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Enhancing the differentiation efficiency of human embryonic stem cells into insulin-secreting Î²-cells

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ENHANCING THE DIFFERENTIATION EFFICIENCY OF HUMAN EMBRYONIC STEM CELLS INTO INSULIN-SECRETING β-CELLS

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A thesis submitted to the Honors College in partial fulfillment of the requirements for the Honors Bachelor of Science degree in Biological Engineering

By

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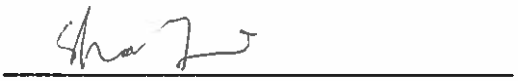
University of Arkansas

ABSTRACT

Recent studies have shown that human embryonic stem cells posses the ability to differentiate into almost any cell type in the body and they can self-renew in culture conditions (Baetge, D'Amour et al. 2006). As the number of Americans with Type I Diabetes continues to increase, innovative methods of treating this chronic illness must be developed in order to improve the quality of life for the affected individuals. Human embryonic stem cells (hESCs) have shown the potential to differentiate successfully into insulin-secreting β-cells, and these cultured β-cells can be used for cell-treatment therapies to replace the deficient β-cells in diabetes patients (Jiang et al. 2007 and Kroon et al. 2008). However promising the capabilities of hESCs seem in treating Type I Diabetes, they still contain different genetic information than that of the recipient, therefore the donated β-cells risk rejection. To overcome this problem, induced pluripotent stem cells (iPSCs) can be compared to hESCs with respect to their differentiation into β-cells since they also can differentiate into almost any cell type (Nishikawa et al. 2008). Induced pluripotent stem cells are derived from reprogramming adult somatic cells back to an embryonic cell-like state, so they would be especially effective in disease treatment since the new β-cells produced would contain the patient's own genetic information and therefore the threat of rejection is low.

This thesis is approved for recommendation to the Graduate Council.

Thesis Director:



Dr. Sha Jin

ACKNOWLEDGEMENTS

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Also, thanks to Huantong Yao for helping supply the undifferentiated hESC cultures used for the collagen membrane tests, and for the collaboration on the hESC and iPSC comparison experiment.

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INTRODUCTION

According to the American Diabetes Association, 7.8% of the population in the United States has Diabetes mellitus and 1.6 million new cases are diagnosed each year. Type 1 Diabetes occurs due to an autoimmune response in which the T cells attack and destroy the β-cells in the pancreas, and these β-cells are important for a human to be healthy and functional because they produce insulin in response to raised glucose levels in order to lower the blood glucose concentration. People with Type 2 diabetes have working β-cells, but their tissues are either insulin resistant or not enough insulin is secreted to lower the blood glucose concentration. A patient suffering from Type 1 diabetes must constantly be monitoring the glucose level in their blood, and they must inject insulin into their bodies when the concentration gets too high. This method is not ideal due to the high initial input of insulin in one area of the body, so alternative methods of treatment have been explored such as cell-replacement therapy or transplants from donated organs. Although these methods are generally effective, a person receiving the transplant must take immunosuppressive drugs for the rest of their lives, and the availability of cells and organs for transplants and therapy is limited (Kroon et al. 2008).

In order to meet the needs of the growing diabetic population, human embryonic stem cells (hESCs) can be differentiated into pancreatic islets that contain β-cells and secrete insulin so that the blood glucose concentration can be lowered naturally. Human ES cells can self-renew in culture conditions and can also be directed

to differentiate into almost any cell type, so they are an obvious choice to be used in cell therapy (Baetge, D'Amour et al. 2006).

Unfortunately, studies on hESC differentiation into pancreatic β-cells have gained little progress to date. Also, existing protocols are not straightforward and consistent reproducibility is extremely poor between groups and experiments. In fact, the differentiation of hESCs into insulin-secreting cells is a big challenge due to 1) the lack of knowledge on the pathways involved in human pancreatic development, 2) the limited information about the signals essential for stimulating the development of pancreatic lineage, and 3) the extensive effect of isolating methods during the establishment of a hESC line on the differentiation potential of hESC into certain lineage. Thus, it is highly desirable to develop an innovative technology to direct hESC differentiation into pancreatic lineage and produce insulin effectively.

One method that has been successful in directing hESC differentiation into definitive endoderm (DE) and eventually β-cells is the addition of Activin A in feeder-free culture environments (Sulzbacher et al. 2009). Activin A is a member of the transforming growth factor-β (TGF-β) superfamily and it activates specific receptors responsible for gene expression and ultimately pancreatic lineage formation. The combination of sodium butyrate and Activin A has also been proven to increase the expression of DE marker genes Sox17 and Foxa2 (Jiang et al 2007). The exact mechanism by which sodium butyrate causes increased gene expression in conjunction with Activin A is unclear, but researchers believe that it stimulates an epigenetic change that enables Activin A to increase the pancreatic progenitor gene levels in the hESCs.

Although the applications of hESCs for disease treatment are extremely promising, human embryonic stem cells contain their own genetic information that is different from the recipient of the cell-treatment therapy, therefore immunorejection can occur and the patient must take immunosuppressor drugs. To overcome this problem, the use of induced pluripotent stem cells is being investigated as a replacement. Induced pluripotent stem cells (iPSCs) are derived from directing somatic cells to reprogram and revert back to an embryonic stem cell-like state through the transfection or forced expression of certain genes, therefore they have the potential to differentiate into almost any cell type in the body, and they can be used for patient specific cell-therapy treatment (Nishikawa et al. 2008). The application of iPSCs to disease treatment could revolutionize modern medicine and increase the quality of life for many people in the world.

METHODS AND MATERIALS

Part I. hESC Differentiation into β-cells

Culturing hESCs

Human embryonic stem cells must be maintained at a pure, undifferentiated state in order to procure accurate results once differentiation is induced. The h9 cells cultured in this experiment were obtained from the Wicell Research Institute and cultured in mTeSR1 medium from Stem Cell Technologies. Matrigel coating of the plates insured cell attachment, and the culture dishes were kept at 37°C and 5% CO_2. The mTeSR1 medium was exchanged on a daily basis, and the cells were passaged every 4 days by

detaching with the enzyme dispase (Stem Cell Technologies) along with manual scraping. An image of an undifferentiated hESC colony is show below in Figure 1.

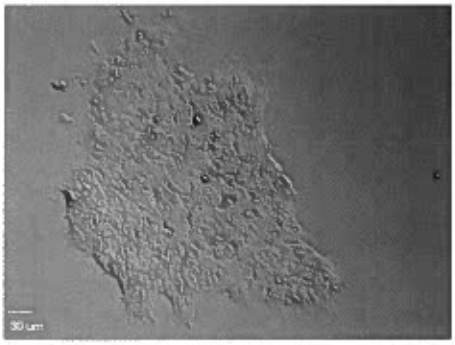

Figure 1. Undifferentiated hESC Colony, scale bar 30 µm.

Differentiation into β-cells

To induce differentiation into β-cells, approximately 2.1 x 10^4 cells/cm^2 were first seeded on a matrigel-coated 48-well culture dish and allowed to incubate overnight in mTeSR1 medium. The following day, the DE medium composed of RPMI1640, B27, 1 mM sodium butyrate, and 4 nM Activin A was added to the culture dish. After 24 hours of incubation, the sodium butyrate concentration was reduced to 0.5 mM since it can be toxic to the cells, and the medium was exchanged every other day for 7 days.

Immunofluorescence staining

To visualize the definitive endoderm markers Sox17 and Foxa2, immunofluorescence staining was utilized. The differentiated cells were first fixed in a 4% Paraformaldehyde solution for 15 minutes with shaking and then washed with PBS buffer. After fixation,

the samples were permeabilized with 0.1% Triton X-100/PBS buffer for 10 minutes and then washed three times with PBS buffer. The cells were incubated in a blocking buffer (composed of 5% sheep serum, 5% donkey serum, 0.05% Tween-20, 0.1% Triton X-100 in PBS) for 1 hour and then, after removing the buffer, the Mouse anti Human Sox17 and Rabbit anti Human Foxa2 primary antibodies were added to the cell cultures and allowed to sit for 1 hour. After the addition of the primary antibodies, the cells were washed with a BSA/PBS wash buffer and then the secondary fluorophore-conjugated antibodies (FITC Sheep anti Mouse and TRITC Donkey anti Rabbit) were added to the wells. The samples were covered with aluminum foil to protect the sensitive fluorescent-conjugated antibodies. After the cells had incubated for an hour, they were washed three times with PBS buffer and then counterstained with the nucleic acid probe, diaminophenylindole (DAPI), to detect the nucleus of the cells. The samples were observed under an Olympus fluorescence microscope, and images were taken and can be seen in the "Results and Discussion" section.

Part II. Membrane Experiment

Culturing hESCs

As mentioned earlier, maintaining hESC at an undifferentiated state is extremely important for proliferation experiments. For details on culture techniques, refer to the "Culturing hESCs" section in Part I.

Membrane Preparation

Collagen Type I membranes of the following characteristics were used for this experiment: Small Pore (~5 µm), Medium Pore (~20 µm), Large Pore (~50 µm), Small Cross-Linked, Medium Cross-Linked, and Large Cross-Linked. See Figure 2 below for images of the membranes.

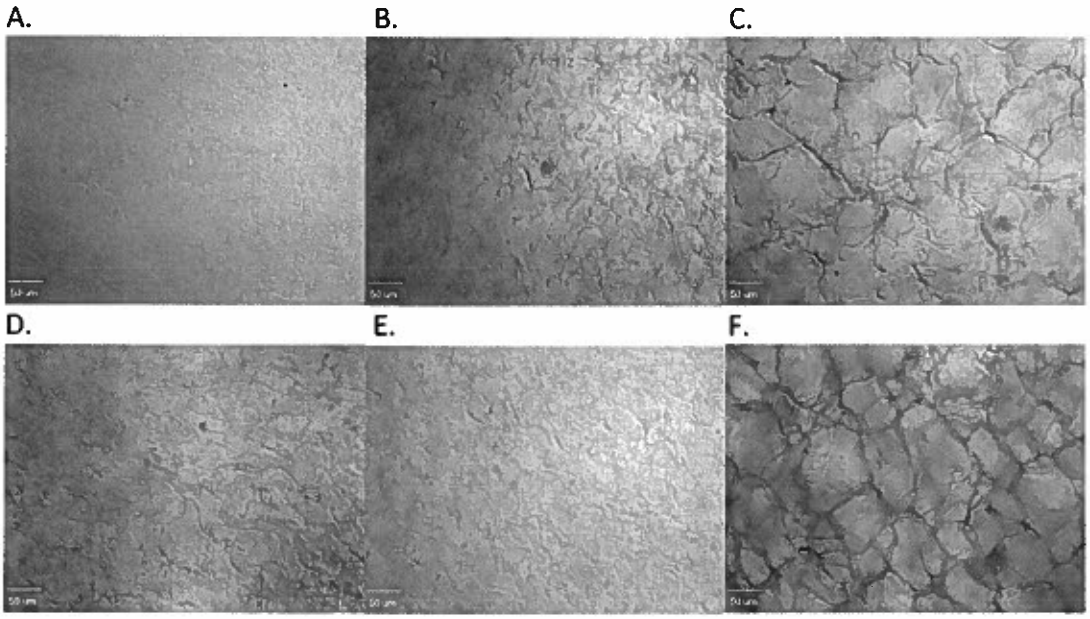

Figure 2. Images of Collagen Membranes; A. Small Pore (~5 µm), B. Medium Pore (~20 µm), C. Large Pore (~50 µm), D. Small Cross-Linked, E. Medium Cross-Linked, F. Large Cross-Linked.

To prepare the experiment, a punch was used to cut each membrane type to fit into a well of a 96-well plate (approximately 0.32 cm^2). The membranes were arranged in the following way to ensure enough samples for Days 1-4 for cell counting:

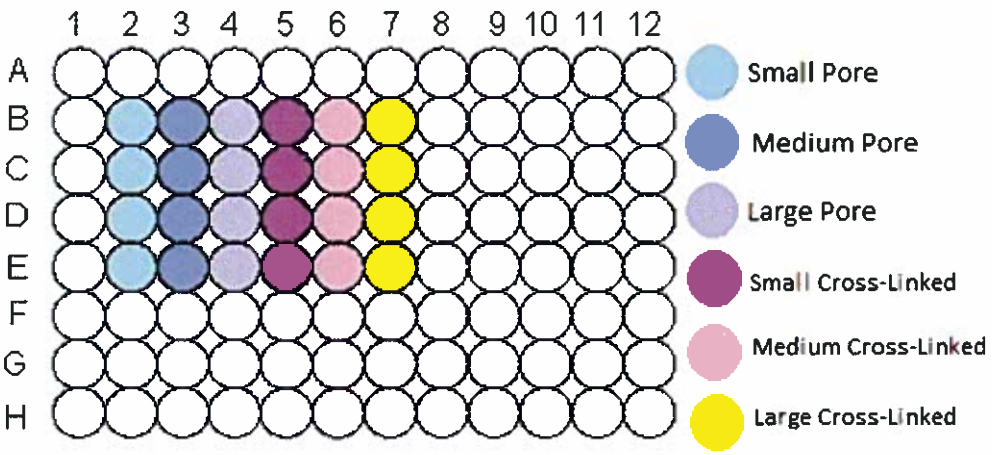

Figure 3. 96-well Plate Configuration for the Membrane Experiment. Row B corresponds to the samples for Day 1, Row C corresponds to the samples for Day 2, Row D contains samples for Day 3, and Day 4 samples were placed in row E. Column 2 has the Small Pore membranes, Column 3 contains the Medium Pore size, Column 4 contains the Large Pore size, Column 5 has the Small Cross-linked membranes, the Medium Cross-linked membranes are in Column 6, and the Large Cross-Linked membranes are in Column 7.

Seeding the Cells

Once the undifferentiated hESC colonies had expanded, the cells were detached from the culture dish using the enzyme dispase as well as manual scraping, and then they were seeded at a density of approximately 2.5 x 10^5 cells/cm^2 of the matrigel-coated 96-well plate containing the membranes. The cells were then fed with mTeSR1 culture medium and incubated at 37°C and 5% CO_2, and the culture medium was exchanged daily.

Counting the Cells

In order to accurately determine the cell-growth kinetics for the hESCs growing on each membrane type, the cell number for Days 1-4 was calculated using Tryphan Blue staining along with a hemacytometer counting chamber. From Days 1-4, the cells cultured on each membrane type for that specific day were detached using the enzyme

collagenase and placed into separate micro centrifuge tubes. Then, 20 µL of each sample was placed in additional separate micro centrifuge tubes and 20 µL of Tryphan Blue was added and mixed thoroughly. After the dye was distributed uniformly in each membrane sample, 10 µL was loaded onto each chamber of the hemacytometer and the cell number was counted two times to guarantee accuracy. To determine the actual cell number per membrane sample, the following equation was used:

$$cell\ number = \frac{number\ counted \times 10^4 \times dilution\ factor}{4} \times volume\ (mL)$$

The dilution factor was 2 since an equal volume of Tryphan Blue and medium containing cells was added to each sample (20 µL of Tryphan Blue and 20 µL of the sample), and the multiplied volume amount was 0.6 mL since that was the quantity of medium in each well of the culture plate. The cell number calculated for each membrane was then plotted for each day of cell culture. A model growth curve is shown below:

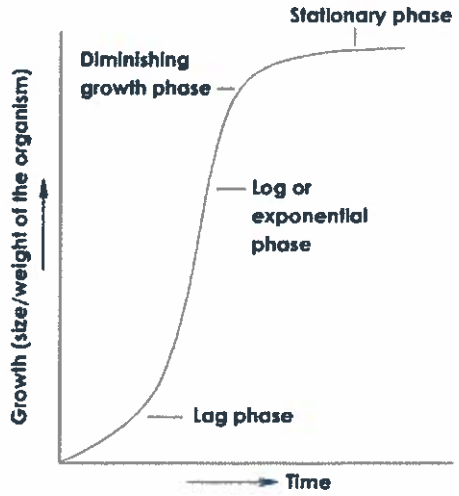

Figure 4. Theoretical Growth Curve for Cells.
(http://www.tutorvista.com/content/biology/biology-iv/plant-growth-movements/growth-curve.php)

Imaging

Images of the membrane cell cultures were taken on Days 1-4 on an Olympus microscope and can be referenced in the Appendix.

Part III. Comparing hESCs and iPSCs

Culturing hESCs and iPSCs

To compare the differentiation potential of both human embryonic stem cells and induced pluripotent stem cells, both h9 (Wicell) and IMR90 (Wicell) cell lines were cultured side by side and maintained at an undifferentiated state by methods explained in "Part I: Culturing hESCs." Since hESC and iPSC are both pluripotent and exhibit similar characteristics, the same procedure for maintaining them at undifferentiated conditions and then inducing differentiation can be used for each cell line. Note the comparable morphology of hESCs and iPSCs seen in Figure 5.

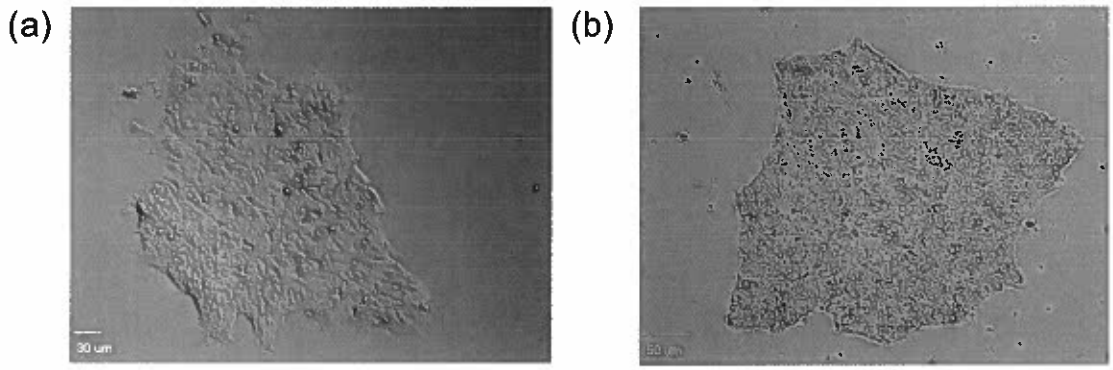

Figure 5. Undifferentiated Pluripotent Cells. (a) hESC colony, scale bar = 30 µm and (b) iPSC colony, scale bar = 50 µm.

Seeding the Cells

Both hESCs and iPSCs were seeded at a density of 5×10^4 cells/cm^2 on a matrigel-coated 6-well culture plate.

Counting the Cells

The timepoints considered for counting cell number were Days 1-3, and the cell number was determined by first detaching the cell samples using Trypsin-EDTA, then using Tryphan Blue and the hemacytometer counting chamber (refer to "Part II: Counting Cells"). This data was then utilized to calculate the specific growth rate of each respective pluripotent cell line by using the Malthusian growth model.

RESULTS and DISCUSSION

Part I. hESC Differentiation into β-cells

Inducing the Stage I differentiation of the hESCs into β-cells was successful, as shown by the marker gene expression in the immunofluorescence staining assay. Foxa2 and Sox17, both definitive endoderm markers, were detected in the Stage I differentiated β-cells (see Figure 6).

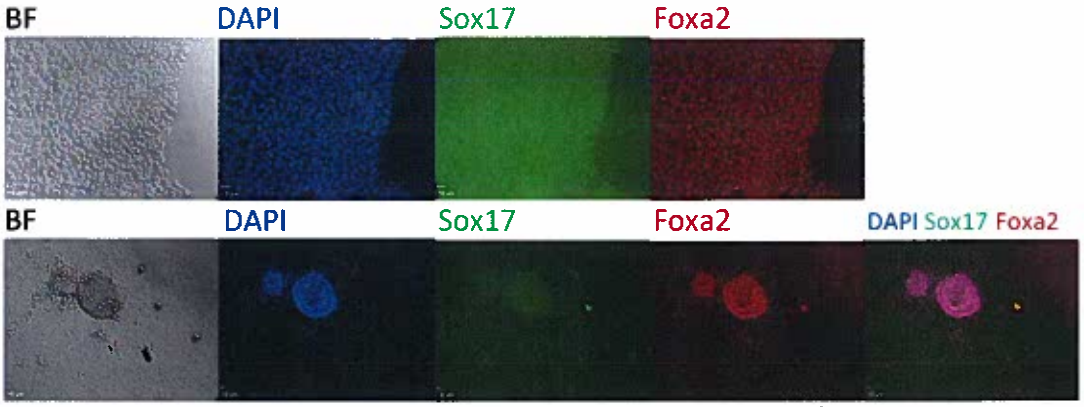

Figure 6. Immunofluorescence Images of Differentiated hESC: Brightfield, DAPI, Sox17, Foxa2, and colocalization of DAPI/Sox17/Foxa2.

It can be concluded from this experiment that providing the correct growth factors such as Activin A in combination with sodium butyrate can result in Stage I differentiation of hESCs into β-cells.

Part II. Membrane Experiment

The collagen membranes in the second stage of the research demonstrate that a 3-D scaffold provides conditions that mimic an *in vivo* environment and also facilitates nutrient transport. The membranes create a greater surface area than a traditional 2-D culture plate, and this allows cells to be seeded at a higher density. The larger pore membranes with cross-linked structures were predicted to have the greatest specific growth rate of the hESCs due to the high stability of the material and increased pore size to allow for mass transfer to occur. The most effective pore size and material structure was determined by counting the cell number on Days 1-4, and the data is presented in Figure 7.

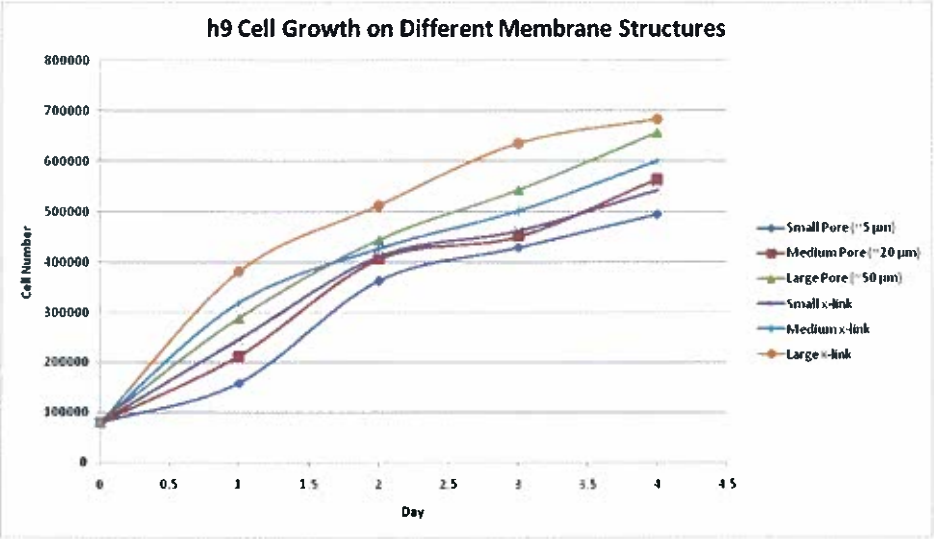

Figure 7. Growth Curves for hESCs on Membranes.

From the graph alone, one can see that the 50 µm cross-linked membranes fostered the greatest growth of cells for each timepoint. The 50 µm pore size surpassed the 20 µm cross-linked membrane after Day 1 of culture, and then after the 20 µm cross-linked membrane, both the 20 µm pore size along with the 5 µm cross-linked membranes exhibited the next best growth numbers. As expected, the 5 µm pore size had the lowest cell numbers for each timepoint since the material does provide a large enough surface area for cell growth as compared to the other membranes. This allows less nutrient transport to occur because of the smaller openings in the membrane, and consequently less cells are able to proliferate.

To quantify the data and compare the specific growth rates (µ) for each membrane, the Malthusian growth model was used:

$$\frac{dx}{dt} = \mu X$$

Where: μ is the specific growth rate

X is the cell number (X_0 is the initial number, X_t is the number at time t)

Rearranging and integrating the original equation:

$$\int \frac{dx}{X} = \mu \int dt$$

$$\ln(X_t - X_0) = \mu t$$

$$\ln(\frac{X_t}{X_0}) = \mu t$$

Solving for μ:

$$\mu = \frac{\ln\left(\frac{X_t}{X_0}\right)}{t}$$

The table below shows the specific growth rates (per hour) of the cell cultures on each membrane, as calculated using the equation above.

Day	5 μm	20 μm	50 μm	5 μm x-Link	20 μm x-Link	50 μm x-Link
1	0.0286	0.0408	0.0534	0.0468	0.0575	0.0650
2	0.0344	0.0268	0.0180	0.0214	0.0122	0.0124
3	0.0070	0.0044	0.0084	0.0049	0.0068	0.0090
4	0.0060	0.0094	0.0079	0.0067	0.0075	0.0030
Average	0.0190	0.0203	0.0219	0.0200	0.0210	0.0224

Table 1. Specific Growth Rates (μ) Calculated at Each Timepoint.

As shown in Table 1, the 50 μm membrane with cross-links had the largest specific growth rate of 0.0224/hour (0.538/day), followed by the 50 μm pore size with a rate of 0.0219/hour (0.526/day). The next largest specific growth rate was the 20 μm cross-

linked membrane with a value of 0.0210/hour (0.504/day), then the 20 μm pore membrane with a rate of 0.0203/hour (0.487/day), and the 5 μm cross-linked had a growth rate of 0.0200/hour (0.480/day). The 5 μm pore size had the slowest growth rate of 0.0190/hour (0.456/day). This data shows that the 50 μm cross-linked membrane has the greatest potential for tissue engineering applications because it has a large surface area for cells to attach, and the big openings in the material allow for essential metabolites to reach all of the cultured tissue. This 3-D culture method also allows waste to be transported from the cells to the culture medium more easily.

Since this experiment focused on undifferentiated hESC proliferation, an interesting continuation could involve inducing differentiation of the hESCs into β-cells on the different membranes and then determining the specific growth rates for differentiated hESCs.

Part III. Comparing hESCs and iPSCs

Since recent studies have demonstrated the potential for iPSCs to replace hESCs in cell-treatment therapies of chronic diseases, concrete experimental evidence had to be generated in order to support that claim. The cell number of both the hESCs and the iPSCs was determined for Days 1, 2, and 3 and then the specific growth rate (μ) was calculated based on the Malthusian growth model explained in Part II of the "Results and Discussion" section. The graphs are shown in Figure 8:

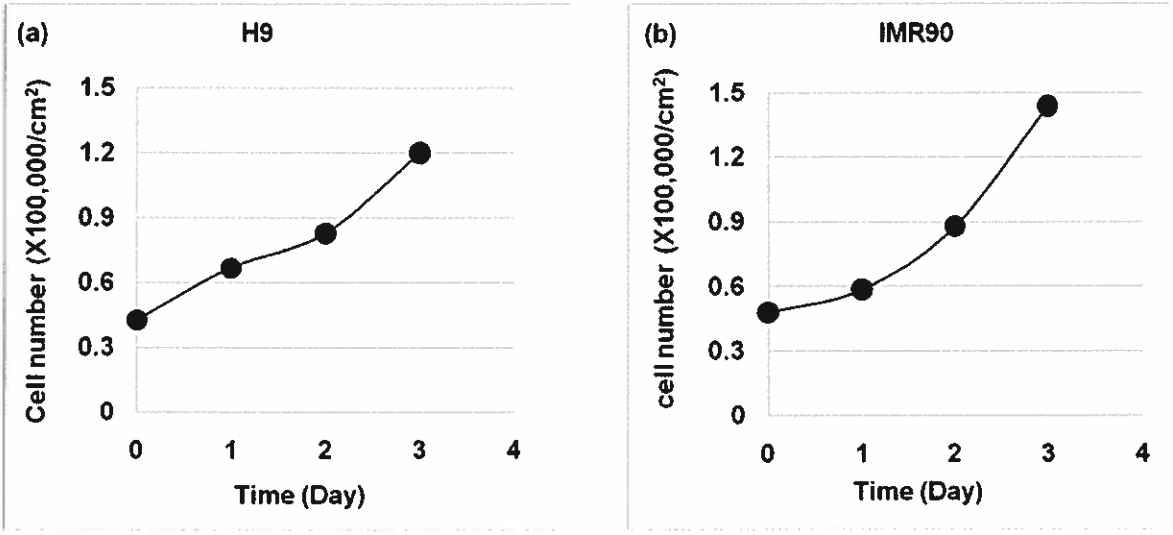

Figure 8. Growth Curves for (a) hESCs and (b) iPSCs on Matrigel-coated tissue culture plates.

The specific growth rate of the hESCs was 0.360/day and the rate of the iPSCs was 0.370/day. This indicates that induced pluripotent stem cells possess the same if not greater potential for applications in cell-treatment therapy as human embryonic stem cells.

CONCLUSION

The results from this experiment show that human embryonic stem cells can differentiate into insulin-secreting β-cells by the addition of the growth factors Activin A and sodium butyrate. The differentiation efficiency can be increased by culturing the cells on 3-D structures such as collagen membranes, and the specific growth rate increases with larger pore sizes and cross-linked stability. The last experiment involving the comparison of human embryonic stem cells to induced pluripotent stem cells

indicates that iPSCs have equivalent potential to differentiate into a desired cell type with the same, if not greater, differentiation efficiency as hESCs. Also, the specific growth rates of the second and third experiments can be analyzed to infer that hESCs grown on membranes have larger specific growth rates than hESCs grown on traditional 2-D culture plates, since the hESCs grown on even the smallest pore size membrane had a much higher growth rate (0.456/day) compared to the hESCs grown on the 2-D surface (0.360/day). Applying iPSCs to disease treatment has the possibility to cure chronic diseases through patient specific cell-treatment therapy, and this could improve the quality of life for people around the world.

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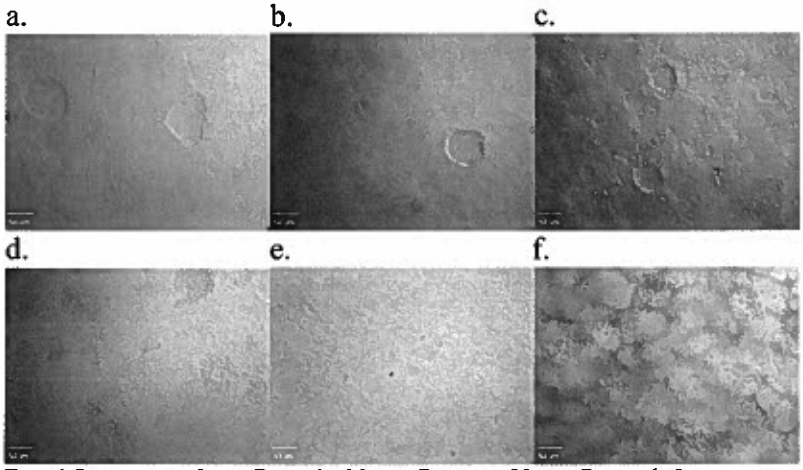
APPENDIX

Part II. Membrane Experiment

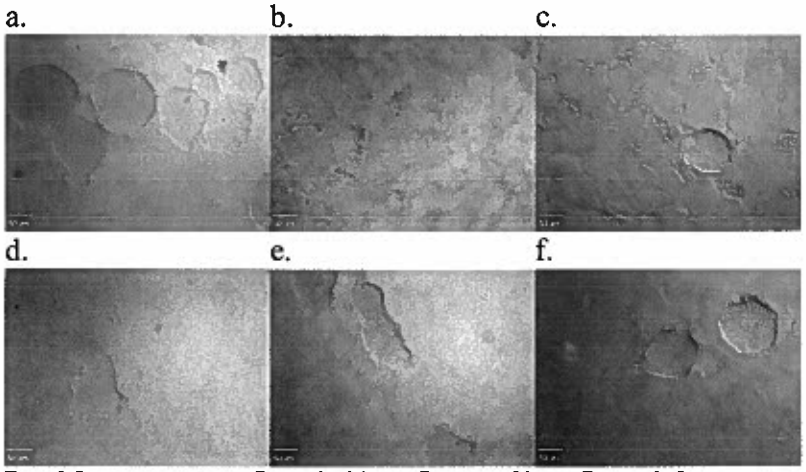
Images

Day 1



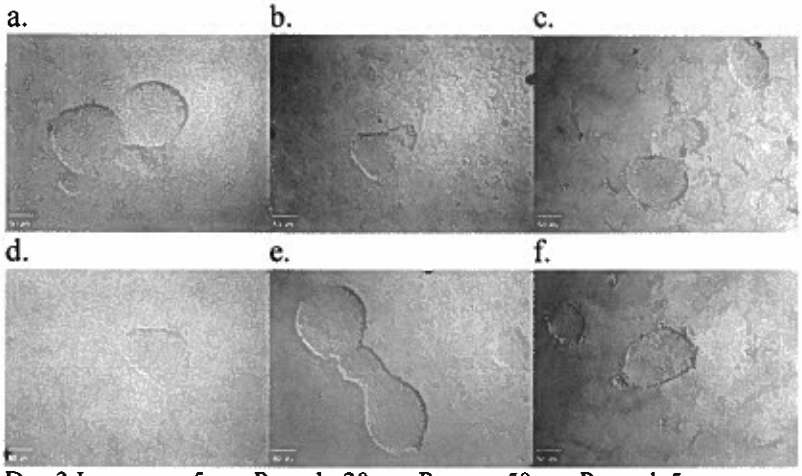
Day 1 Images: a. 5 µm Pore, b. 20 µm Pore, c. 50 µm Pore, d. 5 µm Cross-Linked, e. 20 µm Cross-Linked, f. 50 µm Cross-Linked

Day 2



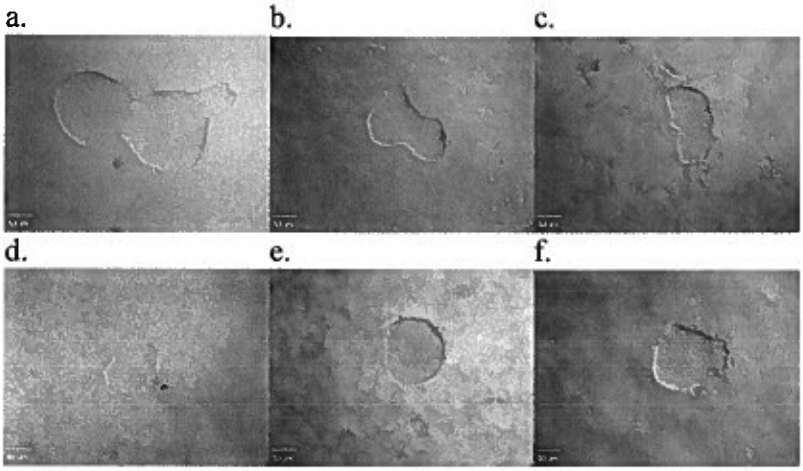
Day 2 Images: a. 5 µm Pore, b. 20 µm Pore, c. 50 µm Pore, d. 5 µm Cross-Linked, e. 20 µm Cross-Linked, f. 50 µm Cross-Linked

Day 3



Day 3 Images: a. 5 µm Pore, b. 20 µm Pore, c. 50 µm Pore, d. 5 µm Cross-Linked, e. 20 µm Cross-Linked, f. 50 µm Cross-Linked

Day 4



Day 4 Images: a. 5 µm Pore, b. 20 µm Pore, c. 50 µm Pore, d. 5 µm Cross-Linked, e. 20 µm Cross-Linked, f. 50 µm Cross-Linked