The Effects of Cell Clustering on Cell Differentiation in Human Pancreatic Ductal Epithelial Cancer Cells

Undergraduate Engineering Research Thesis (Honors)

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CERTIFICATE OF APPROVAL

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FOR

HONORS UNDERGRADUATE SENIOR THESIS

This is to certify that the Senior Thesis of

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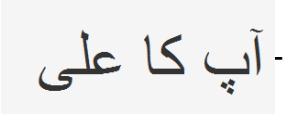
Dedication

I would like to dedicate this thesis to my parents, Amma and Abbu, for all the love and support they have given me as I pursued my ambitions and goals at Ohio State. Without your advice and moral support, I could not have completed this interesting project. Thank you!



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Abstract

For diabetes mellitus type I, regular insulin therapy is not enough to completely control the disease. Ineffective control of blood glucose levels can lead to complications in other parts of the body, including kidney failure, neuropathy, cardiovascular disease, blindness, and acute hypoglycemic attacks. Although pancreatic transplants are one possible solution to reduce the effects of diabetes, it is not the best option. The proposed work of this research project is new and innovative with respect to hypothesis and methodology. It follows the goal to identify the role of cell clustering for differentiation of β cells so that the option of pancreatic islet transplantation can be substituted completely. Work by Boretti and Gooch in their paper "Induced Cell Clustering Enhances Islet β Cell Formation from Human Cultures Enriched for Pancreatic Ductal Epithelial Cells" was relevant to this project as it established the importance of clustering for differentiation of precursors cells into β cells. I used this work as a motivation to further research the *amount* of clustering required for the expression of insulin. My project speculates that the number of cells per cluster is essential for differentiated β cells to form. Further, there also must be a certain number of cells per cluster in order for proper insulin expression to take place.

Upon experimentation, the results showed that cluster sizes of at least 4 cells per cluster were successful in expressing insulin as differentiated β cells. Whereas, small cluster sizes with only 1-2 cells per cluster were not able to express insulin. Further an area-to-perimeter analysis showed that ratios greater than 4.5 were common among insulin-expressing large cluster sizes. Although the results are quite preliminary, the investigation of the role that cluster size has on differentiation of β cells has a lot of potential for further research.

Introduction

Diabetes mellitus is an autoimmune disorder in which the body starts attacking and destroying its own pancreatic β cells. Diabetic patients suffer from the destruction of pancreatic islet β cells, which are required for glucose homeostasis via insulin/glucagon expression. The β cells basically act as sensors: when glucose levels are high, insulin is released.

There are two types of diabetes – Type I and II. Type I diabetes is an autoimmune disorder and occurs when the body turns against itself by killing its own pancreatic β cells, thereby destroying the ability to produce insulin. As a result, glucose levels are not regulated properly, which leads to higher levels than normal patients. A cure for type I diabetes has yet to be discovered. Type II diabetes is different in that cells stop accepting insulin, which leads to high glucose levels. On the other hand, type II diabetes is more reasonable as it can be cured with correct exercise and diet.

For diabetes mellitus type I, regular insulin therapy is not enough to completely control the disease. Ineffective control of blood glucose levels can lead to complications in other parts of the body, including kidney failure, neuropathy, cardiovascular disease, blindness, and acute hypoglycemic attacks [1]. Diabetic neuropathy is a disease that affects the nerves of the body. Damage to nerves can decrease or completely diminish sensation of certain muscles. Decreased sensation may lead to the formation of wounds or ulcers, and possibly amputations. Diabetic retinopathy is another common complication of diabetes, in which cataract formation on the retina leads to decreased vision and eventual blindness.

One solution to this problem utilizes allogenic islet transplantation, which can restore normal blood glucose in normal diabetes. Pancreatic islets are all cells that create insulin, including β cells.

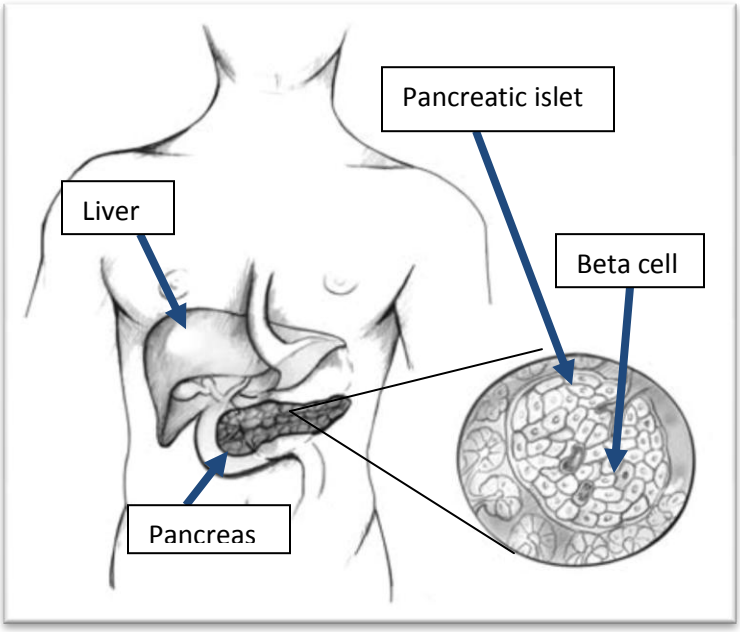

Figure 1. The pancreas is located in the abdomen, adjacent to the liver and behind the stomach. Pancreatic islets contain beta cells, which produce insulin [2].

Although pancreatic transplants are one possible solution to reduce the effects of diabetes, it is not the best option. This solution is a long and limited process that does not reach all of the affected individuals/patients. A very small fraction of the individuals needing transplantation actually receive it. With approximately 35,000 new Type I diabetic patients diagnosed each year in the United States, only about 3000 donor pancreata are available per year. Hence, there is a large discrepancy in the number of patients that could potentially benefit from this therapy and the number that could actually receive treatment [1]. In order to find a solution to reduce this efficiency gap, we are studying how the role of cell clustering of insulin secreting islets can eventually substitute transplantation entirely.

Microfabrication techniques were used to manufacture the microwell array for cell placement. A pre-patterned silicon wafer (provided by Dr. Hansford's laboratory) was used as a mold in which PDMS was applied and cured for a short amount of time. After solidification, the resulting negative PDMS wafer with varying microwell diameter sizes from 50 to 200 μm was

removed. These microwells were used for cell placement in the experimentation part of the project. Cell placement in the bottom of the microwells was essential to observe clustering for this project. In order to investigate the role of cell clustering in differentiated β cells, cell clustering was induced by gravity-settling. PDMS microwells of varied diameters and different cell concentrations were used to capture enough cells to create a monolayer or allow clustering in the microwells before differentiation is observed. Another method apart from gravity settling of cells in the microwells is vacuum-assisted cell seeding [3]. Vacuum-assisted cell seeding is a process in which cell suspensions are placed in a vacuum filtration setup to pull culture medium through the microdevice, trapping the cells in the microwells. Work by Ferrell, et. al. has shown that pancreatic ductal epithelial cells (PANC-1) follow this method closely. However, such a method cannot be used in this experiment as the design of the microwell chips lack vacuum pores.

The role of cell clustering is interesting with respect to the treatment of diabetes mellitus. It is known that differentiated β cells are required for insulin expression. However, recent studies have shown that β cells grown from pre-existing β cells exhibit decreased insulin expression due to dedifferentiation [4]. As a result, research on the redifferentiation of β cells has been a primary focus of study recently. To induce proper differentiation amongst β cells, cell clustering was introduced on a pancreatic cell line in a series of experiments conducted by Boretti and Gooch. The use of commercial Matrigel induced cell clustering (or 3D cysts with multi-cellular islet-like buds) in the PANC-1 cell line. Based on immunohistochemistry, such clustering not only made differentiated β cells, but also proved increased insulin expression in cell clusters. Pancreatic cells, hepatocytes, embryonic stem cells, and cardiomyocytes all exhibit more tissue-like behavior when cultured in 3D clusters [5]. If cell clustering is required for differentiated β cells,

then clustering should be one of the important parameters for the construction of a diabetic patient microdevice in the future. The goal of this research project is to investigate the importance of cell clustering with the hypothesis that cell clustering is required in order to observe insulin expression for permanent treatment of diabetes mellitus.

The proposed work of this research project is new and innovative with respect to hypothesis and methodology. The hypothesis that cell clustering differentiates pancreatic ductal epithelial cells into hormone-expressing endocrine cells is based on the known work that cell clustering enhances islet beta cell formation from human cultures enriched for pancreatic ductal epithelial cells [1]. Dor, et. al. have also shown that differentiated β-cells have significant proliferative potential, whose capability is being explored in this research project [6] (Dor, et. Al.). It is also known that three-dimensional cell-cell interactions are required for differentiation. Further work by Beattie, Itkin-Ansari, et. al. uses the tracing of transcription factor PDX-1+ in ex vivo cultures of pancreatic β cell clusters. The authors reported that human pancreatic endocrine cells can be expanded through 15 cell doublings in vitro for an estimated total 30,000-fold increase in cell number [7].

We know that human pancreatic PANC-1 cells, as well as adult human islet-derived precursor cells (hIPCs), form hormone-expressing islet-like cell aggregates (ICAs) when exposed to a defined serum-free media (SFM) [8]. Since differentiation is observed when serum free media is used to culture the PANC-1 cells, a set of experiments were developed by directly regulating cell clustering using microfabrication techniques (PDMS chip manufacturing) to observe how cell clustering can independently affect cell differentiation. Regardless, once the cells have achieved a differentiated state, immunostaining assays were used to characterize the amount of differentiation observed in both monolayer- and cluster-induced cell cultures. To our

knowledge, this is the first application of microfabrication technology using spatial control of cellular position to study processes relevant to generating tissue-engineered islets.

One advantage of such a device is that it can be placed in any portion of the body as long as it has access to blood vessels, so that β cells can be harvested. Not only will such a device eliminate the need for donor pancreata, but it can also serve as a substitute for regular insulin injections. There are many practical issues related to this idea, for example, bodily rejection of a foreign body – polydimethyl siloxane chip (PDMS), and biological compatibility with other organs/tissues. However, once these logistical issues are addressed in the best possible manner, this device will be able to solve many problems for diabetes-patients for years to come.

Objective: How does cell cluster size affect β cell differentiation?

Hypothesis: Cell clustering is required to differentiate pancreatic ductal epithelial cells into hormone-expressing endocrine cells, and the expression of insulin is directly related to the different cluster sizes.

Rationale: Work by Boretti and Gooch in their paper "Induced Cell Clustering Enhances Islet Cell Formation from Human Cultures Enriched for Pancreatic Ductal Epithelial Cells" highlighted the importance of differentiated β cells in order to detect insulin expression. Along with this finding, the importance of cell clustering was also recognized. I used this work as a motivation to further investigate the *amount* of clustering required for the expression of insulin. The results from my project suggest that the number of cells per cluster is essential for differentiated β cells to form. Therefore, there must also be a required minimum number of cells per cluster in order for proper insulin expression to take place.

Materials and Methods

Materials

The American Type Culture Collection provided PANC-1 (pancreatic ductal epithelial cells), 70% ethanol, 0.05% trypsin, phosphate-buffered saline (PBS), fetal bovine serum (FBS), DMEM, F12-K, insulin transferring selenium (ITS), bovine serum albumin (BSA), 4% formaldehyde, 100% methanol, primary antibody for insulin (I1), secondary antibody for insulin (I2), superblock, medium latex gloves, T-25 vials, and Petri dishes.

Experimental Design.

The seeding density of 10^4 cells was used in the experimental setup for each of the four different diameter microwells on the PDMS chip. Upon incubation, PANC-1 cell differentiation in each of the microwells was analyzed at one time point (4 days after initial cell seeding) qualitatively through DIC microscopy before immunostaining was performed to evaluate the amount of gene expression present in the PDMS chip.

Possible outcomes and interpretation.

In this study, microwells with larger diameters were expected to have more cells present on the bottom of the wells because this design allows for larger clusters to have more differentiation in larger diameter microwells. Similarly, smaller diameter microwells were expected to accommodate single-cell clusters. As a result, the smaller diameter microwells (with fewer expected cells) have slower differentiation rate as well. Hence, this suggests that larger diameter microwells should express pancreatic proteins much more than smaller diameter microwells upon immunohistochemistry statistical analysis.

Methods.

Fabrication of PDMS chips: A silicon wafer consisting of the positive microwell pattern of the four different diameter microwells to create a negative mold of the desired device structure was used. PDMS was poured on to the positive microwell pattern and cured. Following curing, the PDMS stencil was peeled from the master mold and was ready for use. Figure 1 shows the spatial orientation of the PDMS chip where the smallest well is 50 microns wide, followed by 100 and 200 micron-wide diameters. Each of the wells was about 200 microns apart from each other (measured from center-to-center). The differentiation of the target cells was analyzed qualitatively through DIC microscopy. Experimental time was based on observation of sufficient cell differentiation by microscopy. Immunostaining for insulin, one of the pancreatic markers these cells express after clustering takes place, was performed to gauge the amount of successful differentiation by PANC-1 cells. The advantage of using immunostaining is that it gives precise spatial information (to identify which cells are insulin positive) opposed to other techniques that give an average value for a population of cells. Primary antibodies were visualized with fluorescently labeled antibodies using a Leica TSL SL confocal fluorescent microscope.

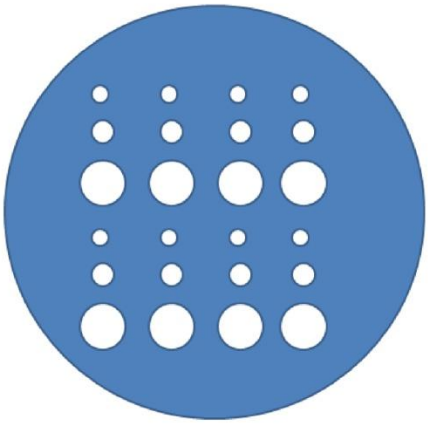

Figure 3. The spatial orientation of the PDMS chip where the smallest well is 50 microns wide, followed by 100 and 200 micron-wide diameters. Each of the wells are about 200 microns apart from each other (measured from center-to-center).

Results

My hypothesis was that cell clustering is required to differentiate pancreatic ductal epithelial cells into hormone-expressing endocrine cells and that the expression of insulin is directly related to the different cluster sizes. After the experiment was completed, I recorded the observations in Table 1 given in the following. I used ImageJ, a computer software that analyzed the images from the experiment, to calculate the area-to-perimeter ratio given in the fourth column. With the exception of picture 2, all area-to-perimeter ratios of cell clusters that do not express insulin are below 4.5. In order to test the hypothesis, an immunostaining procedure was used to detect if insulin was being produced. A green fluorescent marker under Leica TSL SL confocal fluorescent microscopy was used to highlight insulin production. The detection of insulin by fluorescence was performed by Dr. Daniel Gallego-Perez after I prepared the samples for the immunostaining procedure. Although these results are preliminary, Table 1 highlights that indeed larger clusters of PANC-1 cells at the bottom of microwells were more expressive of insulin. Cluster sizes of at least 4 cells per cluster and an area-to-perimeter ratio of at least 4.5 were two important observations among insulin-expressing cell clusters. A green fluorescent marker under Leica TSL SL confocal fluorescent microscopy was used to highlight insulin production.

Table 1: Cell clustering and Insulin expression

Picture	Area	Perimeter	A:P ratio	cells/cluster	Insulin expression (Y/N)
1	250.772	60.852	4.121015	1	N
2	389.351	73.736	5.280338	1	N
3	282.314	72.565	3.890498	1	N
4	323.288	83.777	3.858911	2	N
5	305.42	74.718	4.087636	2	N
6	790.991	142.781	5.53989	5	Y
7	340.268	74.463	4.569625	4	Y
8	727.428	122.726	5.927253	5	Y

*note: all measurements are in micrometers (μm)

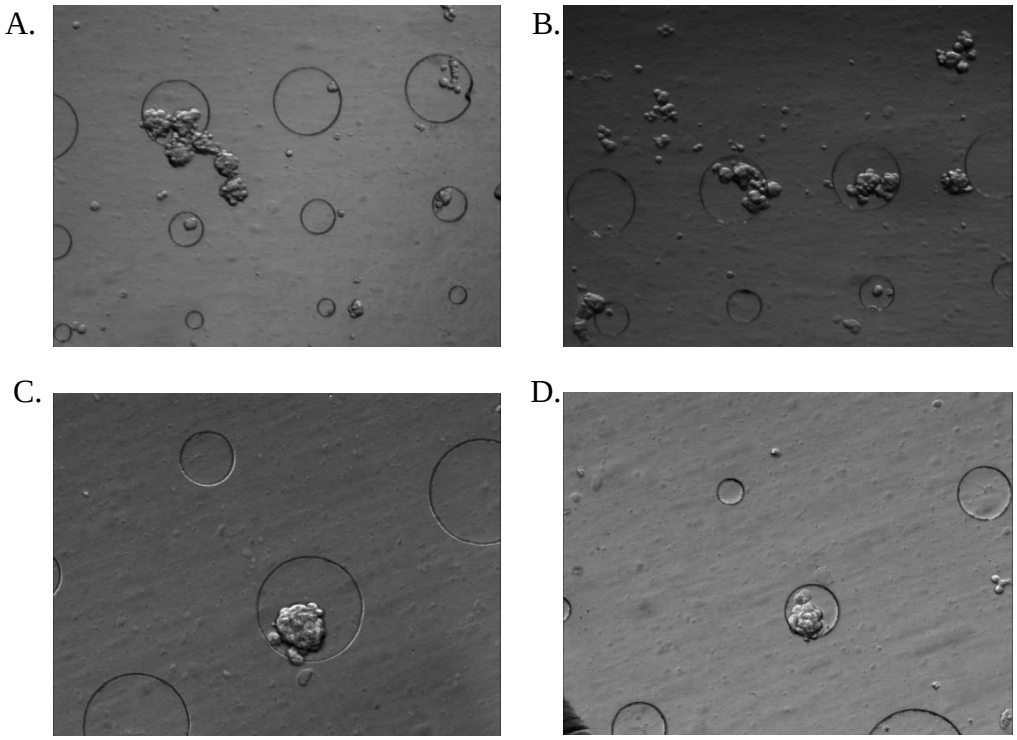

Figure 4. (**A** and **B**) Experiment 1 gravitational seeding; (**C** and **D**) Experiment 2 gravitational seeding.

As described above, the preferential seeding of cells in the microwells can be observed much more closely in pictures C and D of Figure 4, above. After using confocal fluorescent microscopy done by Dr. Gallego-Perez, the pictures in Figure 5 were obtained. The red arrows indicate the expression of insulin from larger cluster sizes as a green fluorescent marker. The green marker is observed in pictures C, D, and E for which the cluster sizes were 4-5 cells per cluster (please see Table 1 on page 18 for detailed information).

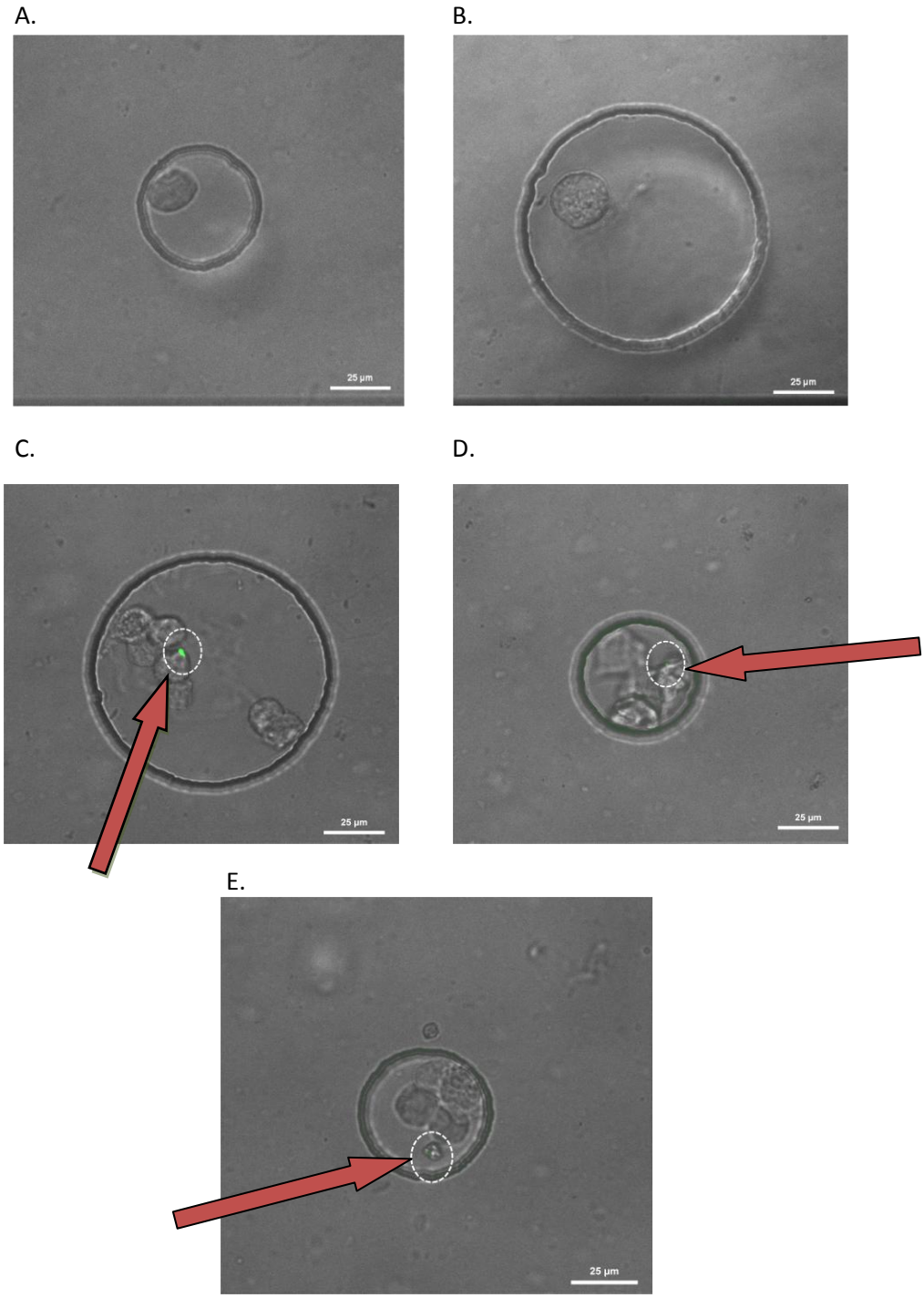

Figure 5. (A) 1 cell per cluster **(B)** 1 cell per cluster **(C)** 5 cells per cluster **(D)** 4 cells per cluster **(E)** 5 cells per cluster. Insulin expression is observed by the green immunostaining marker shown in pictures D, E, and F. The red arrows highlight detection of insulin by confocal fluorescent microscopy.

After running many initial experiments, some common observations were noted under the microscope after 4 days of incubation from initial cell seeding. One of the most important observations was the accumulation of cell clusters on top of the PDMS chip as well as at the bottom of the wells. To address this issue, the media was changed once the cells were allowed to incubate for 2-3 hours after initial seeding. The media was aspirated at an angle so that those clusters in the wells were not disturbed and the superficial clusters were removed. New media was applied twice to the chip at an angle as well to wash off the superficial clusters. Another important observation during experimentation was that cell count done on an incubation of over 96 hours is very high. The cell density of this sample was approximately 910,000 cells per milliliter. To address this high cell concentration, the sample was diluted 1:4 to have a cell concentration that was easier to use. The following figures are DIC microscopy pictures of the microwells after 4 days of incubation. The pictures show preferential seeding in experiment 2, which is not evident in experiment 1.

Discussion and Conclusion

Work by Boretti and Gooch in their paper titled "Induced Cell Clustering Enhances Islet β Cell Formation from Human Cultures Enriched for Pancreatic Ductal Epithelial Cells" highlighted the importance of differentiated β cells in order to detect insulin expression. Along with this finding, the importance of cell clustering was also recognized. We used this work as a motivation to further investigate the amount of clustering required for the expression of insulin. The results of my project suggest that the number of cells per cluster is essential for differentiated β cells to form. Further, there also must be a certain number of cells per cluster in order for proper insulin expression to take place.

Pictures D, E, and F in Figure 5 show that insulin was expressed by observation of the green fluorescent marker. In all of the cases that were observed, the common finding among these pictures was cluster sizes and the data given in Table 1 states that the cluster size was at least 4-5 cells per cluster. Another observation from this experiment is that the area-to-perimeter ratio was observed to be at least 4.5 in order for insulin expression to be present (as shown in pictures D, E, and F). Other pictures A, B and C all have an area-to-perimeter ratio of less than 4.5. Since the results are speculative and quite preliminary, such a conclusion that at least 4-5 cells per cluster are required for differentiated β cells to release insulin is pre-emptive. However, the observations from this project suggest that scientists perform further studies in order to reach a conclusion.

As stated earlier, the results acquired from the experiments relating to this project are, at best, suggestive of the role of cell clustering on the differentiation of β cells. Due to shortage of time in performing further experiments, the results of this experiment could not be duplicated in order to reach a definitive conclusion for the hypothesis. The area-to-perimeter ratio of picture

(see Table 1) does not follow the observation that area-to-perimeter ratios of greater than 4.5 express insulin. So, even though the hypothesis is supported for the most part, further studies are still recommended to reach a solution to this problem. Some modifications are possible to improve the experimentation techniques. One way the experiments could have produced better results is to have a better way to seed cells at the bottom of microwells, without having any clusters outside the wells. The purpose of the different diameter microwells (as shown in Figure 3) was to seed single cell clusters in the small diameter microwells and multiple cell clusters in the larger diameter microwells. But, the cell seeding method did not allow for preferential seeding in the different diameter microwells. As shown in pictures C and D in Figure 4, both large and small diameter microwells have multiple cell clusters, which ignores the purpose of the experimental design of the PDMS chip. So, preferential seeding is essential in order to get meaningful results.

One suggestion to improve the way cells are seeded for this project is to use a microwell array with deeper wells. With deeper microwells, any cells or cluster of cells at the bottom after initial seeding will be less likely to wash out during the washing procedure with serum free media. As a result, most of the wells will be full of cells/cluster and used for experimentation. Additionally, the PDMS chip would be most effectively used for experimentation if the chip would have a tight fit with the container in which the cell seeding is performed. In this project, a 24-well plate was used and there was room around the chip for cells to fall off. Hence, a tighter container for the PDMS chip would prevent cells from being lost to adjacent non-experimental surfaces.

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