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CELL BIOPRINTING: A NOVEL APPROACH FOR ALPHA CELL TO BETA

CELL TRANSDIFFERENTIATION

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Master's Program in Biomedical Engineering

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Dean of the Graduate School

Dedication

This academic achievement is dedicated to the people who have seen me navigate this academic

journey so far, being nothing but unwavering pillars of support.

To my parents

Maria Escudero and Jose Casas

To my siblings:

Airon Casas and Alytzel Casas

To my committee members

Dr. Thomas Boland, Dr. Binata Joddar, and Dr. Siddharta Das

CELL BIOPRINTING: A NOVEL APPROACH FOR ALPHA CELL TO BETA

CELL TRANSDIFFERENTIATION

by

ATZIMBA CASAS ESCUDERO, B.S. Cellular & Molecular Biochem

THESIS

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Abstract

Diabetes is a chronic disease that occurs in the body when the pancreas fails to either produce insulin (TID) or does not effectively use the insulin produced (TIID) and poses further health complications as well as an insurmountable economic impact.[1] Type I diabetes is an autoimmune disease characterized by a deficient amount of insulin production on account of the body's immune system destroying its own β-cells.[2] Current diabetes treatment methods include the administration of insulin via injections or islet transplantation therapy. However, although both are viable options, they come with limitations that make the managing of this disease difficult. It is thus evident that there is a major need for the development of a treatment therapy that is quicker and does not pose much adverse effects on the patient. The purpose of this research project was to see if alpha cells can be transdifferentiated into beta cells when undergoing mechanical stress (bioprinting) as opposed to transdifferentiating alpha cells into beta cells without any added stress (non-printed). In this study, we demonstrated that bioprinted cells produced insulin when exposed to glucose after having undergone the transdifferentiation protocol as compared to cells that had not been subjected to the mechanical stress of the bioprinter. By successfully differentiating α-cells into β-cells, we may pave a path for an alternative cell therapy treatment method that can be used to treat diabetic patients in a health care setting as a means of improving patient's health and quality of life.

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Chapter 1: Introduction and Literature Research

1.1 Diabetes

Diabetes is a chronic disease characterized by elevated levels of glucose in the blood (hyperglycemia) affecting over 463 million people worldwide.[3] This disease is often a result of the pancreas failing to produce enough insulin or the body not being able to use the insulin that is produced to effectively regulate blood glucose levels. There are several types of diabetes that can be divided into type I (insulin dependent), type II (non-insulin dependent), and gestational diabetes.[4] Type II diabetes is usually a result of the body's inability to use the insulin that is produced by the pancreas. This diabetes is a direct culprit of excess body weight and physical inactivity in individuals, which is becoming a problem worldwide as more people's diets and daily habits are changing to more sedentary lifestyles, including children as well. Type II diabetes can usually be corrected by changing daily habits such as diet and exercise.[5] In contrast, type I diabetes is a chronic condition that is not a direct result of health habits. Rather, there are different factors that can contribute to type I diabetes, to include genetics, the exposure of viruses, and other environmental factors. There is no cure for this type of diabetes, so it must be treated for as long as the patient is alive through the management of blood sugar levels with insulin and adhering to a healthy lifestyle to avoid possible complications.[6] A third type of diabetes is known as gestational diabetes that is diagnosed during pregnancy due to the irregularities of hormones changing the way your body processes sugar levels. Although gestational diabetes poses a health threat to the mom and fetus, it usually goes away after delivery and can be managed through a healthy diet, proper exercise, and medication if necessary.[7] All three types of diabetes pose a threat to the public since diabetic prevalence has been rising steadily across the globe. However, due to the nature of our research, we will be focusing on Type I (insulin dependent) diabetes throughout our paper.

1.2 T1D Mechanism of Disease/ Pathogenesis

Type I diabetes is an autoimmune disorder that is characterized by deficient insulin production which can be attributed to the body's auto destruction of pancreatic β-cells.[8] These β-cells are located inside the pancreas where they are responsible for the production of insulin – a "key regulator of glucose homeostasis."[2] The pancreas is a gland that is part of the digestive system whose main role is converting any food that is consumed into fuel for the body.[20] This gland has two populations of cells: exocrine cells and endocrine cells – both with different physiological functions within the body. The endocrine cells are made up of five different hormone secreting cells that aggregate and form the islets of Langerhans.[10] Among these aggregates, both β-cells and α-cells – which secrete insulin and glucagon into the bloodstream, respectively – can be found. Since Type I diabetes is characterized by a loss of β-cell mass (approx. 70 – 100%) and subsequent insulin production, the main strategy for treating this form of diabetes is through the restoration of previously destroyed pancreatic β-cells through cell therapy.[11]

1.3 Diabetes Therapy Treatment

There are currently two proposed therapies for restoring β-cells loss: islet transplantation and α—cell to β-cell transdifferentiation. Islet transplantations occur when islets are taken from a donor and they are purified to then be transferred into a person with type I diabetes. This experimental therapy treatment has been a standard of care for diabetic patients because it decreases the need for insulin injections and provides patients with protection from severe hypoglycemia for a prolonged period.[12] However, there are also several downsides that possess a big risk for the patients. One of these cons involves the risks associated with the transplant procedure itself, since the patient may be subject to hemorrhaging, blood clotting, or subject to the severe side effects of immunosuppressive treatments.[13] Other negative downfalls of this therapy treatment is due to a shortage of islet cells.

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Since islets make up for less than 2% of cells within the pancreas, most of the tissue becomes waste after transplantation.[14] Of the small percentage of cells that can be isolated for treatment, β-cells are subject to being rejected by the body's immune system.[13] On a molecular scale, diabetes also influences the gene expression of the transplanted cells that then induce β-cell dysfunction and contribute to the deterioration of β-cell differentiation. [15, 16]

An alternative therapy treatment for patients with type I diabetes is known as transdifferentiation. Transdifferentiation is a process whereby a differentiated cell is converted into another type of cell.[17] As mentioned above, this alternative is a cell replacement therapy method that is increasing in popularity and further research is being done to better understand the development of β-cell reprogramming from other existing cells in the pancreas such as α—cells, another endocrine cell.[18] Since β-cells possess limited potential for regeneration in adult humans, pancreatic α-cells may represent a promising source of β-cells owing to several reasons. α-cells develop similarly to β-cells, their location in the pancreatic islet, the fact that they commonly undergo hyperplasia in diabetic animals and patients, and the finding that a significant decrease in α-cells in mice does not affect normal glucose metabolism.[20] Although both *in vivo* and *in vitro* research have proven to be successful, this therapy method has yet to be translated into human clinical trials. Another risk that α—cell to β-cell transdifferentiation has shown to possess is that undifferentiated β-cells present intrinsic tumorigenic properties that can pose a risk to the subject.[21] Whereas this method seems to pose a great functional alternative to address the β-cell deficit in type I diabetes, the limited knowledge of "basic processes that control differentiation, proliferation, survival, and physiology of β-cells,"[22] remain unsatisfactory since they still don't offer a cure or sufficient mitigation in preventing the complications associated with type I diabetes.

1.4 α and β cell growth factors

Alpha (α) and beta (β) cells produce glucagon and insulin, respectfully, and employ counter-regulatory actions on glucose metabolism. By stimulating glycogenolysis and gluconeogenesis, glucagon is directly responsible for increasing glucose levels in the body. Similarly, β-cells secrete insulin that then decrease glucose levels in the body by prompting glucose storage in cells and simultaneously lowering the total body glucose secretion.[23] Both alpha and beta cells rely on each other to modulate their activities and maintain a healthy glucose level balance from within the body, since insulin represses glucagon expression/release from α-cells while glucagon stimulates β-cell insulin secretion.[24] Both cells' functions are so dependent of each other that *Morgan, L. Y., et al. 2015* showed that glucagon gene activation is responsible for α-cell fate switch since the blocking of glucagon signaling pathway leads to an immense decrease in β-cell regeneration.[23] As observed in this study, data supports a model where the glucagon/GLP-1 signaling pathway works to elicit β-cell formation from previous α-cells since peptides derived from glucagon genes are crucial for the regulation of this endogenous repair system, suggesting that possible further stimulation of transdifferentiation might lead to a successful and efficient cell replacement therapy in humans.[23,25, 31] Although glucagon is a primary acting hormone aiding in β-cell differentiation, there are other growth factors and transcription factors that further drive this eventual cell differentiation. One such example is Urocorin3 (Ucn3) which is a protein encoding gene and member of corticotropin-releasing factor/ urotensin I family that helps generate the mature peptide hormone that is then secreted by both α and β cells and helps regulate insulin secretion via response to glucose levels in the plasma. As a result, Ucn3 acts as a marker of β-cell maturation as it is found in the intermediate stage of α-cell to β-cell transdifferentiation.[10] Other factors heavily influencing this transdifferentiation include Pdx1 and MafA expression among others. These transcriptional factors are crucial in β-cell development and maturation after initial transdifferentiation process, of which

Pdx1 remains a relevant insulin promoter factor throughout the β-cell's life, and aids in the activation

of transcriptional insulin genes.[18, 19]
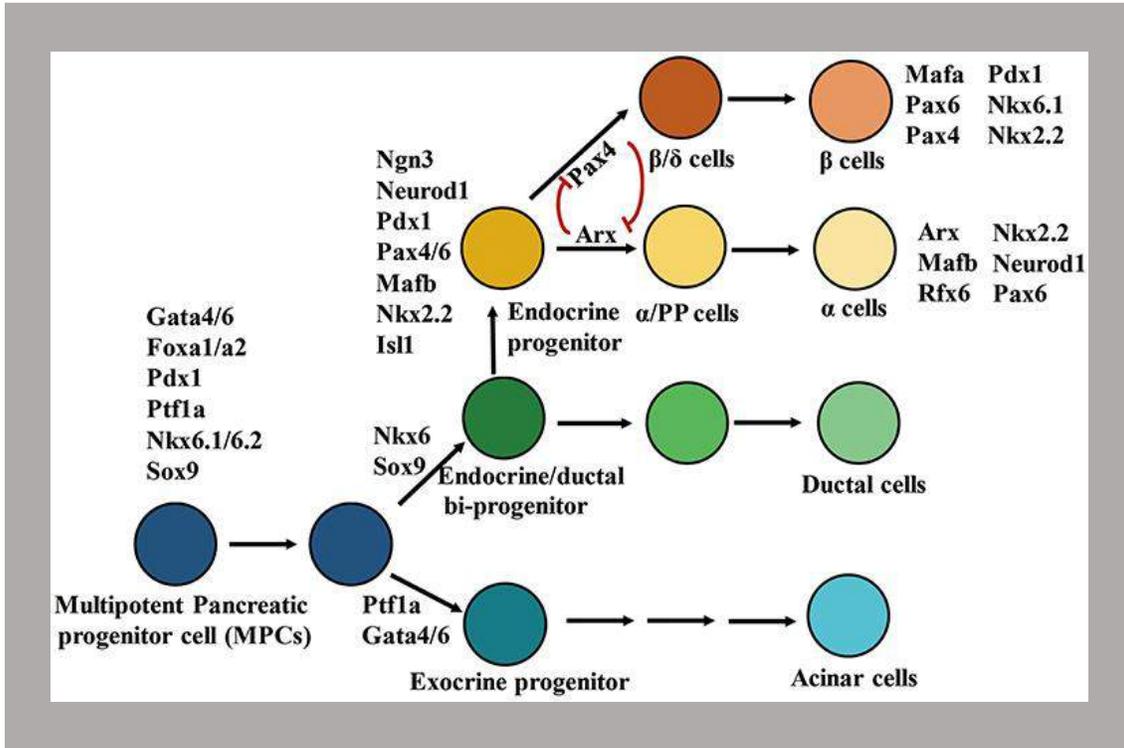
Image 1.4: Imagine highlights transcriptions factors found at different stages of the pancreatic cell lineage. As observed in the image, it also highlights the transcription factors playing a role in the process of α to β cell transdifferentiation.

1.5 Thermal Inkjet Bioprinter (TIB)

A relatively novel research method that has proven to be groundbreaking in several research fields

such as tissue engineering and medicine is known as cell-printing, or bioprinting.[25, 27] Being still

in its infancy stage for biological applications, bioprinting can be defined as the depositing of cells

and other biomaterials in "precise spatial arrangements to enhance cell-cell arrangement and

communication."[26] Currently, there are three main types of bioprinters (inkjet, microextrusion, and

laser-assisted printing) which all involve heat generation that may cause a physiological and

biochemical alteration to cells after having been printed. There are two methods used to form the

drops of ink known as continuous inkjet printing (CIJ) and drop on demand (DOD) printing.[28] Whereas CIJ charges the drops as they form to then be steered into location by electric or magnetic fields, the DOD method focuses on producing drops and placing them in position by hovering the drop generator above the desired position.[28] For the purpose of our experiment, we worked with the DOD method using a Thermal Inkjet Bioprinter (TIB). This bioprinting process exerts a thermal force on the cells, leading to alterations in the cells. At the beginning of the printing process, the thermal actuator/ resistor of the ink cartridges heats up to 300°C which then disperses into the bioink that will be printed and increases the overall temperature of the sample. This heat increase causes the bio-ink bubbles to form on the surface of the resistor. Eventually, enough pressure is exerted on the resistor, the bubbles overcome this surface tension, and bioink droplets containing cells are "printed" into the surface plate at the bottom of the printer.[29] With bioprinting being a relatively novel bio-fabrication process, there is minimal information available providing a clear image of the direct effects that it poses into the printed cells due to the temperature change as well as the stress they are subjected to. As a result, it is possible for these cells to undergo extreme mechanical and heat stressors that, given the right circumstances and experimental protocol, will allow for fully physiological alterations that will then allow us to manipulate printed cells in novel ways.

1.6 ELISA Immunoassays

ELISA Immunoassays are procedures utilized in a lab setting to quantitively measure specific antigens in a sample through the binding of antibodies and proteins. By immobilizing the reactants to the microplate surface, we are able to wash away any non-bound material from the cell whilst leaving specific analytes for measuring. All ELISA assays contain the same elements which start with the direct immobilization of antigens to the surface of the wells along with the addition of irrelevant proteins to cover unsaturated surface-binding sites of the wells. After the initial coating, the next step

is to incubate the wells with antigen specific antibodies that successfully bind to the antigens. Once removing all of the unbound material from the wells, we can quantitatively measure the signal generated from the specific antibodies in the sample using instrumentation such as a spectrophotometer, a fluorometer, or even a luminometer. Currently, there are different ELISA formats which can be categorized as direct, indirect, sandwich, and competitive ELISA detection methods. Direct ELISA assays involve the direct immobilization of the antigen onto the plate while the conjugated detection antibody then binds to the target protein. The substrate added then produces a signal that is proportional to the amount of analyte in the sample (direct correlation). A second type of ELISA assay is the indirect ELISA which is similar to the direct ELISA assay. However, this assay includes an additional amplification detection step in the form of a conjugated secondary antibody directed against the host species of the primary antibody for which the substrate then produces a signal that is, again, proportional to the amount of antigen bound in the well. The third and most common type of ELISA assay used is the sandwich ELISA which is used to determine the analyte concentration in a sample. In this assay, two specific antibodies are used to sandwich the antigen. First, the microplate is covered in capture antibodies which are then bound to the protein or antigen of interest and become immobilized. A secondary antibody is then added to bind to the target protein, in a sandwich manner, and the substrate that is added to the sample emits a signal proportional to the amount of analyte present in the sample. This type of ELISA is most used because the two antibodies required make this assay highly specific.[30]
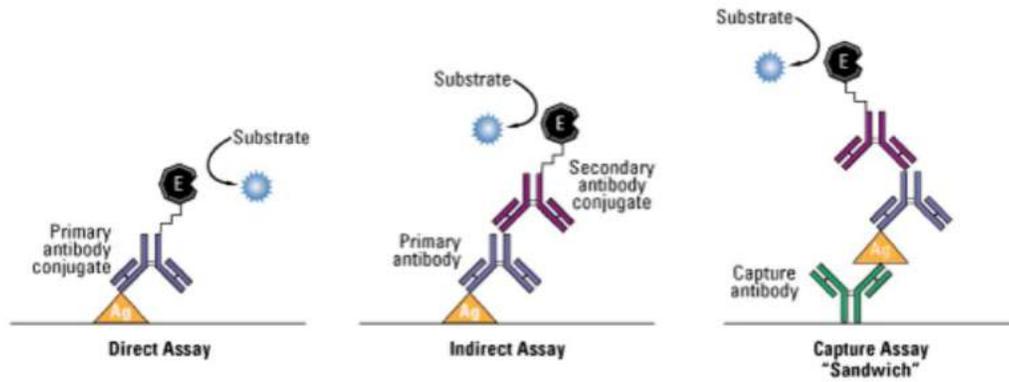
Image 2: Diagram offers a visual of the three different kinds of ELISA assays for comparison. Direct and indirect assays are characterized by the binding of the antigen directly to the plate surface and then having the antibody conjugate(s) of interest attach to the antigen. By contrast, the sandwich assay first binds the capture antibody to the plate surface. The antigen is then bound to this first capture antibody followed by the secondary antibody, entrapping the antigen in a "sandwich" manner.

1.7 Research goal and Significance

Our research goal is primarily focused on determining if there is a success rate of transdifferentiation of α-cells into β-cells when placed under the biomechanical stress of bioprinting as opposed to transdifferentiation into β-cells without having undergone a biomechanical stress beforehand. With diabetes being one of the major causes of death worldwide, a breakthrough in this research field would pose great significance. Not only would it greatly impact the world's economy in a positive manner, but it would also help increase the quality of care in patients in healthcare settings whilst monumentally improving the quality of life for type I and type II diabetic patients.

Chapter 2: Materials and methods

2.1 Reagents

This research is primarily focused on the transdifferentiation of β-cells from α-cells to successfully produce insulin. As a result, a variety of reagents will be used during the research project. For cell differentiation, we used several growth factors aimed at promoting cell proliferation and ensuing better cell survival from environmental stress as well as supporting cell differentiation. A more detailed overview and description of these GF can be observed on Table 1 in the index.

The cell fixing method used reagents aimed at preserving cells and structures (4% paraformaldehyde) while cell staining relied on reagents, used to pull out cell structures and proteins (Triton X-100), and a blocking buffer to use with specific primary and secondary antibodies that bound to cell structures for staining (a detailed chart of the antibodies used can be found on Table 2 in the index). Other reagents used for this protocol include phosphate-buffer saline (PBS) to wash the cells of excess reagents, 1% bovine serum albumin (BSA) in PBST for use with antibodies, and DAPI solution - a dye used to fluorescent DNA staining.

The final ELISA insulin assay relied on reagents who focused on quantitatively measuring the insulin production of both experimental cell samples and mouse insulin. For this assay we used the *Crystal Chem® Ultra Sensitive Mouse Insulin ELISA Kit (Catalog#: 90080)* ordered from Crystal Chem High Performance Assays. The reagents in this kit included the mouse insulin stock solution (to make the insulin standard), the anti-insulin enzyme conjugate, an enzyme substrate solution along with the enzyme reaction stop solution, a sample diluent, and a wash buffer.

2.2 Cell culture and seeding

For this project, pancreatic mouse alpha TC1 cell clone 6 (ATCC® CRL – 2934) were used. These cells were grown in DMEM, low glucose medium which was supplemented with 10% FBS, 12 mM HEPES, 0.1 mM amino acids, and 0.02% bovine serum albumin. The cells were then kept in the incubator at a temperature of 37˚C with a 10% carbon dioxide continuous flow until they were ready to be passaged.

To seed the cells, they were first thawed out and placed in a centrifuge tube along with 10 mL of DMEM media. The centrifuge tube was then centrifuged for 5 min at 2000 RPM until the cells formed a pellet in the bottom. Next, the supernatant was removed from the tube (to remove DMSO and trypsin) and 1 mL of α—cell media was added to mix with the cells. The 1 mL mixture of cells and media was then pipetted into a T-75 cell flask along with another 14 mL of α-cell media which was then placed into an incubator chamber. In order to keep the DMEM media fresh for the cells, the first media change was done 24h after the first seeding and then every other day subsequently until cell confluency was enough for splitting.

2.3 Cell printing

Cell printing is the most critical part of the project, which is done after performing the cell splitting protocol to isolate the cells from the DMEM media through centrifugation. For this project, we utilized a readily available modified HP inkjet bioprinter since we were going to be working with the DOD method to place cells directly into the wells. We decided to use this kind of bioprinter since previous research had proven to be successful and, while cells were stressed sufficiently, it did not induce any major harm or apoptosis on these cells. Once the supernatant was removed, 2 mL of PBS was placed in the tube to break the cell pellet and ensure an even distribution of suspended cells for printing. Before pipetting cells into the printer cartridge, make sure the printer is set up and working

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correctly (a test run with milli-Q water is strongly advised to accurately measure placement and DOD printing). Pipette 150 µL of the cell suspension into the cartridge and run the program to print the cells into a 96 – well cell – adhering plate at the desired rate and time (as shown on Image 3.3 below) and label as "printed experimental group." Along with the printed cells, transfer 12 µL of non-printed cells into another 12 designated wells on cell – adhering plate (again, shown on Image 3.3) and label as "non-printed control group." Add 75 µL of "day 1" differentiation media into all 24 wells and place in incubator (37°C and 10% CO_2) for continued cell growth. Continue β-cell differentiation protocol as stated by protocol guidelines.
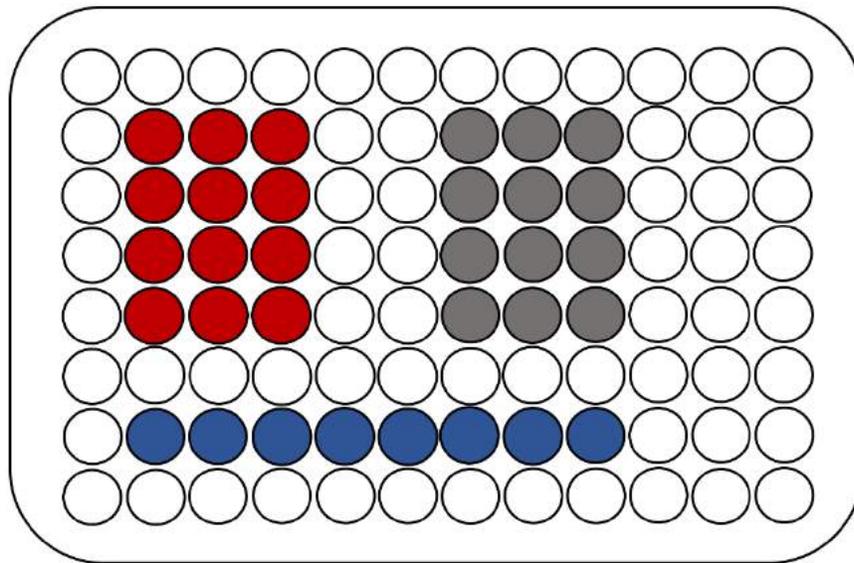
Image 3.3: Image shows the 96 – well cell distribution and how the different experimental and control groups were placed in the plate. As shown above, the red wells were the printed cells while the gray wells were the non-printed cells. These groups would be undergoing the β-cell differentiation protocol. The wells at the bottom (navy blue) represent the control group, which are the α-cells that did not undergo the differentiation protocol.

2.4 Cell differentiation

Cell differentiation was conducted at the same time every day for at least 21 days (minimal duration of protocol). The protocol was divided into 6 different stages where each stage varied with different factors to promote β-cell differentiation from the preliminary printed α-cells. Following the protocol, media changes were done either every day or every other day. These media changes, performed inside a safety hood to prevent contamination, were carefully done with a micropipette to remove all the liquid in the wells, adding 100 µL of the appropriate media to the wells and finally placed in the incubator again to keep promoting cell differentiation. A detailed overview of the β-cell differentiation protocol can be found on Table 2 in the index.

2.5 Cell fixation

Having finished the cell differentiation stage, cells needed to be fixed into the plate before staining to freeze cellular proteins and structures in place for later microscope observation. This fixing protocol is done with all solutions warmed up to 36°C and under a biohazard hood to prevent any health hazards. As shown in Image 3.5 below, we fixed the first three wells from each group (colored in sky blue) while the other wells would be preserved for further experiments.
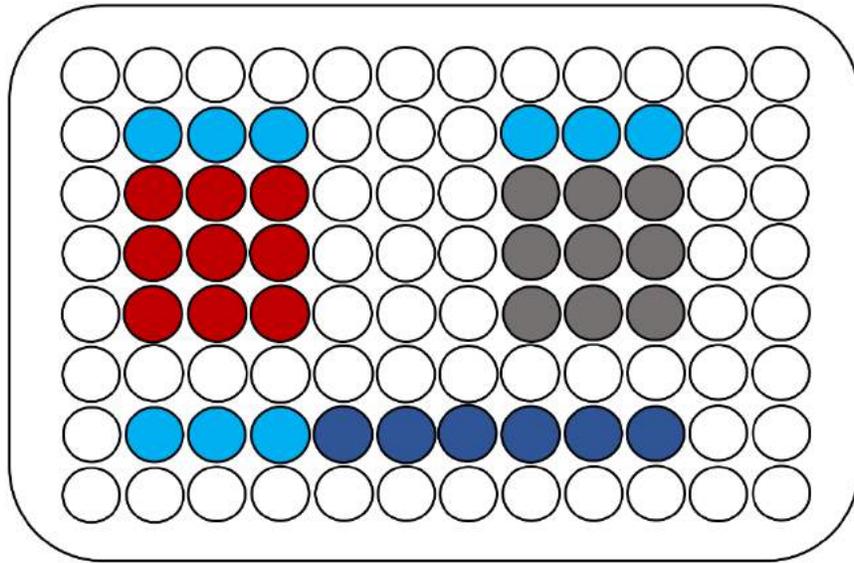
Image 3.5: Image displays the 96-well plate and its cell fixing/ staining distribution. Three wells from each experimental and control group were fixed and scanned for differentiated β-cells under the confocal microscope.

First, the medium inside the wells that would be fixed/ stained were aspirated – careful not to aspirate the cells as well or scratch the bottom of the well. Each well was then rinsed with PBS and then 100 µL of 4% paraformaldehyde solution was added for 20 minutes. In the final stage, we removed the paraformaldehyde and rinsed the wells with PBS. Due to time constraints, we were not able to continue with the cell staining that same day. As a result, we filled each well with 100 µL of PBS and stored in the incubator to preserve the cells that had not been fixed in the other wells. We worked in triplicate to fix cells – where we fixed three wells from each group that where then going to be followed by the staining process.

2.6 Cell staining

Cell staining is done to have a better visual of cells and its components when seen under a microscope. This method was done after fixing the cells in the wells. Since the fixed cells were being hydrated by PBS, the first step was to aspirate the 100 µL of PBS and add another 100 µL of Triton X-100 to each well for 10 minutes. The Triton X-100 was then removed, and the wells were rinsed 3x with PBS, waiting 5 mins in between rinses to remove any residual Triton X-100. After these 3 initial washes, 100 µL of blocking buffer (composed of 1% BSA, 22.52 mg/mL glycine in PBST (PBS+ 0.1% Tween 20) was added to each well for 30 minutes, followed by a 5-minute PBS wash. We then incubated the cells in 50 µL 1% BSA in PBST along with 1 µL of diluted antibody (1:50 dilution) inside the incubator for 1 hour. This first antibody that we used were 3 different primary antibodies that were placed once in each well of the individual groups, which included an antibody for PDX1, glucagon antibody, and NKX6-1 antibody. This solution was subsequently aspirated and kept in conical tubes for later use while the cells were once again washed 3x in PBS for 5 minutes each time. The cells were incubated again with a 1:50 dilution of 1 µL of a second primary antibody (Anti-C peptide) in 50 µL 1% BSA solution in PBST for 1 hour at room temperature in the dark. The solution was again preserved while the cells were once again washed 3x with PBS for 5 minutes. Thirdly, we incubated the cells a third time with 1 µL of a secondary antibody (Goat anti-rabbit igG) in 50 µL 1% BSA in PBST for 1 hour (1:50 dilution) and subsequently washed the cells 3x with PBS for 5 minutes, making sure we saved the antibody supernatant as well. We treated the cells with 1 µL of second secondary antibody (Goat anti-mouse IgG) in 50 µL of 1% BSA in PBST (1:50 dilution) and incubated for 1 hour, followed by the washing of the cells 3x with PBS for 5 minutes each wash. To finalize the cell staining protocol, we added 50 µL of DAPI to each well and waited for 30 minutes. After a final cell wash with PBS (3x for 5 minutes each), the cells were ready to be imaged using the confocal microscope to determine if there had been any β-cell differentiation from the α-cells.

Note: After cells have been fixed and stained, they should be hydrated with PBS. These cells can then be maintained for months and imaged later.

2.7 Mouse insulin assay

This assay is performed for the quantitative determination of insulin produced in the cell culture media from our experiment. To have a more precise and accurate measurement of the insulin production of our wells, it was important to first prepare a mouse insulin standard curve that would graphically show the relationship between the insulin concentrations and the absorbance of a light wavelength. For this standard curve, we first prepared all the reagents we were going to use as specified by the manual. Making sure to use polypropylene microtubes since they have minimal adsorption of insulin, we first pipetted 150 µL of sample diluent and mixed it with 50 µL of the mouse insulin stock solution (25.6 ng/mL) inside a microtube labeled 6.4ng/mL. From this first tube, we micropipetted 50 µL of the sample diluent into 6 polypropylene microtubes and labeled each one depending on the concentrations that we were going to be working with (0.1, 0.2, 0.4, 0.8, 1.6, and 3.2 ng/mL). Using the micropipette, we dispensed 50 µL of the 6.4 ng/mL sample standard into the tube labeled 3.2 ng/mL and mixed it thoroughly. Afterwards, we took 50 µL of the 3.2 ng/mL sample and mixed it with the sample diluent in the 1.6 ng/mL tube. We repeated this step progressively with all the samples for the remaining 4 microtubules. Finally, to obtain a control of the standard curve line, we pipetted 50 µL of the sample diluent into a final polypropylene microtube and labeled it as 0 ng/mL since there would not be any mouse insulin on this sample. Since the mouse curve line was used to establish an insulin baseline and there were no experimental cells involved, we only performed each dilution in a duplicate manner and recorded the mean reading.

After having prepared the working mouse insulin standard, we performed the assay with the experimental glucose concentrations, which we performed in triplicates of each concentration,

obtained the insulin values, and recorded the mean absorbance value from each insulin concentration. These different glucose concentrations are important since the goal of the assay is to measure total insulin production when exposed to different concentrations of glucose solutions and determine if α-cells did undergo β-cell transdifferentiation. The three different glucose amounts that we worked with were 0.009 g, 0.025 g, and 0.150 g. However, these amounts of glucose called for a 50 mL islet media solution, and we needed a minimal amount of glucose solution (5 mL), thus changing the amount of glucose used to 0.0009 g, 0.0025 g, and 0.015 g respectively to maintain the same concentration. Using 3 – 15 mL conical tubes and a scale, we weighed out the powdered glucose and then mixed it with 5 mL islet media until we had a homogeneous mixture that we then filtered out for impurities. Our next step was to begin the assay by inducing the first reaction. After making sure all reagents and working materials were room temperature, we removed the antibody coated microplates from their pouch and placed them in the supporting frame where we then added 95 µL of the sample diluent in each well that we were going to be using. ***Note: To eliminate any kind of incorrect absorbency reading, every sample (for both experimental glucose concentrations and mouse insulin standards) had to be assayed in duplicate.*** We then added 5 µL samples of both glucose and insulin working standards into the wells and covered both the plate and the 96-well plate and incubated them for 2 hours at 4°C. After the two hours were up, we aspirated the contents in the wells and washed them 5x with the provided wash buffer using 300 µL per well and removed any excess wash buffer by inverting the plates and tapping the wells firmly onto a paper towel. Having finished all 5 washes, we then pipetted 100 µL of anti-insulin enzyme conjugate, covered the plates once more, and incubated it for 30 minutes at room temperature. The next step was to, once again, wash the well 7x using 300 µL of wash buffer and remove any excess wash buffer. Following the wash, we added 100 µL of enzyme substrate solution and let it react for 40 minutes at room temperature making sure to not expose it to light or cover it in aluminum. After the 40 minutes were up, we stopped the mechanism of the enzyme

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substrate solution by immediately adding another 100 µL of enzyme reaction stop solution so that we could measure the insulin production based off of the absorbency levels indicated on the samples. *Note: After adding the enzyme reaction stop solution, we had 30 minutes to measure the absorbance of the samples before any significant change occurred in the wells.*

The final step involved the use of a spectrophotometer to run the different dilution samples that we collected, record the absorbance of light based off the insulin concentration, and create a standard curve off the measurements to compare the mouse insulin standard vs experimental insulin production. This step was done by first measuring the absorbency of the sample under the 430 nm wavelength and then repeating the readings using the 630 nm wavelength and finally subtracting the A_{630} readings from the A_{450} to obtain the values to plot into the graph.
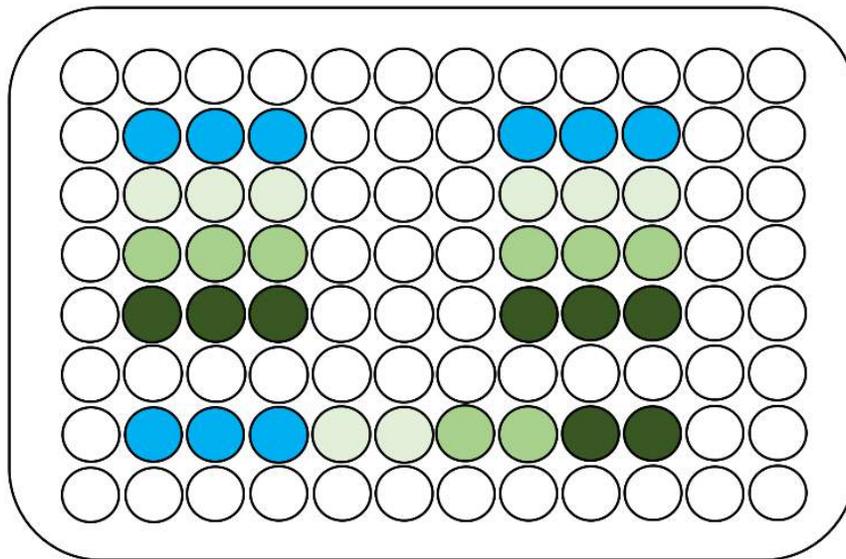
Image 3.7: Image provides an overview of the 96 – well plate for the ELISA insulin assay. As depicted on the picture above, the progressive darkening of color shows the different glucose concentrations (in triplicates) that the cells in each experimental group were exposed to. The lightest green denotes a concentration of 0.00018 g/ mL while the middle triplicate has a glucose concentration of 0.0005 g/mL and the darkest green accounts for the highest glucose concentration of 0.003 g/mL.

Chapter 3: Results and Discussion

3.1 Confocal Microscopy Imaging

Image 3.1.1a: α-cell control
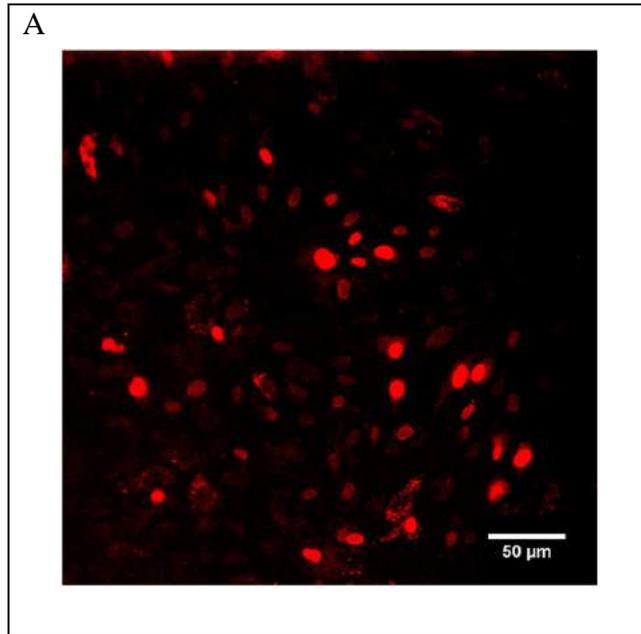
Image 3.1.1a: This image provides a visual of the control α-cell group under when viewed under the confocal microscope. Image A shows cells stained with glucagon antibody.

Image 3.1.1b: α-cell control
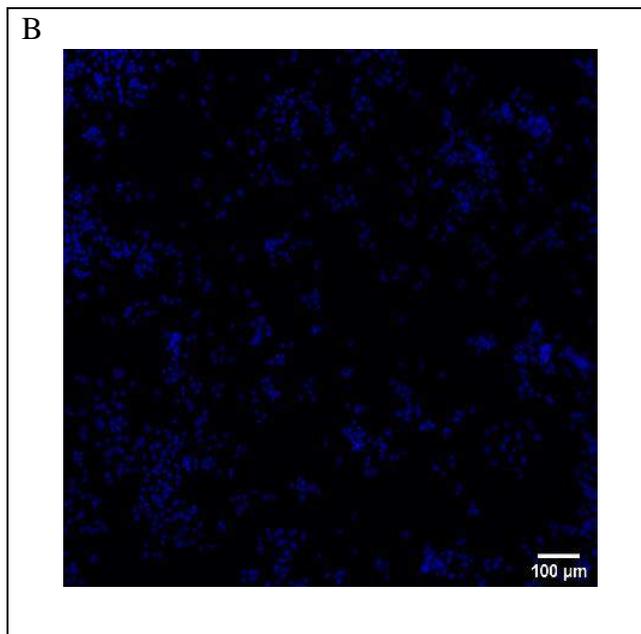
Image 3.1.1a: This image provides a visual of the control α-cell group under when viewed under the confocal microscope. Image B shows cells stained stained with DAPI blue dye.

Image 3.1.1a and image 3.1.1b highlight two different regions taken from the control wells (α-cells) as well as their morphological properties as seen through confocal microscopy using immunofluorescence staining. Image A shows cell samples that were stained with glucagon antibody and subsequently reacted with a secondary antibody in order to test for the presence of glucagon production in the cells. Image B shows cells stained with DAPI blue dye which binds to the A-T regions of dsDNA helix of live cells. This stain was done to show that the samples we were working with were alive at the time of fixing. We also tested for insulin presence in the α-cells by staining the wells with both PDX1 and NKX6.1 antibodies but did not find any insulin production from within these cells. Insulin is a peptide hormone that directly affects α-cells by regulating glucagon secretion from these cells, so it would be normal to find insulin within these cells. However, since both the PDX1 and NKX6.1 antibodies are insulin promoter factor genes, the stain would be retained in the cellular nucleus if the cell was producing insulin.

Image 3.1.2: Printed cell (PDX1 antibody)
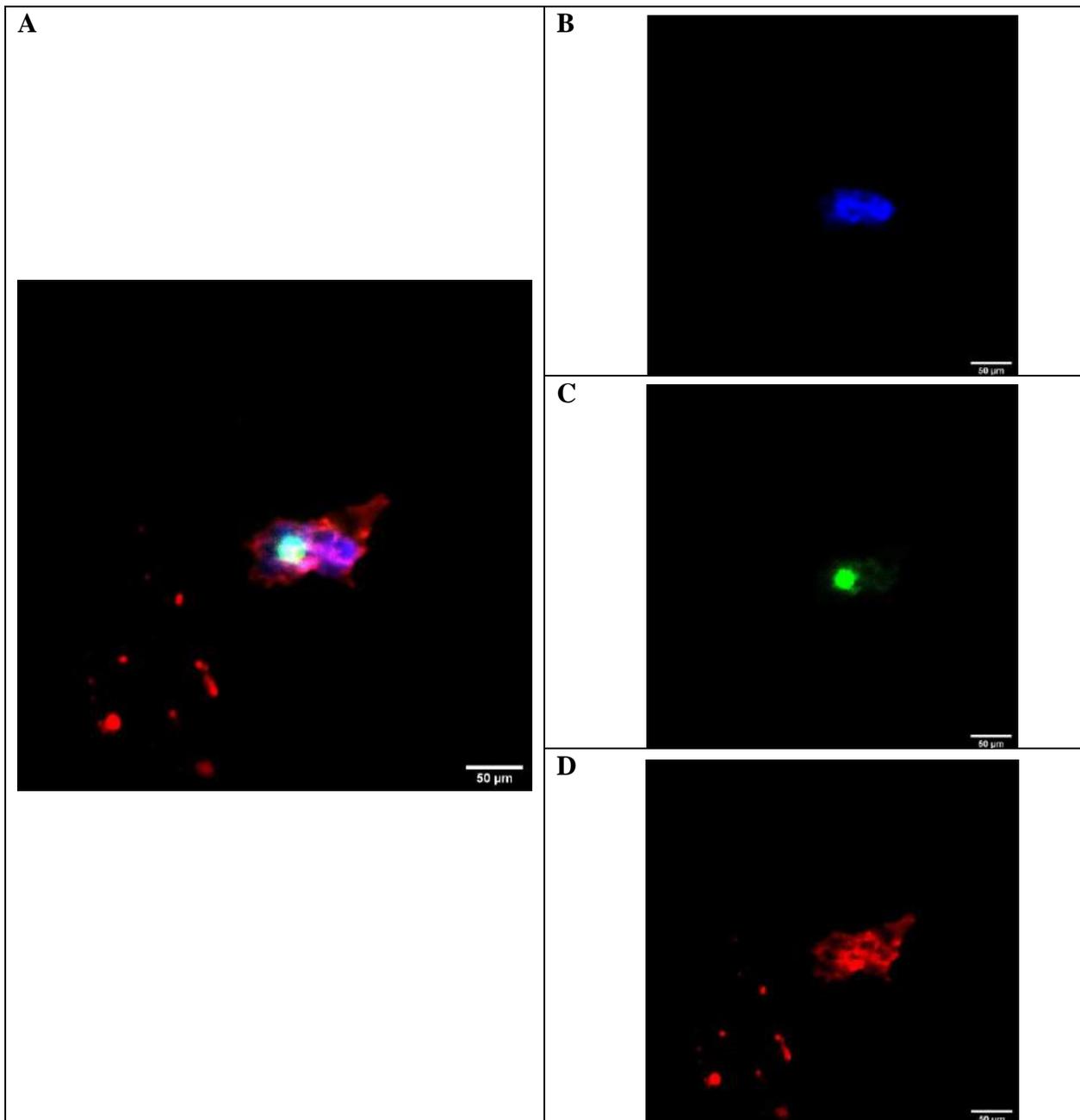
Image 3.1.2: This image provides a visual of a printed cell after having undergone the transdifferential protocol. From these images, A represents a composite of the cell with all three dyes bound to the cell. Image B shows the cell stained with DAPI blue dye while image C was checking for the presence of Pdx1 antibody and image D depicted the presence of C – peptide antibody in the cell.

Image 3.1.3: (NKX6.1 antibody)
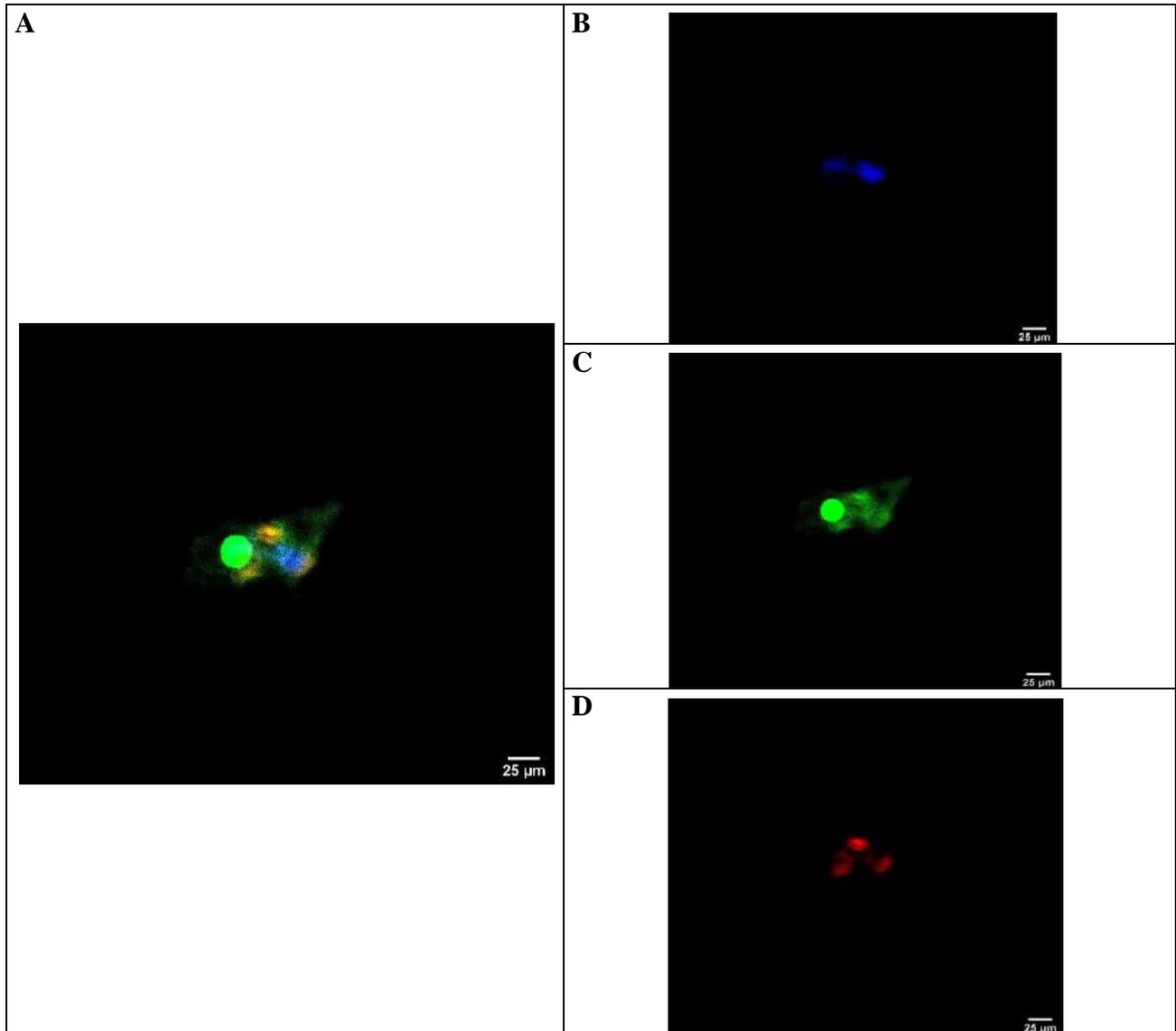
Image 3.1.3: This image provides a visual of a printed cell after having undergone the transdifferential protocol. From these images, A represents a composite of the cell with all three dyes bound to the it. Image B shows the cell stained with DAPI blue dye while image C was checking for the presence of Nkx6.1 antibody and image D depicted the presence of C – peptide antibody in the cell.

Image 3.1.2 shows printed cells treated with PDX1 antibodies with the C – peptide antibodies (to check for insulin production). Whereas the glucagon antibody was used in cells to look for the presence of glucose (i.e. alpha cells), both PDX1 and NKX6.1 were chosen for other specific reasons.

As cited in literature work, PDX1 was chosen as a transcription factor of interest because it is a critical transcription factor that is present in all stages of cell differentiation/ maturation into β – cells.[46] As opposed to Pdx1, NKX6.1 is a transcription factor that is solely found in differentiated beta cells and can therefore help us infer that the cells successfully differentiated into β-cells.[47]

As seen on image A, a composite image is produced showing all of the dyes reacting within the cell's components such as proteins and hormones. Image B is the cell stained with DAPI blue binding to the A-T regions of the dsDNA while image C portrays the binding of the primary antibody used, PDX1 (pancreatic and duodenal homeobox 1), which is a protein coding gene that is heavily expressed in β-cells as it encodes a transcriptional activator of genes that include insulin. This transcription factor also plays an important role in pancreatic β-cell function and survival. During the early stages of differentiation, it has been observed that β-cells overexpress this transcription factor to keep stimulating cell differentiation. As it can be observed, the cell has a high concentration and green contrast in the center of the cell which indicates there is an overexpression of PDX1 taking place in the nucleus of the cell, further supporting the notion that the previous α-cells have undergone cell transdifferentiation into insulin producing β-cells. Finally, image D is linked to the primary antibody used to check for C-peptide antibody. C-peptide is a byproduct of insulin formation that takes place in the pancreas, oftentimes being a prohormone precursor to insulin that is produced in β-cells. Usually, higher levels of C-peptide serve as an indicator of endogenous insulin production in pancreatic islets. As seen on image A and D, the red dye seems to expand further than the cell. This may be an indicator that the cell is not only successfully producing insulin but that it has also begun to secrete it to the extracellular matrix.

Similarly, image 3.1.3 shows a cell treated with NKX6.1 antibodies (instead of NKX6.1) as well as C-peptide antibodies (to check for insulin production). As seen with the previous image, image A utilizes DAPI blue dye to check for live cells as the dye binds to the dsDNA in live cells. For

this image, the green dye is linked to the primary antibody NKX6.1, another transcription factor encoding for the activation of insulin genes. The NKX6.1 transcription factor plays a crucial role in β-cell function and proliferation and is exclusively found in β-cells and no other cells in the islet within the pancreas as it inhibits glucagon gene transcription. Once again, as observed in the previous image, there is a really high concentration and green contrast in the center of the cell which indicates the presence of NKX6.1 transcription factor taking place in the nucleus of the cell, further supporting the notion that the previous α-cells have undergone a cell transdifferentiation into insulin producing β-cells. For this well, image D shows the red dye that is also linked to the C-Peptide antibody to check for C-Peptide production and subsequently the production and secretion of insulin from within the cell, which is also evident. **

*** Note: Distance in shape and colors might indicate a difference in differential stages where the cell is barely starting to produce and secrete insulin into the extracellular matrix space.*

3.2 ELISA Sandwich Assay

Further tests were performed to establish a quantitative analysis on the amount of insulin secreted by the different experimental group of islet cells when exposed to specific glucose concentrations.
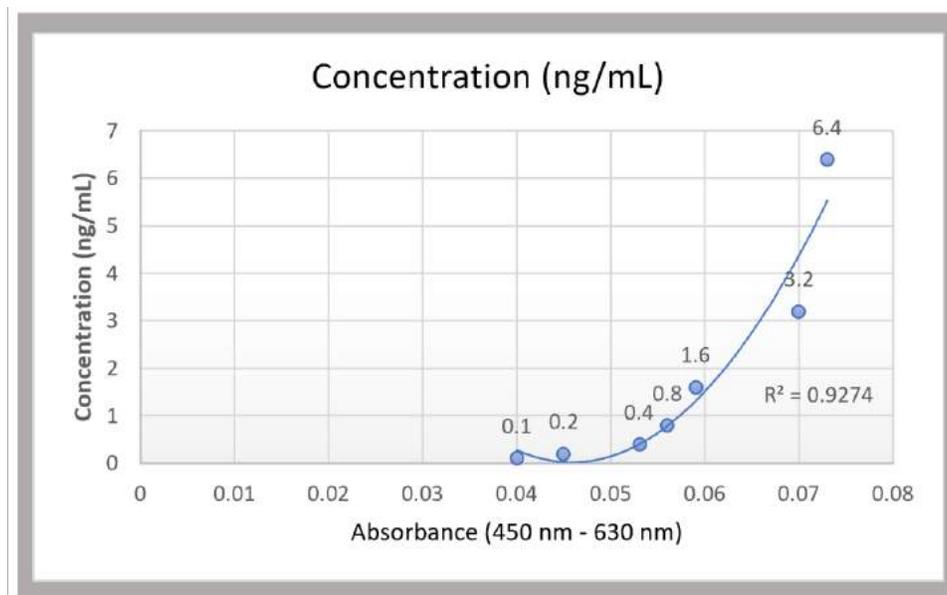
Graph 3.2.1: Provides an overview of the Mouse Insulin Standard curve performed with the ELISA insulin assay. Standard curves are important in experiments to ensure the precision and accuracy of the experiments performed subsequently.
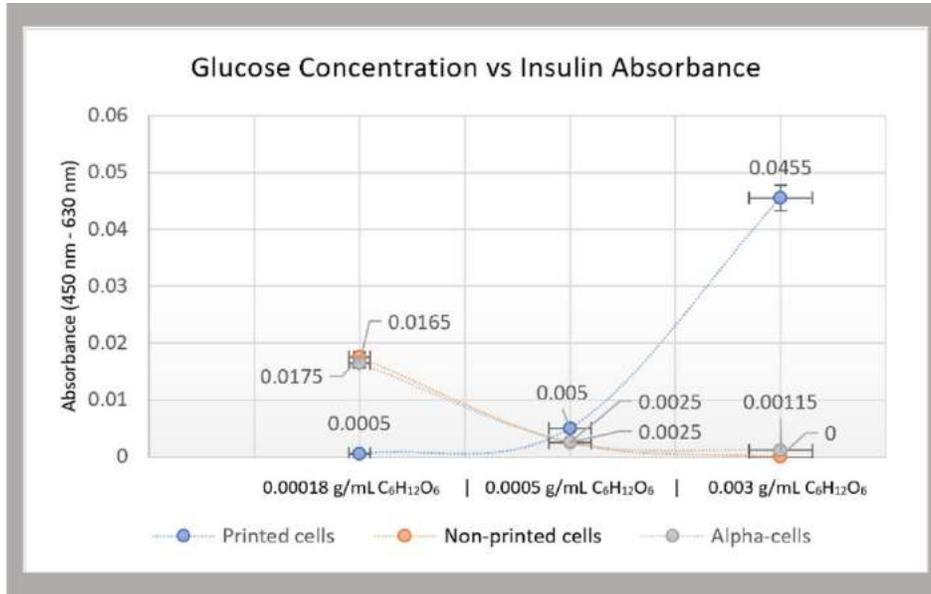
Graph 3.2.2: Portrays the values expressed as means ± SE of the insulin absorbance when exposed to increasing glucose concentrations. As noted on the graph, some values increase while others decrease as the glucose concentration increases.
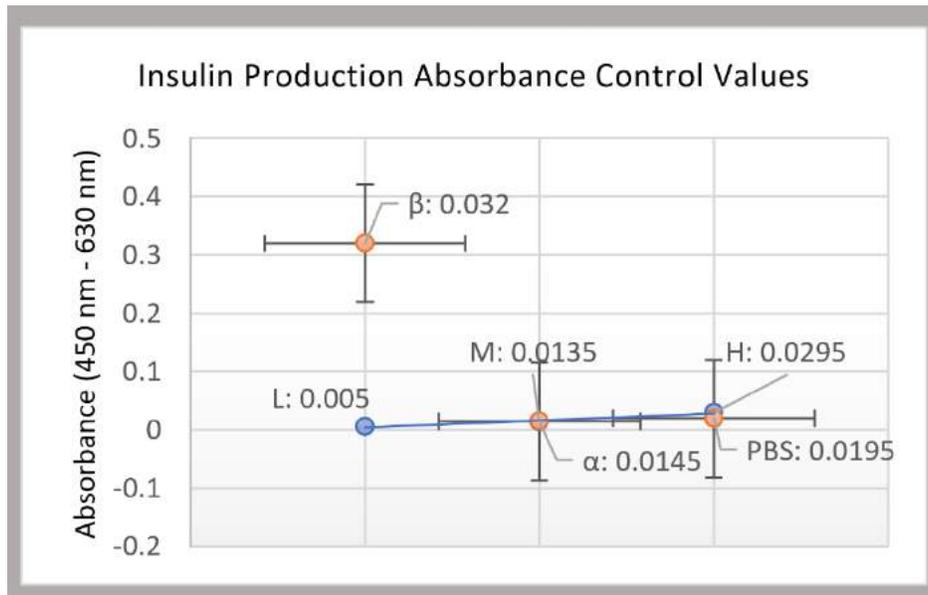
Graph 3.2.3: Provides an overview of the mean insulin production and absorbance of the control values ± SE. As seen on the graph, the mean absorbance values for all three glucose concentrations are recorded as well as the controls for stand alone α-cells, β-cells, and PBS.

Graph 3.2.1 logged the absorbance values of the mouse insulin standard curve (ng/mL) for different concentration dilutions. Starting at a concentration of 0, which was simply milli-Q water, we then recorded the two-fold increasing absorbance values all the way to 6.4 ng/mL. As seen from the graph, there is an exponential correlation line, meaning the reading absorbances increases in relation to the concentration values across the graph.

The second graph, graph 3.2.2, displays the mean values ± SE of the insulin absorbance levels vs. glucose concentration levels. As shown above, we graphed the correlation of the average absorbency values for printed, non-printed, and α-cells. From the graph, we can see that the printed cells insulin absorbed is directly proportional to the increasing glucose concentrations, meaning that the insulin secretion of the cells increased as it became exposed to higher amounts of glucose. Similarly, it can also be noted that both the non-printed and α-cells shared similar characteristics when measuring the insulin absorbance of increasing glucose concentration. Rather than a direct increasing proportion, both experimental groups exhibited an indirect proportion where the recorded values of insulin decreased as the glucose concentration increased. This may be attributed to the fact that insulin production remains relatively constant as opposed to the increasing glucose concentration. As a result, the overall insulin concentration decreases as well. Our findings point to the fact that the cells that were printed underwent a transdifferentiation phase change to β-cells and began secreting insulin as opposed to both the non-printed and α-cells.

Finally, graph 3.2.3 plots the average mean values for both the stand-alone glucose concentrations as well as the control values for α-cells, β-cells, and PBS solution which are all presented for comparison to the experimental groups. As seen above on graph 3.2.3, insulin absorbance values slightly increase linearly as the glucose concentrations increase as well. When comparing PBS solution to the cell lines, we can see that both the PBS and the α-cells presented similar absorbance values while the absorbance in the β-cell control was a little bit more than double

as compared to the α-cells. This graph serves to further support our initial hypothesis that bioprinting

α-cells would aid successfully in the transdifferentiation into β-cells. **

** *NOTE: Any insulin markers found in ELISA assay where there shouldn't be may be due to insulin precursors found in*

cells/ samples or possible minimal contamination of samples when measuring absorbance values in the cuvette.

Chapter 4: Conclusion

In conclusion, our research hypothesis was supported by the data gathered in this project as we demonstrated that bioprinted cells were able to undergo the transdifferentiation protocol into β-cells and secrete insulin. Due to the supporting evidence collected, it is apparent that cells endure mechanical stress during the bioprinting process that ultimately leads to both physiological and chemical alterations whereas non printed cells do not have the same fate and remain unaltered by the transdifferentiation protocol. This is a significant observation since further testing and optimized bioprinting research could lead to the potential development of an alternative cell therapy treatment that could improve and help eradicate diabetes worldwide.

Chapter 5: Limitations and Future work

5.1 Limitations

A limitation that can be addressed for the project involves the use of the bioprinter and the bioprinting process. Since the DOD method relies on cartridges, some of these nozzles could lead to becoming blocked when attempting to print cells into the wells due to cellular debris or sedimentation. This could lead to a minimal number of cells being printed into the wells and thus hindering the transdifferentiation process. Another limitation that was presented during the project was the failure to further adhere the cells to the bottom of their wells in order to prevent them from being washed away during the differentiation protocol with all the media handling and washing.

5.2 Future work

With this research being preliminary, some of the future work that must be accomplished is the successful repeat of this project and procedure with a bigger sample size for both the printed and the non-printed cells. This project would eventually lead to *in vivo* work with appropriate protocols to ensure a controlled, successful project. Additionally, it would also be wise to investigate and promote further research so that differentiated β-cells are not targeted by the immune system or slow the gradual loss of their function. The ending vision for this project is to be able to successfully use bioprinting technology to significantly promote the transdifferentiation of α-cells into β-cells and then inject these same cells in diabetic patients as an alternative, successful therapy treatment.

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Growth Factors Chart

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Glossary

a. **Transdifferentiation** – The conversion of a fully differentiated cell into another cell type.

b. **Alpha (α) cells** – Endocrine cells in pancreatic islets composing 20% of human islet cells and secreting peptide hormone glucagon.

c. **Beta (β) cells** – Endocrine cells that make and secrete insulin.

d. **Glucagon** – A hormone produced by alpha cells that is involved in controlling blood glucose levels by promoting the breakdown of glycogen to glucose.

e. **Insulin** – A hormone produced by beta cells that controls and allows for glucose to enter the body's cells for energy.

f. **Confocal Microscopy** – A fluorescence imaging technique that utilizes laser point source to scan the sample.

g. **IF (Immunofluorescence)** – A laboratory technique based on the use of specific antibodies conjugated to fluorescent dyes that bind to cellular antigens.

h. **PBS (Phosphate-buffered saline)** – A buffer solution used in biological applications such as cell washing and transportation of tissues and dilutions.

i. **PBST (Phosphate-buffered saline/Tween)** – PBS with a low concentration of detergent solution used as a wash solution that does not damage antigen/antibody complex. This solution is commonly used in western blots or ELISA assays.

j. **BSA (Bovine Serum Albumin)** – A serum albumin protein used as a protein concentration standard in labs, as a cell nutrient, and has the ability to stabilize enzymes.

k. **dsDNA** – double stranded DNA

Appendix

8.1 Table 1: Transdifferentiation Growth Factors

Growth Factor	Purpose
Activin A	A member of the TGF-β (transforming growth factor) superfamily. It is made up of two β subunits. A cytokine with roles in inflammation regulation, fibrosis and wound repair.[32]
CHIR99021	An aminopyrimidine derivative that is an extremely potent glycogen synthase kinase (GSK) 3 inhibitor. Promotes self-renewal and enhances derivation efficiency of mouse ESCs. Stabilizes β-catenin and signaling pathways such as TGF-β, Notchm and MAPK.[33]
LDN193189	A potent inhibitor of the bone morphogenetic (BMP) pathway and its induced signaling cascades which regulate cell fate decisions during embryogenesis.[34]
KGF	An epithelial cell specific mitogen secreted by stromal fibroblasts. A potent EGF in promoting cellular proliferation and differentiation.[35]
SANT-1	Cell-permeable antagonist that inhibits Sonic (Shh) Hedgehog signaling pathway. Inhibits the proliferation of glioma stem-like cells. Antagonist promotes beta cell differentiation from human embryonic stem cells. [36]
PDBu	A protein kinase C (PKC) activator. Playing a role in the proliferation and differentiation of various cell types.[37]
Y27632	A specific inhibitor of Rho-associated kinases (ROCK). Improves survival of human ES cell monolayers at the initiation of differentiation protocols. [38]
RA	An analogue of vitamin A. Has a key role in cell growth and differentiation by activating nuclear receptors. [39]
XXI	Analogue inhibitor of γ-secretases and Notch processing. Lowers Aβ levels in APP transgenic mice [40]
Alk5	Small molecule inhibitor protecting cells from loss of key β cell transcription factors. Restores mature β – cell identity after exposure to prolonged/ severe diabetes. [41]
T3	Stimulates respiratory activity at mitochondrial level. [42]
Betacellulin	Is a member of EGF family and plays significant role in regulating growth differentiation of pancreatic β -cells [43]

8.2 Table 2: Cell Staining Antibodies

Antibodies	Reaction/ Purpose
MBS 395603 (Pdx1 antibody)	Primary antibody; Insulin promoter factor 1 that is a transcriptional activator of genes (insulin). The development of β-cells overexpresses Pdx1 TF.
MBS 9600104 (Glucagon antibody)	Primary antibody; Stimulates gluconeogenesis and glycogenolysis in the cell.
MBS 9211953 (Nkx6.1 antibody)	Primary antibody; Transciption factor playing an important role in pancreatic β-cell function/ proliferation. This TF is found solely in β-cells and inhibits glucagon gene transcription.
NB 110-2554 C – Peptide antibody	Primary antibody; A peptide byproduct of insulin formation by the pancreas. Released from β-cells during the cleavage of insulin from insulin. High C-peptide molecules indicate high levels of endogenous insulin production.
NB 7159 (Goat anti-rabbit IgG)	Secondary antibody; Antibodies characterized for specificity for rabbit immunoglobulin classes, subclasses, and fragments.
NB 120-6786 (Goat anti-mouse IgG)	Secondary antibody; Antibodies characterized for specificity for mouse immunoglobulin classes, subclasses, and fragments.
DAPI Blue	A blue-fluorescent DNA stain exhibiting a fluorescence enhancement when binding to the A-T regions of the dsDNA.

8.3 Cell counting:
Cell counting is crucial for experiment success since it allows researchers to accurately assess the cell concentration in a solution. Subsequently, we used this technique to determine the number of cells that we were going to be plating into each well in order to ascertain an equal number of cells.

$406 / 16 = 25.1875 * 10^4$
➔ $2.51875 * 10^5$
➔ $2.52 * 10^5$ cells per mL

For printing
150 µL of cells (into 12 wells)
   ➔ $(150 \, µL / 1000 \, µL) * (\text{'x'} / 2.52 * 10^5 \text{ cells}) = 37,500 \text{ cells} \approx$ <mark>$3.75 * 10^4$ cells</mark>
   ➔ $3.75 * 10^4$ cells / 12 wells $\approx$ <mark>$3.125 * 10^3$ cells per well</mark>
For seeding
12 µL of cells per well
   ➔ $(12 \, µL / 1000 \, µL) * (\text{'x'} / 2.52 * 10^5 \text{ cells}) = 3000 \text{ cells}$
   ➔ $\approx$ <mark>$3 * 10^3$ cells per well</mark>

α-cells
$284 / 16 = 17.75 * 10^4$
➔ $1.775 * 10^5$
➔ $1.78 * 10^5$ cells per Ml

α-cells control
18 µL of cells per well
   ➔ $(18 \, µL / 1000 \, µL) * (\text{'x'} / 1.78 * 10^5 \text{ cells}) = 3,204 \text{ cells} \approx 3.2 * 10^3 \text{ cells}$
   ➔ $\approx$ <mark>$3.2 * 10^3$ cells per well</mark>

*** Incubate 96 – well plate at 37 °C ; 10% CO_2 to maintain cells ***

8.4 B – cell media formulation differentiation schedule
This procedure is spaced out over 21 days where the distinct growth factors are implemented to promote β-cell differentiation.

Stage	Day	Basal Media	Factor	Concentration	Company	Part Number
Stage 1	Day 1	S1	Activin A CHIR99021	100 ng/mL 14 µg/mL	R&D Systems Stemgent	338-AC 04-0004-10
	Day 2	S1	Activin A CHIR99021	100 ng/mL None	R&D Systems	338-AC
	Day 3		No Feed			

Stage 2	Day 1	S2	KGF	50 ng/mL	Peprotech	AF-100-19
	Day 2		No Feed			
	Day 3	S2	KGF	50 ng/mL	Peprotech	AF-100-19
Stage 3	Day 1	S3	LDN193189	200nM	Thermo Fisher Scientific	NC0054000
			KGF	50 ng/mL	Peprotech	AF-100-19
			SANT1	0.25 μM	Sigma Aldrich	S4572
			PdBU	500 nM	EMD Millipore	524390
			Y27632	10 μM	Abcam	ab120129
			RA	2 μM	Sigma Aldrich	R2625
	Day 2	S3	LDN193189	None		
			KGF	50 ng/mL	Peprotech	AF-100-19
			SANT1	0.25 μM	Sigma Aldrich	S4572
			PdBU	500 nM	EMD Millipore	524390
			Y27632	10 μM	Abcam	ab120129
			RA	2 μM	Sigma Aldrich	R2625
Stage 4	Day 1	S3	KGF	50 ng/mL	Peprotech	AF-100-19
			SANT1	0.25 μM	Sigma Aldrich	S4572
			Y27632	10 μM	Abcam	ab120129
			Activin A	5 ng/mL	R&D Systems	338-AC
			RA	0.1 μM	Sigma Aldrich	R2625
	Day 2		No Feed			
	Day 3	S3	KGF	50 ng/mL	Peprotech	AF-100-19
			SANT1	0.25 μM	Sigma Aldrich	S4572
			Y27632	10 μM	Abcam	ab120129
			Activin A	5 ng/mL	R&D Systems	338-AC
			RA	0.1 μM	Sigma Aldrich	R2625
	Day 4		No Feed			
	Day 5	S3	KGF	50 ng/mL	Peprotech	AF-100-19
			SANT1	0.25 μM	Sigma Aldrich	S4572
			Y27632	10 μM	Abcam	ab120129
			Activin A	5 ng/mL	R&D Systems	338-AC
			RA	0.1 μM	Sigma Aldrich	R2625
Stage 5	Day 1	BE5	SANT1	0.25 μM	Sigma Aldrich	S4572
			RA	0.1 μM	Sigma Aldrich	R2625
			XXI	1 μM	EMD Millipore	595790
			Alk5i	10 μM	Axxora	ALX-270-445-M005
			T3	1 μM	EMD Biosciences	64245
			Betacellulin	20 ng/mL	R&D Systems	261-CE-050
	Day 2		No Feed			
	Day 3		SANT1	0.25 μM	Sigma Aldrich	S4572
			RA	0.1 μM	Sigma Aldrich	R2625
			XXI	1 μM	EMD	595790
			Alk5i	10 μM	Millipore	ALX-270-445-M005
					Axxora	
			T3	1 μM	EMD	64245
			Betacellulin	20 ng/mL	Biosciences	261-CE-050

					R&D Systems	
	Day 4		No Feed			
	Day 5		SANT1 RA XXI Alk5i T3 Betacellulin	None 25 nM 1 µM 10 µM 1 µM 20 ng/mL	Sigma Aldrich Sigma Aldrich EMD Millipore Axxora EMD Biosciences R&D Systems	S4572 R2625 595790 ALX-270-445-M005 64245 261-CE-050
	Day 6		No feed			
	Day 7		SANT1 RA XXI Alk5i T3 Betacellulin	None 25 nM 1 µM 10 µM 1 µM 20 ng/mL	Sigma Aldrich Sigma Aldrich EMD Millipore Axxora EMD Biosciences R&D Systems	S4572 R2625 595790 ALX-270-445-M005 64245 261-CE-050
Stage 6	EOD	S6	Alk5i T3	10 µM 1 µM	Axxora EMD Biosciences	ALX-270-445-M005 64245

EOD: Feed every other day

Vita

Atzimba Casas earned her B.S in Cellular and Molecular Biochemistry with a minor in Biomedical Engineering from The University of Texas at El Paso (UTEP) back in 2017. It was at this school where she continued her education, now striving to earn her M.S. in Biomedical Engineering. During her graduate career, Atzimba worked under the direct supervision of Dr. Thomas Boland in his Biomedical Device, Delivery, and Diagnostic (B3D) Lab.

During her lab years research, Atzimba's interests were wide, and she was undecided on her area of research. Eventually, she realized she wanted to work in the field of tissue engineering addressing diabetes' health issue, to be more specific. Her project began to gain direction after deciding to combine both cellular and molecular biology fundamentals with TIB (thermal inkjet bioprinter) in order to alter differentiated cells to address the autoimmune disease that is Type 1 Diabetes.

Before being accepted into the BME Master's program, Atzimba did research in several labs during her undergraduate years. As a junior, Atzimba became part of the first BUILDing scholars' first cohort at the University of Texas at El Paso, which is a prestigious training program funded by the NIH and directed at students with strong research motivation in distinctive STEM fields. It was during this program that Atzimba gained valuable research experience and internships in other labs specializing in BME, microfluidics, and radiology imaging (UTSW). During her undergraduate years, Atzimba also became an author for a science publication focused on multi-walled carbon nanotube alginate hydrogels (DOI: https://doi.org/10.1038/srep32456) in 2016.

Atzimba also worked as a Teaching Assistant for one year of her graduate education in the BME department at UTEP.

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