Research Article

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Pancreatic Islet Composites Secrete Insulin in Response to a Glucose Challenge

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ABSTRACT

This study was designed to test the hypothesis that decellularized pancreatic matrices seeded with adult-derived endogenous stem cells and donor islets provide an optimal environment for islets to secrete insulin in response to a glucose challenge. Adult animals were euthanized following the guidelines of Fort Valley State University-IACUC and Mercer University-IACUC. Adult porcine pancreases were decellularized using a mixture of detergents. Adult rat pancreatic islets were obtained by lipase digestion followed by Ficoll gradient sedimentation. Control cultures consisted of decellularized matrices, clonal populations of naïve adult totipotent and pluripotent stem cell populations, and rat islets, all cultured individually. Experimental groups consisted of islets co-cultured with clonal populations of pluripotent stem cells and totipotent stem cells seeded on decellularized matrices. Control and experimental cultures were challenged with the insulin secretagogue glucose. The control and culture media were removed and stored at -20oC until assayed using a RIA specific for rat insulin. The culture media, containing bovine insulin, were assayed using a RIA specific for rat insulin. No detectable levels of insulin (bovine, rat, human, or porcine) were noted in media only, the stem cell populations or the decellularized matrices, respectively. Native pancreatic islets secreted nanogram quantities of insulin per nanogram of DNA. Pancreatic islets co-cultured with naïve stem cells and matrices demonstrated increased insulin secretion in the range of milligram quantities of insulin per nanogram of DNA, i.e., a 250-fold increase in insulin secretion in response compared to pancreatic islets alone. These studies suggest that native islets in combination with decellularized matrices and adult-derived pluripotent and totipotent stem cells could provide more tissue for pancreatic islet transplants than donor islets alone.

Keywords
Pancreatic Islet, Insulin, Glucose

Introduction

Diabetes mellitus is a metabolic syndrome with a diversity of etiologies, clinical presentations, and outcomes. It is characterized by insulinopenia, fasting or postprandial hyperglycemia, and insulin resistance. Type 1 diabetes mellitus, referred to as juvenile or insulin-dependent diabetes mellitus (IDDM), is typically characterized by insulinopenia, hyperglycemia, and secondary

insulin resistance [1]. It is an autoimmune disease whereby CD8+ T-cells destroy the insulin-producing pancreatic β-cells [2]. Type 2 diabetes mellitus, referred to as adult onset or non-insulin-dependent diabetes mellitus (NIDDM), is characterized by hyperglycemia and varying degrees of primary insulin resistance with elevated plasma insulin concentrations, but a decreased insulin response to challenge by a secretagogue [3,4]. Diabetes mellitus need not be overt and grossly hyperglycemic to induce detrimental metabolic changes. A growing body of evidence suggests that there are detrimental consequences to normal physical challenges such as aging, which may be inherently linked to alterations in body composition. Such challenges may result in subclinical diabetogenic changes. It is becoming increasingly clear that loss of physical strength, functional status, and immune competence are related to decreases in lean body mass observed in diabetogenic states [5-7].

Walsh and colleagues in 1933 showed that the protein wasting that was present in type-I diabetes mellitus could be eliminated by administration of insulin [8]. Later studies suggested that the degree of protein wasting may be related to insulin availability and the degree of pancreatic function [9]. A single mechanism of action, which describes the effect of insulin on proteolysis or proteogenesis, remains to be clearly elucidated. Decreased lean body mass in diabetes mellitus may be due to decreased number and translational efficiency of ribosomes [10,11] and to alterations in peptide chain elongation and termination [12]. Several studies additionally suggest that these effects may be modulated in part by modifications in insulin-like growth factor I (IGF-I). Streptozotocin diabetic rats that are insulin-deficient lack IGF-I [13]. Growth retardation in diabetic infants has been ascribed to a lack of proper insulinization [14]. More recent studies suggest that protein nutrition, insulin, and growth may be modulated via IGF-I [15,16].

While temporary control of hypoglycemia, euglycemia and hyperglycemia can occur in diabetic mellitus patients with the injection of insulin, exogenous insulin administration is not equivalent to the sensitive monitoring of glucose and release of insulin or glucagon by endogenous pancreatic islets to facilitate euglycemia [17]. Unless constant glucose monitoring occurs, even tightly controlled patients are at risk for potentially fatal hypoglycemic episodes due to the inability of pancreatic islets to correct for a decrease in insulin release and an increase in glucagon release due to a decrease in circulating glucose [18]. Unregulated hypoglycemia and/or hyperglycemia can lead to long term complications including cardiovascular disease, neuropathies, nephropathies, and retinopathies [19].

Tobin et al. [20,21] demonstrated that transplantation with normal pancreatic islets of Langerhans completely restores normal body protein levels in rats. The transplantation of pancreatic islets rather than the entire pancreas has been investigated as a possible treatment for type-1 diabetes mellitus in selected patients that were unresponsive to exogenous insulin therapy [22]. The Edmonton group [23-26] reported that sufficient islet mass from as few as two pancreases, in combination with a regimen involving a glucocorticoid-free immunosuppressive protocol, engendered sustained freedom (>1 year) of insulin independence in eight of eight [24] and 12 of 12 [25,26] patients with type-1 diabetes mellitus. Their findings indicated that islet transplantation alone was associated with minimal risk and resulted in good metabolic control [24,27]. However, due to the shortage of cadaver organ donors, less than 0.5% of patients with type-1 diabetes mellitus are able to receive an islet transplant at the present time. Thus, alternative sources of insulin-secreting tissue were urgently needed [23]. Reports by Cornelius et al. [28], Ramiya et al. [26], and Bonner-Weir et al. [30] suggest that reversal of insulin-dependent diabetes mellitus can be accomplished using chemically induced islets generated in vitro from pancreatic ductal endodermal stem cells. In addition, Lumelsky et al. [31] and Rajagopal et al. [32] reported the formation of pancreatic beta cells that spontaneously differentiated from embryonic stem cells.

Young and colleagues [33,34] discovered primitive stem cell populations that were located within the connective tissue compartments of adult mammals, including humans. Six populations of primitive stem cells discovered thus far were termed totipotent stem cells (TSCs) [35], transitional-totipotent/pluripotent stem cells (Tr-T/PSCs) [34], pluripotent stem cells (PSCs) [36,37], mesodermal stem cells [38], ectodermal stem cells, and endodermal stem cells [36,37]. A pluripotent stem cell clone was derived from male outbred Sprague-Dawley rats by single cell by repetitive limiting serial dilution clonogenic analysis. Scl-4β could be induced to form 63 objectively verifiable cell types across all three germ layer lineages after incubation in a general induction medium [33,36,37]. A transitional-totipotent/pluripotent stem cell clone, Scl-40β, derived from a single cell by repetitive limiting serial dilution clonogenic analysis could be induced to form a minimum of 64 objectively verifiable cell types across all three germ layer lineages after incubation in a general induction medium [33,34]. Totipotent stem cell clones, Scl-9β and Scl-44β, were derived from male outbred Sprague-Dawley rats by single cell by repetitive limiting serial dilution clonogenic analysis. The totipotent stem cell clones could be induced to form 66 objectively verifiable cell types after incubation in a general induction medium [33,39]. Pluripotent stem cells were noted to be 6-8 microns in size [36,37], totipotent stem cells were noted to be less than two microns in size [35]; and the transitional-totipotent/pluripotent stem cells were noted to be 3-5 microns in size [34,35]. These primitive adult-derived stem cells demonstrated other unique qualities that set them aside from other more differentiated adult stem cell populations (Table 1) [33-37].

Based on the studies of Lumelsky et al. [31] and Rajagopal et al. [32] in which embryonic stem cells were allowed to spontaneously differentiate in vitro into insulin secreting pancreatic β-cells, Young and Black [40] began a series of preliminary studies to ascertain the ability of adult-derived pluripotent stem cells to be induced de novo into pancreatic-like structures as a method to supplement existing pancreatic islet tissue for transplant. Beginning with a clonal population of adult-derived pluripotent stem cells these

Charac.		DC[1]	PC[2]	GLSC[3]	PSC[4]	Tr-T/PSC[5]	TSC[6]
Size:							
Rat (R)		Var[7]	Var	8-10 µm	6-8 µm	3-5 µm	0.1-2.0 µm
Human (H)		Var	Var	8-10 µm	6-8 µm	3-5 µm	0.1-2.0 µm
Viability P-Mortem[8]	R	6-12 hr	24 hr	3 days	7 days	14+ days	30+ days
	H	6-12 hr	24hr	3 days	7 days	14+ days	30+ days
Presence							
in STs[9]		Yes	Yes	Yes	Yes	Yes	Yes
in CTs[10]		Yes	Yes	Yes	Yes	Yes	Yes
in BM[11]		Yes	Yes	Yes	Yes	Yes	Yes
in Bld[12]		Yes	Yes	Yes	Yes	Yes	Yes
Serum-Free Medium		Qui[13]	Qui	Qui	Qui	Qui	Qui
Commitment		NA[14]	DC	PC	GLSC	PSC	Tr-T/PSC
Tel'ase[15]		Absent	Absent	Present	Present	Present	Present
Trypan Blue	R	neg	neg	neg	neg	pos/neg	positive
	H	neg	neg	neg	neg	pos/neg	positive
PD[16]	R	8-10	8-10	Extensive	Extensive	Extensive	Extensive
	H	50-70	50-70	Extensive	Extensive	Extensive	Extensive
PR[17]	R	D-W[18]	D-W	18-24 hr	12-14 hr	12-14 hr	12-14 hr
	H	D-W	D-W	18-24 hr	12-14 hr	12-14 hr	12-14 hr
CD[19]	R	NYD[20]	NYD	> 400	> 300	> 100	> 300
	H	50	NYD	> 690	> 400	> 100	> 300
CD after Commit to Cell Lineage	R	NA	8-10	8-10	8-10	8-10	8-10
	H	NA	50-70	50-70	50-70	50-70	50-70
CI-C[21]	R	Yes	Yes	Yes	No	No	No
	H	Yes	Yes	Yes	No	No	No
GT-1C[22]	R	Yes	Yes	Yes	Yes	Yes	Yes
	H	Yes	Yes	Yes	Yes	Yes	Yes
G-S[23]	R	No	No	No	No	No	0.1-1.0 µm Yes
	R						1.0-2.0 µm No
G-S	H	No	No	No	No	No	0.1-1.0 µm Yes
	H						1.0-2.0 µm No
R-Prolif[24]	R	Yes	Yes	Yes	Yes	Yes	Yes
	H	Yes	Yes	Yes	Yes	Yes	Yes
R-Prog[25]	R	NA	Yes	No	No	No	No
	H	NA	Yes	No	No	No	No
R-Induc[26]	R	NA	No	Yes	Yes	Yes	Yes
	H	NA	No	Yes	Yes	Yes	Yes
R-Inhib[27]	R	NA	Yes	Yes	Yes	Yes	Yes
	H	NA	Yes	Yes	Yes	Yes	Yes
#Cs-Fr[28]		10^6	10^6-7	10^6-7	10^6-7	10^6-7	10^9-10
Freeze Agent: [29]		Liquid Nitrogen	Liquid Nitrogen	Ultra-Pure DMSO[30]	Ultra-Pure DMSO	Ultra-Pure DMSO	Ultra-Pure DMSO
Temp[31]	R	-196C	-196C	-70C	-80C	-80C	-80C
	H	-196C	-196C	-70C	-80C	-80C	-80C
FrProc[32]	R	Flash	Flash	Slow	Slow	Slow	Slow
	H	Flash	Flash	Slow	Slow	Slow	Slow
ThwProc[33]	R	Flash 37C	Flash 37C	Flash 37C	Flash 37C	Flash 37C	Flash 37C
	H	Flash 37C	Flash 37C	Flash 37C	Flash 37C	Flash 37C	Flash 37C
Recov[34]	R	> 90%	> 95%	> 98%	> 98%	> 98%	> 98%
	H	> 95%	> 95%	> 98%	> 98%	> 98%	> 98%
RC-1C[35]	R	NYD	NYD	A2A2	Scl-4β	Scl-40β	Scl-44β, Scl-9β
	H	NYD	NYD	NYD	NYD	NYD	NYD
CsFmd[36]	R	NA	DC	PC	GLSC	ELSC	CLSC
	H	NA	DC	PC	GLSC	ELSC	CLSC
Genes Expressed		NYD	NYD	Telom	Telom Oct-4 Sonic-hh	Telom Oct-4 Sonic-hh	Telom Bcl-2 Nanog Nanos CXCR-4
CD-Ms[37]	H	Cell Spec	Cell Spec	CD13+, CD56+, CD90+, MCH Class-1+, CD1a-, CD2-, CD3-, CD4-, CD5-, CD7-, CD8-, CD9-, CD10-, CD11b-, CD11c-, CD14-, CD15-, CD16-, CD18-, CD19-, CD20-, CD23-, CD24-, CD25-, CD31-, CD33-, CD34+/-, CD36-, CD38-, CD41-, CD42b-, CD45-, CD49d-, CD55- CD57-, CD59-, CD61-, CD62e-, CD65-, CD66-, CD68-, CD71-, CD79-, CD83-, CD95-, CD105-, CD117-, CD123-, CD135-, CD166-, GlycophorinA-, HLA-DR-II-, FMC-7-, Annexin-V-, Lin-	CD10+, CD1a-, CD2-, CD3-, CD4-, CD5-, CD7-, CD8-, CD9-, CD11b-, CD11c-, CD13-, CD14-, CD15-, CD16-, CD18-, CD19-, CD20-, CD22-, CD23-, CD24-, CD25-, CD31-, CD33-, CD34-, CD36-, CD38-, CD41-, CD42b-, CD45-, CD49d-, CD55-, CD56-, CD57-, CD59-, CD61-, CD62e-, CD65-, CD66-, CD68-, CD71-, CD79-, CD83-, CD90- CD95-, CD105-, CD117-, CD123-, CD135-, CD166-, GlycophorinA-, MHC-I-, HLA-DR-II-, FMC-7-, Annexin-, Lin-	CD10+, CD66e+, CD1a-, CD2- CD3-, CD4-, CD5-, CD7-, CD8-, CD9-, CD10-, CD11b-, CD11c-, CD13-, CD14-, CD15-, CD16-, CD18-, CD19-, CD20-, CD22-, CD23-, CD24-, CD25-, CD31-, CD33-, CD34-, CD36-, CD38-, CD41-, CD42b-, CD45-, CD49d-, CD55-, CD56-, CD57-, CD59-, CD61-, CD62e-, CD65-, CD68-, CD71-, CD79-, CD83-, CD90-, CD95-, CD105-, CD117-, CD123-, CD135-, CD166-, GlycophorinA-, MHC-I-, HLA-DR-II-, FMC-7-, Annexin-, Lin-	CD66e+, CD1a-, CD2- CD3-, CD4-, CD5-, CD7-, CD8-, CD9-, CD10-, CD11b-, CD11c-, CD13-, CD14-, CD15-, CD16-, CD18-, CD19-, CD20-, CD22-, CD23-, CD24-, CD25-, CD31-, CD33-, CD34-, CD36-, CD38-, CD41-, CD42b-, CD45-, CD49d-, CD55-, CD56-, CD57-, CD59-, CD61-, CD62e-, CD65-, CD68-, CD71-, CD79-, CD83-, CD90-, CD95-, CD105-, CD117-, CD123-, CD135-, CD166-, GlycophorinA-, MHC-I-, HLA-DR-II-, FMC-7-, Annexin-, Lin-

Table 1: Characteristics of Differentiated Cells, Progenitor Cells and Stem Cells.

1. DC, differentiated cells, 2. PC, progenitor cells, 3. GLSC, germ layer lineage stem cell, 4. PSC, pluripotent stem cell, 5. Tr-T/PSC, transitional-totipotent/pluripotent stem cell, 6. TSC, totipotent stem cell, 7. Var, variable, 8. Viabil P. Mortem, viability of tissue post mortem (after removal from the body) stored at 4oC, 9. STs, solid tissues, 10. CTs, connective tissues, 11. BM, bone marrow, 12. Bld, blood, 13. Qui, quiescence, 14. NA, not applicable, 15. Tel'ase, telomerase, 16. PD, population doublings, 17. PR, proliferation rate, 18. D-W, days to weeks, 19. CD, cell doublings, 20. NYD, not yet determined, 21. CI-C, cell inhibition at confluence, 22. GT-1C, growth on type-1 collagen substrate, 23. G-S, growth in cell suspension cultures, 24. R-Prolif, response to proliferation factors, 25. R-Prog, response to progression agents, 26. R-Induc, response to induction factors, 27. R-Inhib, response to inhibitory factors, 28. #Cs-Fr, number of cells frozen, 29. Freeze Agent, cryopreservation agent, 30. DMSO, dimethylsulfoxide, 99.99% pure, 31. Temp, optimum freezing temperature in centigrade, 32. FrProc, freezing process, 33. ThwProc, thawing process, 34. Recov, % recovery from freezing process, 35. RC-1C, rat clones derived from one cell, 36. CsFmd, cells formed, 37. CD-Ms, cluster of differentiation (CD) markers.

investigators utilized three separate and selective inductive cocktails to systematically induce the pluripotent stem cells to form endodermal stem cells then to form pancreatic progenitor cells and finally to form three-dimensional pancreatic islet-like structures that stained for insulin, glucagon, and somatostatin.

Following the protocol of Lumelsky et al. [31] for a sequential glucose challenge, except using a Rat-specific insulin radioimmunoassay (RIA) instead of the non-specific insulin-RIA as used by Lumelsky [31], Young and Black [40] demonstrated that the three-dimensional structures, generated de novo from adult-pluripotent stem cells, secreted one-quarter to one-half the amount of insulin secreted by native islets. This was in contrast to de novo-generated pancreatic progenitor cells that secreted one-half to 100% of the insulin secreted by native islets.

These results contrasted with those of Lumelsky et al. [31] and Rajagopal et al. [32] in which they demonstrated that pancreatic

beta cells derived from embryonic stem cells would produce 1/20th (5%) of the amount of insulin as compared to native islets. Their suggestion was to implant more embryonic stem cell-derived beta-cells to increase the amount of insulin output from the transplant.

The procedure developed by Young and Black [40] to generate pancreatic islet-like structures de novo utilized three induction cocktails, i.e., conditioned media from endodermal stem cells to convert pluripotent stem cells to endodermal stem cells; conditioned medium from pancreatic progenitor cells to convert endodermal stem cells to pancreatic progenitor cells; and a defined cocktail of known pancreatic islet inductive agents [30] to convert pancreatic progenitor cells into pancreatic islet-like structures consisting of glucagon secreting alpha-cells, insulin secreting beta-cells, and somatostatin secreting delta-cells. Unfortunately, due to the conditioned media containing quantities of unknown animal proteins, the procedure was deemed problematic as a basis for therapy in humans. To avoid the use of conditioned media, the current study utilized the co-culture of (recipient, autologous) adult pluripotent stem cells and adult totipotent stem cells with (donor, allogeneic) adult native islets on a decellularized porcine pancreatic matrix. The resultant cultures were challenged sequentially with glucose to determine their respective insulin response.

Materials and Methods

Animal Use

The porcine experiments followed the guidelines of Fort Valley State University's IACUC. The rodent experiments followed the guidelines of Mercer University's IACUC. Their respective IACUC guidelines reflect the criteria for humane animal care of the National Research Council as outlined in "Guide for the Care and Use of Laboratory Animals" prepared by the Institute of Laboratory Animal Resources and published by the National Institutes of Health (National Academy Press, 1996).

Pancreatic Tissue Harvest

Porcine

Two porcine experiments were conducted simultaneously. One experiment [42] examined the effect of trauma on the appearance of pluripotent and totipotent stem cells within the peripheral vasculature. The second experiment, reported herein, describes the use of xenogeneic decellularized native porcine pancreatic extracellular matrix (ECM) as a scaffold for endogenous pluripotent and totipotent stem cells co-cultured with donor islets creating composite pancreatic islet organoids as a potential treatment modality for insulin-dependent diabetes. The combined procedure is as follows.

Twenty adult 120 lb. female Yorkshire pigs (n=20) were anesthetized with tiletamine and zolazepam, then prepared for surgery with a Betadine wash and draped in a sterile fashion. A 10-mL pre-trauma blood sample was obtained from the jugular vein. The blood was collected in a 10-ml EDTA hemovac tube (Becton-Dickinson), inverted several times to mix and placed on ice until further processing to isolate endogenous stem cells within the peripheral blood [41]. A midline laparotomy incision was performed. A piece of the right rectus abdominis muscle was obtained (pre-trauma) and placed into ice-cold transport solution (Moraga Biotechnology Corp., Los Angeles. CA). The spleen was isolated and resected. The abdominal aorta was isolated above the celiac trunk and below the renal arteries for insertion of a vascular cannula for infusion of ice cold Hank's buffered salt solution (Grand Island Biological Company [GIBCO], Grand Island, NY), pH 7.4. The pancreas was then resected, placed into ice cold Hank's buffered salt solution and placed on ice for further processing. Isolation and resection of both the spleen and pancreas represented the trauma model to ascertain whether massive trauma would initiate release of pluripotent stem cells and totipotent stem cells into the peripheral vasculature [41]. A piece of left rectus abdominis muscle was obtained (post-trauma) and placed into ice-cold transport solution. A 10-mL post-trauma blood sample was obtained from the jugular vein and processed similarly as the pre-trauma blood sample. The pigs were euthanized by anesthetic overdose. The time period from pre-trauma blood draw to post-trauma blood draw approximated 90 minutes.

Rodent

For pancreatic islet tissue, 200 × 150 mm pancreatic islet equivalent units were isolated from pancreases taken from ~300g breeding age adult male Wistar Furth rats (n = 8) following the procedure described by Tobin and colleagues [13,20,21]. The rats were anesthetized with ketamine/Xylazine, then prepared for surgery with a Betadine wash and draped in a sterile fashion. A midline laparotomy incision was performed. A cannula was placed into the pancreatic duct and the pancreas infused with ice cold Hank's Buffered Salts Solution (GIBCO), pH 7.4. The pancreases were then resected and the rats euthanized by anesthetic overdose [40].

Decellularization of Porcine Pancreas

The porcine pancreases were divided and placed into either sterile Decellularization Solution-A (Moraga Biotechnology Corporation, Inc., Los Angeles, CA) or sterile Decellularization Solution-B (Moraga) on a tissue rotator at 4oC for 3.5 months. Sterile solutions were changed every two weeks to assist in the decellularization process. The decellularized matrices were removed from the solutions and washed with medium to ensure that the decellularization solutions were removed from the matrices. Each wash solution was assessed with a cytotoxicity proliferation assay for primitive stem cell viability. It was noted that 11 wash solutions were necessary to rid the matrices of decellularization solutions for cell viability to equal the viability seen with pristine wash solution. After decellularization in solutions A and B, the matrices were completely denuded of cells and composed of both thick and thin filaments.

Isolation of Rat Pancreatic Islets

Adult rat pancreatic islets were isolated as previously described [13,20,21,40]. Adult rat pancreases were minced. The tissue was weighed and digested with the enzyme liberase. Islets were separated from the remaining pancreatic material by Ficoll gradient centrifugation utilizing a layered solution of 25%, 23%, 20%, and 11%, Ficoll. Pancreatic islets used for human transplant studies

appear as a band of cells between the 20% and 23% gradients of Ficoll. Aliquots from the band between 11% and 20%, between 20% and 23%, between 23% and 25%, and the cell pellet were stained with antibodies to pro-insulin, SSEA, and CEA-CAM-1 to confirm the identity of the isolated tissue, and we noted something peculiar. Pancreatic islets were located both in the band between 20% and 23% Ficoll and in the cell pellet. In addition, besides the pancreatic islets in each of these two locations there were also SSEA+ pluripotent stem cells and CEA-CAM-1+ totipotent stem cells. The heaviest concentration of SSEA+ stem cells, CEA-CAM-1+ stem cells and pancreatic islets was located in the cell pellet rather than in the band between 20% and 23% Ficoll. We therefore used the material from the cell pellet for the composite pancreatic organoids to maximize our chances for optimizing insulin release.

Adult-Derived Pluripotent Stem Cells and Totipotent Stem Cells

Two adult outbred Sprague-Dawley rat-derived stem cell clones were utilized for this study, Scl-40β [36,37] and Scl-44β [35]. Based on the distinctive characteristics of each stem cell clone [33] (Table 1), including staining with antibodies selective for pluripotent stem cells (SSEA) and totipotent stem cells (CEA-CAM-1), Scl-40β (CEA-CAM-1+ / SSEA+) was designated as an adult-derived transitional-totipotent/pluripotent stem cell clone and Scl-44β (CEA-CAM-1+ only) was designated as an adult-derived totipotent stem cell clone. Both clones, Tr-T/PSC-Scl-40β and TSC-Scl-44β, were utilized in combination in an attempt to obtain the maximal beta-cell generation and thus insulin response possible.

Parameters for Glucose Challenge Study

Fifteen parameters were examined for the glucose challenge study: 1) Equine-derived type-I collagen matrix only (our standard substratum for the growth of endogenous stem cells); 2) decellularized porcine pancreatic matrix-A only; 3) decellularized porcine pancreatic matrix-B only; 4) equine-derived type-I collagen matrix + Tr-T/PSC-Scl-40β; 5) equine-derived type-I collagen matrix + TSC-Scl-44β; 6) equine-derived type-I collagen matrix + Tr-T/PSC-Scl-40β + TSC-Scl-44β; 7) equine-derived type-I collagen matrix + native rat pancreatic islets + native SSEA+/CEA-CAM-1+ cells; 8) porcine pancreatic matrix-A + Tr-T/PSC-Scl-40β; 9) porcine pancreatic matrix-A + TSC-Scl-44β; 10) porcine pancreatic matrix-A + Tr-T/PSC-Scl-40β + TSC-Scl-44β; 11) porcine pancreatic matrix-B + Tr-T/PSC-Scl-40β; 12) porcine pancreatic matrix-B + TSC-Scl-44β; 13) porcine pancreatic matrix-B + Tr-T/PSC-Scl-40β + TSC-Scl-44β; 14) porcine pancreatic matrix-A + Tr-T/PSC-Scl-40β + TSC-Scl-44β + native rat pancreatic islets + native SSEA+/CEA-CAM-1+ cells; 15) porcine pancreatic matrix-B + Tr-T/PSC-Scl-40β + TSC-Scl-44β + native rat pancreatic islets + native SSEA+/CEA-CAM-1+ cells.

Equine-derived type-I collagen matrix and decellularized porcine extracellular matrices A & B were seeded with the TSC-Scl-44β and Tr-T/PSC-Scl-40β clones, both singly and in combination, and grown for seven days to allow integration of the clonal stem cells with the porcine matrices. All 15 parameters examined were incubated in 24-well plates in general culture medium for three days. The 15 parameters were challenged sequentially with glucose following the protocol developed by Lumelsky et al. [31] and practiced by Young and Black [40]. In brief, the general culture medium was removed. The 15 parameters examined were washed with Dulbecco's Phosphate Buffered Saline (DPBS), and incubated with medium containing 5 mM glucose for 24 hours. After 24 hours, the first neutral medium was removed. The 15 parameters were washed with DPBS, and then incubated with medium containing 5 mM glucose for one hour. After one hour, the second neutral medium was removed. The 15 parameters were washed with DPBS, and then incubated with medium containing 25 mM glucose for one hour. After one hour the third medium, the challenge medium, was removed. Their respective neutral and challenge media were removed and assayed using a rat-specific insulin-radioimmunoassay (insulin-RIA) with positive and negative controls. The 15 parameters were washed with DPBS and returned to the normal culture medium. Normal culture medium was replaced every other day. The glucose challenge reported herein was performed 3 days after initial seeding with the rat pancreatic islets. However, only the combinatorial constructs (i.e., porcine ECM + TSC & Tr-T/PSC + pancreatic islets) survived past seven days in culture. The native rat islets on type-I collagen matrix began to disintegrate after the first glucose challenge and had completely disintegrated by 7 days in culture.

Insulin-RIAs

The amount of secreted insulin was determined by double antibody competitive binding radioimmunoassay using rat insulin standards and antibodies raised against rat-specific insulin (Linco, St. Louis) [40]. A series of positive and negative controls was performed to ensure that the RIA measured only rat insulin secreted into the medium and not bovine insulin taken up and subsequently released by the cells [32] or potentially porcine insulin from the decellularized porcine pancreatic matrices. The positive controls consisted of a concentration range of rat insulin standards included with the rat-specific RIA kit. The negative controls consisted of serum-free defined BLSC medium (Moraga Biotechnology Corporation, Los Angeles, CA) with and without the insulin secretagogues in a cell-free system. Because our glucose challenge media also contained a small amount of bovine insulin, its presence was monitored using the same concentration range (0.1 to 10 ng/ml bovine insulin) as the rat insulin standards in the rat-specific RIA kit. Because our derived pancreatic organoids contained a porcine pancreatic matrix, the presence of porcine insulin was monitored using the same concentration range (0.1 to 10 ng/ml porcine insulin) as the rat insulin standards in the rat-specific RIA kit. No insulin was detected in any of the negative controls analyzed. DNA analysis was performed in each well of the tissue culture plate to standardize the amount of insulin released [40,42].

Cryosectioned Naive Pancreatic Islets

Breeding age outbred adult Sprague-Dawley rats were anesthetized with ketamine/xylazine, then prepared for surgery with a Betadine

wash and draped in a sterile fashion. A midline laparotomy incision was performed. A cannula was placed into the pancreatic duct and the pancreas infused with ice cold Hank's Buffered Salts Solution (GIBCO), pH 7.4. The pancreas was then resected and the rats were euthanized [40]. The resected pancreases were then placed in ELICA fixative (Moraga Biotechnology Corporation, Los Angeles, CA). The tissue was fixed for 2 weeks and then rinsed with GIBCO's Dulbecco's Phosphate Buffered Saline (DPBS) (Invitrogen Corporation, Carlsbad, CA).

The pancreases were embedded in Tissue Tek OCT Compound 4583 (Miles Laboratory, Ames Division, Elkhart, IN) and then frozen at -20oC. The tissue was then sectioned on a Tissue Tek Cryostat II (GMI, Ramsey, MN) to a thickness of 7 microns. The sections were applied to positively charged slides (Mercedes Medical, Sarasota, FL) and stored at -20oC until stained.

The tissue sections were stained with the CEA-CAM-1 antibody for totipotent stem cells and the SSEA-4 antibody (Developmental Studies Hybridoma Bank, Iowa City, IA) for pluripotent stem cells. We used 1A4 (Sigma, St Louis, MO) as a positive procedural control to stain smooth muscle alpha-actin in the tunica media of the blood vessels within the pancreas. Negative procedural controls consisted of either PBS only, no primary antibody, no secondary antibody (Rat Adsorbed Biotinylated Anti-Mouse IgG, Vector Laboratories Inc., Burlingame, CA) or no tertiary probe (avidin HRP, Vector). The substrate used was AEC (Sigma). Five percent sodium azide (Sigma) and 30% hydrogen peroxide (Sigma) were used as blocking agents to inhibit endogenous peroxidases. The slides were removed from -20oC storage, allowed to come to ambient temperature and fixed with 95% Ethanol for 30 minutes and rinsed with PBS. The slides were then blocked in sodium azide and hydrogen peroxide for a total of 90 minutes and rinsed with PBS. The slides were next incubated with the primary antibodies for 60 minutes and rinsed with PBS. The slides were then incubated with the secondary antibody for 60 minutes and rinsed with PBS. The slides were next incubated with the tertiary probe for 60 minutes and rinsed with PBS several times. The substrate was applied and allowed to incubate for 60 minutes. It was then rinsed off and the slide was coverslipped using VectaMount (Vector).

The slides were photographed using a COOLPIX 995 digital camera and a Nikon Phase Contrast Fluorophot microscope. The digital photographs were cropped using Adobe Photoshop 7.0.

Results

Porcine pancreases prior to decellularization consisted of cells and extracellular matrices (Figure 1). The decellularized matrices were removed from the detergent solutions and washed 11 times with medium to ensure that the cytotoxic decellularization solutions were removed from the matrices, as assessed by a cytotoxicity proliferation assay of primitive stem cells incubated in the wash solutions (Figure 2). After decellularization in solutions A and B, the matrices were completely denuded of cells and composed of both thick and thin filaments (Figure 3).

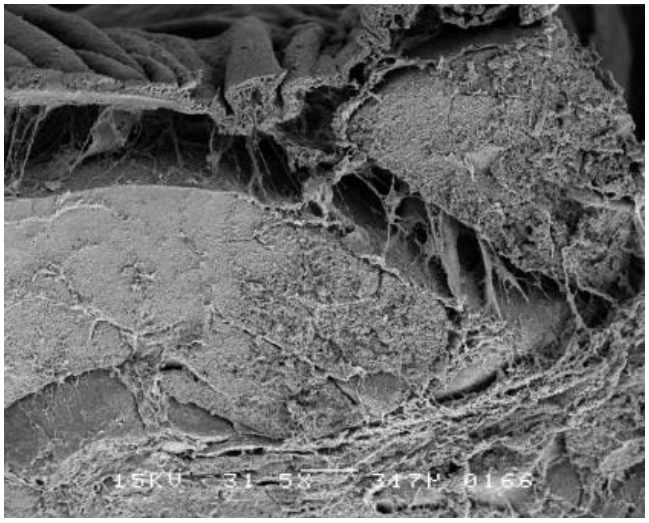

Figure 1: Scanning electron micrograph (SEM) of a cut section of native porcine pancreas before immersion into a decellularization solution.

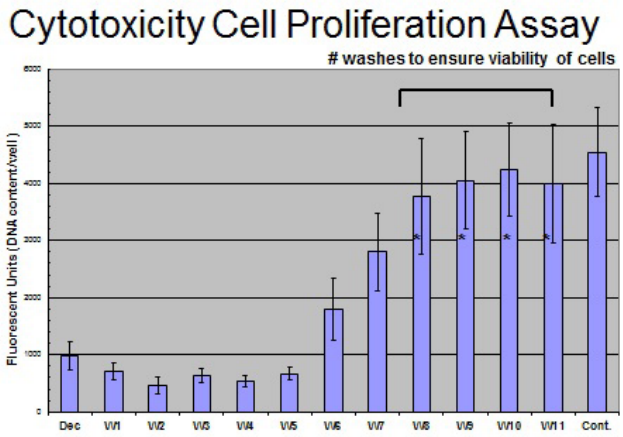

Figure 2: Cytotoxicity cell proliferation assay. Cultures of BLSCs and ELSCs were plated, allowed to attach for 24 hours, washed with buffer, and then incubated with either naïve detergent (Dec), with culture medium incubated with decellularized pancreatic matrix and changed every 24 hours (W1-W11), or with naïve culture medium (Cont.). Solutions were replaced every other day. After 14 days, the cultures were assayed for total DNA content using standard fluorometric procedures (Young et al., 1992). Statistical analysis demonstrated that 8 to 11 media changes (asterisk) were necessary to remove the cytotoxic detergents from the decellularized matrices to allow cell viability.

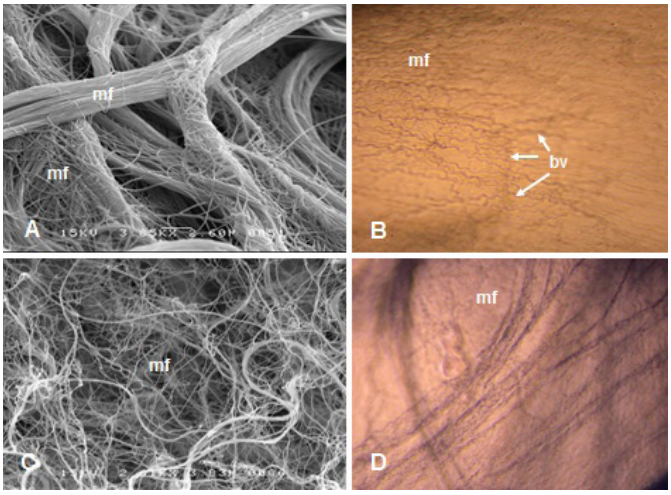

Figure 3: Decellularized pancreatic matrices. Scanning electron microscopy of pancreatic matrices after decellularization with detergent-A (3A) and detergent-B (3C). Note both thick (type-I collagen) and thin (glycoprotein) matrix filaments (mf). Bright field microscopy of pancreatic matrices after decellularization with detergent-A (3B) and detergent-B (3D). Note presence of blood vessels (bv) and both thick and thin matrix filaments (mf).

Ficoll gradient separation of pancreatic isolates noted islets present in the interface between 20% and 23% Ficoll. In contrast, the heaviest concentration of the primitive stem cells and the pancreatic islets were located in the cell pellet rather than in the band between 20% and 23% Ficoll. We therefore used the material from the cell pellet for this study to maximize our chances for optimizing insulin release (Figure 4).

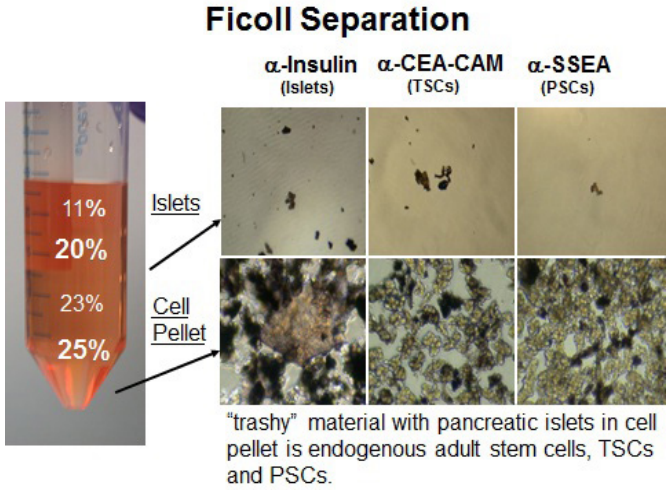

Figure 4: Ficoll gradient separation of native pancreatic islets from adult sexually mature ~220g Wistar-Furth rats. After liberase digestion the wash material was loaded onto a Ficoll density gradient (11%, 20%, 23%, and 25%) and centrifuged. The fraction between 20% and 23% and the cell pellet below 25% Ficoll were stained with antibodies to pro-insulin to identify islets containing insulin secreting beta cells, carcinoembryonic antigen-cell adhesion molecule-1 (CEA-CAM-1) to identify totipotent stem cells (TSCs), and stage specific embryonic antigen (SSEA) to identify pluripotent stem cells (PSCs). Note that the "trashy" material (DAB substrate, black-stained material) within the cell pellet contains insulin secreting islets (column 1), totipotent stem cells (column 2), and pluripotent stem cells (column 3).

The glucose challenge reported herein was performed 3 days after the original plating. However, only the combinatorial constructs survived past seven days in culture (Figures 5 and 6) and actually increased in size with continued culture. The native rat islets on type-I collagen matrix began to disintegrate after the first glucose challenge (Figure 7) and had completely disintegrated by 7 days in culture.

Twelve of 15 parameters examined demonstrated absence of rat insulin after the glucose challenge. Only three parameters, i.e., 7) equine-derived type-I collagen matrix + native rat pancreatic islets + native SSEA+/CEA-CAM-1+ cells, 14) porcine pancreatic matrix-A + PSC-Scl-40β + TSC-Scl-44β + native rat pancreatic islets, and 15) porcine pancreatic matrix-B + Tr-T/PSC-Scl-40β + TSC-Scl-44β + native rat pancreatic islets, demonstrated measurable quantities of secreted rat insulin after the glucose challenge. Indeed, the combinatorial structures, i.e., decellularized porcine pancreatic matrices, Scl-40β clone of stem cells, Scl-44β clone of stem cells, and the pelleted material containing native islets and associated endogenous pluripotent and totipotent stem cells, demonstrated a statistically significant increase (denoted by the presence of asterisks) in insulin secretion normalized to DNA content after a glucose secretagogue challenge, compared to native islets with associated endogenous pluripotent and totipotent stem cells on type-I collagen matrix alone (Figure 8).

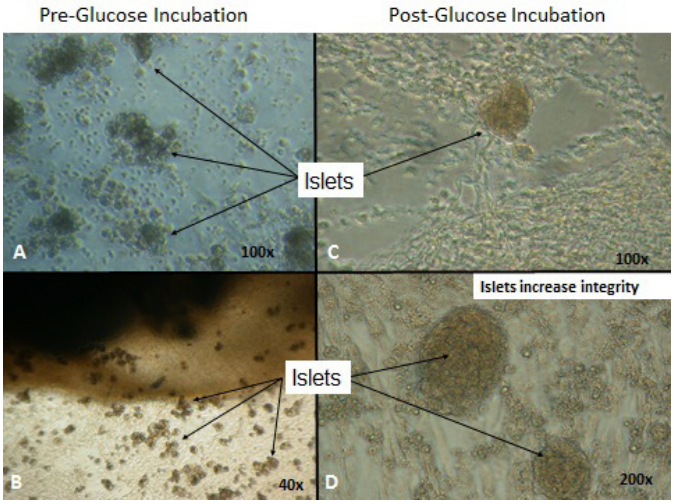

Figure 5: Isolated rat pancreatic islets with endogenous TSC and PSC from cell pellet after Ficoll density gradient centrifugation. Cell pellets were grown on the TSC-PSC-pancreatic matrix-A constructs utilizing the standard TSC-based culture medium. A & B are rat islets 48 hours after plating. C & D are rat islets 72 – 96 hours after plating. Note that islets in C & D are increasing their three-dimensional integrity. These composites consisting of decellularized pancreatic matrix-A, Scl-44β TSCs, Scl-40β PSCs, and Ficoll cell pellet material demonstrated an increase in their integrity throughout 30 days in culture, before termination of the experiment.

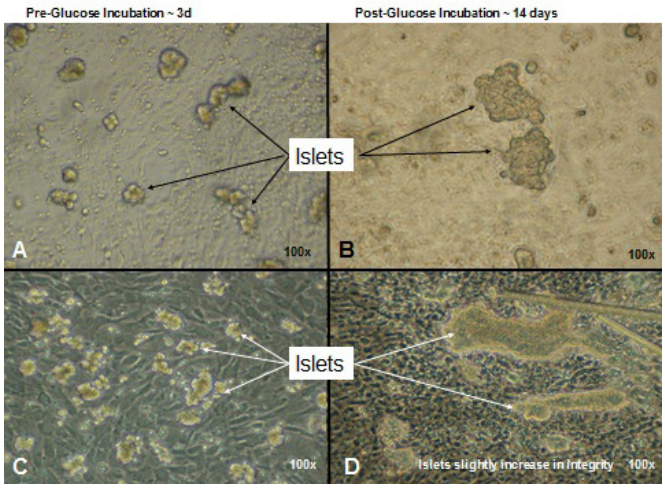

Figure 6: Isolated rat pancreatic islets with endogenous TSC and PSC from cell pellet after Ficoll density gradient centrifugation. Cell pellets were grown on the TSC-PSC-pancreatic matrix-B constructs utilizing the standard TSC-based culture medium. A & C are rat islets approximately three days after plating. B & D are rat islets 14 days after plating. Note that islets in B & D are increasing their three-dimensional integrity. These composites consisting of decellularized pancreatic matrix-B, Scl-44β, Scl-40β, and Ficoll cell pellet material demonstrated an increase in their integrity throughout 30 days in culture, before termination of the experiment.

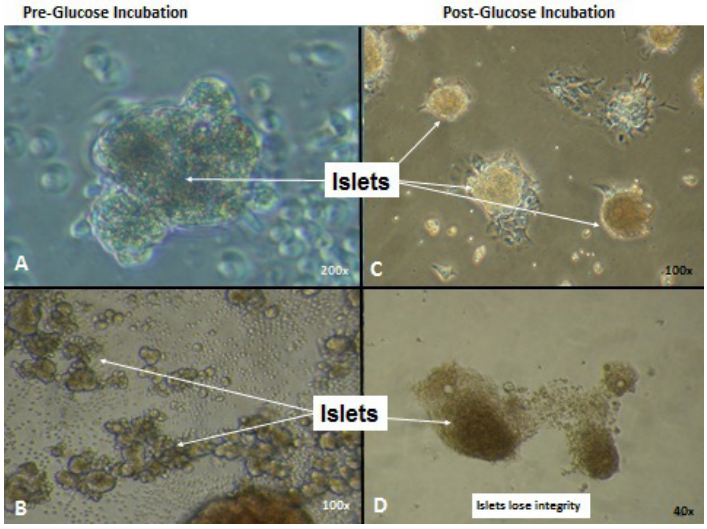

Figure 7: Native pancreatic islets, isolated utilizing Ficoll separation (interface between 20 and 23% Ficoll) were plated onto type-I collagen-coated tissue culture plates. A & B are rat islets 48 hours after plating. C & D are rat islets 72 – 96 hours after plating. Note appearance of native islets pre- (A and B) and post- (C and D) incubation with glucose challenge. Magnifications, as noted.

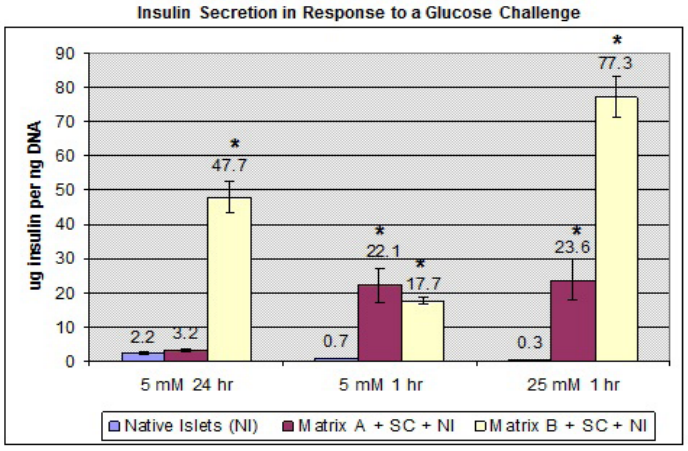

Figure 8: Histogram of radioimmunoassay of insulin secretion in response to a glucose challenge, measured in micrograms of insulin per nanogram of DNA, of native islets, matrix-A composites, and matrix-B composites three days' post plating. Sample sizes are n = 8 for islets alone and n = 6 for combinatorial constructs: Matrix (A or B) + SC (stem cells) + NI (native islets). Note, combinatorial constructs produced a greater insulin response per nanogram DNA than native islets by themselves. This occurred at all glucose concentrations examined: 5 mM glucose for 24 hours [Matrix A: 1.4x, Matrix B: 22x], 5 mM glucose for 1 hour [Matrix A: 31.6x, Matrix B: 25.3x], and 25 mM glucose for 1 hour [Matrix A: 78.7x, Matrix B: 257.7x].

In addition, as shown in Figures 5 and 6, there was an increase in the integrity of the native pancreatic islets after incubation on a substratum of decellularized native pancreatic matrix embedded with clonal populations of primitive pluripotent and totipotent stem cells.

Cryosectioning of pancreases from naïve animals have confirmed the existence of pluripotent (SSEA+) and totipotent (CEA-CAM-1+) stem cells located within the pancreatic islets of healthy unoperated animals (Figures 9 and 10).

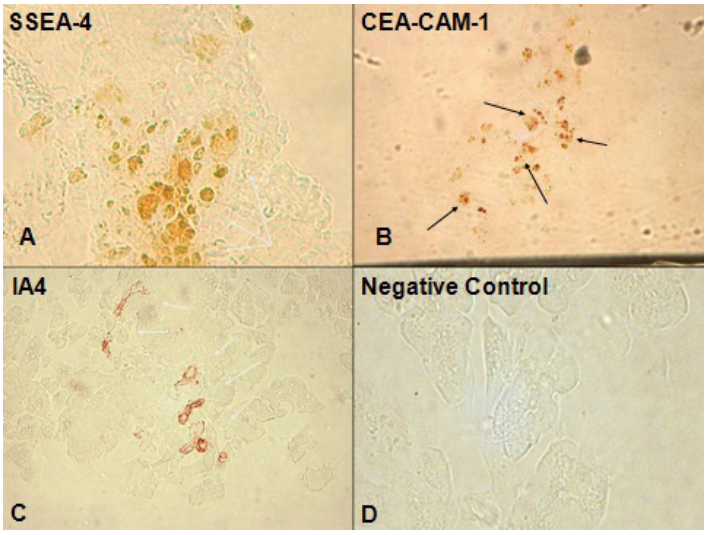

Figure 9: Stage specific embryonic antigen (SSEA) and carcinoembryonic antigen-cell adhesion molecule-1 (CEA-CAM-1) staining of cryosectioned pancreas from adult porcine male (PM0010, porcine, male, 10th animal examined), visualized by bright field microscopy. A. SSEA positive stained cells overlying both islets and acinar cells. B. CEA-CAM-1 positively stained cells overlying pancreatic islets and acinar cells. C. IA4 positive staining for smooth muscle alpha-actin in small blood vessels within pancreas, utilized as the positive procedural control. D. No primary antibody, utilized as the negative procedural control.

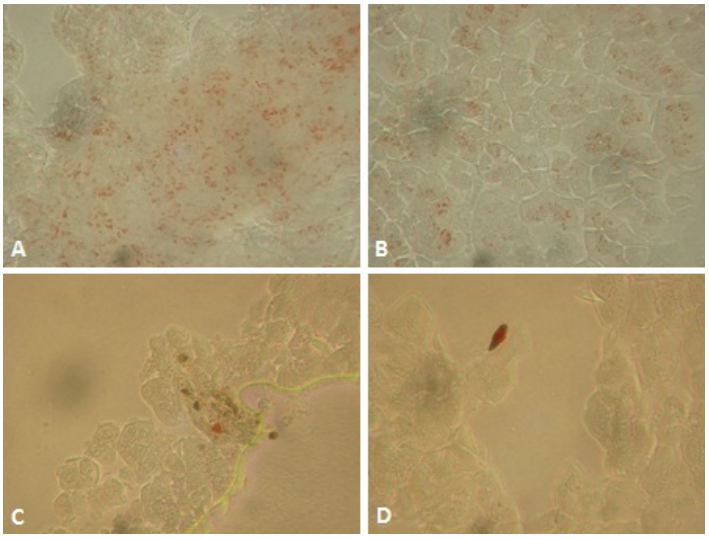

Figure 10: Carcinoembryonic antigen-cell adhesion molecule-1 (CEA-CAM-1) and stage specific embryonic antigen (SSEA) staining of cryosectioned pancreas from adult male rat, visualized by bright field microscopy. A. Predominantly pancreatic islet overlain with (red positive) CEA-CAM-1+ cells, 200x mag. B. Pancreatic acinar cells overlain with (red positive) CEA-CAM-1+ cells, 200x mag. C. Predominantly pancreatic islet overlain with (red positive) SSEA+ cells, 200x mag. D. Pancreatic acinar cells overlain with (red positive) SSEA+ cells, 200x mag.

Discussion

Insulin-dependent type-I diabetes mellitus currently afflicts 16 million Americans; whose health care expenses exceed $92 billion per year. The mortality and morbidity due to diabetes, along with the associated costs of treatment, are a testament to the failure of traditional therapy using exogenous insulin. A

promising experimental cure for type-I diabetes mellitus involves the transplantation of insulin-producing pancreatic islet cells. However, potential obstacles exist that may delay using islet transplantation as a cure for diabetes. One obstacle is the amount of tissue necessary for a successful islet transplant [23]. At the present time, two cadaveric pancreases are necessary to supply the number of pancreatic islets for a successful transplant. Despite these limitations, the clinical benefits of islet transplantations have led to setting up a number of islet transplantation centers around the world [43].

The stem cell community has stepped up to meet the paucity of islet tissue for transplant by developing both in vitro and in vivo technologies for the generation of insulin-secreting beta cells from pluripotent stem cells. Predominantly though, this has occurred using pluripotent embryonic stem cells as the starting material. They have shown that by using recombinant inductive agents, small molecules, and/or miRNAs, recapitulation of development from an embryonic stem cell to an insulin secreting beta cell is theoretically possible [17]. Indeed, following the ongoing studies from Melton's group [32], recent reports from Melton and colleagues [44-51] suggest that pancreatic beta cells can be generated from embryonic stem cells using a defined cocktail of inductive agents. Recently, VitaCyte was given FDA approval to begin clinical trials with embryonic stem cell-induced beta cells immuno-protected by alginate as an implantation treatment regimen for insulin-dependent diabetes.

While using induced pluripotent embryonic stem cells as a source of implantable insulin-secreting beta cells for transplant is one approach, we have attempted an alternative approach to generating more islet tissue for transplant. We started with endogenous pluripotent stem cells derived from an adult individual, rather than pluripotent embryonic stem cells, and used these adult-derived pluripotent stem cells to generate pancreatic islets consisting of multiple cell types rather than just pancreatic beta cells. Initially, clonal populations of endogenous adult-derived stem cells were derived by repetitive single cell clonogenic analysis. The clonal populations of endogenous adult-derived stem cells were characterized by their differentiative potential using both in vitro and in vivo methods. The in vitro method entailed induction with either a general induction agent, such as dexamethasone at varying molar concentrations, or with specific inducing agents, such as neural growth factor, bone morphogenetic protein-2, vascular endothelial cell growth factor, hepatocyte growth factor, etc., to generate cell types from all three germ layer lineages. The phenotypic expression of various cell types was visualized using both specific antibodies and histochemical staining procedures [33,37,42]. Utilizing this combinatorial analysis, it was shown that clone Scl-40β would form 64 unique and identifiable cell types across all three germ layer lineages but would not form gametes, and thus was designated as a transitional-totipotent/pluripotent stem cell [36,37], whereas clone Scl-44β would form 66 unique and identifiable cell types, including spermatogonia and thus was designated as a totipotent stem cell [35].

Previous reports demonstrating in vivo pluripotency occurred after the implantation of embryonic stem cells into nude mice with the demonstration of teratoma formation [52]. The teratomas consisted of a jumbled mixture of cell types from all three germ layer lineages. This has been set as the gold standard that every stem cell must achieve to be designated as "pluripotent" [17]. We attempted a variation of this method by embedding the endogenous stem cell clones into collagen sponges and transplanting them under the skin in an immunologically competent outbred animal model. While there was no immunological rejection response from implanting cells from outbred animals into outbred animals, the results were less than desired. There was no teratoma formation and actually no discernible change in morphology of the stem cells. After being removed from the animals, released from the collagen sponges and placed into culture, the stem cells responded to induction factors by forming their respective cell types in a similar fashion as naïve stem cells [37]. Clearly, these naïve endogenous adult-derived stem cells were decidedly different from embryonic stem cells, with their inherent pre-programming.

We next attempted to demonstrate pluripotency by implanting genomically-labeled clones of stem cells into animal models of tissue damage. We chose three outbred animal model systems, cardiovascular disease [36,37] for mesodermal lineage cells; Parkinson disease [53] for ectodermal lineage cells; and Type-I diabetes [40] (current and future studies) for endodermal lineage cells. For the cardiovascular disease model the heart tissue was damaged by either freezing the apex of the heart with liquid nitrogen or performing transient ligation of the left anterior descending coronary artery. The frozen hearts were injected directly into the apex with genomically-labeled stem cells while the transient ligation model was perfused systemically with similar stem cells via injection into the tail vein. Both models received the genomically-labeled transitional-totipotent/pluripotent stem cell clone Scl-40β. The results from both studies noted that the genomically-labeled stem cells replaced damaged cardiac myocytes, the connective tissue skeleton of the heart and the vasculature, the cell types normally present in the heart and the particular cell types damaged in the two model systems. In addition, no other genomically-labeled non-cardiac cell types were present within the stem cell injected hearts [36,37].

For the Parkinson disease model the genomically-labeled transitional-totipotent/pluripotent stem cell clone Scl-40β or vehicle control were stereotactically implanted ipsilaterally into the midbrains of outbred rats two weeks after the unilateral stereotactic injection of the neurotoxin 6-hydroxydopamine into their respective substantia nigra. The vehicle control injected animals showed the formation of only a glial scar at the needle injection site. The experimental animals were shown to have tyrosine-containing genomically-labeled neurons at the injection site. Additional and unexpected results showed genomically-labeled pyramidal neurons, interneurons, and astrocyte and oligodendrocyte glial cells in all areas that had sustained previous damage due to the stereotactic injections of the neurotoxin and the stem cells. In addition, genomically-labeled endothelial cells

were also present lining blood-filled capillaries in the areas where tissue damage occurred. These results suggested that the injected stem cells replaced the particular cell types (ectodermal-origin and mesodermal-origin cell types) that were damaged in the procedures used to create and treat the Parkinson disease model. No other genomically-labeled cell types were present within the tissue sections [53].

For the Type-I Diabetes model streptozotocin-induced diabetic animals were to be treated with the genomically-labeled stem cell clone Scl-40β to restore euglycemia in these animals. However, we first needed to prove that the Scl-40β clone would form functionally active pancreatic islet structures. The first step was accomplished using mixtures of conditioned media and known inductive agents to step-wise induce pluripotent stem cells to become endodermal stem cells, induced by using conditioned medium from endodermal stem cells; then to become pancreatic progenitor cells, induced by using conditioned medium from pancreatic progenitor cells; and finally to become pancreatic islet-like structures, induced by using the islet inductive cocktail of Bonner-Weir et al. [30]. The formed islet-like structures contained glucagon-secreting alpha cells, insulin-secreting beta cells and somatostatin-secreting delta cells [40]. Upon sequential challenge with glucose following the protocol by Lumelsky et al. [31], the induced islet-like structures secreted insulin at 25% to 50% insulin secretion of native islets challenged sequentially with glucose.

Our next step was to create functionally active implantable scaffoldings for pancreatic islet induction. As noted herein, we utilized decellularized xenogeneic porcine extracellular matrices (Figure 3) in conjunction with the outbred Sprague-Dawley clones, transitional-totipotent/pluripotent stem cell clone Scl-40β and totipotent stem cell clone Scl-44β, and donor inbred Wistar-Furth islets and associated SSEA+ and CEA-CAM-1+ stem cells. These entities formed a combinatorial organoid-like structures (Figures 5 and 6) that secreted insulin in response to a glucose challenge at far greater levels than control Wistar-Furth islets (Figure 7) with their associated stem cells by themselves (compare insulin responses, Figure 8).

The current study demonstrated that insulin secreting pancreatic islet-like constructs could be formed from native (donor) islets, autologous primitive adult stem cells (derived from the recipient), and decellularized porcine pancreatic matrices and that these composite organoids are responsive to a secretagogue glucose challenge by secreting more insulin per nanogram DNA than individual native islets and their associated endogenous stem cells alone. Thus, these constructs have the potential for use in supplementing individual native islets for transplantation therapy, by reducing the amount of donor islet tissue required for transplantation. Also, we noted that the material routinely discarded for islet transplantation therapy, i.e., the pelleted containing the "trashy" material actually contained more viable islets and that they were invested with both CEA-CAM-1+ cells (totipotent stem cells) and SSEA+ cells (pluripotent stem cells) (Figure 4). While these culture experiments need to be proven in large animal studies to demonstrate restoration of euglycemia in diabetically-induced large animal models, we would hypothesize that by using composites composed of decellularized FDA-approved xenogeneic pancreatic matrices, autologous pluripotent and totipotent stem cells from the recipient, allogeneic pluripotent and totipotent stem cells from the donor, and allogeneic pancreatic islets from the donor, more individuals could be transplanted per donor pancreas than has been previously shown. In addition, since these composite pancreatic islet organoid structures contained at least three of the cell types present in pancreatic islets, i.e., glucagon-positive alpha cells, insulin-positive beta cells, and somatostatin-positive delta cells, that a higher degree of euglycemia might be achievable when using these composites.

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