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Characterization of Dental-Pulp Derived Islet-like Cell Aggregates and the Role of GABA

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Characterization of Dental-Pulp Derived Islet-like Cell Aggregates and the Role of GABA

A thesis submitted in partial satisfaction

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in Oral Biology

by

Jennifer Lynn Egli

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ABSTRACT OF THE THESIS

Characterization of Dental-Pulp Derived Islet-like Cell Aggregates and the Role of GABA

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Jennifer Lynn Egli

Master of Science in Oral Biology

University of California, Los Angeles 2012

Professors Eung-Kwon Pae & Mo Kang, Co-Chairs

Diabetes Mellitus (DM) is a growing health concern with 8.3% of Americans suffering from the effects of the disease. The use of multipotent mesenchymal progenitor cells, known as stem cells from human exfoliated deciduous teeth (SHED), to generate insulin-releasing islet-like cell aggregates (ICAs) is considered to be a potential treatment mode for Type-I DM. ɤ-Aminobutyric acid (GABA) has recently been shown to be a strong secretogogue of insulin and increases β-cell mass, reversing DM in rats. This investigation focused on the use of insulin-producing ICAs to examine the roles of GABA in an autocrine excitatory secretory mechanism in pancreatic β-cells. The aim of this study was to investigate the role of GABA on insulin secretion utilizing the *in vitro* system, insulin-secreting ICAs derived from SHED.

SHED was propagated using a 10-day differentiation protocol to generate ICAs. Day-10 ICAs were stained with dithizone (DTZ) to confirm zinc-positive β-cell phenotype. Real-time quantitative reverse transcriptase PCR (qRT-PCR) on Day-5 and Day-10 ICAs was used to confirm the presence of pancreatic markers PDX-1, NeuroD, Nkx6.1, Isl-1, Glut-2, insulin, and $GABA_A$ receptors, and to demonstrate temporal changes in expression levels. Day-10 ICAs were challenged with glucose and GABA to demonstrate dose-dependent insulin secretion using enzyme-linked immunosorbent assay (ELISA).

Results demonstrate that SHED ICAs were able to differentiate toward the pancreatic lineage. Day-10 ICAs demonstrated a weak positive DTZ staining. A statistically significant temporal increase of the β-cell markers was shown from Day-5 to Day-10 ICAs ($p<0.05$) using qRT-PCR; albeit, Day 10 ICAs did not reach pancreatic marker transcript levels previously reported. Day-10 ICAs did secrete insulin in response to glucose, however, not in a dose-dependent manner, possibly due to insufficient storage of insulin because of immaturity of the aggregates or a lack of intracellular zinc concentration. Day-10 ICAs demonstrated a statistically significant increase in the transcript levels of GABA receptors (i.e. GABAα-1, GABAα2, GABAα3, and GABAα6), indicating that the aggregates may respond to GABA during challenge ($p<0.05$). Preliminary results utilizing GABA in addition to glucose challenge indicates that GABA may increase insulin secretion by the ICAs; however, further studies need to be done to confirm this finding. We conclude that SHED is capable of differentiating toward the pancreatic lineage and producing insulin; therefore, these cells may be a possible source for β-cell replacement therapy in Type-I DM patients.

The thesis of Jennifer Lynn Egli is approved.

Ki-Hyuk Shin

Yong Kim

Eung-Kwon Pae, Committee Co-Chair

Mo Kang, Committee Co-Chair

University of California, Los Angeles

2012

DEDICATION:

I would like to first dedicate this thesis to my wonderful husband, Benjamin Egli, for his continuous support and encouragement during my educational endeavors and to our beautiful children, Logan, Breanna, Megan, and Kylee who all make my world a brighter place. I would also like to dedicate this thesis to my parents, Neil and Susan Helgeson, for encouraging me to set my goals high and reach for the stars.

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Diabetes Mellitus: Incidence, Prevalence, Morbidity & Mortality

Diabetes Mellitus (DM) is a metabolic disease characterized by uncontrolled blood glucose levels, which results from defects in production and/or in insulin sensitivity of the tissue[1]. There are many subtypes of diabetes depending on how and when symptoms appear: Type I DM, also known as Insulin-Dependent Diabetes Mellitus (IDDM), Type II DM, Maturity Onset Diabetes of the Young (MODY), Gestational diabetes, and so on. Type I DM is a multifactorial disease process, involving factors from the environment, genetics, and/or viral infection; it is known to be caused by an autoimmune response which destroys the insulin producing pancreatic β-cells, resulting in the need for life-long insulin supplementation[1]. Type II DM is the result of inadequate amounts of insulin being produced by pancreatic β-cells or due to the lack of cell membrane permeability to take up glucose from blood in response to insulin[1]. Gestational diabetes is due to an increased blood sugar that occurs during pregnancy, otherwise normal, which may precede the development of Type II diabetes[1]. MODY is a group of hereditary forms of diabetes which is caused by mutations in an autosomal dominant gene, many of which are transcription factors, which causes a disruption in insulin production[1].

Data from the 2011 National Diabetes Fact sheet (2011)[2] indicates that DM is a growing health concern, with 1.9 million new cases of diabetes having been diagnosed in people 20 years and older in 2010. 8.3% of the population (25.8 million people) in the United States, including children and adults, has DM and it is estimated that 79 million people are pre-diabetics. DM can result in serious, life-threatening, acute complications. These acute complications include

hypoglycemia, diabetic ketoacidosis, and hyperosmolar hyperglycemic nonketotic syndrome. Serious long term complications of DM, due to chronic elevation in blood glucose levels, include cardiovascular disease and stroke, high blood pressure, blindness due to retinal damage, chronic renal failure, neuropathy, and non-traumatic limb amputation. In 2007, morbidity and mortality rates show that DM was the primary cause of death in 71,382 people in the United States and it was a contributing factor for an additional 160,022 deaths. Therefore, it is of extreme importance that diabetics carefully monitor blood glucose levels, maintain adequate treatment, and control contributing factors such as diet and body weight.[2]

Current Treatment Modalities for Type I Diabetes Mellitus

The current treatment modality for Type I DM is based on management of the condition, not a cure for the disease[3]. Patients with Type I DM have to closely monitor blood glucose levels and administer insulin, which is done through subcutaneous injection of insulin or through an insulin pump, in order to avoid hypoglycemic and hyperglycemic episodes[1]. Unfortunately, even when patients with DM tightly adhere to the conventional treatment modalities, in many cases there is a failure for sufficiently tight control of blood glucose levels to avoid serious long term complications[4].

Islet Cell Transplantation Therapy

With the total costs of care for diabetics in the United States in 2007 being $174 billion, the rise in new cases of DM places a significant financial burden on society[2]. It is therefore important to focus efforts into finding a cure for the disease. A potential cure for Type I DM is transplantation of new pancreatic β-cells from a donor[5]. In this method of treatment, donor β-

cells are injected into the hepatic portal vein, where they lodge themselves and secrete insulin. Over the course of treatment, multiple donor organs are required for each patient. Transplantation is repeated 2-3 times over the course of a few months in order for sufficient numbers of β-cells to take up residence and begin to secrete enough insulin to improve blood glucose levels. With this modality of treatment 80% of recipients achieve insulin independence after transplantation, but only 10% remain insulin independent after long term follow up.[6] A difficulty with this treatment is that there are insufficient numbers of suitable donor organs available for this to be a practical therapeutic option. Another disadvantage is that donor cells are required and thus the side-effects of life-long immunosuppressive therapy need to be considered. Therefore, an alternative treatment needs to be developed, preferably one where β-cells that are generated are derived from the patient's own stem cells to avoid the need for post-operative immunosuppressive therapy. [7]

Generation of β-Cells from Stem Cells

The human body is comprised of over 200 cell types which all are derived from the fertilized oocyte[8]. These distinct cell types are derived from specialized cell types within the fertilized egg known as embryonic stem cells. Embryonic stem cells have the potential for unlimited self-renewal and to differentiate into all derivatives of the primary germ layers: ectoderm, endoderm, and mesoderm[8]. Because of their tremendous differentiation potential, embryonic stem cells are a promising source for generation of β-cells for islet cell transplantation therapy.

Multiple studies have shown that embryonic stem cells are capable of generating insulin producing islet-like cell clusters. Lumelsky (2001) generated three-dimensional clusters of cells

that resemble the topology of pancreatic islet cells from mouse embryonic stem cells, which release insulin in response to glucose[9]. Assady (2001) used human embryonic stem cells to generate cells with characteristics of insulin-producing β-cells and observed insulin secretion in a differentiation-dependent manner associated with the appearance of other β-cell markers [10]. D'Amour (2006) converted human embryonic stem cells into endocrine cells which produced insulin, glucagon, somatostatin, pancreatic polypeptide and ghrelin and found that the insulin producing cells that were generated had insulin content that approached that of adult islets [11]. Jiang (2007) developed a novel serum-free protocol to generate insulin-producing islet-like clusters from human embryonic stem cells and found that cells grown using this protocol contained higher levels of insulin compared to human fetal islets [12]. Research in the last decade has shown that embryonic stem cells have the potential to lead to a cure for Type I DM; however, the ethical and legal controversies regarding the use of embryonic stem cells in research limit their use in medicine.

In the adult, mesenchymal stem cells reside in some tissues and act as a source for regeneration in tissue repair[8]. In comparison to pluripotent embryonic stem cells, these cells are multipotent and can only produce a limited number of cell types. Without the ethical and legal implications of their use, mesenchymal stem cells are being widely evaluated for their use in β-cell replacement therapy. Chen (2004) found that functional islet-like cells can be derived from rat bone marrow mesenchymal stem cells and these cells could down-regulate glucose levels in diabetic rats[13]. In a study by Koblas (2009), human umbilical cord cells were shown to differentiate into insulin-producing cells in immunocompromised mice[14]. Yang (2002) showed that adult rat hepatic oval stem cells cultured in high-glucose induction media could be induced

to differentiate into three-dimensional islet-like clusters containing pancreatic endocrine hormone-producing cells[15]. Chandra (2009) developed a 10-day differentiation protocol to generate insulin-expressing islet-like cell aggregates from murine adipose tissue-derived stem cells [16] and using that protocol, generated islet-like cell aggregates from human adipose tissue derived stem cells and demonstrated that the islet-like cell aggregates can rescue diabetic mice[17].

Stem Cells of Neural Crest Origin

Neural crest stem cells are a population of transient, migratory cells that originate from the neural tube in vertebrates[18]. These cells ultimately travel through the embryo to give rise to multiple lineages, including neurons and glia of the peripheral and enteric nervous systems, endocrine cells, connective tissue cells, and melanocytes. It has previously been demonstrated that neural crest cells of the cranium have a higher level of plasticity, compared to their trunk, vagal/sacral, and cardiac counterparts, where with appropriate stimulation they have a greater ability to become cells of endodermal, ectodermal and mesodermal nature[19]. The neural crest stem cells have also been shown to be found within various derivatives and help with tissue regeneration and repair throughout adult life.

Stem cells of neural crest origin have been shown to reside within the periodontal ligament and within the cell rich zone of the dental pulp in adults. Huang (2009) demonstrated that stem cells derived from the periodontal ligament express embryonic and neural crest stem cell markers, including Oct4, Sox2, and Nanog, and Nestin, Slug, p75, and Sox10, respectively, and are capable of differentiating into neurogenic, cardiomyogenic, chondrogenic and osteogenic lineages[20]. Gronthos (2002) characterized the stem cell properties of human dental pulp stem

cells (DPSCs) and demonstrated that these cells are capable of forming ectopic dentin and pulp tissue, as well as forming adipocytes and neural-like cells[21]. When DPSCs from the permanent dentition were compared with DPSCs from the deciduous dentition, also known as stem cells from human exfoliated deciduous teeth (SHED)[22], the pluripotency of the cells from deciduous teeth was maintained for longer periods and they also demonstrated higher expression of endodemal and mesodermal markers; conversely, DPSCs from the permanent dentition had higher expression levels of neural/ectodermal markers[23].

The plastic and pluripotent characteristics of the stem cells found within dental tissues may allow them to be a possible source for the generation of insulin producing cells. In fact, research has shown that stem cells of dental origin have the potential to differentiate into pancreatic cells. Stem cells derived from the periodontal ligament when grown in suspension culture express genes of pancreatic origin, including PDX-1, Somatostatin, GLUT-2 , and Glucagon[20]. Stem cells derived from the dental pulp of the deciduous dentition have also been shown to form islet-like cell aggregates and release insulin in response to glucose challenge[24].

Role of ɤ-Aminobutyric Acid (GABA) in Insulin Secretion

ɤ-Aminobutyric acid (GABA) is the principle inhibitory neurotransmitter in the adult central nervous system (CNS)[25]. Within the neuron, GABA is synthesized from glutamate by the enzyme glutamic acid decarboxylase (GAD) and it is stored in synaptic vesicles. The pre-synaptic neuron releases GABA into the synaptic space when voltage-sensitive calcium channels cause an increase in cytosolic calcium levels. GABA then interacts with ionotropic receptors, primarily $GABA_A$ receptors, on the membrane of the post-synaptic neuron[26]. This leads to an

influx of chloride, which causes membrane hyperpolarization, and nerve inhibition. However, in the developing brain, GABA has been shown to play an excitatory role, for an example, in the early neonatal hippocampus. In the neonatal hippocampus, when GABA stimulates $GABA_A$ receptors on the post-synaptic neuron, chloride effluxes from the cells causing membrane depolarization and excitation[25].

In addition to its presence in the CNS, GABA has also been found to be present in other tissues, including the male and female reproductive organs, kidney, urinary bladder, stomach, intestine, liver and the pancreas[27]. The endocrine pancreas contains high levels of GABA, as well as the enzyme responsible for its production, GAD, in the core of the islets of Langerhans[28]. Within the islets of Langerhans, GABA has been shown to be localized to and produced by pancreatic β-cells[29]. When GABA is released it acts on the neighboring pancreatic α-cells in a paracrine fashion. This causes hyperpolarization of the α-cell membrane through interaction of GABA with $GABA_A$ receptors, which results in the inhibition of glucagon release[30]. In addition to α-cells, rather recently, β-cells have also been shown to express membrane $GABA_A$ receptors[31]. Adeghate (2002) demonstrated that stimulation of normal rat pancreatic tissue by GABA results in a significant increase in insulin secretion compared to streptozotocin induced diabetic rat pancreatic tissue[29]. The autocrine role of GABA on the β-cells was recently elucidated by Soltani (2011) and GABA was determined to have a depolarizing effect on the membrane of β-cells, and the resultant calcium influx activates the PI3-K/Akt-dependent growth and survival pathways[32]. Soltani also demonstrated that in severely diabetic mice, administration of GABA was able to restore β-cell mass and reverses the disease[32].

Summary

Diabetes Mellitus is a metabolic disease which affects a significant number of the population and is characterized by uncontrolled blood glucose levels, which results from defects in production and/or use of insulin. Currently, the treatment involves management of the disease through the use of medications to control blood glucose levels and supplement insulin. However, the ideal treatment modality for type-1 diabetes is autologous β-cell replacement therapy. Current investigations focus on the use of stem cells for the generation of physiologically competent, insulin-producing β-cells. The dental pulp contains multipotent mesenchymal progenitor cells known as dental pulp stem cells (DPSCs), which have been shown to form islet-like cell aggregates (ICAs) and release insulin in response to glucose challenge. GABA has been shown to be involved in an autocrine excitatory mechanism in β-cells and when administered *in* vivo causes increased insulin release, however, the direct effects of GABA on *in vitro* ICAs have not been elucidated.

Rationale

With the rise in the number of people with Diabetes Mellitus, and the huge financial burden that this disease places on society, finding a cure for the disease is important. β-cell replacement therapy through donor organs has been shown to have limited success, therefore finding another source, preferably autologous and homologous in nature, is needed. DPSCs have been shown to be capable of forming insulin producing ICAs which respond to glucose challenge, but whether or not the ICAs express $GABA_A$ receptors and respond to GABA by secreting higher levels of insulin, as seen *in vivo*, has not been elucidated. The aim of the current study is to generate

insulin-expressing ICAs from SHED and to determine the effect of GABA on the levels on insulin secretion in vitro. The results of this study will help improve our understanding of the potential for SHED as an autologous β-cell replacement therapy for diabetic patients.

HYPOTHESIS AND SPECIFIC AIMS:

Purpose:

To investigate the potential role of GABA on insulin secretion utilizing the *in vitro* system, insulin-secreting ICAs derived from SHED. We hypothesize that ICAs generated from SHED express $GABA_A$ receptors and respond to GABA during glucose challenge by increasing insulin secretion.

Specific Aims:

1. To generate ICAs from SHED utilizing the protocol previously established by Chandra (2009) and Govindasamy (2011).
2. To determine if the ICA induction protocol directs SHED away from the odontogenic lineage through Alkaline Phosphatase staining.
3. To determine if ICAs express $GABA_A$ receptors.
4. To determine if ICAs generated from SHED respond to GABA during glucose challenge by increasing insulin secretion levels.

MATERIALS AND METHODS:

Preparation of Samples:

SHED was harvested from sound, intact primary canines, of patients who had serial extractions performed at UCLA School of Dentistry, Los Angeles, CA. After extraction, the teeth were stored at 4˚C in α-Modified Eagle's Medium (MEM) (Invitrogen, Carlsbad, CA) supplemented with 10% Fetal Bovine Serum (FBS) (Invitrogen) and 5 µg/mL gentamicin sulfate (Gemini Bio-Products, West Sacramento, CA), to maintain freshness until extraction of the stem cells was performed (Basal Medium).

The extracted teeth were cleansed with a povodine iodine stick, in order to kill bacterial and fungal contaminants, and placed into a 100 mm tissue culture dish. The teeth were subsequently washed with 3-4 mL of sterile Phosphate Buffer Saline (PBS). The PBS was aspirated and the wash procedure was repeated three times, in order to remove residual povodine iodine. External tissues were removed from the samples (i.e. the attached fibers from the periodontal ligament, Stem Cells of Apical Papilla (SCAP), and gingival tissues) with a scalpel and discarded. With a large, disinfected wire cutter, the tooth was sectioned horizontally along the cementoenamel junction.

The pulp was then removed from the pulp chamber and root canals with a sterile dental explorer and placed in a sterile 100 mm tissue culture dish. 3 mL of dispase (4mg/mL PBS) was added to 3 mL collagenase (6 mg/mL PBS), and 1-2 drops of this enzyme mixture was added to the pulpal tissue (6 mL of enzyme solution per pulp) (Gibco, Life Technologies, Grand Island, NY). A

sterile scalpel was used to mince the pulpal tissue into small pieces until the tissue became paste-like. The minced tissue was placed into a 50 mL centrifuge tube, mixed with the remaining enzymatic solution, and incubated at 37˚ C for 1 hour. The reactions were vortexed every 10 minutes during incubation. After incubation, 6 mL of Basal Medium was added in order to neutralize the enzymatic reaction, and the solution was filtered through a 100 micron cell strainer. The filtered media was centrifuged at 1200 rpm for 4 min, at 4˚ C to pellet the stem cells. The media was aspirated off of the pellet, the cells were resuspended in fresh Basal Medium, and plated on 100 mm tissue culture dishes with a total volume of 6.5 mL per plate. Cultures were incubated in a humidified environment with 5% CO_2, at 37˚C for 24 hours and then the media was changed to fresh Basal Medium. The cells were allowed to grow until sub-confluence (~7-10days) and then were trypsinized.

1mL of Trypsin (Invitrogen, Carlsbad, CA) per 100 mm plate was added to each culture, the trypsin was swirled over the cells to collect unattached/dead cells, and then aspirated. Another 1 mL of Trypsin was added, swirled, and the plates were placed in a humidified environment with 5% CO_2, at 37˚C for 3-5 min (checking every 30 seconds). When the cells were detached from the plate, 2 mL of Basal Medium was added to neutralize the enzymatic reaction. The solution was washed over the plate to collect all of the cells and then transferred to a 15 mL centrifuge tube. Cells were counted using a hemocytometer and then centrifuged at 1200 rpm for 4 min, at 4˚ C. When passing the cells to 100 mm plates, $2x10^5$ cells were plated in 7 mL of Basal Medium, and when passing the cells to 6 well plates, $1x10^5$ cells were plated to each well in 3 mL of Basal Medium. All cultures in the study were in passage 3 through 5. SHED cultures used for this study were provided by UCLA School of Dentistry, Los Angeles, CA.

Fibroblasts isolated from the gingival tissue of a 36 year old male (11-36MOF) (UCLA School of Dentistry, Los Angeles, CA) were used as a negative control for this study and were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% Fetal Bovine Serum (FBS) (Invitrogen) and 5 µg/mL gentamicin sulfate (Gemini Bio-Products, West Sacramento, CA).

Differentiation of SHED into Islet-like Cell Aggregates (ICAs)

The SHED and Fibroblast cultures were grown to confluence and then trypsinized, as described as above. At the time of induction the SHED cultures were in passage 5 and the Fibroblast cultures were in passage 3. Differentiation experiments were carried out as previously described with slight modifications[16, 24]. After centrifugation of the trypsinized cultures, they were re-suspended in 4 mL of Serum-Free Medium–A (SFM-A) [Dulbecco's Modified Eagle's Medium/F-12 Knock Out, 17.5 mM Glucose, 1% Bovine Serum Albumin Cohn Fraction V (fatty acid free), 1X Insulin-Transferrin Selenium, 4 nM Activin A, 1 mM Sodium Butyrate, 50 µM 2-Mercaptoethanol, 5 µg/mL gentamicin sulfate] and plated on 60 mm borosilicate glass plates (Kimble-Kimax, Fisher Scientific, Pittsburgh, PA). The cultures were incubated in a humidified environment with 5% CO_2, at 37˚C for 48 hours. On day 3, the media was changed to Serum-Free Medium B (SFM-B) [Dulbecco's Modified Eagle's Medium/F-12 Knock Out, 17.5 mM Glucose, 1% Bovine Serum Albumin Cohn Fraction V (fatty acid free), 1X Insulin-Transferrin Selenium, 0.3 mM Taurine, 5 µg/mL gentamicin sulfate]. The plates were slowly swirled at 45˚ to pellet the cells with gravity (the pellet of cells should form on the trailing edge of the medium). The media was then gently aspirated, leaving only 200-300 µL of medium in the dish, and was replaced with 4 mL of SFM-B. After another 48 hours of incubation, the

medium was changed to Serum-Free Medium C (SFM-C) [Dulbecco's Modified Eagle's Medium/F-12 Knock Out, 17.5 mM Glucose, 1.5% Bovine Serum Albumin Cohn Fraction V (fatty acid free), 1X Insulin-Transferrin Selenium, 3 mM Taurine, 100 nM glucagon-like peptide-1, 1X Non-Essential Amino Acids, 5 µg/mL gentamicin sulfate]. The cells were incubated in SFM-C, which was refreshed every 48 hours for the next 10 days. Reagents were purchased from Invitrogen, Carlsbad, CA; Sigma-Aldrich, St. Louis, MO; and R&D Systems, Minneapolis, MN.

Odontogenic Differentiation of SHED

SHED and Fibroblasts (11-36 MOF) were allowed to grow to complete confluence in 6 well tissue culture dishes. When cultures were confluent, the medium was aspirated and then replaced with Odontogenic Induction Medium (α-MEM, 10% Fetal Bovine Serum, 100 mM Ascorbic Acid, 1M β-Glycerophosphate, 9mM Potassium Phosphate, 50 µM Dexamethasone, and 5 µg/mL gentamicin sulfate). Reagents were purchased from Invitrogen, Carlsbad, CA; and Sigma-Aldrich, St. Louis, MO. The plates were incubated in a humidified environment with 5% CO_2, at 37˚C for 10 days and the Odontogenic Induction Medium was refreshed every 2 days.

Dithizone (DTZ) Staining of ICAs

Dithizone (DTZ) is a zinc-chelating agent, which is known to selectively stain zinc containing cells, thus, pancreatic beta cells[33]. To stain ICAs, Millipore's Pancreatic Cell DTZ Detection Assay was used (Millipore, Billerica, MA). The protocol provided with the kit was modified slightly to work with small cultures of non-adherent, floating clusters. The DTZ Enzymatic Reaction was prepared by adding 40 µL of 100X DTZ Stain Solution to 4 mL SFM-C, for each

Day-10 ICA culture to be stained. The enzymatic reaction was filtered through the 0.22 µm Millex syringe-filter unit into a sterile 50 mL centrifuge tube. The media was carefully aspirated off of the ICAs, as described above, and 4 mL of the staining solution was added to each culture. The cultures were incubated in a humidified environment with 5% CO_2, at 37˚C for 30 minutes. Non-specific stain was removed by rinsing the culture with DTZ Rinse Solution (Hanks Balanced Salt Solution) 3 times. The cells were then examined for the presence of crimson red stain, as previously described, using light microscopy[33]. To reverse the live DTZ stain, the cells were incubated in a humidified environment with 5% CO_2, at 37˚C in SFM-C, for approximately 5 hours.

Odontogenic Staining Using Alkaline Phosphatase

SHED and Fibroblasts (11-36 MOF) were allowed to grow to complete confluence on 6 well plates. At the time of induction the SHED cultures were in passage 5 and the Fibroblast cultures were in passage 3. When cultures were completely confluent, the medium was aspirated and replaced with induction medium. The cells were either induced toward odontogenic lineage or toward ICAs, or they were maintained in their respective basal media (negative control). The plates were incubated in a humidified environment with 5% CO_2, at 37˚C for 10 days and the induction medium was refreshed every 2 days, as described in the protocols above.

On Day 10, the cultured plates were prepared for staining to measure alkaline phosphatase activity, using Alkaline Phosphatase Staining Kit (Sigma-Aldrich, St. Louis, Mo). The induction medium was carefully aspirated and each well was washed twice with phosphate buffer saline (PBS). The PBS wash was decanted and 2 mL of fixative solution (containing acetone, citrate,

and formaldehyde) was added to each well for 30 seconds and then decanted. The wells were rinsed with double distilled water (ddH_2O). The alkaline phosphatase staining solution was then prepared (containing Sodium Nitrate, FRV Alkaline, ddH_2O, Hapthol As-Bl ALK) and covered in aluminum foil (i.e. the staining solution is light sensitive). The rinse water was decanted from the wells, 2 mL of alkaline phosphatase staining solution was quickly added to each well and the plates were placed in the dark for 15 minutes. The stain solution was decanted and the wells were washed with ddH_2O. If alkaline phosphatase was present within the cell cultures, a magenta pink color appeared.

RNA Isolation

The adherent cell cultures were trypsinized, as described above, and collected in a 15 mL Falcon tube and the ICAs were transferred to a 15 mL Falcon tube. The cultures were centrifuged at 1200 rpm for 4 min, at 4˚ C. The supernatant was carefully aspirated, the cultures were resuspended in 1 mL of sterile PBS and transferred to a microfuge tube, and then centrifuged at 8000 rpm for 3 minutes. The wash buffer was aspirated and 1 mL of Trizol (Ambion, Life Technologies, Grand Island, NY) was added to lyse the cells. The lysate was passed through the pipette several times until no visible pellet was present. The homogenized cell lysate solution was incubated for 5 minutes at room temperature to allow the complete dissociation of nucleoprotein complexes. Next, 0.2 mL of chloroform was added, the sample was tightly sealed, and mixed vigorously by hand for 60 seconds, followed by another 10 minutes of incubation of the samples at room temperature. The samples were centrifuged at 12,000 rpm for 15 minutes, at 4˚ C and the aqueous phase (containing the RNA) was transferred to a clean microfuge tube. 0.5 mL of isopropyl alcohol was added to the aqueous phase, the samples were mixed by hand, and

allowed to incubate at room temperature for 10 minutes. The samples were centrifuged at 12,000 rpm for 15 minutes, at 4˚ C to pellet the RNA. The supernatant was aspirated, the RNA pellet was washed twice with 0.7 mL of 70% ethanol, and the RNA pellet was allowed to dry completely. Finally, 50 µL of RNase-free water was added to resuspend the RNA.

Real-Time Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR)

RNA concentration and purity was measured for each of the samples using the NanoDrop Spectrophotometer (ND v3.8.1). Human Pancreas Total RNA (Clontech Laboratories Inc., Mountain View, CA), was purchased and used as the positive control, as was done by Govindasamy.[24] The iScript cDNA Synthesis Kit was used to generate cDNA, as per the manufacturer's directions (BioRad, Hercules, CA) and 1.0 µg of RNA was used for cDNA synthesis per 20 µL reaction. The primers provided by the kit were a mixture of oligo(dT) and random primers. After cDNA synthesis, the 20 µL reaction volumes were diluted with 180 µL of nuclease-free water. The cDNA was amplified using Roche Lightcycler 480 SYBR Green Reverse Transcription Kit (Roche Applied Science, Indianapolis, IN). The primers were supplied by Integrated DNA Technologies, Coralville, IA and the sequences that were used for qRT-PCR are listed in Table 1. The reactions were performed in duplicate and triplicate with 10 µL reaction volumes. The reactions consisted of 4 µL of cDNA, 0.5 µL of forward primer (100 mM), 0.5 µL of reverse primer (100 mM), and 5 µL of SYBR Green I Master (Roche Applied Science). PCR amplification was carried out using the Roche Light Cycler 480 II (program: pre-incubation- 10 minutes at 95˚C; amplification- 60 cycles of 10 seconds at 95˚C, 45 seconds at 55˚C, 10 seconds at 72˚C; melting-curve- 5 seconds at 95˚C, 1 minute at 65˚C; cooling- 30

seconds at 40˚C). All qRT-PCR results were normalized to 18S RNA, which was carried out in duplicate reactions to correct for differences in the amount of RNA added.

Total Insulin Content and Release Assay: Glucose and ɤ-Aminobutyric Acid (GABA) Challenge

The total insulin content and release assay was carried out as previously described by Chandra [16] with slight modifications. 2 samples of approximately 200-300, Day-10 ICAs were selected, placed in an Eppendorf tubes and washed three times with sterile PBS. Each ICA sample was incubated in 100 µL of fresh Krebs-Ringer bicarbonate HEPES (KRBH) buffer (120 mM NaCl, 5 mM KCl, 2.5 mM $CaCl_2$, 1.1. mM $MgCl_2$, 25 mM $NaHCO_3$, 10 mM HEPES Buffer, and 0.1% BSA) without glucose for 4.5 hours at 37˚C. The supernatant was discarded and then each ICA sample was incubated with 100 µL of KRBH buffer with 5.5 mM glucose for 1 hour at 37˚C. For one ICA sample, the buffer was replaced with 100 µL of KRBH buffer with 22 mM glucose and the ICAs were incubated for another hour at 37˚C and then the supernatant was collected. For the other ICA sample, the buffer was replaced with KRBH buffer with 22 mM glucose and 100 µM GABA and the ICAs were incubated for another hour at 37˚C and then supernatant was collected. All of the supernatant samples were stored at -80˚C. After the glucose and GABA challenges were completed, the intracellular insulin was isolated by incubating the ICAs in 200 µL acid-ethanol (10M HCl + 70% Ethanol) overnight at 4˚C, followed by sonication of the ICAs. All reagents for the KRBH were supplied by Sigma-Aldrich, St. Louis MO.

Insulin concentrations of the supernatant samples were measured using Millipore's Human Insulin Enzyme-Linked Immunosorbent Assay (ELISA) kit (Millipore, Billerica, MA) as per the manufacturer's directions. Samples were run in duplicate. The absorbance of samples at 450 nm

was determined using spectrophotometry. The values for the standards were plotted using Excel to generate a dose-response curve and a line of best fit was created. From this line, the insulin concentration of the unknown samples was determined.

Statistics

The cDNA expression levels (normalized to 18S RNA, as was done by Govindasamy (2011)[24]), obtained from qRT-PCR, were analyzed by one-way Analysis of Variance (ANOVA), followed by post hoc Tukey analysis (Version 10.0, Statistica; Tulsa,OK; http://www.statsoft.com). Between group differences were considered statistically significant when $p < 0.05$.

Values for the Glucose/GABA Challenge and ELISA are expressed as the mean insulin secretion, standard deviation, and standard error of the mean. Due to the small sample size (n=2), statistical analysis was not performed.

Results:

Characterization of SHED

Undifferentiated SHED cultures displayed fibroblastic-like morphological characteristics similar to basal fibroblast cultures and bone marrow mesenchymal stem cells (BM-MSCs), as expected (Figure 2A & B). qRT-PCR analysis demonstrated that SHED basal cultures had a positive expression of pluripotent stem cell markers Oct3/4, Sox2, and Nanog, of which Oct3/4 and Nanog were was significantly higher than the expression levels found in Fibroblast basal cultures ($p<0.05$) (Table 2). Comparison of these markers between SHED basal cultures and Fibroblast basal cultures indicates that SHED has a four-fold higher expression level of Oct3/4 and Nanog in comparison to Fibroblasts (Figure 3). This finding demonstrates that the SHED cultures used in this study do express markers of undifferentiated embryonic stem cells, and therefore are stem cell-like in nature.

Differentiation of SHED into Pancreatic Lineage

As demonstrated by Govindasamy (2011), SHED that proliferated as an adherent monolayer clustered into spherical aggregates when transferred to non-adherent tissue culture conditions (Figure 2D). Fibroblast cultures also demonstrated the ability to form spherical aggregates in non-adherent tissue culture conditions (Figure 2C). On Day 10 of the ICA Induction, the majority of the SHED ICA clusters stained weakly positive for the presence of zinc with DTZ (Figure 2H). The red color, indicating a positive DTZ stain, was only visible by the naked eye. However, light microscopy did reveal morphological differences between the fibroblast clusters (Figure 2G) and SHED ICAs. Day-10 SHED ICAs were more tightly clustered and had a more

defined border than fibroblast clusters. Day-10 SHED ICAs also had more dense granules within the cluster compared to fibroblast clusters.

Staining of Day-10 SHED odontogenic induction cultures demonstrated that the cultures had positive activity for alkaline phosphatase (Figure 4A). Staining of Day-10 SHED ICA induction cultures demonstrated the loss of alkaline phosphatase activity (Figure 5A), demonstrating the ability of the ICA induction protocol to drive the cells away from the odontogenic lineage. Day-10 Fibroblast odontogenic (Figure 4B) and ICA (Figure 5B) induction cultures were negative for alkaline phosphatase activity, which indicates the fibroblasts lack the potential to differentiate into the odontogenic lineage.

Results of qRT-PCR Assays

A brief overview of the temporal sequence of pancreatic marker expression is seen in Figure 6 [24]. qRT-PCR demonstrated that SHED Day-5 ICAs compared to the SHED basal culture had a greater transcript abundance for a few of the pancreatic markers: NeuroD (5-fold), Isl-1 (6-fold), and Glut-2 (1.7-fold). At Day-10, however, cultures had greater transcript abundance for all of the pancreatic markers analyzed: PDX-1 (3-fold), Neuro-D (11-fold), Nkx6.1 (1.5-fold), Isl-1 (8-fold), Glut-2 (10-fold) and insulin (2.8-fold) (Figure 7). SHED Day-10 ICAs had significantly higher expression levels of PDX-1, Nkx6.1, Isl-1, Glut-2 than the SHED basal culture, SHED Day-5 ICAs, and Total Pancreatic RNA ($p<0.05$) (Table 4). Neuro-D expression levels of SHED Day-10 ICAs were significantly higher than the SHED basal culture and Total Pancreatic RNA ($p<0.05$) (Table 4). Insulin levels for SHED Day-10 ICAs were significantly higher than the

SHED basal culture and SHED Day-5 ICAs, however, they were significantly lower than Total Pancreatic RNA ($p<0.05$) (Table 4).

Fibroblast Day-10 ICA cultures had a minimal increase in transcript abundance for a few of the pancreatic markers: PDX-1 (1.7-fold), NeuroD (2-fold), Isl-1 (2-fold); however, insulin levels were almost 9-fold higher than the basal culture (Figure 8). The differences in expression of PDX-1 between the Fibroblast basal culture, Fibroblast Day-10 ICAs and Total Pancreatic RNA were not statistically significant ($p>0.05$) (Table 5). Insulin expression was not statistically significant between the Fibroblast basal culture and Fibroblast Day-10 ICAs; they were, however, statistically lower than the purchased Total Pancreatic RNA ($p>0.05$) (Table 5). Expression of Neuro-D, Nkx6.1, Isl-1, and Glut-2 was significantly higher in Fibroblast Day-10 ICAs, compared to the Fibroblast basal culture and Total Pancreatic RNA ($p>0.05$) (Table 5).

The expression of the endodermal markers was compared between the SHED basal culture, SHED Day-5 ICAs and SHED Day-10 ICAs using qRT-PCR analysis. At Day-5 an increase in transcript levels for GATA4 was detected (3.4-fold), and by Day-10, Sox17 had increased 2.3-times basal levels and GATA4 had increased 55.9-times basal levels (Figure 9). The expression levels of Sox-17 and GATA-4 seen in SHED Day-10 ICAs was statistically higher than the expression levels seen in the SHED basal culture and in SHED Day-5 ICAs ($p<0.05$) (Table 6).

qRT-PCR was also used to evaluate the expression levels of $GABA_A$ receptors. Results demonstrate increased transcriptional activities for all four of the receptors analyzed: GABAα-1 (7.2-fold), GABAα2 (7.6-fold), GABAα3 (2.3-fold), and GABAα6 (4.3-fold) (Figure 10). The

increased expression levels of these receptors in SHED Day-5 and Day-10 ICAs was significantly higher than SHED basal cultures ($p<0.05$) (Table 7).

Total Insulin Content and Release Assay: Glucose and ɤ-Aminobutyric Acid (GABA) Challenge

The absorbance values for ELISA are listed in Table 7. The concentration of insulin release was calculated based on a line of best fit drawn on the ELISA Insulin Dose- Response Curve generated from the known insulin concentrations provided by the kit (Table 7, Figure 11 & 12). The volume of the samples for the challenge was 100 μL for all of the samples, except sample #2- 22 μM glucose and 100 nM GABA challenge, which was 120 μL; thus, for a more accurate comparison of the change in insulin secretion, the mean total amount of insulin per sample was calculated (Table 8, Figure 13). The mean amount of insulin released by SHED Day-10 ICAs when they were exposed to basal glucose concentrations (5.5 mM) for sample #1 was 7.27 μU of insulin and sample #2 was 8.53 μU of insulin. When sample #1 was challenged with 22 μM glucose, the mean total insulin release was 3.79 μU. Sample # 2 released 5.85 μU of insulin during the 22 μM glucose and 100 nM GABA challenge. The mean total intracellular insulin content of sample #1 and sample #2 SHED Day-10 ICAs was 5.55 μU and 4.34 μU, respectively. To determine if GABA had any effect of insulin secretion, the reduction in insulin release between basal and challenge conditions were calculated; the total insulin release was reduced by 3.5 μU for sample #1 and by 2.7 μU for sample #2 (Table 9, Figure 14).

Discussion:

Initial experiments were conducted to investigate the ability of the ICA induction protocol provided by Govindasamy (2011)[24] to drive SHED away from the odontogenic lineage, by evaluating the cultures for alkaline phosphatase activity. The cells were allowed to grow in adherent conditions to complete confluence before the induction protocol was initiated. SHED grown in odontogenic and basal conditions stained positive for alkaline phosphatase activity. Basal conditions caused SHED cultures to stain positive for alkaline phosphatase activity because cell-to-cell contact stimulates the cells to begin differentiation toward the odontogenic lineage. Confluent SHED cultures grown in the ICA induction medium (SFM-A, SFM-B, and SFM-C) were negative for alkaline phosphatase activity, demonstrating the ability of the ICA induction protocol to drive the cells away from the odontogenic lineage. However, the remaining experiments to confirm the positive ICA phenotype and genotype demonstrated by Govindasamy, indicate that our ICAs were immature in comparison.

There are a couple of possibilities why Govindasamy's (2011) results were not able to be repeated and our cultures appear to be immature ICAs in comparison. The first possibility is that the SHED cells used in this study were significantly older. Govindasamy reports that all of the experiments utilized cells that were in passage 2[24]. For the current study, however, due to the limited supply of SHED cells, SHED cells in passage 5 were utilized for the induction. The cells may have been too close to senescence and lost some of their pluripotent abilities, preventing the ICAs from fully maturing[34]. Another explanation may be that the protocol that was reported in the literature did not contain all of the details; therefore, a key step, or ingredient may have been missed.

The non-adherent SHED cultures grown according to the 10-day ICA induction protocol were able to form ICAs. Although, the experiments to confirm that they are in fact insulin producing aggregates, again, lead us to believe that the aggregates were immature in comparison to the aggregates that Govindasamy was able to produce. The first experiment to confirm the production of insulin by the ICAs, DTZ staining, was inconclusive. The clusters appeared to have a light red stain which was visible by the naked eye. This indicated that the aggregates may be expressing low levels of insulin, giving them a light red color, but the insulin production over the 10-Day differentiation protocol was not sufficiently high to produce the crimson red color. The phenotypic differences seen between the aggregates formed by Fibroblasts and ICAs formed by SHED may be indicative of SHED ICAs differentiating toward pancreatic β-cells. The larger granules seen in SHED ICAs may represent cells within the cluster that were further differentiated toward β-cells. These cells may have taken up more stain than the surrounding cells, making them appear dark, however, not enough stain was taken up to show the expected red color.

The qRT-PCR analysis done by Govidasamy (2011), showed that the Day-10 ICAs had 55-fold increase of PDX-1, 49-fold increase of Nkx6.1, 32-fold increase of Isl-1, 128-fold increase of Glut2, and 26-fold increase of Insulin[24]. The qRT- PCR assay in the current study did indicate that by Day-10 the ICAs were headed toward the pancreatic lineage with 3-fold increase in PDX-1, 1.5-fold increase of Nkx6.1, 8-fold increase of Glut-2, and 2.8-fold increase of Insulin compared to basal levels. However, even though all of the markers analyzed showed increased

transcript abundance, compared to the expression generated by Govindasamy (2011) the amount of increase indicates that the aggregates were immature.

Insulin ELISA demonstrated that the Day-10 ICAs secreted insulin in response to glucose. However, when the Day-10 ICAs were challenged with 22 mM glucose concentration, they continued to secrete insulin, but at a reduced level compared to insulin secretion at basal glucose concentrations. This finding is consistent with the DTZ staining and qRT-PCR data. If the ICAs are immature, as we presume, the amount of insulin stored within the aggregates is expected to be lower than if they were completely differentiated into the pancreatic β-cells. It is thought that due to the low insulin levels stored within the ICAs, when the aggregates were challenged with 22 mM glucose concentration, the insulin storage had been mostly depleted with the basal glucose challenge, preventing the ICAs to respond with increased insulin secretion. To confirm the presence of insulin within the ICAs, it would have been ideal repeat the induction, with younger cells, and run Western Blot assay. Therefore, future studies should include Western Blot experiments for confirmation on insulin production by ICAs.

This investigation also examined the effects of GABA on insulin secretion. With the recent demonstration that β-cells express $GABA_A$ receptors[31], and the finding that GABA is a strong secretagogue of insulin from the pancreas in rats[29], we assessed whether or not ICAs generated from SHED express $GABA_A$ receptors through qRT-PCR and if they respond by increased insulin secretion with the addition of GABA to the glucose challenge experiments. qRT-PCR demonstrated that Day-10 ICAs had increased expression of GABAα-1 (7.2-fold), GABAα2 (7.6-fold), GABAα3 (2.3-fold), and GABAα6 (4.3-fold) compared to basal levels. This finding

is consistent with expression of $GABA_A$ receptors in pancreatic β-cells and indicates that the Day-10 ICAs should respond to GABA by increased insulin secretion. However, the reduction of insulin secretion for the Glucose + GABA challenge compared to basal glucose for ELISA made it difficult to assess whether GABA had any effect of insulin secretion. For a preliminary evaluation of the effect of GABA in this study, the reduction in insulin secretion between the basal glucose and 22 mM glucose/22 mM glucose + GABA for the two samples were compared. Sample #2, 22 mM glucose + GABA challenge, had less of a difference compared to the basal, than Sample #1, 22 mM glucose challenge. This finding suggests that GABA may increase insulin secretion. However, to confirm this, the induction protocol needs to be re-evaluated and then utilized with young SHED cells and the glucose + GABA challenge needs to be repeated with fully mature ICAs.

Conclusion:

We conclude that:

1) SHED is capable of differentiating toward the pancreatic lineage and producing insulin. In the future these cells may be a potential source for β-cell replacement therapy in Type I diabetic patients.
2) Day-10 ICAs have positive expression of $GABA_A$ receptors and therefore are capable of responding to GABA.

Future Direction of Research:

The first step for future research should be to collect and isolate fresh SHED from the pulp of deciduous teeth. The cells in passage 2 should be utilized for the induction protocol to determine if the results from Govindasamy (2011) are repeatable. Once the protocol has been elucidated, another step would be to add GABA into the induction medium to determine if it has any effect on insulin production. Soltani (2011) reports that GABA has a trophic effect on β-cells and in diabetic mice GABA is able to restore β-cell mass and reverse the disease[32]. Therefore, including GABA into the induction protocol for SHED may increase β-cell mass of the ICAs and enhance insulin production, making ICAs a stronger candidate for Type-I diabetes β-cell replacement therapy.

Tables:

Table 1: Sequence of Specific Primers used in qRT-PCR and PCR

Primer:	Forward:	Reverse:
18S RNA	5'CGG CTA CAT CCA AGG AA3'	5'TCG CGC GCC ATT AAG GTC G3'
Oct3/4	5'CGA CCA TCT GCC GCT TTG AG3'	5'CCC CCT GTC CCC CAT TCC TA3'
Nanog	5'CCT TCC TCC ATG GAT CTG CTT ATT CA3'	5'TCT TCA CCT GTT TGT AGC TGA3'
Sox2	5'CCC CCG GCG GCA ATA GCA3'	5'TCG GCG CCG GGG AGA TAC AT3'
Sox17	5'CGC ACG GAA TTT GAA CAG TA3'	5'GGA TCA GGG ACC TGT CAC AC3'
GATA4	5'CTC CTT CAG GCA GTG AGA GC3'	5'GAG ATG CAG TGT CGT GC3'
PDX-1	5'GTC CTG GAG GAG CCC AAC3'	5'GCA GTC CTG CTC AGG CTC3'
NeuroD	5'AAG CCA TGA ACG CAG AGG AGG ACT3'	5'AGC TGT CCA TGG TAC CGT AA3'
Nkx6.1	5'GTT CCT CCT CCT CCT CTT CCT C3'	5'AAG ATC TGC TGT CCG GAA AAA G3'
Isl-1	5'GAT TTC CCT ATG TGT TGG TTG C3'	5'CTT CCA CTG GGT TAG CCT GTA A3'
Glut-2	5'GGT TTG TAA CTT ATG CCT AAG3'	5'GCC TAG TTA TGC ATT GCA G3'
Insulin	5'AGC CTT TGT GAA CCA ACA CC3'	5'GCT GGT AGA GGG AGC AGA TG3'
$GABA_A$ α-1	5'GCC TCA GCT AAA AGC CCC CAC A3'	5'TGC TTT GCT CCA GAC TGC CAG A3'
$GABA_A$ α-2	5'TGC AAC CAC GCC AGA ACC CAA3'	5'TGG CTG TTT TCG CAT GGA GTG CTT3'
$GABA_A$ α-3	5'TGC ACT CTG CCC GCT GCA AAA3'	5'AGG TGG CAC GCA GGA TCA CA3'
$GABA_A$ α-6	5'TTG AAT AGC TTG CGG CCA GGA CAA3'	5'TTT GCC CAC ACA TGC TTA CGG A3'

Table 2: One-Way ANOVA with Post-hoc Tukey Analysis to Compare Expression of Stem Cell Markers in Basal Fibroblast and SHED Cultures. $p < 0.05$ is red.

df = 4	Fibroblast-Basal	SHED-Basal
Oct3/4	mean = 0.16626	mean = 0.69059
Fibroblast-Basal		0.017246
SHED-Basal	0.017246	
Nanog	mean = 0.08965	mean = 0.36448
Fibroblast-Basal		0.027618
SHED-Basal	0.027618	
Sox-2	mean = 0.00271	mean = 0.01044
Fibroblast-Basal		0.008017
SHED-Basal	0.008017	

Table 3: One-Way ANOVA with Post-hoc Tukey Analysis to Compare Expression of Pancreatic β-cell Markers in Basal SHED Cultures, SHED Day-5 ICAs, SHED Day-10 ICAs & Total Pancreatic RNA. $p < 0.05$ is red.

df = 8	SHED-Basal	SHED- D5 ICAs	SHED- D10 ICAs	Total RNA
PDX-1	mean = 2.0329	mean = 2.8460	mean = 7.9867	mean = 0.39103
SHED-Basal		0.449688	0.000234	0.053892
SHED- D5 ICAs	0.449688		0.000252	0.006745
SHED- D10 ICAs	0.000234	0.000252		0.000231
Total RNA	0.053892	0.006745	0.000231	
Neuro-D	mean = 0.03540	mean = 0.19196	mean = 0.40567	mean = 0.00767
SHED-Basal		0.244439	0.005543	0.982292
SHED- D5 ICAs	0.244439		0.086931	0.149407
SHED- D10 ICAs	0.005543	0.086931		0.003618
Total RNA	0.982292	0.149407	0.003618	
Nkx6.1	mean = 15.213	mean = 15.342	mean = 22.927	mean = 2.5504
SHED-Basal		0.999544	0.001048	0.000240
SHED- D5 ICAs	0.999544		0.001156	0.000239
SHED- D10 ICAs	0.001048	0.001156		0.000231
Total RNA	0.000240	0.000239	0.000231	
Isl-1	mean = 0.03902	mean = 0.24459	mean = 0.33600	mean = 0.01009
SHED-Basal		0.000231	0.000231	0.344490
SHED- D5 ICAs	0.000231		0.002302	0.000231
SHED- D10 ICAs	0.000231	0.002302		0.000231
Total RNA	0.344490	0.000231	0.000231	
Glut-2	mean = 0.02279	mean = 0.03869	mean = 0.24943	mean = 0.00986
SHED-Basal		0.215148	0.000231	0.360221
SHED- D5 ICAs	0.215148		0.000231	0.018970
SHED- D10 ICAs	0.000231	0.000231		0.000231
Total RNA	0.360221	0.018970	0.000231	
Insulin	mean = 3.2222	mean = 3.6463	mean = 9.0550	mean = 93.395
SHED-Basal		0.974428	0.002087	0.000231
SHED- D5 ICAs	0.974428		0.003261	0.000231
SHED- D10 ICAs	0.002087	0.003261		0.000231
Total RNA	0.000231	0.000231	0.000231	

Table 4: One-Way ANOVA with Post-hoc Tukey Analysis to Compare Expression of Pancreatic β-cell Markers in Basal Fibroblast Cultures, Fibroblast Day-10 ICAs & Total Pancreatic RNA. $p < 0.05$ is red.

df = 6	Fibroblasts-Basal	Fibroblasts-D10 ICAs	Total Pancreatic RNA
PDX-1	mean = 0.41876	mean = 0.72168	mean = 0.39103
Fibroblasts-Basal		0.108363	0.972759
Fibroblasts-D10 ICAs	0.108363		0.081463
Total Pancreatic RNA	0.972759	0.081463	
Neuro-D	mean = 0.05725	mean = 0.12162	mean = 0.00767
Fibroblasts-Basal		0.007088	0.023038
Fibroblasts-D10 ICAs	0.007088		0.000529
Total Pancreatic RNA	0.023038	0.000529	
Nkx6.1	mean = 6.3450	mean = 9.7799	mean = 2.5504
Fibroblasts-Basal		0.019575	0.012580
Fibroblasts-D10 ICAs	0.019575		0.000638
Total Pancreatic RNA	0.012580	0.000638	
Isl-1	mean = 0.03506	mean = 0.07639	mean = 0.01009
Fibroblasts-Basal		0.014786	0.103594
Fibroblasts-D10 ICAs	0.014786		0.001594
Total Pancreatic RNA	0.103594	0.001594	
Glut-2	mean = 0.01687	mean = 0.03343	mean = 0.00986
Fibroblasts-Basal		0.032041	0.374543
Fibroblasts-D10 ICAs	0.032041		0.006672
Total Pancreatic RNA	0.374543	0.006672	
Insulin	mean = 0.22161	mean = 1.9647	mean = 93.395
Fibroblasts-Basal		0.406830	0.000227
Fibroblasts-D10 ICAs	0.406830		0.000227
Total Pancreatic RNA	0.000227	0.000227	

Table 5: One-Way ANOVA with Post-hoc Tukey Analysis to Compare Expression of Endodermal Markers in Basal SHED Cultures, SHED Day-5 ICAs, & SHED Day-10 ICAs. $p < 0.05$ is red.

df = 6	SHED- Basal	SHED- D5 ICAs	SHED- D10 ICAs
Sox-17	mean = 0.33698	mean = 0.25074	mean = 0.78782
SHED- Basal		0.289717	0.000492
SHED- D5 ICAs	0.289717		0.000315
SHED- D10 ICAs	0.000492	0.000315	
GATA-4	mean = 0.00413	mean = 0.01412	mean = 0.23112
SHED- Basal		0.854645	0.000246
SHED- D5 ICAs	0.854645		0.000254
SHED- D10 ICAs	0.000246	0.000254	

Table 6: One-Way ANOVA with Post-hoc Tukey Analysis to Compare Expression of $GABA_A$ Receptors in Basal SHED Cultures, SHED Day-5 ICAs, & SHED Day-10 ICAs. $p<0.05$ is red.

df = 6	SHED- Basal	SHED- D5 ICAs	SHED- D10 ICAs
$GABA_A$-α1	mean = 1.4913	mean = 1.7687	mean = 10.772
SHED- Basal		0.933942	0.000253
SHED- D5 ICAs	0.933942		0.000260
SHED- D10 ICAs	0.000253	0.000260	
$GABA_A$-α2	mean = 0.20300	mean = 0.27967	mean = 1.5462
SHED- Basal		0.064968	0.000227
SHED- D5 ICAs	0.064968		0.000227
SHED- D10 ICAs	0.000227	0.000227	
$GABA_A$-α3	mean = 1.0687	mean = 1.2294	mean = 2.4781
SHED- Basal		0.789648	0.002801
SHED- D5 ICAs	0.789648		0.005019
SHED- D10 ICAs	0.002801	0.005019	
$GABA_A$-α6	mean = 0.09976	mean = 0.06044	mean = 0.42665
SHED- Basal		0.581135	0.000510
SHED- D5 ICAs	0.581135		0.000373
SHED- D10 ICAs	0.000510	0.000373	

Table 7: Absorbance Values for Human Insulin Enzyme-Linked Immunosorbent Assay, Concentrations Generated from the ELISA Insulin Dose-Response Curve (y = 0.16x + 0.1962) for each sample, and the Total Insulin Secreted during Glucose/GABA Challenge.

	Abs @ 450 nm	Concentration (µU/mL)	Total Insulin (µU)
Sample 1- 5.5mM Glu	1.417	75.82608696	7.582608696
Sample1-5.5mM Glu	1.318	69.67701863	6.967701863
Sample 1- 22 mM Glu	0.787	36.69565217	3.669565217
Sample 1- 22 mM Glu	0.827	39.18012422	3.918012422
Sample 1- Total Insulin	0.665	29.11801242	5.823602484
Sample 1- Total Insulin	0.621	26.38509317	5.277018634
Sample 2- 5.5 mM Glu	1.593	86.75776398	8.675776398
Sample 2- 5.5 mM Glu	1.546	83.83850932	8.383850932
Sample 2- 22 mM Glu + 100 µM GABA	0.921	45.01863354	5.402236025
Sample 2- 22 mM Glu + 100 µM GABA	1.043	52.59627329	6.311552795
Sample 2- Total Insulin	0.47	17.00621118	3.401242236
Sample 2- Total Insulin	0.621	26.38509317	5.277018634

Table 8: The Mean, Standard Deviation, and the Standard Error of the Mean for Total Insulin in each Sample Generated in the Glucose/GABA Challenge

Mean Sample 1- 5.5 mM Glucose	7.27515528
Std Deviation	0.434804791
Std Error of the Mean	0.307453416
Mean Sample 1- 22uM Glucose	3.79378882
Std Deviation	0.175678703
Std Error of the Mean	0.124223602
Mean Sample 1- Total Insulin	5.550310559
Std Deviation	0.386493147
Std Error of Mean	0.273291925
Mean Sample 2- 5.5 mM Glucose	8.529813665
Std Deviation	0.386493147
Std Error of the Mean	0.273291925
Mean Sample 2- 20mM Glu + 100 µM GABA	5.85689441
Std Deviation	0.642984054
Std Error of the Mean	0.454658385
Mean Sample 2- Total Insulin	4.339130435
Std Deviation	1.326374211
Std Error of the Mean	0.937888199

Table 9: Reduction in Insulin Released from Basal Conditions to Glucose/GABA Challenge

Samples:	Difference (Basal - Challenge)
Sample 1 (Glucose Only)	3.481276 µU
Sample 2 (Glucose + GABA)	2.67292 µU

Figures:

Figure 1: Schematic Representation of the Stepwise Protocol used to generate ICAs

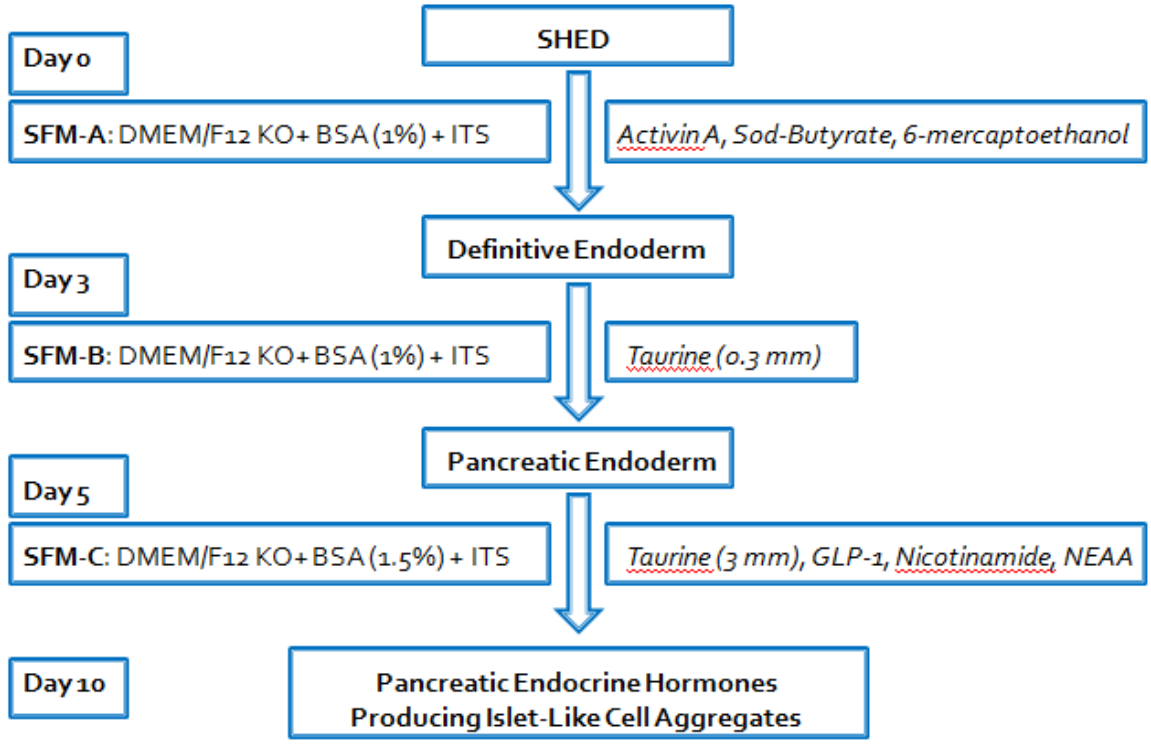
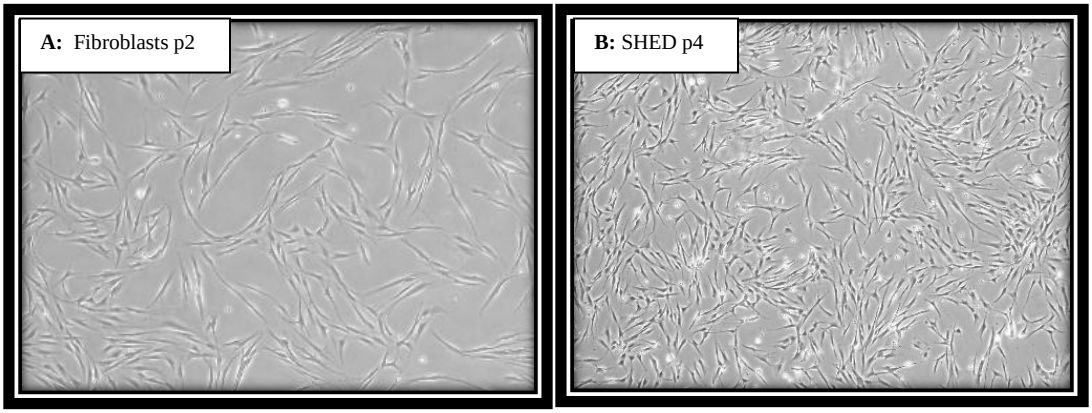


Figure 2: Phenotypic Changes seen in Fibroblast and SHED over the 10-day Differentiation Protocol



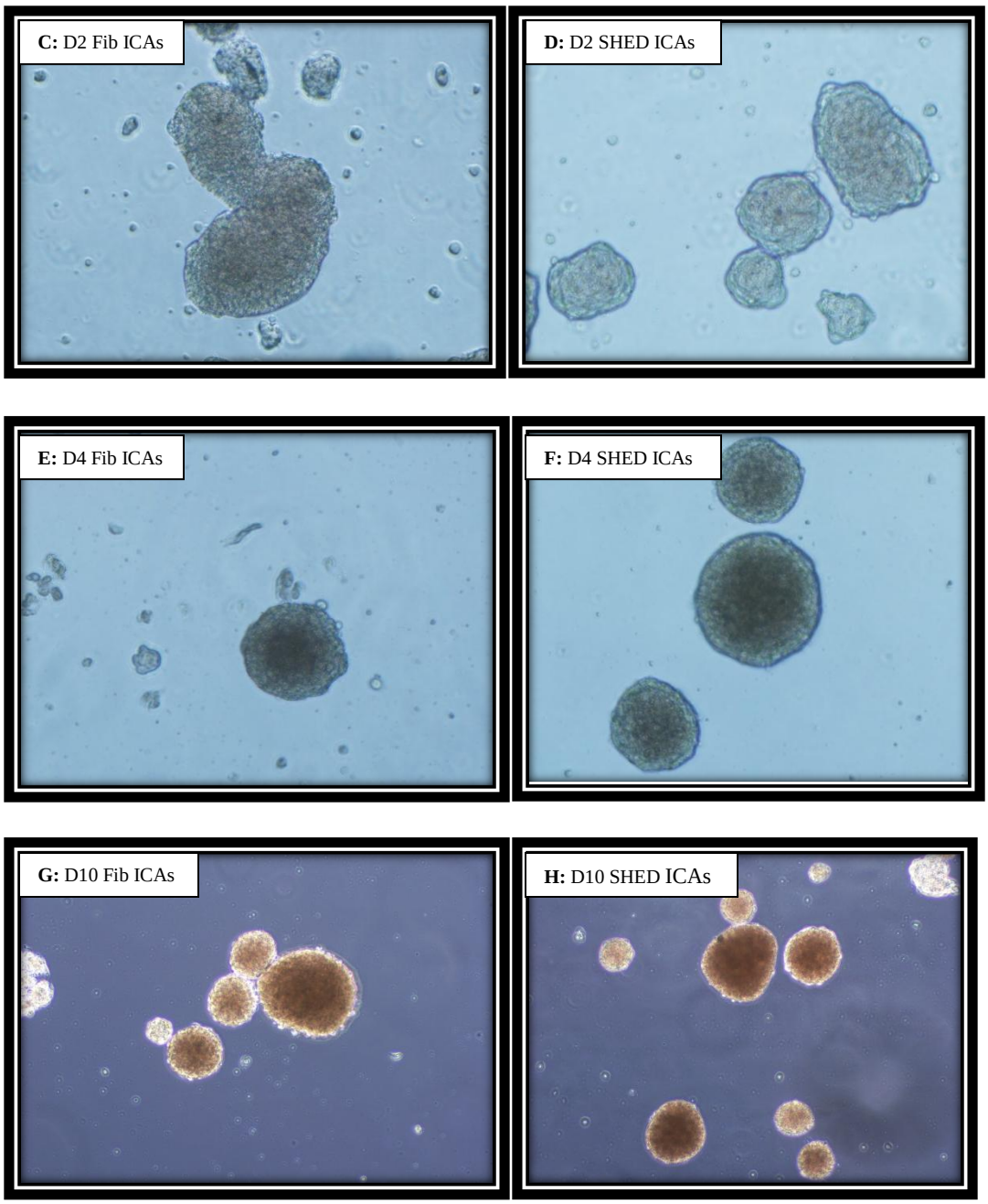

C: D2 Fib ICAs
D: D2 SHED ICAs
E: D4 Fib ICAs
F: D4 SHED ICAs
G: D10 Fib ICAs
H: D10 SHED ICAs

Figure 3: Real-time Quantitative RT-PCR Results for Stem Cell Markers

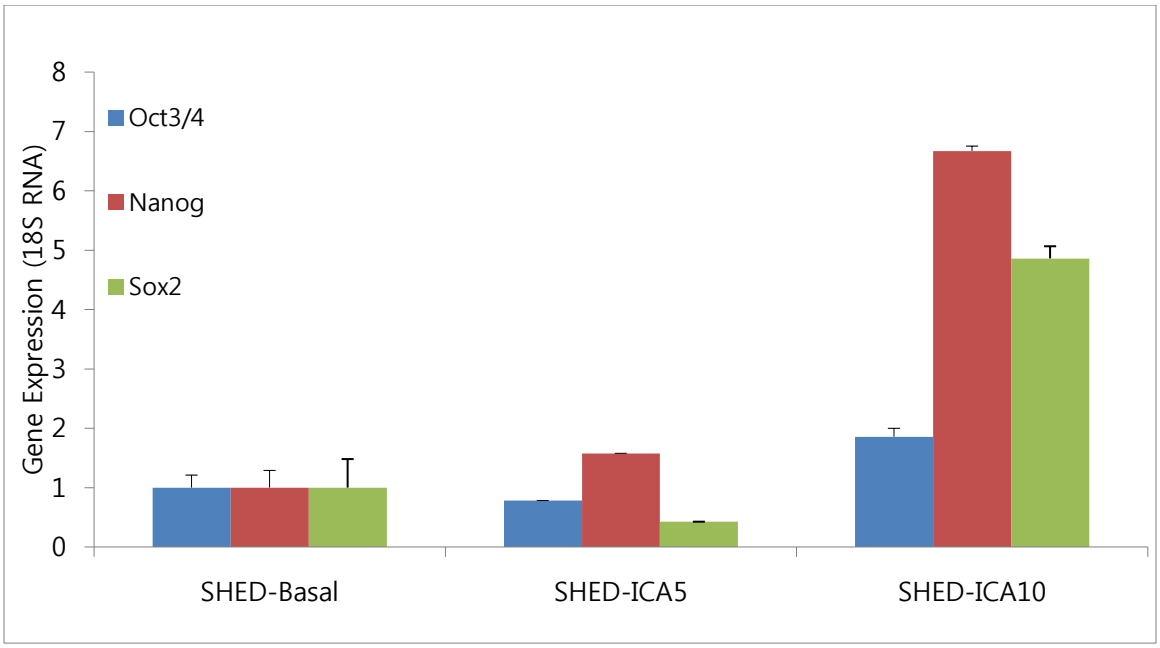


Figure 4: Alkaline Phosphatase Staining of Adherent Odontogenic Cultures

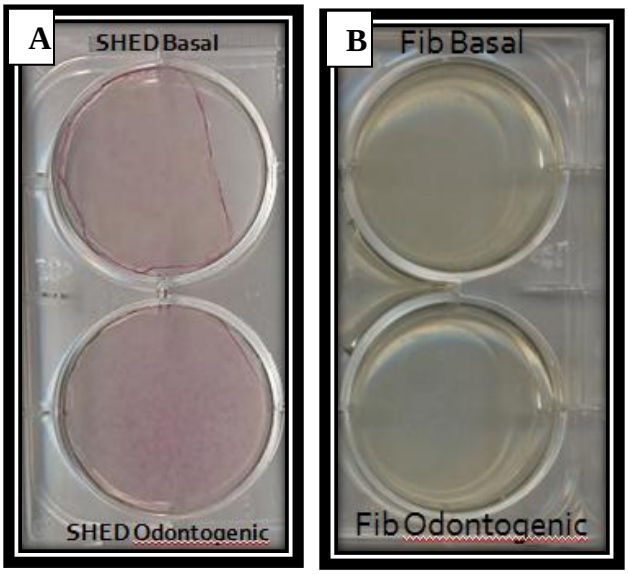


Figure 5: Alkaline Phosphatase Staining of Adherent "ICA" Cultures

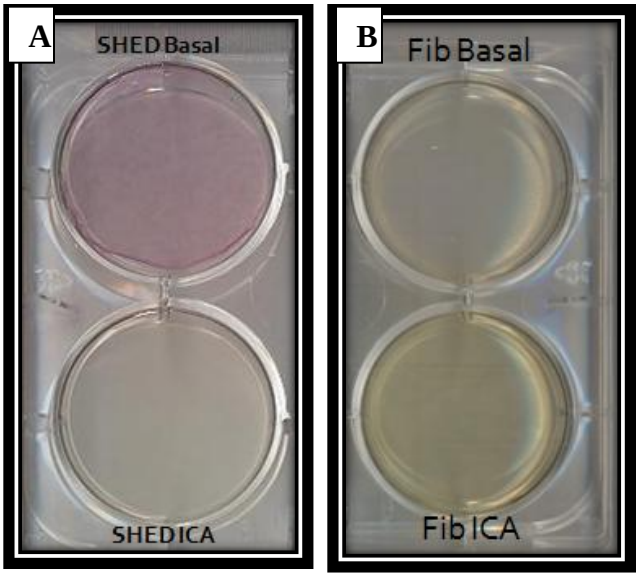


Figure 6: Schematic Representation of the Temporal Sequence of Pancreatic Transcription Factors known to be expressed during development[24]

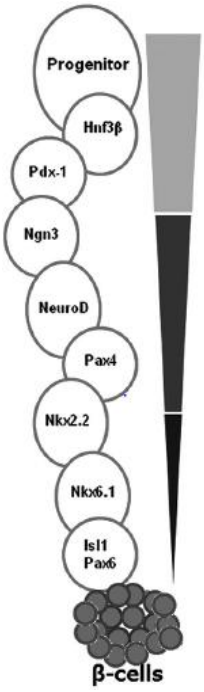


Figure 7: Real-time Quantitative RT-PCR results- SHED Beta Cell Markers

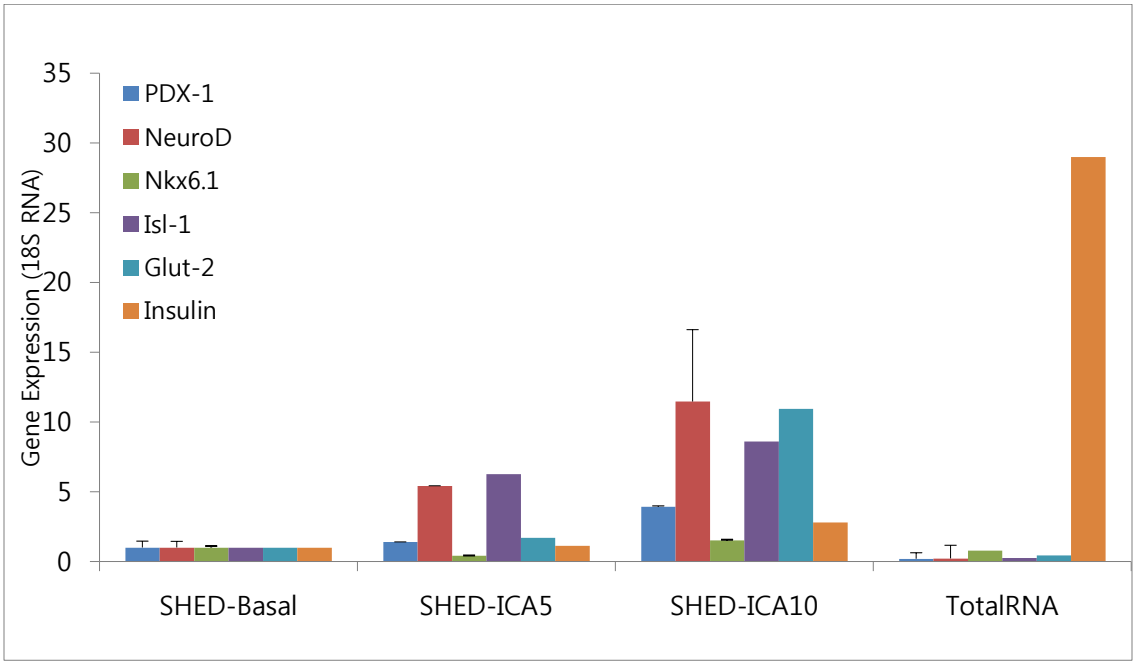


Figure 8: Real-time Quantitative RT-PCR results- Fibroblast Beta Cell Markers

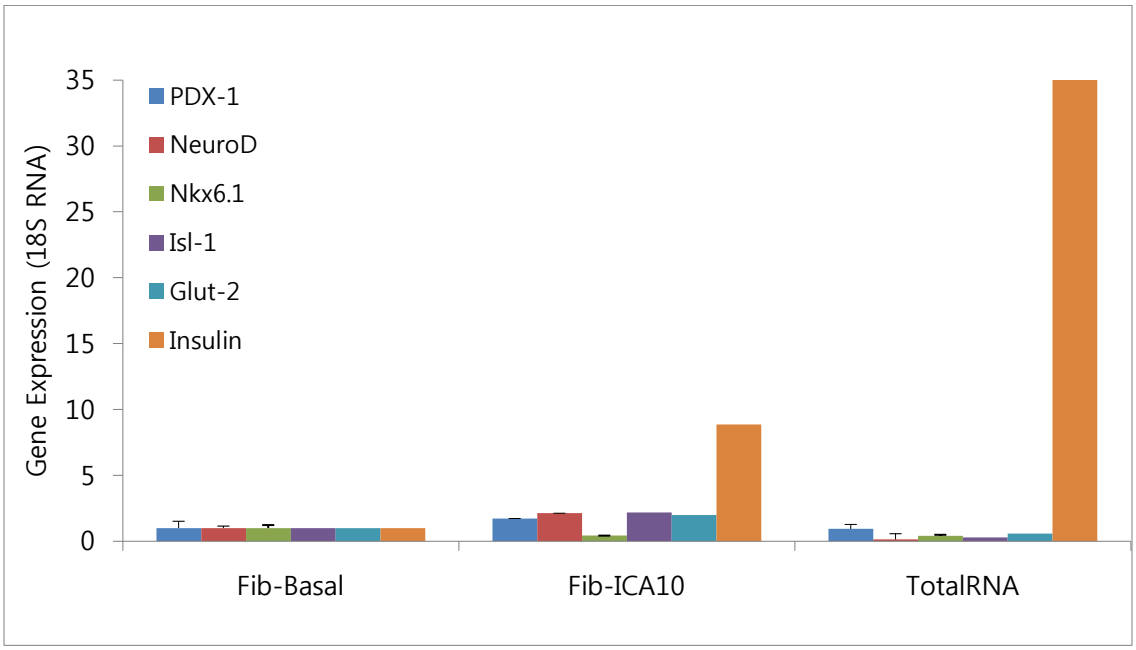


Figure 9: Real-time Quantitative RT-PCR results- SHED Endodermal Markers

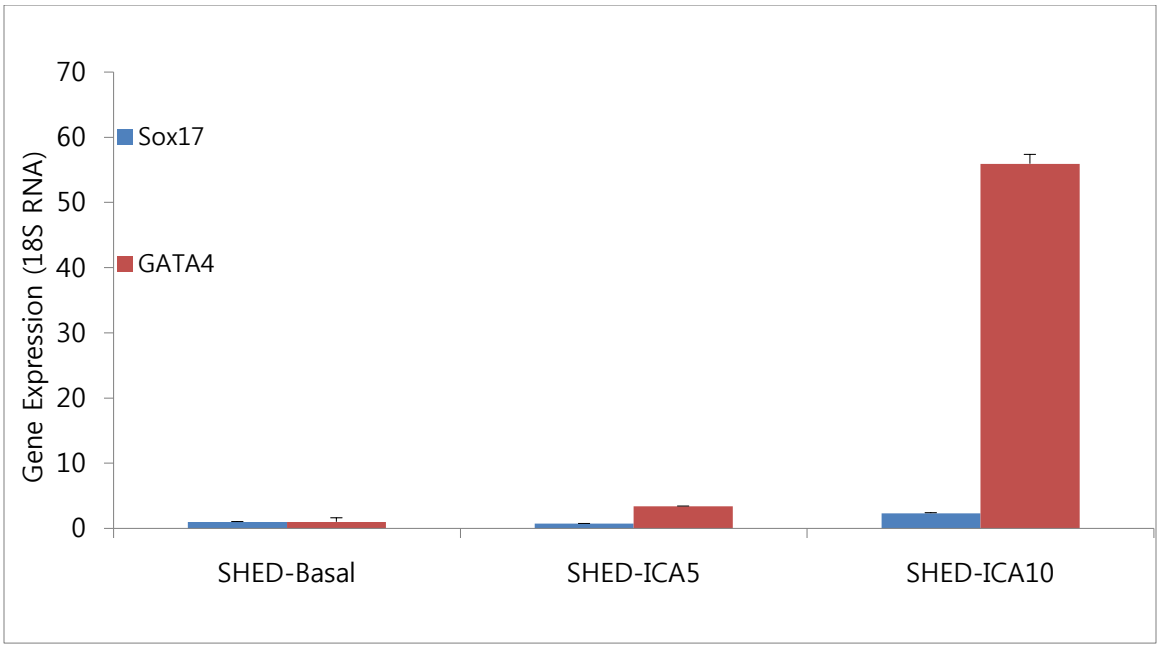


Figure 10: Real-time Quantitative RT-PCR results- SHED $GABA_A$-α Receptors

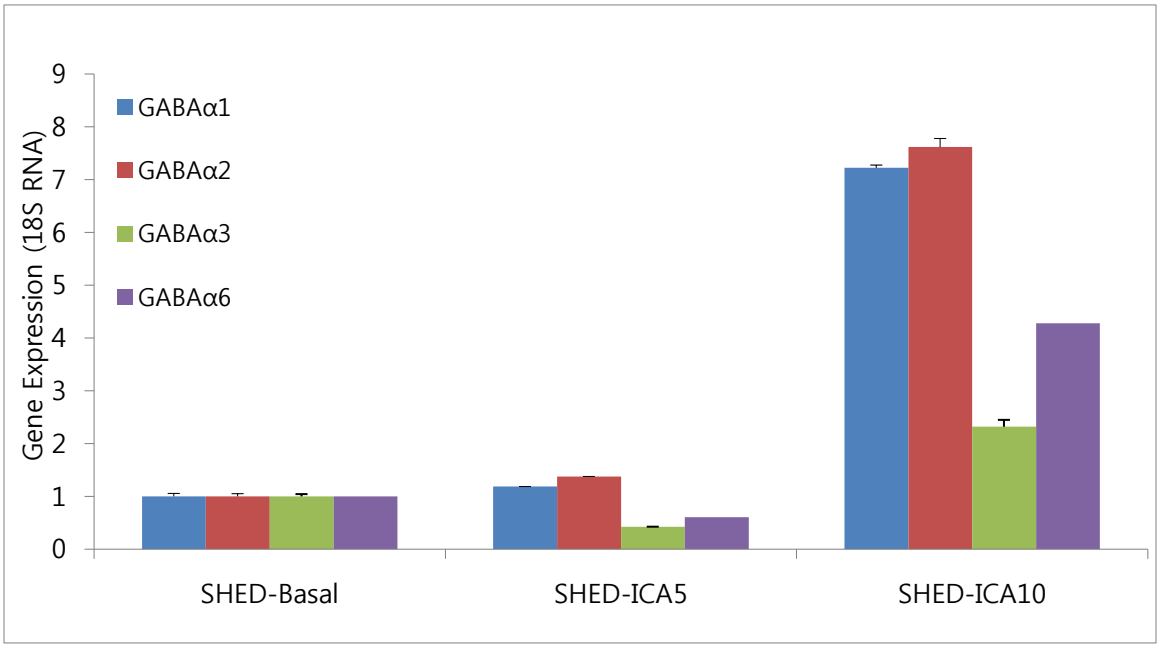


Figure 11: ELISA Insulin Dose-Response Curve

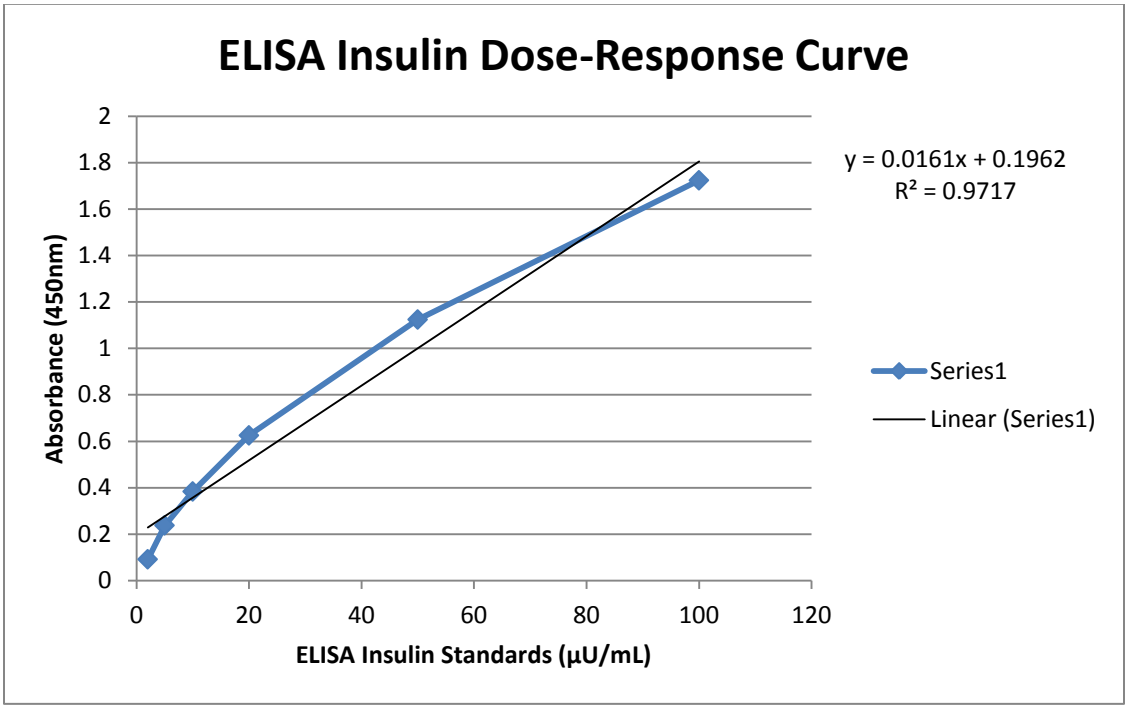


Figure 12: Insulin Concentration of Samples from Glucose/GABA Challenge and Intracellular Insulin Assays

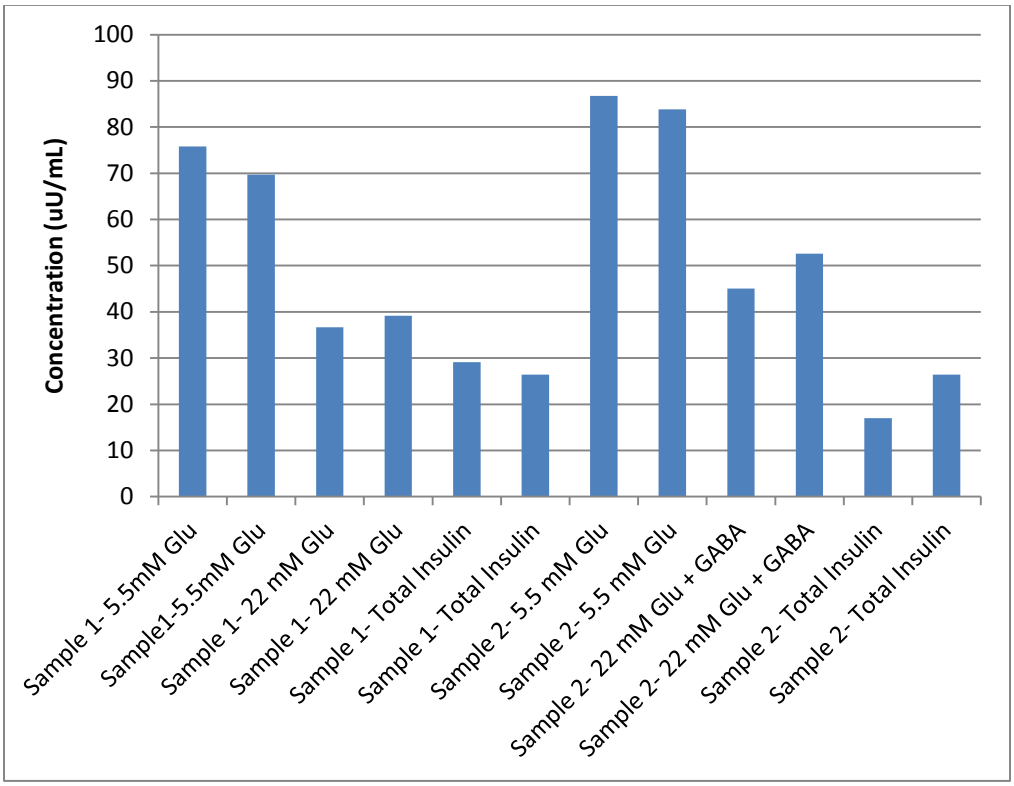


Figure 13: Total Insulin Release during Glucose/GABA Challenge

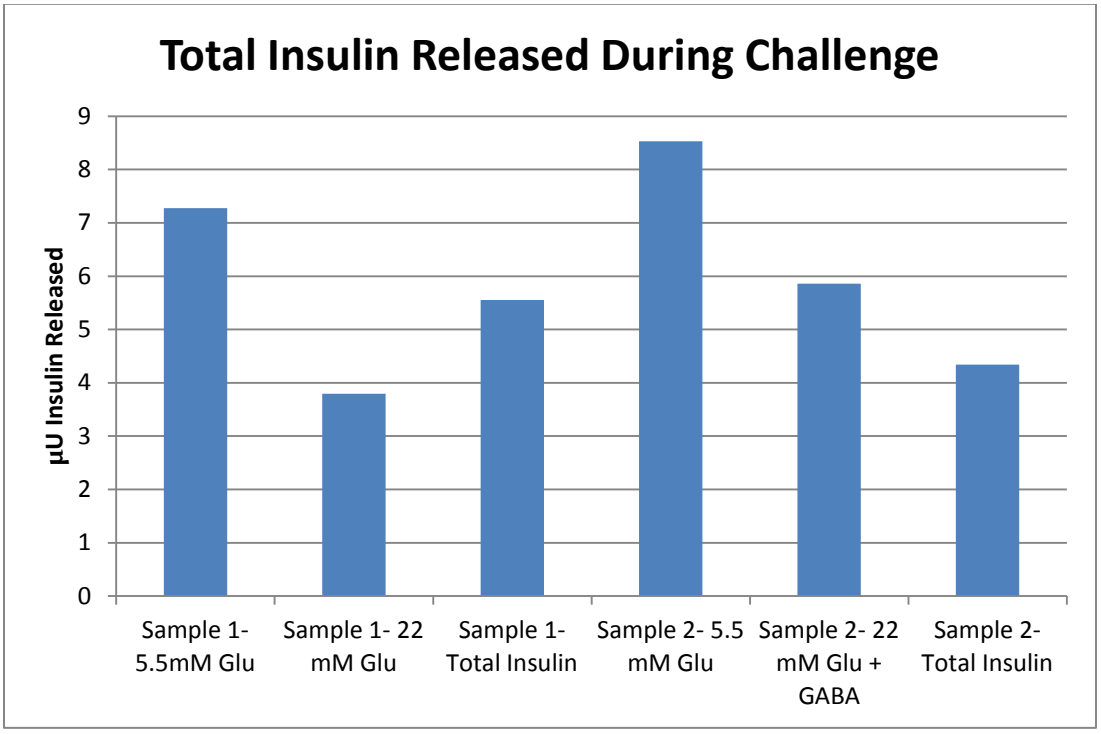
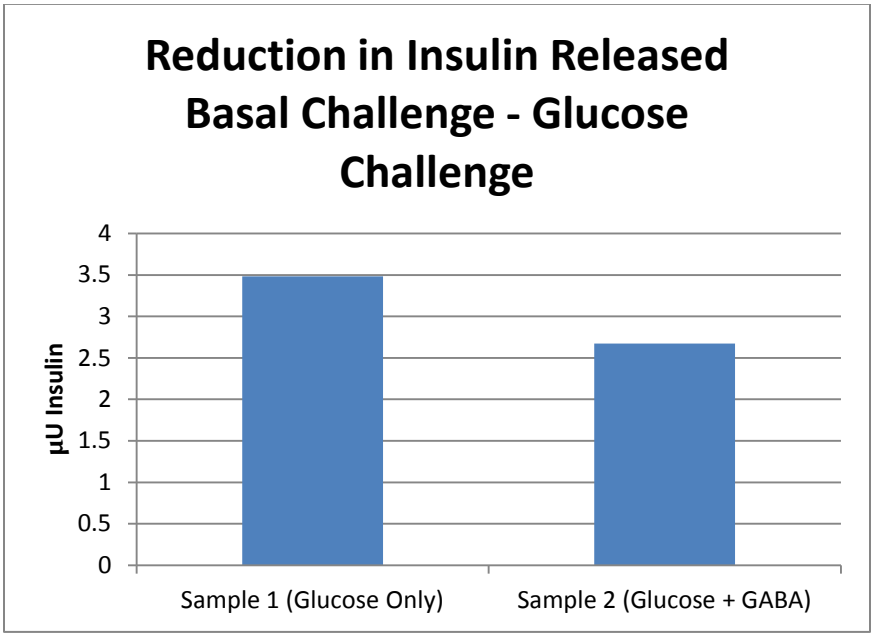


Figure 14: Reduction in Insulin Released from Basal Conditions to Glucose/GABA Challenge



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