Sca-1 Positive Pancreatic Progenitor Cells: A Replacement for Transplanted Islets

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ABSTRACT

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(Under the direction of David A. Gerber)

A major challenge in the treatment of Type I diabetes is the lack of a restorative therapy to replace lost islet mass. Islet or pancreas transplant has proven effective at curing diabetes but its use is severely limited due to donor shortage. An alternative therapy would involve transplantation of a robust, easily accessible cellular population to replace damaged islets and restore insulin secretion. We have isolated a murine pancreatic progenitor cell, using stem cell antigen 1 (Sca-1), a marker of hematopoietic stem cells. These cells are capable of in vitro expansion and differentiation into pancreatic lineage, demonstrate and maintain expression of pancreatic transcription factors, and produce basal amounts of insulin. We theorize that this cell population used, in conjunction with a bioreactor-type culture system that mimics the niche of the pancreas in vivo will generate a viable cell source that has potential for use in the treatment of diabetes.

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ever I see that family is the most important thing we can have and I am grateful everyday that all of you are part of mine.

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LIST OF ABBREVIATIONS

ADA	American Diabetes Association
AU	arbitrary units
BrdU	Bromodeoxyuridine
BSA	Bovine serum albumin
Ca^{2+}	Calcium
Ck19	cytokeratin 19
CO2	Carbon dioxide
DMEM	Dulbecco's Modified Eagle's medium
EDTA	Ethylenediaminetetraacetic acid
ELISA	enzyme linked immunoabsorbant assay
ES cell	embryonic stem cell
FBS	fetal bovine serum
FEP	transparent fluoro ethylene propylene
G	gauge
GDM	gestational diabetes mellitus
GFP	green fluorescent protein
g/L	grams per liter
Hes1	hairy and enhancer of split 1
HRP	horseradish peroxidase
iPS cell	induced pluripotent stem cell
Klf4	Krüppel-like family of transcription factor 4
MACS®	magnetic activated cell sorting

Mg^{2+}	magnesium
mg/dL	milligrams per deciliter
mL	milliliter
mm	millimeter
mM	millimolar
NASA	National Aeronautics Space Administration
ng/mL	Nanograms per milliliter
Oct3/4	octamer 3 / 4
PBS	Dulbecco's phosphate buffered saline
PCR	polymerase chain reaction
PDX1	pancreas and duodenal homeobox gene 1
PE	Phycoerythrin
PP	pancreatic polypeptide
PPC	pancreatic progenitor cell
rpm	revolutions per minute
RWV	rotating wall vessel
Sca-1	stem cell antigen 1
Sox2	SRY (sex determining region Y)-box 2
T1D	Type 1 diabetes
T2D	Type 2 diabetes
WHO	World Health Organization
WT	Wildtype
α	Alpha

β	beta
δ	delta
µg	microgram
µg/ml	micrograms per milliliter

INTRODUCTION

DIABETES PATHOGENESIS

Diabetes is a disease characterized by the body's inability to produce insulin or an inability to respond to insulin's actions. Insulin is a hormone produced by the pancreas which regulates the amount of glucose circulating in the blood. It acts by stimulating cells to absorb circulating glucose and use it for energy. When cells are unable to absorb circulating glucose, it accumulates in the blood, a state known as hyperglycemia and fat is burned as an alternate source of energy. The breakdown of fat for fuel causes toxic acids, known as ketones, to be left behind in urine and blood. The opposite can also occur with insufficient amounts of glucose circulating in the blood leaving cells unable to function due to lack of an energy source. This state is known as hypoglycemia, or low blood sugar. Normal blood glucose levels are expected to be in the range of 80-140 mg/dl. Diabetics have fragile regulatory mechanisms with respect to glycemic control and therefore are unable to prevent hyper- and hypo-glycemic events. It is not uncommon for diabetic patients to experience blood sugar levels ranging from 30-600 mg/dl.

There are several forms of diabetes, all having one thing in common- hyperglycemia. However, the types of diabetes are extremely different and the disease progression and presentation dissimilar. Type 1 diabetes (T1D), also known as insulin-dependent diabetes, results from an autoimmune attack on the Beta (β) cells of the islets of Langerhans, destroying their function and leaving them unable to produce even small amounts of insulin.

The cause of the autoimmune attack on pancreatic β-cells in T1D is unknown. There is some evidence for a genetic factor in the disease as it is seen in families. However, environment and random developmental factors seem to be key players[1]. T1D is the main focus of this research.

Type 2 diabetes (T2D), or non-insulin-dependent diabetes, is due to a decrease in the body's sensitivity to respond to insulin or secondary to an insufficiency in insulin production. This form of the disease does not result from an autoimmune attack on the islets of the pancreas as seen in T1D. While not fully understood, this form of diabetes is strongly associated with obesity and sedentary lifestyles and currently accounts for over 90% of the total cases of the disease[2]. It is also associated with advancing age, with increased risk occurring after age 45. T2D, can progress to the point of islet exhaustion. This results from the normally functioning islets over-compensating for the loss of function or loss of sensitivity to insulin and their eventual hypertrophy and cessation of activity. When this occurs T2D effectively becomes T1D.

Gestational diabetes mellitus (GDM) occurs in 5-9% of pregnancies in the United States[3]. In this type of diabetes, women who have previously shown no signs or symptoms of the disease suffer from hyperglycemia during pregnancy. The exact cause of gestational diabetes is unknown, although there appears to be a genetic factor as the disease occurs often within family members. The following is a brief list of genes in which variations have been suggested to increase the risk of acquiring GDM- glucokinase, insulin receptor, insulin-like growth factor, plasminogen activator inhibitor, and hepatocyte nuclear factor-4α[4]. There is an increased risk of women who suffer from GDM to acquire T2D post-pregnancy[5].

PANCREAS ANATOMY AND FUNCTION

The pancreas is an organ that has both exocrine and endocrine function. Embryonic pancreatic development in vertebrate animals occurs from the dorsal and ventral protrusions of the primitive gut epithelium, which grow, branch, and eventually fuse to form the whole gland around the seventh week of gestation[6]. The human pancreas is located in the abdomen posterior to the stomach, attached to the small intestine at the duodenum and extending to the hilum of the spleen. The human pancreas is roughly 20 centimeters in length and weighs 85-90 grams[7].

The exocrine portion of the pancreas functions in digestion. The cells that compose this portion include acinar cells that secrete numerous enzymes from the pancreas into the duodenum aiding in digestion and breakdown of food as it passes through the digestive tract. These pancreatic enzymes include trypsin, lipase, amylase, and carboxypeptidase. The exocrine portion of the pancreas accounts for 80-85% of the organ's makeup and is thus a major component of pancreas anatomy[7].

The endocrine portion of the pancreas is key in the study of diabetes because it is here that insulin is produced. In the human there are roughly 1 million clusters of cells, termed islets of Langerhans. The islets, making up 1-2% of the organ's total mass, are composed of 100-1,000 endocrine cells. There are four distinct types of endocrine cells: alpha (α) cells producing glucagon, beta (β) cells producing insulin, delta (δ) cells making somatostatin, and pancreatic polypeptide (PP) producing cells. Of the endocrine cells of the pancreas, the β-cells, which produce all the insulin made in the body, account for roughly 60% of the human islet and 80% of the mouse islet[8]. It is these β-cells that are the target of autoimmune attack resulting in T1D.

COMPLICATIONS AND SIDE EFFECTS OF BLOOD SUGAR IRREGULARITIES

All diabetic patients are at risk for serious life threatening complications secondary to poor glucose regulation. The Diabetes Control and Complications Research Trial demonstrated that maintaining blood sugar levels within defined ranges and keeping a close monitor of the disease can greatly decrease the risk of developing life-threatening complications and aide in slowing diease progression[9].

Several acute complications of the disease can affect diabetics on a daily basis. These include bouts of hypoglycemia leading to confusion, headache, fatigue, nausea, numbness, irritability, slurred speech, ataxia, and impaired judgment. Untreated hypoglycemia can ultimately lead to loss of consciousness, diabetic coma, and possible death. Conversely, if blood sugar levels rise above normal, patients can experience polyuria, dizziness, confusion, cardiac arrhythmia, blurred vision, lethargy, and diabetic ketoacidosis as a result of ketones spilling into the blood and urine.

The long-term pathogenesis of diabetes is believed to occur through three main pathways- (1) formation of advanced glycation end products, (2) activation of protein kinase C, and (3) intracellular hyperglycemia with disturbances in polyol pathways[7]. Advanced glycation end products target the extracellular matrix of endothelial cells altering matrix-cell interactions. The glycation end products trap proteins inside cells and contribute to plaque formation leading to cardiovascular events. Advanced glycation end products can also trigger the release of pro-inflammatory factors-causing endothelial cell dysfunction, leading to vascular injury. Activation of protein kinase C leads to increased vasoconstriction and decreased vasodilatation, increased deposition of extracellular matrix, vascular occlusion, and creation of pro-inflammatory cytokines. This contributes to the growth of new

vasculature, as seen in diabetic retinopathy[7]. Intracellular hyperglycemia with disturbances in polyol pathways leads to oxidative stress which causes damage to all cellular components and affects organs like the kidneys, vasculature, nerves, and eyes.[7].

TREATMENT OPTIONS FOR DIABETICS

Treatment for diabetes, especially T1D, is limited. T2D and GDM can often be treated successfully with oral medication, diet, and lifestyle changes. However, T1D treatment is more invasive as deficiency of insulin production requires the injection of exogenous insulin. Insulin was discovered in the 1920's and proven effective at lowering blood glucose levels in test animals and experimental patients. This form of insulin was generated via extraction from animal sources and its use was limited. As a result, treatment remained largely centered around diet and exercise and the disease continued to lead to premature death. It was not until the 1950's that the amino-acid structure of insulin was elucidated and then the 1970's before the first synthetic insulin was produced. With its production came the commercial sale of the medication in the 1980's. Insulin has since been the standard of care for type 1 diabetics with many patients receiving multiple injections daily in order to maintain glucose levels as close to normal as possible. Insulin can be administered via hypodermic injection or given mechanically through an external insulin pump that maintains a steady release of drug subcutaneously.

Islet transplantation is another treatment option reserved for select cases of T1D. This procedure continues to be a clinical focus secondary to a successful protocol where seven patients were left insulin-independent after transplantation with cadaveric islets[10]. While the immediate impact of islet cell transplant is profound, it has several limitations. One

major problem is the fact that it takes more than one organ donor to yield enough islets for a successful transplant. The average donor generates approximately 500,000 viable islets but the typical recipient requires roughly 1 million healthy islets to be successfully weaned from exogenous insulin. Another drawback to the procedure is the requirement for immunosuppressive treatment after transplant to prevent rejection of the transplanted allogeneic cells. Longstanding immunosuppression is complicated by an increased risk of systemic infections and malignancies in the recipient. A final limitation to islet transplantation is the autoimmune nature of the disease that destroyed the patient's original islets. Over time this same pathologic process attacks the transplanted islets, ultimately leaving the patient insulin-dependent again. The Collaborative Islet Transplant Registry reported that 13% of islet recipients experience total loss of transplant function by 6 months and this increases to 42% by 3 years post-transplant[11]. Other studies are more pessimistic with reports that by 2 years post-transplant 76% of patients require exogenous insulin due to failure of the transplanted cells[12].

STEM-CELLS AND DIABETES

Diabetes is a unique disease because not many other diseases are caused by loss of function of a single known cell type. In addition, there is already a preexisting transplant protocol used to treat the disease that shows a clinically successful outcome[13]. Finding an alternate cell source to replace the limited number of islets available for transplant would alleviate the donor shortage as well as provide a potential unlimited source of cells that could ameliorate post-transplant declines in transplanted cell function. This cell source should be

highly proliferative, easily accessible, and unlimited, all qualities of a stem/progenitor cell population.

EMBRYONIC STEM CELLS

Embryonic stem cells (ES cells), were first cultivated in 1981 from mouse blastocysts[14]. Later experiments yielded ES cells obtained and cultivated from human tissues[15]. Embryonic stem cells are a potential candidate for islet replacement because they are pluripotent, capable of self-renewal and have the potential to differentiate into any cell type within the body. Subsequently, focus has been aimed at committing ES cells to a pancreatic β-cell fate. Early experiments with ES cells used the formation of embryoid bodies, or three dimensional aggregates, which were differentiated into insulin-producing cells[16, 17]. These approaches generate low numbers of insulin-producing cells and led researchers to experiment with over-expressing transcription factors that were known to force β-cell formation in an effort to yield robust insulin-producing populations[18]. Recent studies examined ES cells in conjunction with a myriad of signaling factors, growth factors, feeder cells, and/or culture conditions[19, 20]. Groups have also independently reported differentiating human ES cells into pancreatic lineage cells by recapitulating endocrine development. These cells were differentiated via a protocol generating definitive endoderm, posterior foregut, pancreatic endoderm and finally hormone expressing endocrine cells[19, 21, 22].

No ES cell population or process has been developed that can replace the low numbers of available donor islets. Even if that particular population is identified, multiple factors will have to be overcome before translating the therapy into clinical practice. These include the tumorigenic risk of ES cells; immunogenicity of the transplanted cells; and

ethical, political, and moral concerns as ES cells are derived from embryos and many people hold strong religious and moral beliefs about obtaining them.

ADULT DERIVED STEM CELLS

Because of the myriad of political, ethical, and tumorigenic issues surrounding ES cell use, focus has diverted to the use of adult-derived stem cells. These cells, which could potentially be derived from the patient, expanded, and re-implanted, circumvent some of the controversy surrounding ES cells. Adult stem cells, or somatic stem cells, reside in many parts of the body including bone marrow, neural tissues, testis, and adipose. Because of success with other organ systems it is feasible that an adult stem cell potentially resides in the pancreas. In fact, distinct cell populations have been isolated from adult pancreas and pancreatic duct tissue which can clonally proliferate in vitro, express pancreatic markers of all endocrine cell types, and produce insulin in response to glucose stimulation[23]. Other groups have looked at injury models as a means of stimulating proliferation of quiescent adult stem cells. One such experiment showed that after 90% pancreatectomy in rat there is a 45% recovery of tissue that occurs via the pancreatic ducts [24]. This observation suggests that an adult stem cell resides in the rich ductal network of the adult pancreas. Others have approached the possibility of an adult pancreatic stem cell by looking to the exocrine cells around the islets. In fact, insulin-producing cells have been generated from stellate and acinar cells, suggesting that these cells may express potential as endocrine progenitor cells[25-28].

INDUCED PLURIPOTENT STEM CELLS

Induced pluripotent stem cell (iPS cell) technology uses a somatic cell taken from the body and driven backwards in development using select embryologic factors. This brings the cell(s) to a phenotypic state similar to ES cells where they are capable of differentiation into any cell type of the body. This technology was initially reported by Takahashi and Yamanaka in 2006 when they were able to retro-virally induce Oct3/4, Sox2, Klf4, and c-Myc into mouse fibroblast cells and generate cells with properties similar to ES cells[29]. In 2008 human skin fibroblast derived iPS cells were differentiated into insulin-secreting islet-like clusters[30]. Although this technology creates a highly proliferative, functional cell source it does have its drawbacks. First and perhaps most importantly, these cells, because they are induced into ES-like cells with unlimited proliferative ability, give rise to teratomas when transplanted into animals. Because of their unlimited and unregulated proliferative potential there is no way to inhibit the formation of cancerous growths. Also, in order to get the transcription factors needed into the cells, retroviruses or lentiviruses are used. The implications of these technologies remain unknown but there is concern that after placing these cells back into humans the viral vectors could activate unwanted gene expression.

TRANSDIFFERENTIATIONS OF STEM CELLS

Another potential cell population for transplantation includes cells which normally reside in organs other than the pancreas. Much work has been done looking at transdifferentiation of cells that share a common embryologic origin with the developing pancreas. Cells of the liver, lung, and pancreas all arise from the foregut endoderm and differentiate in response to signals from their adjacent germ layers[31]. Therefore the cells of

these tissues may be able to transform into a pancreatic cell type if given the correct signals and growth factors which are conducive to pancreatic differentiation. The theory of transdifferentiation states that tissues derived from the same region of a developing embryo share transcription factors and only differ in the expression of a few factors which determine their ultimate fate[32]. There has been limited success with transdifferentiation of liver cell populations to a pancreatic phenotype via over-expression of pancreas and duodenal homeobox gene 1 (PDX1)[33, 34]. In addition, groups are beginning to see a relationship between the biliary system of the liver and the pancreas[35]. It has been shown that Hes1-null mice display conversion of their common bile duct into pancreas tissue expressing the full array of pancreatic cell types[36].

BIOREACTORS FOR CELL CULTURE AND TISSUE ENGINEERING

For any proposed cell type to be optimized the most efficient culture system must be employed. For decades cells have been grown reproducibly in 2-D petri dishes. However, in live organisms cells are embedded in extracellular matrix forming 3-D structures and tissues. Primary cells growing under two-dimensional culture conditions lose their original features and differentiate or dedifferentiate due to the lack of a 3-D environment like that present during organogenesis[37]. To circumvent monolayer limitations, growing cells in a 3-D system that better mimics the environment provided by nature and allowing spatial freedom facilitates growth and differentiation of cells in a way not seen in 2-D culture.

EARLY ATTEMPTS WITH BIOREACTOR DEVICES

Bioreactor devices have proven to be effective in creating an optimal 3-D environment for cell culture. Early bioreactor experiments were pioneered by the National Aeronautics Space Administration (NASA). They recognized that space flight offers the potential for zero gravity culture conditions which closely mimic the developmental process during embryogenesis. In 1984 human embryonic kidney cells were attached to microcarriers in the microgravity of space and found to have the potential for enhanced attachment and proliferation compared to cultures grown on Earth[38]. The success of the NASA space flight experiments led to the conceptual development of Earth-bound microgravity bioreactor systems.

Many early suspension culture systems used stirring or mechanical agitation as a method of keeping cells and microcarrier beads in suspension. These agitated cultures were found to be detrimental to the cells due to the high amounts of shear force and turbulence produced during culture which often damaged cells and lead to cell death[39]. This lead researchers to examine different types of bioreactor cultures that involved less agitation, such as bubble free oxygenation and higher viscosity medium[40]. It was also noted that in large scale bioreactor devices, less cell damage was seen in cells and microcarriers farthest from mechanical agitators, because the cells came into contact with the agitation devices less frequently[41]. All of this culminated with NASA's development of a small incubator size device that suspended microcarrier beads and cells in culture without the need for induced turbulence or sheer force, much like the environment of space flight[41]. This device was called a rotating wall vessel (RWV) and it worked via solid body rotation and oxygenation via a silicone rubber membrane.

RATIONALE AND GOALS FOR PROJECT

Clinical advances in cell transplant techniques have made islet transplant a successful means of temporarily relieving diabetic patients of the need for administration of exogenous insulin. However, because of the limited success and the need for multiple cell infusions, islet transplant is reserved for only select cases. It is with this in mind that we have set out to identify an adult cell source which can be used to replace islets currently used for transplant.

We have isolated an adult derived murine progenitor cell from pancreas tissue purified for stem cell antigen 1 (Sca-1). We theorize that this pancreatic progenitor cell (PPC) population can be expanded in culture and is capable of differentiation into a cell with β-cell characteristics. We propose that we can grow a Sca-1 positive PPC population in 2-D culture and maintain them over long periods of time while identifying their potential for differentiation and their potential to produce insulin in response to glucose increases. Because of this cell's proliferative capability and pancreatic genotype and phenotype we postulate that it could be a suitable alternative cell type for transplant therapies for diabetes treatment.

Bioreactors have shown improved islet differentiation, insulin secretion and cellular maintenance of endocrine precursor cells[42-45]. As a result, we set forth that by using a well established mouse β-cell line we can create a 3-D culture system allowing the needed 3-D spatial freedom required to mimic embryologic development which optimizes cell growth for unique populations, such as pancreatic progenitors.

Materials and Methods

MICE

All mice were obtained from The Jackson Laboratory (Bar Harbor, ME), including the standard C57BL/6 strain (stock # 000664; for our purposes termed Wild Type or WT), and mice from a C57 background with an enhanced EGFP cDNA constitutively expressed using the β-actin promoter (stock # 003291, termed GFP+). Animals were maintained on standard rodent chow under a constant 12 hour day/12 hour night cycle. C57BL/6 and GFP positive mice were used for isolation of PPCs. Care and use of animals was approved by the Institutional Animal Care and Use Committee at the University of North Carolina at Chapel Hill in accordance with the principles and procedures outlined in the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

ISOLATION OF PANCREATIC PROGENITOR CELLS

PPCs were isolated by a multi-step digestion process beginning with excision of the entire pancreas. Pancreatic tissue was manually sheered and disassociated using a 22G needle and forceps in a buffer of Liberase TL (Roche; Indianapolis, IN). Enzymatic digestion was accomplished in a 37ºC water bath for 20 minutes with agitation every 5 minutes. Subsequently, disruption of remaining pancreatic tissue was accomplished by pipetting with a 10mL pipette. Serum was added to quench the enzymatic activity of Liberase. The

dissociated tissue was filtered through a 70µm filter to yield a single cell suspension, centrifuged for 5 minutes at 1100 rpm, and then resuspended in calcium and magnesium chloride free Dulbecco's Phosphate Buffered Saline (PBS; Sigma; Saint Louis, MO) containing 0.5% BSA and 2mM EDTA prior to sorting by MACS®.

CELL ENRICHMENT AND CULTURE

Isolated cell populations were enriched for Sca-1 positivity using magnetic activated cell sorting technology (MACS®, Miltenyi Biotec Inc.). The cells were incubated with Sca-1 antibody conjugated to mini-magnetic beads (Miltenyi Biotec, cat # 130-092-529; Auburn, CA) according to the manufacturer's instructions. Sca-1 positive (Sca-1+) cell selection was confirmed with a purity of >94% by flow cytometry. Sca-1+ cells were cultured on 35mm tissue culture dishes. Dishes were coated with 0.5mL of 2µg/mL fibronectin solution for 1 hour, washed with 1mL PBS, then coated with 0.5mL of 1µg/mL concavalin A solution for one hour, followed by a final wash with 1mL PBS. Culture medium consisted of DMEM (Dulbecco's Modified Eagle's medium; Gibco; Carlsbad, CA) containing 10% fetal bovine serum (FBS), 20mM HEPES, 10mM Nicotinamide, 30mg/L L-proline, 2µM Dexamethasone, 1X antimycotic-antifungal, supplemented with 10ng/ml BMP-4 (R&D Systems; Minneapolis, MN), 1400U/ml ESGRO (Milipore; Temecula, CA), and 20µL/mL B27 supplement (Gibco; Carlsbad, CA). Cells were plated at a starting density of approximately 1 x 10^6 cells per 35mm dish. The cells were maintained in a 5% CO2 incubator at 37ºC. Cells were allowed to become approximately 80% confluent before being passaged using 1X Trypsin/EDTA at a 1:2 ratio.

FLUORESCENT IMMUNOPHENOTYPING AND FLOW CYTOMETRY

Cells were stained for immunofluorescense using Sca-1 antibody conjugated to Phycoerythrin (PE) (BD Biosciences; San Jose, CA). Cells stained with an IgG2a antibody (BD Biosciences) were used as isotype controls and unstained cells used as a negative control. Cells were suspended at 1 X 10^6 cells per ml in phosphate buffered saline (PBS) containing 2% FBS at room temperature. Cells were incubated with antibody for 20 minutes, centrifuged and resuspended in cold buffer twice to wash, before being analyzed on a Beckman-Coulter CyAn ADP flow cytometer provided by the UNC flow cytometry core facility.

HISTOLOGY AND IMMUNOHISTOCHEMISTRY

Immunohistochemistry was performed by an indirect immunoperoxidase procedure. Primary antibodies against Sca-1and CK19 were used (BD Biosciences; San Jose, CA). ABC Elite and DAB kits (Vector Labs; Burlingame, CA) were used for signal detection. DAB was toned with DAB enhancer (Vector Labs) and counterstained with hemotoxylin (Sigma Aldrich; St. Louis, MO). Slides were blocked with Protein Blocking Agent (Thermo Electron Solutions; Pittsburgh, PA). Negative controls included sections stained with isotype control antibodies. All stained slides were viewed with a Nikon Microphot-FXA microscope equipped with an Optronics DEI 750 3-chip CCD camera and Q Imaging Micropublisher CCD camera for digital image acquisition. Images were captured on an Apple Power Macintosh G3 computer utilizing Q imaging software and saved using Adobe Photoshop CS2 software, all located in the Microscopy Services Lab core facility at UNC.

For fluorescent immunohistochemistry, indirectly labeled antibodies to Sca-1 (rat anti mouse Ly-6A/E; BD Biosciences; San Jose, CA), and rabbit anti-PDX-1 (Millipore; Temecula, CA) were used. Secondary antibodies used include goat anti-rat Texas Red (Molecular Probes; Carlsbad, CA), and goat-anti rabbit AF594 (Invitrogen; Carlsbad, CA) respectively. DAPI was used to counter stain nuclei (Sigma Aldrich; St Louis, MO).

RT-PCR

Polymerase chain reaction (PCR) analysis was performed on total RNA extracted from freshly isolated and cultured Sca-1+ cells. Islet RNA was used as a control. A total of 2µg of total RNA was used as a template to create complementary DNA using RETROscript® kit (Ambion; Austin, TX) per the manufacturer's protocols. The following genes of pancreatic progenitors were selected; Nestin, Hnf6, Ptf1alpha, PDX-1; markers of endocrine progenitors; Pax6, Nkx2.2, PDX-1, Ptf1alpha, Ngn3, Neuro D; and differentiated cell markers; insulin 1, insulin 2, and glut 2. The presence of CK19, a ductal cell marker, was also assayed. Primer sequences are listed in Table 1. Basic conditions included a 94 degree denaturation following by 35 cycles of denaturation at 94 degrees, annealing at specified melting points, and elongation at 72 degrees, all followed by a final elongation at 72 degrees and a final hold of 4 degrees.

WESTERN BLOT ANALYSIS

Cells were lysed in buffer containing 50mM Tris-Cl pH 7.6, 150 mM NaCl, 1% Nonidet P40 (Roche; Indianapolis, IN), 0.5% deoxycholate, 1mL protease inhibitor cocktail for every 100ml (5µg/mL aprotinin, leupeptin, pepstatin, and soybean trypsin inhibitor), and

Table 1: Semi-quantitative PCR primers.

Primer	5'-3'	Annealing Temp	Size
Ptf1alpha	catagagaacgaaccacccttgag cttgagacaggtcctttgaggcacg	60	294
Nkx2.2	aaaggtatggaggtgacgcct tcatgttgcgggtcatgtcga	54	190
Ngn3	aagagcgagttggcactgagc gcgtatcgcctggtgtcgaa	56	223
Insulin 1	tagtgaccagctataatcagagac agccaaggtctgaaggtc	70	288
Insulin 2	ccctgctggccctgctctt aggtctgaaggtcacctgct	60	213
Nestin	ggagagtcgcttagaggtgc gaagagaaccgaaaggactg	58	327
Glut 2	ggataaattcgcctggatga tcaaggtctttggtttcctt	53	298
NeuroD	cttggccaagaactacatctgg ccgttgaagagaaagtttgtgc	53	430
CK19	ctgcagatgacttcagaacc ggccatgatctcatactgac	62	299
Pax 6	tcacagcggagtgaatcag cccaagcaaagatggaag	58	332
Hnf6	gcaatggaagtaattcagggcag cgtgacagtcgttgaagaagtac	60	471
PDX-1	ctttcccgtggatgaaatcc gtcaagttcaacatcactgcc	60	205

1mL phosphatase inhibitor for every 100mL. Lysates were purified by centrifugation at 14,000g for 2 minutes and stored at -20ºC. Protein concentrations were determined by using a standard Bradford assay on a BioTek microplate reader. Total cell proteins (50µg/lane) were separated by SDS-PAGE, transferred to nitrocellulose membrane, incubated with blocking buffer (5% nonfat dry milk in Tris Buffered Saline with 0.05% Tween 20, pH 7.5), and probed with primary antibodies overnight. Antibodies were directed against Glucagon (1:1000), Amylase (1:2000), Insulin Receptor alpha (1:1000), Ngn3 (1:1000) (Santa Cruz Biotechnology; Santa Cruz, CA), Pdx1 (1:5000), and Glut-2 (1:1000) (Milipore; Billerica, MA). After 1 hour incubation with appropriate HRP secondary antibodies (Dako; Carpinteria, CA), peroxidase activity was detected by enhanced chemiluminescence. Densitometric signals from western blots were analyzed with NIH-ImageJ software (http://rsb.info.nih.gov/ij/). Protein levels were calculated in arbitrary units (AU) normalized with β-actin protein levels.

INSULIN ELISA

Insulin secretion of cultured cells from both neonatal and adult mice was measured using an enzyme linked immunoabsorbance assay (ELISA). Experiments began by placing cultures in high and low glucose medium (40mM and 2.8mM respectively) for 48 hour durations. Supernatants were collected and placed in -20º storage. Because the presence of FBS in culture media has the potential to affect readings our later experiments used Krebs Ringer Bicarbonate Buffer (Zenbio; Research Triangle Park, NC) with added glucose at low 5.55mM concentrations, and high 25mM concentrations, over a 4 hour time point. Supernatants were collected at 30 minutes, 1, 2, and 4 hours of culture and frozen at -20º. A

final set of experiments were performed in an attempt to generate a large difference in insulin produced by using low glucose (0mM) and high glucose (100mM) conditions over 1 hour of culture. All samples were analyzed for insulin concentration according to manufacturer's protocol for the Mercodia Mouse Insulin ELISA (Alpco Diagnostics; Salem, NH). Assays were performed by the UNC Center for Gastrointestinal Biology and Disease (CGIBD) immunotechnology core facility. Values were expressed in ng/mL.

CELLULAR PROLIFERATION ASSAY

Cell growth and proliferation were measured using the CyQUANT® NF Cell Proliferation Assay Kit (Invitrogen Molecular Probes™; Eugene, OR) which measures DNA content. Cells were allowed to proliferate in 96 well culture dishes for set time points of 0, 1, 4, 7, 14, and 21 days. On test day growth medium was removed and 50µl of 1X binding dye from kit added. Cells were further incubated for 30 minutes and assayed on a BioTek Microplate reader at emission wavelengths of 485 and 530nm. Absorbance values obtained were correlated to cell numbers using a standard curve (created previously with a rapidly proliferating cell line).

MICROCARRIER PREPARATION

Cytodex 3 microcarrier beads were purchased from GE HealthCare (Piscataway, NJ). Beads were rehydrated and prepared according to manufacturer's protocol. Dry microcarriers were hydrated in Ca^{2+} and Mg^{2+} free PBS at 50ml/g for 3 hours at room temperature. They were sterilized by removing supernatant and adding 70% isopropyl alcohol. The carriers were washed twice in this manner and incubated overnight in the 70% ethanol solution,

50ml/g. The next day the solution was removed and the beads washed three times with sterile PBS. Before use with PPCs beads were suspended in 2mL of 2µg/mL fibronectin solution, washed with 2mL PBS, then resuspended in 2mL of 1µg/ml concavalin A followed by washing with PBS. Cell lines were mixed with beads immediately after third PBS wash.

BIOREACTOR CULTURE

Bioreactor cultures were contained in VueLife™ transparent fluoro ethylene propylene (FEP) closed culture bags. (American Fluoroseal Corporation; Gaithersburg, MD). These culture bags are designed specifically for cell culture and allow high permeability to oxygen and carbon dioxide, thus allowing cells to respire normally without being open to contamination. Microcarrier beads were added to bags at a concentration of 0.02g/bag in 1mL fresh warmed culture media. β-TC6 cells (catalog numberCRL-11506) in standard DMEM with 15% FBS (American Type Tissue Culture; Manassas, VA) where added, at a concentration of 1-2x10^6 cells per bioreactor. Bags were then placed into a rotating culture device set to approximately 25 revolutions per minute (rpm) and continuously rotated. The bioreactor is capable of rotation rates of 2-30 rpm. Media was changed in bioreactors twice weekly by stopping rotation, allowing cells and beads to settle, and then removing the media via a 22G syringe through the bags port. Fresh media was then substituted through the same port via another 22G syringe.

Results

ISOLATION OF A PANCREATIC PROGENITOR CELL

Cellular Localization Within Murine Pancreas

Sca-1 has been demonstrated as a stem cell marker of murine hematopoietic, endothelial, adipose, kidney, and hepatic cells[46-50]. Because of the common lineage of pancreatic and hepatic tissues during embryonic development of these organs, Sca-1 was chosen as a candidate progenitor marker for murine pancreas. To test for Sca-1 expression in the pancreas, tissue sections were obtained from mice of varying ages. Murine pancreas was analyzed from: neonatal, 1-2 weeks old, and adult (>5 week old) animals. In adult mice, Sca-1 expression is seen along the ductal epithelia and in the peri-islet space (Figure 1). In neonatal mice Sca-1 expression is seen along the ductal epithelia of the organ and scattered around the pancreatic acini. No expression is observed within the β, α, δ, PP, or somatostatin cells that compose the islet. Because Sca-1 expression is seen along the peri-ductal space of the pancreas, pancreatic tissue was stained for the expression of CK19, a known ductal epithelial marker. Figure 1C shows CK19 staining in the pancreas and delineates the ductal areas, which correspond to those areas that demonstrate cells which are positive for Sca-1 expression.

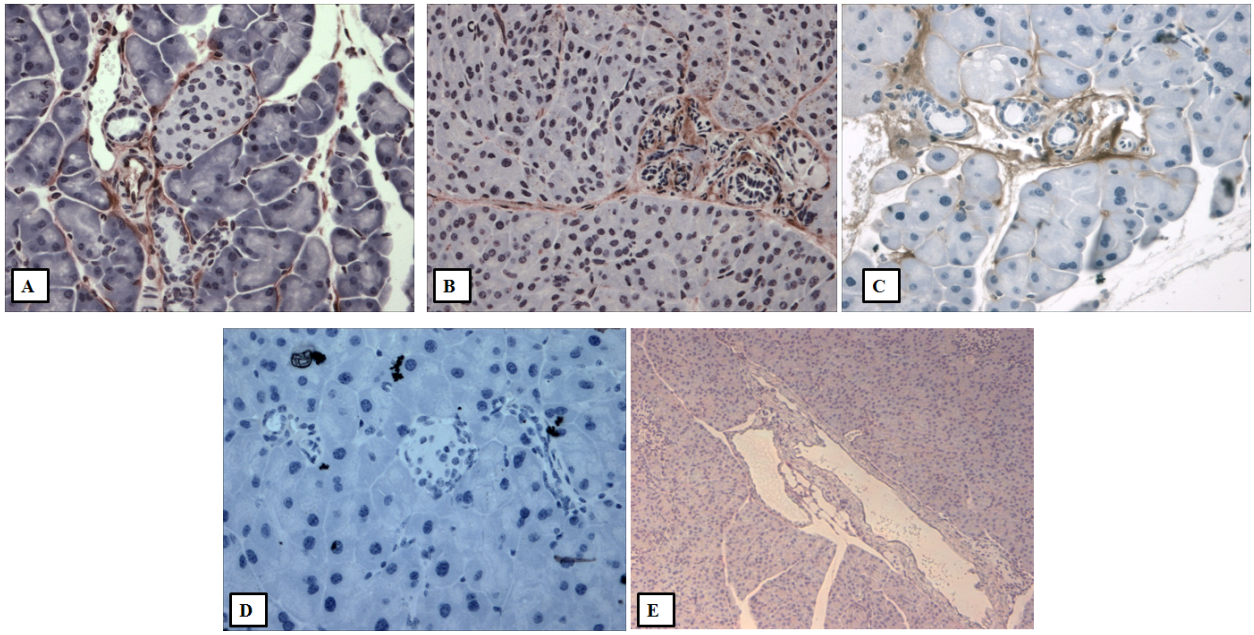

Figure 1. Sca-1 expression in normal murine pancreas tissue. Immunohistochemical staining shows Sca-1 expression along the ductal epithelium as well as in the peri-islet space of adult mice 6weeks of age (A). Sca-1 is also seen along the ductal network of neonate mice 2 weeks of age (B). Cytokeratin19 is a common marker of ductal epithelium and serves as a control to delineate the rich ductal network of the pancreas (C). Images (D) and (E) represent isotype controls for Cytokeratin 19 and Sca-1 respectively. 20X (A,C,D), 10X (B,E).

Enrichment and Purification of Sca-1+ PPCs

Fluorescence-activated cell sorting analysis was utilized to quantitatively assess the number of the total Sca-1+ cells in the murine pancreas. Results show that 1.13 ± 1.28% and 2.3 ± **0**.21% of neonatal and adult pancreatic cells are Sca-1+ respectively. Adult mice generated a higher percentage of Sca-1+ cells but fewer cells overall (690 compared to 16,950 from neonates). For this reason, neonatal mice, ~2 weeks of age were used in all experiments because they gave a higher yield of Sca-1+ cells. Adult mice were used in selected comparison studies.

Immunomagnetic separation was utilized to isolate Sca-1+ cell fractions instead of flow sorting as the former technique produces a higher yield of viable cells during the isolation and purification process. Immunomagnetic technology works by labeling our cell population with a magnetic microbead specific for a given antigen (e.g., Sca-1). The cells are passed through a magnetic filter and the Sca-1 population is retained while the negative population is washed out. Given that the parameters are not as tightly controlled with immunomagnetic technology (compared to flow sorting), the purity of resulting cell preparations underwent confirmatory tests by flow cytometry analysis. As seen in Figure 2, flow cytometry analysis shows that the Sca-1+ population obtained using immunomagnetic separation technology is an enriched and distinct population. This technique yields an average of 94% of the cell preparation staining positive for Sca-1.

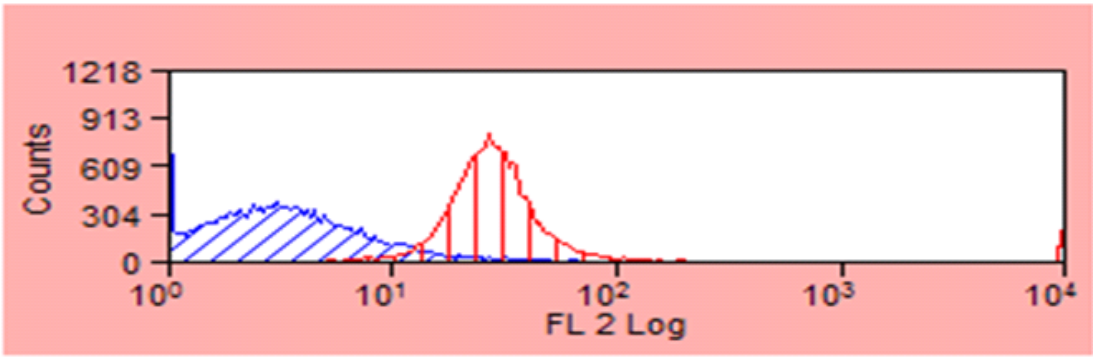

Figure 2. Flow cytometry analysis of immunomagnetically separated Sca-1+ PPCs. MACS© separation generates a reliable Sca-1+ cell population. Flow cytometry analysis shows unlabeled cells (blue) as a distinct population from PE labeled Sca + cells (red), verifying purity in magnetic separation.

Two Dimensional Culture of Sca-1+ Pancreatic Progenitor Cells

Figure 3 demonstrates cell colony morphology. These images are representative of the colonies demonstrated when Sca-1+ PPCs are grown using modified cell propagation medium conditions[51]. Our modified conditions include low glucose DMEM with 10% FBS, 1.4 units ESGRO, 1µl/mL BMP4, and B27 supplement. Using the immunomagnetic separation technique, MACS®, approximately 1.5 million Sca-1+ cells are recovered from a single murine pancreas. Upon initially plating the cells they are seen as individual, single cells. Very early in culture (day 1-3), small colonies form (colony diameter ranging from 50-100µm) (Figure 3a). The colonies are comprised of small and tightly packed cells with a high nuclear to cytoplasm ratio. The cells comprising these colonies rapidly proliferate and expand to form larger colonies several hundred microns in diameter. The morphology of the cells changes while they proliferate. Within two weeks the cells exhibit increasing cytoplasm and their morphologic features are consistent with an epithelial appearance while the colony rapidly expands across the tissue culture dish (Figure 3b). By day 21 of culture, the colonies approach confluence and require passage to maintain viability. After the first passage, cells acquire a fibroblastic phenotype and lose their colony-forming capacity. While small colonies are no longer seen, cells continue to rapidly proliferate and can be maintained through 35+ passages (Figure 3C).

CHARACTERIZATION OF SCA-1+ PANCREATIC PROGENITOR CELL

Immunohistochemical Analysis of Sca-1+ Colonies In-Vitro

MACS® separation is limited by allowing non-specific cells to contaminate the Sca-1+ cultures. To prove that the contaminating cells were not forming the colonies, we assessed

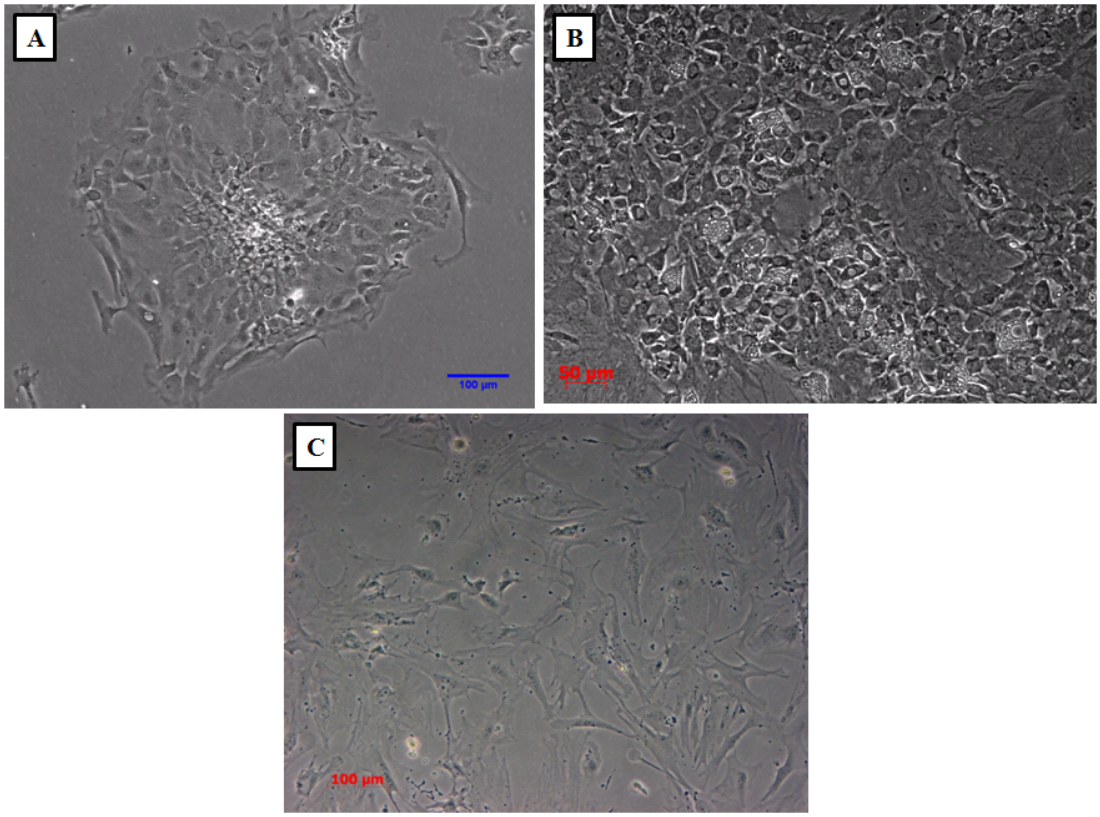

Figure 3. Changes in Sca-1+ colony morphology throughout culture. Sca-1+ colony morphology changes over the duration of standard culture. Initial colonies are typical of those seen with other stem cell sources. Colonies are tightly packed with individual small cells each containing a high nuclear to cytoplasm ratio (A). By day 21 of culture colonies spread over 90% of dishes and have a cobble stone type appearance (B). After passage cells take on a fibroblastic appearance, no longer forming stem cell-like colonies throughout the duration of culture (C). All images 10X magnification. (A) Day 3 of culture, (B) Day 21 of culture (C) Passage 1, day 2 of culture.

progenitor colonies by fluoro-immunohistochemistry for Sca-1 expression and confirmed that the colonies are composed of Sca-1+ cells (Figure 4).

PPC Expansion and Capacity to Proliferate

The CyQuant proliferation assay was utilized to assess PPC proliferation. The assay is based on fluorescent dye binding to DNA of viable cells. As evidenced by the graph in Figure 5, twenty-four hours after isolating and plating the cells there is a decrease in viable cell number to 60% of the starting cell population. This decrease likely reflects the fact that the isolation procedure leads to cellular necrosis due to the fragile nature of this primary cell population. Mechanical and enzymatic stress as well as removing cells from their natural environment contributes to cell death. Additional cells are lost from the culture as some cells do not attach to the dishes after plating. Cell numbers continue to decline through day 4 and at that point our cell population represents approximately 36% of cells compared with the starting population. After day 4 the viable cells undergo a period of recovery and notable proliferation. By day 7 there is an increase to 102% of cells present compared with Day 4. Cell numbers continue to increase and by day 14 there is an 8.7-fold increase compared with the numbers seen at day 4. By day 21, cell numbers plateau and remain constant at 11x the numbers seen on day 4. This coincides with visible confluence of cells on the dish and likely cell-cell contact inhibition secondary to a depletion of space for the cells to further proliferate.

Interim Conclusions

A novel stem cell population that is positive for Sca-1 has been identified in the murine pancreas. In vivo the cell is localized around the peri-islet space as well as along

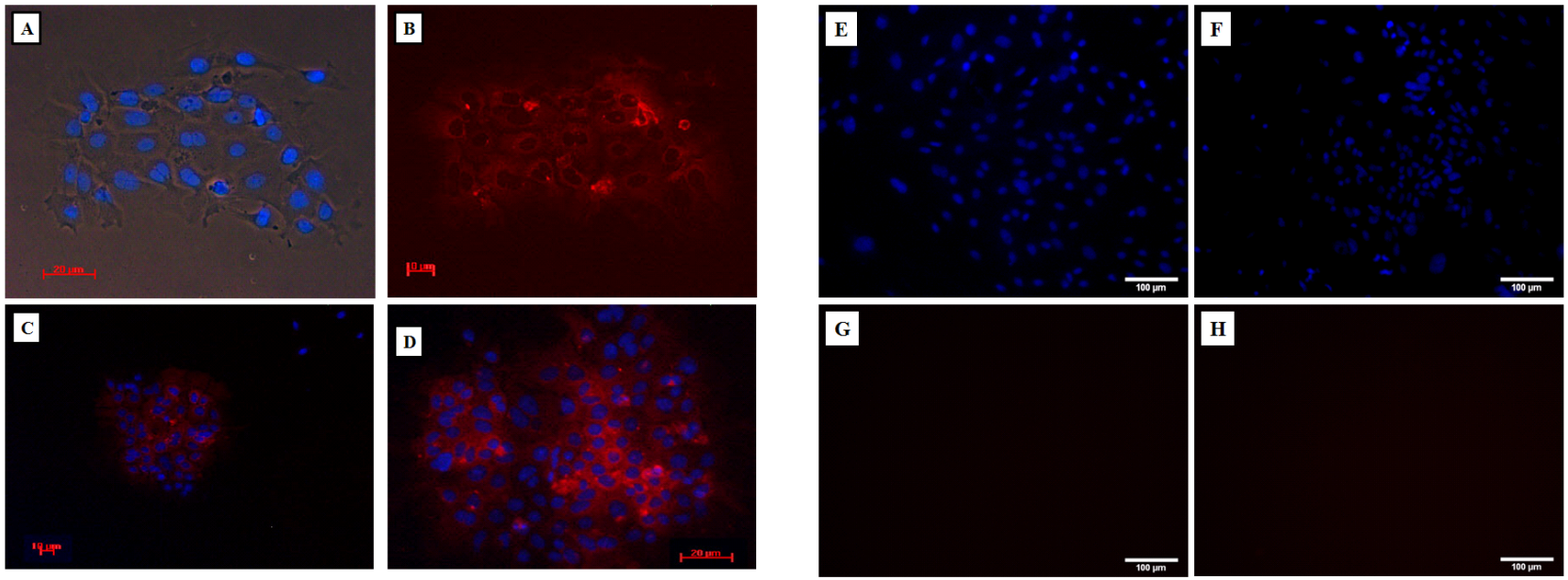

Figure 4. Sca-1 immunohistochemistry in culture. Colonies in standard culture are formed from Sca-1+ cells, not contaminating cell types. DAPI staining delineates nuclei within a common colony at day 3 of culture (A, E, F). Sca-1 expression of colony (B). Merged DAPI and Sca-1 staining (C,D). Staining control-isotype control (G), Staining control,-secondary alone (H). (A,B,D,E,F,G,H) 20X, (C) 10X.

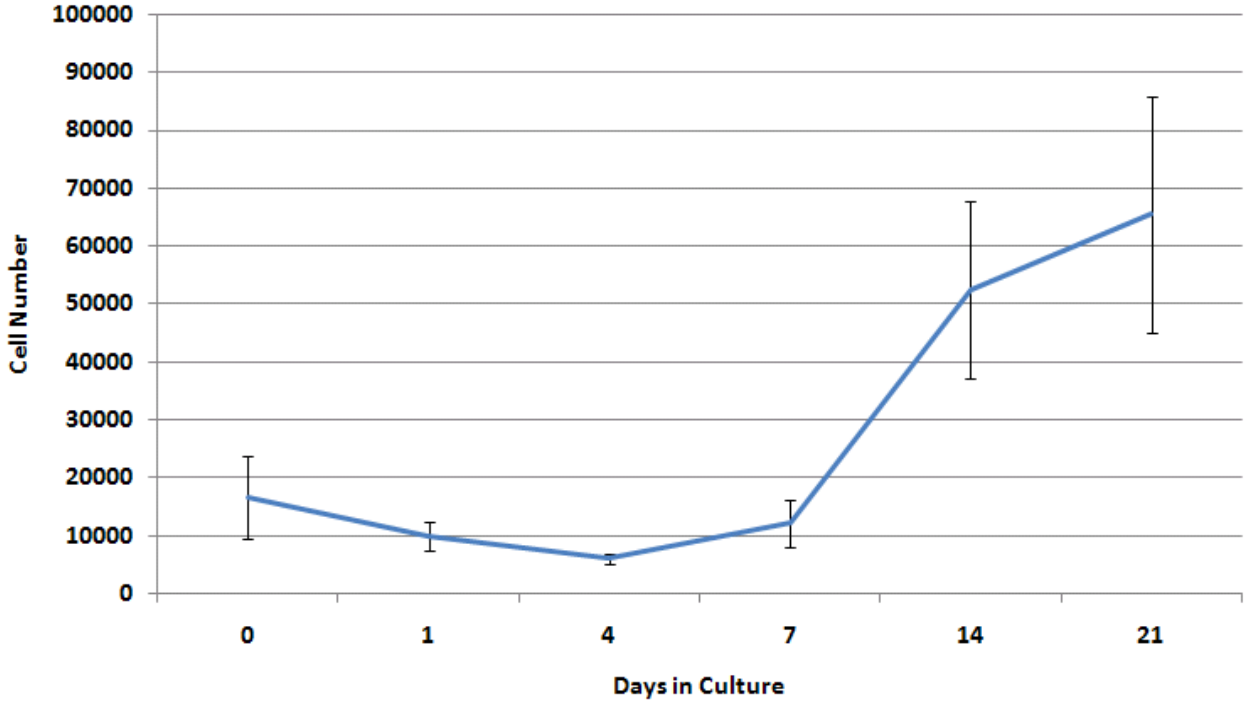

Figure 5. Proliferative ability of Sca-1+ PPCs. After an initial phase of degredation following plating, Sca-1+ PPCs are a highly proliferative, robust cell population. Cells undergo rapid recovery and proliferation over a 3 week culture period. Error bars represent standard error.

ducts of the pancreas. There is no Sca-1 expression seen within the islets or within the acinar cells of the organ. We are able to consistently isolate and purify this cell population using immunomagnetic cell separation technology specific for Sca-1 and obtain a population with 94% purity. Sca-1+ cells form rapidly proliferating colonies of tightly packed small cells when placed in culture. Initially these colonies are compact and composed of cells that have a high nuclear to cytoplasm ratio. However, as the cells continue to proliferate their morphology changes and they develop an epithelioid appearance with larger cytoplasm. Once cells are confluent they are capable of passage. When passaged morphology changes again and cells appear fibroblastic in nature for the remainder of culture. Cells positive for Sca-1 maintain their expression in culture after forming stem cell colonies. Sca-1+ PPCs are a highly proliferative cell population. Upon initial plating cells undergo a period of cell death. After 4 days in culture cells recover and a period of rapid proliferation ensues with ultimate cell numbers being 11 times higher than their lowest numbers on day 4 of culture.

TRANSCRIPTION AND TRANSLATION OF SCA-1+ PPC'S

PPCs Express Transcription Factors Indicative of β-Cell Lineage as well as all Other Cell Fates of Adult Pancreas

Transcription factors of interest were selected based on a review detailing necessary factors required during differentiation of embryonic endoderm to liver and pancreatic fates[52]. Distinct genes are required for differentiation to particular cell types of the pancreas. Figure 6 is a schematic of selected genes and their significance in the developmental pathway leading to the pancreas. Sca-1+ cell populations were evaluated immediately after isolation (P0) and during in vitro culture after select passages (P2 and P4). Murine islets were used as a control population. Immediately after isolation the PPC population expresses

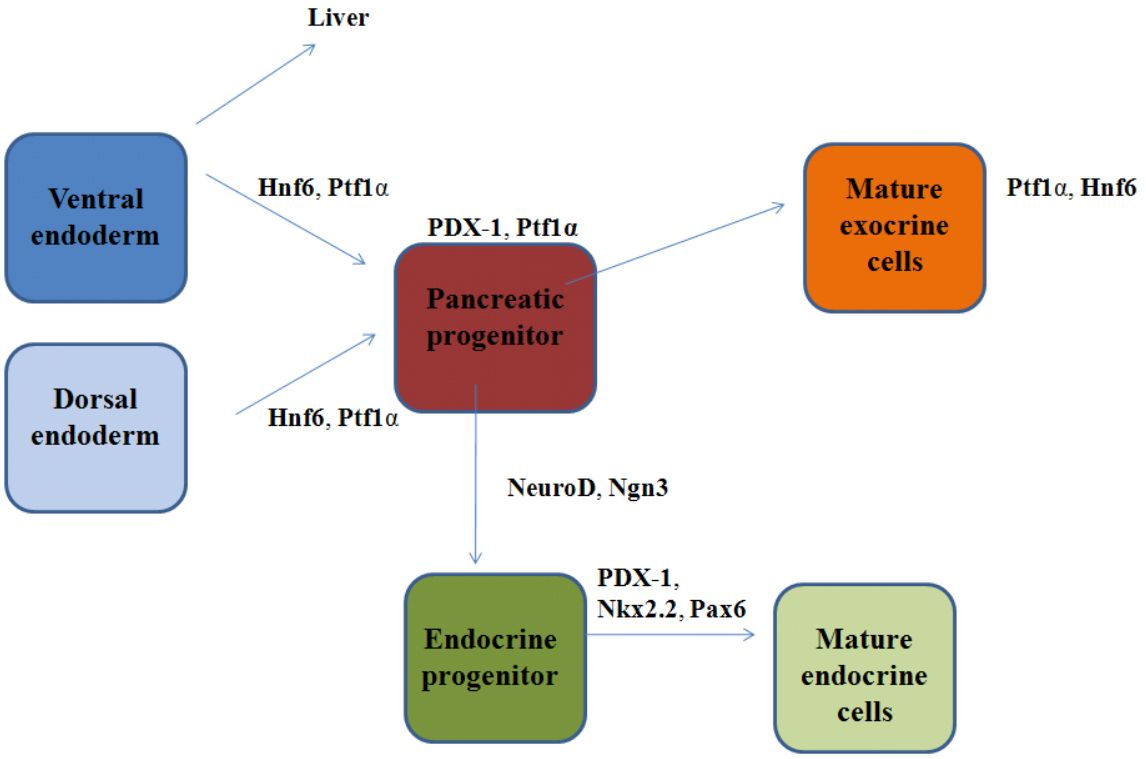

Figure 6. Transcription factors of interest in pancreatic progenitor development. Liver and Pancreas share a common lineage in embryogenesis with specific transcription factors being essential to drive pancreatic development. Pancreas is formed from fusion of the dorsal and ventral endoderm under the influence of factors shown. As pancreas tissue grows and develops specific factors are needed to drive formation of individual cell types- progenitor, endocrine, and exocrine. Transcription factors assayed at each stage in our experiments are listed.

developmental markers Hnf6, Ptf1α, and PDX-1 (Figure 7). During cell culture (passage 2) the markers Hnf6 and PDX-1 are down-regulated. Hnf6 is subsequently up-regulated by P4.

Expression of markers leading to an endocrine progenitor fate Nkx2.2, Ptf1alpha, Ngn3, and NeuroD were analyzed. Nestin, which has been shown to be necessary to differentiate embryonic stem cells toward a pancreatic cell fate was also analyzed[53]. These genes persist throughout passage, while Pax6 and PDX-1 disappear as passage number increases. Markers of endocrine cells, Insulin 1 and 2 are present at P0 and P2, but decrease by P4. Another differentiated cell marker, Glut 2 is very weakly expressed at P2 and P4. Because prior histological analysis showed Sca-1+ cells correlating with ductal areas we subsequently evaluated expression levels of CK19, which was present at all time points in culture and passage.

PDX-1 expression is required for differentiation of pancreatic stem cells toward a pancreatic β-cell[54]. Based on this information and the fact that we see transcription of the gene we analyzed the Sca-1+ progenitor population for co-expression of PDX-1. PDX-1 expression in the Sca-1+ colonies was analyzed by fluoro-immunohistochemistry. Figure 8 shows that Sca-1+ progenitor colonies do exhibit PDX-1 in their cytoplasm. PDX-1 is normally found located in the nucleus of cells. Expression in the cytoplasm of PPCs would suggest transcription but not translation of the protein into an active form. As seen by other investigators, in rat and human islets PDX-1 is found in an inactive form in the cytoplasm of the islet when glucose concentrations are low. PDX-1 is then activated by phosphorylation when glucose concentrations increase and the active form is translocated to the cell nucleus[55-58].

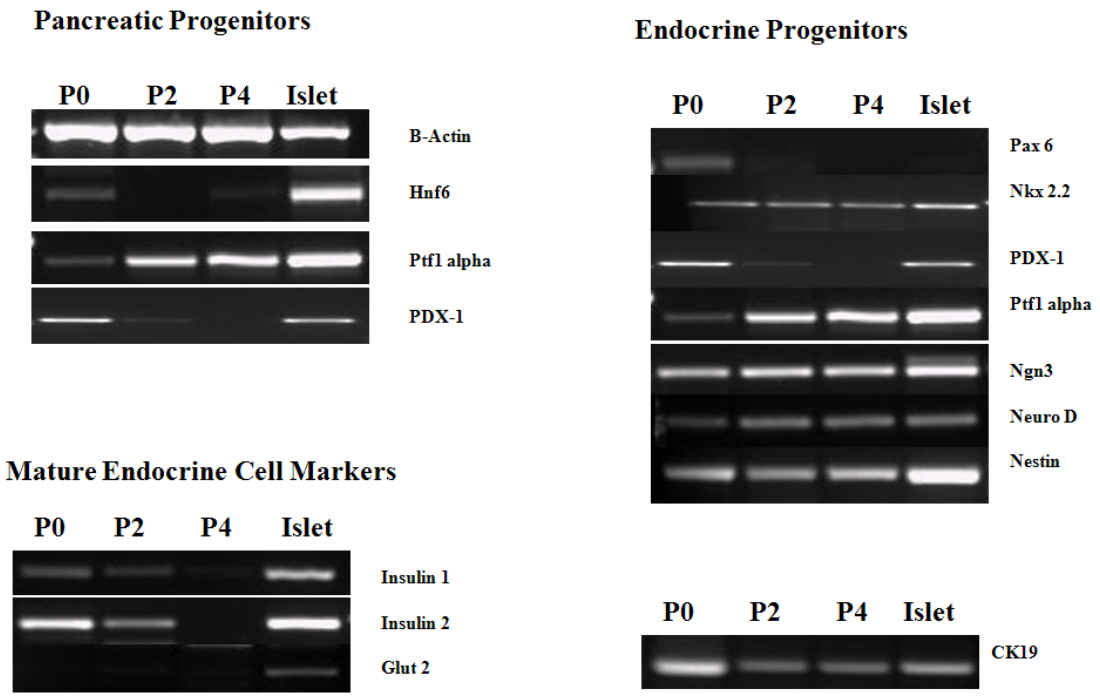

Figure 7. Semi quantitative PCR analysis of Sca-1+ PPCs. PCR analysis shows that PPCs differentiate toward multiple lineages of pancreatic fate and not toward simply endocrine β-cell fate. Cells were assayed at passage 0, 2, and 4 for the listed pancreatic transcription factors. Ck19 was also analyzed since it's expression was seen in staining in vitro.

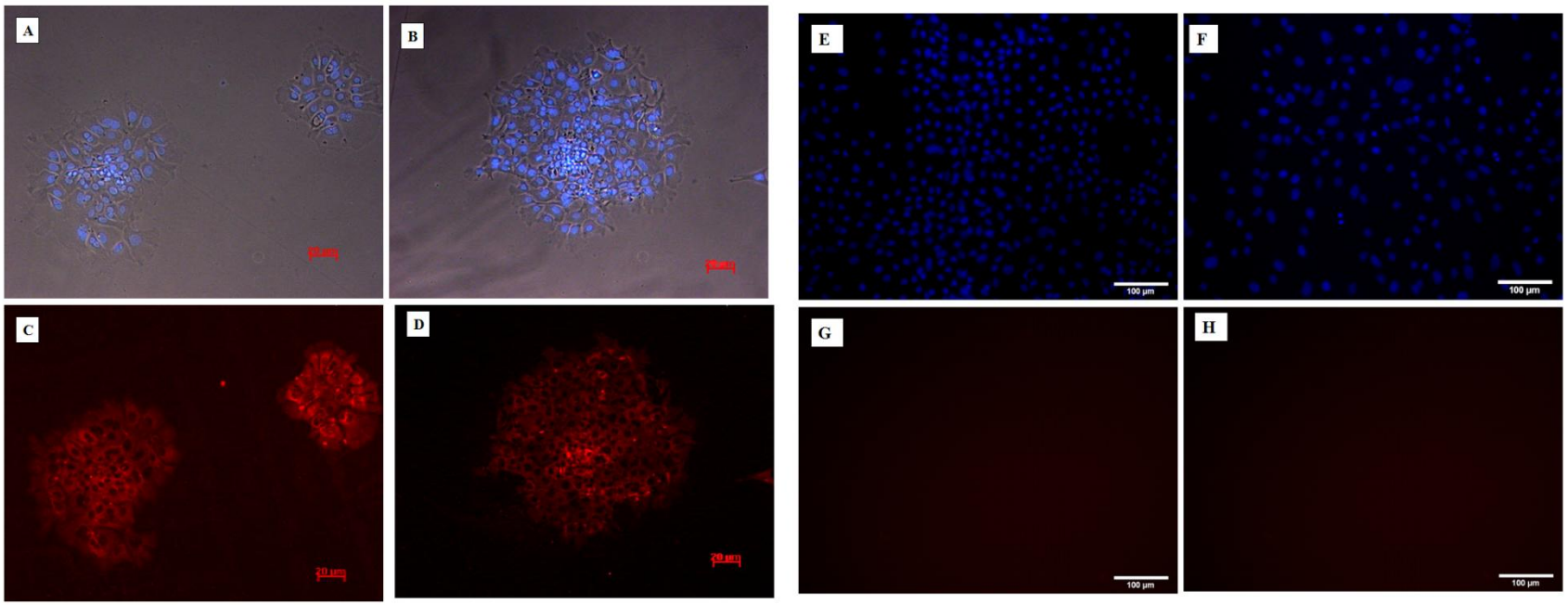

Figure 8. PDX-1 expression of Sca-1+ colonies. PDX-1 is expressed by all β-cells of the pancreas and is necessary in formation of β-cell tissue from progenitor cell origins. PDX-1 is expressed on day 3 Sca-1+ colonies. Expression is strongly shown in the cytoplasm and not the nuclear region of the cells. Transmission image with DAPI delineating nuclei (A,B), PDX-1 expression of the same colonies using PE conjugated antibody (C,D). DAPI transmission (E,F). Controls corresponding to DAPI transmission, isotype control (G), secondary alone (H). (A-D) 10X, (E-H) 20X.

Differentiation of PPCs into Multiple Pancreatic Cell Fates

Western blot analysis was employed to analyze cell cultures to determine differentiation toward the endocrine and exocrine features of the pancreatic population. Cells were assayed at passage 0, 2, 4, and 7. Ngn3 and Pdx1 were chosen as products of early cell differentiation associated with β cell fate; insulin receptor alpha, a marker of fully developed Beta cells; glucagon a marker of alpha cells; and amylase a product of the acinar cells. Final analysis shows cellular expression of PDX-1 and Ngn3 at low levels after isolation and throughout the time points assayed. Insulin receptor alpha expression was higher at all time points but there was no significant variation throughout culture. Amylase was likewise observed at low levels throughout the culture period. Results are summarized in Figure 9.

Interim Conclusions

Transcriptional analysis of Sca-1+ PPCs during culture shows variability in the markers expressed. Initially cells express many of the developmental markers associated with pancreatic and endocrine progenitor cells, including Hnf-6, Ptf1α, PDX-1, Pax6, Nkx2.2, Ngn3, NeuroD, and Nestin. These markers vary in expression, at times down-regulated at passage 2, but are ultimately up-regulated and expressed throughout the culture period. Cells also express many differentiated cell markers such as Insulin1, Insulin2, Glut2, and CK19. There are again fluctuations in the degree of expression of these transcription factors but they are persistent throughout the culture duration. The reason for the fluctuation in progenitor and differentiated cell factors could be due to differentiation as a result of cell to cell-interactions. Although the cells are purified for Sca-1 expression other cells do get mixed in with the cell preparations. MACS® technology while allowing for greater purity

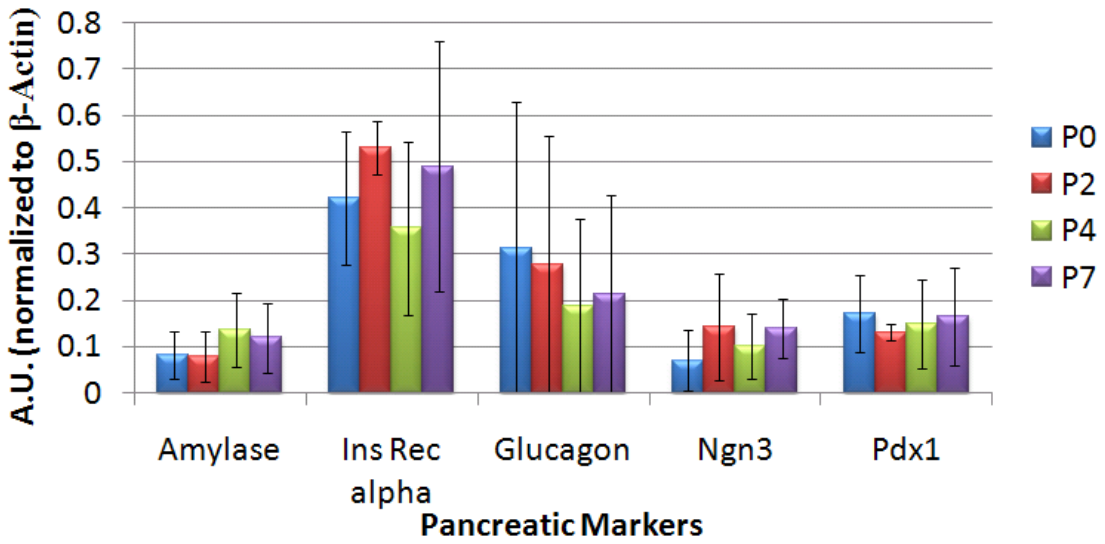

Figure 9. Protein expression of Sca-1+ PPCs. Western blot analyses performed at passage numbers 0, 2, 4, and 7 for pancreatic proteins Amylase, Insulin Receptor Alpha, Glucagon, Ngn3, and Pdx-1. Values are reported in arbitrary units. Results correspond with transcription data in that there is no clear line of differentiation. No statistical significance was observed. Error bars reflect standard error. Arbritraty units are normalized for expression of control protein β-actin.

does not provide a 100% pure population of Sca-1+ cells. In addition, maintaining the cells in a 2-D environment limits their ability to develop normally and forces the cells to differentiate based on constraints of the culture vessel as well as causing cell death due to limited space. PDX-1 is a transcription factor that is present not only in early development in the pancreas but is also essential for the differentiation of mature β-cells. For this reason its presence would be essential in any cell population with potential of becoming an islet-like cell. PPCs do in fact express PDX-1 throughout culture at the transcriptional level. We also analyzed cells in culture for the presence of PDX-1 after colony formation and found that the colonies do express PDX-1 in their cytoplasm.

Protein analysis of the Sca-1+ PPC population was done via western blot. Analysis shows that cells express the developmental markers Ngn3 and PDX-1 throughout culture and passage. The cells also express mature endocrine proteins Glucagon and Insulin Receptor α. Likewise, mature exocrine cell marker Amylase is expressed throughout culture. The expression levels of each protein vary throughout the culture duration showing the ability for endocrine and exocrine function, but a significant trend is in a particular direction is not observed.

FUNCTIONAL ANALYSIS OF SCA-1+ PPC'S

PPCs Release Basal Amounts of Insulin In Vitro

ELISA was performed to assay insulin production secondary to glucose stimulation. Neonatal mice and adult mice 20+ weeks of age were used to compare functional differences. Cells from both age groups produce a basal level of insulin in both high and low glucose conditions over a 48 hour period (Figure 10). While cells from adult mice produce a

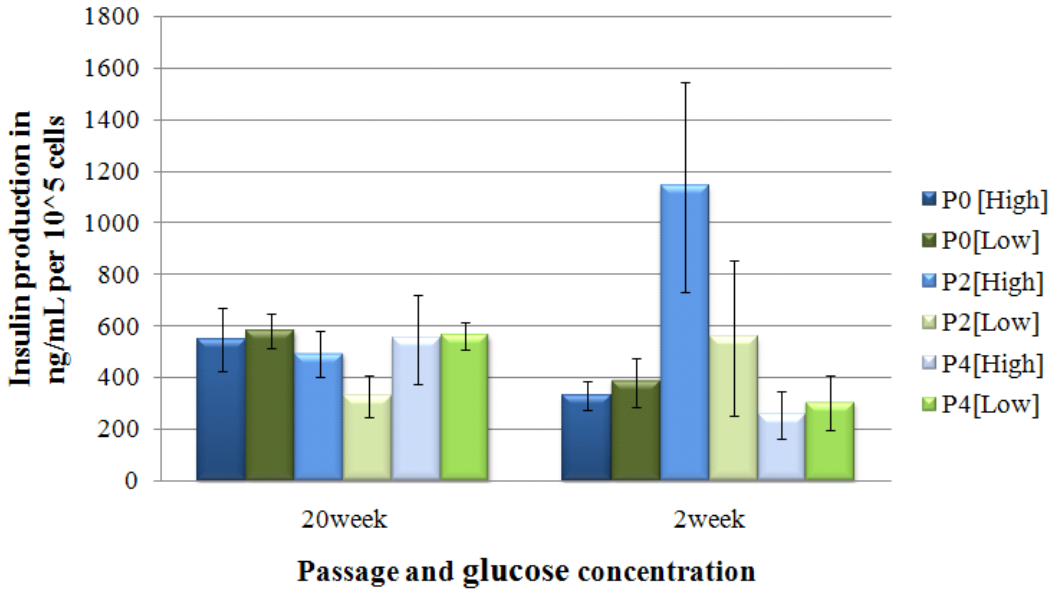

Figure 10. Functional ability of Sca-1+ PPCs to produce insulin. ELISA was utilized to measure sustained secretion of insulin by both adult (20 week) and neonate (2 week) Sca-1+ donor cells. Cells were stimulated with low (2.8mM) and high glucose (40mM) conditions for a 48 hour period and supernatant collected at the conclusion. Error bars represent standard error. No statistical significance was noted.

relatively constant amount of insulin across time points and glucose concentrations, neonatal cells spike in insulin production at P2 and then return to immediate post isolation (P0) levels by P4. There is no statistically significant increase in insulin secretion from low to high glucose conditions at any passage for either age group.

Initial ELISA data was collected allowing 48 hours for the cells to be glucose stimulated. As insulin secretion is typically measured by analyzing the rapid spike in response to acute changes in glucose concentration this physiologic cellular response could be missed over longer collection times like those delineated in Figure 10. To test the cells' acute phase response an experiment was performed using a 4 hour assessment with sample collections at 30 minutes, 1 hour, 2 hours, and 4 hours after glucose challenge (Figure 11). Islets were used as a control to demonstrate normal rapid response to changes in glucose conditions. Sca-1+ cells produced basal amounts of insulin throughout the process, without a significant increase in production as a response to increased glucose concentrations in the media. PPCs in low glucose conditions produced slightly more insulin than their high glucose counterpart.

Interim Conclusions

Sca-1+ PPCs have the functional ability to produce insulin in vitro. However, this ability is not responsive to glucose stimulation. Sca-1+ cells from adult mice produce a steady state of insulin across passages and over a 2 day period of culture. Release of insulin is independent of glucose concentration. Neonatal mice are capable of responding to glucose spikes at passage 2, but are not able to maintain this ability after passage 4. Acute ability to secrete insulin was also analyzed. Over the course of 4 hours there is no increase in insulin secretion in Sca-1+ cells in high glucose conditions. In fact, cells in low glucose conditions

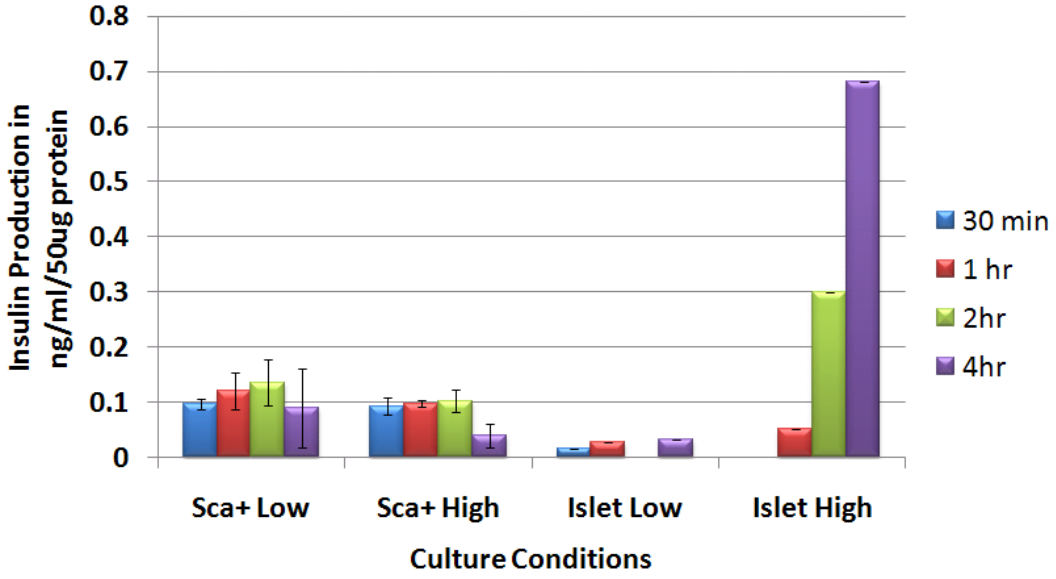

Figure 11. Rapid response of Sca-1+ PPCs to high glucose conditions. Insulin ELISA was utilized to measure rapid response to increased glucose conditions. Neonatal cells were stimulated for a 4 hour duration with either high (40mM) or low (5.55mM) glucose conditions in the absence of FBS. Supernatants were collected at 30 min, 1, 2, and 4 hours. Cells were assayed at Passage 0 of culture during the time period when small colonies are visible. Mature islets were used as controls. Units are expressed in ng/ml normalized for 50µg of protein. Error bars represent standard error.

released more insulin than their high glucose counterparts. This was in stark contrast to islets which showed a drastic increase in insulin secretion at high concentrations and only a minor increase in low glucose conditions.

THREE DIMENSIONAL CULTURE

Bioreactor Culture of Sca-1+ PPCs

Bioreactor culture devices were designed and created with assistance from Dr Robert Dennis of the department of Biomedical Engineering at UNC (Figure 12). The base design is a rotating wall vessel which circles around a fixed point creating conditions of zero gravity and allowing cells to remain suspended throughout the duration of culture. The device measures 8 X 8 X 6 inches and is maintained inside a carbon dioxide infused incubator.

Initial experiments integrated the bioreactor devices with our Sca-1+ PPCs isolated from neonatal mice. Experiments were designed to assess the cells' potential for growth and viability in the bioreactor cultures. Cells are cultured on Cytodex 3 microbeads (Figure 13A) in FEP culture bags that allow diffusion of gases across their surface and thus do not require the infusion of biological gases through mechanical methods (Figure 13B). Initially PPCs attach to microbeads at single contact points. Over the course of 2-3 weeks the cells begin to spread and eventually cover the entire bead. After the beads are covered, the cells begin to form bridges with adjacent beads, attaching and forming aggregates of multiple beads. This indicates that the cells remain viable in this unique 3-D culture condition. GFP-positive cells were used to enable cellular visualization over the course of the two week culture period (Figure 14).

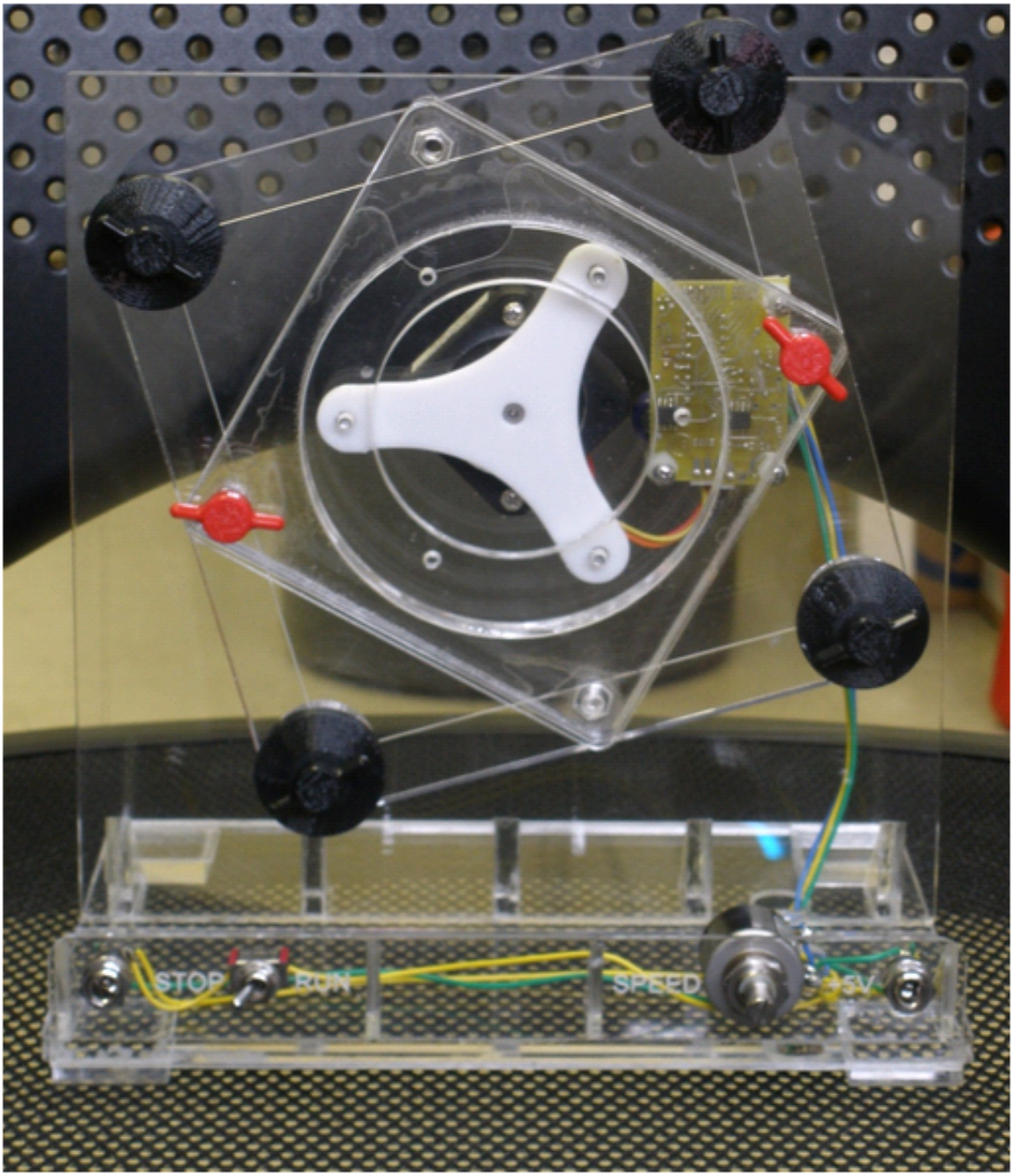

Figure 12. Design of 3-D tissue culture bioreactor. The bioreactor consists of a pivoting platform that rotates around a fixed point. A small motor behind the platform controls power and speed of rotation.

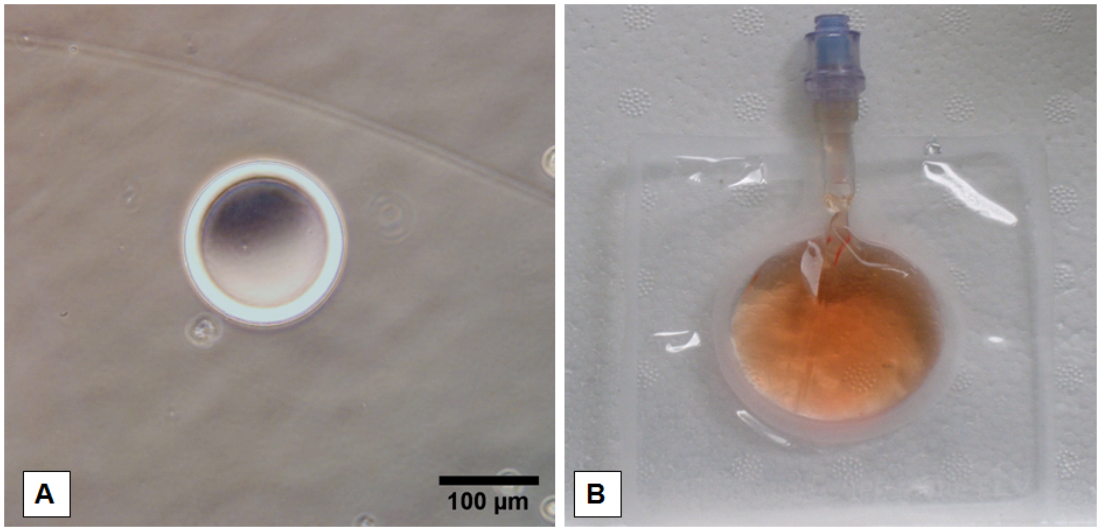

Figure 13. Microcarriers and culture bags for bioreactor experimentation. Cytodex 3 microcarrier beads are used as a substrate for cells to adhere in rotating culture (A). Beads and cells are loaded into fluoroethylene propylene culture bags (B) prior to being attached to bioreactor platforms. 20X (A).

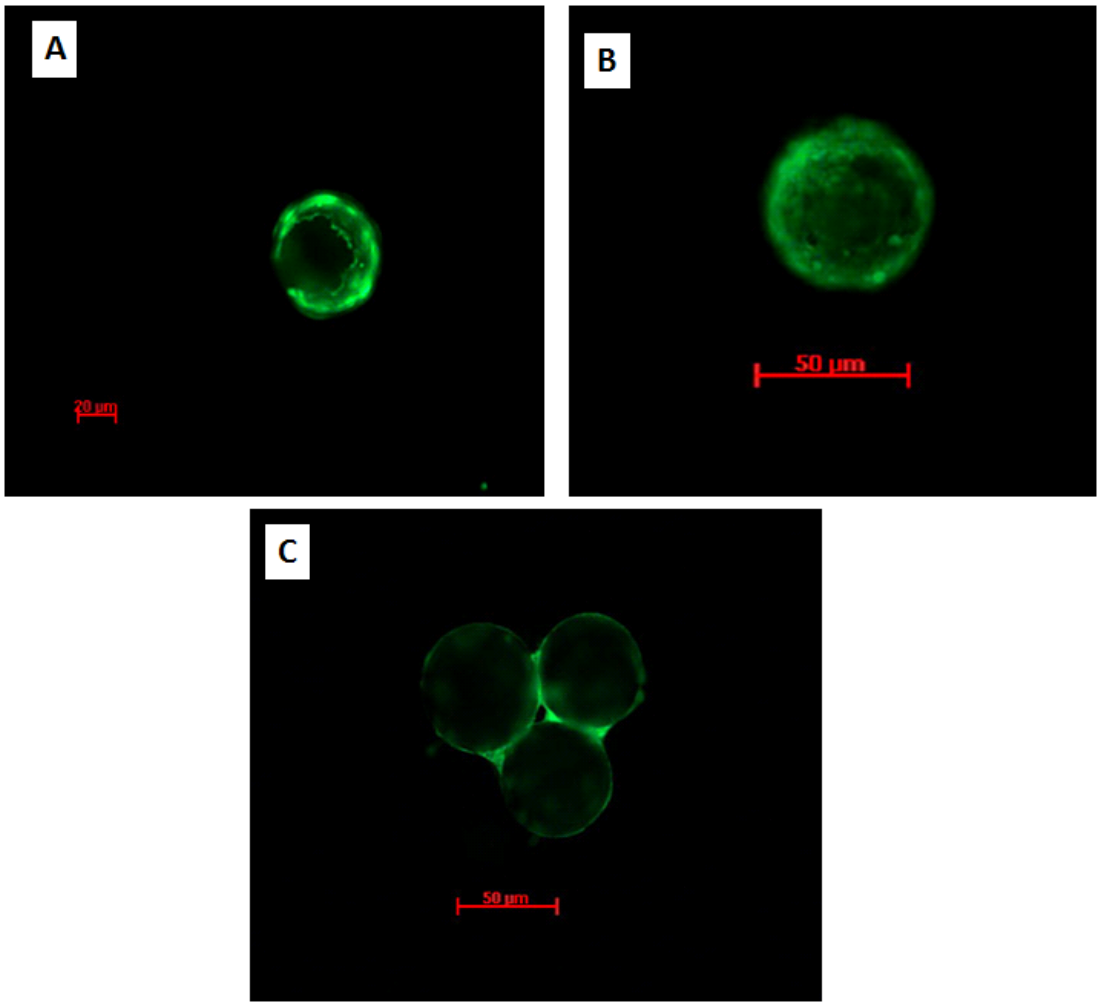

Figure 14: Attachment of Sca-1+ PPCs to microcarrier culture beads. Sca-1+ PPCs have the ability to adhere and proliferate in 3-D bioreactor cultures. Initially cells attach to cytodex beads at individual points. Image at day 5 (A). Cells are then observed to spread and cover the entire bead surface. Image at day 10 (B). Once bead surfaces are covered cells begin to branch out and attach to neighboring beads, forming aggregates. Image at day 13(C). 10X (A), 20X (B,C).

Insulin Secretion of Sca-1+ PPCs in Bioreactors

Cultures were kept in bioreactors until PPCs proliferated to the point where they covered the microcarrier beads. At this point they were placed in high glucose conditions and analyzed for their ability to secrete insulin over 4 hours. Insulin levels increased over a 2 hour time period but levels then declined by 4 hours of increased glucose stimulation (Figure 15). This pattern, which is similar to that seen in fully functioning islets, was a new observation. All previous attempts at glucose challenge of the PPCs resulted in a steady release of insulin over the entire experimental time and no spike in secretion in response to increased glucose. By simply removing PPCs from a monolayer culture and placing them into a 3-D environment they demonstrate a physiologically ability to function in a more normal manner.

Bioreactor Culture of β-TC6 Cell Line

Further experimentation to analyze PPC differentiation was attempted on multiple replicates but due to limited numbers of primary cells and the necessity of a large number of cells for bioreactor culture we identified an alternative endocrine pancreatic cell line that we could use to study the bioreactor's effects. The β-TC6 cell line was chosen for subsequent bioreactor experiments because of its proven endocrine function[59, 60]. The β-TC6 line is derived from β-cell tissue of a mouse pancreatic tumor. This cell line is highly proliferative and easily maintained making it a suitable cell source to analyze an ex vivo culture system's effects on pancreatic cells. β-TC6 cells initially attach to Cytodex beads at single points and begin to cluster on their surface. Over time, clusters detach from beads and form free floating suspended clusters (Figure 16). These clusters vary in size and have been observed to reach

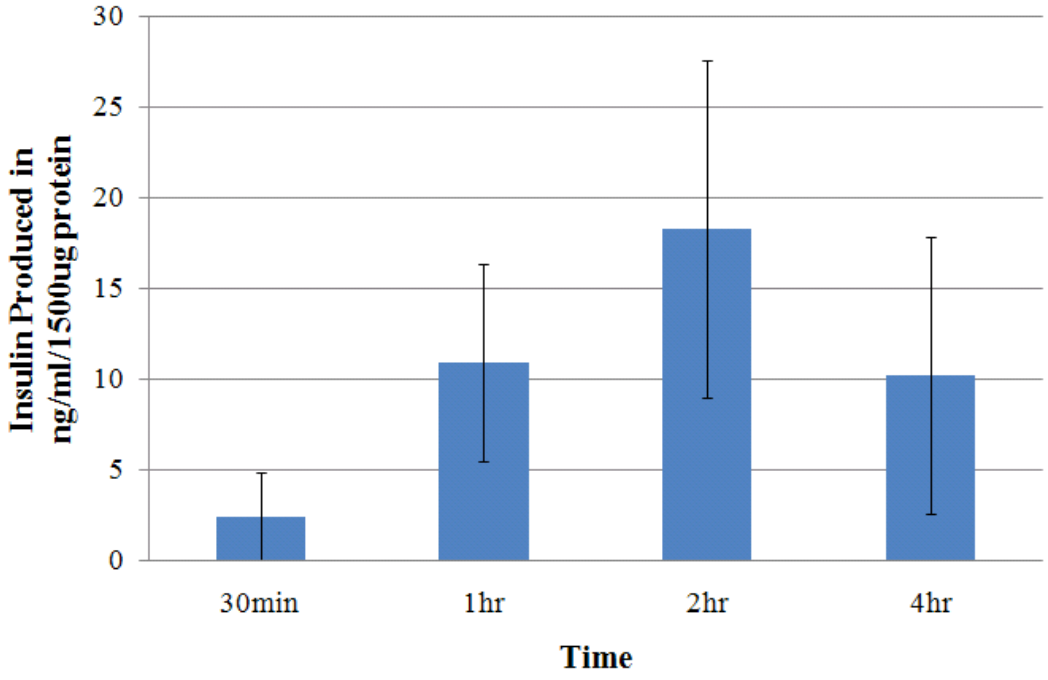

Figure 15. Sca-1+ PPCs respond to increased glucose in 3-D bioreactor cultures. Insulin secretion was measured in response to a 55.5mM concentration of glucose over a 4 hour time period. Insulin levels increase over a 2 hour period and then decline by the 4th hour. Error bars represent standard error.

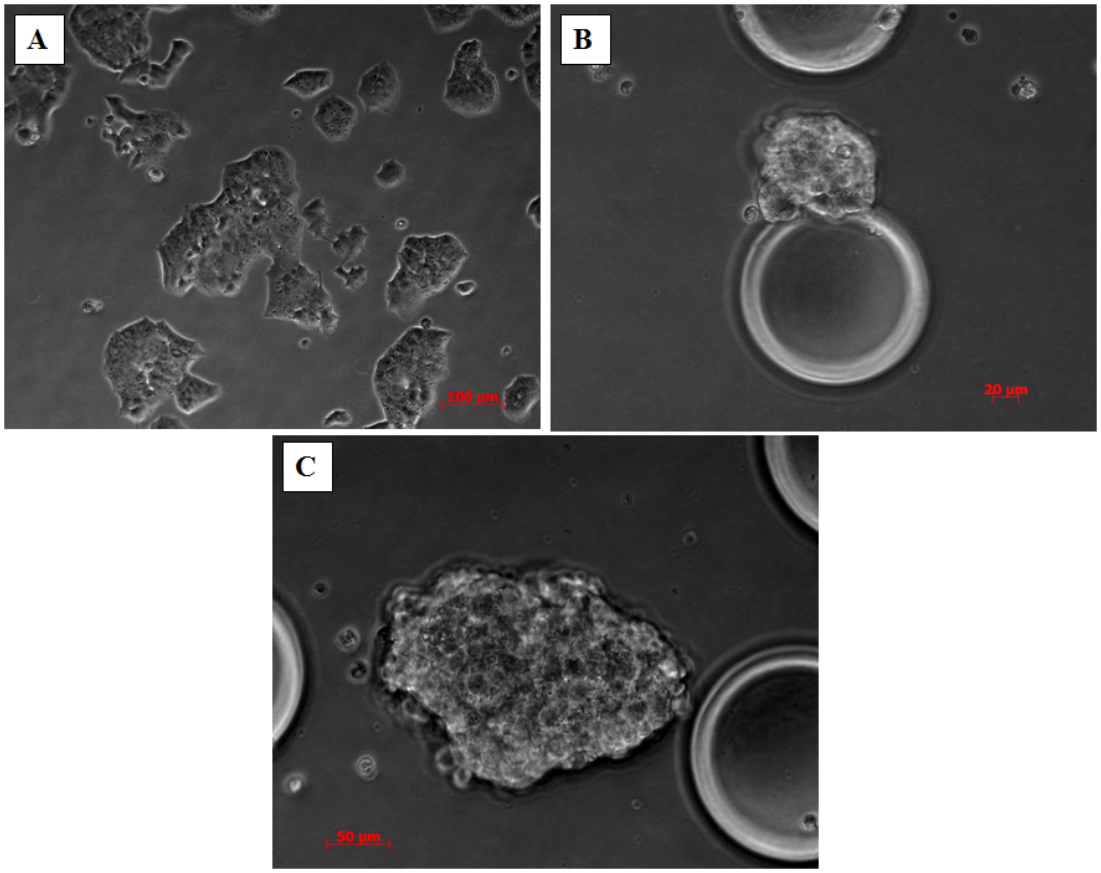

Figure 16. Morphology of β-TC6 cell when grown in monolayer versus bioreactor culture. β-TC6 cells in adherant cultures attach to the dish and form small monolayer colonies (A). In bioreactor cultures cells initially form clusters while attached to the surface of cytodex beads (B). Over time these clusters break off and form self standing structures (C). All images are day 5 of culture.

up to 200 μm. As evidenced in Figure 17, the 3-D architecture of these cell clusters resembles that of viable islets and is strikingly different than the monolayer dish architecture.

Comparison of 2-D and 3-D Transcription

Semi-quantitative PCR shows that over the course of 12 days transcription of pancreatic markers is up-regulated in bioreactor cultures compared to monolayer dish cultures. At day 5 the amounts of mRNA expressed are similar. However, by day 12 there is a trend towards increased mRNA expression in bioreactor cultures across all transcription factors studied (PDX1, NeuroD, Insulin1, Insulin2, Isl1, Pax4, Pax6, GATA4, GATA6). Also of interest is the observation that developmental markers Nestin and Ngn3, and exocrine markers Amylase and Ptf1α are expressed by day 12 in bioreactors, but not expressed in 2-D static dish cultures. Figure 18 graphically illustrates these results.

Comparison of 2-D and 3-D Translation

Western blot analysis reveals that islet proteins Glut2 and PDX-1 are expressed in greater quantities in the bioreactor cultures over 2 weeks of culture. As seen in Figure 19, at day 5 there is a ten-fold increase in PDX-1 expression in bioreactors compared to 2-D dishes. By day 7, expression is reduced in both dishes and bioreactors, but the bioreactors still express twice the amount of PDX compared with the dishes. By day 14 expression has increased again in both conditions with bioreactors having 4 times the expression. The pattern is similar for Glut2 where day 5 dishes have 1/5 the expression of bioreactors, 0.31 AU compared with 1.5 AU, respectively. By day 7 dishes increase to 0.54 AU and bioreactors decrease to 0.84 AU. Finally by day 14 dishes express Glut2 at 0.54 AU and

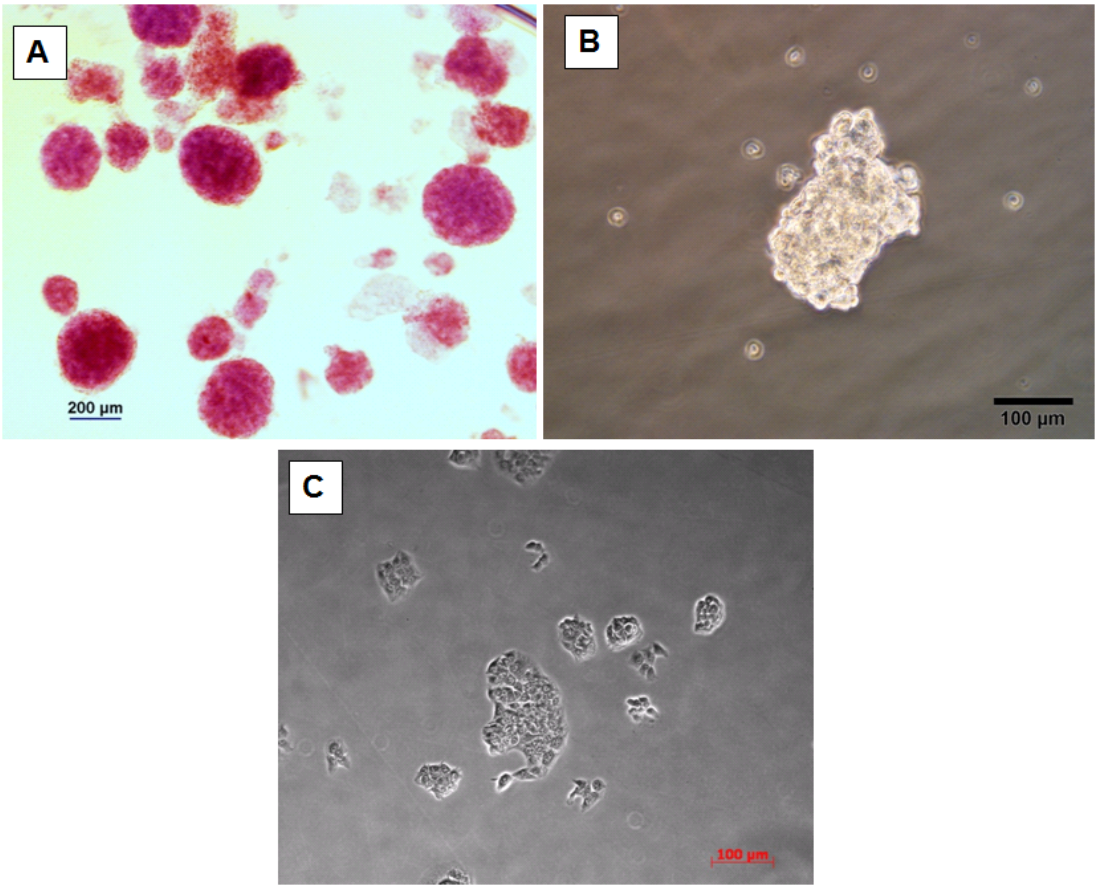

Figure 17. Cell clusters formed by β-TC6 cells in bioreactors resemble normal islets in architecture. Islets from human pancreas are stained with DTZ a common marker of islet tissue (A). Clusters of β-TC6 cells formed in 3-D bioreactor after 5 days of culture are similar to normal islets (B) while β-TC6 cells in dishes are flat and one dimensional (C). 4X (A), 20X (B), 10X (C).

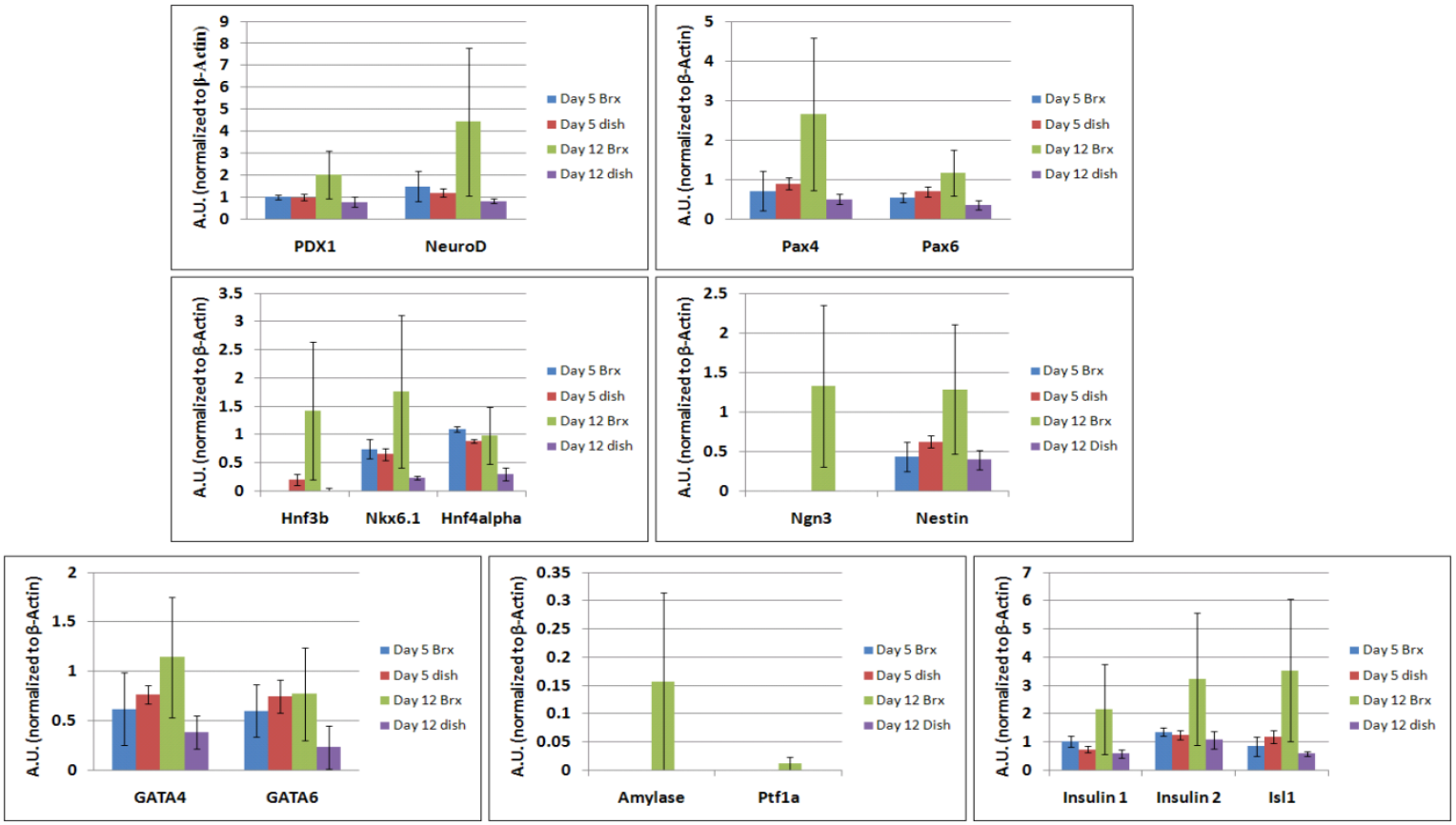

Figure 18. Semi quantitative PCR results for β-TC6 cell line in monolayer and bioreactor culture. Cells were cultured in either dish or bioreactor cultures for 5 and 12 days and RNA collected. PCR analysis was then performed to assay the amount of mRNA present for the pancreatic makers listed. Trending was toward up-regulation of expression in bioreactors by day 12. No statistical significance was observed**.** Error bars represent standard error. Arbitrary units (A.U.) are normalized for expression of control mRNA β-actin.

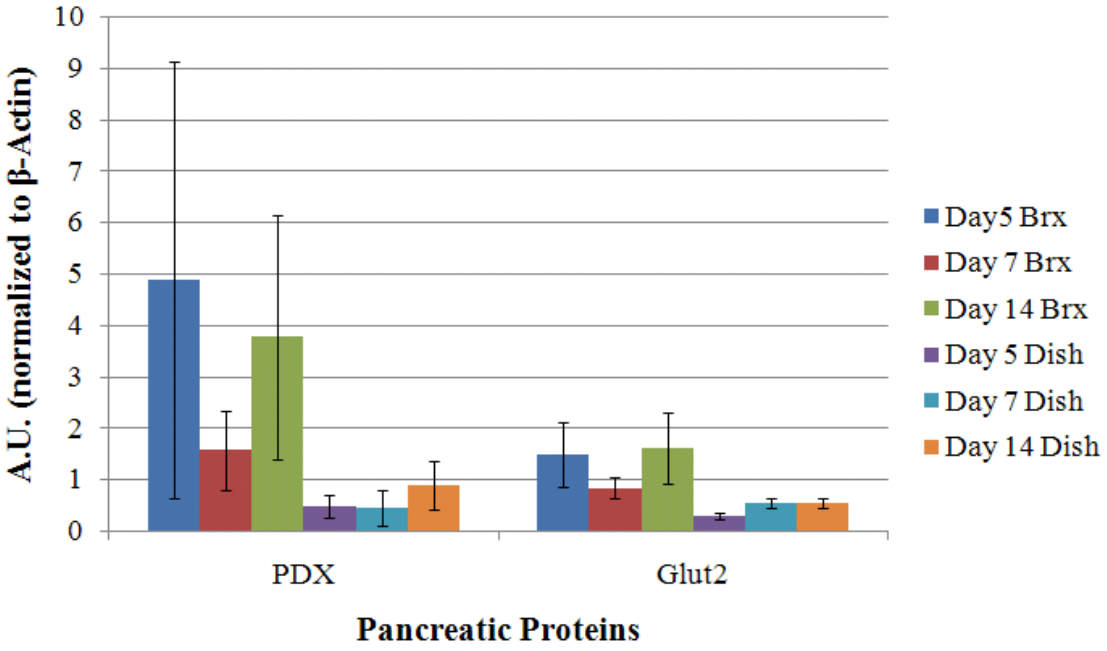

Figure 19. Comparison of protein expression from β-TC6 cells contained in culture dishes and 3-D bioreactors. Expression of both PDX-1 and Glut2 is increased in bioreactor cultures compared to dish cultures. No statistical significance was noted. Arbritraty units (A.U.) are normalized for expression of control protein β-actin. Error bars represent standard error.

bioreactors 1.61 AU. Although expression differences were different between bioreactor and dish cultures the values did not reach statistical significance.

Improved Insulin Secretion in Bioreactor

β-TC6 cell lines were cultured in standard monolayer dish cultures and 3-D bioreactor cultures. We then measured the ability of the cells to secrete insulin in response to glucose stimulation. Figure 20 shows that over the course of an hour there was only a minor response by β-TC6 cells on dishes to an increased glucose concentration; 22.1ng/ml at 30 minutes, 25.3ng/ml at 1 hour. This was surprising as cells cultured in the absence of glucose (0nM) produced higher amounts of insulin 26.9ng/ml at 30 minutes, 45.7 ng/ml at 1 hour than cells in high glucose conditions (100mM). Alternately, cells cultured in bioreactors showed a measurable increase in insulin production in high glucose conditions with 51.6ng/ml at 30 minutes, 182.5ng.ml at 1 hour. All low glucose conditions in bioreactors resulted in lower readings of insulin production 55.8ng/ml at 30 minutes, 78.4ng/ml at 1 hour.

Interim Conclusions

The bioreactor device provides a 3-D microgravity culture environment that changes proliferation and function in a cell population. The device uses technology modeled after rotating wall vessel bioreactors and allows cells to attach to microcarrier beads while being constantly rotated around a fixed point, and thus never settling to the bottom of the culture vessel. Initial experiments with Sca-1+ PPCs in the bioreactor show that the cells successfully attach to beads and are capable of proliferation and maintenance in culture. Cells initially attach to beads at single points and then begin to cover the entire bead surface. Once beads are covered cells begin to reach out and form aggregates of cells/bead combinations.

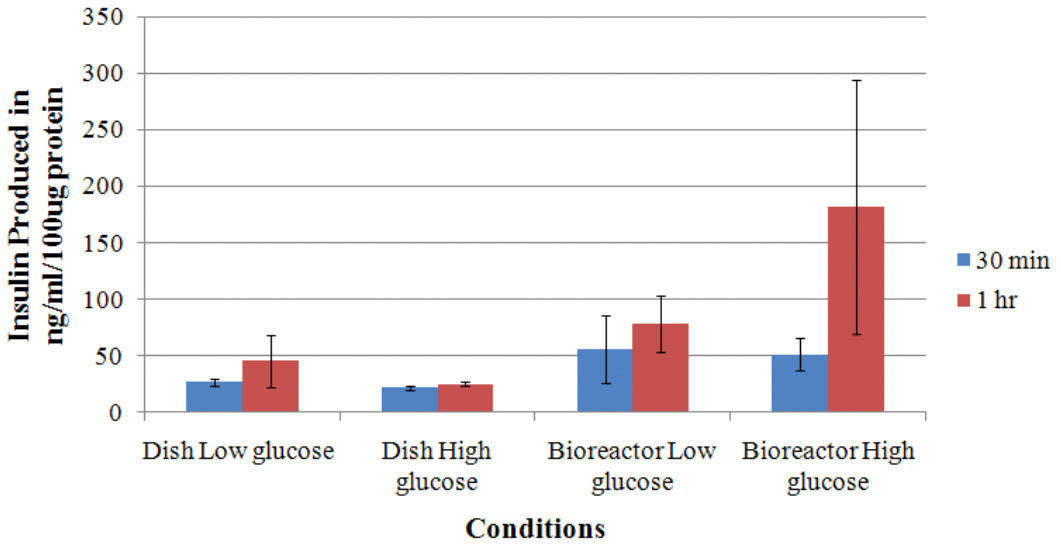

Figure 20. Bioreactors allow for increased secretion of insulin in response to glucose compared to monolayer cultures. Cells cultured in monolayer dishes produce very little insulin in response to increases in glucose concentrations while cells in 3-D cultures produce measurable amounts. Cells were stimulated with low glucose (0mM) and high glucose (100mM) concentrations for 2 hours with samples taken at 30 minutes and 1 hour. No statistical significance was observed**.** Error bars represent standard error.

When assayed for their ability to release insulin PPCs in bioreactors demonstrate function not seen in dish culture. Over the course of 2 hours the amount of insulin released steadily increased by 6 fold in response to continuing culture in high glucose surroundings.

The β-TC6 cell line was used for the majority of bioreactor experiments because of its high proliferative ability and similarities to our endocrine progenitor cell type, notably its ability to produce insulin. When placed in 3-D bioreactors the β-TC6 cells form aggregates on microcarrier beads. These aggregates then break away from the beads and form free standing structures that remain suspended in the culture medium. Aggregates are very similar to islets in their architecture and morphology. In contrast, the same cells on culture dishes form flattened layers of cells that look endothelial in nature.

Transcriptional data shows that β-TC6 cells consistently up-regulate transcription factors required for development and differentiation of the endocrine pancreas. Over the course of 12 days in culture developmental genes NeuroD, Pax4, Pax6, Hnf3β, Hnf4α, GATA4, GATA6, and Nestin were consistently expressed at higher concentrations in bioreactor cultures compared to dishes. Likewise, the developmental marker Ngn3 was not seen in dish cultures but was up-regulated in bioreactors. Differentiated cell markers Insulin1, Insulin2, and Isl1 were also increased in bioreactors after 12 days. Bioreactors also showed increased protein expression. Over the course of 14 days PDX-1 protein was expressed more robustly in bioreactors compared to dishes. While both culture conditions fluctuated in expression, with levels decreasing at day 7, by day 14 expression in bioreactors was 4 times higher than that of dishes. The same pattern was observed with Glut2 with 3 times the amount of protein expression observed at day 14.

The functional ability of the β-TC6 cell line was also enhanced in bioreactor conditions. While dishes show no response to glucose stimulation, bioreactors show a 3.5 fold increase in the amount of insulin released over a 30 minute period. Although largely different, values did not reach statistical significance.

Discussion

SUMMARY

Diabetes is currently the seventh leading cause of death in the US, the leading cause of kidney failure, lower limb amputations, and blindness, as well as a major contributor to heart disease and stroke[61]. Over 220 million people worldwide currently have diabetes[62]. Twenty-six million people (or 8.3% of the population) have diabetes in the United States alone[61]. It is predicted that by the year 2025, 333 million people worldwide will suffer from the disease[63].

Current treatment options for Type 1 diabetics do not offer strict enough control of blood sugar levels to prevent the development of the previously mentioned complications. Islet cell transplant is a clinically proven method to treat Type I diabetes but it is challenged by severe limitations in donor availability, poor long term success rate, and requirement for immunosuppression. For these reasons an alternative cell source that could provide physiologic glucose responsiveness while replacing islet transplantation could offer a cure to the approximately 2 million newly diagnosed T1D patients each year in the US alone[61].

The primary goals of this research project were (i) to identify a novel progenitor cell source from pancreas tissue, (ii) to characterize this cell population and its functional ability to produce insulin and (iii) determine whether a three dimensional culture mechanism would be beneficial in propagation of these pancreatic cells, with the ultimate goal of one day using them as a replacement for transplanted islets.

ISOLATION OF A NOVEL SCA-1+ PANCREATIC PROGENITOR CELL

There is much debate in the field of stem cell biology whether an adult derived progenitor cell exists in pancreatic tissue. Initial studies showed that new β-cells arise as a result of division of pre-existing β-cells throughout life and after pancreas injury, thus casting doubt on the idea of stem cell contributions[64]. Subsequent reports aimed to confirm this phenomenon verify that β-cells are maintained by replication of already differentiated β-cells[65-67]. Alternately, others have shown that a stem cell does in fact exist in the pancreas. Seaberg et al. reported in 2004 that pancreatic precursors could be isolated from adult mouse pancreas[23]. They were able to demonstrate that the cells are positive for many β-cell markers and capable of releasing insulin in response to glucose stimulation. However, the amount of insulin produced by these cells was much lower than what is produced by a normal β-cell. Recent reports have shown that endocrine precursors can be derived from surgically resected portions of human pancreas and that islets themselves contain a population of mesenchymal stem cells[68, 69].

We have shown that it is possible to isolate a tissue derived progenitor cell from murine pancreas and that the cell can be identified and purified by its expression of Sca-1. Sca-1 has been identified as one of the early cell surface markers of hematopoietic stem cells[70]. Other reports have found Sca-1 expressed in progenitor cells of the kidney, liver, cardiac and skeletal muscle, and mammary tissue but to our knowledge this is the first report of a Sca-1+ progenitor cell residing in murine pancreas[46, 50, 71-73]. Our population produces a large amount of insulin in vitro but the cells do not appear to be responsive to glucose stimulation. This is different than most other published findings where insulin secretion is very limited.

BIOREACTOR CULTURE FOR IMPROVED PANCREATIC PROGENITOR CELL FUNCTION

Three dimensional culture offers unlimited potential in cultivation of cell populations. Unlike static dish cultures, 3-D systems allow for more robust growth of cell populations while enhancing cell to cell interaction and cell migration. Cells have expanded surface area to differentiate and divide without the predefined basement membrane of a culture dish. Normal cell division occurs through a series of events that relies on spatial and temporal organization as well as mechanical cues, communication between cells and their matrix, and communication between individual cells, all of which are enhanced in a 3-D environment[74].

Previous research has shown that pancreatic cell lines from rat exocrine tissue are highly proliferative when placed in a rotating 3-D culture and that they are able to maintain their cell markers and potential for differentiation[43]. Porcine pancreatic tissue has also been cultured in 3-D and demonstrated the ability to form islet-like structures and secrete inslin[75]. Similarly, islets have been placed in 3-D culture devices along with co-cultures of sertoli rat cells and show preservation of physiologic function[76].

Many types of bioreactors are commercially available for medical research. One of the most popular is the stirred bioreactor which uses propeller type appendages to keep media and cells in suspension. These are particularly useful for applications such as production of microbes or vaccines because they can be built on large scale[77]. However, mechanical agitation in this type of reactor can cause cell death when they come into contact with the propellers. For this reason we chose to model our bioreactor device after a RWV. In addition our model allows the addition of a culture bag that provides efficient gas exchange. This circumvents the need to filter biologic gases through ports or other mechanical means.

Using our bioreactor a murine endocrine cell line, β-TC6, forms islet-like structures not seen in static monolayer culture. We have also demonstrated up-regulation of transcription and translation of progenitor and differentiated pancreatic markers as well as improved insulin secretion ability of this cell line when compared to dish cultures. Initial experiments with our Sca-1+ PPC population in a 3-D environment demonstrate cell stability and proliferation in culture while the cells gain the ability to respond to changes in glucose in their environment. We have shown a physiologic change in the ability to produce insulin for the β-TC6 cell line when moving it from a static 2-D culture to a dynamic 3-D bioreactor.

FUTURE DIRECTIONS

One major obstacle to overcome with the Sca-1+ PPC population is the fact that there is no ortholog for Sca-1 in humans. The Sca-1+ cells are co-localized in the peri-ductal region of the pancreas and we postulate that they may also be positive for CK19, a ductal marker in the pancreas. Recent reports have identified progenitor cells along the ductal epithelia of murine pancreas[78-80]. Further investigation into this staining may offer a key to finding and isolating these cells in other mammals.

Studies also need to be done with our Sca-1+ population to test their ability to function after transplantation into a diabetic model. We have initiated transplant studies but factors such as site and method of transplant, animal model selection, engraftment, and optimal cell number are all major issues that need to be fully elucidated to design a robust series of experiments. After we identify a reproducible transplant methodology the cells will be tested for viability and restoration of function in a disease model.

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