Differential of Human Deceased Donor, Adipose-Derived, Mesenchymal Stem Cells into Functional Beta Cells

Rao P*, Deo D*, Marchioni M*

Abstract

There is an emerging need for the rapid generation of functional beta cells that can be used in cell replacement therapy for the treatment of type 1 diabetes (T1D). Differentiation of stem cells into insulin-producing cells provides a promising strategy to restore pancreatic endocrine function. Stem cells can be isolated from various human tissues including adipose tissue (AT). Our study outlines a novel, non-enzymatic process to harvest mesenchymal stem cells (MSC) from research-consented, deceased donor AT. Following their expansion, MSC were characterised morphologically and phenotypically by flow cytometry to establish their use for downstream differentiation studies. MSC were induced to differentiate into insulin-producing beta cells using a step-wise differentiation medium. The differentiation was evaluated by analysing the morphology, dithizone staining, immunocytochemistry, and expression of pancreatic beta cell marker genes. We stimulated the beta cells with different concentrations of glucose and observed a dose-dependent increase in gene expression. In addition, an increase in insulin and c-Peptide secretion as a function of glucose challenge confirmed the functionality of the differentiated beta cells. The insulin and c-Peptide secretion as a function of glucose challenge confirmed the functionality of the differentiated beta cells. Consequently, these cells can be a promising therapeutic approach for cell replacement therapy to treat patients with T1D.

Key Words: Adipose; Deceased Donor; Mesenchymal; Diabetes

Introduction

Among the US population, approximately 1.4 million adults (20 years or older) and 187,000 children/adolescents have been diagnosed with type 1 diabetes (T1D), also known as juvenile diabetes or insulin-dependent diabetes[1]. T1D is an autoimmune disease in which the T cells attack and destroy the beta cells, the cell population within islet cells that produce and secrete insulin[2]. There is currently no cure for T1D[3]. Patients with T1D need to introduce their own insulin manually and have an increased risk of problems such as heart disease and stroke[4].

An option to treat T1D is through pancreatic islet cell transplantation[5]. One approach is the Edmonton protocol. The Edmonton protocol requires 1-3 deceased donor pancreata to obtain the dosage of islet cells needed for transplantation. These islet cells are infused into the portal vein. Patients typically receive a dose of at least 10,000 islet cells per kilogram of weight and often require two transplants[6]. The patient is then maintained on immunosuppression to keep the cells from being destroyed[7]. The major limitations of this procedure is the scarcity of transplantable human islets[8] and the need for lifelong use of immuno-suppressive drugs[9]. This has evoked a large-scale search for a physiologically competent and renewable source of primary human pancreatic islets.

Many alternative sources have been proposed to replace the damaged beta cells. These include the use of pancreatic beta cell lines[10], transplanting native islet cells that have regenerating potential[11], or the use of extra-pancreatic stem cell therapy.

Stem cells have the potential to differentiate into insulin-producing cells both in vivo and in vitro. This makes them an ideal candidate for the treatment of T1D as they have the ability to recognize the blood glucose levels and secrete the correct amount of insulin in the body[12]. Various sources have been identified to isolate stem cells and differentiate them into insulin-producing cells. Embryonic stem cells have a tremendous differentiating potential; however, due to ethical and legal considerations, there is limited use of these cells in translation medicine[13]. Adult stem cells isolated from bone marrow[14], Wharton’s jelly[15], liver[16], pancreatic stem/progenitor cells[17] and adipose tissue from human living donors[18] provide more reliable and preeminent candidates to differentiate into pancreatic beta cell lineage for use in cell-based therapies for T1D. The present study describes, for the first time, the use of deceased donor adipose tissue as a source for the creation of beta cells.

Deceased donors are routinely HLA-typed and infectious disease screened. Adipose tissue can be easily procured from research-consented deceased donors during the routine deceased donor workup without the pain, morbidity and mortality associated with living donor collection[19]. In the present study, we demonstrate that mesenchymal stem cells (MSC) isolated from deceased donor adipose tissue retain their stem cell characteristics and can be expanded to provide a ready source for cell differentiation studies. We outline for the first time, the process of differentiation and characterization of MSC isolated from deceased donor adipose tissue into functional insulin-producing beta cells. We hypothesize that adipose tissue could be harvested routinely from deceased donors for beta cell production. These HLA-typed beta cells could be cryopreserved and banked. Clinicians would then be able to

Author Names in full: Prakash Rao*, Dayanand Deo*, Misty Marchioni*

1Personalized Transplant Medicine Institute at NJ Sharing Network, New Providence, NJ, USA

#These authors contributed equally

Received 01 Aug 2020; Accepted 14 Oct 20; Published online: 11 December 2020

Copyright © Journal of Stem Cells and Regenerative Medicine. All rights reserved
choose an allogeneic beta cell transplant with the advantage of knowing the degree of HLA mismatch ahead of time, allowing for better planning of immunosuppression regimens. Our findings represent an important step towards cell-replacement therapy for T1D.

Materials and Methods

Adipose Tissue

Adipose tissue was excised from the abdomen of a research-consented deceased donor and placed in a sterile transport container. The container was transported to the laboratory on wet ice.

In the laboratory, a fraction of the adipose tissue was collected and processed using a non-enzymatic method for stromal vascular fraction (SVF) isolation. The adipose tissue was minced in an individual one-time use, disposable AC:Px® Systems (Axocell Laboratories, Inc., Cambridge, MA, USA). The finely minced tissue was washed with 0.9% sodium-chloride (B. Braun, Bethlehem, PA, USA) saline and filtered through the series of bags of the AC:Px® System. The cell suspension was centrifuged at 430 x g for 30 minutes. The cell pellet was resuspended in phosphate buffered saline (PBS) (Thermo Fisher Scientific, Waltham, MA, USA) to a final volume of 20 ml and represented the SVF.

The minimal criteria for the phenotyping of MSC is the expression of cell surface markers CD73, CD90, CD29, CD44, and CD105 accompanied by the lack of expression of CD11b, CD34, CD45, CD79a, and HLA-DR[28].

A cell count for the SVF was performed on a Guava easyCyte™ HTS flow cytometer (Luminex, Austin, TX, USA) using the ViaCount™ assay reagent as per manufacturer’s instructions. A well-mixed sample was taken and aliquoted in separate tubes for antibody staining. Antibodies used in our study were APC conjugated mouse anti-human CD73, PerCP-CyTM5.5 conjugated mouse anti-human CD105, PE conjugated mouse anti-human CD44 (BD Biosciences, San Jose, CA, USA), APC conjugated mouse anti-human CD90, PE conjugated mouse anti-human CD29, FITC conjugated mouse anti-human CD45, FITC conjugated mouse anti-human CD11b/MAC-1 (BD Pharmingen; BD Biosciences, San Jose, CA, USA). All the above antibodies were added to the aliquoted sample and incubated in the dark for 30 minutes at room temperature. The cells were washed twice with wash buffer (PBS containing 1% Fetal Bovine Serum) and resuspended in wash buffer for analysis on the flow cytometer. The sample was gated on cells negative for FITC (CD11b/MAC-1, CD45) and positive for APC (CD73, CD90), PerCP-CyTM5.5 (CD105), and PE (CD44, CD29).

Cell Growth and Proliferation

MSC were grown in a CELLstart™ CTS™ (Thermo Fisher Scientific, Waltham, MA, USA) coated flask as follows. CELLstart™ CTS™ was diluted 1:100 in 10 ml PBS and added to a 75cm² tissue culture flask (Falcon®, Corning, Corning, NY, USA) gently swirled to ensure complete surface coverage. The flask was incubated in a humidified incubator set at 37°C and 5% CO₂ for a total of 14 days or until the cell confluency reached 80%, with replacement of the complete growth medium in the flask every 2-3 days. For subculturing the cells, the media was aspirated and cells were washed once with pre-warmed PBS. Cells were detached from the flask by adding 5 ml of TrypLE™ Select CTS™ (Thermo Fisher Scientific, Waltham, MA, USA) and incubated at 37°C for 5 minutes. Upon detachment, 5 ml of PBS was added to the flask and the cell suspension was transferred to a 15 ml conical tube, followed by centrifugation at 200 g for 5 minutes. The cell pellet was resuspended in minimal volume of complete growth medium for cell counting. A total of 4 x 10⁵ viable cells were added to a CELLstart™ CTS™ pre-coated 75cm² tissue culture flask containing StemPro® MSC SFM CTS™ complete growth medium, 2% L-Glutamine and 1% antibiotics. The cells were incubated in a humidified CO₂ incubator as above with media replacement carried out every 2-3 days for optimal cell growth and proliferation.

Beta Cell Differentiation

MSC from passage 2 were harvested using TrypLE™ Select CTS™ and plated at a concentration of 3 x 10⁵ cells/well in CELLstart™ CTS™ coated 6-well culture plates in triplicate. The cells were grown in StemPro® MSC SFM CTS™ complete growth medium until they reached 80% confluency. Differentiation of MSC to beta cells was carried out in three stages using the process described by Chandra, V. et al.[31]. Briefly, MSC were induced to differentiate into the definitive endoderm (first stage) for 2 days using serum-free DMEM/F12 containing 17.5mM Glucose (Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 1% BSA, 4nM Activin A, 1mM sodium butyrate, 50µM 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA), and 1x insulin-transferrin-selenium (ITS) (Thermo Fisher Scientific, Waltham, MA, USA). On the third day, differentiation into the pancreatic endoderm (second stage) was induced by adding fresh serum-free DMEM/F12 media containing 1% bovine serum albumin (BSA), 1x ITS, and 0.3mM taurine (Sigma-Aldrich, St. Louis, MO, USA), then incubated for another 2 days. The third and final stage of differentiation into pancreatic hormone-expressing beta cells was carried out by replacing the media with fresh serum-free DMEM/F12 media containing 1.5% BSA, 1x ITS, 1mM nicotinamide, 3mM taurine, 100mM human Glucagon-Like Peptide (GLP)-1 (amide fragment 7-36) (Sigma-Aldrich, St. Louis, MO, USA) and 1x nonessential amino acids (Thermo Fisher Scientific, Waltham, MA, USA). The differentiated cells were maintained in this media for 5 days with replacement of fresh media after every 2 days.

Dithizone Staining

Beta cells that were differentiated from MSC were stained with the zinc-chelating dye dithizone (DTZ). Briefly, DTZ stock solution was prepared by dissolving 10mg of DTZ in 1ml of dimethyl sulfoxide (DMSO) (Sigma-Aldrich, St. Louis, MO, USA) and filtered through a 0.2mm filter. The cells were stained with DTZ using 10µl of the stock solution in 1ml of culture medium and incubated at 37°C for 30 minutes. The cells were then washed 3 times with PBS and crimson-stained stained clusters were observed under the microscope.

Immunocytochemistry

MSC were seeded onto single chamber glass slides (Lab-Tek® II Chamber Slide™ System, VWR, Radnor, PA) pre-coated with CELLstart™ CTS™. The cells were grown in StemPro® MSC SFM CTS™ complete growth medium until they reached 80% confluency. Differentiation of MSC to beta cells was carried out as above. The differentiated beta cells were washed with PBS, fixed using 4% paraformaldehyde for 10 minutes at room temperature and permeabilized in 0.1% Triton X-100 (Sigma-Aldrich, St. Louis, MO, USA) at room temperature for 10 minutes. The cells were washed thrice with PBS and blocked with 2% BSA and PBST (1x PBS containing 0.1% Tween 20) (Sigma-Aldrich, St. Louis, MO, USA) at room temperature for 60 minutes. The blocked cells were incubated with human primary antibodies (insulin, c-Peptide, glucagon,...
Beta Cells Differentiated from Deceased Donor MSC

PDX-1), or their respective isotype control antibodies (Abcam, Cambridge, MA) in 1% BSA and PBST at the manufacturer’s recommended dilutions in a humidified chamber for 2 hrs at 37°C. The cells were washed thrice in PBS and incubated with the secondary antibody labelled with fluorescent Alexa Fluor 488 (Abcam, Cambridge, MA) in the dark for 60 minutes at room temperature. After washing the cells with PBS, fluoroshield mounting media containing DAPI to visualize the nuclei was added onto the slides and cover slipped. Images of the fluorescence-labelled cells were captured using fluorescent microscope (Leica, Mannheim, Germany).

Glucose-stimulated Insulin and c-Peptide release

The differentiated beta cells were washed twice with PBS and incubated for 12 hrs in DMEM-low glucose fasting medium (Thermo Fisher Scientific, Waltham, MA, USA) containing 0.5% BSA. Glucose stimulation was achieved by incubating cells for 2 hrs in DMEM (no glucose) media containing various glucose concentrations ranging from 0mM glucose (control cells) to 100mM glucose (Sigma-Aldrich, St. Louis, MO, USA). Secreted insulin and c-Peptide levels in the media were determined using human insulin and c-Peptide ELISA kits according to manufacturer’s instructions (Sigma-Aldrich, St. Louis, MO, USA).

Gene Expression: qRT-PCR

Differentiated beta cells in 6-well plates were stimulated for 2 hrs with glucose at concentrations ranging from 0 mM to 100 mM glucose. Undifferentiated MSC were used as control. The media was aspirated and cells were detached from the wells by adding 0.5 ml of TrypLE™ Select CTS™ (Thermo Fisher Scientific, Waltham, MA, USA) and incubated at 37°C for 5 minutes. Upon detachment, 1 ml of PBS was added to the wells, cell suspension was pooled and transferred to a 15 ml conical tube, followed by centrifugation at 200 x g for 5 minutes. The cell pellet was resuspended in TRizol® Reagent (Sigma-Aldrich, St. Louis, MO, USA) and total RNA was extracted by the phase separation procedure[22]. 1 µg of total RNA was reverse transcribed to cDNA using the qScript™ cDNA SuperMix first-strand synthesis system kit (Quanta Biosciences, Gaithersburg, MD, USA). The cDNA was added to SsoAdvanced™ Universal SYBR® Green Supermix and overlaid onto custom 96-well PCR plates (Bio-Rad Laboratories, Hercules, CA, USA) containing transcripts of genes that are specific for beta cells. qRT-PCR was performed using the CFX96™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) transcript for normalization was measured. Lease System (Bio-Rad Laboratories, Hercules, CA, USA). The relative expression of genes related to beta cell differentiation and function were examined after stimulating the beta cells at various glucose concentrations. Gene expressions in undifferentiated MSC were used as controls.

MSC were Successfully Differentiated in vitro Into Beta Cells

Beta cell differentiation of MSC was carried out using cells at passage 2 that retain all stem cell characteristics. The morphology of MSC changed greatly when they were stimulated to differentiate to beta cells as observed under a phase contrast inverted microscope (Leica Microsystems Inc., Buffalo Grove, IL, USA). The spindle shaped fibroblast-like morphology changed gradually to cells that were more flattened and developed round-shaped islet-like clusters (Figure 2).

Beta cells in the islets contain large amounts of zinc and can be specifically stained with the zinc-chelating dye DTZ[24,25]. We used this characteristic of DTZ and identified a distinctly-stained crimson red population of beta cell clusters in the differentiated cells, while undifferentiated cells were negative for DTZ (Figure 2).

Determined the functional capability of the differentiated beta cells was assessed by detecting the presence and amount of free insulin and c-Peptide released into the culture media. We observed that secretion of both insulin and c-Peptide by the differentiated beta cells increased with an increase in glucose stimulation. Increasing the glucose concentration from 5 to 75 mM induced the beta cells to secrete insulin from 175.8 ± 8.3 U/ml to 384.7 ± 7.9 U/ml. However, there was no significant increase of insulin release (389 ± 2.3 U/ml) when the cells were stimulated at the highest 100 mM glucose concentration (Figure 4A). We observed a similar dose-dependent increase in c-Peptide release by the differentiated beta cells upon increasing glucose stimulation. As shown in Figure 4B 5 mM glucose stimulated a release of 2.18 ± 0.06 ng/ml free c-Peptide. As the concentration of glucose stimulation was increased, we observed an increase of c-Peptide released in the media, reaching a level of 4.2 ± 0.6 ng/ml c-Peptide at 75 mM glucose. No significant c-Peptide release (4.37 ± 1.8 ng/ml) was observed at the highest 100 mM glucose concentration. Glucose-stimulated release of insulin and c-Peptide was observed to be statistically significant (p<0.01) compared to unstimulated control cells.

Increased Expression of Beta Cell Specific Genes was Observed in Response to Glucose Challenge

The relative expression of genes related to beta cell differentiation and function were examined after stimulating the beta cells at various glucose concentrations. Gene expressions in undifferentiated MSC were used as controls.

All values are expressed as mean ± standard deviation (S.D.). Statistical analysis was performed using the One Sample t-test in GraphPad QuickCalcs software. Differences were considered statistically significant at p<0.05.

Results

MSC Isolated from Deceased Donor Adipose Tissue Displays Standard Morphology and Phenotypic Markers Characteristic of Mesenchymal Stem Cells

The initial culture of SVF isolated after processing deceased donor adipose tissue yielded a small group of adherent cells showing homogeneous morphology of fibroblastoid cells within 2 days. As per Bourin P et al[23], the amount of MSC found in adipose tissue is 1%-10% of total nucleated cells. In deceased donor adipose tissue, we have observed the MSC concentration to be 4.5% of the total nucleated cells[19]. The adipose-derived MSC were expanded in fresh media to yield cells that had spindle shape fibroblast-like morphology.

The cells had high capacity of proliferation and grew to about 80% confluency within 2 weeks. Flow cytometric analysis showed that the cells were positive for MSC surface markers CD73, CD90, CD105, CD44, and CD29 and negative for CD45 and CD11b/MAC-1 (Figure 1A). The specificity of the antibody binding was assessed using their respective isotype control antibodies (Figure 1B). The isolated MSC from deceased donor adipose tissue maintained their differentiation capacity. After addition of appropriate growth factors, MSC could be differentiated to form adipocytes, chondrocytes and osteocytes as described earlier[19].

Statistical Analysis

The isolated MSC were subsequently differentiated in vitro into beta cells.

The differentiated beta cells were examined morphologically for the presence of insulin and c-Peptide using specific staining with alkaline phosphatase and CTS™ (Thermo Fisher Scientific, Waltham, MA, USA). The differentiated beta cells were subjected to immunocytochemistry. As shown in Figure 3 the differentiated beta cells stained positive for insulin, c-Peptide, glucagon, and PDX-1. The red population of beta cell clusters in the differentiated cells, while undifferentiated cells were negative for DTZ (Figure 2).

Glucose Stimulation of Differentiated Beta Cell Induced Release of Insulin and c-Peptide

Beta cells in the islets contain large amounts of zinc and can be specifically stained with the zinc-chelating dye DTZ[24,25]. We used this characteristic of DTZ and identified a distinctly-stained crimson red population of beta cell clusters in the differentiated cells, while undifferentiated cells were negative for DTZ (Figure 2).

To confirm the presence and localization of beta cell markers, the differentiated cells were subjected to immunocytochemistry. As shown in Figure 3 the differentiated cells stained positive for insulin, c-Peptide, glucagon, and PDX-1. We observed that both insulin and glucagon were localized near the nucleus. Both c-Peptide and PDX-1 were observed to be dispersed in the cytoplasm. Absence of non-specific staining of cells was confirmed by staining with the respective isotype control antibodies.

Increased Expression of Beta Cell Specific Genes was Observed in Response to Glucose Challenge

The relative expression of genes related to beta cell differentiation and function were examined after stimulating the beta cells at various glucose concentrations. Gene expressions in undifferentiated MSC were used as controls.
Figure 1. Flow cytometric analysis for MSC surface markers.
(A) Expression of cell surface markers was assessed in MSC isolated from human deceased donor adipose tissue. The sample was gated on cells negative for CD11b, CD45 and positive for CD73, CD90, CD29, CD44 and CD105 corresponding with stem cell-specific characteristics of mesenchymal cells. Abbreviations: FITC, fluorescein isothiocyanate; APC, allophycocyanin; PE, phycoerythrin; PerCP, peridinin-chlorophyll protein. (B) Specificity of antibody binding of MSC markers using their respective isotype controls.

Figure 2. Morphological changes in adipose-tissue derived MSC during their differentiation to beta cell clusters.
(A) Undifferentiated MSC showing spindle shaped fibroblast-like morphology; (B) Differentiated beta cells showing islet-like clusters; (C) Beta cell clusters showing positive crimson red staining with DTZ.
Figure 3. Immunocytometry detection and localization of beta cell differentiation markers. Differentiated beta cells were fixed and stained with primary antibodies for the beta cell markers or their respective isotype control antibodies (A) Insulin, (B) c-Peptide, (C) Glucagon and (D) PDX-1. The cells were further stained with the fluorescent secondary antibody Alexa Fluor 488 and the cytoplasmic localization of these markers was observed under the fluorescent microscope. The nuclei were counter stained with DAPI (4',6'-diamidino-2-phenylindole). Representative images of three separate staining procedures are shown.
Figure 4. Functional capability of differentiated beta cells.
Beta cells differentiated from adipose-derived MSC were observed to release increasing amounts of human insulin (A) and c-Peptide (B) in the culture media in response to increasing glucose concentrations. Control cells were not exposed to glucose. Data represents the mean ± SD of three independent experiments (*** p<0.01 [A] and p<0.01 [B]).

Figure 5. Expression of beta cell-specific genes after differentiation from adipose-derived MSC.
Differentiated beta cells were stimulated with increasing concentrations of glucose and analysed for the expression of beta cell related genes. Expression of genes was normalized to the internal control GAPDH. The gene expression was expressed as fold increase over undifferentiated control cells. An increase in gene expression was observed with increasing glucose stimulation of the differentiated beta cells with no further increase observed after 25mM of glucose stimulation. Data is represented as mean ± SD of three independent experiments. (** p<0.03). GCG - Glucagon, GCK - Glucokinase, INS - Insulin, SST - Somatostatin, ISL1 - ISL LIM homeobox 1, NEUROG3 - Neurogenin 3, NKX2-2 - NK2 homeobox 2, NKX6-1 - NK6 homeobox 1, PAX4 - Paired box 4, PAX6 - paired box 6, PDX1 - Pancreatic and duodenal homebox 1, PPY - Pancreatic polypeptide, SLC2A1 - Solute carrier family 2 member 1, SLC2A2 - Solute carrier family 2 member 2.
All gene expression data was normalized to GAPDH used as an internal control. Interestingly, we observed that increase in gene expression levels was directly correlated with stimulating the cells with an increase in glucose concentration (Figure 5). Transcript levels of genes specific for mature beta cells such as INS, GCG, GCK and SST increased with an increase in glucose exposure. Various transcription factors specific for beta cell development such as PDX1, ISL1, NEUROG3, NKX2-2, NKX2-6, PAX4 and PAX6 were also seen to increase similarly. A dose dependent increase in gene expression of other pancreas related gene such as PYY, SLC2A1 and SLC2A2 was also observed. The relative gene expression of all beta cell differentiation genes was statistically significant (p<0.03) compared to undifferentiated control cells.

Discussion

T1D is a manifestation of the autoimmune destruction of beta cells leading to a decrease in insulin production and hyperglycaemia[29]. Stem cell therapy for the treatment of T1D is gaining importance due to their potential for differentiation into insulin-producing cells and immunomodulatory properties[3]. Recently, a preferred cell source for isolation of allogenic stem cells for research and clinical applications has been adipose tissue[32]. These stem cells retain the ability to secrete trophic factors, have immunomodulatory properties and low immunogenicity which make them ideal cell source for a wide range of therapeutic and regenerative applications[33,34]. Adipose tissue may be procured on a routine basis from deceased donors during the normal deceased donor workup without the pain, morbidity and mortality associated with living donors. We have shown earlier that by using the adipose tissue obtained from deceased donors, we were able to isolate plastic-adherent MSC populations from the SVF having multi-lineage differentiation capacity[19]. As seen in Figure 1, flow cytometric characterization of MSC show that these cells express the stem cell-specific combination of surface markers such as CD73, CD90, CD29, CD44, and CD105 and the lack of expression of CD11b, CD45[29]. This suggests that MSC isolated from the adipose tissue of deceased donors can be used for downstream regenerative applications.

Insulin-producing cells have been successfully differentiated from MSC isolated from the bone marrow, umbilical cord and adipose tissue obtained from liposapirantes[18]. Differentiation of MSC isolated from deceased donor adipose tissue into insulin producing beta cells has not been reported previously. In the present study, we demonstrate the potential of MSC from deceased donor adipose tissue to differentiate into insulin-secreting beta cells in vitro. One of the factors needed to differentiate adipose-derived MSC to functioning beta cells is the presence of high glucose concentration in the growth medium. We used basal growth medium containing 17.5 mM glucose throughout the differentiation process[20]. The first stage of differentiation was carried out by supplementing the growth medium with Activin A, sodium butyrate, ITS and 2-mercaptoethanol. These components have specific function that enable the differentiation of the mesodermal MSC to the endoderm lineage. Activin A, a member of the transforming growth factor – beta family is responsible for hormone synthesis, cell count management, and inducing undifferentiated stem cells to differentiate to the endodermal lineage cells[31]. Sodium butyrate, a histone deacetylase inhibitor[32] is another reagent that aids Activin A in the endodermal differentiation of stem cells. ITS and the reducing agent 2-mercaptoethanol support growth of cells and act as an antioxidant and reduce the toxic levels of oxygen radicals and peroxides[33,34]. Further differentiation to the pancreatic endoderm cells was induced by the addition of physiological levels of taurine (a non-essential amino acid required for the development and maturation of functional beta cells)[35] to the basal growth medium. Maturation of cells into hormone expressing beta cells was performed by supplementing the basal medium with supra-physiological levels of taurine, nicotinamide and GLP-1 peptide. Nicotinamide helps to protect the beta cells from dying or differentiating into other cell types and also enhances the secretion of various hormones including insulin[36-38]. GLP-1 is an incretin hormone responsible for the increase in expression and stabilization of insulin mRNA, increasing insulin synthesis and stimulation of insulin secretion in the pancreas[37].

The differentiated beta cells were observed to be morphologically similar to the pancreatic beta cell clusters and was further confirmed by DTZ staining. Immunofluorescence studies showed that the differentiated beta cells expressed insulin, c-Peptide, glucagon and PDX-1. Intracellular localization of these beta cell markers suggest that our observations are in accordance with the normal pancreatic development and expression of specific pancreatic hormones[39-40].

T1D is characterized by reduction in the number, area and density of pancreatic islets, inhibition of free radical scavenger enzymes, and loss of cellular organization in the endocrine region of the pancreas. This leads to the dysregulation of beta cells and their corresponding loss of capacity to secrete insulin[41]. Transplantation of insulin-producing cells in vivo has been shown to help in the regeneration of pancreatic islets by secreting insulin[42] and the expression of c-Peptide that is used as a marker of insulin secretion[43]. To evaluate whether the differentiated beta cells retained their functional capacity, we stimulated them with different concentrations of glucose. Glucose uptake by pancreatic beta cells has been shown to induce secretion of insulin by these cells. The amount of insulin secreted is dependent on the concentration of glucose stimulation. This suggests that sensitivity of beta cells to glucose underlie the glucose dose dependence in islets[44]. We observed that both insulin and c-Peptide were released into the growth media upon glucose stimulation, suggesting that the differentiated beta cells retained their functional characteristics. Our observations are in accordance with earlier reports measuring an increase in insulin secretion between low and high glucose stimulation[43]. We hypothesized that insulin release by differentiated beta cells could be dependent on the amount of glucose simulation and that there is a limit to the amount of stimulation that the cells could withstand and reach a saturation point. Interestingly, we observed that there was a step-wise increase in insulin as well as c-Peptide release as a function of increasing glucose concentration. This indicates that the differentiated beta cells were glucose-sensitive and insulin-responsive. In addition, when the cells were stimulated with 75 mM and 100 mM glucose, there was no further increase in the secretion of both insulin and c-Peptide. This observation suggests that altering the stimulation of cells leads to a corresponding change in the functionality of the cells to release insulin and c-Peptide. Further, a saturation of stimulus is reached where no further increase in exposure to glucose would induce the cells to increase the secretion of insulin and c-Peptide.

We studied the relative gene expression of the differentiated beta cells that would support our observations that the beta cells are of the pancreatic endocrine lineage. After differentiation, we observed that all the relevant pancreatic endocrine genes were expressed in accordance with earlier observations[45]. In addition, we observed that expression of different glucose concentrations led to a dose-dependent increase in gene expression, reaching a plateau at the higher glucose exposure levels. To the best of our knowledge, we report for the first time the dependence of gene expression pattern on the amount of glucose stimulation in the beta cells that were differentiated from MSC isolated from deceased donor-derived adipose tissue.

Overcoming barriers to cell therapy in T1D

Replacement of beta cells in T1D has the potential to prevent hypoglycemic episodes in patients, insulin independence, and long-term graft function with improvement of quality of life. However,
experimental trials clearly highlight several obstacles that remain to be overcome before the procedure could be proposed to a much larger population. The vulnerability of differentiated beta cell grafts to host immune and autoimmune attack is a primary barrier that is associated with islet graft rejection in T1D. Strategies have been proposed to promote immune tolerance by either selectively deleting memory T-cells or promoting immune modulation via Treg cell therapy.[47] Search for a minimally invasive and well vascularized site for implanting stem cell-derived beta cells is gaining importance. Camillo Ricordi suggests omentum as a potentially advantageous site for implanting stem cell-derived beta cells. The initial success of this strategy has promoted omentum as a potential site for transplanting of both free and micro-encapsulated stem cell-derived islet clusters.[48] A novel approach to implant decellularized pancreatic grafts that are reseeded with stem cell-derived islets, ductal cells, and endothelial cells is showing promise; however, more studies are required to examine such grafts. Non-viable, free of thrombosis, and capable of sustaining functional islet tissue in vivo.[49] Obtaining favourable outcomes in clinical islet cell transplantation depends on improved distribution strategies between the islet cell processing facility and the distant islet cell transplant programs. Optimization of shipping techniques that allow maximizing preservation of islet mass, viability, potency, and sterility is being investigated.[50]

Conclusion

The success of cell therapy depends on the functional activity of the transplanted cells. We clearly demonstrate that MSC can be easily isolated from deceased donor adipose tissue and differentiate into insulin-producing beta cells that respond to glucose stimulation. We hypothesize that, despite current barriers, differentiated cells can be used as an alternative source of beta cells for the cell replacement therapy in T1D. Further studies are needed to evaluate the immunogenicity and clinical applicability of these differentiated cells, and the minimum number of cells required to achieve the same glucose regulation as in a normal pancreas.

References

1. Prevention CDC. National Diabetes Statistics Report. In: Services USDHHS, editor. Atlanta, GA 2020.
2. Diabetes.co.uk. Beta Cells. Diabetes Digital Media Ltd; 2019 [cited 2020 2/14/20]; Available from: https://www.diabetes.co.uk/body/beta-cells.html.
3. Shiroi A, Yoshikawa M, Yokota H, Fukui H, Ishizaka S, Tatsumi SW, Shyr YM, Tang KT, Chen TH. The role of the innate immune system in the development of type 1 diabetes in NOD mice. Clin Immunol 2014;153(1):187-98.
4. Trucco M. Is facilitating pancreatic beta cell regeneration a valid option for clinical therapy? Cell Transplant 2006;15 Suppl 1:575-83.
5. Naujok O, Francis F, Picton S, Bailey CJ, Lenzen S, Jorns A. Changes in gene expression and morphology of mouse embryonic stem cells on differentiation into insulin-producing cells in vitro and in vivo. Diabetologia 2009;52(5):640-76.
6. Jiang J, Au M, Lu K, Ebsheter A, Korbett G, Fisk G, Majumdar AS. Generation of insulin-producing islet-like clusters from human embryonic stem cells. Stem Cells 2007;25(8):1940-53.
7. Isokvich S, Kaminitz A, Yafe MP, Mizrahi K, Stein J, Yaniv I, Askenasy N. Participation of adult bone marrow-derived stem cells in pancreatic regeneration: neogenesis versus endogenosis. Curr Stem Cell Res Ther 2007;2(4):272-9.
8. Wang HS, Shyu JF, Shen WS, Hsu HC, Chi TC, Chen CP, Huang SW, Shyr YM, Tang KT, Chen TH. Transplantation of insulin-producing cells derived from umbilical cord stromal mesenchymal stem cells to treat NOD mice. Cell Transplant 2011;20(3):455-66.
9. Yang L, Li S, Hatch H, Ahrens K, Cornelius JG, Petersen BE, Peek AB. In vitro trans-differentiation of adult hepatic stem cells into pancreatic endocrine hormone-producing cells. Proc Natl Acad Sci U S A 2002;99(12):8078-83.
10. Seaberg RM, Smukler SR, Kieffer TJ, Enikolopogol G, Asghar Z, Wheeler MB, Korbett G, van der Kooy D. Clonal identification of multipotent precursors from adult mouse pancreas that generate neural and pancreatic lineages. Nat Biotechnol 2004;22(9):1115-24.
11. Timper K, Seboek D, Eberhardt M, Linscheid P, Christ-Crain M, Keller U, Muller B, Zaleski H. Human adipose tissue-derived mesenchymal stem cells differentiate into insulin, somatostatin, and glucagon expressing cells. Biochem Biophys Res Commun 2006;341(4):1135-40.
12. Rao PN, Deo DD, Marchioni MA, Taghizadeh RR, Cetulo K, Sawezak S, Myrick J. Structural and Functional Characterization of Deceased Donor Stem Cells: A Viable Alternative to Living Donor Stem Cells. Stem Cells Int 2019;2019:5841587.
13. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006;8(4):315-7.
14. Chandra V, G S, Phadnis S, Nair PD, Bhonde RR. Generation of pancreatic hormone-expressing islet-like cell aggregates from murine adipose tissue-derived stem cells. Stem Cells 2009;27(8):1941-53.
15. Chomczynski P, Mackey K. Short technical reports. Modification of the TRI reagent procedure for isolation of RNA from polysaccharide- and proteoglycan-rich sources. Biotechniques. 1995;19(6):942-5.
16. Bourin P, Bunnell BA, Castella I, Dominici M, Katz AJ, March KL, Redl H, Rubin JP, Yoshimura K, Gimble JM. Stromal cells from the adipose tissue-derived stromal vascular fraction and culture expanded adipose tissue-derived stromal/stem cells: a joint statement of the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ICTST). Cytotherapy 2013;15(6):641-8.
17. Shiroi A, Yoshikawa M, Yokota H, Fukui H, Ishizaka S, Tatsumi K, Takahashi Y. Identification of insulin-producing cells derived from embryonic stem cells by zinc-chelating dithizone. Stem Cells 2002;20(4):892-9.
18. Wang N, Adams G, Buttery L, Falcone FH, Stolnik S. Alginate from embryonic stem cells by zinc-chelating dithizone. Stem Cells 2007;25(8):1940-53.
19. Ryan EA, Lakey JR, Paty BW, Imes S, Korbutt GS, Kneteman KL, Redl H, Rubin JP, Yoshimura K, Gimble JM. Stromal cells and glucagon expressing cells. Biochem Biophys Res Commun 2006;341(4):1135-40.
20. Timper K, Seboek D, Eberhardt M, Linscheid P, Christ-Crain M, Keller U, Muller B, Zaleski H. Human adipose tissue-derived mesenchymal stem cells differentiate into insulin, somatostatin, and glucagon expressing cells. Biochem Biophys Res Commun 2006;341(4):1135-40.
21. Wang HS, Shyu JF, Shen WS, Hsu HC, Chi TC, Chen CP, Huang SW, Shyr YM, Tang KT, Chen TH. Transplantation of insulin-producing cells derived from umbilical cord stromal mesenchymal stem cells to treat NOD mice. Cell Transplant 2011;20(3):455-66.
22. Yang L, Li S, Hatch H, Ahrens K, Cornelius JG, Petersen BE, Peek AB. In vitro trans-differentiation of adult hepatic stem cells into pancreatic endocrine hormone-producing cells. Proc Natl Acad Sci U S A 2002;99(12):8078-83.
23. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, Deans R, Keating A, Prockop D, Horwitz E. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006;8(4):315-7.
24. Chandra V, G S, Phadnis S, Nair PD, Bhonde RR. Generation of pancreatic hormone-expressing islet-like cell aggregates from murine adipose tissue-derived stem cells. Stem Cells 2009;27(8):1941-53.
Abbreviations

AT : Adipose Tissue
BSA : Bovine Serum Albumin
cDNA : Complimentary Deoxyribonucleic Acid
DAPI : 4’-6’ Diamidino-2-Phenylindole
dMSO : Dimethyl Sulfoxide
DTZ : Dye Dithizone
ELISA : Enzyme-Linked Immunosorbent Assay
GAPDH : Glyceraldehyde-3-Phosphate Dehydrogenase
GLP-1 : Glucagon-Like Peptide – 1
HLA : Human Leukocyte Antigen
ITS : Insulin-Transferrin-Selenium
MSC : Mesenchymal Stem Cells
PBS : Phosphate Buffered Saline
PBST : Phosphate Buffered Saline Containing Tween
PDX-1 : Pancreatic and Duodenal Homeobox 1
qRT-PCR : Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction
SVF : Stromal Vascular Fraction
T1D : Type 1 Diabetes
Potential Conflicts of Interests

None

Acknowledgements

First and foremost, we would like to thank all of the donor families for giving their consent for research which made this study possible. We wish to acknowledge the clinical staff at the NJ Sharing Network for tissue recovery.

Corresponding Author

Prakash Rao, Transplant Laboratory, NJ Sharing Network & Personalized Transplant Medicine Institute, 691 Central Avenue, New Providence, NJ 07974, prao@njsharingnetwork.org