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The Effects of Mechanical Rocking Motion on Transdifferentiating Feline Adipose Multipotent Stromal Cells into Functioning Pancreatic β-cell like Islets

by

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Undergraduate honors thesis under the direction of

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Submitted to the LSU Roger Hadfield Ogden Honors College in partial fulfillment of the Upper Division Honors Program.

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Baton Rouge, Louisiana

Abstract

Diabetes mellitus (DM), or type two diabetes, is a growing threat to feline health. Traditional palliative treatments for diabetes involve daily insulin injections. Feline adipose multipotent stromal cells (fASCs) isolated from routine surgeries' medical waste, are reported to have robust proliferation and multipotential. These fASCs, when differentiated into insulin-producing β-like cells, may substitute impaired β-cell function. However, these cells do not possess all the characteristics of true β-cells. Current research suggests cells differentiated towards β-like cells under a mechanic rocking stimulus have improved differentiative capabilities.

Our objective was to compare and quantify cell cluster size and shape, quantify pancreatic cell-associated gene expression, detect synthesized intracellular insulin, and quantify insulin released upon glucose stimulus in cells cultured with and without the presence of mechanical rotation. Our hypothesis was that mechanically stimulated fASCs will form more, smaller clusters than their stationary counterparts, differentiate into β-like cells, and secrete insulin in response to a glucose stimulus in defined medium.

The fASCs were isolated from adipose tissue from six donors (three male, three female) and were differentiated towards insulin-producing β-like cells at passage 0 in a 3-stage induction protocol. The cells were cultured with identical defined mediums with the control group culturing in static medium whereas the experimental group was exposed to mechanical rocking for stages 1 and 2. Viability of the cells was evaluated with calcein-AM/ethidium-homodimer-1 staining. Expressions of β-cell specific genes were evaluated with qRT-PCR. Synthesis of pancreatic hormones were evaluated with immunolabeling. Insulin response to glucose

stimulation was evaluated using ELISA. Cell structures were analyzed using scanning electron microscopy and transmission electron microscopy. Cell clusters were counted and measured using ImageJ.

Collectively our results demonstrated that mechanical rocking induced the cells to exhibit β-cell pancreatic islet structures and behaviors. The mechanically rocked cell cultures demonstrated more cell clusters with larger diameters and demonstrated an upregulation of insulin and glucagon expressing genes. In conclusion, exposing differentiating cell cultures to mechanical rocking motion promotes differentiation physically and genetically which may allow these cells to be an effective treatment for diabetes mellitus in feline patients and beyond.

Introduction

Feline diabetes mellitus, or type two diabetes, is a growing threat to feline health. This endocrine disorder effects between 0.2 and 1% of client owned population[1]. The disorder is characterized by a lack of pancreatic response to fluctuating sugar levels in the body and can lead to more serious health issues. [2] Pancreatic tissues contain several types of cells, one of which is β- cells. These cells are responsible for responding to high sugar levels by releasing insulin, a hormone responsible for sugar uptake. In a diabetic pancreas, β-cells are unable to manufacture their own insulin, which leads to drastic fluctuations in sugar levels. Type I diabetes is characterized by an inability to create or secrete insulin, whereas type II diabetics can produce pancreatic insulin, but it is not adequately secreted in response to blood glucose levels. If left untreated, diabetes can lead to malnutrition, ketoacidosis and eventually death. [3] [4] This condition can come from a variety of sources including diet, genetics, and environmental factors such as activity levels. [5]

Stem cell differentiation is a process through which stem cells are harvested and transformed into specialized cells. This process occurs *in vivo* and involves three types of stem cells: totipotent, pluripotent, and multipotent stem cells. Totipotent and pluripotent stem cells are embryonic stem cells whereas multipotent stem cells are a type of adult stem cell and can be found in various tissues. Multipotent stem cells differentiate into many, but not all types of tissues. Current knowledge supports that adult stem cells have greater differentiative capabilities than previously thought. In *vitro* experimentation has proven multipotent adult stem cells can be differentiated into many cell types with medicinal applications. [6] Multipotent stem cells are more accessible and have less ethical concerns than their counterparts. [7] The ability for stem cells to differentiate into a variety of cells may allow for effective treatments to be developed for a variety of ailments, including diabetes.

Traditional treatments for diabetes involve either oral hypoglycemic medications, or daily insulin injections. However, only approximately 25% of diabetic felines can achieve glycemic control with the medications, leaving approximately 75% relying on injections. [8] [9] The injection process is expensive, painful, and palliative. [10]Stem cell therapy treatments have been used in human and murine trials with remarkable success. [11] [12][13]Using differentiated stem cells may prove to be a beneficial treatment for cats as it requires less injections and may be able to provide longer lasting insulin responses. Previous studies indicate that adipose derived stem cells (ASCs) have been transdifferentiated into insulin producing cells (IPCs). [13, 14] These previous studies imply that these cell therapies will be an efficient and effective treatment for type II diabetes.

One advancement of medical technology is the development of organoids. Organoids are microstructures developed from differentiating stem cells into cells of a specific organ. [15]

These organoids can come from a variety of sources including embryonic cells as well as induced pluripotent cells. However, new technologies have arisen that allow for adult stem cells to be transdifferentiated into organoids rather than inducing undifferentiated cells. The process of transdifferentiation involves transforming one cell into another without having to revert to its original form or dedifferentiate. [16] This transdifferentiate process has been effective in differentiating adult mesenchymal stem cells into different cell linages and creating organoids. [17] Transplantable pancreatic islet organoids derived from transdifferentiated adipose tissue have shown to have diabetic corrections when transplanted into mice. However, these organoids were temporary, and lacked all the abilities of true β-cells. [18] Another issue with these transplantable organoids is size. Healthy feline pancreatic islets are approximately 85μm in diameter. [19] However, traditional *in vitro* organoids are often larger than their *in vivo* counterparts and are prone to necrosis. [20] Being able to combat this tendency of larger clusters may yield more effective and viable islets. Unlocking the aspects of β-cell differentiation and islet size may provide more dynamic β-cells to treat insulin resistance and repair damaged pancreatic islets.

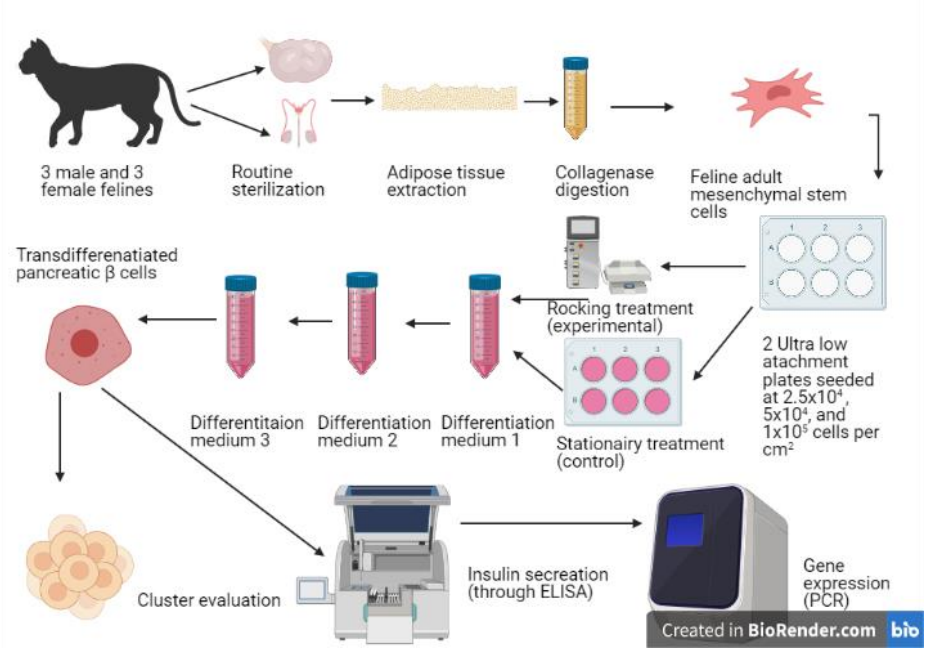
When cells are differentiating, they rely on a network of environmental cues. [21] These cues include chemical, physical, metabolic, and secretory factors that influence cell development. [22] Physical cues that generate shear stress on the cells can prompt development of the extracellular matrix (ECM). [23] This microstructure contains specific protein markers and acts as the scaffolding to guide cells to differentiate and organize into organoid structures. [24] The ECM develops *in vivo* after exposure to several factors including fluid motion. [22] Replicating this motion may prompt ECM development in *in vitro* experimentation. Prior research found that using a mechanical stimulus caused a change in the microstructure of

differentiated cells and led to upregulation of target genes. [21] Mechanical stimulation may prove to be an effective method to unlock the characteristics of true β-cell pancreatic islets. We hypothesize that culturing fASCs into feline pancreatic islet organoids using a mechanical rocking motion stimulus 1) form more viable clusters with smaller size, 2) upregulate mature β-cell related genes *Insulin*, Glucagon *(GCG*), Somatostatin *(STS)* and, 3) increase production and release of insulin upon glucose stimulation.

Methods

Study Design

Feline adipose stem cells (fASCs) isolated from 6 donors (3 male, 3 female) were transdifferentiated into pancreatic organoids composed of β-like cells at passage 0 using a 3-stage differentiation protocol with defined medium identically, except for the presence or absence of rocking in the initial 2 stages. At stage 3, the cells from both treatments were replated onto a 24-well laminin coated plate and were not exposed to mechanical motion. The rate of mechanical stimulus was determined to be the standard setting on the rocking machine used. Cells were compared for cluster number and size using ImageJ. The structures of the cell clusters were observed using scanning and transmission electron microscopies. Viability was evaluated using calcein-AM/ethidium-homodimer-1 staining. The multipotentiality of the cells was evaluated using varied differentiation media. Expression of genes associated with β-cells was evaluated using qrt-PCR and was expressed as a fold change between the control and experimental treatment. Insulin release upon glucose stimulation was evaluated using ELISA. Immunolabeling was used to confirm the synthesis of insulin, glucagon, and somatostatin.



Cell Isolation

Adipose tissues were harvested from the epididymis and broad ligament of reproductive organs removed from sterilization procedures and cells were then isolated. The adipose tissues were minced and then digested with 0.3% type I collagenase (Worthington Biochemical Corporation, Lakewood, NJ) in Kreb's Ringer buffer (KRB) for 30 minutes, while being stirred at 1,000 rpm with a stir bar in an incubator at 37°C. After filtering with 100 μm nylon cell strainers (BD Falcon, Bedford, MA) and centrifugation (260 × g, 5 minutes), the stromal vascular fraction (SVF) pellets were then resuspended in 5 ml red blood cell lysis buffer (0.16 M NH_4Cl, 0.01 M $KHCO_3$, 0.01% ethylenediaminetetraacetic acid) for 5 minutes. The SVF pellets were collected after centrifugation (260 × g, 5 minutes) and seeded in 10-cm cell culture dishes in stromal medium (Dulbecco's modified Eagle's medium F-12 (DMEM/F-12, Hyclone, Logan, UT), 1% antibiotic/antimycotic solution (MP Biomedical, Irvine, CA), 10% fetal bovine serum (FBS, Hyclone)). Stromal medium was refreshed after 24 hours and then every 3 days. After 70 – 80% confluence, the SVF cells were detached with 0.05% trypsin (Hyclone) and cells were

seeded in 9.6 cm ultralow attachment plates (Corning Inc) for stages 1 and 2 with varying densities of $2.5x10^{4,}$ $5x10^{4}$, and $1x10^{5}$ cells per cm^{2}. Once stage 3 of the induction protocol was reached, the cells were replated onto a 24-well laminin coated plate (Corning Inc). This plate was not exposed to motion. In this study, P0 is the cell passage of primary cells.

Multipotentiality

To evaluate plasticity, the P0 cells were cultured in stromal medium for 7 days and then fixed with 4% paraformaldehyde (PFA). The fixed cells were stained with 0.1% toluidine blue. For adipogenesis, cells were cultured in stromal medium until 70 – 80% confluence was reached, washed with phosphate buffered saline (PBS, Hyclone) and cultured in adipogenic medium for 10 days. The induced cells were fixed with 4% PFA and stained with oil red O. For osteogenesis, the cells were cultured in osteogenic preinduction medium for 10 days and then in osteogenic induction medium for another 10 days. The cells were fixed with 70% ice cold ethanol and stained with 2% alizarin red.

Mechanical Rocking Motion

Paired samples of cells from each donor were either exposed to mechanical rocking motion or kept static. The cells exposed to the mechanical stress were rocked back and forth rate of 17 oscillations per minute using the Vari-Mixer™ test tube rocker (Thermo Scientific, Waltham, MA) for stages 1 and 2 of the differentiation protocols. The cells were continuously exposed to this mechanic rocking motion during stage 1 and 2. Static cells were placed in the same incubator as the experimental cells with no exposure to motion to be used as a control.

Differentiation of feline ASCs into insulin producing cells (IPCs).

The fresh isolated fASCs were cultured and expanded until 70 – 80% confluence and then seeded to 6-well ultralow attachment plates (Corning Inc., Corning, NY) with $2.5x10^4$, $5x10^4$, and $1x10^5$ cells per cm^2 respectively for 1 – 2 days in stromal medium until visible round clusters were formed. The fASCs were induced to transdifferentiate into pancreatic β-cell islet-like clusters in a three-stage protocol using β cell induction medium.

Medium	Composition
β-cell induction medium 1	Serum free medium (SFM) 1: DMEM/F-12, 1% BSA (bovine serum albumin, fraction V, fatty acid free, Fisherbrand Scientific, Pittsburgh, PA), 1 × insulin-transferrin-selenium (ITS, Gibco BRL, Gaithersburg, MD), 4 nM activin A (R&D Systems Inc., Minneapolis), 1 mM sodium butyrate, 50 μM 2-mercapethanol, 1% N-2 supplement (R&D Systems Inc.), 2% B-27 supplement (Gibco), 5 μg/ml laminin (Corning), 50 ng/ml recombinant human hepatocyte growth factor (HGF, EMD Millipore, Temecula, CA), 20 ng/ml basic fibroblast growth factor (bFGF, Gibco);
β-cell induction medium 2	SFM 2: DMEM/F12, 1% BSA, 1 × ITS, 0.3 mM taurine (ACROS Organics, Morris Plains, NJ), 5 μg/ml laminin, 20 ng/ml bFGF, 1% N-2 supplement, 2% B-27 supplement, 50 ng/ml HGF, 1 mM nicotinamide.
β-cell induction medium 3	SFM 3: DMEM/F-12, 1.5% BSA, 1.5 × ITS, 3 mM taurine, 100 nM glucagon-like peptide 1 (GLP-1, TOCRIS bioscience, Ellisville, MO), 1 mM nicotinamide (ACROS Organics), 1 × non-essential amino acids (NEAA, Gibco), 10 nM pentagastrin (TOCRIS bioscience), 1% N-2 supplement, 1% B-27 supplement, 50 ng/ml HGF, 20 ng/ml bFGF, 5 μg/ml laminin, 20 ng/ml betacellulin (R&D Systems), 10 nM extendin-4 (TOCRIS bioscience)

Cell Morphology

Cells were evaluated for quantity, morphology and diameter of clusters. Images were taken of the clusters in each well for each plate using ImageJ and a standard microscope. A group of cells was determined to be a cluster if it had a diameter greater than 25μm. [25] Each cluster was analyzed for size, shape, and quantity. The cell cluster diameters were measured using ImageJ and the mean per treatment by concentration was graphed using GraphPad. The number of clusters for each well were recorded and plotted using JMP. Cluster counts were averaged by well per treatment and the mean and least squared mean (LSM) were graphed using GraphPad.

Live/Dead Cell Imaging Assay

The cells were washed with PBS and centrifuged (350 ×g, 8 minutes) to form cell pellets. Live/Dead™ cell imaging kit (Invitrogen Corp., Carlsbad, CA) was then used, and the cell pellets were suspended with staining solution. After incubating for 15 minutes at room temperature in the dark, a confocal laser scanning microscopy (CLSM, Leica TCS SP2, Leica, Wetzlar, Germany) was used to obtain images of each well for both plates.

Immunohistochemistry

The induced cell clusters were washed with PBS and fixed overnight in 4% paraformaldehyde (PFA). The cell clusters were embedded in paraffin and sectioned (5 μm). The sections were incubated with xylene to remove paraffin and rehydrated in a series of ethanol-distilled water solutions. Rehydrated sections were blocked with 4% Bovine Serum Albumin (BSA) in Tris-buffered saline (0.1% Tween-20, 20 mM Tris, 137 mM NaCl, pH = 7.6). The

sections were stained with the primary antibodies (insulin (1:1000), glucagon (1:2000), and somatostatin (1:1000)) specific feline antigens or validated for feline cross reactivity for 1 hour at room temperature. The sections were then washed with PBS and incubated with secondary antibodies (goat anti-mouse IgG1-FITC (1:100) and goat anti-rabbit CF™-594 (1:500)) for 1 hour at room temperature. 4',6-diamidino-2-phenylindole (DAPI) was used to stain the nuclei. Photomicrographs were obtained for all labeled cells with CLSM. The static cells were used as controls and the sections incubated with secondary antibodies alone were used as secondary antibody controls.

Glucose Challenge Assay

The induced islet-like cell clusters were collected and washed twice with PBS. The islet-like cell clusters were incubated with KRB buffer without glucose for 4 hours at 37°C, then incubated with KRB buffer supplemented with different glucose concentrations (5.5, 25, and 55 mM) for 3 hours at 37°C. The supernatant was collected and stored at – 80°C until further use. The stored medium was analyzed for insulin content using a feline specific enzyme-linked immunosorbent assay (ELISA) kit (Mybiosource, San Diego, CA). The stromal cultured cells were used as a control.

Transmission Electron Microscopy

The islet-like cell clusters were rinsed with PBS and then fixed in 2% PFA and 2.5% glutaraldehyde in 0.1 M PBS for 10 minutes. After centrifugation, the supernatant was removed, and the samples were fixed with the same fresh fixative and shaken for 2 hours. The samples were mixed with equal amounts of 3% agarose and the mixture was placed to a light microscopy slide. After the mixture solidified, it was cut into cubes and the cubes were placed into a glass

vial filled with 0.1 M PBS. The cubes were then washed with 0.1 M PBS and 0.08 M glycine 5 times (15 minutes/time), followed by fixing cells with 2% osmium tetroxide in 0.1 M PBS in the dark for 1 hour. The samples were washed with H_2O and dehydrated in a series of ethanol-distilled water solutions. The dehydrated samples were infiltrated with 1:1 ethanol and LR white resin for 2 hours, and then infiltrated with 100% LR white resin for another 2 hours. Embedded samples were placed into the bottom of a beem capsule and incubated in an 18^{o}C oven for 24 hours. Ultra-thin sections (90 nm) were cut and stained with 2% uranyl acetate and lead citrate. Some sections were directly evaluated with transmission electron microscopy (JEOL JEM-1400, Japan). The other sections were blocked in 5% BSA in PBS for 30 minutes and then incubated with goat anti insulin in 1% BSA in PBS (1:20) for another 90 minutes. After incubation, the sections were washed with 1% BSA buffer and PBS, respectively. The sections were then fixed in 2% glutaraldehyde in PBS for 5 minutes and contrasted with 2% uranyl acetate and lead citrate after being thoroughly washed in distilled water. The gold labeled sections were observed with the TEM. Stromal cells were used as a control.

Scanning Electron Microscopy

The islet-like cell clusters were collected by filtration and fixed in 2% PFA and 2% glutaraldehyde in 0.1 M PBS for 15 minutes. The solution was extracted into a 10 ml syringe with a Swinney filter holder fitted with a 13 mm diameter 2 μm pore polycarbonate, and then filtered and fixed for another 15 minutes using the same fixative. The samples were rinsed with 0.1 M PBS and distilled water. The filter was removed from the syringe and dried with hexamethyldisilazane (HMDS, Electron Microscopy Sciences, Fort Washington, PA) for 30 minutes, 1:1 100% ethanol and HDMS, and 2 changes for 30 minutes each with 100% HDMS. Finally, the HDMS was removed, and the samples were placed in a hood overnight to air-dry.

The dried samples were mounted onto aluminum SEM stubs, coated with platinum in an EMS 550X sputter coater and imaged with JSM-6610 High vacuum mode SEM (JEOL Ltd., Japan).

RT-PCR – Gene Expression

Total RNA was isolated from the cells harvested from each induction stage (EZNA® MicroElute Total RNA kit, Omega, Bio-Tek, Norcross, GA). The concentration of RNA for each sample was determined spectrophotometrically (NanoDrop ND-1000; NanoDrop Technologies, Wilmington, DE), and cDNA synthesized (Maxima First-Strand cDNA synthesis kit, Thermo Scientific, Waltham, MA). Feline pancreatic target gene levels (*insulin, STS, and GCG)* were quantified with real-time RT-PCR using the Thermo Fisher Absolute^{TM} Blue QPCR Rox Mix technology and an ABI Prism 7900 HT Sequence Detection System (Applied Biosystems, Foster City, CA). The $2^{-\Delta\Delta Ct}$ values were determined relative to the reference gene *β-actin*.

Statistical Analysis

All results are presented as least squares (LS) mean ± SEM. Statistical analyses were performed with the JMP statistical package (v 13.0.0, SAS Institute Cary, NC). Mixed ANOVA models were used to evaluate insulin secretion between glucose concentrations within conditions and between concentrations within glucose concentrations. The same models were used to evaluate target gene expression among induction stages within conditions and between concentrations within induction stages. Tukey's post hoc tests were applied for multiple group comparisons ($p<0.05$).

Results

Multipotentiality

The cells demonstrated a fibroblastic shape when cultured in the stromal medium. When cultured in the osteogenic medium, calcium deposits formed. Additionally, when cultured in adipogenic medium, the cells formed large lipid droplets.

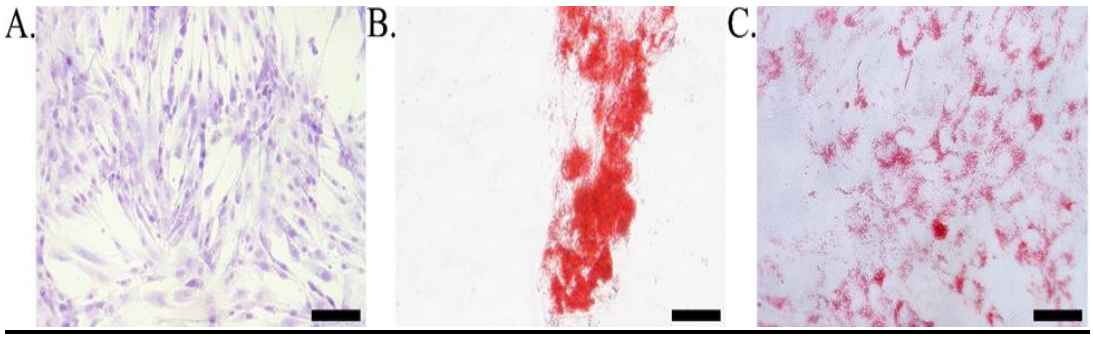


Figure 1 Photomicrographs of feline ASCS after culturing in stromal medium (A), with alizarin red staining of calcium deposition after cultured in osteogenic medium (B), and with oil red O lipid staining after culture in adipogenic medium (C). Scale bars =200 μm

Cell Morphology

Cell clusters formed in both the static control cultures as well as the mechanically induced experimental culture (Figure 2). The experimental treatment had a larger LSM of cell clusters and number of cell clusters at the $2.5x10^4$ concentration (Figure 3 and 4). The $5.0x10^4$ and $1.0x10^5$ concentrations had larger numbers of cell clusters in the control samples (Figure 3). However, the control clusters were smaller and irregular in shape when compared to the experimental treatments. The cluster diameters were larger for all the rocking treatments with an average of 50μm larger than their stationary counterparts by concentration. The difference in results for cluster numbers were statistically significant between rocking and stationary treatments ($p<0.05$) for the $2.5x10^4$ concentration and not statistically significant between the other concentrations. The differences between the rocking treatment and stationary treatment

cell cluster diameters were statistically significant ($p<0.05$) for all concentrations. There was no significant difference between concentrations.

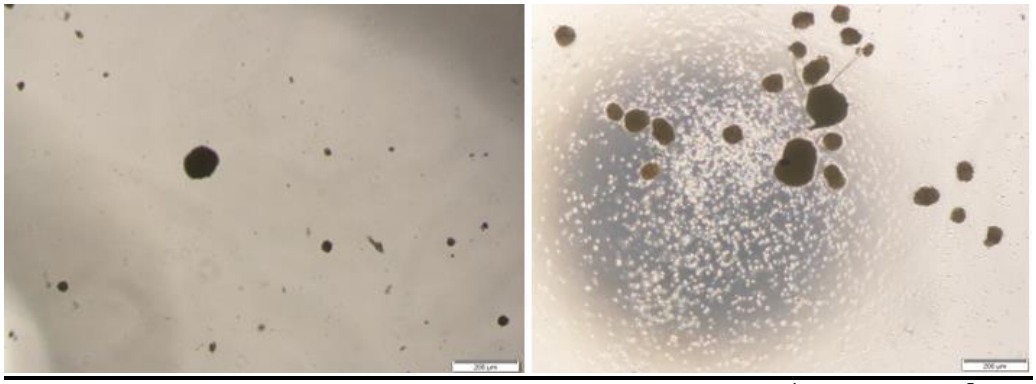


Figure 2 Photomicrographs of P0 fASCs at a concentration of $2.5x10^4$ cells per cm^2 after exposure to static (left) or rocking motion (right) and culturing in pancreatic β-cell induction medium. Scale bars=200μm

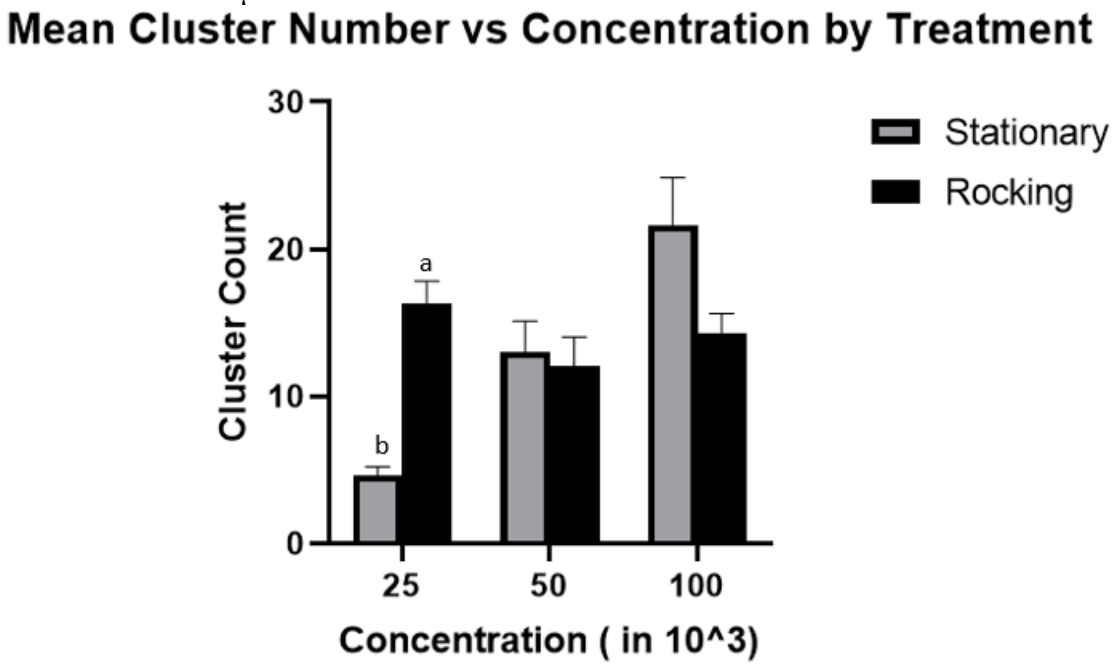


Figure 3 Mean cluster count per well (±SEM) of fASCs cultured in differential medium in stationary and rocking treatments by concentration.

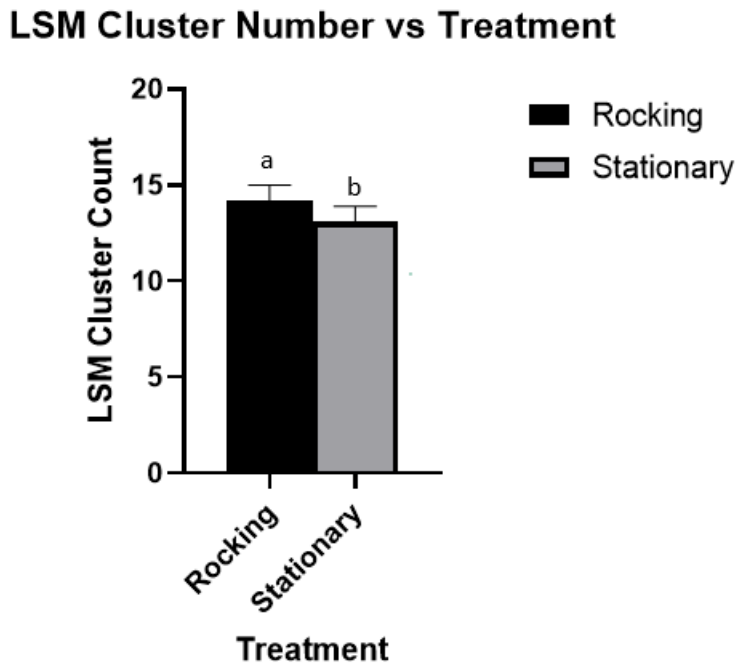


Figure 4 LS Mean (± SEM) cluster count by treatment of fASCs cultured in differential medium in stationary and rocking treatments.

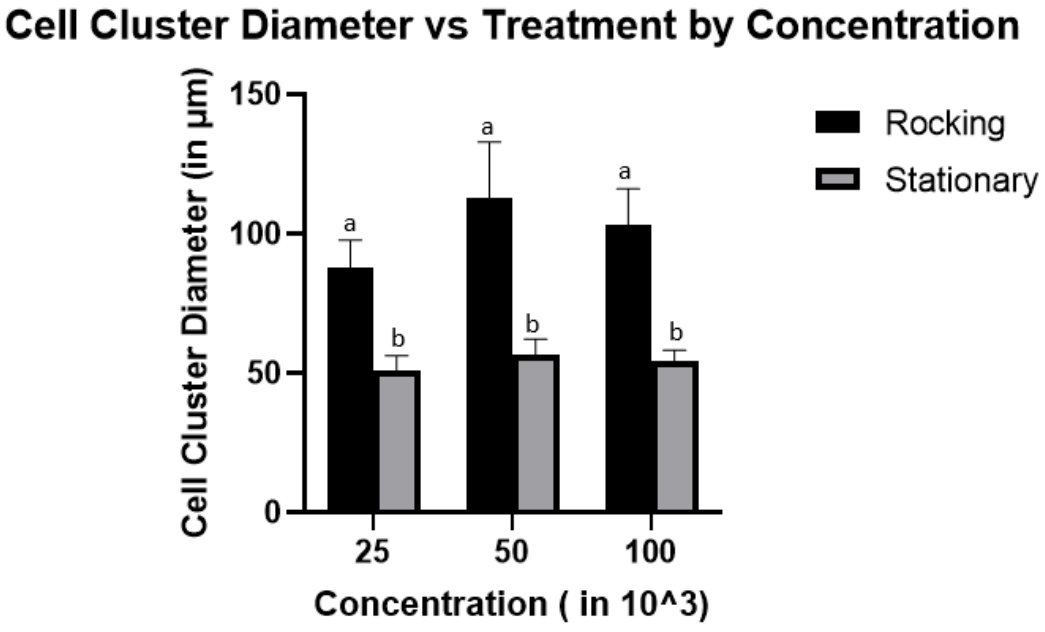


Figure 5 Mean cell cluster diameter (±SEM) of fASCS cultured in differentiating medium under stationary or rocking treatments by concentration.

Live/Dead Cell Imaging Assay

Most of the cells from both treatments were alive. Both treatments had an increasing number of dead cells with increasing cluster size. Cell clusters smaller than 100μm had a higher proportion of live cells than dead cells (Figure 6).

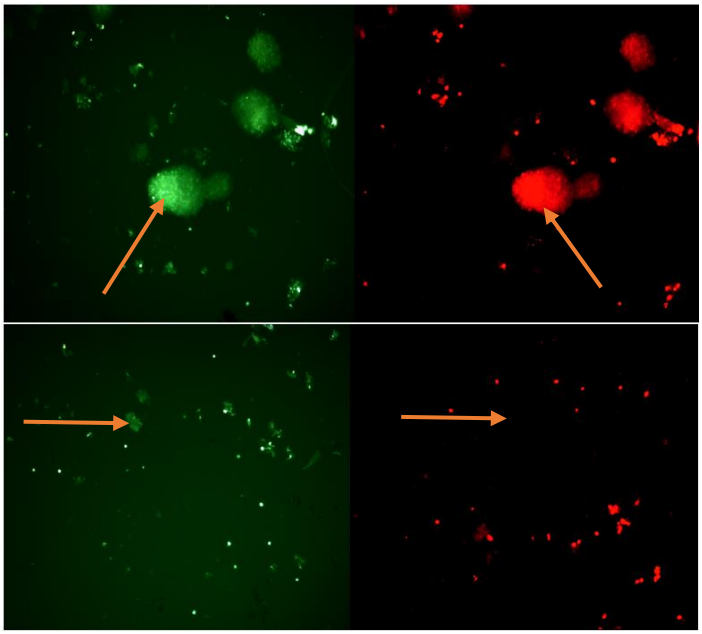


Figure 6 Fluorescent photomicrographs of static (Bottom left and right) and mechanically induced (Top left and right) live fASCS labeled with calcein (green) and dead cells labeled with ethidium (red). Images taken at 10x magnification with a confocal laser scanning microscope.

Immunohistochemistry

Insulin, glucagon, and somatostatin expression was confirmed with immunocytochemistry in induced cell clusters (Figure 7). Limited insulin and somatostatin expression was detected in the cells cultured in stromal medium. However, there was no unspecific expression when the induced cell clusters were incubated with secondary antibodies only. Both the cells cultured in stromal medium and the clusters incubated with only secondary antibodies did not express glucagon and somatostatin.

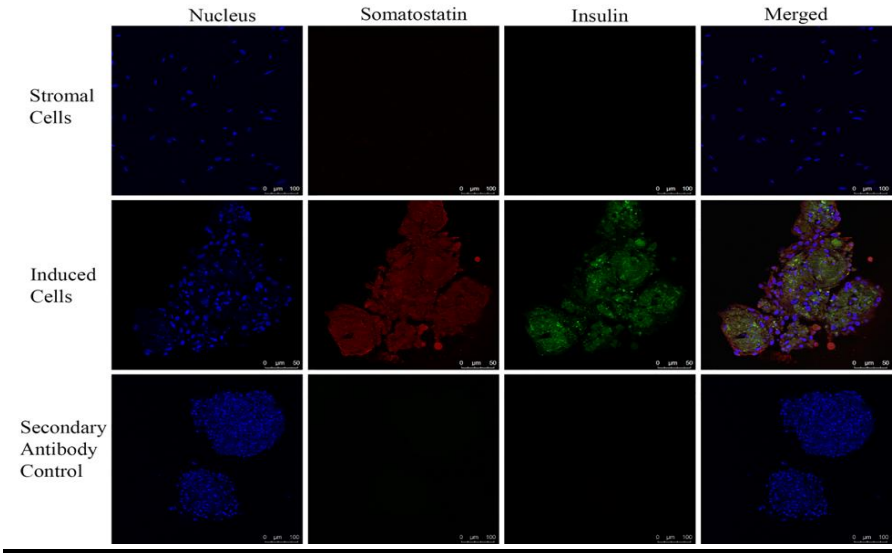


Figure 7 Fluorescent photomicrographs of induced cell clusters from feline ASCs (middle row) labeled with somatostatin (red) and insulin (green). The cells cultured in stromal medium were used as negative control (top row) and the induced cell clusters incubated with secondary antibodies were used as the secondary antibody control (bottom row). Scale bars = 50 μm (middle row) and 100 μm (top and bottom rows).

Glucose Challenge Assay

The induced cell clusters from male and female donors were examined for their insulin secretion potential through the glucose challenge assays (Figure 8). The glucose challenge tests showed that the insulin secretion depended on the glucose concentration with the highest level of insulin secretion released when the clusters were cultured in the medium contained 55mM glucose for 3 hours. The differences between genders were statistically significant ($p<0.05$).

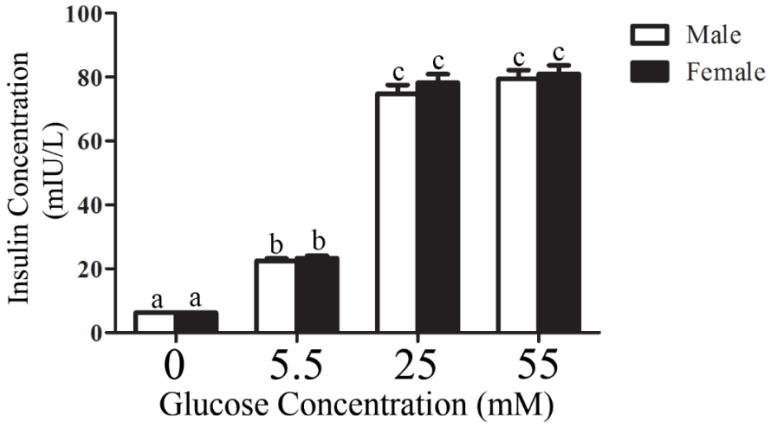


Figure 8 The extracellular insulin secretion from differentiated islet-like cell clusters before and after stimulated with a series of glucose concentrations (5.5, 25, and 55 mM) are illustrated by

the glucose challenge assay. Columns with distinct superscripts are significantly different among glucose concentrations within genders ($p < 0.05$).

Transmission Electron Microscopy

Transmission Electron Microscopy showed islet-like cell clusters were characterized with numerous vesicle formation throughout the cytoplasm (Figure 9). However, this prominent vesicle formation was not evident in the cytoplasm of undifferentiated cells.

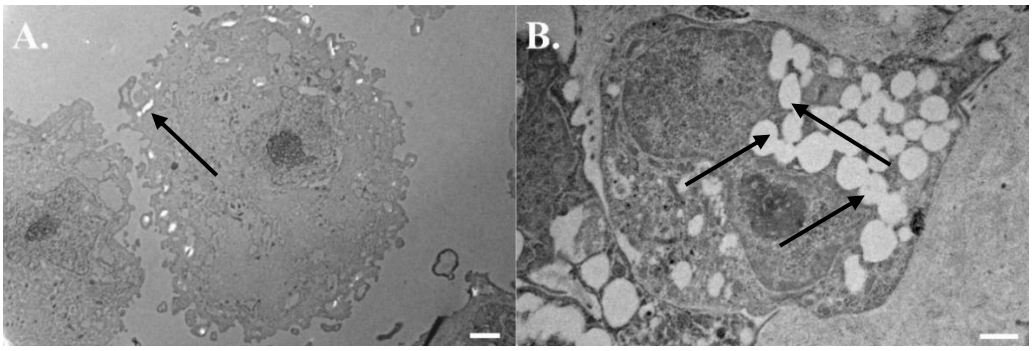


Figure 9 Transmission electron microscopy (TEM) images of undifferentiated (A) and differentiated islet-like cell clusters (B) from fASCs. Scale bars A 2μm, B 1μm.

Scanning Electron Microscopy

Scanning Electron Microscopy demonstrated that undifferentiated and islet-like cell clusters had distinctly different size and surface morphology (Figure 10). The islet-like cell clusters were significantly larger than undifferentiated cells and contained particle depositions not found on the undifferentiated cells. However, the undifferentiated cells did demonstrate some spheroid formation.

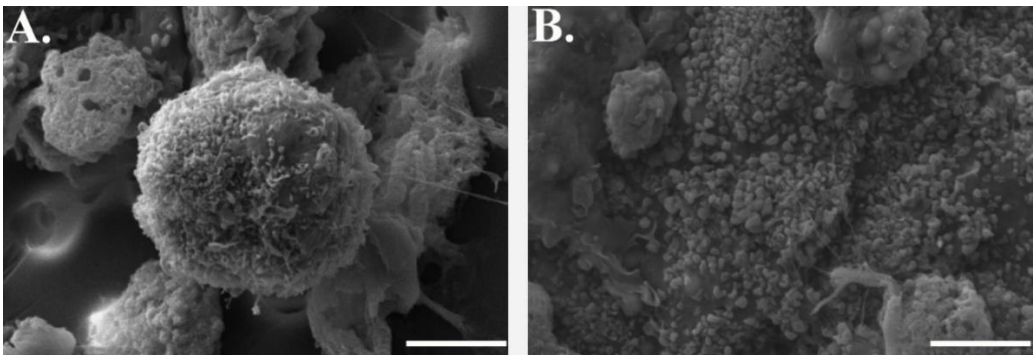


Figure 10 Scanning electron microscopy (SEM) of undifferentiated (A) and differentiated islet-like cell clusters (B) of fASCs. Scale bar 5μm.

Islet-cell Target Gene Expression

The *GCG*, *Insulin*, and *STS* expression were observed to be the highest at Stage 3 (Figure 11). The mRNA levels of these genes increased with each induction stage. Fold changes increased with each stage and were observed to be higher in female cell cultures. Stromal cultures expressed nearly no fold change for these genes. Fold change for expression of Insulin and GCG between rocking and stationary treatments was higher for both genes at both the $5x10^4$ and the $1.0x10^5$ concentrations (Figure 12). However, the target gene fold change for the $2.5x10^4$ concentration was unable to be calculated because the smaller quantity of cells did not have enough RNA to run PCR.

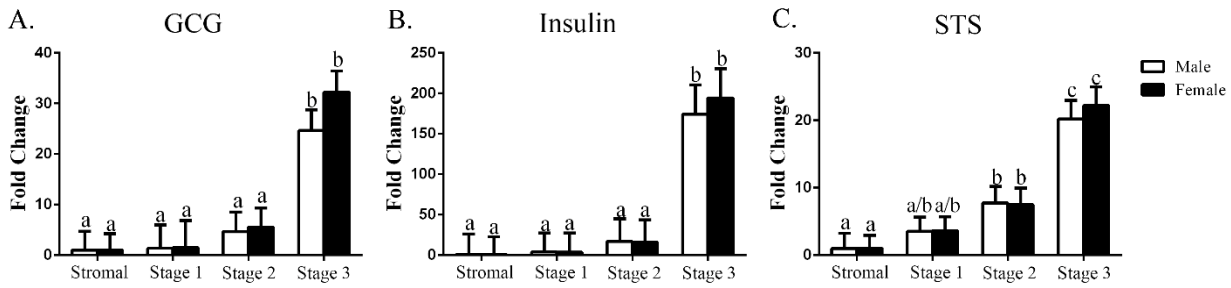


Figure 11 Feline glucagon (A), insulin (B), and somatostatin (C) from three induction stages. Columns with different distinct superscripts are significantly different among induction stages within genders ($p < 0.05$).

Gene	Cell Concentration per cm^2	Fold Changes (2^-ΔΔCT)
GCG	$5x10^4$	1.71
	$1x10^5$	75.37
Insulin	$5x10^4$	0.46
	$1x10^5$	1.44

Figure 12 Calculated fold change of *GCG* and *Insulin* genes between rocking and stationary treatments at stage 3 of the induction protocol.

Discussion

The overall results of this study demonstrate that feline adipose derived stem cells cultured in a defined medium secrete insulin, upregulate β-cell specific genes and demonstrate morphological changes. When these differentiated cells are exposed to mechanical rocking, they are then able to form numerous large cell clusters and further upregulate β-cell specific genes. This supports some aspects of our hypothesis that culturing fASCs into feline pancreatic islet organoids using a mechanical rocking motion stimulus 1) form more viable clusters with smaller size, 2) upregulate mature β-cell related genes *Insulin*, Glucagon *(GCG*), Somatostatin *(STS)* and, 3) increase production and release of insulin upon glucose stimulation. Our hypothesis was true with the caveat of cell cluster size in the mechanically rocked cells. The variance in cluster size and count between cell concentrations disproves that there would be more small clusters in the experimental treatment than the control. The changes to the fASCs were a result of the differentiation medium, mechanical rocking, and cell concentration.

The differentiation medium used in this experiment produced results indicative of transdifferentiation of fASCs to β-cells. These changes in morphological structure were shown in the results of the transmission electron microscopy, and scanning electron microscopy. The cells successfully transdifferentiated into multiple cell types, indicating a possibility of a successful transdifferentiation into β-cells. Islet-like cell clusters exhibited vesicle formation in their cytoplasm, indicating that the original adipose cells were able to transdifferentiate into β-like-cells. [26] Vesicles are used by cells to export secretions such as hormones out of the cell. Prominent vesicles are indicative of the presence of β-cells, since β-cells have numerous vesicles which they use to export insulin when exposed to glucose. [27] In addition to vesicle formation, the differentiated cells also demonstrated drastic morphological changes between clusters when

observed under SEM. The differentiated clusters demonstrated closer knit clusters and formed a desirable spheroid shape. These morphological changes prompted by the differentiation medium allow the differentiated cells to display some of the characteristics of β-cells. Another one of the defining characteristics of β-cells is their ability to secrete insulin. The results from the rt-PCR, immunohistochemistry, and glucose challenge assay indicate that the differentiated cells from this experiment demonstrate these characteristics. Insulin, glucagon, and somatostatin were detected in the immunohistochemistry assays, indicating that the induced cell clusters differentiated into β-cells, α-cells and δ-cells respectively. [28] These results are conducive to the formation of pancreatic islet clusters capable of secreting their characteristic hormones. The results of the glucose challenge assay showed that these cells were able to secrete insulin when exposed to glucose. Insulin concentration increased proportionally with the glucose concentration, indicating that the differentiated cells were able to mimic the abilities of true β-cells. [11] These changes were observed in cells cultured in the differentiation medium we have developed. Therefore, these differential changes are to be expected in both the static and rocking cells from this study.

In addition to supporting the benefits of mechanical motion, the results of the qrt-PCR, cluster number and cluster diameter assays also demonstrate that the concentration of cells also influenced the cells differentiative capabilities. In both the control and experimental cultures, a higher concentration of cells resulted in a larger upregulation of target genes *Insulin* and *GCG*, and larger cell cluster diameters. However, the mechanically rocked cells showed a more drastic change in diameter with each increase in cell concentration while the stationary cells demonstrated slight increases in diameter between concentrations. The difference in diameter for the experimental treatment between concentrations was statistically significant indicating that

a combination of mechanical rocking and higher cell concentration resulted in larger clusters. The fold change in gene upregulation increased with cell concentration. It is also worth noting that the lowest concentration of cells was unable to produce enough RNA to run the PCR test and therefore did not possess the necessary gene upregulation to be considered differentiated. These results are supported by previous studies that indicate a link between cell concentration and differentiation potential. [29] The results from the viability staining also highlighted the effects of cell concentration. The cells were mostly alive with dead cells increasing with cluster size. This indicates that the smaller cell clusters in both the control and the experimental treatment may be more effective in viability and, in turn, differentiation than their higher concentration counterparts.

Overall, exposure to mechanical rocking motion resulted in statistically significant changes in cluster size and number in comparison to the stationary control. Both the static control and mechanical rocking motion cultures formed cell clusters after the three stages of pancreatic induction medium. However, the experimental mechanically induced cells demonstrated an overall higher LSM of clusters. This increase in cluster counts in the experimental cultures indicate that the mechanical stimulus prompted more cluster formation in the experimental treatment. [25] These mechanotransduced cells also formed larger clusters than the stationary control. This does not support our original hypothesis that these experimental clusters would be smaller. The cluster sizes were larger than the desired 85μm across islets we had hoped for. This increase in size may have caused some cell necrosis in the larger clusters from both treatments. [20] These large cluster results were most likely a side effect of the mechanical rocking motion pushing the cells against one another over time. Another notable difference between the two treatments was the shape of the cell clusters. The experimental cell

clusters demonstrated a more uniform shape across all concentrations whereas the stationary cells did not. Since the stationary cells were not exposed to any motion, they may have been able to grow and divide in clusters in random pattern since they did not have any motion to shape where and how the clusters would form. [30] Cluster uniformity was significant between treatments. These results indicate that future formation of pancreatic islets from stem cells could be expedited with mechanical motion.

Another observation from this study was the changed in gene regulation in cells exposed to mechanical rocking motion. The rocking cells demonstrated a higher level of expression for the insulin and glucagon genes. These changes indicate that the mechanically induced cells were able to differentiate better than their stationary counterparts. The high fold change in GCG may indicate that some α-cells formed in addition to β-cells. [31] While this result was not anticipated, injections of mixed cell islets that contain α and β-cells may be more effective than a single cell type. [32] Experimentation with mechanical motion has been conducted with various cell types with similar morphological and genetic results. These studies included mesenchymal stem cells differentiated into cells that had higher gene expression of TGF-β linked genes [33] as well as stem cells that demonstrated morphological nuclear changes when exposed to mechanical rocking motion. [34] Using mechanical rocking on cells cultured in differentiation medium yielded further differentiated cells than their stationary counterparts.

Several limitations existed within this study which, with future research, may provide further insight into the effects of mechanical stimulation on cell development, and how this stimulation could be utilized to improve stem cells used treat a variety of ailments. One limitation for this study was the limit of mechanical motion that could be applied to the cells. This limit prevented the cells from being exposed to more motion which could have encouraged

a more thorough differentiation. This limited side-to-side rocking may have also caused the larger clusters to form in the higher concentrations of cells as the cells were constantly pushed into one another. Previous studies have used electromagnetic forces to successfully generate motion in the cell cultures. [35] Creating a different mechanical stimulus apparatus that can vary the pattern and rate of motion may remedy this limitation in the future. Another limitation was the limit to how many genes were we able to test with PCR. In the future, testing for more β-cell specific genes as well as genes related to the *YAP/TAZ* pathway would be beneficial. The *YAP/TAZ* pathway is a cell signaling pathway that cells use to communicate. It can affect cell adhesion and mechanical signaling. Previous research on mechanical stimulation and the *YAP/TAZ* pathway has been conducted, which demonstrated that mechanical motion prompted upregulation of *YAP/TAZ* specific genes. [36] Being able to test for a higher frequency of these genes would allow us to paint a better picture of the genetic profile of the cells we are studying and better understand which aspect of the differentiation process are affected by mechanical stimulation. Ideas for future research include remedying these two limitations by creating a more complete mechanical motion and testing more genes to better understand the full scope of influence mechanical motion has on the genetic development of cell cultures. Another idea for future research would be to use an assay to detect ECM proteins in cells cultured with and without motion to observe the direct effects of mechanical motion on cell cluster development. ECM proteins include laminin, integrin and many more, detecting the presence of these proteins would demonstrate a stronger matrix had been built under the guidance of mechanical motion. [35]

Conclusion

Collectively our results signify the positive effects of mechanical rocking on pancreatic islet development. Exposure to mechanical rocking prompted numerous, large uniform cell clusters to form. These cell clusters demonstrate β-cell characteristics and may prove to be an effective replacement for the failing pancreatic islets in diabetic felines. Future studies should be conducted to evaluate the patterns indicated by this study. These studies may include different types of mechanical rocking motion to observe the varying effects different mechanical stresses may have on the development and transdifferentiation of adipose mesenchymal stem cells. Under the guidance of mechanical motion, these pancreatic islet organoids derived from feline adipose tissue have the potential to treat diabetic patients in veterinary medicine and beyond.

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