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Distinct Human Progenitor Subtypes Promote Islet Regeneration via Activation of Endogenous Angiogenic or Neogenic Programs

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Distinct Human Progenitor Subtypes Promote Islet Regeneration via Activation of Endogenous Angiogenic or Neogenic Programs

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ABSTRACT

Transplanted murine bone marrow (BM) progenitor cells stimulate endogenous beta cell proliferation and insulin production. To enrich for analogous human progenitor cells, we purified human umbilical cord blood (UCB) and BM based on high aldehyde dehydrogenase activity ($ALDH^{hi}$), a conserved function in multiple progenitor lineages. Transplantation of $ALDH^{hi}$ mixed progenitor cells or culture-expanded multipotent stromal cells (MSC) into streptozotocin-treated NOD/SCID mice revealed endogenous islet regeneration occurred via distinct mechanisms. Transplantation of $ALDH^{hi}$ cells improved systemic hyperglycemia via increased islet vascularization and proliferation resulting in increased islet size, but not islet number. Contrary to mixed-progenitor cells, transplantation of cultured MSC stably reduced hyperglycemia via an increase in islet number directly associated with the ductal epithelium without evidence of $NGN3^{+}$ endocrine precursor activation. Further understanding of these endogenous angiogenic or putative neogenic programs activated by distinct human progenitor subsets may provide new approaches for combinatorial cellular therapies to treat diabetes.

Keywords: Diabetes, Transplantation, Progenitor Cells, Multipotent Stromal Cells, Beta Cells, Islet Regeneration

Co-Authorship

The following thesis contains material from a submitted manuscript co-authored by Gillian Bell, Heather Broughton, Krysta Levac, David Allen, Anargyros Xenocostas and David Hess. Gillian Bell performed all the experimental work presented in the thesis. Heather Broughton, Hess lab animal technician, performed all cell transplantations into hyperglycemic mice and helped with animal care. Krysta Levac, Hess lab research associate, isolated and characterized MSC from BM samples and collected UCB samples. David Allen from the University of Ottawa provided UCB samples. Anargyros Xenocostas from the London Health Sciences Centre provided BM samples. Original manuscripts, versions of which appear in this thesis, were written by Gillian Bell and edited by David Hess.

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LIST OF ABBREVIATIONS

7-AAD – 7-Aminoactinomycin D

AAA - Aminoacetaldehyde

ALDH – Aldehyde dehydrogenase

ANOVA – Analysis of variance

BM – Bone marrow

BMP-7 – Bone-morphogenic protein-7

CAB – Citric acid buffer

CD – Cluster of differentiation

CK19 – Cytokeratin 19

NOD – Non-obese diabetic

ECFC – Endothelial colony forming cell

EdU – 5-ethynyl-2'-deoxyuridine

EGM – Endothelial growth media

EPC – Endothelial progenitor cells

ES – Embryonic stem

FACS – Fluorescence activated cell sorting

FBS – Fetal bovine serum

FGF – Fibroblast growth factor

GLP-1 – Glucagon-like peptide-1

GLUT2 – Glucose transporter 2

HCFC – Hematopoietic colony forming cell

HGF – Hepatocyte growth factor

HLA – Human leukocyte antigen

HSC – Hematopoietic stem cell

IDO – Indolleamine-2,3-dioxygenase

IL – Interleukin

i.p. – Intraperitoneal

iPS – Induced pluripotent stem

IRS – Insulin-receptor substrate

MCFC – Mesenchymal colony forming cell

MNC – Mononuclear cell

MPSVII – Mucopolysaccharidosis Type VII

MSC – Multipotent stromal cell OR Mesenchymal stem cell

Ngn3 – Neurogenin 3

OCT – Optimal cutting temperature media

PBS – Phosphate buffered saline

PDX1 – Pancreatic and duodenal homeobox 1

PGE_2 – Prostaglandin E2

RCF – Relative centrifugal force

SCID – Severe combined immunodeficiency

SDF-1 – Stromal-derived-factor-1

STZ – Streptozotocin

TGF-β – Transforming growth factor beta

Tregs – Regulatory T cells

UCB – Umbilical cord blood

VEGF – Vascular endothelial growth factor

vWF – von Willebrand factor

Chapter 1

Introduction

1.1 The Human Pancreas

The human pancreas is an organ composed of exocrine, ductal and endocrine cells, and has two main functions: digestion and glucose homeostasis. Exocrine cells, which compose 98% of the pancreas, are responsible for digestion, and release a mixture of digestive enzymes and bicarbonate via pancreatic ducts into the duodenum. The endocrine cells, which compose 1-2% of the pancreas, are clustered throughout the exocrine tissue in islets of Langerhans, and release hormones into the systemic circulation via the portal vein. The islets of Langerhans were first described by Paul Langerhans in 1869, but their involvement in endocrine secretion was not discovered until 1893 by Edouard Laguesse (1).

Adult islets contain four major endocrine cell types: α-cells, β-cells, δ-cells, and PP cells, as well as more recently described Epsilon or Ghrelin cells (1) (Figure 1). The α-cells, which comprise 15-20% of the islet, secrete glucagon, a 29-amino acid peptide with hyperglycemic action, into the circulation when low blood glucose is detected. Upon release and binding to glucagon receptors on hepatocytes, blood glucose levels are increased by stimulating breakdown of stored glycogen (glycogenolysis) and by activating hepatic gluconeogenesis. β-cells compose the majority of the islet, representing 70-80% of the endocrine cells. β-cells uptake glucose through the GLUT2 transporter, sense blood glucose levels, and secrete insulin, a 51-amino acid peptide with hypoglycemic action, into the circulation when high blood glucose levels are detected. Upon insulin release, insulin responsive cells in the peripheral tissues are stimulated to

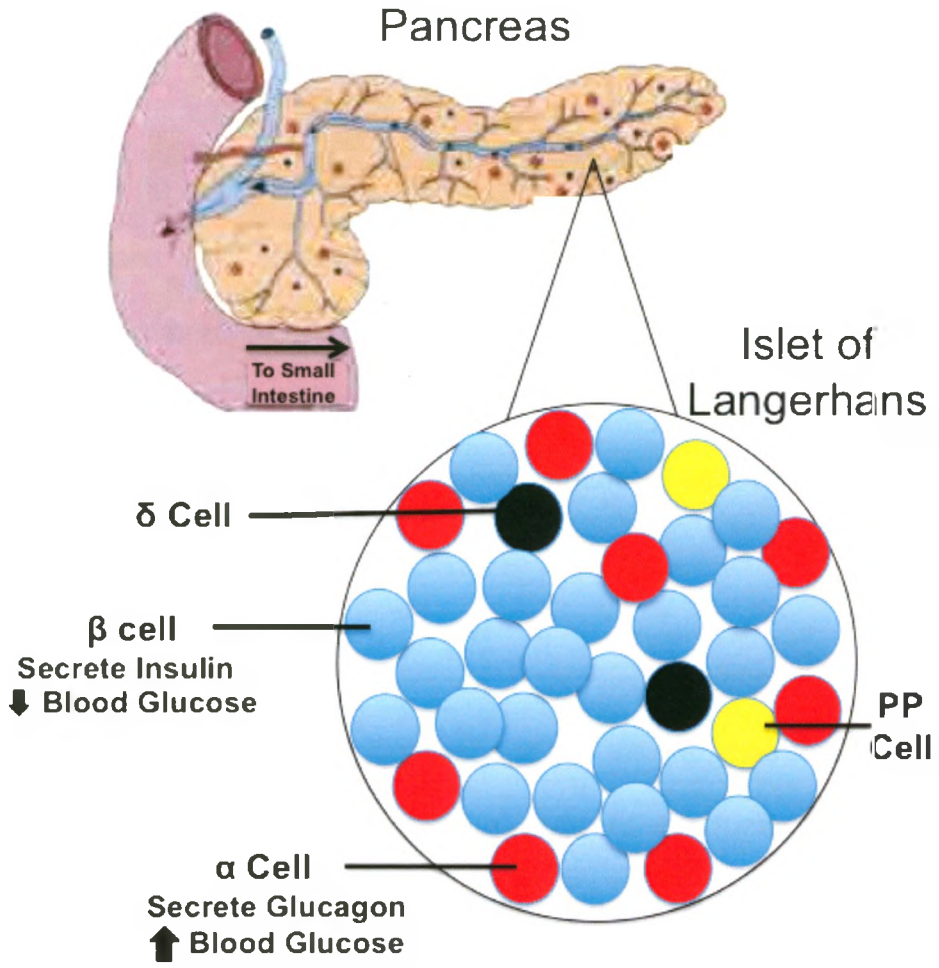

Figure 1. The endocrine cells of the islets of Langerhans. Schematic representation of the pancreas containing the islets of Langerhans in which the endocrine cells are clustered. In order to maintain glucose homeostasis, the α cells secrete glucagon to increase blood glucose levels, and the β cells secrete insulin to decrease blood glucose levels.

uptake, store and suppress the production of glucose. δ-cells secrete somatostatin, an inhibitor of glucagon and insulin release. The least well-studied endocrine cell in the pancreas is the PP-cell, which releases pancreatic polypeptide (PP) following a protein-rich meal, fasting, or exercise. PP inhibits gall bladder contractions and regulates pancreatic endocrine and exocrine secretions (2, 3). The most recent hormone secreting cell to be discovered in the pancreas, the Epsilon or Ghrelin cell, is thought to be important in growth hormone release, metabolic regulation and energy balance, although it's exact role in the islet is not yet known (4).

1.2 Diabetes

The cells of the islets of Langerhans are responsible for the maintenance of glucose homeostasis. Reduced ability of beta cells to produce insulin, or resistance to insulin in peripheral tissue leads to a disruption in glucose homeostasis and development of diabetes mellitus, a disease of which there are two types: type 1 diabetes, also known as early onset or juvenile diabetes, and type 2 diabetes, also known as adult onset diabetes (Figure 2).

1.2.1 Type 1 diabetes

Type 1 diabetes is an auto-immune disease where destruction of the insulin secreting β-cells is mediated by a cellular immune response. Although it may be diagnosed at any age, type 1 diabetes is the most common auto-immune disorder in childhood (5, 6), and accounts for 10% of diabetic patients. Incidence rates in western countries is 8 to >50 per 100 000 individuals per year and the

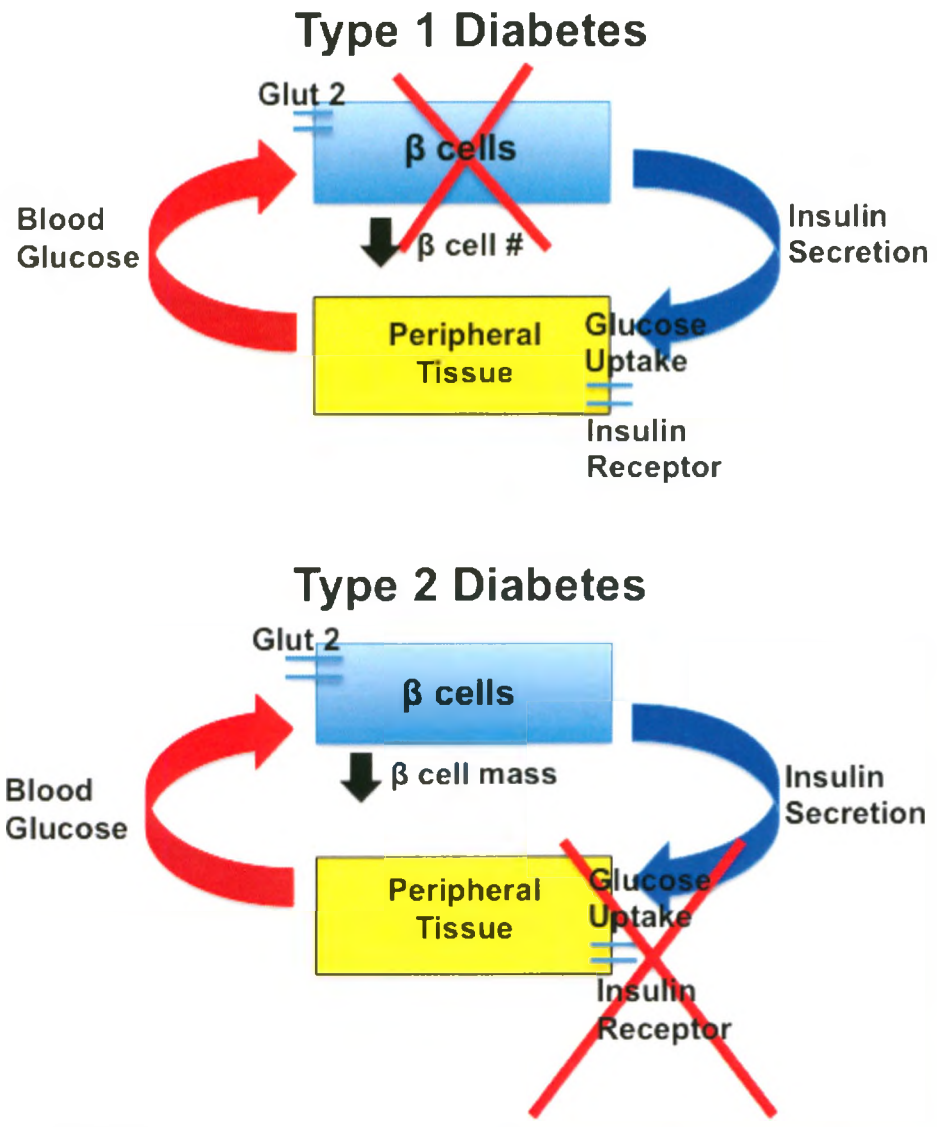

Figure 2. The mechanisms of type 1 and type 2 diabetes. Type 1 diabetes is an autoimmune disease where pancreatic beta cells are destroyed leading to reduced beta cell number and impaired insulin secretion. Type 2 diabetes involves reduced sensitivity to insulin at insulin receptors on peripheral tissues, impairing the uptake of glucose into tissues. Although different mechanisms are involved that impair glucose homeostasis, both types of diabetes can lead to sustained elevated blood glucose levels requiring intervention.

prevalence in children under 14 is expected to rise to 1 million worldwide by 2025 (7).

In 1965, W. Gepts showed that the pancreata of patients with inflammatory infiltrants (insulitis) contained lesions only in islets where some β-cells were still present. Islets without lesions were pseudoatrophic and contained only non-β endocrine cells. These seminal findings indicated that type 1 diabetes was an inflammatory disease of autoimmune etiology (8). These inflammatory infiltrants were later identified as mainly $CD8^+$ T-cells and macrophages (9, 10), and could be detected in patients at clinical presentation of type 1 diabetes (11).

Type 1 diabetes results from a combination of genetic and environmental factors. The genes most associated with risk of type 1 diabetes are located in the HLA class II locus on chromosome 6, however less than 10% of individuals with this susceptibility develop diabetes (12). Environmental factors such as infectious agents, dietary factors, and environmental toxins are also thought to trigger the onset of diabetes in genetically predisposed individuals (13).

Prior to the discovery of insulin, patients with type 1 diabetes suffered from severe hyperglycemia and ketoacidosis, became comatose and inevitably died. However, poorly managed diabetes over extended periods of time is still associated with many secondary complications that can be life threatening. It is a leading cause of blindness, renal failure and limb amputation, as well as a risk factor for heart disease and stroke (14).

1.2.2 Type 2 diabetes

Type 2 diabetes mellitus is a metabolic disorder characterized by resistance to insulin action on glucose uptake in peripheral tissue, impaired insulin action to inhibit hepatic glucose production, and dysregulated insulin secretion (14). Type 2 diabetes is increasing at an alarming rate and accounts for >90% of diabetic cases. A worrisome trend is that this disease previously predominantly found in adults is now widely diagnosed in children and adolescents, paralleling the increase in childhood obesity (15). Up to 50% of newly diagnosed diabetic children and adolescents in the US are diagnosed with type 2 diabetes (16).

Prolonged presence of type 2 diabetes is also accompanied by a decrease in β cell mass, due to increased β cell apoptosis, and is further exacerbated by amyloid plaque deposits in the islets (17, 18). Susceptibility to insulin resistance and defects in insulin secretion are at least partially genetically determined, and the insulin-receptor substrates (IRS proteins), particularly IRS-2 may be involved, since decreased IRS signaling leads to insulin resistance as well as β cell apoptosis (19-21). IRS is important in promoting β cell survival (22) and replication (23), however chronic hyperglycemia and hyperlipidemia found in type 2 diabetic patients, leads to IRS2 degradation and therefore β cell apoptosis (24, 25).

Environmental factors such as diet, physical activity and age play a large role and interact with genetic predispositions in development of the disease. Obese individuals will initially exhibit an increase in beta cell mass to compensate for the

increased demand for insulin and obesity-associated insulin resistance, however this adaptation eventually fails, leading to an overt type 2 diabetic state that must be treated with insulin therapy (14, 17, 26, 27).

1.3 Insulin therapy

Insulin was first isolated and used clinically in 1923 by Banting, Best and Collip, providing a major medical breakthrough for the treatment of diabetes (28, 29). In 1921 Banting and Best demonstrated that intravenous injections of extract from dog pancreas, removed following 7-10 weeks of ductal ligation, reduced blood glucose and sugar excreted in the urine in diabetic dogs. With the aid of Collip, ox pancreatic extracts, were first administered into a diabetic patient at the Toronto General Hospital on January 11, 1922. Although these first extracts were impure, and caused adverse reactions, extracts were further purified by Collip by removing inert material and precipitating the insulin, resulting in a purified extract which worked successfully in decreasing the signs of diabetes in treated patients.

Animal-sourced insulin remained the standard until the first sythentic insulin was produced in the early 1960s (30, 31). Synthetic human insulin became available commercially in 1982 under the brand name Humulin. Home glucose monitoring and self-administration of exogenous insulin became widely available in the 1970s (32) and remains effective treatment for many diabetic patients. Although advances in blood glucose monitoring have been made, particularly with continuous glucose monitoring systems (32), and continuous subcutaneous insulin infusion (insulin pumps) (33, 34) this method of treatment remains

insufficient for a small group of patients who experience life-threatening hypoglycemic episodes with exogenous insulin therapy (35). Current treatment for this subgroup of patients with brittle diabetes involves either whole pancreas transplantation or islet transplantation.

1.3.1 Pancreas transplantation

Transplantation of cadaveric pancreata into diabetic patients, either as a single organ transplant or in combination with kidney transplantation, can restore euglycemia. Over 30 000 pancreata have been transplanted since the first procedure was performed in 1966 (36, 37). In 1979 the first living donor pancreas transplant using a segmental graft was performed and restored insulin independence in the diabetic recipient (38). In 2008, 155 segmental pancreas transplants from living donors were also reported and demonstrated advantages over whole cadaveric pancreas transplants, such as closer HLA match, reduced immunosuppression, and increased graft survival. This also lead to the much needed expansion of the potential donor pool (39). However, living donor transplants are not likely to become common practice as the pancreas is an unpaired organ, and there are risks of serious organ-specific complications such as pancreatitis, as well as possible deterioration of glucose metabolism in the donor over their lifetime (40).

1.3.2 Pancreatic islet transplantation

As an alternative to whole pancreas transplant, allogeneic islet transplantation has also been studied as a less invasive treatment for severe diabetics. The first

islet transplantation was performed in 1990 (41), but since then, overall success has been around 10% (42). In 2000, a landmark study from Edmonton showed that 7 of 7 patients became insulin independent following islet transplantation using a corticoid-free immunosuppressive regimen (43). This dramatically increased the number of patients undergoing islet transplantation following this newly developed protocol. In 2006, Shapiro et al published "International Trial of the Edmonton Protocol for Islet Transplantation" in the New England Journal of Medicine, revealing the successes as well as the many challenges and shortcoming of this procedure. One year after transplantation, 44% of recipients were still insulin independent, however only 1 of 36 subjects remained insulin independent at 3 years. Islet transplantation remains a therapy limited to few patients since there is a critical shortage of cadaveric islets available for transplantation (many patients require islets from more than one donor), islets lose their ability to function over time, and there are many adverse side effects experienced due to immune-suppressant therapy (44). Future direction for the treatment of diabetes is shifting from the replacement of whole pancreas, or pancreatic islets, to the development of cellular therapies using stem cells for beta cell replacement, or for the endogenous regeneration of beta cell function.

1.4 Stem cells

Stem cells, first identified in 1963, have two distinguishing features. They can self-renew while remaining undifferentiated, and can differentiate into multiple cell types (45) (Figure 3). Embryonic stem (ES) cells are pluripotent cells derived from the inner cell mass of 4-5 day old embryos, that can proliferate indefinitely

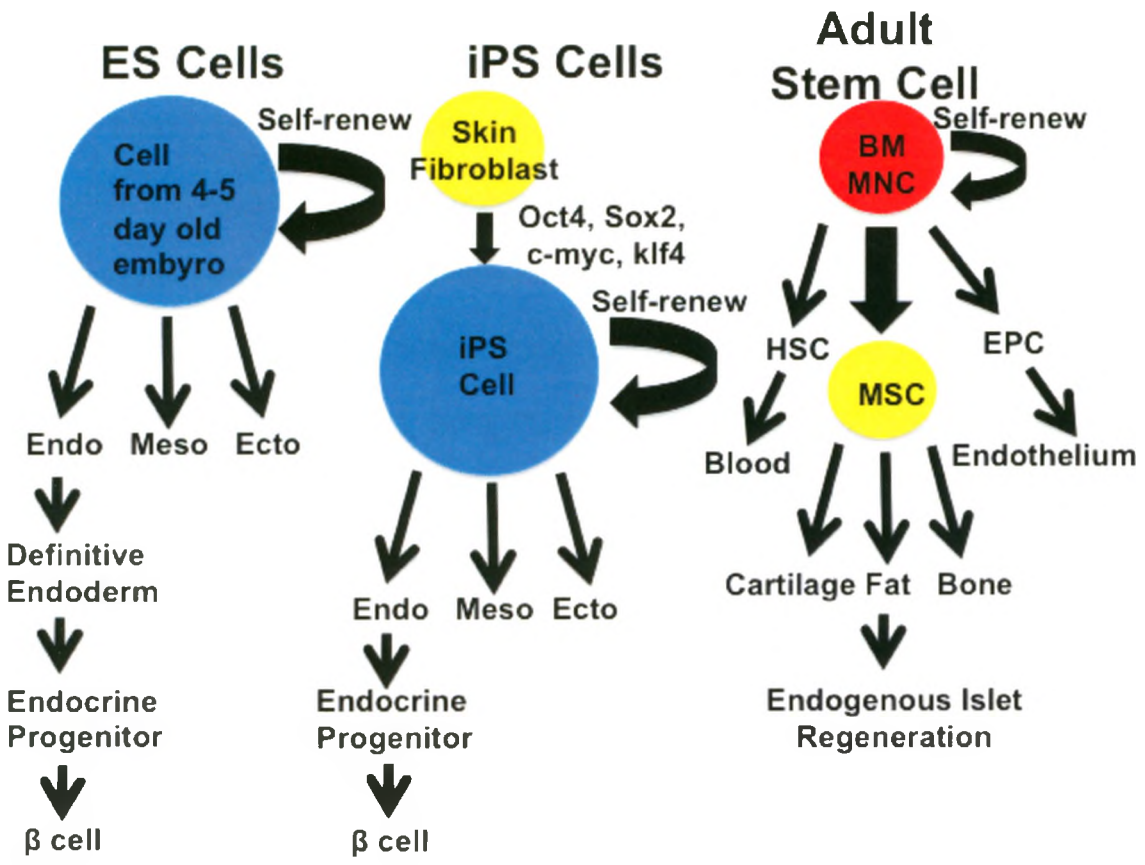

Figure 3. Differentiation of regenerative stem cells. Schematic of the differentiation of human embryonic stem (ES) cells, induced pluripotent stem (iPS) cells, and adult stem cells. ES cells and iPS cells can self-renew as well as develop into cells of all 3 germ layers, including towards a beta cell fate. Adult stem cells such as those found in bone marrow (BM) are typically germ-layer specific in their differentiative ability, but contain stem and progenitor cells of the hematopoietic (HSC), endothelial (EPC) and mesenchymal (MSC) lineages, which can further differentiate into multiple regenerative cell types.

while maintaining the capacity to differentiate into cells of all three germ layers. ES cells were initially isolated and cultured from mouse embryos in 1981 (46), and then later from human embryos in 1998 (47). In contrast to pluripotent ES cells, adult stem cells with multipotent differentiation ability are found in most adult organs where their function may be to maintain and regenerate tissues after damage. A newly developed type of stem cell, the induced pluripotent stem cell (iPS cell), has been derived from human skin fibroblasts through retroviral transduction of transcription factors Oct4, Sox2, c-myc, and Klf4 that modifies the epigenetics and phenotype of the cells (48-50). iPS cells show pluripotency similar to ES cells, including their ability to differentiate into all germ layers. There are currently no approved therapies using ES or iPS cells due to difficulties in directing the production of functional effector cells, and their undifferentiated tumor (51), and teratoma forming capacity (52). In contrast, adult stem cells are used for a growing list of medical procedures such as bone marrow transplantation to treat hematopoietic malignancies.

1.5 Development of regenerative therapies for diabetes

There are currently two major initiatives underway to combat the beta cell deficiency in diabetes: beta cell replacement, and endogenous beta cell regeneration (53).

1.5.1 Cell replacement strategies for diabetes

1.5.1.1 Ex vivo expansion of adult beta cells

Ex vivo beta cell expansion from donor pancreatic tissue to increase the number of beta cells available for transplantation is currently under investigation. Beta cells are however relatively quiescent and proliferate infrequently at a rate of about 0.1-0.3%/day (54). Since under certain conditions, such as pregnancy, beta cell mass can expand *in vivo,* it may be feasible to use external stimuli to expand primary beta cells *ex vivo.* However, culturing human islets in serum free media with growth factors such as FGF and HGF, GLP-1 (55), or overexpressing nuclear protein p8 (56, 57), results in limited expansion (58) and beta cell apoptosis. Beta cells of cultured islets have been reported to dedifferentiate, expand, and redifferentiate, however these beta cells quickly lose their ability to produce and secrete insulin. With insulin mRNA levels only 0.01% that of human beta cells (59), this approach is currently limited as a feasible strategy to develop large numbers of beta cells for transplantation into diabetic patients.

1.5.1.2 Derivation of beta cells from ES or iPS cells

ES and iPS cells may provide a promising therapy for the treatment of diabetes. If these cells could be efficiently directed to a beta cell fate, customized patient-specific cellular therapy without the need for immune-suppressant therapy could potentially be used to treat diabetic patients. However, a lack of reliable methods of generating specific lineages from ES or iPS cells, and the risk that transplantation of undifferentiated cells may produce teratomas, has limited the use of these cells for therapy. Recently improvements have been made in effectively differentiating ES cells towards the pancreatic lineage. In 2005, D'Amour et al showed that ES cells could be differentiated into definitive

endoderm cells (60), and then in 2006 that the definitive endoderm cells could be differentiated into hormone expressing endocrine cells through a 5-step series of directed intermediates similar to those that occur during embryonic pancreatic development (61). Furthermore, these endoderm cells derived from human ES cells were shown to generate glucose-responsive endocrine cells after transplantation into mice (62). However, although β-like cells derived from ES cells have shown the ability to reverse hyperglycemia when transplanted into hyperglycemic NOD/SCID mice, this hyperglycemic correction was maintained for less than 3 weeks, with insulin production lost while teratoma formation progressed (52).

Like human ES cells, iPS cells also have the potential to differentiate into islet-like clusters through definitive and pancreatic endoderm, that release c-peptide upon glucose stimulation (63). However, these protocols are still inefficient as they produce heterogeneous cell populations with a low frequency of insulin producing cells, and differentiated progeny that demonstrate only a fraction of the insulin content found in endogenous beta cells. In addition, iPS protocols which use retroviral or lentiviral-mediated expression of the necessary reprogramming transcription factors, are currently not suitable for therapy due to the risk of *mutagenesis* and *tumorigenesis*. Improvements in protocols to generate iPS cells have been made that include non-integrating methods of gene delivery such as plasmid transfection (64, 65), adenoviral transduction (66, 67), the *piggyback* transposon system (68), or membrane-soluble reprogramming factors (69, 70). However, clinical cell-replacement therapy for diabetes has not yet been

achieved due to the continued obstacle of differentiating ES and iPS cells towards fully mature endocrine cells.

1.5.2 Endogenous beta cell regenerative therapies for diabetes

In the context of regenerative therapies for the treatment of diabetes, the potential contributions of transplanted stem cells are not limited to the direct replacement of damaged beta cells (Figure 4). The repair of damaged islets or the generation of new islets *in vivo* has also been proposed (53).

1.5.2.1 Bone marrow-derived stem cells

Adult, or post-natal stem cells can be obtained from several sources including umbilical cord blood (UCB) and bone marrow (BM). These stem cells have the potential to self-renew as well as develop into a wide range of regenerative cell types serving as easily procured sources to facilitate repair of damaged or diseased tissues (71). Human BM contains cells of the hematopoietic and endothelial lineages involved in revascularization (72), as well as cells of the mesenchymal lineage, involved in revascularization, regeneration and immunomodulation (72, 73). An early study using transplanted mouse BM-derived stem cells showed that these cells could differentiate *in vivo* into insulin producing beta cells. Donor BM cells were able to specifically repopulate the pancreatic islets without evidence of cell fusion with endogenous insulin producing cells (74). However, similar experiments by several other groups did not support this finding of transdifferentiation of BM cells into beta cells (75-77).

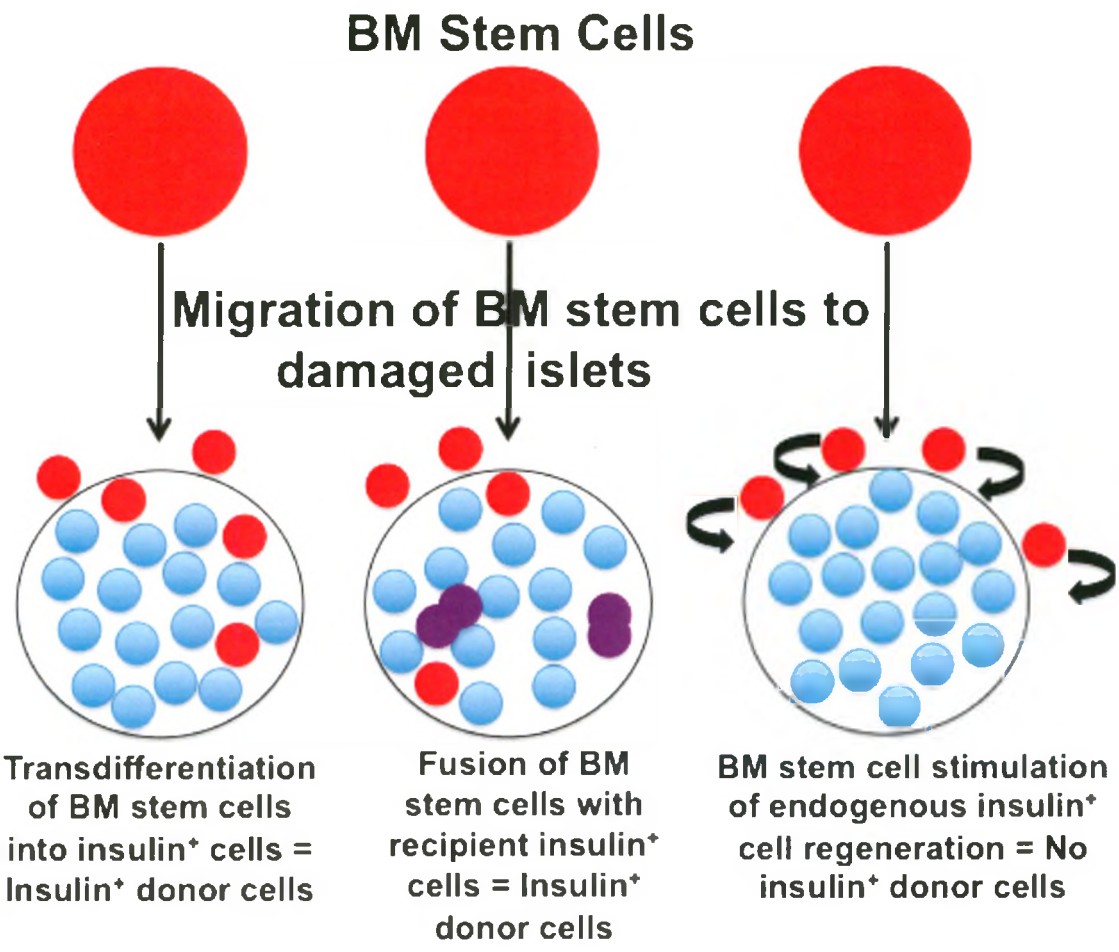

Figure 4. Proposed mechanisms of beta cell regeneration following transplantation of murine BM cells into hyperglycemic mice. Increased beta cell regeneration has been attributed to transdifferentiation of transplanted BM cells into insulin$^+$ donor cells. Fusion of transplanted cells with endogenous beta cells, leading to insulin$^+$ donor cells has also been suggested. The third proposed mechanisms for beta cell regeneration involves the transplanted cells secreting factors which stimulate endogenous beta cells to replicate in order to restore beta cell numbers.

Hess et al has previously shown that transplantation of murine BM-derived mononuclear cells, or further purified c-kit$^+$ progenitor cells, stimulate the recovery of streptozotocin (STZ)-damaged islets by inducing proliferation of recipient beta cells, augmenting glycemic control via the endogenous regeneration of beta cell function (75). Donor mouse cells with hematopoietic and endothelial phenotypes were recruited to ductal regions, surrounded damaged islets, and stimulated beta cell proliferation and insulin production in recipient-derived beta cells. Several groups have extended these findings to show that islet recovery can be induced by the induction of hematopoietic chimerism in overtly diabetic NOD mice (78), and that simultaneous infusion of murine BM mononuclear cells (MNC) with allogeneic mesechymal stem cells (MSC) optimized islet repair and protection against T-cell mediated beta cell deletion (79). Recently, Lee at al have shown that multiple high-dose intercardiac infusion of human BM-derived MSC into STZ-treated immune-deficient NOD/SCID mice have the ability to repair islets with minimal engraftment in the pancreas (73). Thus, human MSC and other potentially regenerative BM-derived cell populations represent an accessible population of expandable progenitors with regenerative and immunomodulatory properties (80-82) for the development of regenerative therapies for diabetes, with phase 2 clinical trials using MSC to treat recently diagnosed type 1 diabetics currently being performed by Osiris Therapeutics.

1.5.2.2 MSC for islet regeneration

The International Society of Cellular Therapy defines mesenchymal stem cells, or multipotent stromal cells (MSC) using three defining criteria: First, MSC are

isolated in culture by plastic adherence, second, after culture >95% of cells express CD73, CD90 (Thy-1), and CD105 (endoglin), but do not express hematopoietic markers such as CD45, and third, MSC are able to differentiate into multiple mesenchymal lineages (83). Specifically, they have multipotent differentiative ability into osteocytes, adipocytes and chondrocytes in secondary differentiation assays (84).

MSC migrate towards areas of damage and inflammation through their expression of a variety of chemokine receptors and adhesion molecules. *In vitro*, pancreatic islets attract MSC, mediated by CX3CL1-CX3CR1 and CXCL12-CXCR4 interactions (85). This same group also confirmed MSC migration to islets *in vivo*. MSC have also been shown to play an important role in modulating the immune response. In damaged tissues, MSC are able to stimulate reduced T-cell (86) and B-cell proliferation (87), inhibit maturation as well as differentiation of dendritic cells (88) and decrease the production of inflammatory cytokines by immune cells (89). MSC can exert their effects by secreting immunosuppressive molecules such as TGF-β, IL-10, IDO and PGE_2 (89). However, MSC require the proper microenvironment, with a recent study finding that only MSC that had exposure to activated T-cells displayed immunosuppressive effects (90), via the induction of regulatory T cells (82). Exhibiting immunomodulatory effects and having the capacity to escape immune recognition makes MSC an attractive cell type to treat autoimmune or inflammatory diseases including type 1 diabetes (82).

MSC also contain properties that aid in the repair of damaged tissues. MSC may modulate the microenvironment during injury and stimulate a shift from

inflammatory response to a repair response (91). MSC also aid in repair through exerting anti-fibrotic effects by secreting HGF and BMP-7 (92), and by stabilizing vessels during neoangiogenesis to allow for adequate perfusion.

Phase 2 clinical trials are currently underway using intravenous infusion of the drug PROCHYMAL®, in which the active ingredient is *ex vivo* cultured adult human MSC, to treat patients with newly diagnosed type 1 diabetes. The trial, performed by Osiris Therapeutics Inc in collaboration with the Juvenile Diabetes Research Foundation, involves administration of $2x10^6$ allogeneic MSC per kg body weight at 3 time points 30 days apart. The aim of the study is to determine whether transplanted MSC can protect the pancreatic tissue from further autoimmune attack and/or repair damaged pancreatic tissue, and is expected to be completed by December 2010.

1.5.2.3 UCB-derived stem cells for islet regeneration

In addition to the use of BM-derived stem cells or MSC, umbilical cord blood (UCB) derived stem cells have also been proposed to support the endogenous regeneration of beta cell function. In the past, clinical application of UCB-derived stem cells has been limited to transplantation in the fields of hematology and oncology, however an increasing number of studies support the use of these cells for non-hematopoietic disorders, including diabetes (93, 94).

Advantages of UCB-derived stem cells include: 1.) non-invasive collection procedure from the umbilical vein following separation of the umbilical cord from the newborn, 2.) it is a readily available source due to over 100 million births

worldwide annually (95), 3.) UCB is isolated early in ontogeny, with UCB cells having longer telomeres and containing telomerase activity (96), and 4.) UCB cells demonstrate a lower incidence of graft-versus-host disease following transplantation due to the lower number of T-cells, immaturity of B-cells and decreased function of dendritic cells (97). In addition to being a rich source of hematopoietic stem cells, UCB also contains endothelial progenitor cells (98) similarly to BM. However, only 1 in 10 UCB samples contain MSC (99), a cell type that is present in BM and has previously been implicated in beta cell regeneration. Therefore differences between adult or post-natal stem cell sources should not be overlooked when considering optimal cell source for therapy for diabetes.

In vivo studies involving UCB cell transplantation into diabetic mice have shown improvement in hyperglycemia and increased survival rates post-transplantation (100) as well as delayed onset of autoimmunity and insulitis in a model of type 1 diabetes (101). Although the mechanisms by which UCB stem cells regenerate the pancreas following transplantation are not yet known, in one study, insulin-producing cells of human origin were found in the islets of NOD/SCID mice following intravenous transplantation (102). Some insulin producing cells contained strictly human chromosomes, while other contained human and mouse chromosomes, leading these authors to suggest that generation of insulin-producing cells can be mediated through fusion-dependent as well as fusion independent mechanisms. A clinical trial using autologous UCB cells is currently underway in children with recently diagnosed type 1 diabetes, who have

autologous UCB stored (94). Thus far a slowing of the loss of endogenous insulin production and enhanced glucose control has been reported, and found to be due to a highly functional population of regulatory T cells (T-regs) within UCB (103, 104). These Tregs are proposed to suppress activation of the immune system and increase tolerance to self-antigens (94). Tregs preferentially localize to the sites of inflammation (105, 106) where they suppress recruitment and proliferation of effector T cells, and a decrease in frequency of naturally occurring Tregs can contribute to the onset of autoimmune diabetes (107). Therefore, both BM and UCB-derived cells partially function to improve and/or prevent autoimmune diabetes by promoting generation or expansion of endogenous Treg cells (82), or through provision of donor-derived Treg cells respectively. Thus, human BM and UCB represent promising sources of stem cells for regenerative therapy to treat diabetes in the future.

1.6 Endogenous pancreatic regeneration

The relevant mechanisms whereby the post-natal endocrine pancreas can undergo repair or regeneration after various injury stimuli remain highly controversial (Figure 5). In the healthy pancreas endogenous beta cell turnover is low. However lineage tracing studies by Dor et al have established that after partial pancreatectomy there is an increase in mature beta cell replication, and this represents the primary mechanism responsible for restoring beta cell number (108). In contrast several groups have suggested that beta cells can be regenerated via islet neogenesis, the budding of new islets from the ductal epithelia following partial pancreatectomy (109), or ductal ligation (110).

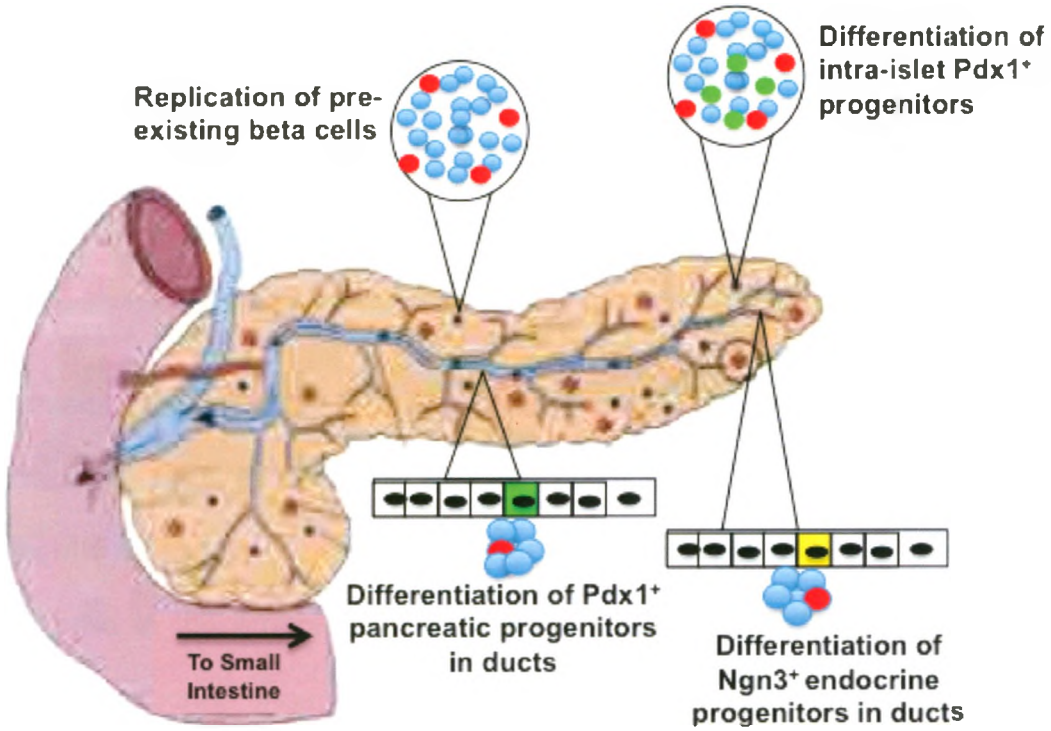

Figure 5. Mechanisms of pancreatic regeneration following injury. Following injury such as partial pancreatectomy, ductal ligation, or STZ-treatment, several regenerative mechanisms have been suggested to restore beta cell mass. Proliferation of remaining beta cells or neogenesis from intra-islet Pdx1$^+$ pancreatic progenitors has been demonstrated. Neogenesis from Pdx1$^+$ or Ngn3$^+$ progenitor cells in ductal regions has also been implicated in restoring beta cells.

Although the existence and identity of a pancreatic progenitor cell have been debated, Seaberg and colleagues have clonally isolated proliferative multipotent pancreatic precursor cells from adult pancreatic islets and ductal regions (111). Intra-islet $PDX1^+$ precursors (112-114) and $NGN3^+$ endocrine precursors associated with the ductal lining have also been reported (115, 116).

Overexpression and ablation studies have shown that pancreatic endocrine progenitor cells require transient expression of both PDX1 and NGN3 in development (117, 118). PDX1 is expressed early in development in pancreatic progenitor cells in the embryonic pancreatic ducts, differentiating towards both endocrine and exocrine lineage (119). PDX1 is subsequently repressed until re-expression in mature beta cells. $NGN3^+$ endocrine progenitor cells are a subset of $PDX1^+$ cells first appearing at embryonic day (E) 8.5, peaking at E15.5, and becoming undetectable with hormone gene expression (120). $NGN3^+$ cells are unipotent committed cells, with each cell differentiating into only one endocrine cell type.

In light of these current controversies, the regenerative programs activated during islet recovery after STZ-mediated beta cell ablation and human progenitor cell transplantation *in vivo* are largely uncharacterized and remain the key to understanding endogenous beta cell regeneration.

1.7 Isolation of progenitor cell subtypes from UCB and BM

In order to specify the human progenitor cell lineages that facilitate the regeneration of beta cell function after transplantation and to further investigate

the potential endogenous regenerative mechanisms involved in immune-deficient mice human UCB and BM stem cells were purified. With the exception of human HSC expressing CD34, prospective purification of infrequent UCB- or BM-derived stem cells for use in targeted regenerative therapy using cell surface markers is currently ineffective because many nascent stem cell subtypes demonstrate the absence of specific markers of differentiation *in situ* (121). Stem cell surface markers also vary between species, stem cell source, lineage and cell cycle progression during culture *in vitro* (122). In order to simultaneously isolate progenitor cells from multiple lineages, these progenitors were purified based on a conserved stem cell function, aldehyde dehydrogenase (ALDH) activity, an enzyme highly expressed in primitive hematopoietic and neural progenitor cells. In addition, ALDH1A1 activity is involved in intracellular oxidation of vitamin A to retinoid acid, and implicated in the protection of long-lived cells from oxidative stress (123, 124).

Relative ALDH activity can be detected using a fluorescent substrate for ALDH, Aldefluor™ (124). Aldefluor™ is a Bodipy fluorochrome conjugated to an aminoacetaldehyde (AAA) moiety, is uncharged and can freely diffuse across cell membranes. Once in the cell, cytoplasmic ALDH1A1 converts Aldefluor™ into a metabolized substrate that becomes trapped in the cell due to its negative charge. Under pharmacological inhibition of the ABC transporters with Aldefluor™ buffer, cells with high ALDH activity retain the Aldefluor™ and fluoresce (Figure 6). The integrity and function of the cell are not

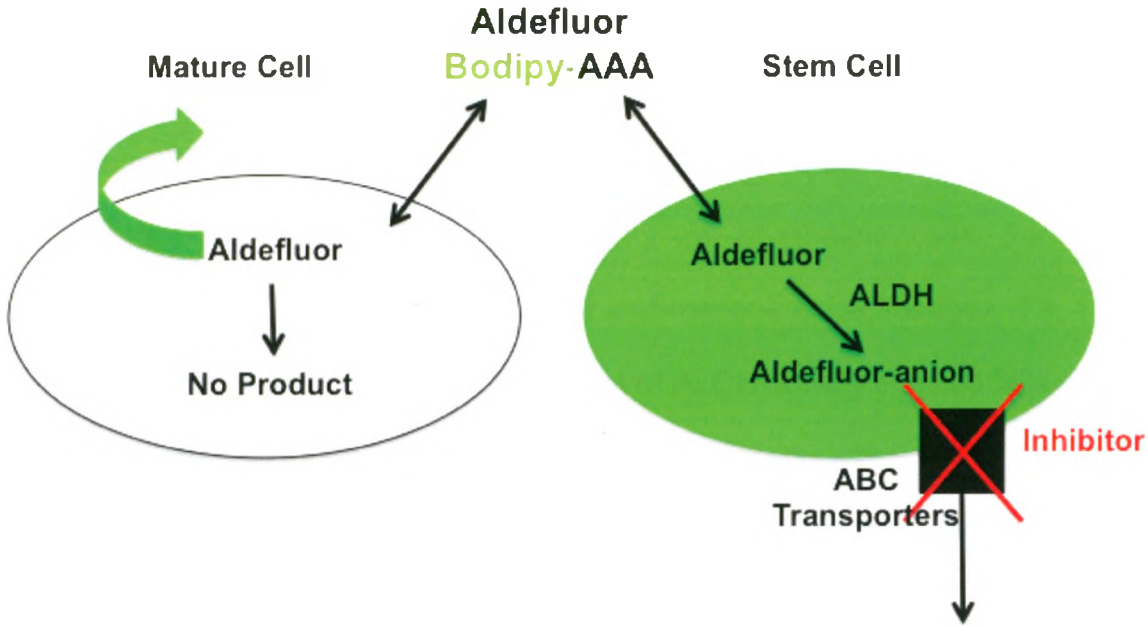

Figure 6. Isolation of stem cells based on aldehyde dehydrogenase activity. Diffusion of Aldefluor™ reagent into mature and stem cells, where only the stem cells contain high ALDH activity which converts the Aldefluor into a negatively charged substrate, trapping it within the cell and causing the cell to fluoresce. Stem cells can then be isolated using fluorescence activated cell sorting.

compromised by this procedure since upon removal of the Aldefluor™ buffer the ABC transporters become reactivated and Aldefluor™ can be pumped out of the cell returning the cell to it's original state. Therefore, this procedure is clinically applicable for the simultaneous enrichment of progenitor cells from the hematopoietic (122, 125), endothelial (126) and mesenchymal lineages (72, 126) for use in regenerative therapies. The amount of ALDH activity in a cell falls along a spectrum from low ALDH activity to high ALDH activity, where cells with high ALDH activity possess enriched progenitor function, are less committed to differentiation, and maintain multipotency (122). In contrast $ALDH^{lo}$ cells contain primarily mature T and B cells and demonstrate reduced colony forming capacity (72).

We have recently shown that after hematopoietic reconstitution in sublethally irradiated NOD/SCID/MPSVII mice, hematopoietic and non-hematopoietic progeny of transplanted human UCB-derived $ALDH^{hi}$ cells traffic efficiently to the ductal and peri-islet regions of the pancreas (127) and transplantation of human BM-derived $ALDH^{hi}$ progenitor cells promote endogenous revascularization in a mouse model of critical limb ischemia (72). Thus, is was hypothesized that transplantation of these prospectively purified mixed progenitor cells may also aid in the regeneration or damaged islets via increased beta cell proliferation, increased islet vascularization and increased insulin released systemically (128).

1.8 Islet vascularization

Adequate islet vasculature is critical for maintaining glucose homeostasis due to the high oxygen consumption of beta cells, as well as the need for timely responses to changes in glucose concentrations and subsequent release of islet hormones into the circulation. Islets have a dense capillary network that has been shown to be 5-times higher than in exocrine tissue (129). Each beta cell is in contact with at least one capillary, and they show polarity, with the secretory granules being adjacent to the vessels to facilitate hormone release (130). Islet capillaries also contain up to ten-fold more fenestrations than exocrine capillaries due to the necessity of close interactions between endocrine cells and the circulation. To ensure adequate islet capillary juxtaposition, pancreatic β-cells release VEGF-A, an angiogenic cytokine, which acts in a paracrine manner on neighbouring endothelial cells to establish a network of fenestrated capillaries (131). Pancreatic endocrine cells can upregulate VEGF-A during islet formation (132), as well as following injury (133), to aid in hormone secretion. Therefore transplantation of pro-angiogenic $ALDH^{hi}$ cells may stimulate increased islet vascularization through further support of these endogenous processes.

1.9 Hypothesis and Objectives

The regenerative potential of UCB and BM-derived adult stem cells on islet regeneration was investigated. The human source (UCB or BM) and cellular populations ($ALDH^{lo}$ or $ALDH^{hi}$) most effective at regenerating endogenous islets after transplantation into STZ-treated, immune-deficient recipients was sought to

be determined. Moreover, the lineage-specific contributions of transplanted progenitor subpopulations to support islet regeneration via putative pro-angiogenic or neogenic mechanisms were investigated.

It was hypothesized that human UCB and BM ALDH^{hi} cells will establish expandable cultures of hematopoietic, endothelial and mesenchymal lineages in vitro. Due to the increased progenitor frequencies, it was further hypothesized that transplantation of ALDH^{hi} cells will mediate recovery from hyperglycemia in immune-deficient mice with STZ-induced diabetes.

The first objective was to determine the in vitro colony forming frequency of UCB and BM ALDH^{lo} and ALDH^{hi} populations, and to establish expansion cultures of mesenchymal lineages for future transplantation.

The second objective was to determine the capacity of UCB and BM ALDH^{lo} and ALDH^{hi} cells to improve beta cell function when transplanted into immune-deficient STZ-treated mice, and to investigate the mechanisms by which transplanted cells augment beta cell function and insulin production.

Chapter 2

Materials and Methods

2.1 Progenitor cell isolation from human bone marrow and umbilical cord blood. Human bone marrow (BM) samples (15 ml) were drawn from the iliac crest with informed consent from healthy allogeneic donors and human umbilical cord blood samples (50 ml) were drawn with informed consent by venipuncture of the umbilical vein following scheduled C-sections at the London Health Sciences Centre, London, ON (Appendix 1). Samples were diluted with PBS (1:2 for BM, and 1:1 for UCB) and layered onto Hypaque-Ficoll (Pharmacia Biotech, Uppsala, Sweden) for centrifugation to isolate a layer of mononuclear cells (MNC). MNC were removed and resuspended in lysis buffer (ammonium chloride) to remove remaining red blood cells. MNC were then re-suspended in Aldefluor™ buffer (StemCell Technologies, Vancouver, BC) and incubated with Aldefluor reagent (5 µl/ml of cells) for 30-60 minutes at 37°C. Cells were centrifuged and re-suspended in Aldefluor™ buffer for purification. $ALDH^{lo}$ and $ALDH^{hi}$ cells with low side scatter were selected by fluorescence activated cell sorting (FACSVantage or FACSAria, BD Biosciences) at the London Regional Flow Cytometry Facility. Following sorting, Aldefluor™ labeled cells were washed with PBS to remove accumulated fluorescent substrate by reactivating transporters previously inhibited by Aldefluor™ buffer, leaving the cells in their original state.

2.2 Progenitor Cell Assays. Human UCB and BM $ALDH^{lo}$ and $ALDH^{hi}$ cell populations were enumerated for human hematopoietic colony forming cell (HCFC) activity following 10-12 days incubation in methylcellulose media (Methocult® H4434, StemCell Technologies). $ALDH^{lo}$ cells were plated at

densities of $2x10^3$ – $4x10^3$ cells/well, and $ALDH^{hi}$ cells were plated at densities of 100 - 200 cells/well of a 12-well plate. Endothelial colony forming cell (ECFC) activity and multipotent mesechymal stromal cell (MSC) activity were enumerated by plastic adherence following 12-14 days incubation in endothelial growth media (EGM-2 + Single Quots, Cambrex Biosciences, Inc., Walkersville, MD) and Amniomax™ media + supplement (Invitrogen, Burlington, ON, Canada) respectively. For non-hematopoietic colony formation, $ALDH^{lo}$ cells were plated at densities of $1x10^6$ – $2x10^6$ cells per T25 flask, and $ALDH^{hi}$ cells were plated at densities of $1x10^5$ – $3x10^5$ cells per T25 flask (Figure 7). Single endothelial or mesenchymal colonies were expanded *in vitro* for 2 passages and frozen in liquid nitrogen for future expansion.

2.3 Progenitor cell transplantation into hyperglycemic immune-deficient NOD/SCID mice. Adult (7-9 weeks) NOD/SCID (Jackson Laboratories, Bar Harbour, ME) mice were intraperitoneally (i.p.) injected with 35 mg/kg streptozotocin (STZ) (Sigma-Aldrich) in citric acid buffer (CAB) daily for 5 days (Day 1-Day 5) to induce hyperglycemia, or injected with CAB alone for normoglycemic controls. Mice reaching hyperglycemia (3-5 fold baseline blood glucose concentrations, or 15-25 mmol/L) by day 10, were sublethally irradiated (300 cGy) and transplanted by tail vein injection with PBS as control, UCB- or BM-derived MNC ($20x10^6$), $ALDH^{lo}$ ($10x10^6$) cells, or $ALDH^{hi}$ ($2x10^5$) cells, or cultured BM-derived $ALDH^{lo}$ or $ALDH^{hi}$ MSC ($5x10^5$). Blood glucose concentrations were monitored by tail venipuncture weekly until day 42 using a standard blood glucose monitor (FreeStyle mini, Abbott Diabetes Care Inc,

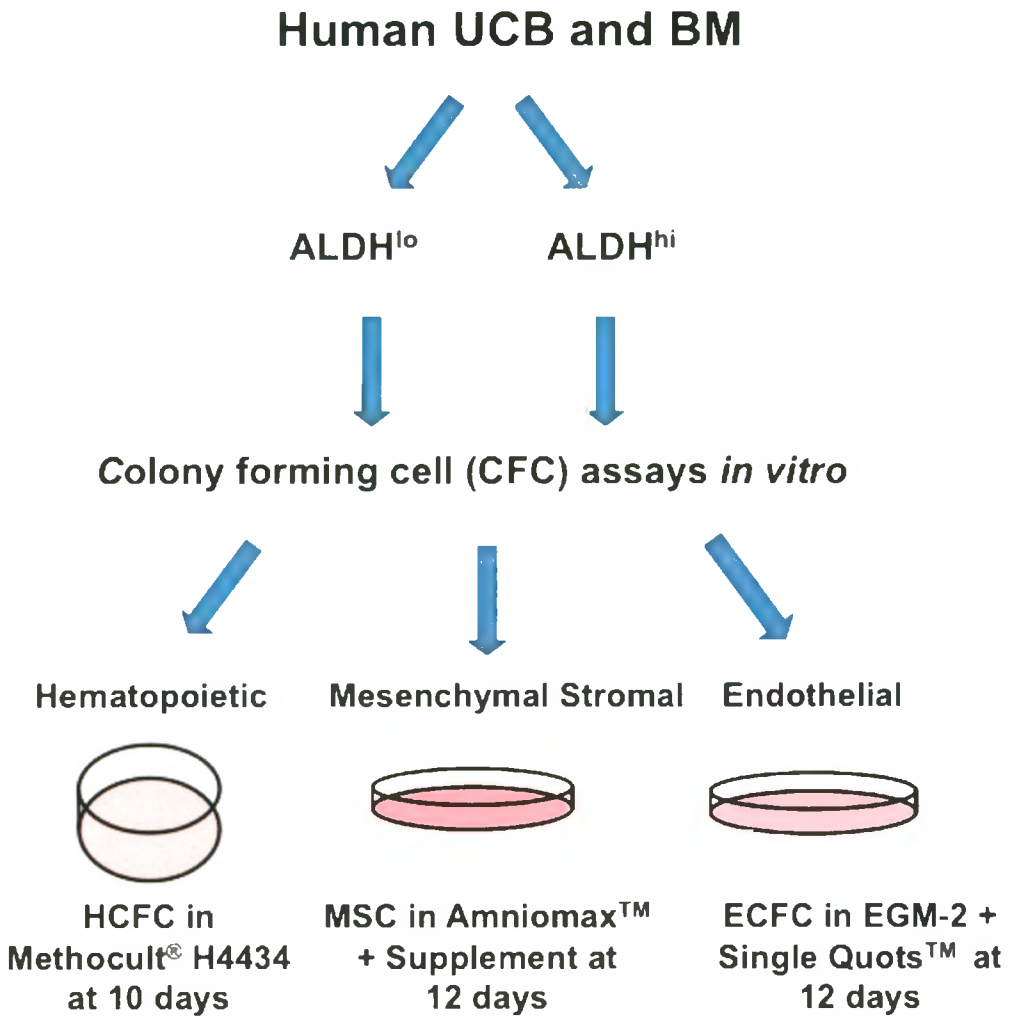

Figure 7. Schematic of *in vitro* colony forming assays. Human UCB and BM cells are sorted based on ALDH activity into ALDH^{lo} and ALDH^{hi} populations prior to being plated into media for hematopoietic, mesenchymal. and endothelial colony forming assays.

Alameda, CA) 24 hours prior to sacrifice, mice were i.p. injected with 200 µg of EdU (Invitrogen, Burlington, ON) to label dividing cells (Figure 8).

2.4 Human cell engraftment of mouse tissues. Isoflurane anesthetized (Baxter Corporation, Mississauga, ON) NOD/SCID mice were sacrificed on day 14, 17 or 42 (4, 7 or 32 days post-transplantation) by cervical dislocation. Blood was drawn immediately from the left ventricle, allowed to clot at room temperature for 30 min, and centrifuged for 15 min at 1.5 rcf to separate the serum layer. Serum was removed and frozen for subsequent serum insulin quantification. The pancreas was extracted and weighed, and the splenic portion of the pancreas was frozen in optimal cutting temperature media (OCT, Tissue Tek, Sakura Finetek, Torrance, CA). The remainder of the pancreas, the spleen, and a section of liver were extracted and mechanically digested in 1 ml PBS with 5% FBS using a 16 gauge needle and syringe. The BM was flushed from the femur and tibia. All digested tissues were filtered, centrifuged, and re-suspended in PBS with 5% FBS before being stained for the human cells using pan-leukocyte marker CD45 and HLA-A,B,C, for 30 min and for 7-AAD to identify dead cells, prior to analysis of human cell engraftment by flow cytometry (FACSCaliber, BD Biosciences). Engraftment of human cells were compared to matched isotype controls (all BD Biosciences).

2.5 Immunohistochemistry and immunofluorescence. The splenic portion of the pancreas was sectioned at 10 µm such that each slide contained 3 sections 150 µm apart. Pancreatic sections were analyzed by colorimetric immunohistochemistry for insulin$^+$ beta cells to determine islet size, islet number

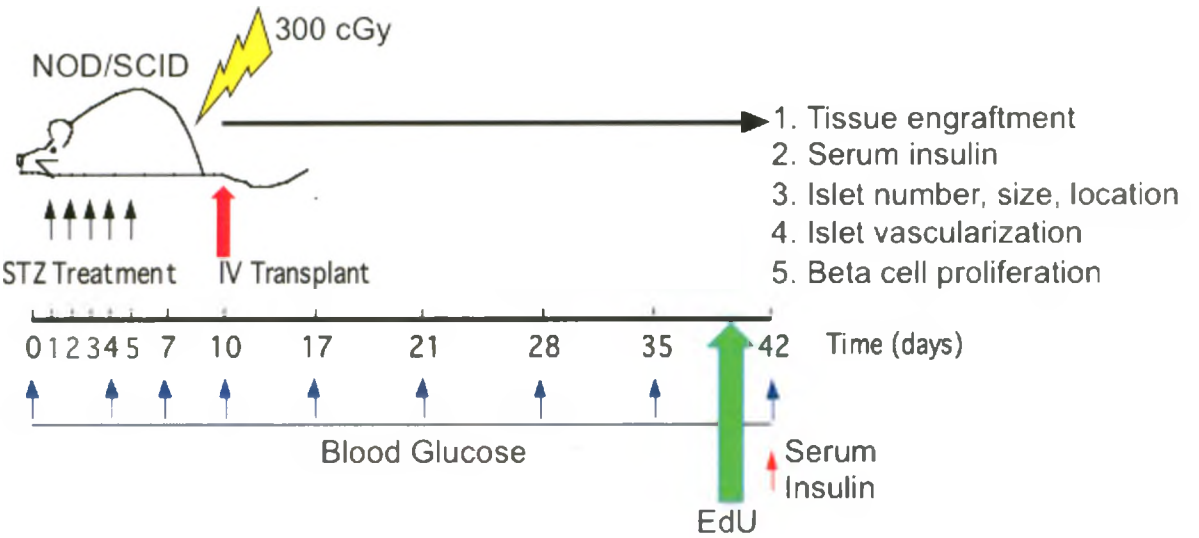

Figure 8. Schematic of *in vivo* beta cell regeneration model. Immune-deficient NOD/SCID mice were i.p. injected with STZ for 5 days to induce hyperglycemia. Hyperglycemic mice are sublethally irradiated and tail vein injected with PBS or UCB or BM ALDH-purified cells, or BM-derived ALDH-purified MSC on day 10. Blood glucose was monitored weekly until day 42, with EdU being administered 24 hours prior to sacrifice, and serum insulin being collected at sacrifice on day 42 for subsequent analysis of islet regeneration (Adapted from Hess et al., Nat Biotechnol, 2003).

per mm^2, and total beta cell mass. Immunofluorescence analysis was also performed for insulin$^+$ beta cells in islets, vWF$^+$ endothelial cells in blood vessels, CK19$^+$ epithelial cells in ducts, NGN3$^+$ endocrine progenitor cells, and EdU$^+$ proliferating cells as detailed below. All tissues stained for immunofluorescence were mounted in VectaShield with DAPI (Vector).

2.6 Quantification of islet size, number, and total beta cell mass. Frozen sections were fixed in 10% buffered formalin (Sigma), washed in PBS, and blocked with mouse-on-mouse reagent (Vector Labs, Burlingame, CA) and peroxidase block. Mouse anti-mouse insulin antibody (1 in 1000 dilution, Sigma) was detected with peroxidase labeled rabbit anti-mouse IgG antibody (1 in 500 dilution, Vector) and ImmPACT DAB (Vector) and counterstained with hematoxylin. Size and number of islets were quantified by light microscopy (Axioscope Z2, Zeiss, Germany) analyzing 4 areas (1 mm^2) selected randomly per section from a total of 3 sections per mouse using Northern Eclipse software. Beta cell mass was calculated by [beta cell area/(total area – beta cell area)] X pancreas weight.

2.7 Immunofluorescent islet blood vessel density quantification. For mouse vWF$^+$ endothelial cell detection, rabbit anti-mouse vWF antibody (1 in 200 dilution, Chemicon, Temecula, CA) was detected with fluorescein labeled goat anti-rabbit IgG secondary antibody (1 in 200 dilution, Vector). For mouse insulin$^+$ beta cell detection, mouse anti-mouse insulin antibody (1 in 1000 dilution, Sigma) was detected with Texas Red labeled rabbit anti-mouse IgG antibody (1 in 200 dilution, Vector). Intra- and extra-islet blood vessel densities at day 42 were

quantified using fluorescence microscopy (Axioscope Z2, Germany) and AxioVision software. vWF^+ vessels per islet and vWF^+ vessles per section area, not including islet bound vessels, were quantified for 4 islets randomly selected per section for each of 3 sections per mouse.

2.8 Immunofluorescent quantification of islet and duct association. To quantify percentage of islet directly associated with ductal epithelium, pancreatic tissue was stained with rabbit anti-mouse ck19 (1 in 200 dilution, Abcam Inc, Cambridge, MA) and detected with fluorescein labeled goat anti-rabbit IgG (1 in 500 dilution, Vector) to identify ductal epithelial cells, and co-stained with anti-mouse insulin as previously described. Each islet in 3 pancreatic sections per mouse was designated as either not associated with, or in direct contact with $CK19^+$ ducts at day 14, day 17 and day 42.

2.9 Immunofluorescent analysis of $Ngn3^+$ endocrine progenitor activation. Isoflurane anaesthetized pregnant NOD/SCID mice were sacrificed at 19 days gestation by cervical dislocation. E19 mouse fetuses were dissected and pancreata, brain and spinal cord were removed and frozen in OCT media. Tissues were cryostat sectioned at 10 µm and stained as positive controls for NGN3 expression. For mouse $Ngn3^+$ progenitor cell detection, goat anti-mouse NGN3 antibody (1 in 200 dilution, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) was detected with fluorescein labeled rabbit anti-goat IgG antibody (1 in 5000 dilution, Vector). BM-derived $ALDH^{lo}$ or $ALDH^{hi}$ MSC transplanted mice were stained for NGN3 at day 14, 17, and 42 to look for activation of endogenous endocrine progenitor cells.

2.10 Analysis of proliferating pancreatic cells. Mice were i.p. injected with 200 µg EdU 24 hours prior to sacrifice. EdU^+ proliferating pancreatic cells were detected using the Click-iT™ imaging kit (Invitrogen). Mouse testis were cryostat sectioned at 10 µm and stained as positive control for a highly proliferative tissue. Pancreata were co-stained for EdU and anti-mouse insulin or vWF as previously described to identify dividing islets and vessels respectively. Percentage of dividing beta cells was quantified by counting the number of $EdU^+Insulin^+$ cells per number of $insulin^+$ cells in each of 3 sections per mouse.

2.11 Serum insulin quantification. Mouse serum insulin was quantified at day 42 (32 days post-transplantation) using mouse insulin ultra-sensitive EIA ELISA kits (ALPCO, Salem, NH). 20 µl of serum was analyzed in duplicate per cell-transplanted mouse, and compared to PBS transplanted control and CAB injected normoglycemic control insulin levels.

2.12 Statistical Analysis. Data were expressed as mean ± standard error of the mean (SEM). Analysis of statistical significance was performed using an unpaired t-test for CFC frequencies, by two-way ANOVA for blood glucose data and by one-way ANOVA followed by Tukey's post-hoc test for all other analyses.

Chapter 3

Results

3.1 Isolation of ALDH^{lo} and ALDH^{hi} cells from human UCB and BM. In order to simultaneously purify cellular populations potentially enriched for multiple progenitor phenotypes, we FACS-purified human UCB (Figure 9A) and BM (Figure 9B) based on low side scatter, to remove granulocytes, and low versus high Aldefluor ™ fluorescence. Cells with high ALDH-activity (ALDH^{hi}) were rare in human UCB (0.3±0.1%) and BM (0.8±0.2%), whereas cells with >5-fold lower ALDH-activity (ALDH^{lo}) represented 34.3±2.6% of erythroid-depleted UCB or 8.2±1.3% of BM mononuclear cells (MNC) respectively. We have previously shown that both UCB-ALDH^{hi} cells (122), and BM-ALDH^{hi} cells (72), demonstrated enriched expression of primitive cell surface markers (CD34, CD133, c-kit), whereas ALDH^{lo} cells demonstrated enriched expression of cell surface markers of mature T-lymphocytes (CD3, CD4, CD8) and B-lymphocytes (CD19, CD20).

3.2 UCB-derived ALDH^{hi} cells were enriched for hematopoietic and endothelial colony forming cells. In UCB samples, both ALDH^{lo} and ALDH^{hi} cell populations contained hematopoietic colony forming cells (HCFC), capable of forming colonies of erythroid (Figure 10A), macrophage and granulocyte lineages. ALDH^{hi} cells were enriched for hematopoietic colony forming cells (~1 HCFC in 4 cells) compared to ALDH^{lo} cells (~1 HCFC in $7x10^3$) (Figure 10C). Similarly, both populations of cells contained endothelial colony forming cells (ECFC) (Figure 10B), with ALDH^{hi} cells being enriched (~1 ECFC in $4x10^4$) compared to ALDH^{lo} cells (~1 ECFC in $1x10^5$) (Figure 10D). Neither ALDH^{lo} nor

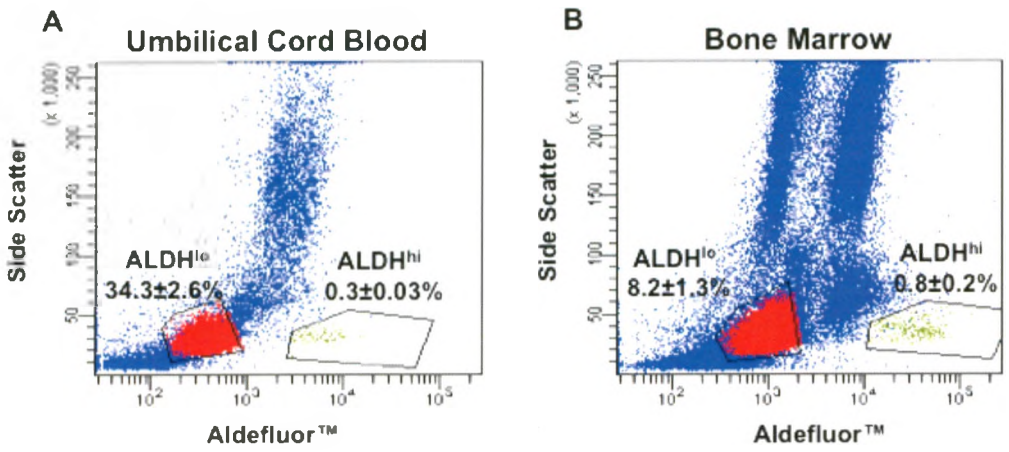

Figure 9. Isolation of ALDH^{lo} and ALDH^{hi} cell populations from human UCB and BM. Representative FACS sort plots for the isolation of cells from **(A)** human umbilical cord blood (n=9) and **(B)** human bone marrow (n=8), with low side scatter and low vs. high Aldefluor ™ fluorescence.

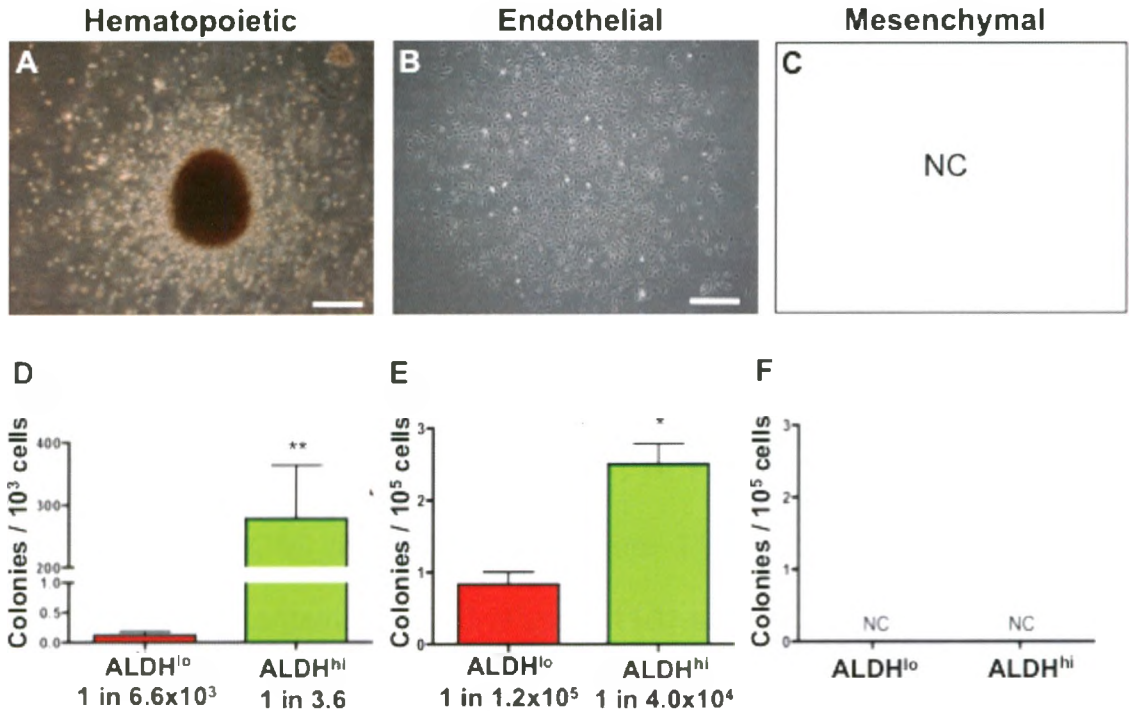

Figure 10. UCB-derived ALDH^{hi} cells were enriched for hematopoietic and endothelial colony forming cells. Representative photomicrographs of **(A)** hematopoietic, **(B)** endothelial, and **(C)** mesenchymal colonies 14 days following plating of UCB-derived ALDH^{hi} cells under hematopoietic, endothelial, or mesenchymal growth conditions respectively. **(D)** UCB-derived ALDH^{hi} cells are enriched for hematopoietic colony forming cells (n=4). **(E)** UCB-derived ALDH^{hi} cells are enriched for endothelial colony forming cells (n=4). **(F)** UCB-derived ALDH^{lo} and ALDH^{hi} cells were devoid of mesenchymal colony forming cells (n=4) (NC= No Colonies). Scale bar = 200μm for hematopoietic colonies, and 500μm for endothelial colonies. Data represent mean ± SEM (*P<0.05, **P<0.01).

$ALDH^{hi}$ UCB-derived populations established mesenchymal colonies (MCFC) in UCB samples isolated by venipuncture.

3.3 BM-derived $ALDH^{hi}$ cells were enriched for hematopoietic, endothelial and mesenchymal colony forming cells. Similar to UCB-derived cells, BM-derived $ALDH^{lo}$ and $ALDH^{hi}$ cell populations could establish hematopoietic colonies of erythroid (Figure 11A), macrophage and granulocyte lineages, with the $ALDH^{hi}$ cells being enriched for HCFC (~1 HCFC in 8 cells) compared to the $ALDH^{lo}$ cells (~1 HCFC in $2x10^3$ cells) (Figure 11D). $ALDH^{lo}$ and $ALDH^{hi}$ BM-derived cells also contained ECFC (Figure 11B) and MCFC (Figure 11C), with $ALDH^{hi}$ cells being enriched for ECFC (~1 ECFC in $1x10^3$) compared to $ALDH^{lo}$ cells (~1 ECFC in $4x10^5$) (Figure 11E), as well as being enriched for MCFC (~1 MCFC in $1x10^3$) compared to $ALDH^{lo}$ cells (~1 MCFC in $3x10^4$) (Figure 11F) in lineage selective media *in vitro*. Collectively, these analyses suggest that both UCB- and BM-derived $ALDH^{hi}$ cells contain progenitors with putative pro-angiogenic functions (72), whereas human BM-derived $ALDH^{lo}$ or $ALDH^{hi}$ cells also contained expandable MSC, previously implicated in islet regeneration (73).

3.4 Transplantation of UCB- or BM-derived $ALDH^{hi}$ cells reduced blood glucose in STZ-treated hyperglycemic mice. To evaluate the capacity of human UCB or BM progenitor cells to restore islet function after injury, STZ-treated (35 mg/kg/day, days 1-5) hyperglycemic (15-25 mmol/L at day 10) NOD/SCID mice were intravenously injected with human $ALDH^{lo}$ cells ($10x10^6$), $ALDH^{hi}$ cells ($2x10^5$) or MNC ($20x10^6$) at day 10 and blood glucose concentrations were monitored until day 42. Cell doses were chosen according to

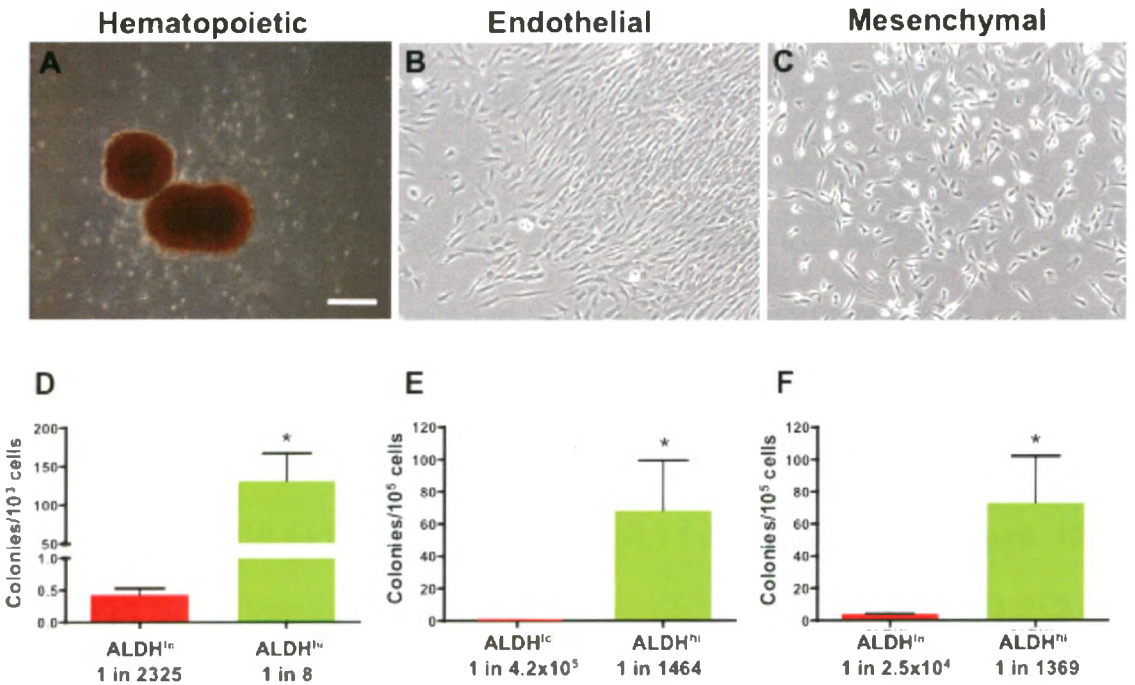

Figure 11. BM-derived ALDH^{hi} cells were enriched for hematopoietic, endothelial, and mesenchymal colony forming cells. Representative photomicrographs of **(A)** hematopoietic, **(B)** endothelial, and **(C)** mesenchymal colonies 14 days following plating of BM-derived ALDH^{hi} cells in hematopoietic, endothelial or mesenchymal growth conditions. Scale bars = 200μm. **(D)** BM-derived ALDH^{hi} cells were enriched for hematopoietic colony forming cells (n=4). **(D)** BM-derived ALDH^{hi} cells were enriched for endothelial colony forming cells (n=4). **(E)** BM-derived ALDH^{hi} cells were enriched for mesenchymal colony forming cells (n=4). Data represent mean ± SEM (*P<0.05, **P<0.01).

the availability of sorted cell populations and the relative frequencies of progenitor colony formation in vitro. Compared to PBS-injected controls that remained severely hyperglycemic (24.4±2.9 – 31.9±2.1 mmol/L) after day 17, transplantation of $10x10^6$ UCB-derived $ALDH^{lo}$ cells lead to a temporary reduction in hyperglycemia (18.1±1.9 mmol/L) at day 17, with a gradual return of equivalent hyperglycemia from days 21-42 (Figure 12A). Transplantation of 50-fold fewer ($2x10^5$) UCB-derived $ALDH^{hi}$ cells induced a modest but lasting relative reduction in hyperglycemia (18.4±1.8 – 23.2±3.1 mmol/L) from days 17-42 (Figure 12A). Interestingly, mice transplanted with low-dose ($2x10^5$) $ALDH^{lo}$ cells (24.2±3.4 – 3.5±3.4 mmol/L, n=5), or $20x10^6$ unpurified UCB MNC (20.9±2.4 – 26.1±2.8 mmol/L, n=9) did not improve hyperglycemia compared to PBS controls (Data now shown).

Mice transplanted with equivalent doses of human BM-derived cells showed a similar pattern of hyperglycemic reduction. Transplantation of BM-derived $ALDH^{lo}$ cells transiently reduced hyperglycemia at day 21 (17.3±2.4 mmol/L) and 28 (21.5±3.1 mmol/L) compared to temporally matched PBS controls (Figure 12B). Likewise, transplantation of BM-derived $ALDH^{hi}$ cells induced significant and stable reductions in hyerglycemia (16.0±2.1 – 20.1±3.6 mmol/L) from days 17-42.

For serum insulin concentrations at day 42, all STZ-injected mice showed reduced serum insulin compared to citric acid buffer (CAB)-injected vehicle controls (**$P<0.01$, Figure 12, C and D). Compared to PBS controls (0.04±0.01 ng/ml), only BM-derived $ALDH^{hi}$ cell transplanted mice had increased serum

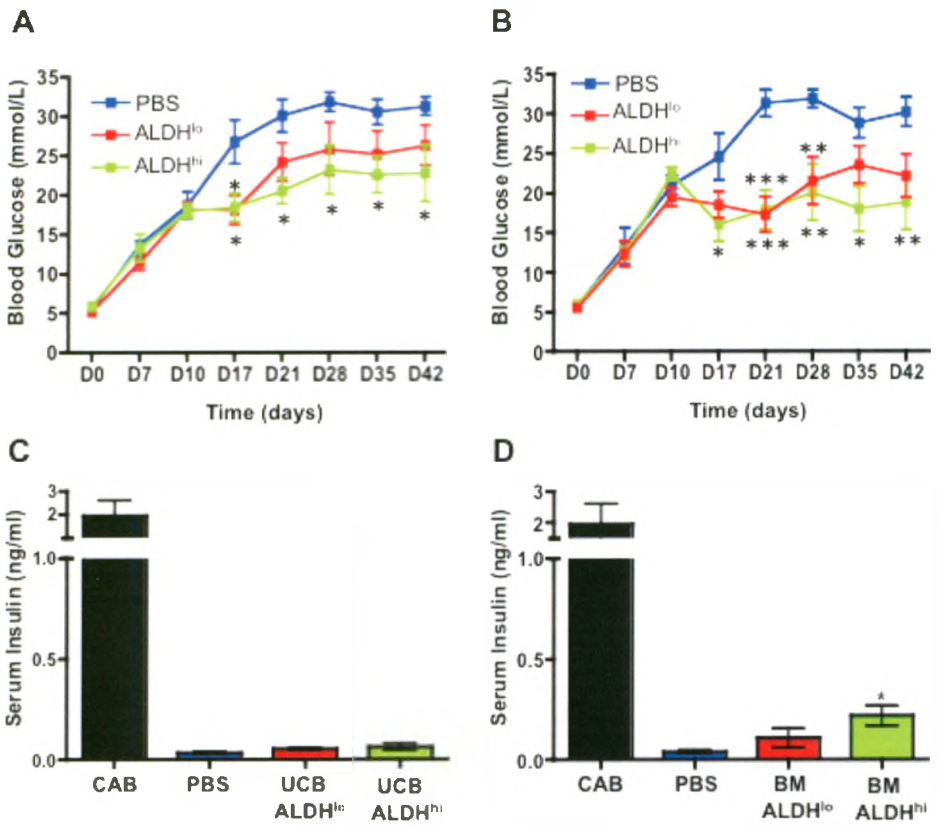

Figure 12. Transplantation of UCB- or BM-derived ALDH^{hi} cells reduced blood glucose in STZ-treated hyperglycemic mice. Compared to PBS-transplanted controls (n=7), mice transplanted with **(A)** UCB ALDH^{lo} cells (n=9) had temporarily reduced hyperglycemia at day 17 with subsequent return of hyperglycemia while mice transplanted with UCB ALDH^{hi} cells (n=7) had stably reduced hyperglycemia from day 17-42. **(B)** Mice transplanted with BM ALDH^{lo} cells (n=10) had transiently reduced hyperglycemia days 21-28, while mice transplanted with BM ALDH^{hi} cells (n=8) had stably reduced hyperglycemia. **(E-F)** At day 42, only STZ-treated mice transplanted with BM-derived ALDH^{hi} cells had increased serum insulin compared to PBS controls. Data represent mean ± SEM (*P<0.05, **P<0.01, ***P<0.001).

insulin (0.22±0.05 ng/ml) at day 42 (Figure 12D), corresponding to the observed decreases in blood glucose concentrations. Thus, BM-ALDH^{hi} cell transplantation stably reduced systemic hyperglycemia and increased serum insulin. Collectively, transplantation of low-dose ALDH^{hi} cells was more effective than high-dose ALDH^{lo} cells at sustaining glycemic control, and BM-derived ALDH^{hi} cells were superior to UCB-derived ALDH^{hi} cells in terms of the magnitude of improved hyperglycemia and serum insulin release.

3.5 Engraftment of human cells into recipient mouse BM and pancreas. To promote human hematopoietic reconstitution after transplantation, hyperglycemic NOD/SCID mice were sub-lethally irradiated (300 cGy) on day 10 prior to human UCB- or BM-derived cell transplantation. CD45^{+}/HLA-A,B,C^{+} human cell engraftment in recipient mouse BM and pancreas was detected by flow cytometry (Figure 13 A and B). There was robust human hematopoietic reconstitution at day 42 after transplantation with equivalent doses ($2x10^5$) of ALDH^{hi} cells from UCB (28.0±6.0%) or BM (12.0±3.0%) (Figure 14 A and B). In contrast, 50-fold higher doses ($10x10^6$) of ALDH^{lo} cells from human UCB or BM did not consistently engraft recipient BM (Figure 14A and B).

Pancreatic damage due to pre-treatment with multiple low-dose STZ injections has previously shown to increase pancreas recruitment of murine BM cells after hematopoietic progenitor cell transplantation (75). Despite high levels of human hematopoietic reconstitution, intravenously injected ALDH^{hi} cells from human UCB showed inconsistent (4 or 9 transplanted mice) and low frequency (<0.4%) human hematopoietic (CD45^{+}) cell engraftment in the murine pancreas (Figure

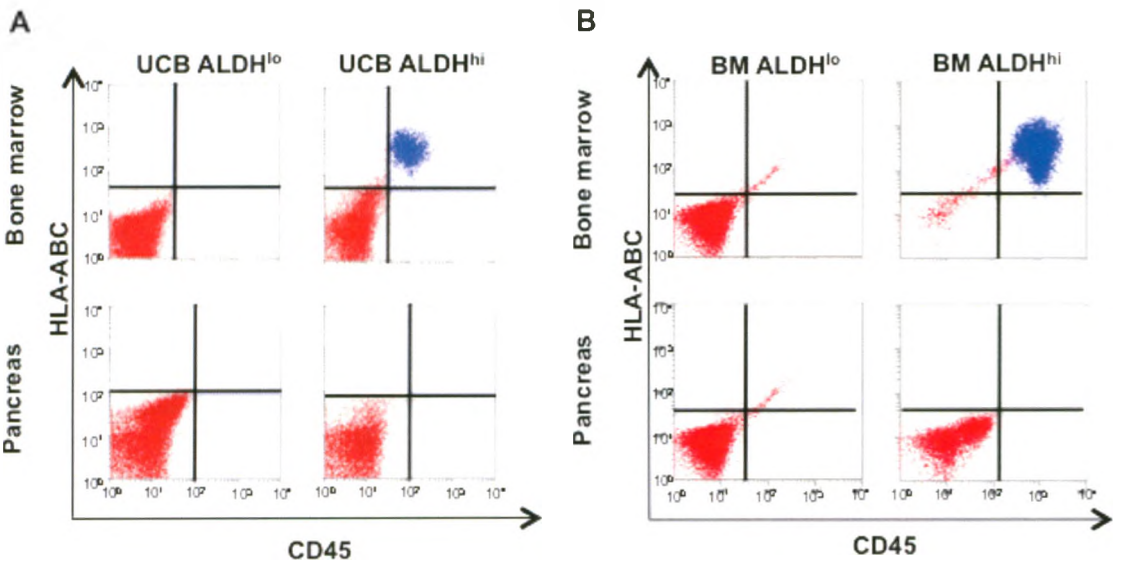

Figure 13. UCB and BM ALDH^{hi} cells engraft recipient mouse bone marrow. Representative flow cytometric quantification of human cell engraftment at day 42 in the BM and pancreas of recipient mice intravenously transplanted with 10x10^6 ALDH^{lo} or 2x10^5 ALDH^{hi} cells from human **(A)** UCB or **(B)** BM. ALDH^{hi} cells from both sources highly engrafted recipient BM, and had low levels of engraftment in recipient pancreas, while ALDH^{lo} cells did not engraft recipient tissues.

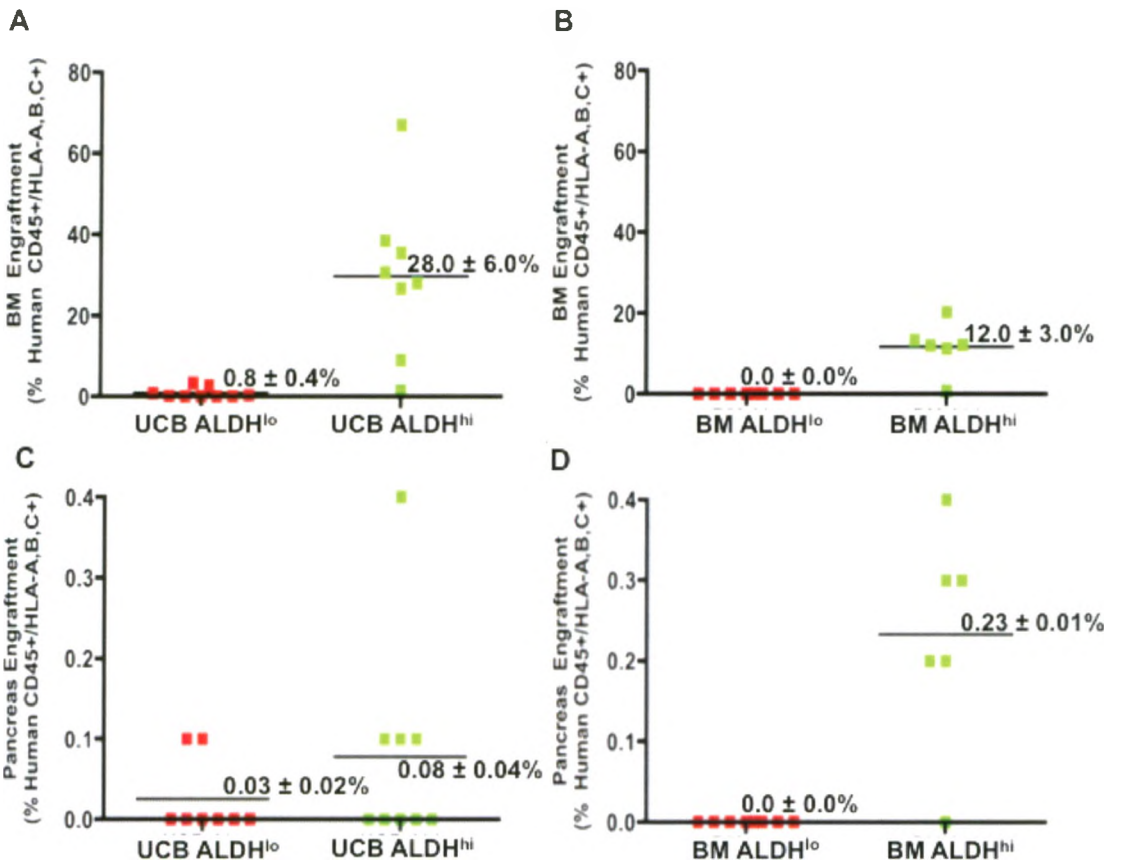

Figure 14. Transplantation of human ALDH^{hi} cells resulted in significant human hematopoietic chimerism in mouse BM. Transplantation of 2x10^5 ALDH^{hi} cells from human **(A)** UCB or **(B)** BM into sublethally irradiated STZ-treated NOD/SCID mice highly engrafted recipient murine BM. ALDH^{lo} cells did not engraft recipient BM. **(C-D)** Transplantation of human ALDH^{hi} cells from BM, but not UCB resulted in consistent human cell engraftment in recipient mouse pancreata.

14C). BM-derived $ALDH^{hi}$ cells consistently (5 of 6 transplanted mice) engrafted the recipient pancreas, however human cell frequency remained low (0.23±0.01%), and pancreas engrafted human cells were largely hematopoietic (Figure 13B). Transplanted $ALDH^{lo}$ cells from human UCB or BM were rarely detected in the mouse pancreas at day 42 (Figure 14, C and D), indicating that transplanted human $ALDH^{hi}$ cells showed enhanced engraftment in the injured mouse pancreas, albeit at low frequencies.

3.6 Early recruitment of human cells to injured murine pancreata. To address the initial recruitment of human cells to the injured pancreas at the time of hyperglycemia reduction, STZ-treated mice were transplanted with $10x10^6$ $ALDH^{lo}$ or $2x10^5$ $ALDH^{hi}$ cells at day 10 and euthanized at day 14 or 17. At 4 days post-transplantation (day 14), human cells were not detected in the pancreas of mice transplanted with either $ALDH^{lo}$ (n=3) or $ALDH^{hi}$ (n=3) cells. By 7-14 days post-transplantation, human cells were detected at low frequency (<0.1% human cells) in 5 of 6 mice transplanted with $ALDH^{hi}$ cells compared to only 1 of 8 mice transplanted with $ALDH^{lo}$ cells. Although low frequency human $ALDH^{hi}$ cell recruitment to the pancreas was sufficient to observe improved hyperglycemia, more efficient delivery and improved survival of progenitor cells in the pancreas may be required to further improve glycemic control.

3.7 Transplantation of ALDH-purified cells increased endogenous islet size, but not islet number. Although we have previously established that transplanted murine BM progenitor cells stimulate endogenous islet recovery (75), the mechanisms by which transplanted human progenitor cells support a

regenerative niche in the pancreas remain uncharacterized. To further investigate the role of endogenous islet regeneration after human progenitor cell transplantation in STZ-treated mice, the pancreata of control and transplanted mice were stained for murine insulin (Figure 15) and analyzed for islet number (Figure 16A), islet size (Figure 16B) and total beta cell mass (Figure 16C), revealing increased islet size following ALDH-purified mixed progenitor cell transplantation.

To confirm severe pancreatic damage at day 10, multiple low-dose STZ administration induced approximately 10-fold reduced islet number (0.16±0.03 islet/mm^2), and a 2-fold reduced islet circumference (225±25 μm), compared to CAB injected vehicle controls. Over the 32 day post-transplantation period, STZ-treated mice injected with PBS at day 10 increased islet number approximately 3-fold (0.54±0.10, *P<0.05), without significant increase in mean islet size (198±18 μm), indicating that endogenous islet recovery could be detected without corresponding control of hyperglycemia (Figure 12 A and B). Compared to CAB-injected normoglycemic mice, islet number remained 2-fold lower and mean islet size was significantly decreased at day 42 (P<0.05, Figure 16, A and B). Compared to PBS-injected controls, transplantation of UCB- or BM-derived ALDH^{lo} or ALDH^{hi} cells had no effect on overall islet number at day 42 (Figure 16A), however there was a significant increase in islet size following UCB ALDH^{lo} or ALDH^{hi} cell transplantation, to 273±22 μm and 266±17 μm respectively (Figure 16B). Mean islet size was further increased following BM ALDH^{lo} or ALDH^{hi} cell transplantation to 337±42 μm and 390±33 μm respectively, and equivalent to

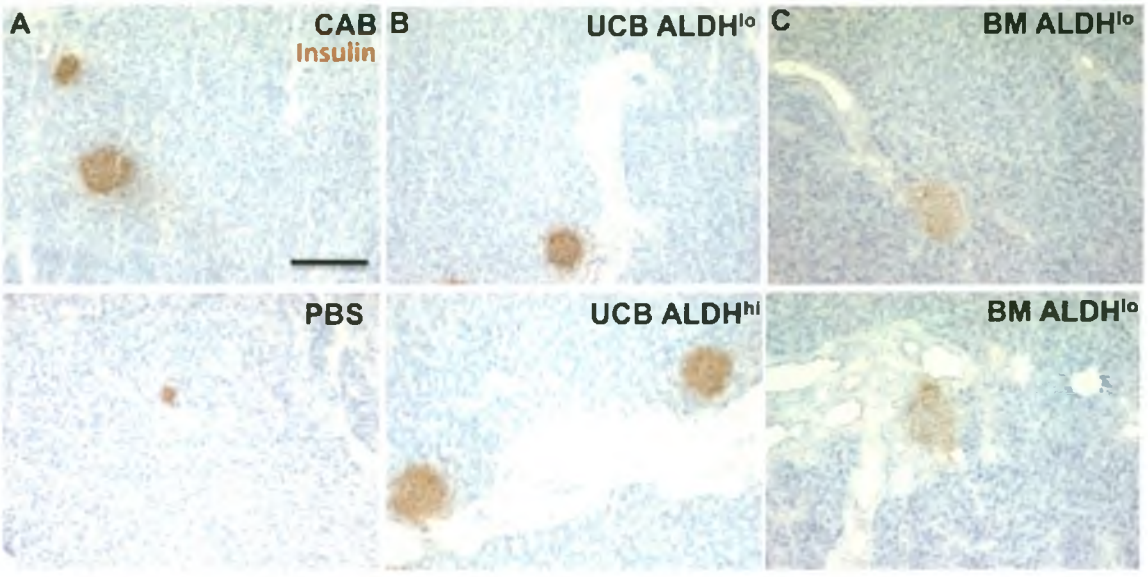

Figure 15. Transplantation of ALDH-purified UCB- or BM-derived cells increased islet size in STZ-treated recipients. Representative photomicrographs of insulin$^+$ islets at day 42 in mice injected with **(A)** citric acid buffer (CAB) vehicle (upper), or STZ (35mg/kg, days 1-5) followed by transplantation at day 10 with PBS (lower), **(B)** UCB ALDH^{lo} or ALDH^{hi} cells, or **(C)** BM ALDH^{lo} or ALDH^{hi} cells. Scale bars = 200μm.

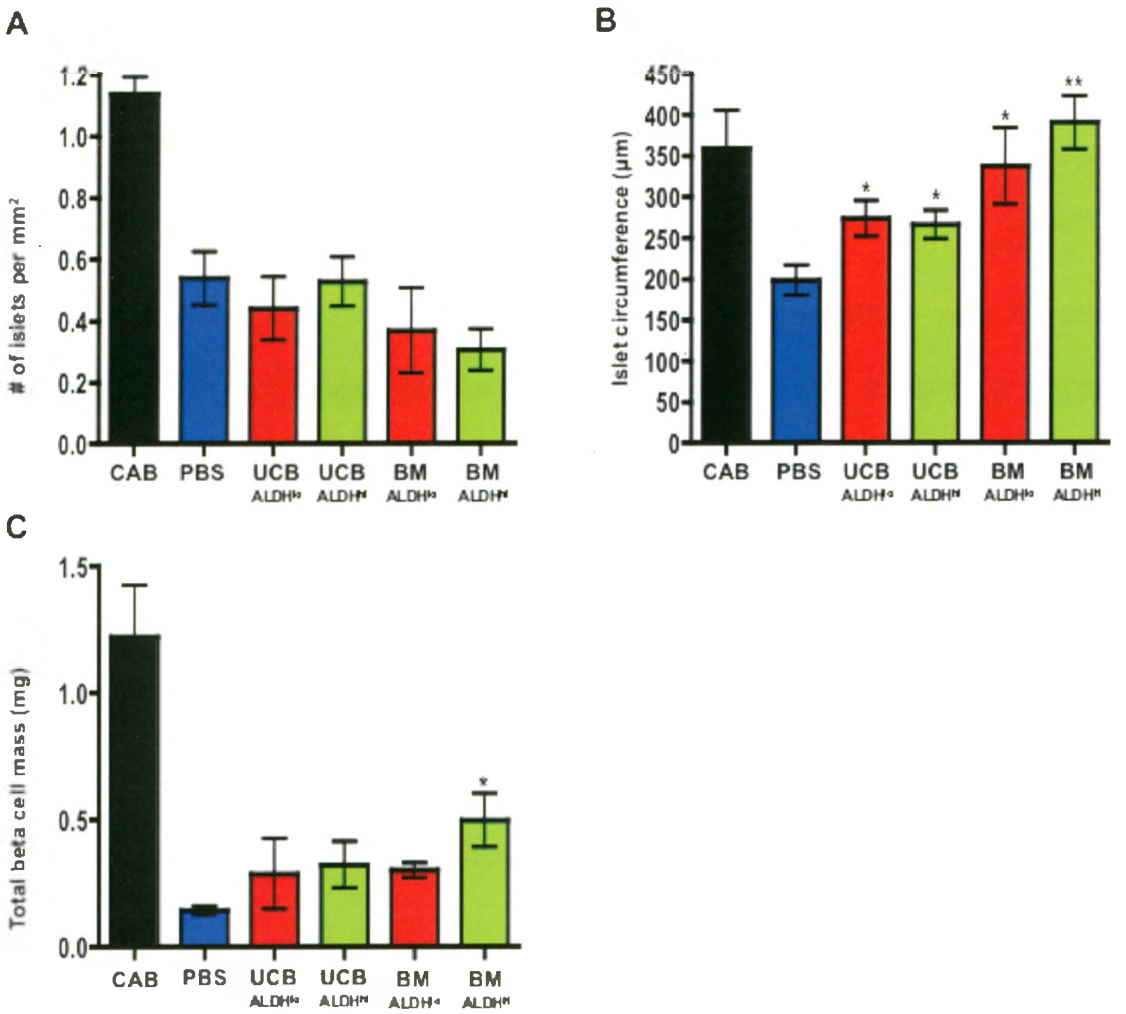

Figure 16. Transplantation of BM-derived ALDH^{hi} cells increased islet size and total beta cell mass in STZ-treated recipients. Compared to PBS-transplanted controls (n=10), mice transplanted with UCB ALDH^{lo} (n=7), UCB ALDH^{hi} (n=7), BM ALDH^{lo} (n=7) or BM ALDH^{hi} (n=7) cells showed **(A)** equivalent islet numbers, and **(B)** increased islet size. **(C)** Mice transplanted with BM ALDH^{hi} cells showed increased total beta cell mass compared to PBS controls. Data represent mean ± SEM (*P<0.05, **P<0.01).

baseline islet size in CAB-treated control mice (358±40 μm). As an overall indication of islet recovery, transplantation of BM ALDH^{hi} cells significantly increased total beta cell mass to 0.50±0.09 mg, compared to PBS controls at 0.14±0.01 mg (Figure 16C). Collectively, only human BM ALDH^{hi} cell transplanted mice demonstrated increased islet size correlated with increased total beta cell mass, augmented serum insulin levels, and prolonged glycemic control.

3.8 Transplantation of *ex vivo* expanded MSC from independent BM samples showed variable capacity to reduce hyperglycemia. Lee et al., has previously shown that intercardiac injection of dual bolus human BM MSC improved hyperglycemia via the endogenous repair of pancreatic islets and renal glomeruli after low-dose STZ-treatment in NOD/SCID mice (73). Since human BM-derived ALDH^{lo} and ALDH^{hi} populations contained cells with mesenchymal colony forming capacity (72), whereas analogous human UCB cells did not (Figure 10E), and human BM cells were superior to human UCB cells in the magnitude of hyperglycemic correction (Figure 12, A and B), we sought to further investigate the capacity of *ex vivo* expanded ALDH^{lo} or ALDH^{hi} MSC to improve hyperglycemia after transplantation.

Clonally established ALDH^{lo} or ALDH^{hi} MSC lines from 6 independent BM samples were expanded for 3-5 days in *ex vivo* culture, and transplanted into hyperglycemic NOD/SCID mice as a single bolus of $5x10^5$ MSC at day 10. After initial screening, transplantation of ALDH-purified MSC showed donor dependent variability in the capacity to improve hyperglycemia (Table 1). Independent of

Table 1. ALDH^{lo} and ALDH^{hi} MSC from independent BM samples showed variable capacity to reduce hyperglycemia. Transplantation of 5x10^5 ALDH^{lo} or ALDH^{hi} BM-derived MSC from 6 independent BM samples into hyperglycemic immune-deficient NOD/SCID mice **(A)** reduced blood glucose, or **(B)** did not reduce blood glucose from day 17-day 42 compared to **(C)** PBS controls. Data shown as mean ± SD. (*p<0.05, **p<0.01, ***p<0.001).

	BM Sample	Cell Population	n	Day 10 Glucose (mmol/L)	Day 17 Glucose (mmol/L)	Day 28 Glucose (mmol/L)	Day 42 Glucose (mmol/L)
A	BM12	ALDH^{lo}	3	21.0±2.6	***6.1±0.7	***11.2±1.0	***12.7±2.6
		ALDH^{hi}	3	21.2±3.2	**13.6±0.0	*18.7±2.7	**17.3±1.0
	BM17	ALDH^{lo}	5	20.6±2.2	***12.2±5.3	***16.0±6.6	***18.1±8.1
		ALDH^{hi}	6	21.9±4.5	*17.8±5.5	*23.6±7.3	**20.5±6.0
	BM20	ALDH^{lo}	6	19.9±2.8	***13.9±6.5	***15.3±6.0	***17.8±6.3
		ALDH^{hi}	7	19.6±2.2	***13.5±4.9	***15.5±4.6	***16.3±7.0
B	BM16	ALDH^{lo}	3	21.4±4.1	18.3±5.6	30.1±4.6	33.0±0.0
		ALDH^{hi}	3	20.5±4.3	28.0±3.7	33.0±0.0	29.8±4.3
	BM18	ALDH^{lo}	7	21.5±4.9	25.4±5.7	33.0±0.0	26.2±2.7
		ALDH^{hi}	6	23.4±3.0	29.4±3.0	31.6±2.3	31.1±2.3
	BM21	ALDH^{lo}	3	22.5±4.6	31.1±1.9	23.9±9.1	33.0±0.0
		ALDH^{hi}	3	21.8±5.1	16.5±4.9	23.2±3.7	33.0±0.0
C	PBS		7	20.6±4.9	25.6±6.8	28.6±7.8	30.7±6.2

whether the MSC were initially derived from $ALDH^{lo}$ or $ALDH^{hi}$ cells, mice transplanted with expanded MSC from 3 independent human BM samples (BM12, BM17, BM20) showed consistent reductions in hyperglycemia (Table 1A), whereas $ALDH^{lo}$ or $ALDH^{hi}$ MSC from 3 alternative human BM samples (BM16, BM18, BM21) showed no capacity to reduce hyperglycemia (Table 1B) when compared to PBS-injected matched controls (Table 1C).

In order to fully characterize MSC-induced islet regeneration, we selected BM18 (Figure 17A) and BM20 (Figure 17B) to represent non-regenerative and regenerative samples respectively. Although BM18 ALDH-purified MSC did not reduce hyperglycemia after transplantation (Figure 17A), mice transplanted with BM20 $ALDH^{lo}$ or $ALDH^{hi}$ MSC showed significantly reduced hyperglycemia between day 17 - day 42 (Figure 17B). Transplantation of both BM20-derived $ALDH^{lo}$ (0.30±0.10 ng/ml) or $ALDH^{hi}$ MSC (0.32±0.05 ng/ml) significantly increased serum insulin at day 42 compared to BM18-derived MSC (0.08±0.04 ng/ml) or PBS-injected controls (0.04±0.01 ng/ml, $P<0.01$) (Figure 17C). Similar to previous findings by Lee et al (73), the functional capacity to improve hyperglycemia was independent of permanent MSC engraftment in the pancreas, where human cells were not detected by FACS at day 42.

3.9 Prolonged expansion of ALDH-purified MSC diminished hyperglycemic reduction after transplantation. In order to quantify the effect of expansion on MSC capacity to impact islet regeneration, highly regenerative $ALDH^{hi}$ MSC from BM20 were expanded for 2-4 passages, 5-6 passages, or 7-8 passages, prior to transplantation into hyperglycemic recipients. Each expanded MSC cohort

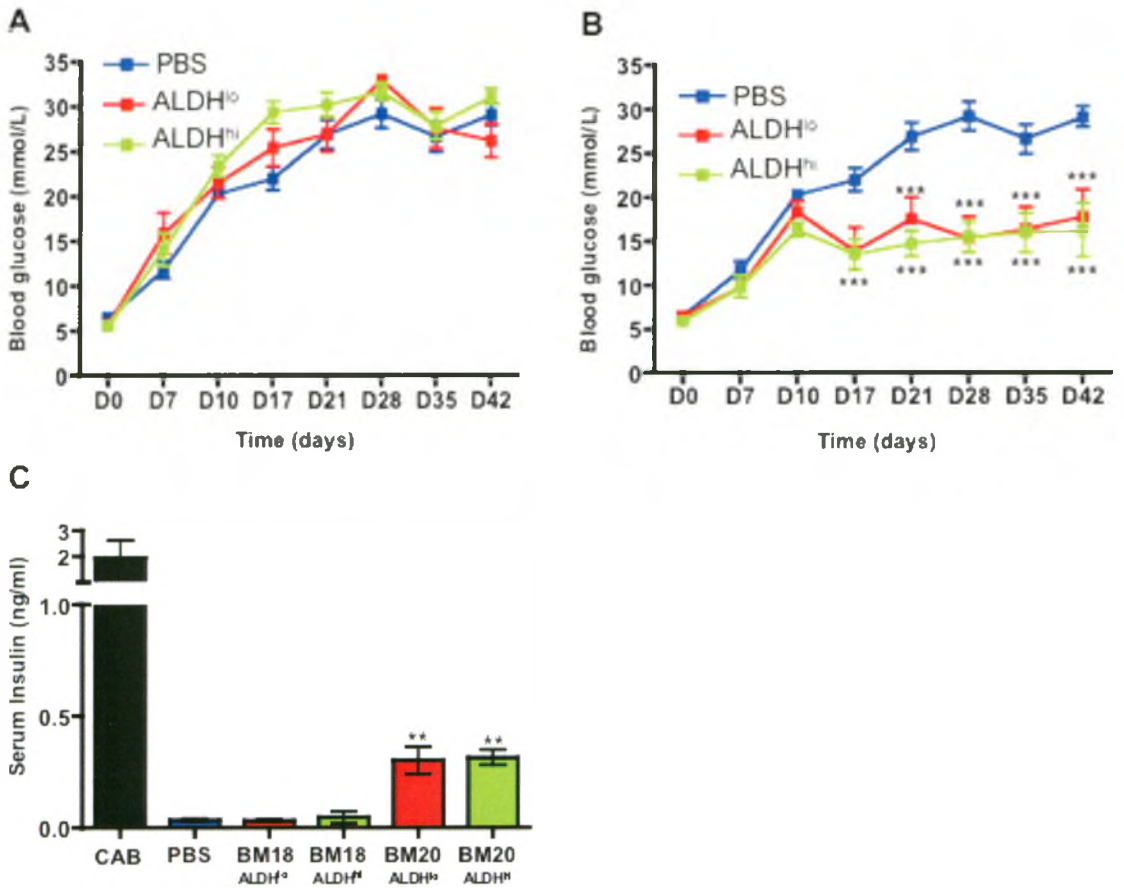

Figure 17. Transplantation of *ex vivo* expanded ALDH^{lo} or ALDH^{hi} MSC reduced hyperglycemia and increased serum insulin in STZ-treated recipients. **(A)** Transplantation of *ex vivo* expanded ALDH^{lo} (n=7) or ALDH^{hi} (n=6) MSC from BM18 did not reduce hyperglycemia compared to PBS controls (n=8). **(B)** Transplantation of *ex vivo* expanded ALDH^{lo} (n=6) or ALDH^{hi} (n=7) MSC from BM20 improved hyperglycemia compared to PBS controls (n=7). **(C)** Compared to BM18-derived MSC transplanted mice or PBS transplanted controls, mice transplanted with BM20-derived ALDH^{lo} or ALDH^{hi} MSC showed increased serum insulin. Data represent mean ± SEM (**P<0.01, ***P<0.001).

possessed similar viability (>90%) at harvest, expressed characteristic MSC surface markers (>95% $CD90^+$, $CD105^+$, $CD73^+$), and retained multipotent capacity for adipocyte, osteocyte, and chondrocyte formation in differentiation cultures (data not shown). Despite no evidence of population-specific differentiation or senescence *in vitro*, prolonged expansion of $ALDH^{hi}$ MSC diminished their capacity to improve hyperglycemia after transplantation (Figure 18). As shown previously, transplantation of early passage (P2-P4) $ALDH^{hi}$ MSC showed robust and prolonged hyperglycemic reduction from day 21 – day 42 (15.5±2.3 – 18.3±2.9 mmol/L) compared to PBS-injected controls (25.1±1.5 – 31.0±1.7 mmol/L). In contrast, transplantation of late passage (P7-P8) $ALDH^{hi}$ MSC did not produce hyperglycemic reduction (24.1±4.0 – 29.0±1.6 mmol/L), whereas transplantation of P5-P6 $ALDH^{hi}$ MSC induced intermediate hyperglycemic reduction (16.2±2.4 – 23.2±2.3 mmol/L) (Figure 18). Collectively, endogenous islet regeneration observed after MSC transplantation was BM sample-dependent, and prolonged MSC expansion prior to transplantation decreased the magnitude of hyperglycemic reduction.

3.10 Transplantation of *ex vivo* expanded MSC increased endogenous islet number but not islet size. In contrast to mice transplanted with uncultured ALDH-purified cells from human UCB or BM that showed an increase in islet size but not islet number (Figure 17, A and B), transplantation of BM20-derived $ALDH^{lo}$ or $ALDH^{hi}$ MSC increased $insulin^+$ islet density at day 42 to 0.80±0.09 and 0.82±0.10 islets/mm^2 respectively compared to PBS-injected controls (0.50±0.08 islets/mm^2, Figure 19A). However, regenerating islet size was similar to PBS-

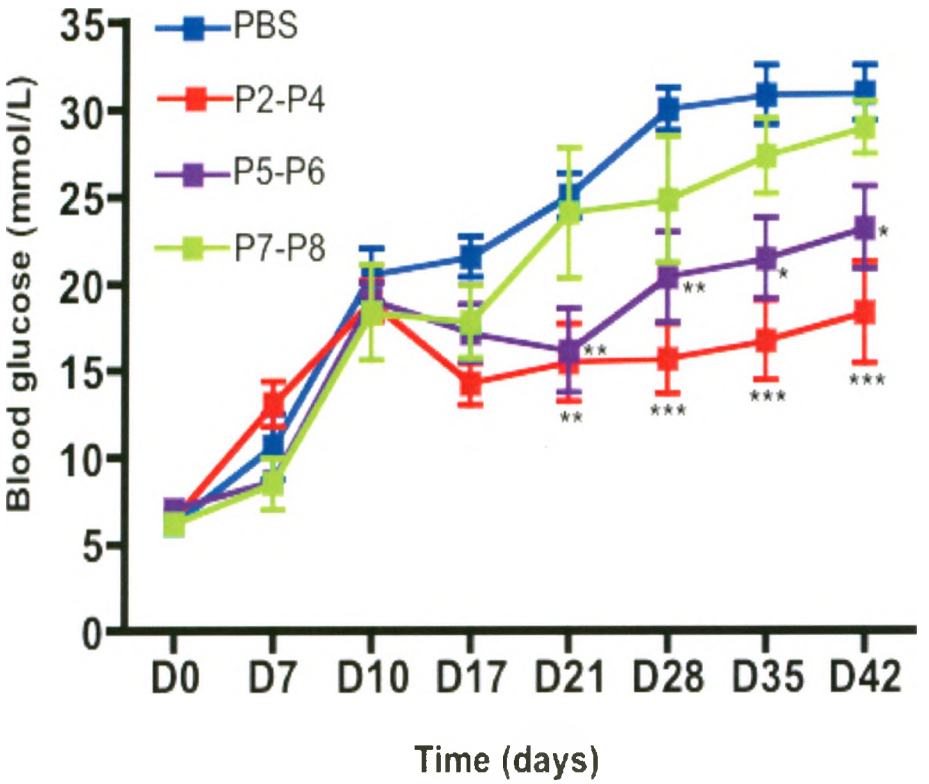

Figure 18. Prolonged expansion of ALDH-purified MSC diminished hyperglycemic reduction after transplantation. Mice transplanted with BM20 ALDH^{hi} MSC showed diminished capacity to reduce hyperglycemia with prolonged *ex vivo* expansion from P2-P4 to P7-P8. Data represent mean ± SEM (*P<0.05, **P<0.01, ***P<0.001).

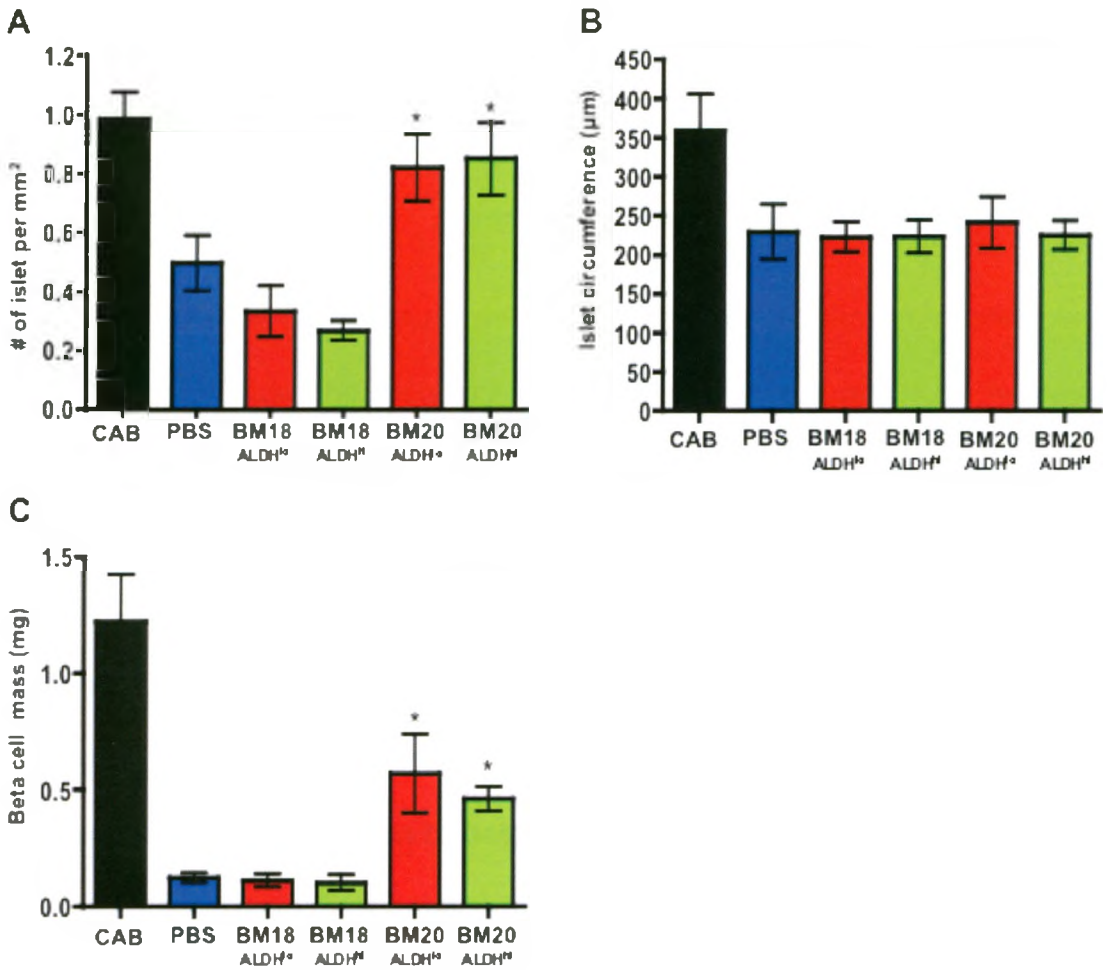

Figure 19. Transplantation of *ex vivo* expanded MSC increased endogenous islet number but not islet size. Compared to BM18-derived MSC transplanted mice and PBS transplanted controls, mice transplanted with BM20-derived early passage ALDH^{lo} or ALDH^{hi} MSC showed **(A)** increased islet number, **(B)** equivalent islet size, and **(C)** increased total beta cell mass. Data represent mean ± SEM (*P<0.05).

injected controls (Figure 19B). As predicted by the lack of blood glucose correction, transplantation of analogous BM18-derived MSC did not increase islet number (Figure 19A) or islet size (Figure 19B). For BM20 $ALDH^{lo}$ or $ALDH^{hi}$ MSC, islet recovery led to a 10-fold increase in total beta cell mass to 0.6±0.2 ng/ml and 0.50±0.06 ng/ml respectively compared to PBS-injected controls at 0.05±0.09 ng/ml. Thus, the recovery of islet function could occur by increased islet size with uncultured ALDH-purified progenitors, or by an increase in islet number with cultured $ALDH^{lo}$ or $ALDH^{hi}$ MSC, and the stimulation of these distinct programs was mediated by the characteristics of the progenitor populations administered. Therefore, we next sought to characterize how specific human progenitor subpopulations support endogenous islet recovery via the potential stimulation of islet angiogenesis or beta cell proliferative programs.

3.11 Transplantation of human $ALDH^{hi}$ cells induced islet-specific vascularization. The efficient coordination of new blood vessel formation is essential for many regenerative processes. In the regenerating adult pancreas, glucose-sensitive insulin secretion into the islet vasculature is a prerequisite to improved systemic hyperglycemia. Since transplanted human BM $ALDH^{hi}$ progenitor cells have been shown to support capillary formation in a model of acute hindlimb ischemia (72), we postulated that reduced hyperglycemia (Figure 12) and increased islet size (Figure 16) after human $ALDH^{hi}$ cell transplantation would be complemented by progenitor-stimulated islet angiogenesis. To investigate islet vascularization after the transplantation of UCB- or BM-derived progenitor populations, pancreata of transplanted mice were co-stained for insulin

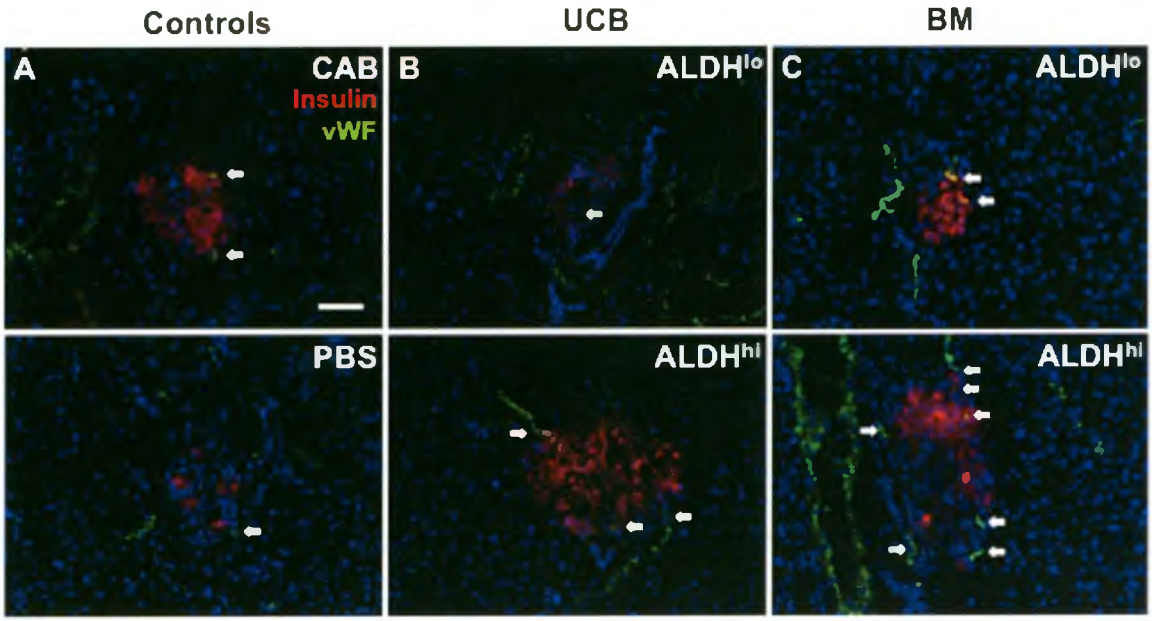

Figure 20. Transplantation of ALDH^{hi} progenitor cells increased islet vascularization in STZ-treated recipients. Representative photomicrographs of vWF^{+} vessels associated with insulin^{+} islets 42 days after injection of **(A)** CAB vehicle (upper), or STZ (35mg/kg/day, days 1-5) followed by transplantation at day 10 with PBS (lower), **(B)** 10x10^{6} UCB ALDH^{lo} or 2x10^{5} ALDH^{hi} cells, and **(C)** 10x10^{6} BM ALDH^{lo} or 2x10^{5} BM ALDH^{hi} cells. Arrows mark vWF^{+} microvessels within islets. Scale bars = 50μm.

and von Willebrand factor (vWF) at day 42 (Figure 20). vWF was used as a specific marker of functional endothelial cells within islet microvessels in vivo (72). CAB-injected (2.0±0.6 vWF^+ vessels/islet) and STZ-injected (2.1±0.8 vWF^+ vessels/islet) control mice showed equivalent islet microvessel density at day 10, indicating that STZ-treatment did not initially target microvasculature in spite of significant beta cell damage. At day 42 islet vascularization in PBS-injected controls was further decreased to 1.0±0.2 vWF^+ vessels/islet, whereas islet vascularization in mice transplanted with UCB-derived $ALDH^{hi}$ cells was increased to 2.8±0.4 vWF^+ vessels/islet, and transplantation of BM-derived $ALDH^{lo}$ cells (3.3±0.7 vWF^+ vessels/islet) or BM-derived $ALDH^{hi}$ cells (5.5±0.7 vWF^+ vessels/islet) further augmented regenerating islet vascularization (Figure 21A). Induced vessel formation in the transplanted mouse pancreas was islet-specific as extra-islet pancreatic tissue vascularization was unchanged after transplantation of $ALDH^{lo}$ or $ALDH^{hi}$ cells from either human source (Figure 21B).

3.12 Transplantation of BM-derived $ALDH^{lo}$ or $ALDH^{hi}$ MSC did not increase vascularization. In contrast to the improved islet vascularization following transplantation of freshly isolated $ALDH^{hi}$ cells from human UCB or BM, transplantation of *ex vivo* expanded $ALDH^{lo}$ or $ALDH^{hi}$ MSC from BM18 or BM20 did not augment vWF^+ microvessel density (Figure 22). Compared to PBS controls, neither intra-islet vasculature (Figure 23A) nor extra-islet vasculature (Figure 23B) were significantly increased following MSC transplantation. These data indicated that the transplantation of UCB- or BM-derived ALDH-purified progenitor cells, but not *ex vivo* expanded MSC, activate an islet-specific

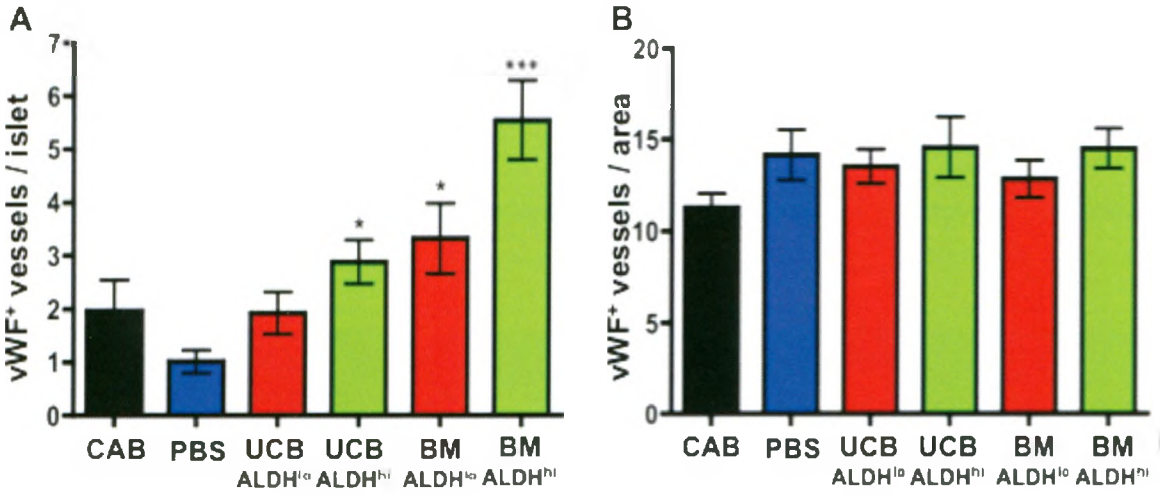

Figure 21. Transplantation of pro-angiogenic ALDH^{hi} progenitor cells into STZ-treated mice increased intra-islet vascularization. (A) Compared to PBS controls (n=6), mice transplanted with UCB ALDH^{hi} (n=5), BM-derived ALDH^{lo} (n=7), or BM-derived ALDH^{hi} cells (n=8) increased vascularization within islets. **(B)** Transplantation of UCB ALDH^{lo} or ALDH^{hi} cells, or BM ALDH^{lo} or ALDH^{hi} cells, did not increase overall pancreas (extra-islet) vascularization. Data are shown as mean ± SEM (*P<0.05, ***P<0.001).

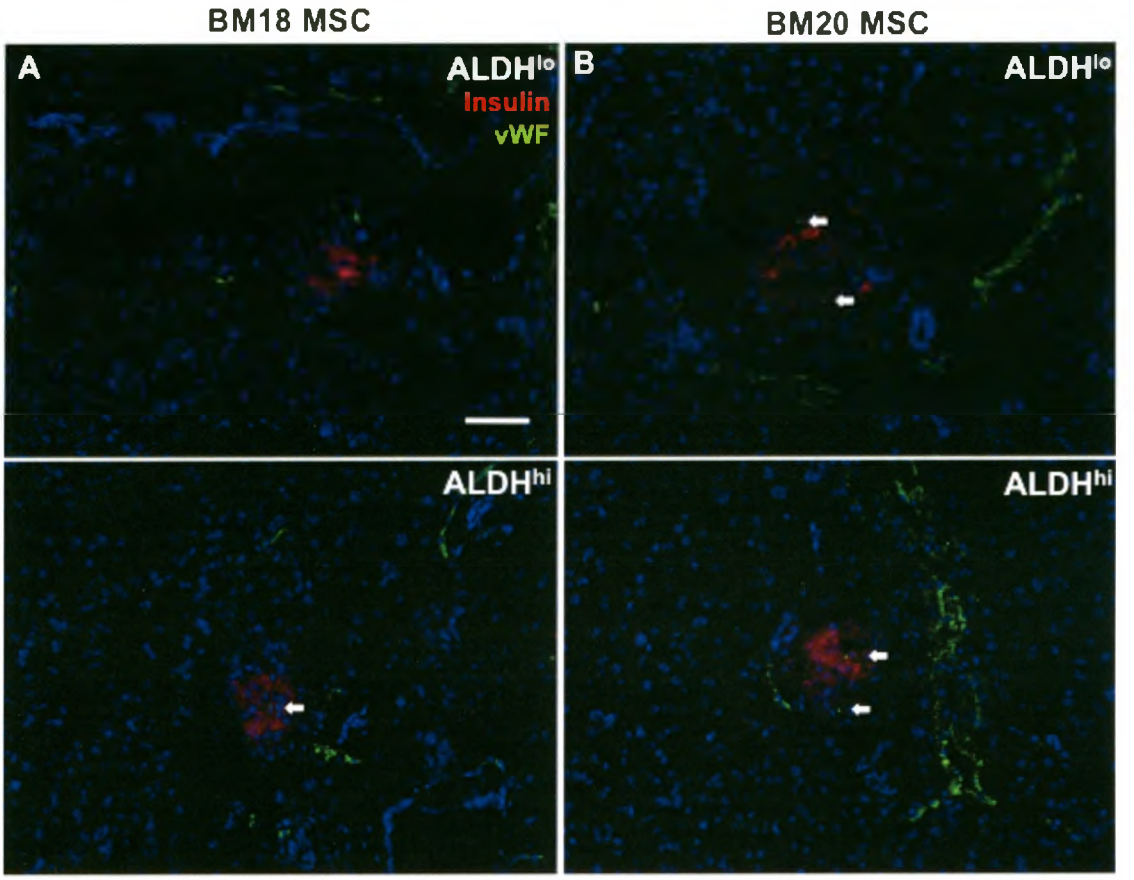

Figure 22. Transplantation of *ex vivo* expanded human ALDH^{lo} or ALDH^{hi} MSC did not increased islet vascularization. Representative photomicrographs of vWF^{+} blood vessels associated with insulin^{+} islets at day 42 after injection of STZ (35mg/kg/day, days 1-5) followed by transplantation at day 10 with **(A)** 5x10^{5} *ex vivo* expanded ALDH^{lo} or ALDH^{hi} MSC from BM18, or **(B)** 5x10^{5} ex vivo expanded ALDH^{lo} or ALDH^{hi} MSC from BM20. Arrows mark vWF^{+} microvessels within islets. Scale bars = 50μm.

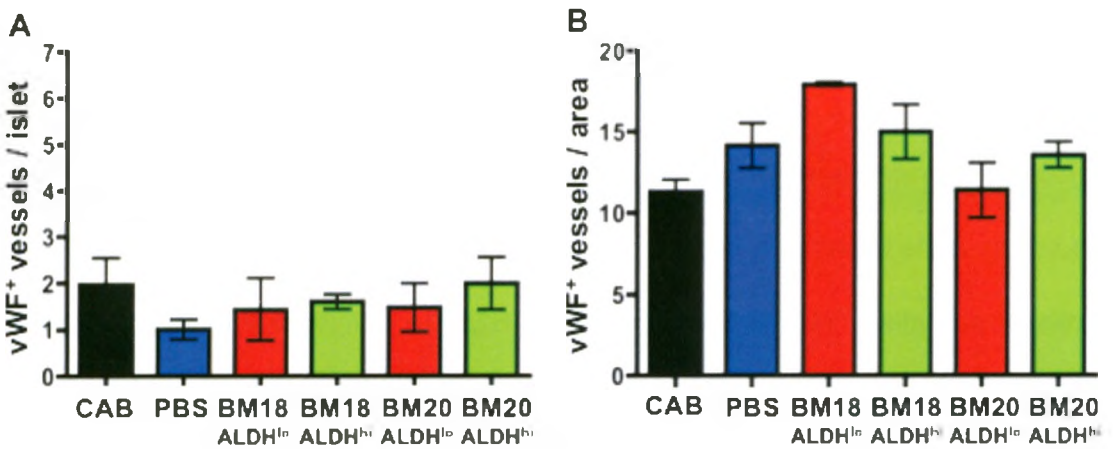

Figure 23. Transplantation of *ex vivo* expanded ALDH^{lo} or ALDH^{hi} MSC had no impact on intra-islet or extra-islet vascularization. (A) Compared to PBS controls, mice transplanted with BM18- or BM20-derived MSC did not increase vascularization within islets. **(B)** Transplantation of BM18- or BM20-derived MSC did not increase overall pancreas (extra-islet) vascularization. Data shown are mean ± SEM.

angiogenic program resulting in increased islet vascularization and release of insulin into the circulation, thereby improving glycemic control.

3.13 Transplantation of ALDH^{hi} progenitor cells induced proliferation of vWF^{+} vessels. To further investigate the increased vascularization of murine islets following transplantation of UCB- or BM-derived ALDH^{hi} cells, we analyzed EdU incorporation into proliferating cells within vessels at day 17 and day 42. In PBS-injected controls, proliferating cells associated with vWF^{+} blood vessels were rare, indicating a slow turnover of endothelial cells within blood vessels after STZ-damage (Figure 24A). However, representative photomicrographs at early (day 17) and late (day 42) time points showed numerous proliferating vWF^{+} endothelial cells following UCB- or BM-derived ALDH^{hi} (Figure 24B) cell transplantation, confirming the induction of endothelial cell activation and sustained blood vessel remodeling. However, transplantation of *ex vivo* expanded ALDH^{lo} or ALDH^{hi} MSC from regenerative marrow samples (BM20) did not stimulate significant microvascular cell proliferation, with EdU incorporation in endothelial cells similar to PBS-injected controls (Figure 24C).

3.14 Transplantation of ALDH^{hi} progenitor cells induced proliferation of insulin^{+} islets. Although alternate modes of beta cell expansion through islet neogenesis have recently been proposed (116), the established mechanism responsible for islet regeneration in the STZ-damaged adult pancreas is through induced proliferation of surviving beta cells (108). To investigate the increased islet size following UCB- or BM-derived ALDH^{hi} cell transplantation, we analyzed EdU incorporation into proliferating cells within the islets at the earliest

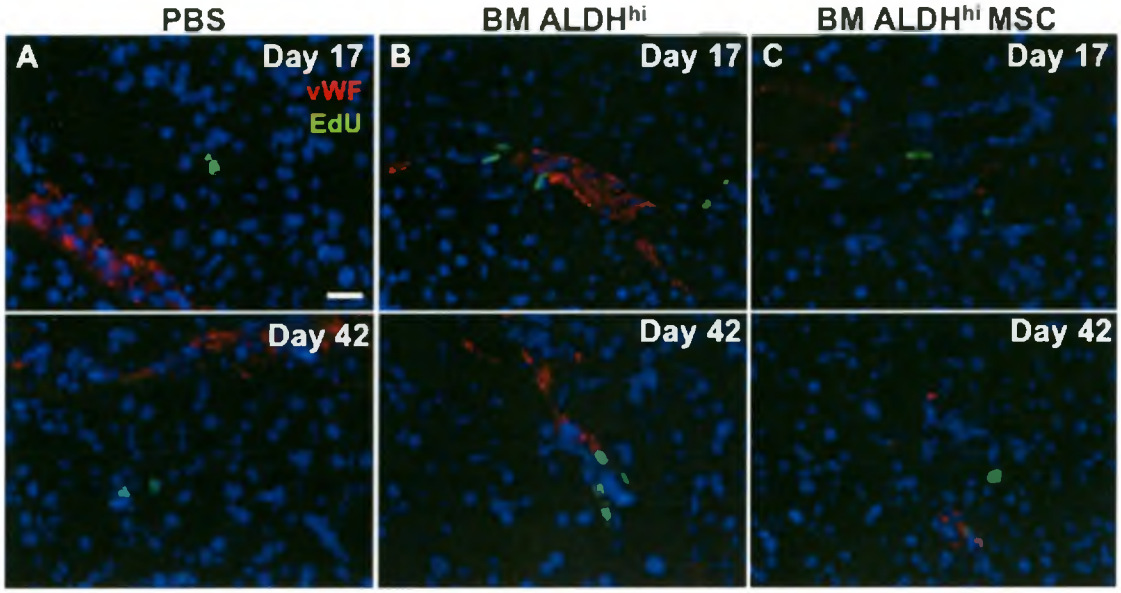

Figure 24. Transplantation of ALDH^{hi} mixed progenitor cells induced rapid proliferation of vWF^{+} vessels in STZ-treated mice. Representative photomicrographs of EdU-labeled proliferating cells associated with vWF^{+} blood vessels at day 17 (upper) or day 42 (lower) after STZ injection (35mg/kg/day, days 1-5) and transplantation at day 10 with **(A)** PBS, **(B)** 2x10^{5} BM ALDH^{hi}, or **(C)** 5x10^{5} *ex vivo* expanded BM20 ALDH^{hi} MSC. Mice transplanted with BM ALDH^{hi} cells showed extensive proliferation of vWF^{+} vessels by day 17 that was maintained until day 42. PBS and MSC transplanted mice showed minimal proliferation directly associated with the vWF^{+} vessels. Scale bars = 20μm.

observation of blood glucose improvement (day 17, 7 days post-transplantation) and at the end of our 42-day protocol (32 days post-transplantation). EdU incorporation within insulin$^+$ islets after transplantation of UCB or BM-derived ALDH^{hi} cells revealed numerous EdU$^+$ beta cell nuclei per islet at day 17 (Figure 26A), and EdU$^+$ proliferating beta cells were still detected in large islets out to day 42 (Figure 25B) (Figure 26B). PBS-injected control pancreata did not contain EdU-labeled insulin$^+$ beta cells within islets at early or late time points (Figure 25A) (Figure 26). Together these data indicate the early stimulation (within 7 days) and sustained beta cell proliferation was associated with the systemic administration of UCB- or BM-derived ALDH^{hi} progenitor cells. EdU$^+$ and insulin$^+$ cells were also observed within small islets or adjacent to single beta cells 7 days following ALDH^{lo} or ALDH^{hi} MSC transplantation (Figure 25C) (Figure 26A), but no islet bound EdU$^+$ cells were detected at later time points (day 42) (Figure 26B). Thus, increased islet size and vascularization following ALDH^{hi} cell transplantation could be attributed to the stimulation of increased beta cell and endothelial cell proliferation respectively, while modest but unsustained beta cell proliferation observed after transplantation of BM MSC resulted in numerous, small islets with arrested beta cell proliferative potential.

3.15 Transplantation of ALDH-purified MSC increased islets associated with ck19$^+$ ducts. We next sought to determine the mechanisms by which ALDH-purified MSC contribute to the endogenous regeneration of islet function. Since transplantation of BM20-derived ALDH^{lo} or ALDH^{hi} MSC increased islet number (Figure 19A), without extensive or prolonged beta cell proliferation (Figure 25C),

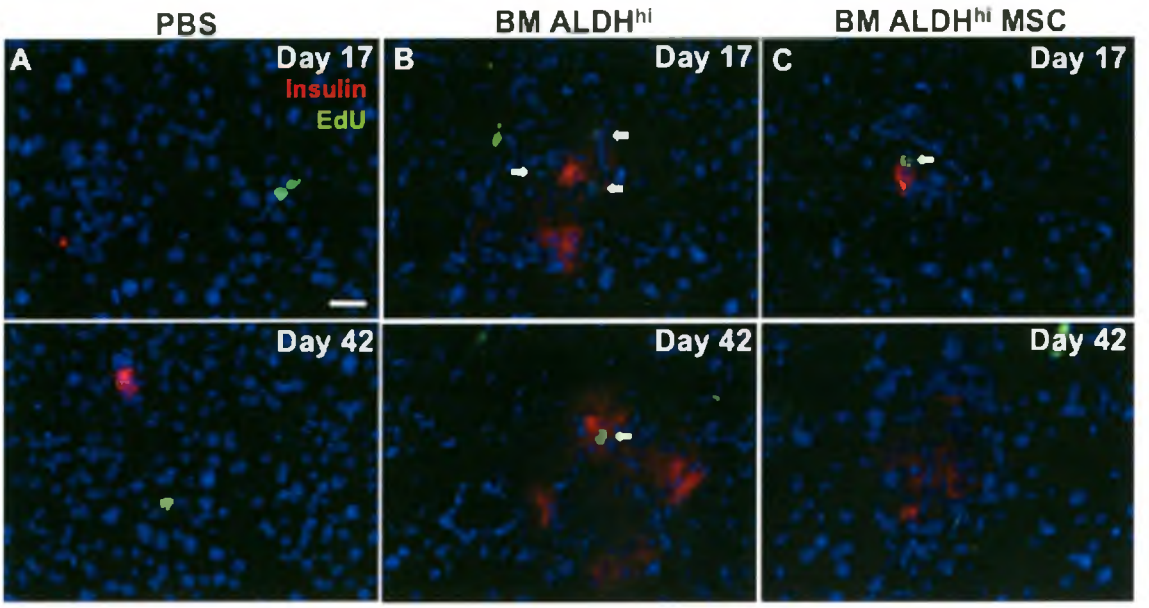

Figure 25. Transplantation of ALDH^{hi} mixed progenitor cells induced proliferation of murine insulin^{+} islets in STZ-treated mice. Representative photomicrographs of EdU-labeled proliferating cells associated with insulin^{+} islets at day 17 (upper) or day 42 (lower) after STZ-injection (35mg/kg/day, days 1-5) and transplanted at day 10 with **(A)** PBS, **(B)** 2x10^{5} BM ALDH^{hi} cells, or **(C)** 5x10^{5} BM20 ALDH^{hi} MSC. PBS injected mice showed no proliferation within insulin^{+} islets at day 17 or day 42. Mice transplanted with BM ALDH^{hi} cells showed beta cell proliferation within islets at day 17 and was still present at day 42. Mice transplanted with BM ALDH^{hi} MSC showed beta cell proliferation within islets at day 17 only. Arrows mark proliferating cells within islets. Scale bars = 20μm.

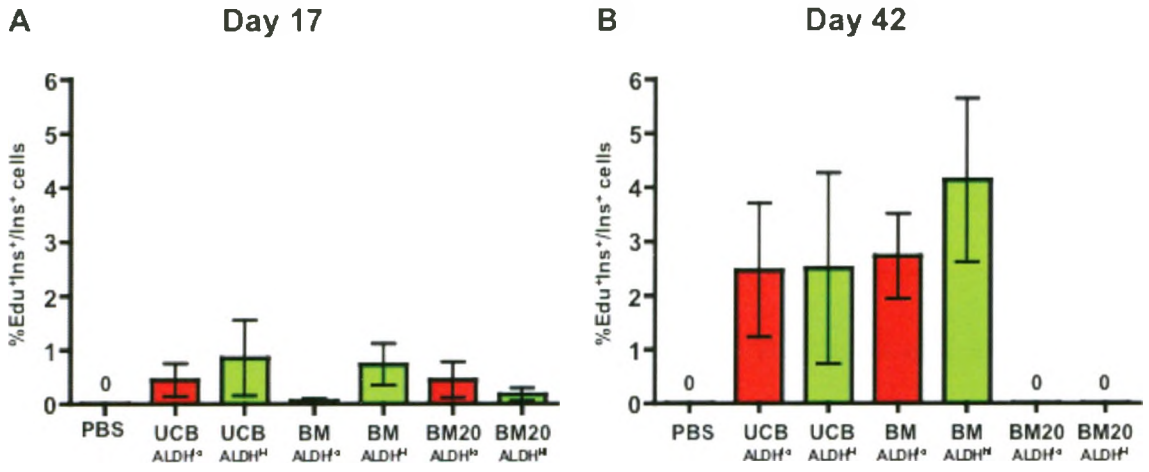

Figure 26. Transplantation of UCB and BM ALDH-purified cells induced sustained beta cell proliferation. (A) Compared to PBS controls which showed no proliferation of beta cells at day 17, mice transplanted with UCB or BM ALDH^{lo} or ALDH^{hi} cells, or BM20 ALDH^{lo} or ALDH^{hi} MSC had increased beta cell proliferation. **(B)** Compared to PBS controls which showed no proliferation of beta cells at day 42, mice transplanted with UCB or BM ALDH^{lo} or ALDH^{hi} cells had increased beta cell proliferation. Mice transplanted with BM20 ALDH^{lo} or ALDH^{hi} MSC had no beta cell proliferation at day 42. (n=3-4).

we postulated that new islet formation may be stimulated in ductal regions following MSC transplantation. Since previous studies have reported islet neogenesis in ductal regions (116) (53) (110), we analyzed the association of regenerating islets with $CK19^+$ ductal epithelial cells at day 17 and day 42 after BM20 $ALDH^{lo}$ or $ALDH^{hi}$ transplantation. Representative photomicrographs show $insulin^+$ islets were in direct contact with $CK19^+$ ductal structures at 7 and 32 days after $ALDH^{lo}$ (Figure 27A) or $ALDH^{hi}$ (Figure 27B) MSC transplantation. Compared to PBS-injected controls at 7 days post-transplantation where only 5.0±0.8% of islets were directly associated with $CK19^+$ ducts, there was a significant increase in islets with direct contact to $CK19^+$ ductal epithelia following regenerative $ALDH^{lo}$ (41.0±8.3% of islets) or $ALDH^{hi}$ (22.5±1.1% of islets) MSC transplantation (Figure 28A). The significant increase in islet and duct association following $ALDH^{lo}$ or $ALDH^{hi}$ MSC transplantation was maintained for >1 month post-transplantation, at 18.5±2.7% and 20.2±1.5% respectively, however, no increase in islet association with ducts was observed following UCB- or BM-derived ALDH-purified progenitor cell transplantation at day 17 (Figure 28A) or day 42 (Figure 28B). Some regenerating islets associated with ducts also contained $CK19^+$ cells (Figure 27A) suggesting putative interplay between ductal-resident endocrine precursors and regenerating islets. Nevertheless, the ductal region represents a potential niche for endogenous islet regeneration after MSC transplantation.

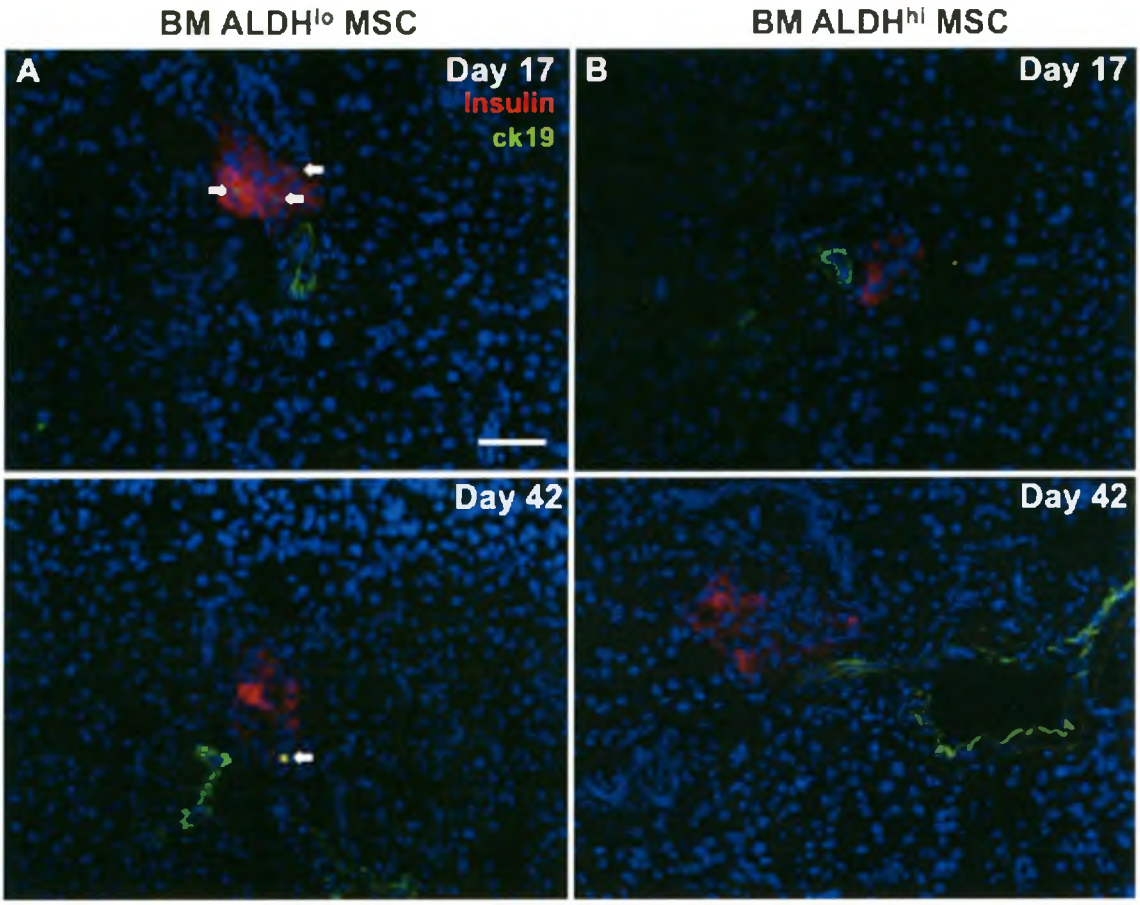

Figure 27. Transplantation of BM-derived ALDH^{lo} or ALDH^{hi} MSC increase islet association with ck19^{+} ducts. Representative photomicrographs of insulin^{+} islets associated with ck19^{+} ductal structures at day 17 (upper) or day 42 (lower) in STZ-treated mice after transplantation at day 10 with 5x10^{5} BM20-derived **(A)** ALDH^{lo} or **(B)** ALDH^{hi} MSC and stained for mouse insulin and the ductal epithelial marker ck19. Arrows mark ck19^{+} cells within islets. Scale bars are 50μm.

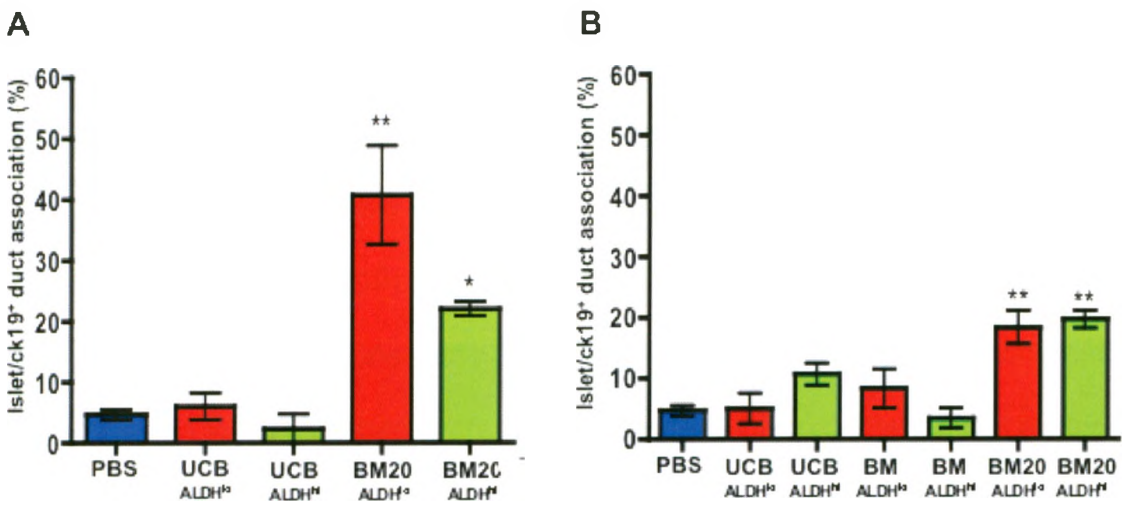

Figure 28. Increased islet/duct association at day 17 and day 42 following transplantation of BM20-derived ALDH^{lo} or ALDH^{hi} MSC. **(A)** Compared to PBS controls, mice transplanted with BM20 ALDH^{lo} (n=4) or ALDH^{hi} (n=4) MSC had significantly increased islets associated with ducts at day 17. **(B)** Compared to PBS controls, mice transplanted with BM20 ALDH^{lo} (n=5) or ALDH^{hi} (n=5) MSC had significantly increased islets associated with ducts at day 42. Data are mean ± SEM (*P<0.05, **P<0.01).

3.16 Transplantation of BM-derived MSC did not activate Ngn3 expression.

Previous studies of islet regeneration have suggested that similar to embryonic development, islet neogenesis could be mediated from endocrine progenitor cells to restore beta cell mass (109) (110). Furthermore, Xu et al has shown that newly formed islets in ductal regions arise from $NGN3^{+}$ endocrine precursor cells after ductal ligation (116). To further characterize regeneration in ductal or islet regions after the transplantation of human MSC, we stained murine pancreata for insulin and NGN3 at several time points throughout the regeneration process (day 14, 17 and 42). NGN3 is expressed by endocrine progenitor cells during embryonic development, appearing first at embryonic day (E) 8.5, peaking at E15.5, and becoming undetectable at birth (120). Representative photomicrographs show nuclear localization of NGN3-expression was abundant in E18.5 pancreas (Figure 29A, upper panel). However, in the adult pancreas, NGN3-expression was absent at day 10 in mice pre-treated with STZ (Figure 29A, lower panel), demonstrating that STZ-treatment alone did not activate NGN3 expression. Although insulin expression was induced in ductal regions as early as day 14, immunofluorescent microscopy did not detect activation of endogenous NGN3-expression in islets or ducts at day 14, day 17 (Figure 29, B and C) or day 42 following $ALDH^{lo}$ or $ALDH^{hi}$ MSC transplantation. Similarly, transplantation of UCB or BM-derived ALDH-purified cells did not activate endogenous NGN3 expression in recipient pancreas. In contrast to the induction of endogenous angiogenic and beta cell proliferative programs characterized after the transplantation of BM-derived $ALDH^{hi}$ progenitor cells, transplantation of ALDH-

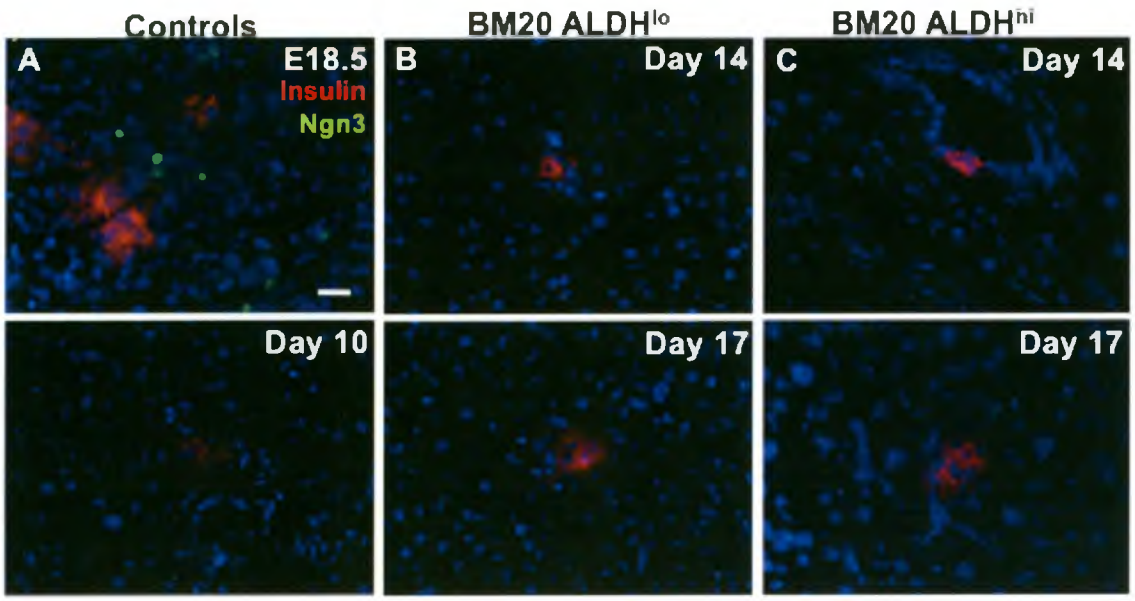

Figure 29. Transplantation of BM-derived ALDH^{lo} or ALDH^{hi} MSC did not activate Ngn3-expression in the pancreas of STZ-treated recipients. (A) Representative photomicrographs of embryonic day 18.5 pancreas (upper) as a positive control for Ngn3 expression, and STZ injected mice at at day 10 prior to cell transplantation (lower). **(B)** Representative photomicrpgraphs of STZ injected (35mg/kg/day, days 1-5) mice transplanted at day 10 with BM ALDH^{lo} MSC and stained for insulin and Ngn3 showed no detectable Ngn3^{+} cells in the pancreas at day 14 (upper) or 17 (lower). **(C)** Representative photomicrographs of STZ-injected (35mg/kg/day, days 1-5) mice transplanted at day 10 with BM ALDH^{hi} MSC and stained for insulin and Ngn3 showed no detectable Ngn3^{+} cells in the pancreas at day 14 or 17. Scale bars are 20μm.

purified MSC specifically augmented the formation of small islets associated with $CK19^{+}$ ducts, without increased islet vascularization or sustained beta cell proliferation. Taken together, these data suggest that islet regeneration in STZ-treated mice after human MSC transplantation was consistent with the induction of an endogenous neogenic program, but without transition through an Ngn3-expressing endocrine precursor.

Chapter 4

Discussion

4.1 Summary

The primary objective of this research was to investigate the mechanisms underlying endogenous islet regeneration after the transplantation of clinically relevant human progenitor cell populations. Transplantation of uncultured human $ALDH^{hi}$ cells or culture-expanded human $ALDH^{lo}$ or $ALDH^{hi}$ MSC into STZ-treated, hyperglycemic NOD/SCID mice showed that activation of distinct islet regenerative programs was dependent on source and lineage-restriction of the progenitor cells administered. UCB and BM-derived $ALDH^{hi}$ mixed progenitor cells contained pro-angiogenic progenitor subtypes (72) that following transplantation into hyperglycemic NOD/SCID mice stimulated beta cell and endothelial cell proliferation leading to increased islet size and vascularization. BM $ALDH^{hi}$ transplant recipients had a subsequent increase in total beta cell mass, serum insulin levels, and a stable reduction of systemic blood glucose. These findings supported the hypothesis that $ALDH^{hi}$ cells will mediate recovery from hyperglycemia following STZ-induced diabetes.

Although transplantation of culture-expanded MSC derived from independent BM donors showed variable capacity to improve hyperglycemia, transplantation of culture-expanded BM-derived $ALDH^{lo}$ or $ALDH^{hi}$ MSC from selected regenerative lines permanently reduced blood glucose via an alternative mechanism. Whereas the freshly isolated mixed progenitor cells reduced hyperglycemia through an increase in islet size and vascularization, cultured MSC did not significantly stimulate proliferative or pro-angiogenic programs, but increased beta cell mass and serum insulin release through an increase in islet number, a large proportion

of which were directly associated with the ductal epithelium. Although $NG3^{+}$ endocrine precursors have been identified in ductal regions after ducal ligation (116), we did not observe NGN3 activation after cell transplantation in STZ-treated recipients. These divergent mechanisms are summarized in Figure 30. We have identified that endogenous islet regeneration can occur by the stimulation of mature beta cell proliferation associated with islet specific angiogenesis, or by the formation of new islet structures in ductal regions suggestive of a neogenic regenerative mechanism.

4.2 ALDH-purified mixed progenitor cells initiate islet vascularization

We have previously shown that BM $ALDH^{hi}$ cells contain pro-angiogenic progenitor cells capable of inducing revascularization of ischemic limbs following transplantation in a mouse model of acute limb ischemia, by transiently homing to the damaged tissue and stimulating endogenous blood vessel regeneration (72). Without integrating into recipient vasculature, tail vein injected human $ALDH^{hi}$ cells migrated specifically to the ischemic tissue and stimulated endogenous capillary formation resulting in the recovery of blood flow after femoral artery ligation. Here we showed that UCB- and BM-derived $ALDH^{hi}$ cells similarly enhanced islet-specific vascularization in the STZ-treated mouse pancreas. Increased vascularization was specific to the islets, rather than extra-islet pancreatic tissue, suggesting that STZ damage to beta cells, followed by $ALDH^{hi}$ cell transplantation was required to optimally induce islet angiogenesis. Damaged tissues secrete growth factors which promote recruitment of hematopoietic and

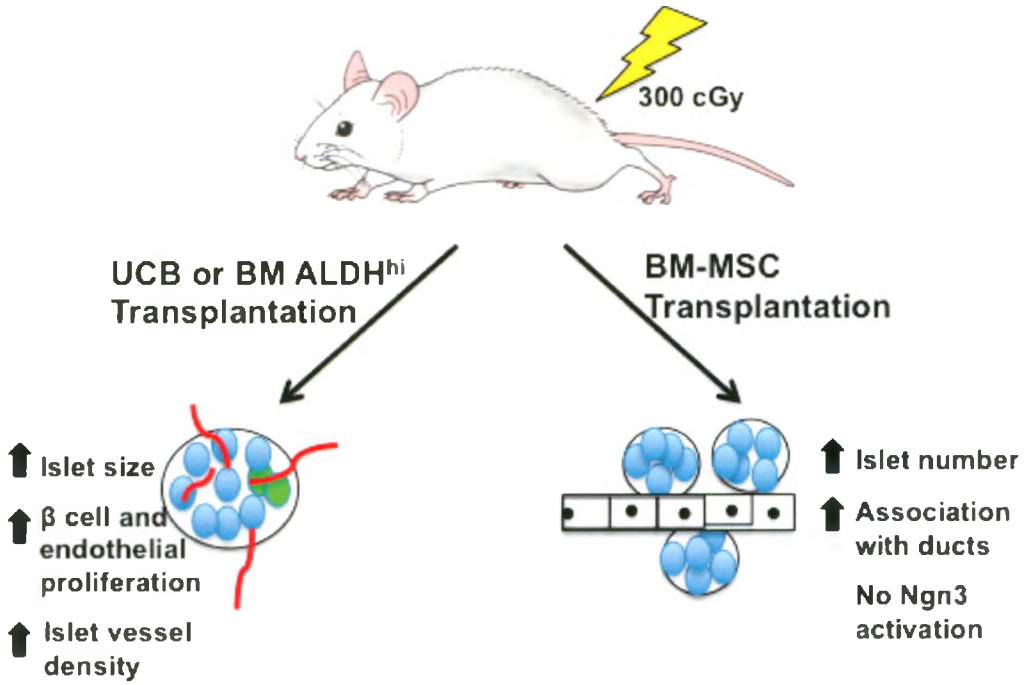

Figure 30. Distinct mechanisms for endogenous islet regeneration following transplantation of human ALDH^{hi} cells or *ex vivo* cultured MSC. Schematic of the divergent mechanisms involved in pancreatic regeneration after progenitor cell transplantation. Transplantation of ALDH^{hi} mixed progenitor cells augmented islet size and vascularization through increased beta cell and endothelial cell proliferation. Transplantation of ALDH- purified MSC induced an increase in islet number associated with ck19^{+} ducts, suggesting that the mechanism involved in pancreatic regeneration depends on the transplanted population of cells initiating regeneration.

endothelial progenitor cells to areas of damage where they may promote and sustain endogenous angiogenesis (133). Islet- derived beta cells (134), and pancreatic ductal cells (135), are known to secrete angiogenic growth factors such as VEGF and chemokines such as stromal derived factor 1 (SDF-1) that promote recruitment of proangiogenic cells to areas of pancreatic damage, where they initiate angiogenesis (136). Interestingly, increased islet vascularization was also associated with increased beta cell turnover and islet hyperplasia, which ultimately contributed to improved hyperglycemia and increased total beta cell mass after transplantation of BM-derived $ALDH^{hi}$ cells. Thus, the pro-angiogenic progenitor subtypes contained within freshly isolated UCB- and BM-derived $ALDH^{hi}$ populations are also implicated in the induction of beta cell proliferation via paracrine interaction (72). Further study of the cellular communication at the islet microvascular and beta cell interface during islet regeneration is warranted to uncover potential target molecules that stimulate endogenous beta cell proliferation and islet vascularization.

4.3 Potential role of human MSC in islet revascularization

In addition to hematopoietic and endothelial cells, MSC contained within the BM $ALDH^{hi}$ cell population may also support revascularization. MSC have recently been shown to have a perivascular origin *in situ*, and cultured $CD146^{+}$ perivascular cells exhibited osteogenic, chondrogenic and adipogenic differentiation potentials, and expressed characteristic stromal cell surface markers after culture (CD73, CD90, CD105) (137). *In vitro*, BM-MSC can stabilize tubule formation in a pericyte-like manner, and release both angiogenic and

stabilizing factors, which act on the vasculature in a paracrine manner, facilitating vessel formation (138). In *in vivo* implants, adipose-derived MSC also function as perivascular cells, where they wrap endothelial cell-formed functional blood vessels and stabilize the vascular network (139, 140). Thus, MSC work in concert with other progenitor subtypes to promote vascularization, as implants of endothelial and adipose progenitors together produced a density and complexity of vascular structures much higher than implants of either cell type alone (140).

When injected as a singular lineage specified by ex vivo culture, transplantation of human MSC alone did not augment islet-specific vascularization. However, human BM-derived $ALDH^{hi}$ cells possessed enriched multipotent mesenchymal colony forming cell capacity *in vitro* (72), and the most potent islet vasculogenic potential after transplantation, suggesting that enhanced islet function after transplantation of BM-derived $ALDH^{hi}$ cells may be synergized by paracrine regenerative or pro-angiogenic stimuli provided by nascent MSC administered within the $ALDH^{hi}$ mixed progenitor population. We hypothesized that the resulting return of hyperglycemia over time following UCB $ALDH^{hi}$ transplantation may be due to the lack of MSC in this source of progenitor cells. UCB $ALDH^{hi}$ cells containing only EPC and HSC may form less stable neo-vessels that lose their function over time. The presence of MSC in the transplanted BM $ALDH^{hi}$ cell population may co-ordinate angiogenesis through direct contact with newly formed vessels, or through the secretion of pro-angiogenic factors, the later of which is supported by our model which shows limited engraftment of transplanted MSC in the recipient mouse pancreas. Increased vascularization of damaged

islets is essential to allow for delivery of nutrients and regenerative stimuli to maintain viability of regenerating tissue, and to allow for secretion of produced insulin into the circulation.

4.4 Potential role of human MSC in islet regeneration

Transplantation of human MSC into STZ-treated mice has previously been shown to reduce blood glucose concentrations via an uncharacterized mechanism (73). Here we show that ALDH-purified MSC had a greater ability to significantly and permanently reduce blood glucose compared to the previously studied unpurified MSC. In addition, we showed that there is sample dependent variability in the capacity of transplanted MSC to stimulate endogenous beta cell regeneration, and that early passage MSC were required in order to obtain maximal blood glucose reduction. Extended passage of regenerative MSC clones showed diminished capacity to reduce hyperglycemia after transplantation, warning that the islet regenerative activities of human MSC may be compromised by expansion in serum rich media under atmospheric oxygen tension (141, 142). Although, MSC did not appear to change phenotypically with increasing passage, it has been reported that there are major differences between early and late stage cultures of MSC, with cell cycle genes upregulated early, and subsequent upregulation of cell development genes at later stages, which may impact their regenerative capacity (143). Nevertheless, optimization of lineage-specific progenitor cell isolation and expansion require further development to assure efficient delivery of the appropriate paracrine stimuli to induce islet regeneration.

Similar to previous findings by Lee et al which have shown that transplanted MSC possess significant capacity to regenerate islets with only minimal long-term engraftment in the pancreas (73), we observed significantly improved hyperglycemia after ALDH-purified MSC transplantation, but did not detect significant human cell engraftment in the recipient mouse pancreas at one week and one month after MSC transplantation, further suggesting that in our model MSC act to enhance endogenous regeneration through paracrine mechanisms. Similarly, in murine transplantation models for cardiac regeneration after infarct, transplanted MSC improve cardiac function via secretion of anti-inflammatory or pro-angiogenic mediators without long-term cardiac engraftment (144, 145). After tail vein injection, adherent MSC accumulate in the pulmonary capillaries of the lungs (146). Although some MSC have previously been shown to migrate to islet and ductal regions following STZ-treatment (73, 85) our data strongly suggests that in our model MSC may act from distant site to exert subsequent paracrine or hormone action in the pancreas. However, the specific factors secreted by MSC that are involved in islet regeneration are yet to be determined and remain the key to fully understanding transplantation-induced endogenous islet regeneration.

Robust beta cell proliferation and sustained islet development appeared attenuated following the initial formation of new islets. Thus, more efficient delivery of regenerative MSC directly to the pancreas to initiate cell-cell contact dependent regeneration, or increased survival of delivered MSC at ectopic sites, may improve the efficiency of endogenous islet recovery. One alternative strategy to improve delivery and retention of MSC in damaged tissues may be to culture

MSC under low oxygen conditions, which upregulates CXCR4 and CX3CR1 expression, enhancing migration of MSC in response to SDF-1α in vitro and enhancing MSC engraftment *in vivo* (141).

4.5 Human MSC enhance endogenous islet formation via a novel neogenic mechanism

Several lines of evidence support that human MSC augment endogenous islet regeneration via a novel post-natal islet neogenic mechanism. Neogenesis is defined as the formation of new islets from endocrine precursor cells in the pancreas. During embryonic development, all pancreatic exocrine and endocrine cells differentiate from the ductal epithelium. Previous studies of pancreatic regeneration suggested that similarly to embryonic development, new islets are formed from endocrine progenitor cells in ductal regions to restore beta cell mass following partial pancreatectomy (119, 147). Neogenesis has been previously documented to occur rapidly in several steps following injury: proliferation of the ductal epithelium, dedifferentiation to a multipotent pancreatic progenitor, and subsequent directed differentiation into endocrine, acini or mature duct cells, where differentiation of the specific cell types was mediated by external stimuli (148). We found a significant increase in the number of islets associated with the ductal epithelium following MSC transplantation into our STZ-treated mice, suggesting MSC could possibly act to stimulate differentiation of ductal progenitor cells to islet cells in order to regenerate the damaged pancreas. However, the identity of these endocrine progenitor cells remains elusive. We did not observe evidence of $NGN3^{+}$ progenitor cell activation in ductal regions as noted in studies

of islet neogenesis by Xu et al employing ductal ligation (116). NGN3 is expressed transiently by endocrine progenitors during embryonic pancreas development, and it cannot be ruled out that NGN3 was not expressed very briefly during MSC-stimulated regeneration. Alternatively, the increased number of islets may have differentiated from $PDX1^+$ progenitors in ductal regions as noted after partial pancreatectomy (147), however PDX1 detection in our STZ model was suboptimal (data not shown).

Evidence supporting that islets may be derived from the ductal epithelial cells following MSC transplantation was the continued presence of $CK19^+$ cells in several duct-associated islets. During islet neogenesis from the ductal epithelium, transitional cells can express both CK19 and insulin, and some beta cells in newly developing islets express duct cell markers not normally expressed in mature beta cells (147, 149). Another possibility is that the ductal region may act as a regenerative niche that is stimulated to support the regeneration of islets following MSC transplantation, possibly through activation of $insulin^-PDX1^+$ intra-islet beta cell precursors, which have previously been shown to escape STZ-deletion and repopulate damaged islets (112). Thus, intra-islet precursors in duct-associated islets may be preferentially activated to restore beta cell mass following MSC transplantation. Furthermore, ductal epithelial cells have been shown to produce VEGF and interleukin-8 (135), angiogenic cytokines or chemokines reported to have beneficial effects on islet survival in rodent transplantation models (150). Therefore islet regeneration in ductal regions may

be partially supported and maintained by the initiation of regenerative stimuli provided by $CK19^{+}$ ductal epithelial cells.

4.6 Clinical Implications

Our data outlines several clinically applicable regenerative therapies for the treatment of diabetes. Autologous human BM or allogeneic human UCB progenitor cells can be isolated in a clinically relevant manner using ALDH-activity, and ALDH-purified MSC are amenable to *ex vivo* expansion to increase regenerative cell numbers. However, serum free expansion conditions that do not result in diminished regenerative capacity require further development.

In order to be a plausible therapy for type 1 diabetes, the issue of continued auto-immune destruction of regenerated beta cells must be addressed. The immunomodulatory properties of MSC, and their ability to escape immune recognition make them a cell type of interest. Phase II clinical trials by Osiris Therapeutics Inc, transplanting allogeneic MSC into newly diagnosed type 1 diabetics are currently underway and are expected to be complete by the end of 2010. In addition to MSC, clinical trials are also being performed with autologous UCB-derived cells, a source containing T regulatory cells, which may further protect beta cells from auto-immune destruction.

A challenge with treating diabetes using endogenous beta cell regenerative strategies may be the requirement of some remaining endogenous beta cells. In patients with advanced type 1 diabetes, beta cell mass may be too low to endogenously regenerate to a sufficient level to reduce hyperglycemia. In these

cases, an exogenous source of beta cell may first need to be administered, followed by *in vivo* expansion and maintenance using endogenous regenerative strategies.

Transplantation of ALDH-purified mixed progenitor cells or MSC may also be a possible therapy for type 2 diabetic patients, who do not have auto-immune destruction of beta cells, but rather have decreased beta cell mass and insulin resistance. Although diet and exercise is still a necessity, and the issue of insulin resistance would need to be addressed, these cells could aid in restoring beta cell mass and increase insulin available to help meet metabolic demand.

There are several limitations to our study and it's application for clinical treatment of diabetes. While type 1 diabetic patients have autoimmune destruction of their pancreatic beta cells leading to hyperglycemia, our model involved chemical destruction of pancreatic beta cells to induce hyperglycemia in NOD/SCID mice, an immune-deficient model. Therefore beta cell regeneration is being studied in an environment free of continued autoimmune attack of the regenerating beta cells. Furthermore, although it is beneficial to study the transplantation of stem cells from human sources, as these would be the cells studies in clinical trials, the function of transplanted human cells into a mouse model is not fully representative of their potential regenerative capacities when transplanted into humans due to species incompatibilities.

4.7 Future Direction

It is essential to determine the origin of regenerating islets, and investigate the regenerative cytokines and chemokines secreted by the transplanted ALDH-purified mixed progenitor cells and MSC to understand the complexity of the regenerative niche and more efficiently direct regeneration. Expanding MSC in low oxygen conditions, and transplanting directly into the pancreas may further improve regeneration by maintaining MSC in a less differentiated state and enabling contact-dependent regeneration in addition to paracrine supported regeneration.

To address auto-immunity, studies could be performed in NOD mice, which spontaneously develop diabetes, to assess the ability of ALDH-purified murine BM cells or MSC to protect against immune-development of diabetes, as well as to regenerate the damaged islets and provide subsequent protection from auto-immune deletion. In addition, it can be investigated whether multiple transplantations of MSC, one to stimulate islet regeneration following damage, and a subsequent transplantation to support continued regeneration may be more effective than a single dose.

The divergent mechanisms presented by freshly isolated UCB or BM-derived $ALDH^{hi}$ mixed progenitor cells, and culture expanded BM-derived ALDH-purified MSC, suggests a combinatorial cellular therapy may be the most effective approach to islet regeneration and optimal glycemic control. MSC could be administered to stimulate islet neogenesis and potentially protect newly formed

islets from auto-immune destruction, followed by $ALDH^{hi}$ cell administration to increase the size and vascularization of these newly formed islets. Further understanding of the specific mechanisms governing these divergent endogenous regenerative programs will aid in the development of cellular therapies to treat diabetes.

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Office of Research Ethics

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Use of Human Subjects - Ethics Approval Notice

Principal Investigator: Dr. D.A. Hess
Review Level: Expedited
Review Number: 12934
Revision Number: 2
Review Date: January 22, 2010
Approved Local # of Participants: 200
Protocol Title: Transplantation of human stem cells for the induction of angiogenesis and the regeneration of beta-cell function
Department and Institution: Vascular Biology, Robarts Research Institute
Sponsor: JUVENILE DIABETES RESEARCH FOUNDATION
Ethics Approval Date: January 26, 2010
Expiry Date: October 31, 2014
Documents Reviewed and Approved: Revised study end date and revised sample size to 200.
Documents Received for Information:

This is to notify you that The University of Western Ontario Research Ethics Board for Health Sciences Research Involving Human Subjects (HSREB) which is organized and operates according to the Tri-Council Policy Statement: Ethical Conduct of Research Involving Humans and the Health Canada/ICH Good Clinical Practice Practices: Consolidated Guidelines; and the applicable laws and regulations of Ontario has reviewed and granted approval to the above referenced revision(s) or amendment(s) on the approval date noted above. The membership of this REB also complies with the membership requirements for REB's as defined in Division 5 of the Food and Drug Regulations.

The ethics approval for this study shall remain valid until the expiry date noted above assuming timely and acceptable responses to the HSREB's periodic requests for surveillance and monitoring information. If you require an updated approval notice prior to that time you must request it using the UWO Updated Approval Request Form.

During the course of the research, no deviations from, or changes to, the protocol or consent form may be initiated without prior written approval from the HSREB except when necessary to eliminate immediate hazards to the subject or when the change(s) involve only logistical or administrative aspects of the study (e.g. change of monitor, telephone number). Expedited review of minor change(s) in ongoing studies will be considered. Subjects must receive a copy of the signed information/consent documentation.

Investigators must promptly also report to the HSREB:

a) changes increasing the risk to the participant(s) and/or affecting significantly the conduct of the study;
b) all adverse and unexpected experiences or events that are both serious and unexpected;
c) new information that may adversely affect the safety of the subjects or the conduct of the study.

If these changes/adverse events require a change to the information/consent documentation, and/or recruitment advertisement, the newly revised information/consent documentation, and/or advertisement, must be submitted to this office for approval.

Members of the HSREB who are named as investigators in research studies, or declare a conflict of interest, do not participate in discussion related to, nor vote on, such studies when they are presented to the HSREB.

Chair of HSREB: Dr. Joseph Gilbert
FDA Ref. #: IRB 00000940

Ethics Officer to Contact for Further Information			
☐ Janice Sutherland	☑ Elizabeth Wambolt	☐ Grace Kelly	☐ Denise Grafton

This is an official document. Please retain the original in your files.

)RE File
LHRI

Western

01.01.10
***This is the 3rd Renewal of this protocol**
*A Full Protocol submission will be required in **12.31.10**

Dear Dr. **Hess**

Your Animal Use Protocol form entitled:

Transplantation of novel stem cells for the regeneration of B-cell function

has had its yearly renewal approved by the Animal Use Subcommittee.

This approval is valid from **01.01.10 to 12.31.10**

The protocol number for this project remains as **2006-122**

1. This number must be indicated when ordering animals for this project.
2. Animals for other projects may not be ordered under this number.
3. If no number appears please contact this office when grant approval is received.
   If the application for funding is not successful and you wish to proceed with the project, request that an internal scientific peer review be performed by the Animal Use Subcommittee office.
4. Purchases of animals other than through this system must be cleared through the ACVS office. Health certificates will be required.

REQUIREMENTS/COMMENTS
Please ensure that individual(s) performing procedures on live animals, as described in this protocol, are familiar with the contents of this document.
The holder of this *Animal Use Protocol* is responsible to ensure that all associated safety components (biosafety, radiation safety, general laboratory safety) comply with institutional safety standards and have received all necessary approvals. Please consult directly with your institutional safety officers.

c.c. Heather Broughton; Melissa Pickering

***The*University *of* Western Ontario**
Animal Use Subcommittee / University Council on Animal Care
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