The HSC/HPC pool in mice deficient in both ALDH1A1 and ALDH3A1, have an increased ROS level, and are more sensitive to DNA damage. As a result, ALDH1A1 and ALDH3A1 deficient mice have reduced HSC/HPC cell numbers. In addition, ALDH1A1 and ALDH3A1 deficient mice had deficiencies in B cell development. Thus, this study illustrates that ALDH plays an important role in B cell development and HSC/HPC survival.

In addition, ALDH1A1 is a critical enzyme in the biosynthesis of retinoic acid (RA), the active metabolite of vitamin A (retinol).[68] ALDHA1 catalyzes the oxidation of retinaldehyde to retinoic acid, the final step in this process.[70] RA, is a non-peptidic lipophilic morphagen that enters the nucleus, and binds to the heterodimer formed by the RA receptor (RAR) and the retinoid-x-receptor (RXR) to drive the transcription of target genes.[70] Human HSC/HPC are intrinsically programmed to undergo differentiation in response to RA, since HSC/HPC highly express ALDHA1 and RARα. Human BM stromal cells highly express CYP26 enzymes inactivates retinoids, and thereby maintains an environment low in retinoids in the human BM. Culturing HSC/HPC *ex vivo* both in the presence and absence of retinoic acid resulted HSC/HPC differentiation.[70,71] Furthermore, blocking the RAR receptor or ALDHA1 during *ex vivo* culture prevented HSC/HPC

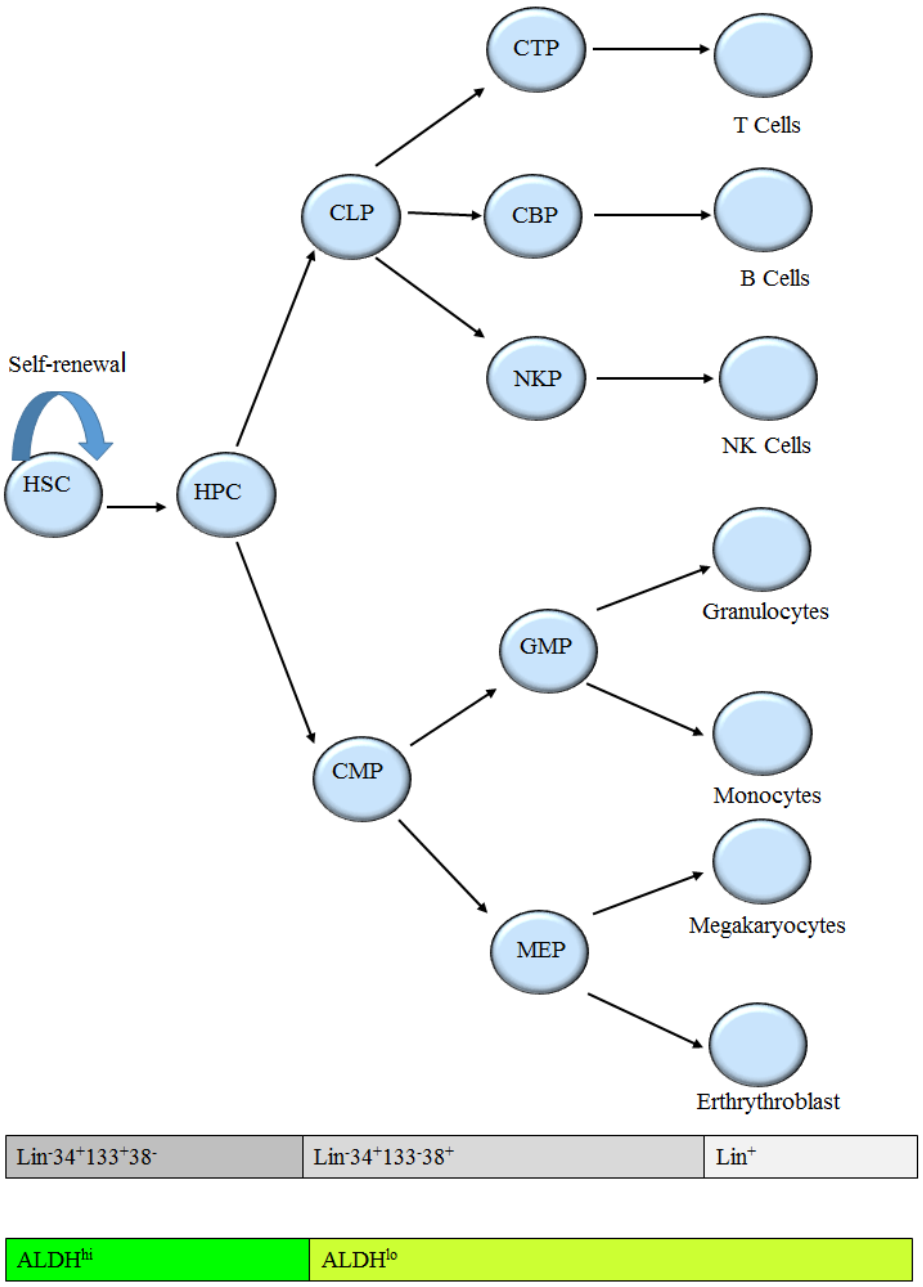

Figure 1.3 Adult Human hematopoietic hierarchy. Schematic representation of the phenotypic markers expressed by hematopoietic stem/progenitor cells (HSC/HPC) and mature blood cells (adapted from Doulatov *et al.*[61]) The expression of cell surface molecules as HSC mature during hematopoiesis is a highly regulated process. Therefore, cell surface markers can be used to separate more primitive HSC/HPC cells from more mature lineage committed blood cells. Alternatively, HSC/HPC can be isolated according to stem cell function via relative aldehyde dehydrogenase (ALDH) activity. HSC/HPC have high ALDH activity, whereas more committed cells have low ALDH activity. Myeloid progenitor (CMP), Lymphoid progenitor (CLP), T cell progenitor (CTP), B cell progenitor (CBP), NK cell progenitor (NKP), granulocyte/macrophage progenitor (GMP), megakaryocytic/erythroid progenitor (MEP).

differentiation, and promoted HSC/HPC self-renewal.[70,71] These studies suggest that, retinoids play an important role in mediating key aspects of HSC self-renewal and differentiation.

The fluorescent substrate for ALDH, BODIPY aminoacetaldehyde developed by Storms *et al*.,[72] and now marketed as Aldeflour™ by Stem Cell Technologies™ can be used to detect relative ALDH activity. Aldeflour™ is a aminoacetadehyde moiety conjugated to a BODIPY (4,4-difluoro-5,7-dimethyl-4-bora-3a,4adiaza-5-proprionic acid) flourochrome. Once the lipophilic Aldeflour™ substrate enters the cells it is converted to an acetaldehyde anion by ALDH. Under pharmacological inhibition of ABC transporters with the Aldeflour™ buffer, cells with high ALDH activity retain a higher amount of Aldeflour™ compared to cells with low ALDH activity. Thereby, cells with high ALDH activity will be brighter compared to cells with low ALDH activity, a property that enables the FACS purification of these two populations [72,73]. The integrity and function of the cell are not compromised by this procedure since upon removal of the Aldeflour™ buffer the ABC transporter becomes reactivated and Aldeflour™ is pumped out of the cell returning the cell to its original state, thus the isolated cells can be used for various downstream applications.

Hess et al.,[73] showed that, UCB progenitor cells with high ALDH ($ALDH^{hi}$) activity isolated from Lin^- UCB have enhanced *in vitro* colonergic progenitor activity, and highly co-express primitive hematopoietic cell phenotypes compared to UCB cells with low ALDH ($ALDH^{lo}$) activity. Furthermore, transplantation of $Lin^-/ALDH^{hi}$ UCB progenitor cells into NOD/SCID and NOD/SCID β2 microglobulin (β2M) null mice resulted in multilineage human cell engraftment in hematopoietic tissues, while $Lin^-/ALDH^{lo}$ UCB cells were devoid of repopulation ability.[74] Further dissection of the $Lin^-/ALDH^{hi}$ UCB progenitor population by using the cell surface molecule CD133 showed that, $Lin^-/ALDH^{hi}/CD133^+$ progenitor cells are significantly enriched for *in vivo* repopulating ability compared to $Lin^-/ALDH^{hi}/CD133^-$ progenitor cells. Limiting dilution analysis demonstrated that, $Lin^-/ALDH^{hi}/CD133^+$ cells have a 10-fold increase in the frequency of NOD/SCID repopulating cells (also known as SCID repopulating cells, SRC) compared with $Lin^-/CD133^+$ cells. Furthermore, the hematopoietic and non-hematopoietic progeny

of $ALDH^{hi}$ UCB trafficked efficiently to various organs including the pancreas after being transplanted into glucuronidase (GUSB)-deficient NOD/SCID/mucopolysaccharidosis type VII mice (MPSVII) mice. Therefore, Aldeflour™ can be used to isolate HSC/HPC.

1.7.3.2 *Ex vivo* Expansion of Hematopoietic Stem and Progenitor Cells

The widespread initiatives to HLA-phenotype and cryopreserve human UCB in North America and Europe, has ensured that UCB represents a readily available source of HSC/HPC for cell therapy applications. However, the low number of HSC/HPC found in UCB limits the widespread therapeutic application of transplanted UCB-derived HSC/HPC in humans.[75,76] Therefore, *ex vivo* expansion of HSC/HPC is one of the ways which the number of UCB can be increased for cellular therapies.

The last two decades, researchers have established protocols aimed at the *ex vivo* expansion of HSC/HPC for clinical application.[75] Under serum free conditions, hematopoietic cytokines are necessary for amplification HSC/HPC *ex vivo*.[77,78] These signaling molecules, are produced by stromal cells in the bone marrow, and induce the proliferation of hematopoietic cells at various stages of development.[76] The rapid discovery of new hematopoietic growth factor's and their receptors enabled researchers to find cytokines that amplified HSC/HPC in culture.[76] Initially HSC/HPC expansion was supported by hematopoietic cytokines and serum. Since, serum is a potential source of bacterial, mycoplasmas, and viral contaminants, many investigators attempted to develop serum free conditions to expand HSC for clinical application.[79] Through systematic analysis it was found that stem cell factor (SCF), fms-related tyrosine kinase 3 ligand (FLT-3L), and thrombopoietin (TPO) were essential cytokines for the expansion of primitive progenitor cells in serum-free culture.[77,78] In contrast, the addition of other cytokines such as: interleukin-3 (IL-3), granulocyte colony stimulating factor (G-CSF), erythropoietin (EPO) and macrophage colony-stimulating factor (M-CSF) supports the expansion of more mature hematopoietic cells, instead of primitive HSC/HPC.[80,81] Furthermore, research has also established that the selection of a purified starting cell population is a key aspect in *ex vivo* expansion protocols. $ALDH^{hi}$, $CD34^{+}/CD38^{-}$, $CD34^{+}/CD133^{+}$ progenitor cells

demonstrate a greater expansion potential than their more heterogeneous cell populations containing mature cell counterparts.[82–88] At the single cell level, multipotent progenitor cells can give rise to 70×10^6 nucleated cells, and more than 90,000 $CD34^+$ progenitor cells, whereas mature erythroid progenitors can only produce 9×10^6 nucleated cells and no more than 5,000 $CD34^+$ progenitor cells.[89] Currently, researchers are attempting to improve *ex vivo* expansion technologies to increase the number of HSC/HPC available for cellular therapies.[85,90–102]

1.8 Stem Cell Therapies for Diabetes

There are two major initiatives in the stem cell biology field to correct the loss of β cell mass in both type 1, and type 2 diabetic patients: (1) generating β cells *ex vivo*, by utilizing pluripotent stem cells; (2) stimulating the regeneration of β cells in the pancreas using adult stem cells.[36]

1.8.1 β Cell Replacement

1.8.1.1 Derivation of β cells from ESC

A number different groups are attempting to differentiate ESC's and iPSC's into functional β cells, to provide an unlimited source of β cells for replacement therapy in diabetic patients.[36] In order to generate glucose responsive pancreatic β cells from hESC, critical signals that regulate endocrine pancreas development needs to be successfully recapitulated *in vitro*. During normal development human epiblast cells first differentiate into definite endoderm during gastrulation. Culturing hESC in Wnt3a and activinA leads to cultures that contain 80% definitive endodermal cells.[103,104] Attempts to efficiently produce β cells from the definitive endoderm has not been as fruitful. In 2005 Novocell (now Viacyte) developed a complex multi-step protocol to generate insulin-producing β cells. However, only 7.3% of the cells generated using this protocol were positive for insulin.[105] By making modification to the Viacyte protocol Nostro *et al.*[106] was able to increase the number of insulin-producing cells to 25%. In both these protocols the insulin producing cells were polyhormonal, and were not glucose responsive. Alternatively, Kroon

et al.[107] differentiated hESC into pancreatic-endocrine progenitor like cells in culture, and subsequently transplanted these progenitor cells into streptozotocin induced (STZ)-diabetic mice. Following transplantation, the endocrine progenitor-like cells matured into various cell types, including functional β cells that normalized the blood glucose levels of these diabetic mice. Although, these results were very encouraging, the composition, organization, and mass of the resulting cellular structure from progenitor cell differentiation *in vivo* was highly unpredictable.[108] Furthermore, signals that stimulate endocrine progenitor cells to differentiate into fully functional β cells remain unknown.[108] Thus, the inability to differentiate hESC into functional β cells in culture, coupled with ethical concerns and tumorigenicity of hESC-derived β cells, has prevented the use of hESC-derived β cells in a clinical setting.

1.8.1.2 Derivation of β cells from iPSC

Similarly, iPSC can be used as a non-embryonic source for the derivation of β-like cells. Murine iPSC–derived β-like cells have the capacity to normalize hyperglycemia in both a type 1 and type 2 diabetic mouse models.[109] Furthermore, iPSCs derived from the epiblast of non-obese diabetic (NOD) mice differentiated into insulin-producing cells that expressed diverse pancreatic β cell markers, and normalized hyperglycemia upon transplantation into diabetic mice.[110] In another study, human iPSCs derived from dermal fibroblasts of patients with type 1 diabetes, were differentiated into cells that expressed insulin, somatostatin, and glucagon *in vitro*.[111] Interestingly, these cells were able to produce insulin in response to glucose.[111] Thus, iPSCs may be an appropriate choice for generating autologous β cells, however like hESC, the clinical utility of iPSC is hampered by incomplete maturation of differentiated cells, chromosomal aberrations, and enhanced tumorigenic potential.[112] Due to these limitations, strategies that stimulate endogenous islet regeneration *in situ* are also under intense investigation.

1.8.2 Endogenous Islet Regeneration Therapies

The β cell mass in adult humans is maintained at a constant level with very low cellular turnover.[113] Interestingly, drastic increases in β cell mass have been observed in response

to the increase in metabolic demand during pregnancy and obesity.[114–116] Studies in mice have attempted to elucidate the mechanism that induces β cells to re-enter the cell cycle. Factors such as epidermal growth factor (EGF), insulin-like growth factor 1 (IGF1), human growth hormone, prolactin, as well as glucose have been shown to stimulate β cell proliferation in the mouse.[117] In Hess and collegues[118] initial publication on islet regeneration, they showed that transplanted murine BM progenitor cells reduced hyperglycemia in STZ-induced NOD/SICD (STZ-NOD/SCID) diabetic mice.[118] Transplanted BM progenitor cells engrafted in the ductal and islet regions of the damaged pancreas, but did not differentiate into insulin producing cells. Instead, BM progenitor cells stimulated β cell proliferation, and enhanced insulin production in recipient-derived islets via undetermined mechanisms.[118] In order for endogenous islet regeneration to be a feasible strategy, the signal receiving cells within the pancreas, and the mechanism by which transplanted cells stimulate β cell expansion needs to be identified.

1.8.2.1 The Medalist Study

The medalist study followed 411 type 1 diabetic patients with disease duration of more than 50 years.[34] The majority (63.4%) of the medalist had c-peptide levels in the minimal, and sustained range.[34] Furthermore, their c-peptide release doubled during a mixed meal tolerance test.[34] Histological analysis of pancreas sections, from the patients that died during the study showed the presence of insulin+ proliferating β cells.[34] The data presented in this study clearly supports that, residual functional β cells can remain in type 1 diabetic patients with long standing diabetes. Thus, the amelioration of autoimmune destruction of β cells, together with stimulating the regeneration of endogenous β cells, may represent a feasible approach to improve endogenous insulin production in patients with type 1 diabetes. In order to develop better therapies to stimulate islet regeneration, the activation of endogenous regenerative mechanisms within the pancreas must be understood.

1.8.2.2 Mechanisms of Endogenous Islet Regeneration

The mechanisms by which new β cells arise in the post-natal pancreas is highly controversial, and is still under intense research (Figure 1.4). To investigate the origin of adult β cells, Dor *et al.*[119] performed a pioneering lineage tracing experiment. These researchers defined mature β cell as post-natal cells transcribing the insulin gene, and used a pulse-chase lineage tracing approach to distinguish stem-cell derived β cells, from the progeny of pre-existing β cells. Firstly, pre-existing mouse β cells were heritably labelled with tamoxifen-inducible Cre transgene, rat insulin promoter (RIP)-CreER, and a human alkaline phosphatase reporter (HAP, the pulse). Then the labelled cells (HPAP+) were tracked over a period of time or after injury, during which cellular turnover occurred (the chase). Using this approach, the cells generated from pre-exiting β cells are labelled, but new β cells derived from any other cells, including stem cells will not be labelled. Thus, if new islets are derived entirely from stem cells, these islets would not contain any labeled β cells. If stem cells replenish β cells within existing islets, the frequency of labelled β cells would gradually decrease. However, if new β cells are derived from pre-existing β cells the frequency of labelled β cell within an islet would remain constant. Interestingly, over time and after injury the total number of β cell increased, but the frequency of labelled β cells remained constant.[119] Thus, it was concluded that β cell expansion was primarily driven by the division of already differentiated cells.

However, Dor *et al.*[119]'s definition of a mature β cell has been questioned by the recent identification of a rare population of insulin expressing cells in the murine and human pancreas that have progenitor like characteristics.[120,121] These cell referred to as pancreas-derived multipotent progenitor (PMP) cells, are able to form colonies *in vitro* and differentiate into all hormone producing cells in the endocrine pancreas. Furthermore, transplantation of both murine and human PMPs improved hyperglycemia in STZ-diabetic mice.[120,121] Thus, it is possible that these insulin+ precursors also contributed to the increase in the number of β cells observed by Dor *et al.*[119] Further research is required to delineate

the relative contributions of mature β cells and β cell precursors during β cell expansion after injury, and during adult life.

In contrast, endogenous islet regeneration has been shown to occur through the activation of ductal-resident progenitor cells that may contribute to new islet formation. Inada *et al*.[122] used a lineage tracing approach to determine if ductal progenitors gives rise to β cells after ductal ligation in the adult pancreas. These researchers used the reporter gene β-galactosidase (β-gal) to trace carbonic anhydrase II+ ductal cells. After ductal ligation, the number of β-gal+ β cells increased, which suggests that islet regeneration occurs through the differentiation of ductal cells, into insulin expressing β cells. Furthermore, Xu *et al*.[123] used pancreatic ligation as an injury model, and a neurogenin 3 (Ngn3) reporter to trace Ngn3+ endocrine precursor present in the ducts. Upon injury, these cells were activated in the ducts and could give rise to all endocrine cell types *in situ*, and in embryonic explant cultures. These studies suggests that the islet neogenic program may also be activated after pancreas injury.

In addition, Rovira *et al*.[124] showed that adult mouse ALDH+ centroacinar and terminal duct (CA/TD) cells highly express progenitor cell markers Sca1, Sdf1, c-Met, Nestin, and SOX9. Notably, these ALDH+ CA/TD cells were able to form pancreatosphere colonies *in vitro*, that demonstrated spontaneous endocrine and exocrine differentiation, as well as glucose responsive insulin secretion. When ALDH+ CA/TD cells were injected into mouse embryonic dorsal pancreatic buds these cells also differentiated into both endocrine and exocrine lineages. Finally, ALDH+ CA/TD cells in the pancreas expanded in the setting of chronic epithelial cell injury. In addition, Li *et al*.[125] showed that ALDH+ human fetal pancreas cells highly express progenitor cell markers. High ALDH expression was observed in newly differentiated insulin+ cells, and overall ALDH-expression was decreased as development progressed. Interestingly, pharmacological inhibition of ALDH activity resulted in reduced endocrine cell differentiation, which was reversed by with co-treatement of an RAR/RXR agonist. Collectively, these studies suggest that ALDH+ cells represent a pool of progenitors that may contribute to the maintenance of pancreatic tissue homeostasis in the adult mouse and human fetal pancreas.

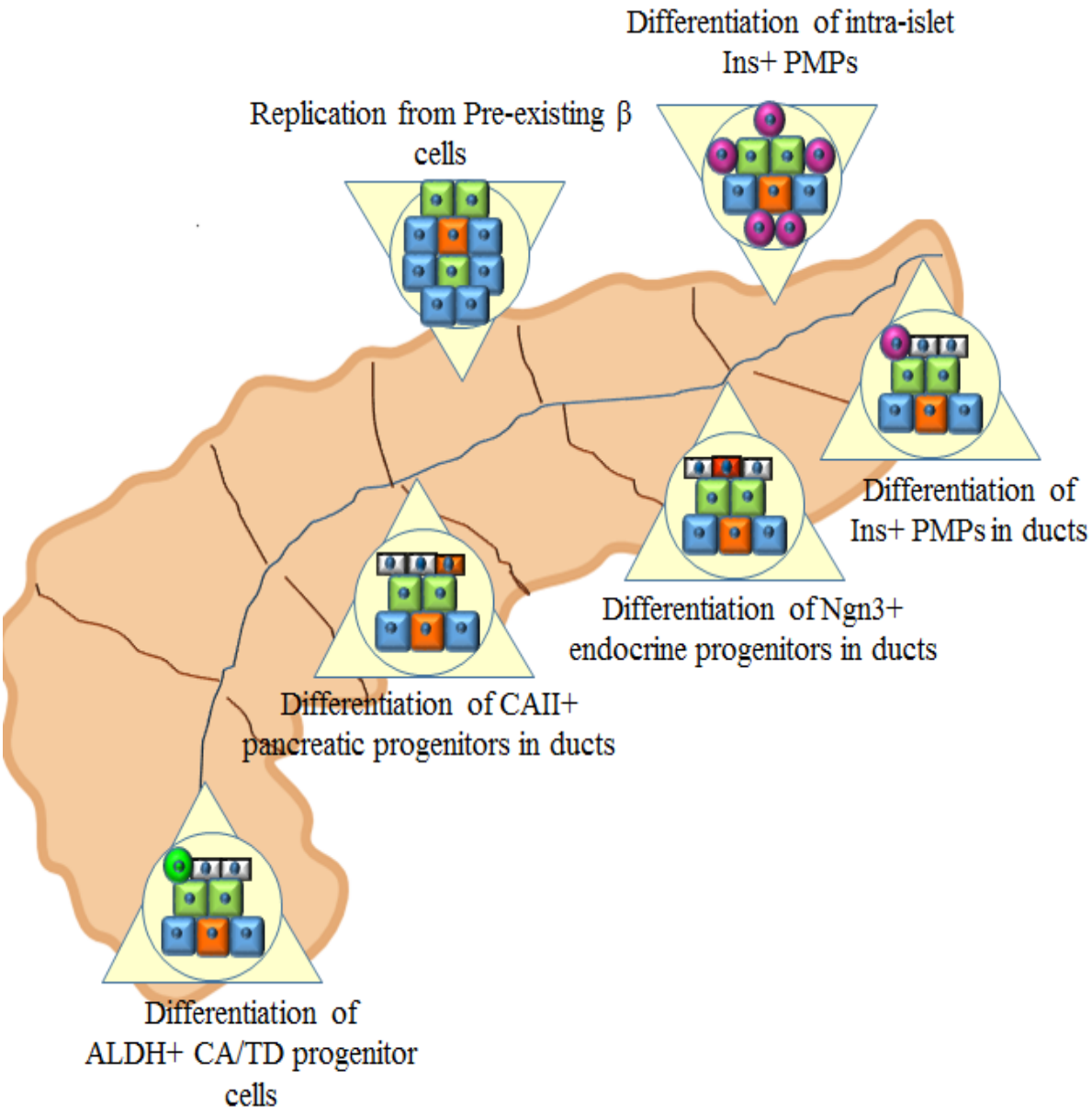

Figure 1.4 Mechanisms of endogenous islet regeneration in the post-natal pancreas. Schematic representation of the proposed mechanisms of endogenous islet regeneration (adapted from Bonner-Weir *et al.*[36]). The proliferation of pre-existing β cells and the differentiation of intra-islet pancreas-derived multipotent progenitor (PMP) cells has been shown to give rise to new β cells. Alternatively, islet neogenesis, via the differentiation of Ins+, Ngn3+, and CAII+ ductal progenitor cells and ALDH+ centroacinar and terminal ductal (CA/TD) progenitor cells has also been implicated in restoring β cells.

Although, research is required to delineate the mechanism of endogenous islet regeneration, finding a therapeutic strategy that "tips the balance" in favour of islet regeneration versus destruction will be beneficial therapeutic strategy to treat diabetic patients.

1.8.2.3 Blood Progenitors Stimulate Endogenous Islet Proliferation and Revascularization

Bell *et al*.'s[126,127] studies provide insight into the potential mechanisms by which BM and UCB derived human progenitor cells stimulate endogenous islet regeneration (Figure 1.5). Bell and colleagues found that, i.v. transplantation of BM derived $ALDH^{hi}$ progenitor cells, and not $ALDH^{lo}$ BM cells improved systemic hyperglycemia and augmented insulin production in STZ-NOD/SCID diabetic mice.[126] In addition, only certain Multipotent Stromal (MSC) samples derived from BM improved the islet function of STZ-NOD/SCID diabetic mice. Interestingly, the islet regenerative program activated depends on the lineage-restriction of the progenitor cell administered. $ALDH^{hi}$ progenitor cells derived from BM engrafted around the damaged islets, and secreted paracrine factors to stimulate islet specific-vascularization and islet-associated cell proliferation. This resulted in an increase islet size (islet circumference), and β cell mass. In contrast, i.v. transplanted MSC did not engraft in the pancreas or stimulate islet proliferative or pro-angiogenic programs. Instead, MSC improved systemic hyperglycemia by stimulating the formation of β cell clusters associated with cytokeratin (CK19) + ductal epithelium, which resulted in an increase in islet number, suggestive putative neogenic mechanism, whereby MSC stimulate CK19+ cells to differentiate into insulin+ β cells.

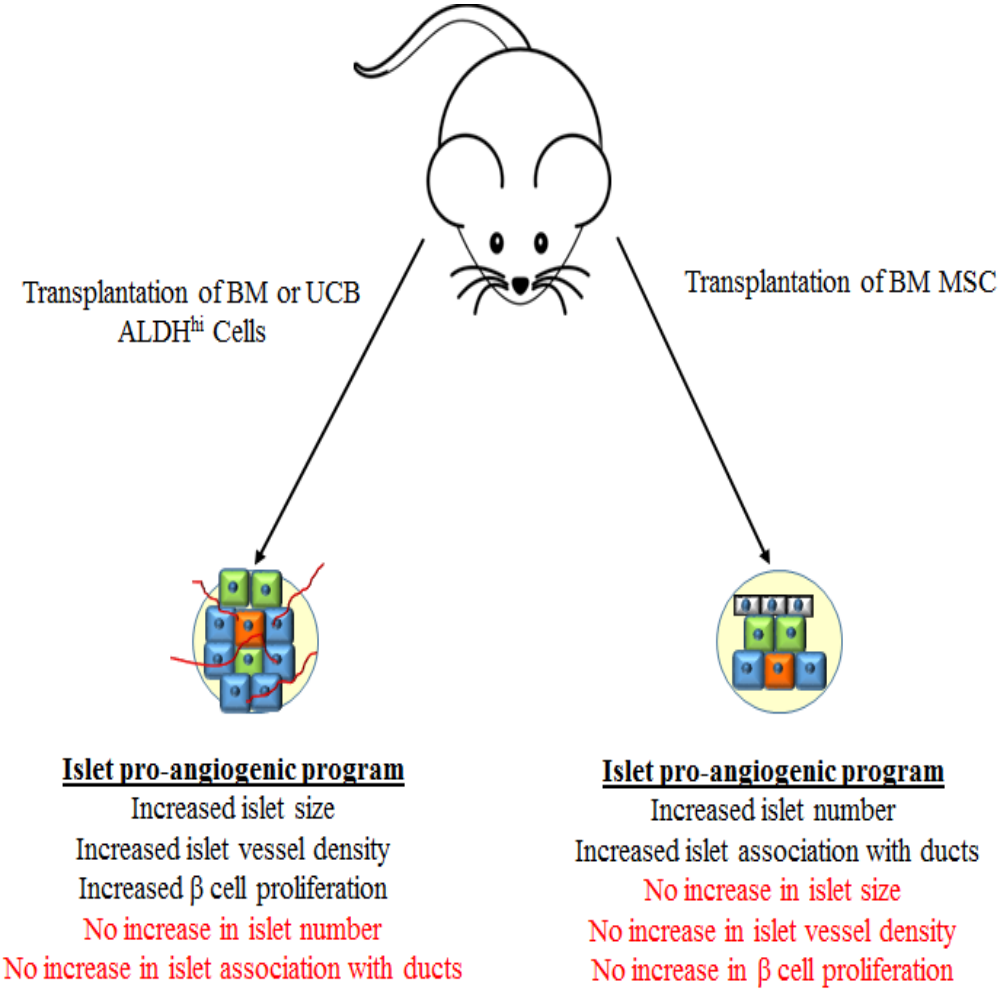

Figure 1.5 Endogenous islet regeneration after the transplantation of human BM/UCB-derived ALDH^{hi} progenitor cells or BM-derived MSC. Schematic representation of mechanisms involved in islet regeneration after the transplantation of progenitor cells (adapted from Bell *et al.*[128]). Transplantation of ALDH^{hi} cells augmented islet size and vascularization, by stimulating β cell and endothelial cell proliferation, but did not increase islet number. Transplantation of MSC induced an increase in β cell clusters associated with CK19+ ducts, which also resulted in an increased β cell mass. Thus, the islet regenerative program activated depends on the lineage restriction of the progenitor cell administered.

In addition, ALDH^{hi} progenitor cells isolated from UCB stimulated endogenous islet regeneration. Intravenously injected ALDH^{hi} UCB progenitor cells demonstrated low-pancreas engraftment, and only transiently improved islet function.[127] Whereas, intra-pancreatic (iPan) transplantation ALDH^{hi} UCB progenitor cells, and not ALDH^{lo} UCB cells improved systemic hyperglycemia, augmented insulin production, and improved glucose tolerance in STZ-NOD/SCID diabetic mice. Similar to ALDH^{hi} progenitor cells isolated from the BM, iPan transplanted ALDH^{hi} UCB cells surrounded the damaged pancreas, and stimulated the islet associated-cell proliferation and islet angiogenesis.[126,127]

To identify paracrine effectors secreted by ALDH^{hi} UCB that stimulated islet regeneration, the mRNA transcripts of islet regenerative ALDH^{hi} UCB cohort was compared to the non-regenerative ALDH^{lo} UCB cohort.[128] ALDH^{hi} UCB highly expressed the ligands of the epidermal growth factor receptor (EFGR) superfamily amphiregulin (AREG), epiregulin (EREG). The EGFR superfamily has been implicated in the regulation of pancreatic β cell mass. Also, EGFR signaling was essential for the β cell mass expansion in response to a high fat diet or pregnancy. Furthermore, ALDH^{hi} UCB highly expressed pro-angiogenic cytokines such as angiopoietin 1 (ANGPT1) and vascular endothelial growth factor A (VEGF A). In contrast, islet regenerative MSC highly expressed the transcripts of fibrillin 2 (FBN2)-a modulator of transforming growth factor β (TGFβ) bioavailability-WISP1 (WNT1 inducible signaling pathway protein 1) and SFRP1 (Secreted frizzled-related protein 1)-matrix modifiers downstream of WNT-signaling. Both the TGFβ signaling, and WNT-signaling are required for pancreatic lineage development. This study suggests that, AREG, EREG, VEGF and ANGPT1 are involved in supporting β cell proliferation, and islet angiogenesis. Whereas, WSP1 and FBN2 are involved in supporting islet neogenesis. However, further research is required to elucidate the progenitor cells and paracrine factors that formulates the islet regenerative niche, to maximize the endogenous regenerative potential of the endocrine pancreas.

1.8.2.4 Clinical Trials Using Blood Progenitor Cells to Treat Diabetic Patients

In 2005 an Argentine team infused mononucleated cells derived from the human BM directly into the pancreas through the splenic artery to stimulate endogenous islet regeneration in type 2 diabetic patients.[129] Eighty-four percent of the patients that received this transplant were weaned off hypoglycemic pharmacological agents. In another study, 25 type 2 diabetic patients received a combination therapy of autologous stem cell infusion and peri-infusion of hyperbaric oxygen treatment. Compared to baseline before treatment metabolic variables such as: fasting glucose, HbA1c, fasting c-peptide, and the insulin requirement improved in these patients 12 months after treatment.[130] Although these preliminary results are exciting, our studies show that it is only blood-derived progenitor cells that stimulate robust endogenous islet regeneration. Whereas, blood-derived mononuclear cells do not have this effect.[126–128] UCB is a readily available source of blood-derived progenitor cells, however there is not enough of these progenitor cells in UCB to treat a human diabetic patient. Thus, in order for stem cell infusion to be used as a curative and sustainable therapy for diabetic patients, progenitor cells must be expanded, and the mechanism by which these cells stimulate endogenous islet regeneration must be understood.

1.9 Hypothesis and Objectives

The overall objective of my project was to characterize UCB-derived, $ALDH^{hi}$ HPC expanded in clinically applicable serum-free culture conditions. In addition, we aimed to determine whether these expanded HPC subsets, selected for low versus high ALDH activity after culture, stimulate endogenous islet regeneration after transplantation *in vivo*.

We hypothesize that HPC retaining high ALDH activity during ex vivo expansion, will possess islet regenerative capacity, and will lead to the recovery of hyperglycemia after transplantation into diabetic mice. We further hypothesize that $ALDH^{hi}$ HPC will secrete developmental cytokines that stimulate β cell proliferation and islet revascularization.

My first objective was to characterize the cell surface phenotype and hematopoietic colony formation of human UCB $ALDH^{hi}$ cells after *ex vivo* expansion over a 9 day time course.

My second objective was to investigate the mechanisms by which culture-expanded $ALDH^{hi}$ HPC could stimulate islet regeneration and recovery from hyperglycemia after transplantation into STZ-NOD/SCID mice.

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2.0 Culture-Expanded Hematopoietic Progenitor Cells Re-Selected for High Aldehyde Dehydrogenase Activity Demonstrate Islet Regenerative Functions

2.1 Introduction

With approximately one trillion (10^{12}) cells arising daily in the adult human BM, blood is one of the most highly regenerative tissues in the body. Therefore, it is a rich source of adult progenitor cells that can be utilized for novel cell therapies.[1] Besides hematopoietic repopulating capacity progenitor cells derived from BM demonstrate tissue regenerative[2–5], immunomodulatory[6], and angiogenic properties.[7,8] Furthermore, in a seminal publication investigating the effect of BM cells on islet regeneration, Hess *et al.*,[9] showed that the transplantation of murine BM progenitor cells reduced hyperglycemia in STZ treated diabetic mice by stimulating β cell proliferation and enhancing insulin production in recipient derived islets *in situ* via undetermined paracrine mechanisms. This concept termed "progenitor cell-stimulated islet regeneration" has become a central process for pancreas repair as evidence now suggest multiple progenitor subtypes induce islet regeneration either by stimulating β cell expansion, or neoislet formation.[10–13]

Curative therapies that re-establish functional β cell mass in patients with diabetes has been an area of intense pre-clinical studies over the past 10 years. There are two distinct types of diabetes; type 1 diabetes is associated with the autoimmune destruction of insulin secreting β cells, while type 2 diabetes is linked to a combination of insulin resistance, β cell dysfunction, and ultimately β cell failure.[14,15] Currently, the symptoms associated with the β cell dysfunction in both type 1 and type 2 diabetic patients are treated using exogenous insulin. Furthermore, oral hypoglycemic agents like metformin are currently used to treat the peripheral insulin resistance observed in type 2 diabetic patients. Since, these treatments do not provide the same "on-demand" glycemic control, diabetic patients have more extreme peaks and troughs in blood glucose levels.[16] As a result, more than 90% of diabetic patients develop severe vascular complications, including peripheral limb ischemia, heart attack, and stroke.[17] In patients with severe diabetes, the success of whole pancreas or isolated islet transplantation via the Edmonton protocol suggests that hyperglycemia can be controlled by the replacement of viable β cells.[18] However, continued autoimmune destruction of donor islets, the need for lifelong immunosuppressant therapy, and the critical shortage of donor pancreata and islets have

prevented the widespread use of these procedures.[19,20] Interestingly, the medalist study has shown that, type 1 diabetic patients with disease duration >50 years have sustained resting c-peptide levels, suggesting residual β cell function.[17] Also, immunohistochemical analysis of 9 patients that died during the study revealed the presence of proliferating insulin+ cells in the pancreas.[17] Thus, stimulating the regeneration of endogenous β cells *in situ*, may represent a feasible approach to improve endogenous insulin production in diabetic patients.

In addition to BM, UCB represents a readily available source of progenitor cells. The widespread initiatives to HLA-phenotype and cryopreserve human UCB in North America and Europe, has ensured that UCB represents a sustainable source of post-natal progenitor cells, for cell therapy applications. In our most recent studies, we used human UCB cells with high aldehyde dehydrogenase activity ($ALDH^{hi}$), a conserved progenitor cell function, to establish that $ALDH^{hi}$ cells stimulate endogenous islet proliferation and revascularization after direct iPan ransplantation into STZ-NOD/SCID mice.[11] However, the low number of $ALDH^{hi}$ cells found in UCB limits the widespread application of transplanted UCB-derived progenitor cells to stimulate islet regeneration in human diabetic patients.

To increase the number of progenitor cells available for clinical application, we expanded the HPC lineage of $ALDH^{hi}$ UCB cells under clinically applicable, serum-free conditions. These conditions resulted in a 15-fold increase in total cell number, and a 3-fold increase in the number of HPC retaining high ALDH activity. HPC that retained high ALDH activity ($ALDH^{hi}$ HPC) highly expressed primitive hematopoietic cell surface markers (CD34, CD133), and demonstrated hematopoietic colony forming capacity *in vitro*. Culture-expanded $ALDH^{hi}$ HPC transplanted STZ-NOD/SCID mice demonstrated improved hyperglycemia, and glucose tolerance, increased islet size, and vascularization. These data identify culture-expanded $ALDH^{hi}$ HPC as a novel cell population for clinical applications, and provide strong justification for the further enhancement of expansion, re-selection, and transplantation strategies using UCB-derived HPC to tip the balance in favour of islet regeneration versus destruction in patients with diabetes.

2.2 Methods

2.2.1 ALDH^{hi} Progenitor Cell Isolation from Human Umbilical Cord Blood

Human UCB (N=10) was obtained with informed consent following cesarian section, by phlebotomy of the umbilical vein, at the Victoria Hospital Birthing Centre in London. Within 24 hours, UCB samples were lineage depleted (Lin$^-$, RosetteSep™ Human Cord Blood Progenitor Cell Enrichment Cocktail, StemCell Technologies, Vancouver, Canada) and the mononuclear cells (MNC) were isolated by Hypaque Ficoll centrifugation. Lin$^-$ depleted MNC were incubated with the Aldeflour^{TM} reagent (StemCell Technologies), and fluorescence activated cell sorting (FACSAria, BD Biosciences, Mississauga, ON) was used to isolate cells with low side scatter and high ALDH-activity.

2.2.2 *Ex vivo* Expansion of UCB ALDH^{hi} Cells

For *ex vivo* HPC expansion, freshly isolated ALDH^{hi} UCB cells were plated into fibronectin coated plates in X-vivo 15 media (Lonza, Basel, Switzerland) supplemented with-SCF, FLT-3L, and TPO-at a concentration of 10ng/mL (Invitrogen, Burlington, ON). UCB ALDH^{hi} cells were cultured for 3, 6, or 9 days, with a media change every 3 days.

2.2.3 Characterization of Cell Surface Marker Expression on Culture-Expanded HPC

Day 0 fresh ALDH^{hi} UCB or day 3, 6, or 9 culture-expanded HPC were collected and stained with anti-human antibodies for CD3 (T cells), CD14 (monocytes), CD19 (B cells) (all BD Biosciences), and HPC markers CD34 (Biolegend, San Diego, CA), CD133 (Miltenyi Biotec, Auburn, CA). Cells were co-stained with Aldeflour™ to determine ALDH activity, and cell viability was assessed by trypan blue viability counts and co-staining with 7-amino-actinomycin D (BD Biosciences). Cell surface marker expression

was acquired using a LSRII flow cytometer at the London Regional Flow Cytometry Facility, and analyzed using FlowJo software.

2.2.4 Methylcellulose Assay

Six day culture-expanded unsorted (Bulk) HPC, $ALDH^{hi}$ HPC or $ALDH^{lo}$ HPC subpopulations were seeded in methylcellulose media (H4434, Stem Cell technologies) at cell density of 1000 in duplicate or triplicate, and the hematopoietic colonies formed were phenotyped by light microscopy, and enumerated after 14 days.

2.2.5 Transplantation of Culture-Expanded HPC Subsets into STZ-NOD/SCID Mice

Adult (7-12 week) NOD/SCID mice (Jackson Laboratories, Bar Harbor ME) were injected with STZ (35mg/kg/day) for 5 days. On day 10 these mice were sub-lethally irradiated (300cGy) and transplanted by i.v. injection with PBS or with 5×10^5 Bulk HPC, 2×10^5 $ALDH^{lo}$ HPC, or 2×10^5 $ALDH^{hi}$ HPC. Systemic blood glucose concentrations were monitored weekly for 42 days. Twenty-four hours prior to sacrifice, mice were injected with 5-ethynyl-2'-deoxyuridine (EdU, Invitrogen) to mark proliferating cells, and glucose tolerance tests (2 hour duration) were performed after intraperitoneal injection of 2mg/kg glucose. The BM, and the duodenal (pancreas head) portion of the pancreas were collected and analyzed for engraftment of human cells expressing human pan-leukocyte specific marker CD45, and human nucleated cell marker HLA-A,B,C by flow cytometry. Previously, it has been shown that islet neogenesis occurs in the splenic (pancreas tail) portion of the pancreas.[21] Therefore, to characterize the islet regenerative program stimulated by transplanted progenitor cells, the tail of the pancreas was harvested frozen and sectioned at 10 μm for immunohistochemical analysis, using a cryostat such that each slide contained 3 sections that were 150 μM apart, belonging to one mouse, to ensure that the same islet was not counted twice. For all subsequent immunohistochemical staining procedures the appropriate controls-primary antibody only, secondary antibody only-were performed to control for background staining.

2.2.6 Islet Size, Number, and β Cell Mass Quantification

For islet analysis each section was fixed with 10% buffered formalin for 15 minutes (Sigma, St. Louis, MO), washed with PBS, and blocked with peroxidase block for 5 minutes and mouse-on-mouse reagent for an hour (Vector, Burlington, ON). Afterwards, sections were incubated for 5 minutes with mouse-on-mouse protein diluents (Vector). Sections were then incubated with mouse insulin antibody (diluted 1/1000, Sigma) for an hour, which was detected with a secondary peroxidase labeled rabbit anti-mouse IgG antibody (1 hour incubation, diluted 1/200, Vector) and *ImmPACT*™ DAB (Vector), then counterstained with hematoxylin and mounted in vectamount (Vector). Islets were detected using a light microscope (BX50, Olympus, Richmond Hill, ON). In each section islets with a circumference above 150µm in 4 randomly selected areas ($1mm^2$) were counted, and analyzed using the Northern Eclipse Software. β cell mass was calculated using the formula: (βcell area)/(total area – β cell area) × pancreas weight.

2.2.7 Islet Blood Vessel Density Quantification

In order to quantify blood vessels, each of the sections were first fixed with 10% buffered formalin for 15 minutes (Sigma), washed with PBS, permeabilized with 1 % Trition X-100 (Sigma) for 20 minutes, and blocked with mouse-on-mouse reagent (vector) for an hour, and incubated for 5 minutes with mouse-on-mouse protein diluents (Vector). Afterwards, sections were incubated with mouse anti-mouse insulin primary antibody (1 hour incubation time, diluted 1/100, Sigma), which was detected using the Texas Red-labeled horse anti-mouse secondary antibody (1/200, Vector). Each section was first blocked with rabbit blocking buffer for an hour, then co-stained with rat anti-mouse CD31 antibody (1 hour incubation, diluted 1/200, Chemicon, Temecula, CA), which was detected using Fluorescein-labeled rabbit anti-rat IgG secondary antibody (30 minute incubation, diluted 1/200, Vector).The slides were then mounted in VectaShield with DAPI (Vector). The fluorescence microscope (Axio Scope Z2) and the AxioVision software were used to detect CD31+ vessels within islets, and in extra-islet regions. In each section intra-islets blood vessels, extra-islet blood vessels, and DAPI stained nuclei within the islet were quantified.

CD31 vessels/islet cell was calculated using the formula: intra-islet CD31+ vessels/intra-islet DAPI stained nuclei.

2.2.8 Quantification of Proliferating Cells

Each section was fixed with 10% buffered formalin (Sigma) for 15 minutes, washed with PBS for 20 minutes, permeabilized with 1 % Trition X-100 (Sigma) for 20 minutes. EdU+ cells were detected using the Click-iT™ EdU imaging kit (Invitrogen) as per manufacturer's protocol, and co-stained for insulin as described in the islet blood density protocol above. The fluorescence microscope (Axio scope Z2, Germany) and the AxioVision software were used to detect proliferating islet cells. EdU+ cells in each islet, the number of islets with at least one EdU+ cell, and DAPI nuclei were then quantified. Percentage of EdU+ islet cells was calculated using the following formula: (EdU+ islet cells/intra-islet DAPI stained nuclei) × 100. Percentage of islets with at least one EdU+ cell was calculated using the following formula: (islets with at least 1 EdU+ islet cell/the total number of islets) × 100.

2.2.9 RNA Isolation and Microarray Analyses

mRNA was isolated from 6 day culture-expanded $ALDH^{hi}$ and $ALDH^{lo}$ HPC in triplicate using the mRNeasy Mini kits (Qiagen, Dusseldorf, Germany). Microarray was performed using human array chips (Affymetrix, Santa Carla, CA) at the London Regional Genomic Center. mRNA transcripts that were significantly ($p<0.05$) increased by 1.5-fold in $ALDH^{hi}$ HPC vs. $ALDH^{lo}$ HPC ($p<0.05$) were identified, and then filtered for documented angiogenic function using the Partek Genomic Suite (Partek, Inc., St. Louis, MO).

2.2.10 Statistical Analysis

All data was expressed as mean ± standard error of the mean (SEM). Analysis of significance was performed by a two-way analysis of variance (ANOVA) for the blood glucose curve, and by one way ANOVA, followed by Tukey's test for all other analyses unless otherwise stated.

2.3 Results

2.3.1 Cells Retaining High ALDH-activity and Primitive Progenitor Phenotype was maximized after 6 Days of Culture

Hematopoietic progenitor enriched human UCB Lin^- cells were FACS purified based on low side scatter and high ALDH activity (Figure 2.1A-C), 1.7 ± 0.3% of UCB Lin^- cells demonstrated high ALDH activity (N=10). We have previously shown purified $ALDH^{hi}$ cells represent a heterogeneous progenitor cell population, primarily composed of primitive hematopoietic and early myeloid progenitor cells, with murine islet regenerative and proangiogenic potential.[7,11] Specifically, $ALDH^{hi}$ UCB highly co-expressed the hematopoietic progenitor cell markers CD34 (91.6±1.8%), and CD133 (62.2±5.7%, Figure 2.2). We typically accrue a total of $2\text{-}4\times10^5$ from a UCB sample, and the low number of $ALDH^{hi}$ cells found in UCB limits the widespread application of transplanted UCB-derived progenitor cells available for regenerative therapies. Therefore, we adapted clinically applicable conditions to expand $ALDH^{hi}$ UCB cells in serum free media using the hematopoietic growth factors: SCF, FLT-3L, TPO.[22–26] Under these hematopoietic cell expansion conditions fresh UCB $ALDH^{hi}$ cells expanded by 2.3 ± 0.4-fold at day 3, 20.7± 2.0-fold at day 6, and 53.5 ± 9.0-fold at day 9 of culture (Table 2.1, N=5-7). Furthermore, the frequency of HPC retaining high ALDH-activity diminished as culture time progressed from 28.7 ± 2.0% at day 3, versus. 14.9 ± 3.9 % day at 6, versus. 2.2 ± 0.3% at day 9, (Figure 2.1D-F). Interestingly, at day 6 the culture expanded HPC population demonstrated the highest number of total $ALDH^{hi}$ (3.09 ± 1.1 fold, Figure 2.1G), $ALHD^{hi}/CD34^+$ (2.24 ± 0.31 fold, Figure 2.1H), and $ALDH^{hi}/CD133^+$ (1.84 ± 0.37 fold, Figure 2.1I) primitive progenitor cells. Therefore, we selected 6 day culture-expanded HPC for all further analyses.

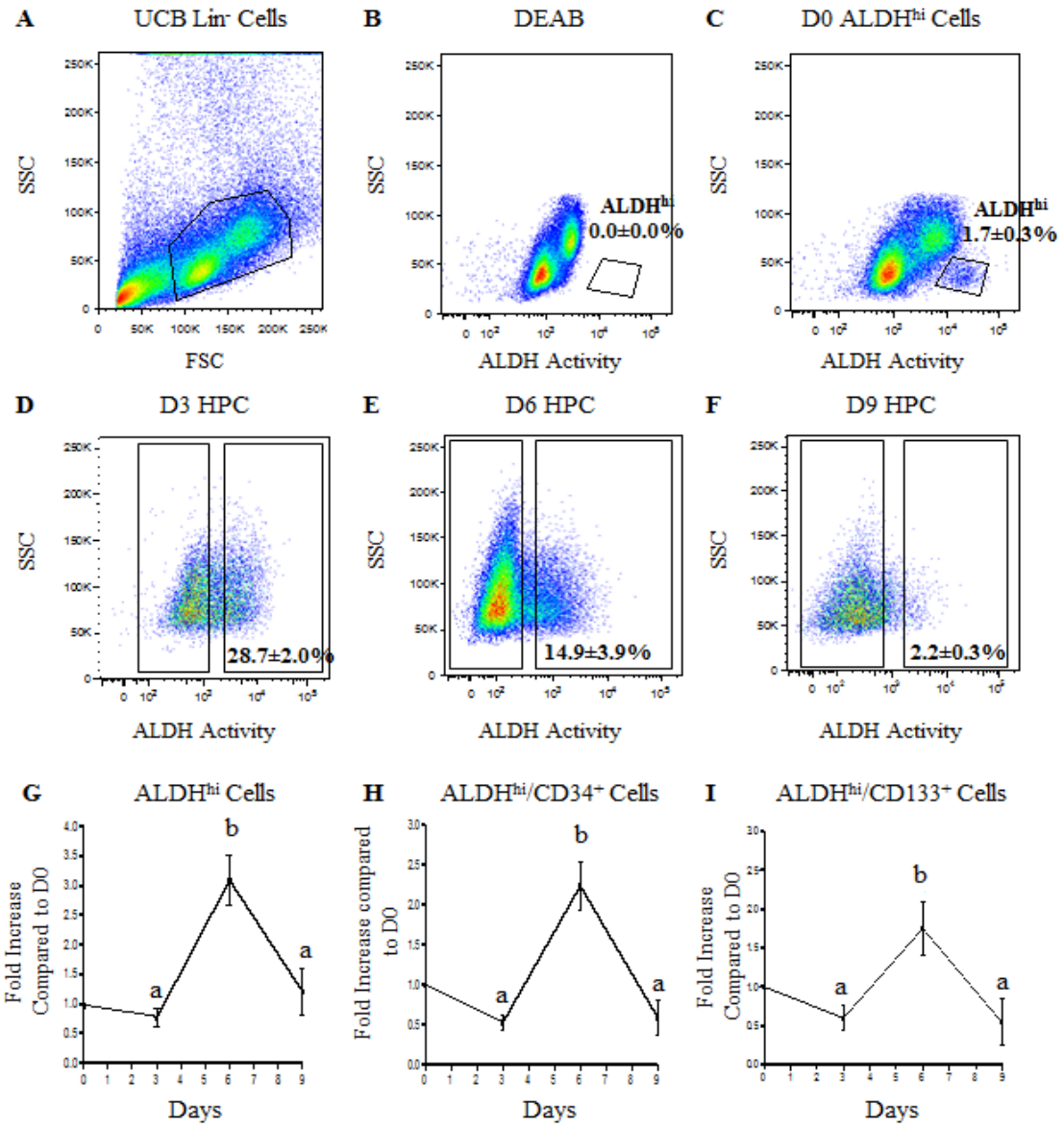

Figure 2.1 Isolation and expansion kinetics of human UCB ALDH^{hi} cells. Representative flow cytometry plots showing the initial purification of human UCB ALDH^{hi} cells. Lineage depleted UCB cells were first gated using (**A**) forward and side scatter properties. (**B**) DEAB-treated controls were used to establish gates for the selection of (**C**) ALDH^{hi} cells using the Aldefluor^{TM} assay. Purified UCB ALDH^{hi} cells represented 1.7±0.3% of the total Lin^{-} cell population. (**D-F**) Representative FACS analysis showing the frequency of culture-expanded HPC that retained high ALDH-activity after culture for 3, 6, or 9. The frequency of HPC retaining high ALDH-activity was diminished as culture time progressed. (**G-I**) Total cell expansion at days 3, 6, or 9 for ALDH^{hi} cells, ALDH^{hi}/CD34^{+} cells, and ALDH^{hi}/CD133^{+} cells compared to the number of cells of each subset seeded at day 0. At day 6 cultured HPC demonstrated highest expansion of ALDH^{hi} cells, ALDH^{hi}/CD34^{+} cells, and ALDH^{hi}/CD133^{+} cells compared to 3 day or 9 day cultured HPC. Data represent mean ± SEM from 5-7 UCB samples (a<b, $p<0.05$).

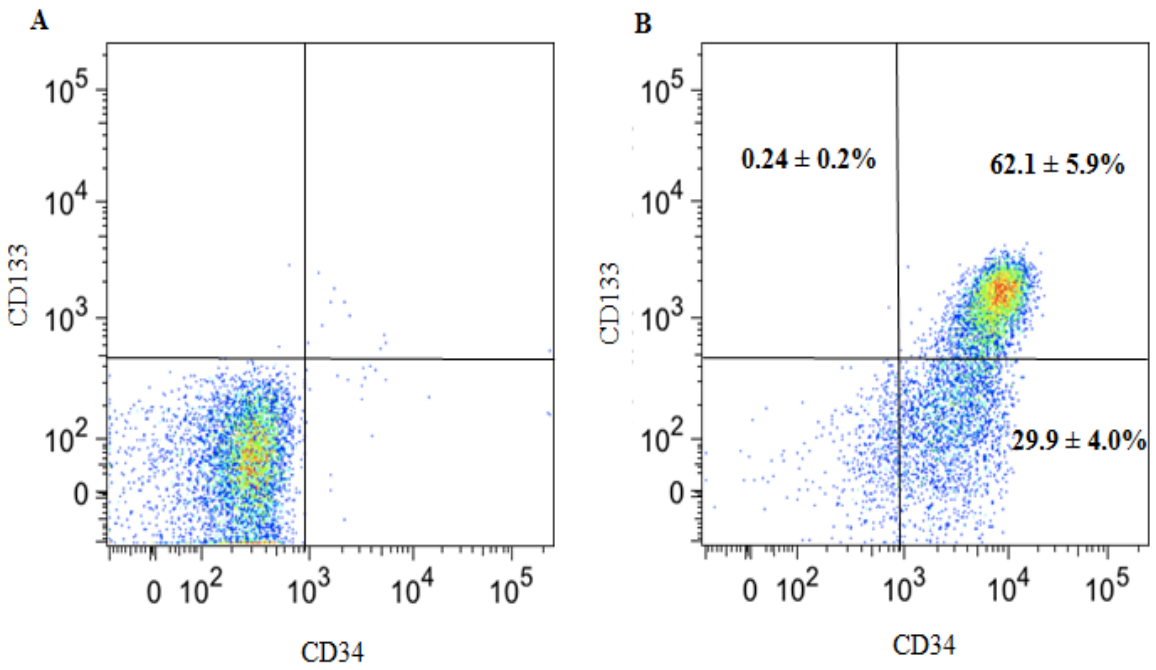

Figure 2.2 . Fresh UCB cells with high ALDH-activity (ALDH^{hi} UCB) co-expressed hematopoetic progenitor cell surface markers. Representative flow cytometry plots showing the analysis of fresh ALDH^{hi} progenitor cells isolated from Lin^{-} UCB. Specifically, flow cytometry plots of (**A**) Isotype control (**B**) of primitive cell surface markers expressed on fresh ALDH^{hi} UCB cells. Data represent mean ± SEM from 3 UCB samples.

Table 2.1 Total cell expansion of ALDH^{hi} UCB cells after 3, 6, or 9 days of culture.

Days of Culture	Sample ID	D0 Cell Number (x 10^5)	Final Cell Number (x 10^5)	Total Cell Expansion (Fold Increase)
	Sample 1	4.0	6.2	1.6
	Sample 2	1.0	1.2	1.2
	Sample 3	0.5	1.3	2.6
	Sample 4	1.1	3.0	2.8
	Sample 5	3.0	10.0	3.3
3	**Mean**	**1.9±0.7**	**4.4±1.5**	**2.3±0.4**
	Sample 6	2.3	30.3	13.4
	Sample 7	1.1	17.6	15.4
	Sample 8	2.9	67.6	23.4
	Sample 9	3.2	76.6	24.3
	Sample 10	5.0	99.2	19.9
	Sample 11	0.6	17.0	28.4
	Sample 12	1.1	21.2	19.8
6	**Mean**	**2.3±0.6**	**47.1±1.3**	**20.7±2.0**
	Sample 13	4.0	312.0	78.0
	Sample 14	0.8	23.8	31.4
	Sample 15	1.1	76.1	71.0
	Sample 16	1.2	62.9	53.8
	Sample 17	2.3	77.8	33.2
9	**Mean**	**1.9±0.6**	**110.5±51.3**	**53.5±9.5**

Human ALDH^{hi} UCB cells were cultured for 3, 6, or 9 days, and total cell number and total cell fold expansion was quantified at each time point, by comparing the number cells at each day to the number of ALDH^{hi} cells seeded at day 0. Data represent mean ± SEM from 5-7 UCB samples.

2.3.2 The Culture-Expanded ALDH^{hi} HPC Subset Co-Expressed Primitive Hematopoietic Progenitor Cell Surface Markers

After 6 days of expansion 14.9±3.9% of cells retained high ALDH activity (Figure 2.1E). Therefore, the 6 day culture-expanded HPC population was categorized as Bulk HPC, ALDH^{lo} HPC, and ALDH^{hi} HPC subsets based on DEAB controls (Figure 2.3A-C), and each population was subsequently analyzed for primitive hematopoietic cell surface markers. Cell surface molecules such as CD34, and CD133 are commonly used to isolate primitive hematopoietic cells with *in vitro* and *in vivo* repopulating function. The expression of these molecules diminish with maturation and differentiation.[1,27,28] These cell surface molecules were highly expressed on freshly isolated BM, and UCB ALDH^{hi} progenitor cells with islet regenerative functions (Figure 2.2).[8,11,12] Compared to the Bulk HPC, and ALDH^{lo} HPC, the ALDH^{hi} HPC subset was highly enriched for primitive hematopoietic cell surface markers (69.3 ± 6.4% CD34^{+}, 40.3 ± 8.1% CD133^{+}, Table 2.2). In addition, the islet regenerative BM and UCB derived ALDH^{hi} progenitor cells are enriched for CD34^{+}/CD38^{-} and CD34^{+}/CD133^{+} primitive progenitor populations (Figure 2.2).[8,27] ALDH^{hi} HPC showed a significantly higher frequency of cells expressing this primitive hematopoietic progenitor cell phenotype (34.8 ± 6.6% CD34^{+}/CD38^{-}, 41.6 ± 7.4% CD34^{+}/CD133^{+}, Figure 2.2D-J) compared to Bulk HPC and ALDH^{lo} HPC subsets. Notably, ALDH^{hi} HPC did not acquire mature monocyte (0.4±0.1% CD14^{+}, Table 2.2) or macrophage markers (0.8 ±0.2% CD11b^{+},Table 2.2). These data suggests that the 6 day culture-expanded ALDH^{hi} HPC sub-population retains a primitive hematopoietic progenitor phenotype, without full differentiation to the monocyte or macrophage lineage.

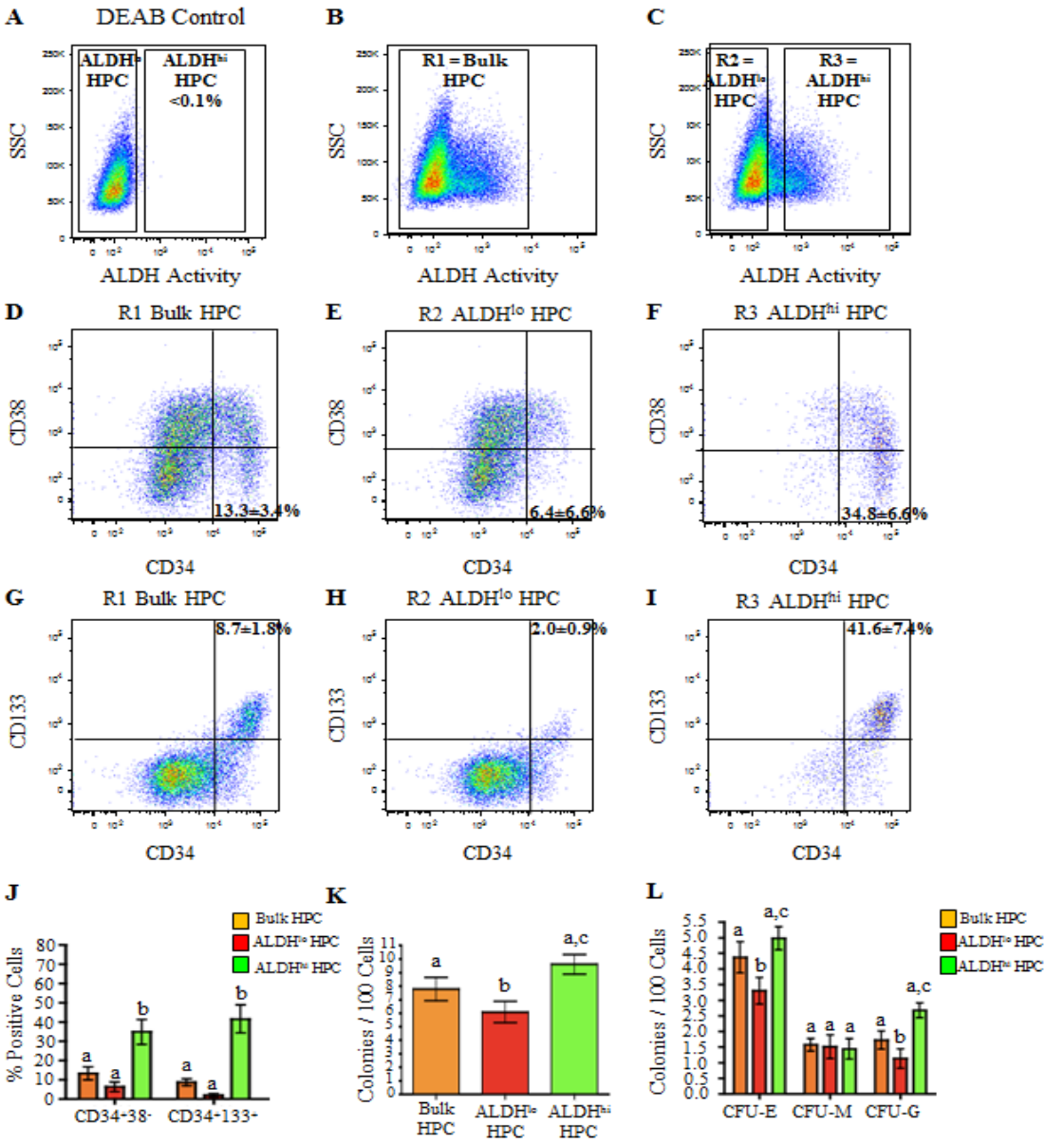

Figure 2.3 Culture-expanded cells retaining high ALDH-activity (ALDH^{hi} HPC) co-expressed progenitor cell surface markers and demonstrated hematopoietic colony forming function *in vitro*. Representative flow cytometry plots showing the analysis of expanded HPC subsets after 6 days culture. (**A**) DEAB controls were used to establish gates for (**B**) Bulk HPC and (**C**) ALDH^{lo} versus ALDH^{hi} HPC subsets. (**D-I**) Representative flow cytometry plots of primitive cell surface markers expressed on expanded HPC subsets. (**J**) ALDH^{hi} HPC showed significantly higher frequency of cells expressing a primitive HPC phenotype (CD34^{+}/CD38^{-}, CD34^{+}/CD133^{+}). (**K-L**) Bulk HPC, ALDH^{lo} HPC, or ALDH^{hi} HPC were cultured in Methylcellulose^{TM} media and colonies of erythrocyte, granulocyte, and macrophage lineages were enumerated after 14 days. ALDH^{hi} HPC were enriched for total hematopoietic colony forming cells, and ALDH^{hi} HPC formed significantly more erythroblast, and granulocyte colonies compared to ALDH^{lo} HPC. Data represent mean ± SEM from 5-6 UCB samples (a<b<c, $p<0.05$).

Table 2.2 Analyses of Bulk HPC, ALDH^{lo} HPC, and ALDH^{hi} HPC for expression of hematopoietic cell surface markers.

	Marker	Total HPC	ALDH^{lo} HPC	ALDH^{hi} HPC
Progenitor	CD34	28.3±4.0%^{a}	15.4±4.7%^{a}	**69.3±6.4%^{b}**
	CD38	37.1±8.9%^{a}	33.7±8.4%^{a}	47.3±9.4%^{a}
	CD133	9.1±1.8%^{a}	2.3±0.9%^{a}	**40.3±8.1%^{b}**
	CD117	2.8±0.5%^{a}	1.9±0.4%^{a}	7.1±1.3%^{a}
Monocyte	CD14	0.4±0.2%^{a}	0.3±0.2%^{a}	0.4±0.1%^{a}
Macrophage	CD11b	0.5±0.2%^{a}	0.4±0.1%^{a}	0.8±0.2%^{a}

ALDH^{hi} HPC demonstrated increased levels of primitive cell surface markers (CD34 and CD133) compared to Bulk HPC and ALDH^{lo} HPC. Data represent mean ± SEM from 4-6 UCB samples ($a<b$, $p<0.05$).

2.3.3 The Culture-Expanded ALDH^{hi} HPC Subset Contained Multipotent Hematopoietic Colony Forming Cells

In order to determine whether ALDH^{hi} HPC retained hematopoietic colony forming cell activity after 6 days of expansion, Bulk HPC or ALDH^{lo} HPC or ALDH^{hi} HPC were plated in methylcellulose media, and the clonogenic hematopoietic colonies formed were enumerated after 14 days. Compared to ALDH^{lo} HPC, the ALDH^{hi} HPC subset was enriched for total multipotent hematopoietic colony cells (9.6 ± 0.7% ALDH^{hi} HPC versus. 6.1 ± 1.7 ALDH^{lo} HPC, Figure 2.3K). Particularly, ALDH^{hi} HPC formed significantly more erythroblast, and granulocyte colonies compared to ALDH^{lo} HPC (Figure 2.3L). Therefore, the ALDH^{hi} HPC subset retained a greater hematopoietic colony forming capacity compared to the ALDH^{lo} HPC subset.

2.3.4 Transplantation of Culture-Expanded ALDH^{hi} HPC Improved Islet function.

Our group has previously found that the transplantation ALDH^{hi} UCB improved the islet function of STZ-NOD/SCID diabetic mice.[11] However, the low number of ALDH^{hi} cells found in UCB limits the widespread application of transplanted UCB-derived progenitor cells to treat diabetes. Therefore, our goal was to expand ALDH^{hi} UCB cells, in hematopoietic culture, while retaining its islet regenerative functions. To investigate the islet regenerative capacity of fresh ALDH^{hi} UCB-derived, culture-expanded HPC populations *in vivo*, STZ-treated NOD/SCID mice were i.v. injected with PBS or 5×10^5 Bulk HPC on day 10, and blood glucose levels were assessed until day 42 (Figure 2.4A). Similar to PBS-injected controls, mice i.v. transplanted with Bulk HPC remained severely hyperglycemic (>25mmol/L,Figure 2.4B), suggesting a loss of islet regenerative function in the Bulk HPC population, as a result of 6 *day ex vivo* expansion. Therefore, the Bulk HPC were re-selected after culture expansion by FACS for the sub-populations with low (ALDH^{lo} HPC) versus high ALDH (ALDH^{hi} HPC) activity (as seen in figure 2.3C). After i.v. transplantation of 2×10^5 cells, mice transplanted with ALDH^{lo} HPC remained severely hyperglycemic (>25mmol/L,Figure 2.4B). Remarkably, mice transplanted with an equal dose ALDH^{hi} HPC demonstrated reduced systemic glucose levels from day 17-42

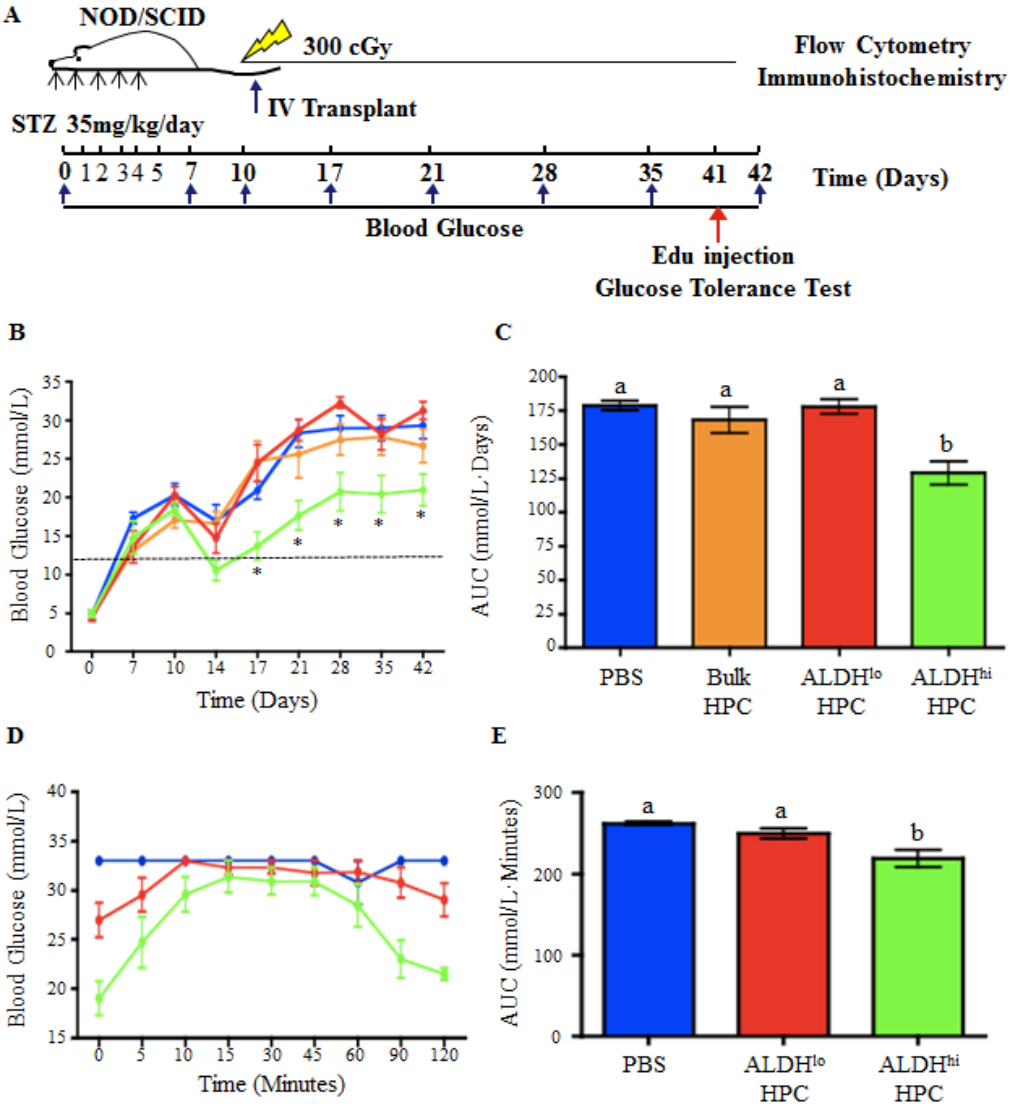

Figure 2.4 Transplantation of culture-expanded ALDH^{hi} HPC improved hyperglycemia and glucose tolerance in STZ-NOD/SCID mice. (**A**) STZ-NOD/SCID mice (35mg/kg/day, days 1-5) were sublethally irradiated and i.v. injected with PBS (blue, n=8 mice); 5x10^5 Bulk HPC (orange, n=9 mice), 2x10^5 ALDH^{lo} HPC (red, n=9 mice), or 2x10^5 ALDH^{hi} HPC (green, n=9 mice) on day 10 and blood glucose levels were monitored for 42 days. (**B**) Compared to mice transplanted with PBS, Bulk HPC or ALDH^{lo} HPC, mice transplanted with ALDH^{hi} HPC showed reduced resting blood glucose levels from days 17-42, and (**C**) reduced area under the curve. (**D**) At day 41 a glucose tolerance test was performed on mice transplanted with PBS (n=3) ALDH^{lo} HPC (n=9) and ALDH^{hi} HPC (n=9). Compared to PBS-injected or ALDH^{lo} HPC, mice transplanted with ALDH^{hi} HPC showed improved glucose tolerance, and (**E**) reduced area under the curve. Data represent mean ± SEM from 5-7 UCB samples (a<b, $p<0.05$; *$p<0.05$).

compared to mice from all other treatment groups (Figure 2.4B). After performing area under the curve (AUC) analyses on all i.v.-transplanted mice, ALDH^{hi} HPC transplanted mice showed significantly reduced hyperglycemia compared to all other treatment groups (Figure 2.4C). Furthermore, compared to mice transplanted with PBS or ALDH^{lo} HPC, mice transplanted with the culture-expanded ALDH^{hi} HPC subset showed improved glucose tolerance (Figure 2.4D-E). This suggests that the i.v. transplantation of ALDH^{hi} HPC subset improved the glucose response in STZ-NOD/SICD mice.

2.3.5 Culture-Expanded ALDH^{hi} HPC Engrafted in the Murine BM

To correlate the improvement of glycemic control with human cell engraftment the BM and the head of the pancreas was collected at day 42, and analyzed for the presence of human CD45+ and HLA-ABC by flow cytometry. Only the culture-expanded ALDH^{hi} HPC subset consistently engrafted in the murine BM, however human cells were not detected in the murine pancreas by flow cytometry at day 42 (Figure 2.5A-C). Although, analysis at earlier time points is required to delineate the recruitment kinetics of ALDH^{hi} HPC to the injured pancreas of STZ-NOD/SCID mice after i.v. injection, our previous studies with freshly isolated ALDH^{hi} UCB cells have documented low level pancreas engraftment at 7 day post-transplantation.[12]

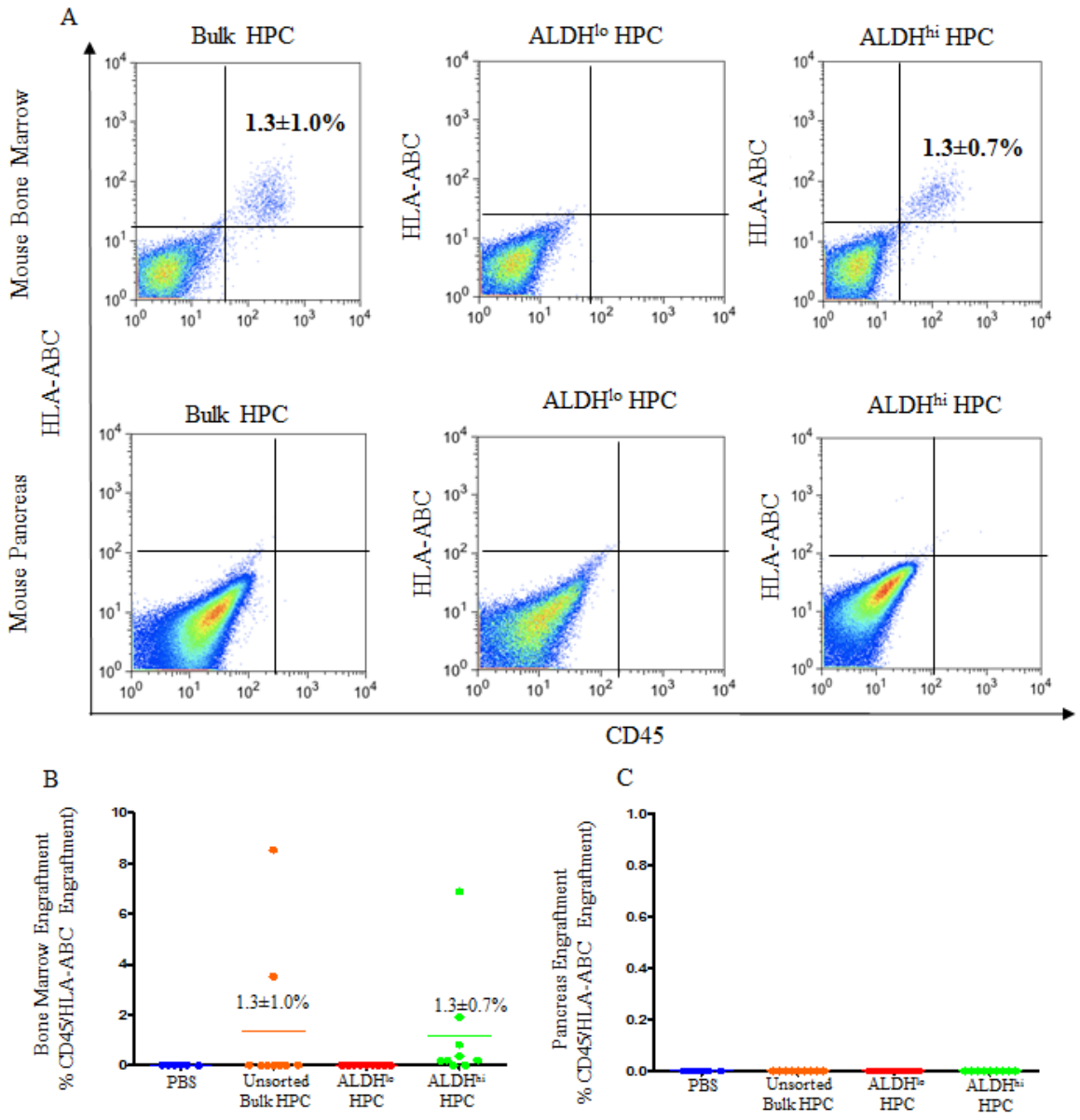

Figure 2.5 Engraftment of human UCB-derived ALDH^{hi} progenitor cells in the BM and pancreas of STZ-NOD/SCID mice. (**A**) Representative flow cytometry plots of human cell engraftment at day 42 in the BM and pancreas of recipient mice i.v. transplanted with Bulk HPC (n=9), ALDH^{lo} HPC (n=9), or ALDH^{hi} HPC (n=9). (**B**) Transplantation of ALDH^{hi} cells, but not Bulk HPC or ALDH^{lo} HPC, resulted in consistent human hematopoietic chimerism in mouse BM. (**C**) Bulk HPC, ALDH^{lo} HPC or ALDH^{hi} HPC were not detected in the mouse pancreas at day 42. Data Represents Mean ± SEM from 5-7 UCB samples.

2.3.6 Transplantation of Culture-Expanded ALDH^{hi} HPC Increased Islet Size, and β Cell Mass

To determine the mechanisms by which ALDH^{hi} HPC improved glycemic control, pancreas sections of transplanted mice were stained for murine insulin (Figure 2.6A-D), and analyzed for islet size (Figure 2.6E), islet number (Figure 2.6F), and total β cell mass (Figure 2.6G). Mice transplanted with Bulk HPC, and ALDH^{lo} HPC had a similar islet size, islet number and β cell mass as PBS injected controls. In contrast, ALDH^{hi} HPC transplanted mice showed a significant ($p<0.05$) increase in islet size (circumference), and β cell mass, but not islet number compared to mice from all other treatment groups. All islets were derived from murine cells suggesting that transplantation ALDH^{hi} HPC improves islet function by stimulating an endogenous islet regeneration.

2.3.7 Transplantation of Culture-Expanded ALDH^{hi} HPC Increased Islet Vascularization

We postulated that improved hyperglycemia (Figure 2.4) and increased islet size (Figure 2.6E) after culture-expanded ALDH^{hi} HPC transplantation may also correlate with progenitor-stimulated islet vascularization.[7,11,12] Thus, mouse pancreas sections were stained with murine insulin and CD31-a marker of endothelial cells within blood vessels at day 42 (Figure 2.7 A-F). The intra-islet and extra-islet microvessel densities in mice transplanted with Bulk HPC and ALDH^{lo} HPC were similar to PBS injected mice (Figure 2.7A-C). Interestingly, mice transplanted with ALDH^{hi} HPC showed augmented islet vascularization with increased numbers of CD31+ cells (Figure 2.7D, arrowheads). Furthermore, higher zoom of the representative inset shows CD31 expressing cells in close association with insulin+ β cells within the regenerating islet (Figure 2.7E), and CD31+ cells were distinct and formed continuous vessels (Figure 2.7F, arrows). Histomorphometric quantification of intra-islet capillary density was significantly increased ($p<0.05$) in ALDH^{hi} HPC transplanted mice, compared to all other treatment groups (Figure 2.7F). Interestingly, similar analyses quantifying extra-islet vascularization were equivalent (Figure 2.7H), when compared to mice from all other treatment groups.

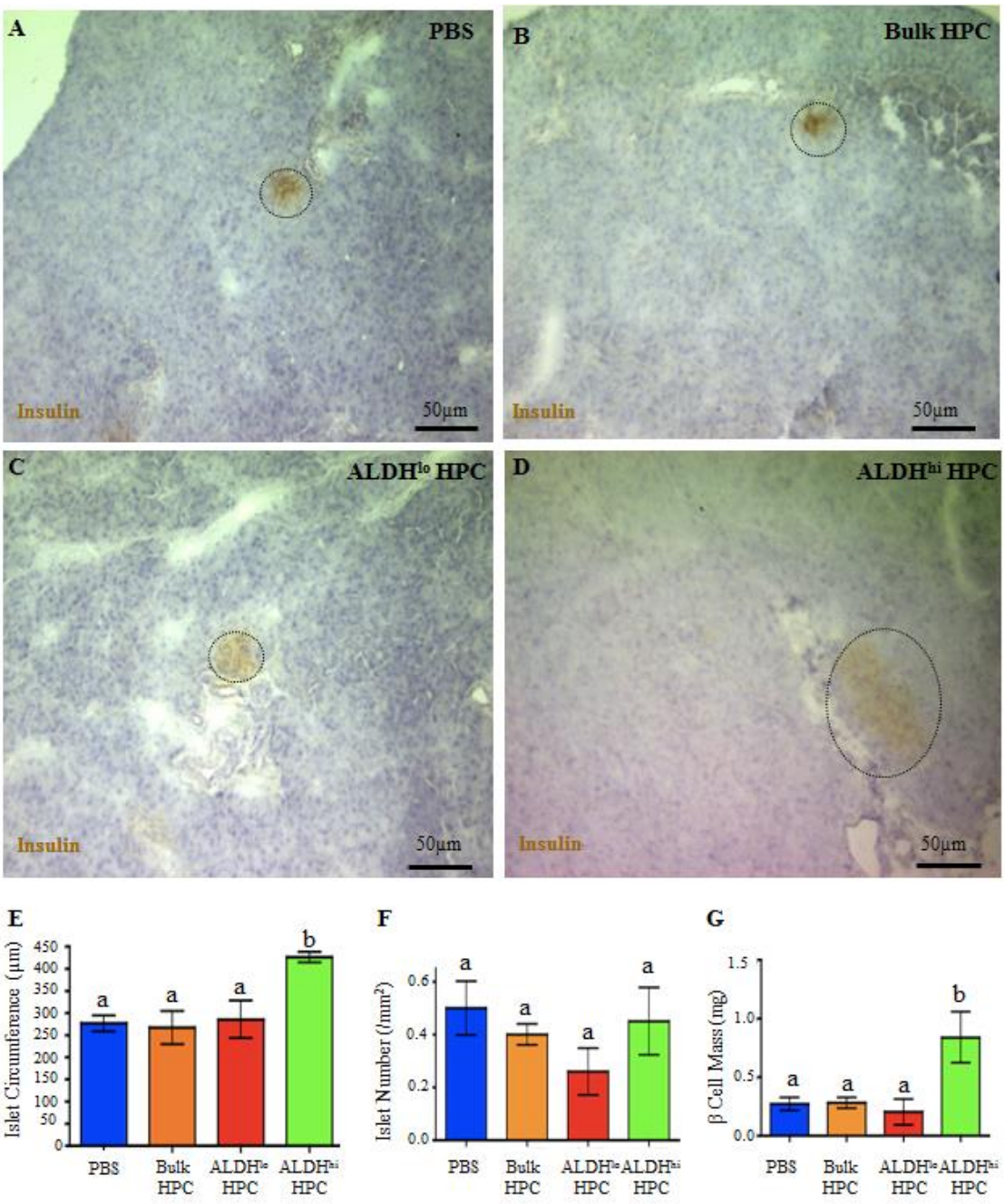

Figure 2.6 Transplantation of culture-expanded ALDH^{hi} HPC increased islet size and β cell mass without increasing total islet number. Representative photomicrographs of islet structures (brown) at day 42 in STZ-NOD/SCID mice i.v. injected with (**A**) PBS (n=6), (**B**) Bulk HPC (n=9), (**C**) ALDH^{lo} HPC (n=5), or (**D**) ALDH^{hi} HPC (n=6). Compared to PBS controls, or mice transplanted with Bulk HPC or ALDH^{lo} HPC, mice transplanted with ALDH^{hi} HPC showed (**E**) significantly increased islet circumference, **(F)** equivalent islet number, (**G**) and increased β cell mass. Data represent mean ± SEM from 3-6 UCB samples (a<b, $p<0.05$).

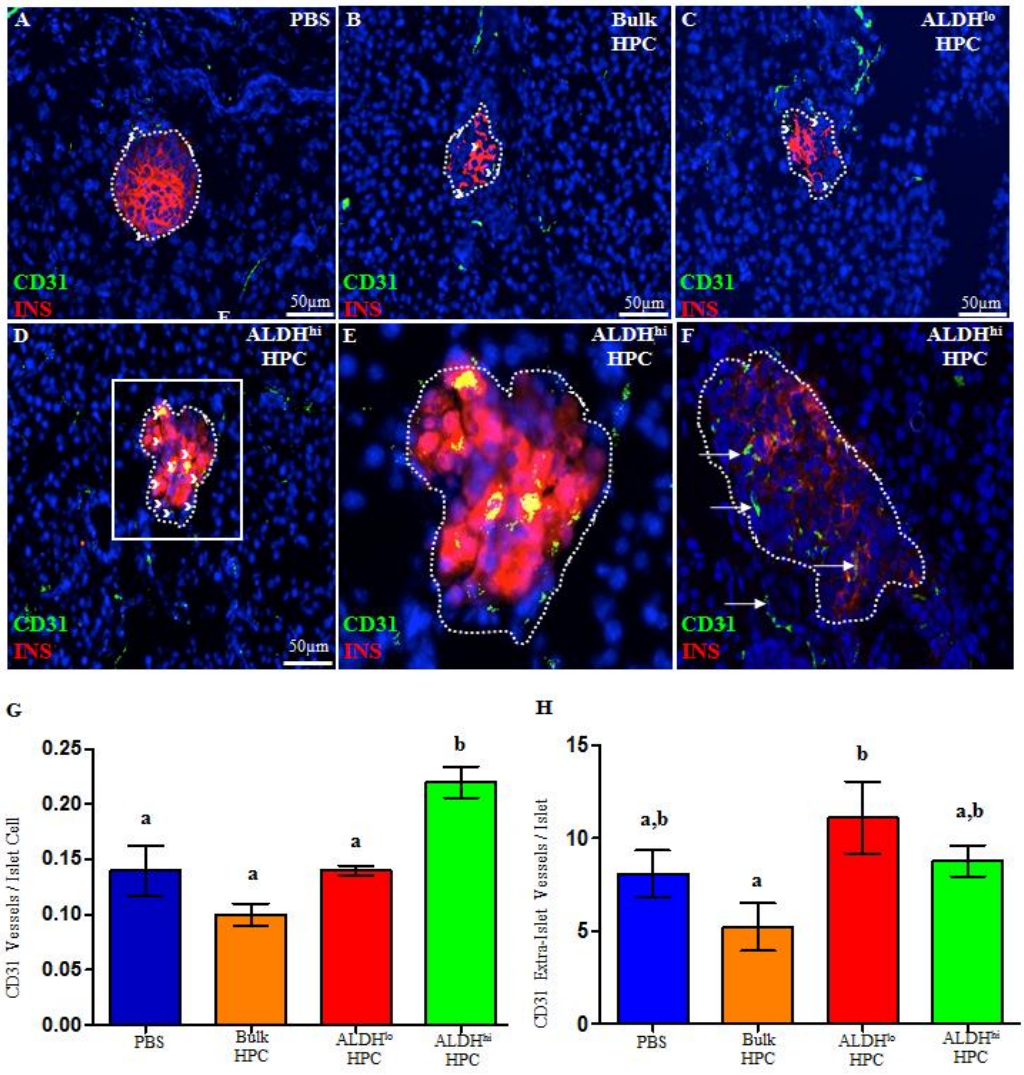

Figure 2.7 Transplantation of culture-expanded ALDH^{hi} HPC increased islet vascularization. Representative photomicrographs of CD31+ blood vessels (green) associated with insulin+ islets (red) at day 42 in STZ-NOD/SCID mice transplanted with (**A**) PBS (n=7), (**B**) Bulk HPC (n=9), (**C**) ALDH^{lo} HPC (n=5), or (**D**) ALDH^{hi} HPC (n=8). Arrowheads mark CD31+ cells within islets. (**E**) Higher zoom of the inset shown in (**D**) clearly shows CD31+ cells in close association with insulin expressing β cells within regenerating islets. (**F**) Transplantation of UCB ALDH^{hi} HPC also induced the appearance of CD31+ vessel structures within and immediately surrounding insulin+ islets. Arrows mark CD31+ continuous vessels. (**G**) Compared to mice transplanted with PBS, Bulk HPC, and ALDHl^{o} HPC, mice transplanted with ALDH^{hi} HPC showed increased vascularization within islets, but not in (**H**) extra-islet areas. Data represents mean ± SEM from N=3-6 UCB samples (a<b, $p<0.05$).

These data suggest that transplantation of ALDH^{hi} HPC stimulated islet-specific revascularization.

2.3.8 Mice Transplanted with Culture-Expanded ALDH^{hi} HPC Demonstrated an Increase in Proliferating Islets

To further investigate an increase in islet size, EdU incorporation into proliferating islets were analyzed at day 42 (Figure 2.8A-D). According to a student's t-test, compared to mice transplanted with PBS, ALDH^{hi} HPC transplanted mice demonstrated a significantly higher % EdU^{+} islet cells (Figure 2.8E), and a significant increase ($p<0.05$) in the % islets with at least one EdU^{+} proliferating cell (Figure 2.8F). Our previous studies with freshly isolated ALDH^{hi} cells have documented augmented islet proliferation at 7 day post-transplantation.[12] Although analyses of islet proliferation at earlier time points were not performed, these data suggest that transplantation of culture-expanded ALDH^{hi} HPC stimulated islet proliferation.

2.3.9 Culture-Expanded ALDH^{hi} HPC Demonstrated a Pro-angiogenic Transcription Profile

Affymetrix microarray analyses were performed on three ALDH^{hi} HPC samples compared to sample matched ALDH^{lo} HPC, and 406 transcripts with significantly ($p<0.05$) increased expression were identified. These samples were filtered for documented angiogenic biological function. As an internal control for the selection of HPC with high ALDH activity, ALDHA1 mRNA expression was higher by 3.7-fold in ALDH^{hi} HPC versus ALDH^{lo} HPC (Table 2.2). Furthermore significantly higher CD34, PROM1 (Table 2.3) mRNA expression in ALDH^{hi} HPC correlated with increased CD34, and CD133 cell-surface protein expression, previously observed by flow cytometry (Table 2.2). Interestingly, transcripts associated with vascular cell signaling, and angiogenic cytokines were increased in ALDH^{hi} HPC, compared to the ALDH^{lo} HPC subset (Table 2.4).

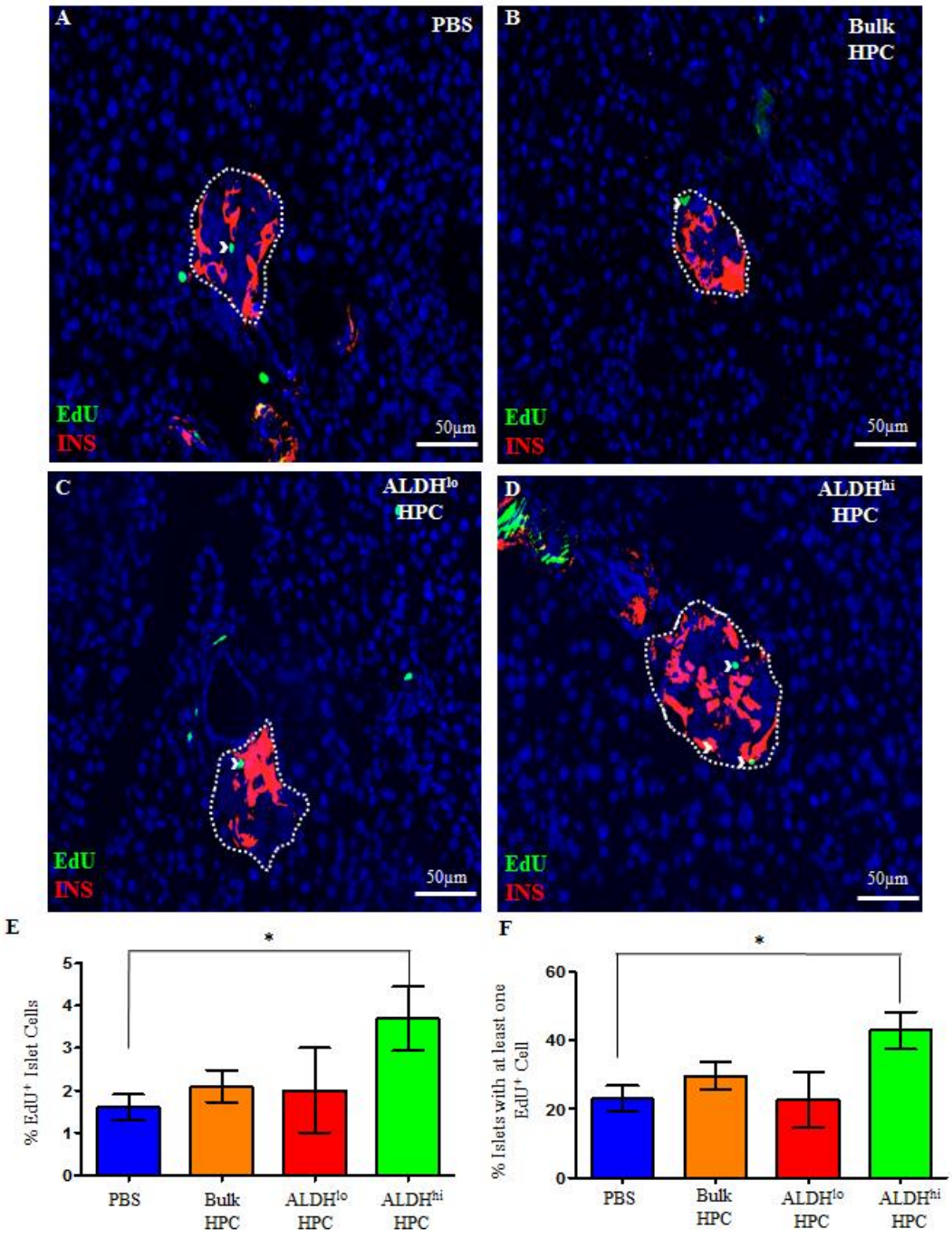

Figure 2.8 Transplantation of culture-expanded ALDH^{hi} HPC increased proliferating Islets. Representative photomicrographs of EdU^{+} proliferating cells (green) within insulin^{+} islets (red) at day 42. STZ-NOD/SCID mice transplanted with (**A**) PBS (n=4), (**B**) Bulk HPC (n=7), (**C**) ALDH^{lo} HPC (n=4), or (**D**) ALDH^{hi} HPC (n=7). Arrowheads mark EdU^{+} proliferating cells within islets. (**E**) Compared to mice transplanted with PBS mice transplanted with ALDH^{hi} HPC showed an increase in percentage of EdU^{+} cells within islets and, (**F**) an increase in the percentage of islets with at least one EdU^{+} proliferating cell. Data represent mean ± SEM from 3-5 UCB samples (*$p<0.05$).

Table 2.3 . Progenitor cell-associated transcripts encoding progenitor cell markers, with increased expression in ALDH^{hi} HPC versus ALDH^{lo} HPC

Gene Symbol	Common Names	Biological Function	Molecular Function	FC vs. ALDH^{lo} HPC	p-value vs. ALDH^{lo}
ALDH1A1	Aldehyde dehydrogenase 1	Retinaldehyde metabolism, retinoic acid production	Cytosolic enzyme	3.71987	0.00408895
PROM1	Prominin 1, CD133	hematopoiesis, vascular development / remodeling	MAPK and Akt signaling	2.8084	2.54E-05
CD34	CD34	hematopoiesis, vascular development / remodeling	adherence to BM matrix and cells	2.60607	0.00839392

FC Fold change **MAPK** mitogen-activated protein kinase **CD** cluster of differentiation.

Table 2.4 Angiogenesis-associated transcripts encoding secreted proteins, and cell surface proteins with increased expression in ALDH^{hi} HPC versus ALDH^{lo} HPC

Gene Symbol	Common Names	Biological Function	Molecular Function	FC vs. ALDH^{lo} HPC	p-value vs. ALDH^{lo}
GAPT	GRB2-binding Adaptor Protien	Intracellular signaling molecule downs stream of angiogenic factors	Active in endothelial cells during migration, and proliferation	2.74167	0.00147562
HOXA7	HomeoboxA7	Endothelial cell differentiation	Transcription Factor	1.93463	0.00318028
TIE1	Tyrosine kinase with immunoglobulin-like and EGF-like domain 1	Angiopoietin dependent angiogenesis	Modulates TEK/TIE2 binding of angiopoietins	1.81964	0.0142529
VAV3	VAV3 guanine gucleotide exchange factor	Linked to EphA2 receptor to Rho family GTPase activation	When activated by EphA2, it activates Rho GTPase	1.81241	4.62E-05
SEMA4A	Semaphorin 4A	VEGF dependent angiogenesis	Enhances VEGFA expression in macrophages	1.77569	3.96E-05
TEK	Tyrosine kinase with immunoglobulin-like and EGF-like domain 2	Angiopoietin dependent angiogenesis	Tyrosine kinase activity binds angiopoietins	1.68008	0.000206671
ANGPT2	Angiopoietin 2	Angiopoitin dependent angiogenesis	Binds to TIE2 and inhibits angiopoietin 1 dependent TIE 2 signaling	1.66868	0.0108526
TIMP2	TIMP metallopeptidase inhibitor 2	VEGF dependent angiogenesis	Binds aplha 3 beta 1. decreases total protein tyrosine phosphatase (PTP) activity.	1.62387	0.000224585
HS6ST1	Heparan sulfate 6-O-Sulfotransferase 1	Involved in angiogenesis	Sulfates glucosamide residues, important in cytokine signaling	1.60044	0.00113202
MFGE8	Milk fat globule-EGF factor 8 protein, lactdherin	VEGF dependent angiogenesis	Interacts with alphavbeta3 and alphavbeta5 integrins to mediate VEGF mediated AKT phophorylation.	1.59494	0.00258047
AKT3	Akt murine thymoma viral oncogene homolog 3	Signaling molecule downstream of VEGF signaling	Stabilized in response to VEGF signaling	1.58394	0.000231637
ELK3	ETS domain-containing protein, ETS-related protein, NET	VEGF dependent angiogenesis	Binds to and stimulates the mouse VEGF promoter	1.56375	0.000144384
TGFBR1	Transofrming growth factor β receptor 1	TGF dependent angiogenesis	Binds TGF β family members	1.53514	0.00367309

FC Fold change **EGF** epidermal growth factor **TIMP** tissue inhibitors of metalloaproteinases **ETS** E26 transformation-specific

2.4 Discussion

These studies demonstrated that the HPC lineage of fresh ALDH^{hi} UCB cells expands efficiently in culture, and that only the HPC sub-population that retains high ALDH activity stimulates islet regeneration when i.v.-transplanted into STZ-NOD/SCID mice. UCB ALDH^{hi} cells expanded in serum-free media supplemented with hematopoietic cytokines SCF, TPO, and FLT-3L. Although, the highest number of total HPC was observed after 9 days of *ex vivo* expansion, the highest number of ALDH^{hi} HPC was observed after 6 days of *ex vivo* expansion. Interestingly, the culture-expanded ALDH^{hi} HPC subset demonstrated primitive hematopoietic progenitor cell phenotype, and multipotent colony formation. In addition, the ALDH^{hi} HPC subset highly expressed pro-angiogenic transcripts. Transplantation of 6 day culture-expanded ALDH^{hi} HPC also improved islet function, increased β cell mass, and augmented islet vascularization in STZ-NOD/SCID mice. Collectively, these data suggest that culture-expanded ALDH^{hi} HPC from human UCB represent a readily available, and novel population for the development of cellular therapies to promote endogenous islet regeneration.

UCB ALDH^{hi} progenitor cells were highly proliferative in culture. We achieved a total cell expansion of 2.3-fold, 20.7-fold, and 53.5-fold after 3, 6, and 9 days of culture, respectively. Studies have shown that culturing UCB-derived progenitor cells with hematopoietic cytokines results in a significant expansion of committed progenitor cells, with only a minimal expansion of primitive progenitor cells.[25] We used relative ALDH activity after culture to distinguish primitive hematopoietic progenitor cells (ALDH^{hi} HPC), from culture differentiated, more committed progenitor cells (ALDH^{lo} HPC). Compared to ALDH^{lo} HPC, ALDH^{hi} HPC demonstrated increased expression of primitive cell surface markers, and were enriched for multipotent colony forming cells *in vitro*. Similar to previous studies[25,26,29,30], we found that the frequency of primitive progenitor cells (ALDH^{hi} HPC) declined as culture progressed. Furthermore, committed progenitor cells in culture have been shown to secrete factors such as TGFβ that limit the proliferation of primitive progenitor cells.[31–36] Due to the accumulation of inhibitory soluble factors in primary HPC cultures, we wanted to determine the time point that yielded the highest number of primitive ALDH^{hi} HPC. These analyses revealed that 6 days of *ex vivo*

expansion, yielded the highest number of $ALDH^{hi}$, $ALDH^{hi}/CD34^{+}$ HPC, and $ALDH^{hi}$/CD133+ HPC, with a drastic decrease in HPC with these primitive phenotypes at day 9. Thus, we characterized the islet regenerative properties using day 6 culture-expanded HPC subpopulations purified using low versus high ALDH activity.

The endocrine pancreas is a highly vascularized tissue, it has 5-7 times more capillaries than the surrounding exocrine pancreas tissue.[37] A dense capillary network is required for β cell to optimally sense blood glucose levels, and the fenestrations in the islet capillary endothelium is the most efficient way for insulin to be secreted by β cells into the systemic circulation.[38] Thus, the islet capillary network is essential for optimal islet function. Furthermore, hepatocyte growth factor (HFG) secreted by endothelial cells, and laminin which is presented on the basement membrane of the islet capillary network have been shown to stimulate β cell proliferation.[39,40] Collectively, these studies suggest that endothelial-endocrine axis plays an important role in supporting β cell function, and β cell turnover. Previously, we have shown that freshly isolated $ALDH^{hi}$ UCB cells contains pro-angiogenic progenitor cells that improve perfusion after transplantation into mice with hind-limb ischemia.[7] Here we show that culture-expanded $ALDH^{hi}$ HPC, derived from freshly isolated UCB cells with high ALDH activity, enhanced islet-specific vascularization in the STZ-NOD/SICD mouse pancreas. Increased vascularization was specific to the islets, rather than extra-islet pancreatic tissue, suggesting that culture-expanded $ALDH^{hi}$ HPC stimulates an islet specific vascularization program after transplantation into STZ-NOD/SCID mice. Interestingly, an increased vascularization was associated with an increased islet size, β cell mass, and an improved islet function. Collectively, our data suggest that $ALDH^{hi}$ HPC improves islet function in STZ-NOD/SCID mice, by re-establishing the endothelial-endocrine axis.

This study also begins to address the transcriptional, and secretory activities of 6 day culture-expanded $ALDH^{hi}$ HPC. The transcripts of the secreted proteins Semaphorin 4A (SEMA4A), Angiopoietin 2 (ANGPT2), and Milk Fat Globule-EGF factor 8 (MFG-E8) were higher in culture-expanded $ALDH^{hi}$ HPC compared to $ALDH^{lo}$ HPC. The proteins encoded by these transcripts are all involved in supporting vascular endothelial growth factor-A (VEGFA) mediated angiogenesis. MFG-E8 also known as lactadherin has been

shown to interact with $\alpha_v\beta3$ integrins on endothelial cells, and has been shown to be critical in VEGFA induced angiogenesis.[41] In the hind-limb ischemic model, injection of anti-MFG-E8 blocked VEGFA induced angiogenesis.[41] The transcript of ANGPT2 was also higher in culture-expanded ALDH^{hi} HPC. Studies have shown that, ANGPT2 is a complex regulator of vascular remodelling that plays a role in both vessel sprouting, and vessel regression. Studies have suggested that ANGPT2 in the presence of VEGF stimulates vessel sprouting.[42–47] Pancreatic β cells express high levels of VEGF.[38,48,49] It is plausible that MFG-E8, and ANGPT2 secreted by ALDH^{hi} HPC stimulate islet vascularization, by supporting a VEGF dependent angiogenic program.

Finally, the transcript of SEMA4A was higher in culture-expanded ALDH^{hi} HPC. SEMA4A has been shown to act on PlexinD1 receptors on macrophages, to stimulate the release of VEGFA from macrophages.[50] The VEGFA subsequently secreted from macrophages, after SEM4A stimulation exerted a pro-angiogenic effect on endothelial cells *in vitro*, and *in vivo*. Mouse macrophages are recruited to pancreas during injury.[51] SEM4A produced by ALDH^{hi} cells may act on the PlexinD1 receptors on these mouse macrophages to stimulate VEGFA release, which subsequently supports angiogenesis in the pancreas of STZ-diabetic mice. Collectively, these data suggest that a pro-angiogenic microenvironment may be established in the pancreas of STZ-NOD/SCID diabetic mice, after the transplantation of ALDH^{hi} HPC.

Intra-pancreatic transplantation of freshly isolated UCB cells with high ALDH activity stimulated islet revascularization, and β cell proliferation in STZ-NOD/SCID diabetic mice, despite the low level of engraftment of UCB ALDH^{hi} cells in the pancreas (0.09 ± 0.03%, n=6 mice), at day 42.[11] The freshly isolated ALDH^{hi} UCB cells created a regenerative niche around the islets 7 days after transplantation, and initiated a regenerative process. Pancreatic β cells, and ductal cells secrete cytokines, and chemokines after damage that recruit angiogenic cells to the pancreas. However, in this study human cells were not detected in the murine pancreas, at day 42, after i.v. transplantation of culture-expanded ALDH^{hi} HPC. It is possible that i.v.-injected culture-expanded ALDH^{hi} HPC are recruited to the damaged islets at early time points, surround these damaged islets and secrete factors that stimulate islet regeneration. Due to the unfavourable environment of the pancreas,

culture-expanded $ALDH^{hi}$ HPC may undergo apoptosis, or migrate away from the pancreas before day 42. IPan transplantation of culture-expanded $ALDH^{hi}$ HPC may further increase pancreas engraftment, and the islet regenerative response even though $ALDH^{hi}$ HPC may not survive, or migrate away from the pancreas after regenerative programs are initiated.

This study shows that it is only primitive $ALDH^{hi}$ HPC that stimulates endogenous islet regeneration. Currently, researchers are attempting to develop protocols aimed at efficiently expanding primitive HPC, and limiting unwanted cellular differentiation during *ex vivo* expansion.[22,52–55] For instance, Boitano et al[56] have found that StemRegenin1 (SR1)-an aryl hydrocardon receptor antagonist-promotes the expansion of human hematopoietic stem and progenitor cells. When added to serum free media supplemented with hematopoietic growth factors this compound increased the number of primitive $CD34^+$ cells by 73-fold, when compared to cells cultured with only growth factors. In addition, the expanded $CD34^+$ cells retain both their multilineage, and long-term engraftment potential. Hence, small molecules such as SR1 may be used in future experiments to increase the number of HPC available for the treatment of diabetic patients.

In Summary, these studies provide proof-of-concept that $ALDH^{hi}$ UCB cells can be expanded under clinically applicable hematopoietic culture conditions, and it is only the culture-expanded primitive progenitor population ($ALDH^{hi}$ HPC) that retains islet regenerative function, after transplantation *in vivo*. Thus, optimization of *ex vivo* expansion strategies, and reselection of primitive cells using ALDH activity, may potentially lead to the development of a cell based therapy to treat diabetes using culture-expanded allogeneic UCB. Further elucidation of the mechanism by which progenitor cells stimulate islet regeneration is also required to maximize islet regenerative therapies to treat diabetic patients.

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3.0 Discussion

3.1 Summary

The primary objective of this research, was to determine whether expanded UCB ALDH^{hi} cells could stimulate endogenous islet regeneration after transplantation into STZ-NOD/SCID mice. UCB ALDH^{hi} cells were cultured for 6 days under hematopoietic serum-free culture conditions containing the obligate growth factors, SCF, TPO, and FLT-3L. Maximal total cell expansion (53.5 ± 9.0-fold) was observed after 9 days of *ex vivo* culture, however, as culture time proceeded the frequency of cells retaining high ALDH activity and primitive cell surface phenotype (CD34, CD133) was diminished. Thus, the highest total number of ALDH^{hi} HPC was observed after 6 days of *ex vivo* expansion, resulting in a 3.09 ± 1.1-fold expansion of total ALDH^{hi} cells. Due to these consistent expansion kinetics, the total HPC population was categorized into Bulk HPC, ALDH^{lo} HPC, and ALDH^{hi} HPC subsets. The ALDH^{hi} HPC subset was highly enriched for primitive progenitor cell surface marker expression (CD34^{+}/CD38^{-}, CD34^{+}/CD133^{+}), and retained multipotent colony forming capacity in clonal methylcellulose colony forming assays *in vitro*. Thus, we characterized the islet regenerative properties using day 6 culture-expanded HPC subpopulations purified using low versus high ALDH activity.

We have previously shown that freshly isolated UCB-derived ALDH^{hi} cells could reduce hyperglycemia after transplantation into STZ-NOD/SCID mice.[1] In order to address whether culture-expanded HPC, derived from ALDH^{hi} UCB cells retained the capacity to reduced hyperglycemia, STZ-NOD/SCID hyperglycemic mice were transplanted with Bulk, ALDH^{lo} or ALDH^{hi} HPC. Mice transplanted with culture-expanded Bulk HPC or ALDH^{lo} HPC remained severely hyperglycemic. Interestingly, transplantation of ALDH^{hi} HPC consistently reduced hyperglycemia via increased islet size, and islet vascularization upon post-mortem histochemical analyses. ALDH^{hi} HPC transplant recipients had a significant increase in total β cell mass, increased islet-specific proliferation, and showed improved response to glucose bolus injection in glucose tolerance tests. Furthermore, the culture-expanded ALDH^{hi} HPC sub-population highly expressed pro-angiogenic transcripts by Affymetrix microarray, compared to the more differentiated ALDH^{lo} HPC

subset. These findings suggest that HPC retaining high ALDH activity mediated the recovery from hyperglycemia, by stimulating an islet proliferative and islet vascularization.

3.2 The HPC Lineage of ALDH^{hi} UCB Progenitor Cells Expanded Efficiently in Culture.

The HPC lineage of UCB ALDH^{hi} cells was highly proliferative in culture. We achieved a total cell expansion of 2.3-fold, 20.7-fold, and 53.5-fold after 3, 6, and 9 days of culture, respectively. Culturing UCB-derived progenitor cells with hematopoietic cytokines results in a significant expansion in committed progenitor cells, with only a minimal expansion of primitive progenitor cells.[2,3] Also, prolonged culture of UCB-derived progenitor cells under serum-free hematopoietic culture conditions, results in unwanted cellular differentiation. For instance, Bhatia *et al.*[3] showed that, UCB-derived CD34$^+$ cells expanded by 4-fold after 4 days of culture. These cells still expressed a primitive progenitor phenotype (CD34$^+$/CD38$^-$), and retained primitive SCID repopulating cells (SRC). Whereas, 9 days of culture resulted in an 8-fold expansion of total cells, but contained no CD34$^+$/CD38$^-$ primitive progenitor cells, and loss of SRC function. We used relative ALDH activity to distinguish primitive hematopoietic progenitor cells, from more differentiated or committed progenitor cells. Similar to Bhatia *et al.*, we found that the frequency of primitive progenitor cells declined as culture progressed.

Furthermore, maturing progenitor cells in culture have been shown to secrete inhibitory factors such as TGF-β that limit the proliferation and expansion of more primitive progenitor cells.[2,4] The TGF-β superfamily are pleotropic factors that regulate somatic tissue development, and regeneration.[5] When TGF-β1 was added to liquid hematopoietic cultures it inhibited the proliferation of the primitive CD34$^+$/CD38$^-$ cells, while stimulating the proliferation of the more mature CD34$^+$/CD38$^+$ cells.[6] Studies investigating the mechanism by which TGF-β1 limits primitive progenitor cell expansion have shown that, TGF-β1 modulates CD-117 (c-kit receptor, binds SCF), TPO receptor, and FLT-3L receptor on primitive progenitor cells, thereby making them less responsive to these important supplemental factors *ex vivo*.[7–9]. Due to the accumulation of inhibitory soluble

factors in primary stem cell cultures, we first needed to determine the time point that yielded the highest number of primitive and presumably islet regenerative ALDH^{hi} HPC. We found that 6 days of *ex vivo* expansion yielded the highest number of ALDH^{hi}, ALDH^{hi}/CD34^{+}, and ALDH^{hi}/CD133^{+} HPC. Thus, we further characterized the multipotent colony forming capacity *in vitro* and islet regenerative properties after i.v.-transplantation using 6 day culture-expanded HPC subpopulations.

3.3 Transplanted ALDH^{hi} HPC Stimulated Islet Angiogenesis

The endocrine pancreas in highly vascularized, it has 5-7 times more capillaries that the surrounding exocrine pancreas tissue.[10] These islet capillary network provides glucose sensing pancreatic islets with nutrients, and oxygen.[11] In addition, the islet capillary network is essential for optimal islet function. A dense islet capillary network is required for β cells to optimally sense blood glucose levels, and the fenestrations in the islet capillary endothelium is the most efficient way for insulin secreted by β cells to enter the systemic circulation.[12] Furthermore, the islet capillary endothelium also supports β cell turnover. In an *in vitro* system, purified islet endothelial cells stimulated β cell proliferation primarily via the secretion of HGF.[13] During pregnancy pancreatic β cells can undergo enhanced turnover. *In vivo* experiments, in pregnant rats showed that HGF levels were highly expressed in the islet capillary endothelium during peak stimulation of β cell proliferation.[13] Another study showed that, laminins expressed on the basement membrane of the islet endothelium, binds to β1 integrin on β cells to stimulate β cell proliferation.[14] Collectively, these studies suggest that endothelial-endocrine axis plays an important role in supporting β cell function, and β cell turnover.

Previously, we have shown that freshly isolated UCB cells with high ALDH activity contains pro-angiogenic progenitor cells that improved the perfusion of the ischemic limb, following transplantation into immunodeficient mice with acute hind-limb ischemia induced by femoral artery ligation.[15] Freshly isolated UCB ALDH^{hi} cells did not integrate into the murine vasculature, instead UCB ALDH^{hi} cells homed selectively to areas of

inflammation in the ischemic limb, and stimulated endogenous murine capillary regeneration.[15] Here we show that a subset of the culture-expanded progeny, ALDH^{hi} HPC, significantly enhanced islet-specific capillary density in the STZ-treated mouse pancreas. VEGF is highly expressed in β cells, and is not expressed in extra-islet tissues.[11,12,16,17] Increased vascularization was specific to the islets, rather than extra-islet pancreatic tissue, suggesting that the VEGF is required for culture-expanded ALDH^{hi} HPC to support an islet specific vascularization program, in the pancreas of STZ-NOD/SCID diabetic mice. Interestingly increased CD31+ capillary density was associated with an increased islet size and, islet cell proliferation, resulting in increased total β cell mass, and improved islet function. Thus, our data suggest that the transplantation of ALDH^{hi} HPC improves islet function in STZ-NOD/SCID mice by re-establishing the endothelial-endocrine axis (Figure 3.1).

Intra-Pancreatic transplantation of freshly isolated UCB cells with high ALDH activity stimulated islet revascularization, and β cell proliferation in STZ-NOD/SCID mice, despite remarkably low level of engraftment (0.09 ± 0.03%, n=6 mice) of freshly isolated ALDH^{hi} UCB cells in the pancreas, at day 42.[1] However, iPan transplantation of ALDH^{hi} UCB cells increased early recruitment of islet regenerative cells immediately surrounding regenerating islets at 7 days post transplantation. Presumably, many of these injected cells were lost by 1 month post-transplantation. Interestingly, iPan transplantation of freshly isolated UCB ALDH^{hi} cells was more effective at improving systemic glycemia, compared to i.v. transplantation for UCB ALDH^{hi} cells. Thus, we postulated that increased exposure of recovering islets to UCB ALDH^{hi} cell secreted stimuli augmented a natural or endogenous islet regenerative process. Pancreatic β cells and ductal cells are known to secrete angiogenic growth factors such as vascular endothelial growth factor (VEGF), and chemokines such as stromal derived factor 1 (SDF-1), that recruit a pro-angiogenic cells specifically to STZ-damaged islets. In these studies human cells were not detected in the murine pancreas, at day 42, after i.v.-transplantation of culture-expanded ALDH^{hi} HPC. Although early time course recruitment experiments have not yet been performed using expanded HPC subsets, we speculate i.v.-injected culture-expanded ALDH^{hi} HPC were recruited to the damaged islets at early time points, surround these damaged islets and secrete factors that stimulate islet regeneration. However, due to the unfavourable

environment of the pancreas culture-expanded ALDH^{hi} HPC may undergo apoptosis, or migrate away from the pancreas before day 42 leaving the regenerative effects observed appear short-term and not sustained. Thus, iPan transplantation of culture-expanded ALDH^{hi} HPC may further increase early pancreas engraftment, and may augment the islet regenerative response *via* the initiation of islet regenerative programs. These important experiments are planned for future studies

3.4 ALDH^{hi} HPC Secrete Cytokines Involved in Angiogenesis

The mRNA levels of the secreted proteins Semaphorin 4A (SEMA4A), Angiopoietin 2 (ANGPT2), and Milk Fat Globule-EGF factor 8 (MFG-E8) were higher in culture-expanded ALDH^{hi} HPC compared to ALDH^{lo} HPC. The proteins encoded by these transcripts are all involved in supporting vascular endothelial growth factor-A (VEGFA) mediated angiogenesis. MFG-E8 also known as lactadherin has been shown to interact with $\alpha_v\beta3$ integrins on endothelial cells, and cooperates with VEGF signaling to activate a potent pro-angiogenic pathway.[18] Interestingly, in the hind-limb ischemic model injection of anti-MFG-E8 blocked VEGFA induced angiogenesis.[18] The transcript of ANGPT2 was also up regulated in culture-expanded ALDH^{hi} HPC. Studies have shown that, ANGPT2 is a complex regulator of vascular remodelling that supports both vessel sprouting and vessel regression. In the setting of angiogenic sprouting ANGPT2 is rapidly induced with VEGF, whereas in the setting of vascular regression, ANGPT2 is induced in the absence of VEGF.[19–24] Studies in mice lacking ANGPT2 shows that, ANGPT2 is essential for postnatal vascular remodeling of the eye. The retina of post natal mice lacking ANGPT2, was devoid of peripheral vessels, and had minimal central vasculature.[25] Pancreatic β cells express high levels of VEGF.[12,16,17] It is plausible that MFG-E8, and ANGPT2 secreted by ALDH^{hi} HPC stimulate islet vascularization, by supporting a VEGF dependent angiogenic program.

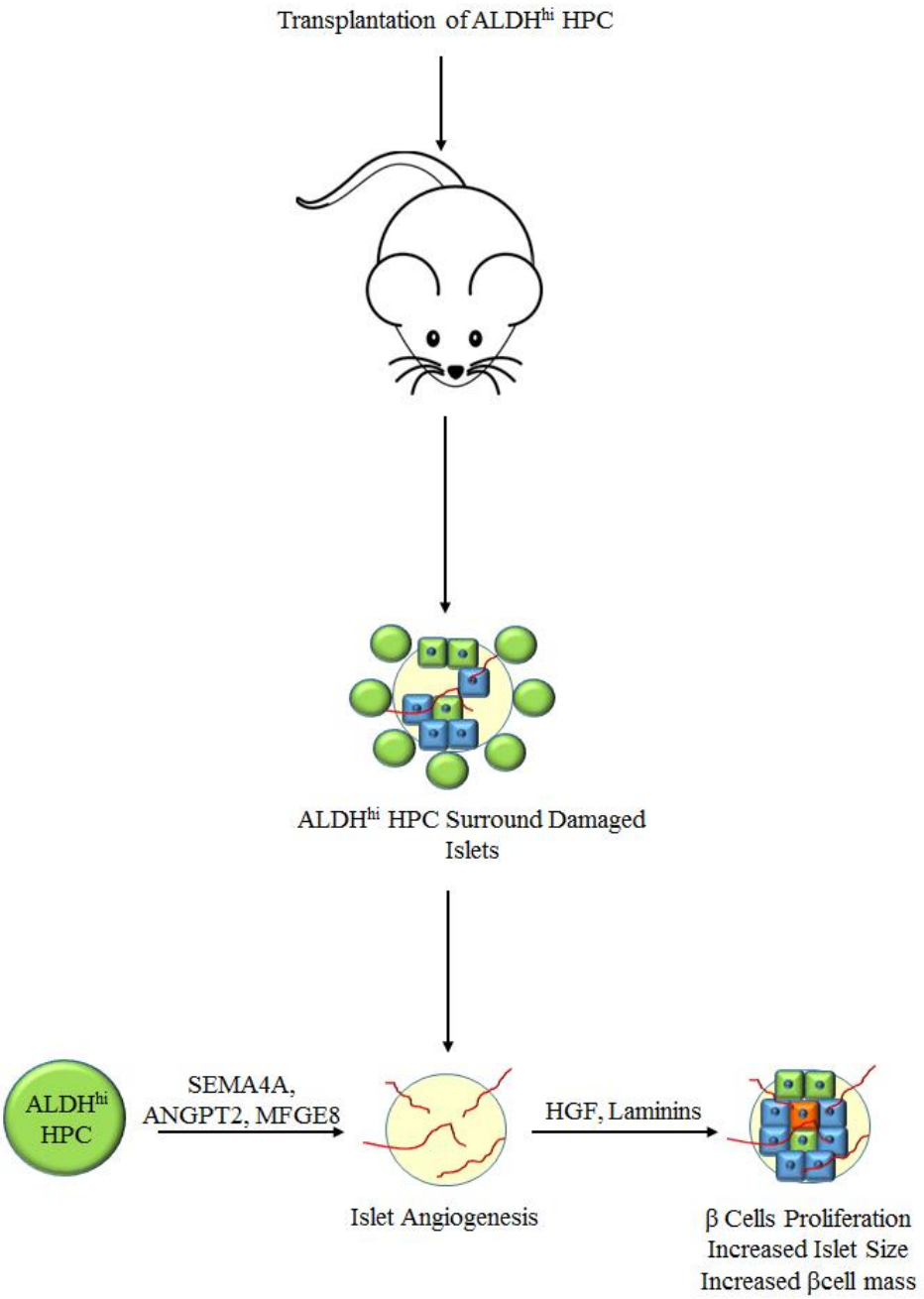

Figure 3.1 Working Model. Schematic of the proposed mechanism by which ALDH^{hi} HPC improves islet function. Our data suggest that transplanted ALDH^{hi} HPC surround damaged islets and secrete factors such as SEMA4A, ANGPT2, and MFGE8 that supports islet angiogenesis, to re-establishing the endothelial-endocrine axis. HGF secreted by endothelial cells, or laminins expressed on the basement membrane of endothelial cells support β cell proliferation, which results in an increased islet size, and β cell mass.

Finally, the transcript of SEMA4A was up-regulated in culture-expanded ALDH^{hi} HPC. SEMA4A has been shown to act on PlexinD1 receptors, to stimulate the release of VEGFA from macrophages.[26] The VEGFA subsequently secreted from macrophages, after SEM4A stimulation exerted a pro-angiogenic effect on endothelial cells *in vitro,* by activating the P13/AKT pathway in endothelial cells. Furthermore, the VEGFA produced by accessory macrophages stimulated angiogenesis in the *in vivo* chorioallantonic membrane (CAM) assay. Interestingly, although the NOD/SCID is completely depleted of T and B-lymphocyte activity, the NOD/SCID does retain residual macrophage and NK-cells of the innate immune system Thus, residual mouse macrophages may be recruited to the pancreas after STZ-induced islet injury in our model and may act as accessory cells in the islet regenerative process.[27] Adoptive transfer of M2 macrophages have been shown to protect NOD mice from developing diabetes, by homing to the inflamed pancreas and secreting anti-inflammatory cytokines.[27] SEM4A produced by ALDH^{hi} cells may act on the PlexinD1 receptors on these macrophages to stimulate VEGFA release, which subsequently supports angiogenesis in regenerating islets of STZ-treated diabetic mice. Collectively, these data suggests that the pro-angiogenic microenvironment established in the pancreas of STZ-NOD/SCID diabetic mice after the transplantation of ALDH^{hi} HPC may be beneficial to islet regeneration through the activity of murine macrophage intermediates.

3.5 Increasing the Number of Primitive Culture-Expanded HPC for a Cell Based Therapy to Treat Diabetes

In this study we show that culture-expanded HPC retaining a primitive progenitor phenotype (ALDH^{hi} HPC) stimulate endogenous islet regeneration in STZ-NOD/SCID diabetic mice. Conversely, more differentiated ALDH^{lo} HPC or even unsorted Bulk HPC could not stimulate similar regenerative processes *in situ.* For hematopoietic transplantation applications using UCB cells, researchers have attempted to increase the number primitive HPC during the *ex vivo* expansion of the HPC lineage. Recent success

has been achieved by, adding small molecules to hematopoietic culture conditions, and expanding UCB progenitor cells in a bioreactor using a batch fed system.[28–34]

For instance, Boitano *et al*.[29] have found that StemRegenin1 (SR1)-an aryl hydrocardon receptor antagonist-promotes the overall expansion of human HPC. When added to serum free media supplemented with hematopoietic growth factors this compound increased the number of primitive $CD34^+$ cells by 73-fold, when compared to cells cultured with growth factors alone (basal culture conditions). Importantly, the number of SRC that retain both multilineage and long-term engraftment potential, increased by 8-fold compared to basal culture conditions.

ALDH is highly expressed in HPC, and is a critical enzyme in the biosynthesis of RA. The addition of RA to hematopoietic culture (SCF, FLT3L, TPO), resulted in HPC differentiation.[32] Chute *et al*.[32] found that inhibiting ALDH with the small molecule DEAB in hematopoietic culture (SCF, FLT-3L, TPO) decreased RA signaling in HPC. Furthermore, when DEAB was added to hematopoietic the number of primitive SRC increased by 6-fold compared to cells cultured in basal hematopoietic culture conditions.

Csaszar *et al.*[28] developed an automated controlled fed-batch dilution approach to expand UCB progenitor cells. UCB cells were cultured in hematopoietic conditions in a bioreactor, and new media was added at a constant flow rate to the hematopoietic culture. Using this approach the factors that inhibit the growth of primitive progenitor cells such as TGF-β are constantly diluted and removed from the culture microenvironment. As a result, the number of CD34+ cells was increased by 80-fold, when compared to UCB cells expanded under basal (non-batch fed) conditions. In addition, the number of SRC increased by 11-fold compared to control conditions. Thus, the number of primitive HPC available to treat diabetes using the stimulation of endogenous islet regeneration could be increased in the future by adding small molecules to hematopoietic culture conditions or by expanding UCB $ALDH^{hi}$ progenitor cells using a batch-fed a bioreactor system.

3.6 Clinical Implications

Our data outlines a clinically applicable regenerative approach for the treatment of diabetes. Allogeneic human UCB progenitor cells can be isolated in a clinically relevant manner using ALDH activity, and the HPC lineage of the $ALDH^{hi}$ UCB cells can be expanded without serum or xenobiotic agents in clinically applicable fashion. High ALDH activity can subsequently be used to re-select cells with islet regenerative properties after culture, and prior to transplantation. Furthermore, the number of primitive HPC with islet regenerative properties can be further increased by adding clinically applicable small molecules to hematopoietic cultures, or by expanding hematopoietic cells using a batch-fed bioreactor system.

In order for culture=expanded $ALDH^{hi}$ HPC to be a plausible therapy for type-1 diabetes, the issue of continued autoimmune destruction of regenerated β cells must be addressed. Solvason *et al*.[35] demonstrated that a plasmid DNA vaccine encoding mouse proinsulin II (BHT-3021) reduced the incidence of diabetes, when administered into NOD mice both before the onset of hyperglycemia (prophylactically), and after the onset of hyperglycemia (therapeutically). BHT-3021 recently completed phase I clinical trials in type-1 diabetic patients diagnosed within the past 5 years.[36] No adverse events were reported in subjected treated with BHT-3021. In fact, the subjects administered with BHT-3021 demonstrated improved c-peptide level compared to placebo treated subjects even without the stimulation of endogenous islet regenerative processes. In addition, the frequency of proinsulin reactive $CD8^+$ T-cells declined in BHT-3021 treated subjects. This trial clearly shows that, BHT-3021 reduces frequency of $CD8^+$ T cells reactive to proinsulin, while preserving c-peptide levels over the course of dosing. However, the β cell mass of type 1 diabetic patients is already reduced by 70-80% at the time of diagnosis.[37] Thus, $ALDH^{hi}$ HPC that increases β cell mass and endogenous glucose secretion, together with BHT-3021 that prevents further β cell deletion and preserves β cell function may represent a highly rationalized potential strategy to treat type 1 diabetes.

As mentioned earlier, the Medalist study revealed that diabetic patients with long standing diabetes demonstrate residual β cell function.[38] Thus, culture-expanded $ALDH^{hi}$ HPC from

widely accessible UCB samples can be potentially utilized to stimulate endogenous β cell regeneration in diabetic patients with long standing diabetes.

Transplantation of culture-expanded $ALDH^{hi}$ HPC may also represent a potential strategy for the therapy for type 2 diabetic patients, who have a decreased β cell mass, and insulin resistance at advanced stages of the disease.[39] Although the issue of insulin resistance in these patients will still need to be addressed by diet, exercise, and hypoglycemic agents, culture-expanded $ALDH^{hi}$ HPC can aid in restoring β cell mass, and increase the insulin available to meet metabolic demand, essentially "tipping the balance" in favour of islet regeneration versus metabolic destruction in end-stage (insulin-dependent). We are currently generating preclinical xenotransplantation models of type 2 diabetes using the longer-lived NOD/SCID gamma (NSG) xenotransplantation model combined with high fat diet and low dose STZ monotherapy

Our study has several limitation that needs to be addressed before our findings can be applied to the clinic. Firstly, the glucometer we used for glucose tolerance testing was only able to detect a maximum glucose concentration of 33mmol/L. Therefore, to better characterize the glucose tolerance of mice transplanted with $ALDH^{lo}$ HPC, and $ALDH^{hi}$ HPC a glucometer with a higher range is required as readings in the mice were maximal after the injection of a glucose bolus. Alternatively, mouse blood samples could be diluted prior to glucose measurement to ensure that readings were within the range of quantification by the glucometer, resulting in more accurate glucose tolerance measurements. Also, type 1 diabetes involves the auto-immune destruction of β cells, whereas our diabetic mouse model involves the chemical destruction of β cells to induce hyperglycemia in immunodeficient NOD/SCID mice. Therefore, β cell regeneration is being studied in a model free of any autoimmune attack on islets. In addition, type 2 diabetes is a metabolic disease usually associated with obesity. Our NOD/SCID diabetic mouse model only depicts the hyperglycemia of type 2 diabetes, thus islet regeneration is studied in the absence of many inflammatory cytokines and metabolites associated with obesity. Finally, studying the islet regenerative properties of human cells is important, because these cells may eventually be used in human clinical trials. However, due to

species incompatibilities, transplantation of human progenitor cells into a mouse model may not fully recapitulate the regenerative capacity of human progenitor cells.

3.7 Future Directions

BHT-3201 preserves β cell function, while $ALDH^{hi}$ HPC stimulates endogenous islet regeneration. Studies could be performed in NOD mice, in order to determine if these therapeutic agents can work in combination to treat type 1 diabetes. In order to determine if $ALDH^{hi}$ HPC can stimulate endogenous islet regeneration in type 2 diabetic milieu, $ALDH^{hi}$ HPC could be transplanted into NSG type 2 diabetic mouse model. After transplantation islet function, β cell mass, and islet vascular density needs to be assessed.

By microarray analysis of $ALDH^{hi}$ HPC and $ALDH^{lo}$ HPC sub-populations this study identified MFG-E8, SEMA4A, and ANGPT2 to be potentially involved in stimulating islet specific angiogenesis. To further gain insight into the islet regenerative niche these microarray hits needs be confirmed by performing ELISA on the supernatants or by western blot on cell lysates from culture-expanded $ALDH^{hi}$ HPC and $ALDH^{lo}$ HPC subsets. In order determine if these secreted proteins are directly involved in islet revascularization, $ALDH^{hi}$ HPC needs to be intravenously transplanted with either MFG-E8, or SEMA4A, or ANGPT2 antibodies individually or with a combination of these antibodies, and islet function, β cell mass, and islet vascular density must be assessed. Further understanding the regenerative niche will aid in the optimization of a cellular regenerative therapy using expanded HPC therapy to promote islet function as a part of a multidisciplinary rational approach to treat both type 1 or type 2 diabetes.

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Curriculum Vitae

Section 1: University Education Background

September 2012-Present: Master's of Science in the department of physiology and pharmacology.
University of Western Ontario, London, Ontario
Thesis Title: Islet Regenerative Properties of *ex vivo* Expanded Hematopoietic Progenitor Cells with High Aldehyde Dehydrogenase Activity.

September 2008-August 2012: Honours Bachelor of Medical Science degree with distinction in the department of physiology and pharmacology.
University of Western Ontario, London, Ontario
Honours Specialization in Physiology
Thesis Title: The Effects that Hematopoietic Progenitor Cells have on β-cell Proliferation and Islet Regeneration

September 2010-June 2011: International Exchange at the University of Leeds, United Kingdom in the department of biomedical science.

Section 2: Research Experience

September 2012-Present: Master's Student in Dr. Hess' Lab of Regenerative Medicine Robarts Research Institute, and Department of Physiology and Pharmacology, Schulich School of Medicine and Dentistry, University of Western Ontario.

- I have shown that *ex vivo* expanded hematopoietic progenitor cells, derived from human umbilical cord blood, with high aldehyde dehydrogenase activity ($ALDH^{hi}$ HPC) demonstrates islet regenerative properties in immunodefficient diabetic mice.
- I performed immunohistochemical analysis on mouse pancreas sections to determine the regenerative program orchestrated by $ALDH^{hi}$ HPC.
- Microarray analysis was performed to gain insight into the paracrine factors that $ALDH^{hi}$ HPC secrete to stimulate islet regeneration.

May 2012-August 2012: Summer Student in Dr. Hess' Lab of Regenerative Medicine Robarts Research Institute, and Department of Physiology and Pharmacology, Schulich School of Medicine and Dentistry, University of Western Ontario.

- I developed, and optimized a seven colour flow cytometry panel to characterize the phenotype of human umbilical cord blood derived *ex vivo* expanded human hematopoietic progenitor cells.

September 2011-April 2012: 4th year Thesis Student in Dr. David Hess's Lab of Regenerative medicine Robarts Research Institute, and Department of Physiology and Pharmacology, Schulich School of Medicine and Dentistry, University of Western Ontario.

- I studied the effects of human umbilical cord blood derived, *ex vivo* expanded human hematopoietic progenitor cells (HPC) on β cell proliferation and islet regeneration.
- I co-cultured *ex vivo* HPC with murine βTC6 cells to determine the effect that this cell lineage has on β cell proliferation.
- I also transplanted HPC into immunodeficient diabetic mice to determine whether this lineage can stimulate islet regeneration *in vivo.*

June 2011-August 2011: Summer Student in Dr. Aaron Schimmer's Lab of Chemical Biology and Drug Discovery Princess Margret Hospital, and Department of Medical Biophysics, Faculty of Medicine, University of Toronto.

- I performed flow cytometry to investigate the ability of the artemisinin family a class of anti-malarial, and the lysomotrophic agent L-Leucyl-L-leucine methyl ester (LeuLeuOMe) to produce reactive oxygen species (ROS) in acute myelogenous leukemia (AML).
- Also looked into the ability of these drugs to synergize with mefloquine to produce ROS, and cause cell death in AML.
- My research was fully funded by the department of Medical Biophysics at the University of Toronto.

May 2010-September 2010: Summer student in Aaron Schimmer's lab of chemical biology and drug discovery Princess Margret Hospital, and Department of Medical Biophysics, Faculty of Medicine, University of Toronto.

- My project focused on the aberrant expression of toll like receptors in AML.
- I performed flow cytometry to determine the expression of toll-like receptors in AML cell lines as well as patient samples.
- I also optimized a stable transfection protocol for the over-expression of the TLRs in HEK 293 cells.
- My research was fully funded by Canadian Institutes of Health and Research (CIHR) program in Biological Therapeutics, as well as the Department of Medical Biophysics at the University of Toronto.

September 2009-May 2010: **Work-Study Student in Dr. Chandan Chakraborty's Lab, Department of Pathology, Schulich School of Medicine and Dentistry, The University of Western Ontario.**

- Lab focuses on intracellular signal transduction mechanisms which play an important role in agonist induced cellular migration
- Assisted MSc and PhD students with cell migration assays, PCR, and gel electrophoresis.

Section 3: Publications

Journal Publications

Bell GI, **Seneviratne A**, Nasri GN, Hess DA. *TRANSPLANTATION MODELS TO CHARACTERIZE THE MECHANISM OF STEM CELL INDUCED ISLET REGENERATION.* In Press at *Current Protocols in Stem Cell Biology*. April 10, 2013, CP-13-0022.R1.

My role in this project was to design and write the manuscript. Overall, my contribution to the team effort for this publication was 30%.

Sukhai MA, Prabha S, Hurren R, Rutledge AC, Lee AY, Sriskanthadevan S, Sun H, Wang X, Skrtic M, **Seneviratne A**, Cusimano M, Jhas B, Gronda M, Maclean N, Cho EE, Spagnuolo PA, Sharmeen S, Gebbia M, Urbanus M, Eppert K, Dissanayake D, Jonet A, Dassonville-Klimpt A, Li X, Datti A, Ohashi PS, Wrana J, Rogers I, Sonnet P, Ellis WY, Corey SJ, Eaves C, Minden MD, Wang JC, Dick JE, Nislow C, Giaever G, Schimmer AD**.** *LYSOSOMAL DISRUPTION AS A THERAPEUTIC STRATEGY FOR ACUTE MYELOID LEUKEMIA. (2013) The Journal of Clinical Investigation*. 123: 315-328.

- January 2013 *JCI* Cover Article
- Editor's Pick: highlighted in *JCI Impact* Digest, January 2013 (inaugural edition)
- Named one of the Canadian Cancer Society's top 10 cancer research stories for 2012:

http://www.cancer.ca/Canada-wide/About%20us/Media%20centre/CW-Media%20releases/CW-2012/Top%2010%20Society-funded%20research%20of%202012.aspx?sc_lang=en

- Highlighted in the *Toronto Star*, January 03, 2013:

http://www.thestar.com/specialsections/cancerresource/article/1309484--hope-in-10-cancer-breakthroughs

My role in this project was to design and perform experiments to determine if the anti-malarial mefloquine induced the production of ROS in AML cells, and synergistically increased ROS production when combined with 3 members of the artmisinin family of anti-malarials. To further explore the anti-leukemic activity of lysosomal disruption, I evaluated the ability of the known lysosomal disrupter L-leucine-leucine methyl ester (LeuLeuOMe) to produce ROS in AML cells. My contribution to the team effort for this publication was 5%.

Abstracts for Publication at International Conferences:

Seneviratne A, Bell GI, Sherman S, Putman DM, Hess DA. A Sub-Population of Expanded Hematopoietic Progenitor Cells that Retain High Aldehyde Dehydrogenase Demonstrates Islet Regenerative Properties. *Accepted for a poster presentation by the Canadian Stem Cell Network for presentation at the 2nd Annual Till and McCulloch Meeting, October 23-25, 2013 Banff, AB.* Recipient of Stem Cell Network Travel Award 2013.

Seneviratne A, Bell GI, Putman DM, Hess DA. A Sub-Population of Expanded Hematopoietic Progenitor Cells that Retain High Aldehyde Dehydrogenase Activity Improves Islet Function. *Accepted for a poster presentation by the International Society of Stem Cell Research for presentation at the 11th Annual ISSCR Conference Boston, MA.* Recipient Ontario Stem Cell Initiative travel award 2013.

Putman DM, Hewitt M, **Seneviratne A**, Hess DA. Ex vivo Expanded Human Umbilical Cord Blood Myeloid Progenitor Cells Stimulate Vascular Regeneration.
Accepted for a poster presentation by the International Society of Stem Cell Research for presentation at the 11th Annual ISSCR Conference Boston, MA.

Seneviratne A, Bell GI, Putman DM, Hess DA. The Phenotype and Islet Regenerative Functions of *ex vivo* Expanded Hematopoietic Progenitor Cells. *Accepted for a poster presentation at the 1st Annual M3: Mostly Mammals in Montreal Meeting, March 21-23, 2011, Montreal, QB.*

Sukhai MA, Hurren R, Rutledge A, Livak B, Wang X, **Seneviratne A**, Cusimano M, Gebbia M, Skrtic M, Sun H, Gronda M, Spagnuolo P, Urbanus M, Eppert K, Dissanayake D, Li X, Sun T, Vizeacoumar F, Datti A, Ohashi P, Wrana J, Rogers I, Minden MD, Wang J, Dick JE, Corey S, Nislow C, Giaever G, Schimmer AD. Lysosomal disruption selectively targets leukemia cells and leukemia stem cells through a mechanism related to increased reactive oxygen species production. *Accepted for oral presentation by the American Society of Hematology for presentation at the 53rdASH Annual Meeting, December 10-13, 2011, Sam Diego, CA.*

Sukhai MA, Li X, Hurren R, Wang X, Skrtic M, Sun H, Gronda M, **Seneviratne A**, Dissanayake D, Chow S, Bremner R, Hedley D, Minden MD, Ohashi P, Schimmer AD. The Anti-Malarial Mefloquine Demonstrates Preclinical Activity In Leukemia and Myeloma, and Is Dependent Upon Toll-Like Receptor Signaling for Its Cytotoxicity. *Accepted for Oral Presentation by the American Society of Hematology for presentation at the 52nd ASH Annual Meeting, December 04-07, 2010, Orlando, FL.*

Manuscripts in Preparation

Seneviratne A, Bell GI, Cooper T, Sherman S, Putman DM, Hess DA. A Sub-Population of Expanded Hematopoietic Progenitor Cells that Retain High Aldehyde Dehydrogenase Demonstrates Islet Regenerative Properties.

My role in this project is project lead. I am significantly involved in collecting, interpreting and analyzing data, and drafting the manuscript. Overall, my contribution to the team effort for this publication is 95%. As of now this manuscript is 70% complete.

Putman DM, Hewitt M, **Seneviratne A**, Hess DA. *Ex vivo* Expanded Human Umbilical Cord Blood Myeloid Progenitor Cells Stimulate Vascular Regeneration.

My role in this project is to design and perform experiments to characterize the phenotype of hematopoietic progenitor cells derived from umbilical cord blood cells with high aldehyde dehydrogenase activity. Overall, my contribution to the team effort for this publication is 15%. As of now this manuscript is 98% complete.

Patel P, Brooks C, **Seneviratne A**, Hess DA and Séguin CA. Investigating Microenvironmental Regulation of Human Chordoma Cell Behaviour.

My role in this project is to design and perform experiments to characterize the phenotype of human chordoma cells cultured in hypoxic or normoxic conditions. Overall, my contribution to the team effort for this publication is 5%. As of now this manuscript is 80% complete.

Section 4: Presentations

Ayesh Seneviratne, Gillian I. Bell, Stephen Sherman, David M. Putman, David A. Hess. A Sub-Population of Expanded Hematopoietic Progenitor Cells that Retain High Aldehyde Dehydrogenase Activity Improves Islet Function by Stimulating Endogenous Islet Regeneration and Vascularization. *Accepted for poster presentation by* **the Molecular Medicine Group at the Robarts Research Institute for presentation at the 1st annual Robarts Research Retreat**, Bellamere Winery, June 9, 2014.

Ayesh Seneviratne, Gillian I. Bell, Stephen Sherman, David M. Putman, David A. Hess. A Sub-Population of Expanded Hematopoietic Progenitor Cells that Retain High Aldehyde Dehydrogenase Activity Improves Islet Function by Stimulating Endogenous Islet Regeneration and Vascularization. **The Collaborative Graduate Program in**

Developmental Biology 6th annual Developmental Biology research day, Civic Garden complex, May 30, 2014.

Ayesh Seneviratne, Gillian I. Bell, Stephen Sherman, David M. Putman, David A. Hess. A Sub-Population of Expanded Hematopoietic Progenitor Cells that Retain High Aldehyde Dehydrogenase Activity Improves Islet Function by Stimulating Endogenous Islet Regeneration and Vascularization. **Schulich School of Medicine and Dentistry London Health research day**, London Convention Centre, March 18, 2014.

Ayesh Seneviratne, Gillian I. Bell, Stephen Sherman, David M. Putman, David A. Hess. A Sub-Population of Expanded Hematopoietic Progenitor Cells that Retain High Aldehyde Dehydrogenase Demonstrates Islet Regenerative Properties. **Schulich School of Medicine and Dentistry 4th annual Diabetes research day**, St. Joseph's Hospital, November 12, 2013.

Ayesh Seneviratne, Gillian I. Bell, Stephen Sherman, David M. Putman, David A. Hess. A Sub-Population of Expanded Hematopoietic Progenitor Cells that Retain High Aldehyde Dehydrogenase Demonstrates Islet Regenerative Properties. **Deparment of Physiology and Pharmacology Gowdey research day**, Western University, November 4, 2013. *1st place award Recipient for the best poster.*

Ayesh Seneviratne, Gillian I. Bell, Stephen Sherman, David M. Putman, David A. Hess. A Sub-Population of Expanded Hematopoietic Progenitor Cells that Retain High Aldehyde Dehydrogenase Improves Islet Function. **The Collaborative Graduate Program in Developmental Biology 5th annual Developmental Biology research day**, Civic Garden complex, May 30, 2013

Ayesh Seneviratne, Gillian I. Bell, Heather C. Broughton, David A. Hess. The Phenotype and Islet Regenerative Functions of *ex vivo* Expanded Hematopoietic Progenitor Cells. **Schulich School of Medicine and Dentistry London Health research day**, London Convention Centre, March 19, 2013.

Ayesh Seneviratne, Gillian I. Bell, Heather C. Broughton, David A. Hess. The Phenotype and Islet Regenerative Functions of *ex vivo* Expanded Hematopoietic Progenitor Cells. **Schulich School of Medicine and Dentistry 3rd annual diabetes research day**, St. Joseph's Hospital, November 13, 2012. *2nd place award Recipient for the best poster.*

Ayesh Seneviratne, Gillian I. Bell, David M. Putman, David A. Hess. The Phenotype and Islet Regenerative Functions of *ex vivo* Expanded Hematopoietic Progenitor Cells. **Deparment of Physiology and Pharmacology Stevenson research day**, Western University, November 6, 2012. *2nd place award Recipient for the best poster.*

Ayesh Seneviratne, Gillian I. Bell, Heather C. Broughton, David A. Hess. The Effect of Transplanted Human Hematopoietic Progenitor Lineage on β cell Proliferation and Islet Regeneration. **Deparment of Physiology and Pharmacology annual 4th year thesis student research day**, Western University, April 2, 2012.

Ayesh Seneviratne, Maria Cusimano, Mahadeo A Sukhai, Bozena Livak, Angela

Rutledge, Rose Hurren, Paul Spagnuolo, Aaron D. Schimmer. Lysosomal Disruption Selectively Targets Leukemia Cells Through a Mechanism Related to Increased ROS Production. **Deparment of Physiology and Pharmacology Gowdey research day**, Western University, November 8, 2011.

Ayesh Seneviratne, Maria Cusimano, Mahadeo A Sukhai, Bozena Livak, Angela Rutledge, Rose Hurren, Paul Spagnuolo, Aaron D. Schimmer. Lysosomal Disruption Selectively Targets Leukemia Cells Through a Mechanism Related to Increased ROS Production. **Department of Medical Biophysics summer student presentation**, University of Toronto, August 18, 2011.

Ayesh Seneviratne, Mahadeo A. Sukhai, Joyce Sun, Xiaoming Li, Aaron D. Schimmer**.** Aberrant Expression of Toll-Like Receptors in Acute Myelogenous Leukemia. **Department of Medical Biophysics summer student presentation**, University of Toronto, August 13, 2010.

Ayesh Seneviratne, Mahadeo A. Sukhai, Joyce Sun, Xiaoming Li, Aaron D. Schimmer. Aberrant Expression of Toll-Like Receptors in Acute Myelogenous Leukemia **CIHR Biological Therapeutics summer student presentation**, July 26, 2010.

Section 5: Teaching Experience

September 2012-Present: Teaching Assistant Physiology 3130Y, Western University

- Responsible for facilitating the pre-lab tutorials, post lab tutorials, and helping students to complete their lab.

September 2009-May 2010: **Tutor for Frontier College, an institution that helps underprivileged individuals with their academics.**

- Helped children from ages 8-13 with reading and assignments

Sept 2007-June 2008: Peer Tutor- Applewood Heights Secondary School, Mississauga Ontario.

- Mentored and guided grade 9 and 10 students to achieve better academic result

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Section 6: Supervisory and Mentorship Experience

September 2013-Present	Mr. Tyler Cooper, 4th year Research Student, Department of Biology, University of Western Ontario, London, Ontario. **Project Title: The Phenotype and Colony Forming Capacity of Hematopoietic Progenitor cells Expanded by the Modulation of the Retinoic Acid Pathway.**
May 2013-August 2013	Mr. Stephen Sherman, Summer Student Robarts Research Institute, London, Ontario

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Section 7: Technical Experience

- Proficient *in vivo* procedures such as: 1) intravenous transplantation, intrapancreatic transplantation procedures; 2) subcutaneous, intramuscular, and intraperitoneal injection procedures; 3) Intravenous, and intracardiac blood collection procedures.
- Proficient *in vitro assays* such as: cell imunotyping using flow cytometry, tissue culture, transfection methodologies, immunohistochemistry, colony formation unit assay

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Section 8: Academic Accomplishments and Awards

Master's

Studentships:

- Recipient, Ontario graduate scholarship (2013-2014; $15,000)
- Recipient, Ontario Graduate Scholarship (2012-2013; $15,000).

Travel Awards:

- Recipient, Stem Cell Network travel award (2013)
- Recipient, Ontario Stem Cell Initiative travel award (2013; $ 1,000)

Poster Awards:

- Recipient, 1st place award Recipient in the Category of Endocrinology and Reproductive Physiology at the Deparment of Physiology and Pharmacology Gowdey research day, Western University (November 4, 2013; $250).
- Recipient, 2nd place award for the best poster at the Schulich School of Medicine and Dentistry annual diabetes research day, St. Joseph's Hospital (November 13, 2012; $200).
- Recipient, 2nd place award Recipient in the Category of Endocrinology and Reproductive Physiology at the Deparment of Physiology and Pharmacology Stevenson research day, Western University (November 6, 2012; $100).

Undergraduate

Studentships:

- Recipient, University of Toronto Summer Undergraduate Studentship Award (2011; $2400).
- Recipient, University of Toronto Summer Undergraduate Studentship Award (2010; $2400).
- Summer Studentship, CIHR strategic training program in biological therapeutics (2010).

Scholarships:

- Recipient, Richard Ivey Foundation International Exchange Scholarship (2010/11; $5,000).
- Queen Elizabeth Scholarship (2008-2012; $3,500/yr).
- University of Western Ontario Scholarship of Excellence (2008/09; $2000).

Academic Accomplishments:

- University of Western Ontario Dean's Honours' list (2008-2012).
- University of Western Ontario Scholar (2008-2012).

Secondary School

- Ontario Scholar (2007-2008)
- Applewood Heights Secondary School Honors list (2006-2008)
- Proficiency, Introduction to Anthropology, Sociology, and Psychology (2006/07)