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3D differentiation enhances the efficiency of differentiation of human induced pluripotent stem cells to insulin producing cells

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3D DIFFERENTIATION ENHANCES THE EFFICIENCY OF DIFFERENTIATION OF HUMAN INDUCED PLURIPOTENT STEM CELLS TO INSULIN PRODUCING CELLS

by

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CERTIFICATE OF APPROVAL

MASTER'S THESIS

This is to certify that the Master's thesis of

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To my dear parents, Gururaj Rotti and Vidya Rotti, to my dear sister, Priyanka Rotti, and to Karthik Iyappan Gunasekaran, my best friend.

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ABSTRACT

Type 1 Diabetes (T1D) is an autoimmune disorder in which the pancreatic β-cells are destroyed by the body's immune system. The reduced number of β-cells leads to inadequate insulin secretion and high glucose levels in the body. The requirement of insulin injection throughout life and lack of donors for islet transplantations has prompted a search for more accessible and available sources of insulin producing cells that can be transplanted in T1D patients. To that end, the discovery of induced pluripotent stem (iPS) cells has provided a potential source of precursors for cell therapy for T1D. iPS cells are reprogrammed somatic cells, which can be transplanted back into the patient, from whom the somatic cells were initially derived, thus potentially avoiding immune rejection when transplanted. As a potential therapy for T1D, we aim to derive insulin producing cells (IPCs) from human iPS cells. In contrast to the conventional two dimensional (2D) cell culture systems used in many iPS derived IPC studies, the inner cell mass (ICM) from which various organs differentiate during embryogenesis is a cluster of cells that enables signaling crosstalk between cells of different types. Three dimensional (3D) cell culture systems allows cells to form cell clusters that promote cell – cell signaling. Hence, we hypothesized that 3D cell culture systems will yield better efficiency of differentiation to functional IPCs *in vitro* than 2D cultures.

Initially, the synthetic polymers sodium alginate and matrigel were analyzed for their ability to enable cell clustering to establish 3D cell culture systems. The 3D cell environment established using matrigel was used for the differentiation of human iPS cells to Insulin Producing Cells (IPC). The cells were first converted to endodermal cells. A mixture of growth factors then induced the differentiation of endodermal cells to pancreatic cells. The pancreatic cells were converted to IPCs that resemble pancreatic β-cells. Our 3D differentiated IPCs strongly expressed pancreatic endocrine transcription factors and pancreatic hormones. The IPCs also produced insulin when exposed to a high glucose

environment. But the number of IPCs obtained at the end of the differentiation was low.

Hence, our results demonstrate that 3D differentiation generates functional IPCs *in vitro* unlike 2D differentiation. In the future we aim to improve the percentage of IPCs that we generate from the 3D differentiation. Our expectation is that these cells will be able to cure hyperglycemia in diabetic mice more rapidly compared to the 2D differentiated cells owing to their proven insulin production in the presence of a high glucose environment *in vitro.*

PUBLIC ABSTRACT

Type 1 diabetes (T1D) is caused by the destruction of pancreatic β-cells. This process results in loss of the β-cell mass and reduced production of insulin. Lack of insulin, the key regulator of glucose, leads to elevated blood glucose levels, which can lead to the complications of diabetes such as blindness, cardiovascular disease, neuropathy and kidney failure. Our goal was to establish an alternative source of pancreatic β-cells. First, human skin cells were reprogrammed to generate induced pluripotent stem cells. These cells are very similar to embryonic stem cells in that they can be driven to generate any desired tissues such as pancreatic β-cells. We established a protocol that utilizes Matrigel to create the three dimensional (3D) environment which facilitates cell differentiation.

During the differentiation process, the cells were first differentiated to endodermal cells, which express CXCR4. Past this stage, the cells transitioned into pancreatic precursor cells, characterized by the expression of Pdx-1, a key pancreatic nuclear factor. At the end of the differentiation procedure, the cells formed three characteristic types of clusters—tight clusters, cyst-like clusters that had a thick edge and cyst like clusters that contained compact clusters at the core. On characterization of the cells at the end of the differentiation process, the tight cell clusters were positive for insulin, C-peptide, Nkx6.1, and Nkx2.2. On glucose stimulation, the cells responded by secreting insulin. However, when compared to human pancreatic islets, the response to glucose stimulation by our cells was very low.

Thus, we have established a method for generating insulin producing cells from human skin cells. However, the protocol needs further improvement to enhance insulin secretion and we need to test our cells in vivo to determine whether they can correct glucose levels.

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LIST OF ABREVIATIONS

2D	-	Two dimensional
3D	-	Three dimensional
4F	-	Four factors
ActrI	-	Activin receptor type I
ActrII	-	Activin Receptor type II
ADSC	-	Adipose derived stem cells
bFGF	-	Basic Fibroblast growth factor
BMP	-	Bone Morphogenic Protein
EGF	-	Epidermal Growth Factor
ELISA	-	Enzyme linked Immunosorbent Assay
ES	-	Embryonic stem cells
FDA	-	Food and Drug Administration
GPR119	-	G protein coupled receptor 119
GSK-3β	-	Glycogen synthase kinase three beta
ICM	-	Inner Cell Mass
IGF-1	-	Insulin-like growth factor-1
IPC	-	Insulin producing cells
iPS	-	Induced pluripotent stem cells
MEF	-	Mouse Embryonic Fibroblasts
NGF	-	Nerve Growth Factor
NOD	-	Non Obese Diabetic
PDGF	-	Platelet Derived Growth Factor
Pdx-1	-	Pancreatic and duodenal homeobox-1
PI3K	-	Phosphoinositide-3-kinase
RIP	-	Rat insulin protein

ROCK	-	Rho-associated protein kinase
RPMI	-	Roswell Park Memorial Institute Medium
Shh	-	Sonic hedgehog
T1D	-	Type 1 Diabetes

CHAPTER 1. INTRODUCTION

1.1 The Pathology of Diabetes

Diabetes is a chronic disease characterized by elevated levels of blood glucose. 8.3% of the American population is diabetic. Out of the 26 million children and adults with diabetes in the US, the JDRF reports 18.8 million people diagnosed with diabetes and 7 million people undiagnosed [1]. Statistical surveys indicate that every 1 in 400 of people younger than 20 years of age have diabetes, type 1 or type 2. About 15,600 people with T1D and 3600 with T2D are newly diagnosed every year [1]. Diabetes is a cause of death for nearly 231,404 Americans annually [1]. Complications that arise due to diabetes include stroke and heart disease, high blood pressure, blindness, kidney disease, retinopathy and amputations of lower limbs. Due to this looming crisis, diabetes has attracted the attention of the research community. A successful treatment of diabetes can also reduce diabetes related deaths and complications.

There are two types of diabetes, namely, Type 1 diabetes (T1D) and Type 2 diabetes (T2D). T1D is an autoimmune disorder diagnosed in children and young adults. Its prevalence in children earned it the name “juvenile diabetes”. In T1D the body’s own immune system destroys the pancreatic β-cells leading to underproduction of insulin. Inadequate insulin production directly affects the glucose regulation in the body leading to hyperglycemia [2-4]. On the other hand, T2D is a result of the inability of the body to regulate glucose levels in the body due to insulin resistance. This occurs either due to disruption of the insulin receptors on the cells or due to glucose insensitivity of the insulin receptors. Insulin is thus not used to transport the glucose into the cells. The persistence of high glucose levels in spite of normal insulin production by the β-cells induces increased production of insulin initially. The functionality of the β-cells is impaired further on when the cells are unable to meet the demand for insulin and regulate hyperglycemia [5].

Although T2D is more prevalent than T1D, due to its prevalence in children, its genetic nature, complications accompanying its occurrence and complications that ensue if treatments are not managed appropriately, T1D has been declared a chronic and potentially fatal disease.

1.2 Existing Methods of Treatment for Type 1 Diabetes

T1D requires exogenous insulin since the β-cells are destroyed by the body's own immune system. Insulin injections have been a long standing treatment. Initially, purified insulin was injected subcutaneously keeping the dose constant. This has now evolved to implantation of devices that can detect high glucose levels in the body and secrete insulin. Insulin injections however have various disadvantages. They can lead to a sudden gain in weight, rashes and hematoma, dizziness, faster heartbeat, wheezing and shortness of breath. These side effects remove normalcy from the life of the patient. This has given way to efforts to transplant islet cells in order to replace the dysfunctional islets or induce islet cell proliferation to increase the number of existing islets.

Allo-transplantation of islets has met widespread success in reducing hyperglycemia and enabling the achievement of insulin independence to T1D patients [6]. Allo-transplantation is defined as transplantation of cells or organs from a donor of the same species. The procedure of allo-transplantation of islets hence heavily relies on the availability of islet donors. The islets from the donors are purified from the pancreas by the use of a mixture of enzymes [6]. Each T1D patient typically receives 400,000 to 500,000 islets per infusion. The transplanted islets mature *in vivo* before they are fully functional [6]. The process of maturation includes adapting to the immune system and inducing the development of blood vessels to make the transplanted islets fully functional grafts. The most important aspect of islet functionality is detection of high glucose before the secretion of insulin. Hence, the patients who receive islets need to receive insulin

injections to maintain normoglycemic levels while the transplanted islets mature *in vivo* [6]. Whole organ transplantation is used as an alternative technique to islet cell transplantation. The advantages of whole organ transplantation include longer functionality sustenance by the organ although surgery for whole organ transplantation is far more extensive than islet transplantation, where the islets are infused under the liver in a minimally invasive manner [6].

Islet transplantation and whole organ pancreas transplantation is still an experimental technique and is not yet classified by the US FDA as a therapeutic technique [6]. The requirement to improve the technique is necessitated by the disadvantages of islet transplantation like the use of immunosuppressive drugs to prevent an immune reaction against the allo-graft, the inability of the technique to provide complete insulin independence to the patients and time taken by the transplanted cells to mature and become functional. Side effects of continued use of immune suppressive drugs includes increased cholesterol levels, high blood pressure, fatigue, anemia, decreased white blood cell counts, decreased kidney function and increased susceptibility to bacterial and viral infections. In 2011, 8000 organ donors were available, but only 1562 pancreases were recovered from donors. In spite of availability of the organs, the functionality needs to be carefully assessed before transplantation [6]. The quality assurance of donor organs eliminates many as they may not meet the criteria for transplantation or the islets might be destroyed while processing. The data on donor islet availability and their need clearly indicates the necessity to have a source of IPCs that is easily available and accessible.

Cell based therapies have emerged as possible treatments for T1D with the potential to be easily available and accessible. One strategy of treatment is inducing islet cell proliferation [7-9]. It was reported that a combination of EGF and gastrin increased the beta cell mass of isolated human adult islets *in vitro* [7]. Similarly in a different study, a xenograft of encapsulated sertoli cells induced beta cell neogenesis which reversed hyperglycemia in NOD mice. Several chemicals that are GSK-3β inhibitors, glucokinase

activators, GPR119 agonists, L-type calcium channel activators and adenosine kinase inhibitors have been used to induce β-cell replication [10].

With the discovery of stem cells, ES cells were extensively used to obtain IPCs [11-13]. The ES cells are isolated from the ICM of the blastocyst [14-16]. ES cells have the potential to differentiate into the three germ layers namely the endoderm, mesoderm and ectoderm and subsequently to any cell type required. The protocols being developed to differentiate ES cells to IPCs attempt to trace the path followed by the cells differentiating into the pancreatic β-cells during embryogenesis [17]. This is enabled by treating the cells with a cocktail of growth factors that are known to play a role in the development of pancreatic β-cells by suppressing the development of the hepatic and gastrointestinal cells [13, 18-21]. The pancreas develops from the endoderm. After the formation of the primitive gut tube, cells from the anterior foregut differentiate into pancreatic progenitors [22]. The pancreatic progenitor cells, however, are precursors to the endocrine, acinar and ductal epithelial cells. Research has shown that the β-cells of the pancreas develop when the pancreatic endocrine progenitor cells come in contact with the dorsal aorta [23]. Research on manipulation of the developmental pathways and signaling crosstalk by the use of growth factors has helped develop protocols for the differentiation of ES cells to pancreatic endocrine progenitor cells which on transplantation into diabetic mice successfully cured hyperglycemia [13, 18-21]. However, current therapies with ES cells have not yet established a protocol that provides mature functional IPCs *in vitro* [24]. The immune reaction after transplantation of the ES cells derived IPCs is another disadvantage to the use of ES derived IPCs as a therapy for T1D.

1.3 Derivation of pluripotent stem cells.

Stem cells can differentiate into various mature cell types of the body. The potential of stem cells to self-renew and replace damaged cells was discovered by Till & McCollouch establishing the basis of stem cell science [25]. In 1998 Thompson at the University of Wisconsin extracted human ES cells and displayed their tremendous ability to replace dysfunctional cells [14, 26]. However, there was a debate on approval and funding for stem cell research due to ethical issues on using the human embryo for research purposes. Researchers were urged to discover alternative sources of stem cells. In 2006 and 2007 Yamanaka established iPS cells [27, 28]. These were a new type of stem cells that were derived from somatic cells. iPS cells are stem cells derived after the reprograming of the somatic cells. It was discovered that the introduction of four transcription factors Oct 3/4, Sox2, Klf4 and c-Myc into fibroblasts converted them into ES cell-like cells that could differentiate into all three germ layers similar to the characteristics of ES cells [28, 29]. This discovery created a new prospect of stem cell therapy for various diseases. Figure 1 portrays the benefit of using iPS cells in the context of T1D. Fibroblasts can be derived from the T1D patients and reprogrammed to become stem cells. These stem cells could then be differentiated into IPCs and transplanted into the diabetic patient. This reduces the possibility of the immune rejection and has the ability to enable the graft to be more successful without the use of immunosuppressive drugs. It hence eliminates the side effects of immunosuppressive drugs and has the potential to improve the quality of life of the patients.

1.4 Existing strategies to produce insulin producing cells from stem cells.

The strategies to convert ES or iPS cells to IPCs attempt to trace the developmental cell mechanisms during embryogenesis. The process of generation of IPCs *in vitro* is divided into four stages – endoderm formation, pancreatic endoderm specification,

pancreatic endocrine cell generation and lastly maturation of β- like cells to form functional IPCs.

First the stem cells are differentiated to endodermal cells. This involves the suppression of PI3K signaling and the subsequent upregulation of Nodal signaling [30, 31]. Nodal pathway plays an important role in formation of endodermal cells [32]. The Nodal ligands

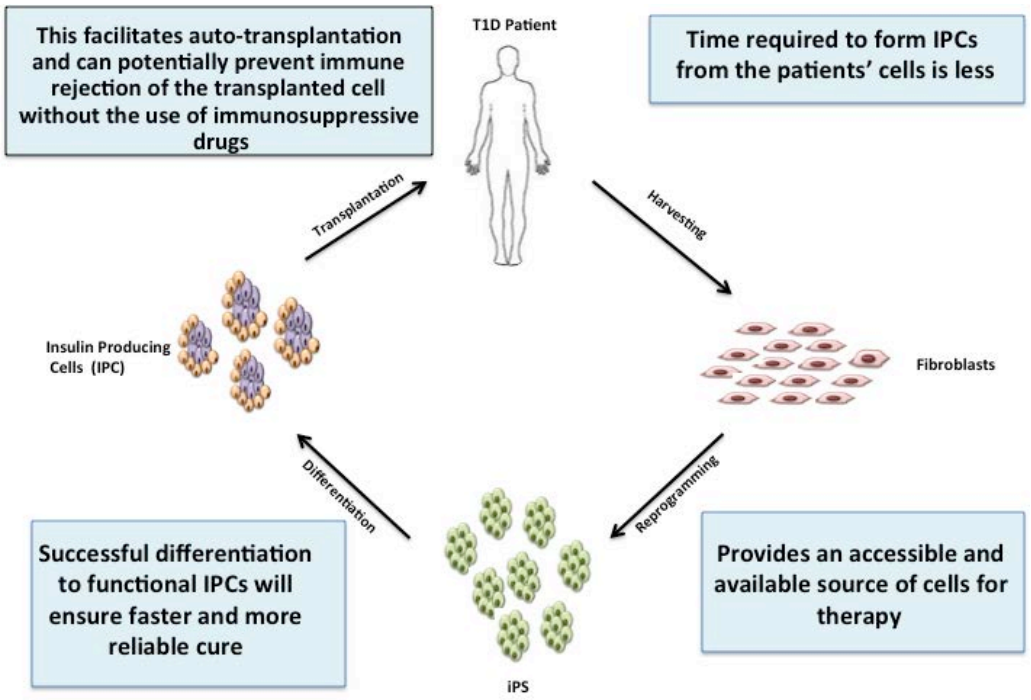


Figure 1. The application of human iPS cells in the context of T1D.
Fibroblasts obtained from T1D patient can be reprogrammed to form iPS cells. These stem cells can be banked and used subsequently, thus making them accessible and available when required. These iPS cells can be differentiated to IPCs. The IPCs generated can be transplanted back into the same T1D patient. Since the precursors to the IPCs are in fact the fibroblasts from the same person, this model has the potential to eliminate the immunological rejection after transplantation of the IPCs, thus providing long term insulin independence.

activate the ActrI/ActrII receptors, which leads to the phosphorylation of Smad 2/3 and

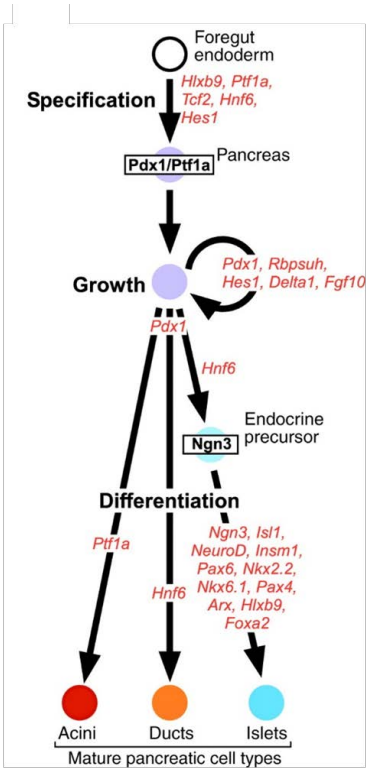
their activation results in the upregulation of transcription of Lefty, Crypto. The transcription of Lefty and Crypto in turn upregulates the Nodal pathway [33-37]. This is a positive feed-back mechanism to sustain the Nodal signaling in order to bring about endodermal cell conversion. The endodermal cells then form the primitive gut tube. Spatial and temporal suppression/ upregulation of signaling pathways is important for organ specification in the gut tube. In order to form pancreatic cells, the suppression of Sonic Hedgehog (Shh) and BMP signaling in congruence with upregulation of Retinoic Acid signaling is required [38-47]. The subsequent suppression of Notch aids the formation of pancreatic endocrine cells [17, 48-56]. Further, the inhibition of Notch must be followed by PI3K upregulation and PARP inhibition to form IPCs and induce cell proliferation by inhibiting apoptosis [57-60]. Figure 2 briefly describes the characteristic transcription factors expressed at each stage during the differentiation of ES or iPS cells to pancreatic cells.

In a study, the development of β-cells was observed in the areas of the developing pancreas that were in contact with the endothelial cells of the dorsal aorta in mouse embryos [23]. Some reports suggest the exocrine tissue of the pancreas can be a source of the endocrine tissue [61, 62]. Recently, it was stated that a co-culture of endothelial cells, hepatic cells and stem cells was a successful system to generate IPCs [63].

Generation of IPC from mouse ES cells was first reported in 2001. However, the insulin expressing cells produced by this protocol was later reported to be due to the uptake of exogenous insulin from the medium and not endogenous production of insulin [64-67]. Emphasis was thus given on elaborate study of stage-wise differentiation of pluripotent stem cells to IPCs. Research groups focused on the differentiation of stem cells to the endodermal stage to maximize the differentiation to pancreatic cell fate and later on methods to induce proliferation of progenitors at each stage in order to increase the size of progenitor pool and eventually increase efficiency of differentiation [68] [69].

But most of the current protocols differentiate stem cells to pancreatic cells in a 2D

culture system [70] . Upon transplantation under the kidney capsule, a small percentage of the 2D differentiated cells form IPCs *in vivo* and cure hyperglycemia over time [71]. The signaling players that have a role in the conversion of the transplanted pancreatic cells to IPCs are unknown and hence efforts to produce high percentage of functional IPCs *in vitro* have failed. Additionally, the transplanted cells are also bi-hormonal. This could imply that the transplanted cells immature and mature *in vivo* after transplantation before they have a reversing effect on hyperglycemia. The time taken for the cells to mature delays their therapeutic effect.



Murtaugh L C Development 2007;134:427-438

Figure 2. Characteristic transcription factors expressed at each stage of differentiation of pancreatic cells from endoderm.

The transcription factors characteristically expressed by cells at each stage during the differentiation to pancreatic β-cells are shown in the figure. In the endoderm, cells expressing Pdx-1 are pancreatic cells. Differentiation of Pdx-1 expressing cells to cells expressing Nkx6.1, Nkx2.2, Pax4, Pax6 indicates of islet cell fate.

Likewise, efforts to produce IPCs from adult stem cells, like mesenchymal stem cells have generated results that require improvement with respect to the number of IPCs generated and their functionality. The mesenchymal stem cells that have been used to differentiate to IPCs are adipose tissue derived stem cells (ADSC), bone marrow derived stem cells and cells derived from the umbilical cord and placenta. ADSC have the advantage of ease of procurement and storage for the patient. The ADSC-derived pancreatic endocrine cells express key pancreatic transcription factors like Pdx-1 and appear to mature faster in a diabetic microenvironment and although the safety of ADSC derived cells has been displayed clinically, the protocol to differentiate ADSC into IPCs requires further investigation for better characterization of cell population generated and the process of their maturation *in vivo* [72]. Bone marrow derived stem cells have been infused to replace dysfunctional cells and initiate *in vivo* differentiation of the stem cells to the required cell type. However, this mode of treatment has not provided substantial results in replacing the β-cells. *In vitro* differentiation of the bone marrow stem cells to IPCs has also not generated a good percentage of conversion to IPC, but generation of IPCs has been reported after transplantation of precursors into diabetic mice, indicating a favorable microenvironment for their differentiation and maturation *in vivo* [73] [74]. Likewise, mesenchymal stem cells derived from the umbilical cord and placentas are convenient to procure and bank but their efficiency to differentiate into IPCs *in vitro* is not very well studied. Generation of IPCs from mesenchymal stem cells has the potential to eliminate the possibility of immune rejection and is easy to obtain. Hence, current efforts are focused towards producing fully functional IPCs *in vitro* to reduce the time needed for the transplanted cells to normalize glucose levels in diabetic mice [75].

To alleviate the problem of organ shortage, our lab has pioneered the differentiation of human iPS cells to IPCs [76]. We found that ES cells engineered to constitutively express Pdx-1 and Rat Insulin Promoter (RIP) driven luciferase differentiated to IPCs and

cured hyperglycemia when transplanted into diabetic mice [77]. Alternate protocols to generate IPCs *in vitro* in our lab included the use of the conventional 2D culture system of human iPS cell differentiation, in which the cells were exposed to a cocktail of growth factors that have been studied carefully for their ability to induce pancreatic differentiation. The 2D iPS differentiation produced pancreatic endocrine cells that were bi-hormonal and the percentage of cells producing insulin was very low. Furthermore, the insulin secretory granules in the *in vitro* generated IPC did not have the same morphology as seen in β-cells when analyzed using transmission electron microscopy. On transplantation of these premature pancreatic cells in diabetic mice, reversal of hyperglycemia was observed only after 4 months. Additionally, the mice transplanted with *in vitro* generated IPCs died after a glucose challenge suggesting that the transplanted cells were not capable of handling high glucose levels. Our data thus indicated that the cells produced by 2D differentiation of iPS cells were not mature and functional IPCs. Changes in the protocol to make the process of differentiation more physiological than the previously used 2D differentiation and robust were necessary to generate functional IPCs *in vitro.*

We hypothesize that the 3D differentiation of stem cells can enhance the process of differentiation to functional IPCs. The ICM in the blastocyst, which consists of stem cells that eventually differentiate to form the different cell types of the body, is a cluster of cells that enables cell-cell interaction and cross signaling between different cell types. Research has shown that the formation of IPCs during embryogenesis is a result of upregulation/ downregulation of various signaling pathways brought about by paracrine effects of different cells. The cell-cell interaction and signaling crosstalk found in nascent environment is absent in 2D cell cultures. Consequently, 2D cultures may not be the ideal model to recapitulate *in vivo* processes *in vitro.*

Our goal was to first establish 3D cultures in polymers and then differentiate human iPS cells to IPCs in 3D cultures.

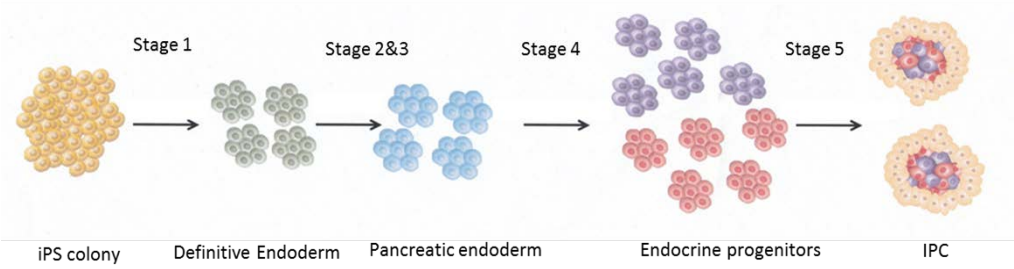


Figure 3. Schematic of the stagewise differentiation of human iPS cells to IPCs
The process of differentiation of iPS to IPCs *in vitro* followed the stagewise formation of the pancreatic β-cells as observed during embryogenesis. Stage 1 of the differentiation was intended to convert the stem cells to endodermal cells. Stage 2, Stage 3 and Stage 4 were dedicated to form pancreatic cells. They involved formation of the pancreatic endoderm and subsequently the progenitors for endocrine cells of the pancreas. Stage 5 of the differentiation was for the maturation of the endocrine IPCs.

CHAPTER 2. MATERIALS AND METHODS

2.1 Human iPS cell lines and culture conditions

The four factor (4F) human iPS cells were used. The 4F iPS cells are derived by reprogramming human fibroblasts using four viral vectors. Reprogramming induces the expression of four transcription factors that revert them to stem cell state. The four transcription factors responsible to revert somatic cells to stem cells are Oct4, Sox2, Klf4 and c-Myc [27, 28]. The 4F iPS cells used for differentiation were generated by Dr E M Kim in Dr Zavazava's lab [78]. These cells were cultured on irradiated mouse embryonic fibroblasts (MEFs). The MEFs are required to maintain the pluripotency and self-renewal capacity of the iPS cells and are generally termed as feeder cells. The media used to culture these cells was supplemented with bFGF, which is known to maintain the pluripotency of the iPS cells by initiating the PI3K pathway [30, 31]. The iPS cells grow as colonies on the MEFs. Maintenance of these colonies is important to prevent differentiation to random cell types. The media was supplemented with a ROCK inhibitor to permit cell survival when dissociated from colonies during splitting and sorting of cells done to maintain their differentiation potential [79].

When the cultures consist of spontaneously differentiated cells, pluripotent stem cells are purified by immunomagnetic bead sorting of CD326+ cells. CD326 is E-cadherin and the cells that express this cell surface marker are pluripotent and grow in colonies. Hence, this process of sorting ensured that the cells used for differentiation were pluripotent and had not differentiated.

2.2 Establishing 3D cultures

Three dimensional (3D) cultures were established by coating plates with Matrigel diluted in media used for differentiation at a 1:1 ratio. This mixture of matrigel and media was added to the culture plates and incubated at 37 degrees Celsius for one hour. This initiated crosslinking of matrigel and coated the culture plates for differentiation. Pluripotent human iPS cell colonies were seeded on the matrigel and then subjected to different differentiation media cocktails.

2.3 Differentiation of human iPS cells into IPCs

The process of differentiation of human iPS cells into IPCs consisted of first differentiating the iPS cells first to endodermal cells. After exposure to pancreatic growth factors, the endodermal cells were converted to pancreatic cells. The pancreatic cells were coaxed into endocrine cells that produced insulin in a high glucose environment. The process of differentiation was divided into five different stages.

Figure 3 describes the growth factors used for cell fate specification at each stage of the differentiation process. In the first stage of the differentiation, the cells were subjected to 20ng/ml Wnt3A and 100ng/ml Activin A for one day followed by exposure only to 100ng/ml Activin A for two days. The presence of Wnt 3A for a day is known to induce the mesendodermal cell fate and the subsequent treatment with Activin A for two days produced endodermal cells. For the second stage of the differentiation cells were treated with 50ng/ml FGF-7 (KGF) for three days. The cells were then differentiated to a pancreatic cell lineage by using a cocktail of 2umol/ml Retinoic acid, 100ng/ml Noggin and 0.25umol/L Sant-1 for four days. Subsequently 1umol/L ALK5i and 50nmol/L TPB was used for three days to promote pancreatic endocrine cell fate [70]. The final and fifth stage was maturation of the pancreatic endocrine cells to IPCs. This was brought about by using nicotinamide and IGF-1 for about 6-10 days.

In contrast to the previously used 2D cultures, this protocol included the use of

Wnt3a to ensure differentiation of the iPS cells to mesendodermal cells. In addition to this, the cells were differentiated when they were in a scaffold formed by polymerized matrigel, which provided the environment that enabled cell clustering.

2.4 Quantitative analysis of differentiation by flow cytometry

Flow cytometry was used to quantify the conversion of cells from stem cells to endodermal cells, pancreatic cells and IPCs. The cells were stained with an antibody for CXCR4 to determine the percentage of endodermal cells in Stage 1 of the differentiation process. Since CXCR4 is a surface antigen, the cells were washed to eliminate residual medium after harvesting and were incubated with the antibody for CXCR4 for 30 min at 4 degrees Celsius. Cells were washed to eliminate unbound antibody and then fixed with 4% PFA before running them on the flow cytometer. The BD FacsScan flow cytometer was used and the data was analyzed on FlowJo.

2.5 Characterization of pancreatic hormones and transcription factors using immunofluorescence and confocal microscopy

Immunofluorescence analysis was used to visualize the presence of pancreatic transcription factors and hormones after differentiation of iPS cells into IPCs. The cells were fixed using 4% paraformaldehyde. The cells were cytospun onto a slide, blocked with a solution containing BSA for 30 minutes and stained with the appropriate primary and secondary antibodies. The antibodies used were specific for insulin (Abcam antibody, ab9569, 1/100), Nkx 6.1(Santa Cruz, sc-15030, 1/50), Nkx2.2 (Santa Cruz, sc-25404, 1/50), c-peptide (Cell Signaling, #4593, 1/100). The cells were incubated with the primary antibody for one hour at room temperature or at 4 degrees Celsius followed by incubation for 30minutes at room temperature with the secondary antibody. Slides were imaged using the Zeiss 710 in the CMRF at the University of Iowa. Undifferentiated human iPS cells were used as a negative control. Human islets generously provided by the Integrated Islet

Distribution Program (IIDP), City of Hope, were utilized as a positive control.

2.6 Assessment of functionality of iPS derived IPCs in vitro using the Glucose Stimulated Insulin Secretion assay

The Glucose Stimulated Insulin Secretion assay is used to analyze the glucose sensitivity of the IPCs. The 3D differentiated IPCs and human islets were maintained to a high glucose solution (20mM) for 60 minutes. The cell lysate was analyzed for the production of insulin by the cells and the supernatant was analyzed to confirm the secretion of insulin in the presence of high glucose levels.

CHAPTER 3. RESULTS

3.1 Introduction

The ICM in the blastocyst forms three germ layers—the endoderm, mesoderm and the ectoderm—that contain multipotent cells which eventually differentiate into different cell types. The endodermal cells are characterized by the expression of a cell surface marker CXCR4 [80]. During embryogenesis, the CXCR4 positive cells indicate mature endodermal cells that further form the primitive gut tube [38]. The gut tube consists of the pancreatic endoderm and the hepatic endoderm. The pancreatic endoderm is characterized by the presence of CXCR4 and Sox17 whereas the hepatic endoderm does not express CXCR4. It has been shown that FGF signaling further potentiates the development of the endodermal cells from the intermediate mesendodermal cell population [81]. At this stage the development of the anterior-posterior axis of the primitive gut tube defines the patterning of the cell fate through the length of the tube for which the TGF-β, Shh, Wnt and FGF signaling pathways play an important role. The anterior primitive gut tube develops into the trachea, respiratory tract, lungs, esophagus, stomach, pancreas and upper duodenum. The pancreas develops from the foregut of the anterior endoderm [17]. The pancreatic bud is characterized by the presence of the transcription factor Pdx-1. Research has shown that mutation in the gene of Pdx-1 leads to suboptimal development of the pancreas [82, 83]. The suppression of Shh, Notch and TGF-β signaling at this stage coaxes the cells to become pancreatic [38]. The pancreas, however, consists of many different non-β-cells. In fact β-cells form a very small percentage of cells within the pancreas. The last stage of development of the β-cells consists of multiple stages. The pancreatic cells first form the endocrine precursor stage where they have the potential to form both α and β-cells. The formation of β-cell has been reported to depend on PARP inhibition. Many reports have also established the dependence of β-cell maturation and proliferation on the presence of endothelial cells [84]. Our efforts were focused on generating β-cells from

human iPS in a physiological manner so as to maximize the efficiency of conversion to IPCs, thus, facilitating a model to generate IPCs as a treatment for T1D.

3.2 Matrigel successfully established 3D cultures

3D cultures in matrigel can be defined as a method of culturing cells in 3D form allowing them to form clusters. Our hypothesis is that in 3D cultures iPS cells better differentiate than in 2D. Sodium alginate and matrigel were the two polymers tested for their ability to establish 3D cultures. βTC3 cells are a mouse insulinoma cells that we used in preliminary studies to determine whether they form spheroids in matrigel and sodium alginate. Three types of constructs of the sodium alginate were used—the spheroid construct, grid construct and the capillary construct. The βTC3 cells were suspended in sodium alginate solution and were then printed to form the constructs in calcium chloride solution. The constructs were cultured for two weeks at 37 degrees Celsius and 5% CO2 and then observed to investigate cell clustering and proliferation. The cells appeared in single cell form and did not proliferate. This demonstrated that the process of printing cells suspended in sodium alginate solution was not successful in enhancing cell clustering and hence was not a good model to establish 3D cultures. Figure 4 (a) shows the cells encapsulated in sodium alginate in a spheroid and grid construct stained with DAPI.

In contrast to the sodium alginate constructs, matrigel cultures consisted of polymerized mixture of matrigel and incomplete medium in a 1:1 (vol/vol) ratio. The cell suspension in medium was then added to the polymerized mixture and incubated at 37 degrees Celsius and 5% CO2 overnight. The cells penetrated the polymerized mixture and formed three dimensional clusters. The clusters of βTC3 cells were tested for cell viability by staining for insulin. They were also cultured for extended periods to test the culture model for sustenance. In contrast to the sodium alginate model, matrigel enabled clustering

of the cells and the cells expressed insulin, hence proving their viability. Figure 4 (b) shows the images of cell clusters in matrigel with cells that expressed insulin.

These initial tests suggested that matrigel was a better polymer for 3D cultures than sodium alginate. The cells were not subjected to processes that could reduce their viability in the matrigel model and clustered a lot faster than that seen in sodium alginate. We concluded that for fragile cells like human induced pluripotent cells, reduced handling and culture time was beneficial and matrigel was thus chosen as the polymer to be used in differentiation.

3.3 3D differentiation displayed better morphological changes and cell organization than 2D differentiation

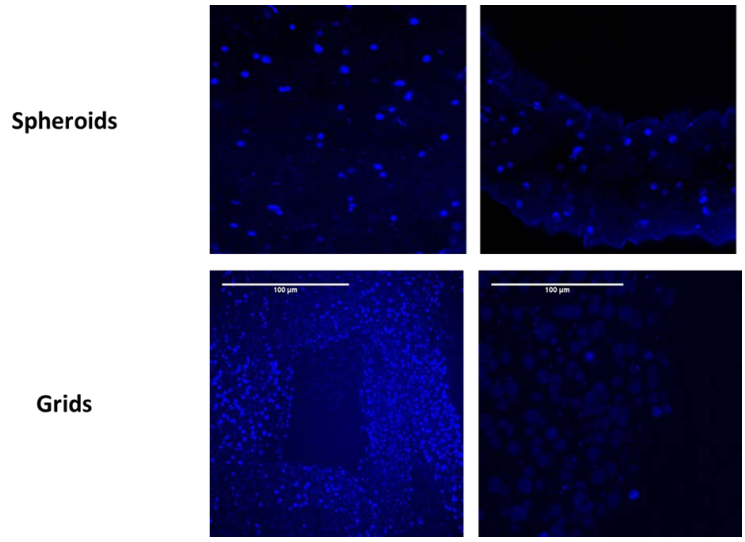
Pilot experiments to compare the 2D and 3D differentiation aimed at observing the morphological changes occurring during the process of differentiation. These initial experiments validated the beneficial effect of 3D differentiation by displaying enhanced cell organization and stark changes in morphology in 3D differentiation when compared to 2D differentiation.

By the end of second stage of the differentiation, single cells appeared around the bigger cell clusters which were indicative of cell proliferation and clustering. There was a definite increase in the cluster size when compared to the clusters in the first stage. At the end of the third stage, three different types of clusters appeared in the culture. There were tight clusters, hollow cysts like clusters and cyst like clusters containing a tight cluster core.

In the 2D cultures, on the other hand, cell proliferation was visible, but the cell organization was not distinguishable during differentiation. It was also noted that the cell density was very high at the end of the differentiation.

Figure 5a illustrates the changes observed in the 3D cell cultures while Figure 5b shows the changes in 2D cell cultures during differentiation. The results suggested that 3D

cell culture model could sustain the extended culturing period while enabling cell proliferation and differentiation.



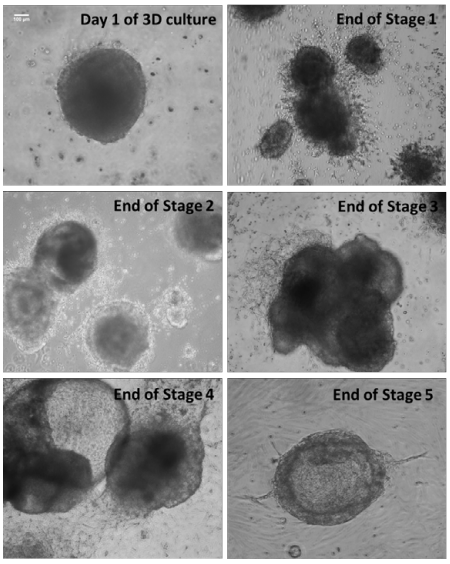
A: βTC-3 cells encapsulated in sodium alginate

B: βTC-3 in matrigel

Figure 4. 3D cultures in sodium alginate and matrigel

The βTC3 cells were used for pilot experiment to establish 3D clusters. The βTC3 cells were suspended in sodium alginate solution and printed to form constructs (4a) did not form clusters even after being cultured for two weeks. Whereas, cells seeded on matrigel scaffolds formed clusters within 24 hours and expressed insulin.

A: 3D cultures



B: 2D cultures

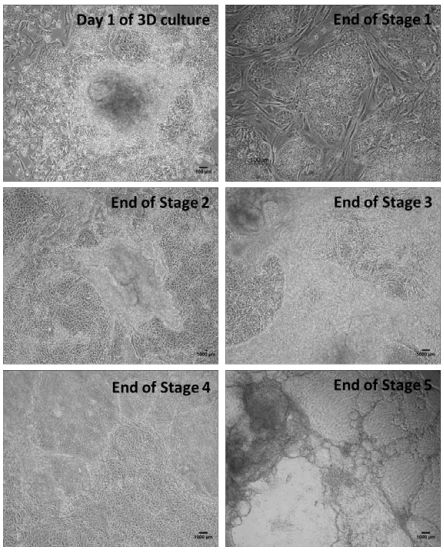


Figure 5. morphology of cells in 2D and 3D cultures at the end of each stage of the differentiation.

When 3D cultures (A) and 2D cultures (B) were analyzed for morphology differences during differentiation**,** better cell organization was observed in 3D cultures than in 2D cultures. The cell clusters in 3D cultures demonstrated characteristic and reproducible morphological changes during the differentiation. The clusters initially had single cells surrounding the cell clusters. Some clusters developed projections whereas some appeared hollow and some others were dense. The 2D differentiation on the other hand, appeared to have very high cell density without the changes in morphology as observed in 3D differentiation.

3.4 Quantitative analysis by flow cytometry revealed high percentage conversion to endodermal cells in 3D cultures

The endoderm is one of the three germ layers formed during embryogenesis. It consists of multipotent cells that differentiate into the lung, stomach, pancreas and duodenum. The cells of the endoderm are characterized by the presence of a surface marker CXCR4. The expression of CXCR4 was quantified by flow cytometry.

Figure 6 shows the results of quantification of CXCR4 expressing cells in both 2D and 3D cultures by flow cytometry. 3D differentiation produced 20% conversion to endodermal cells. This was greater than the percentage of endodermal cells obtained by 2D differentiation (6%). The increase in the endodermal cells obtained was suggestive of successful establishment of an alternative model of differentiation.

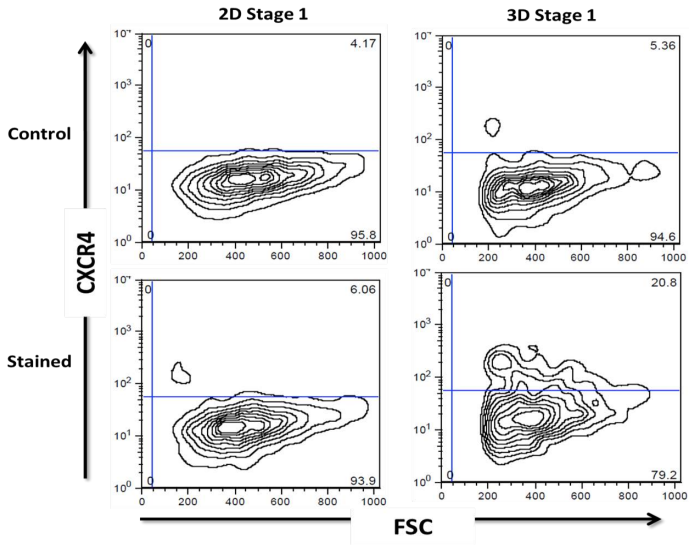


Figure 6. Flow cytometry analysis of expression of CXCR4 in 2D and 3D cultures at the end of stage 1 of the differentiation.
CXCR4 expression by the cells was quantified by flow cytometry. The 2D culture had very few cells expressing CXCR4 when compared to the 3D culture cells. This indicated lesser efficiency of differentiation in 2D cultures compared to the 3D cultures.

3.5 The granularity of tight clusters was shown by the Dithizone stain.

Dithizone staining is used to detect zinc containing granules which are characteristic of β-cells of the pancreas. This staining is used to purify pancreatic islets from the acinar tissue while harvesting donor islets. Dithizone chelates the zinc in the granules of the β-cells and produces a red stain. While this stain is not indicative of insulin production by cells, it is however indicative of existence of zinc granules that are characteristic of the β-cells in the pancreas.

Figure 7 shows the results of dithizone staining on 3D cultures. As seen in the figure, the larger clusters and the hollow cyst like clusters stained negative for dithizone (A&B), whereas the smaller, tighter clusters stained positive (C).

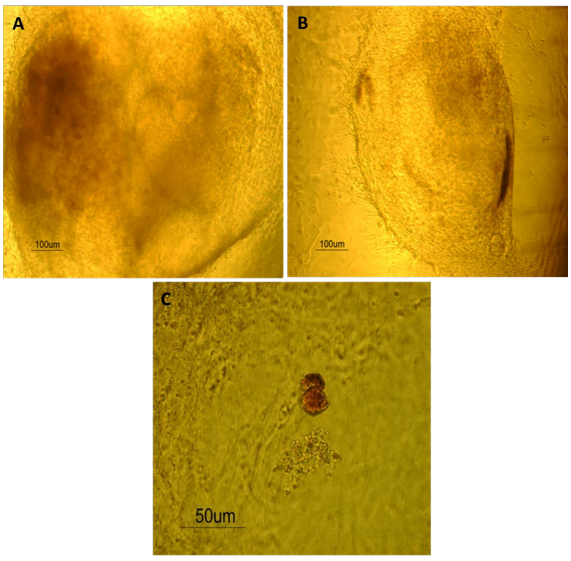


Figure 7. Dithizone staining of clusters in 3D cultures at the end of differentiation. Dithizone staining at the end of the differentiation distinguished the clusters with zinc granules. The small and dense clusters stained red after dithizone staining while the larger clusters remained colorless. This helped identify the number of clusters that could potentially produce insulin.

3.6 iPS-IPCs expressed pancreatic hormones and transcription factors.

IPCs obtained from 3D differentiated human iPS cells were tested for the expression of transcription factors Nkx6.1 and Nkx2.2. The IPCs were tested for presence of important pancreatic hormones like insulin.

While the presence of the Nkx6.1, Nkx 2.2, Glut 2, insulin and glucagon were visualized using confocal microscopy, the presence of insulin and glucagon producing cells were quantified using flow cytometry.

Figure 8-11 show the expression of transcription factors like Nkx6.1 and Nkx2.2, and pancreatic hormones like insulin and c-peptide in the 3D IPCs analyzed using immunofluorescence. The human islets were used as positive controls to determine the expression of these pancreatic transcription factors and hormones.

3.7 iPS-IPCs produced insulin when exposed to high glucose environment in vitro

Pancreatic islets produce insulin when a high glucose environment is detected. High glucose stimulation is hence a proof of functionality of the pancreatic islets. In order to test the functionality of IPCs generated from iPS cells, the cells were maintained in high glucose medium. Insulin was quantified in the cell lysate of the IPCs after exposure to high glucose medium and compared with the lysate of the islets in similar experimental conditions. The insulin concentration was normalized to the total protein content in the cell lysate used.

Figure 12 shows the relative levels of insulin production in 3D IPCs and human islets when maintained in high glucose media

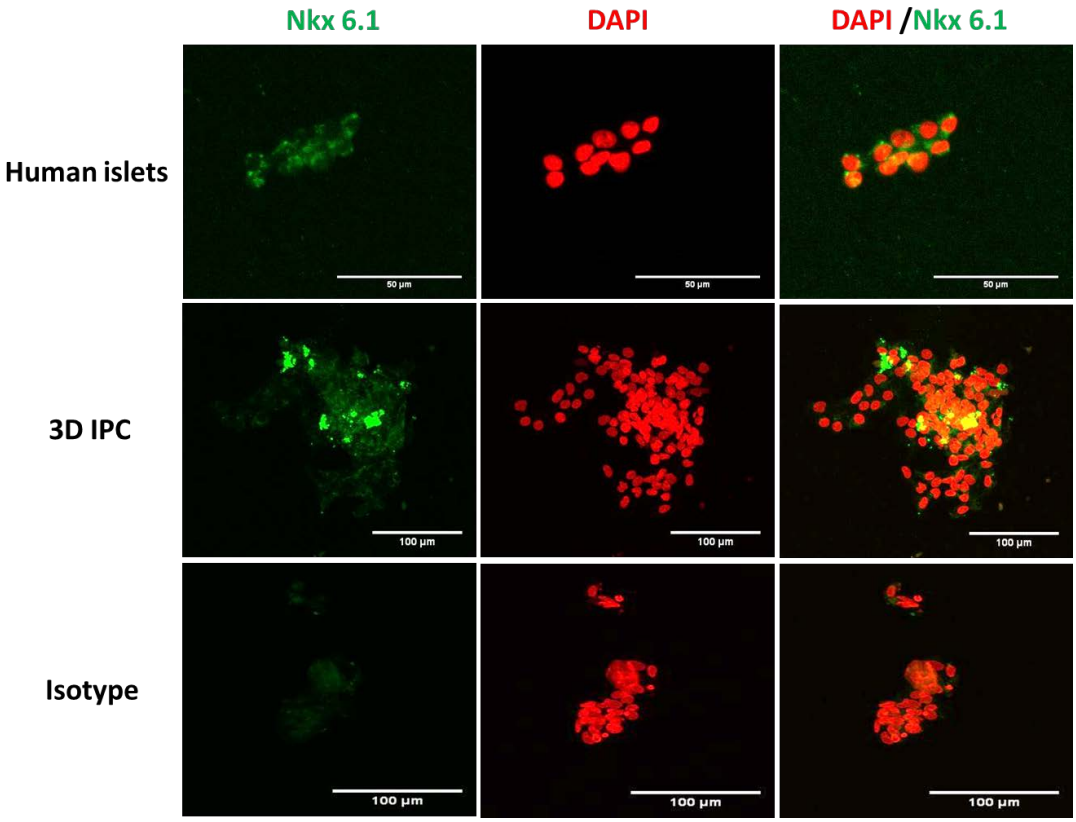


Figure 8. Analysis of Nkx6.1 expression in 3D differentiated IPCs.
Nkx6.1 is a transcription factor critical for β-cell maintenance and proliferation. The presence of Nkx6.1 was indicative of transcription machinery of the 3D differentiated IPCs being similar to that of pancreatic β-cells.

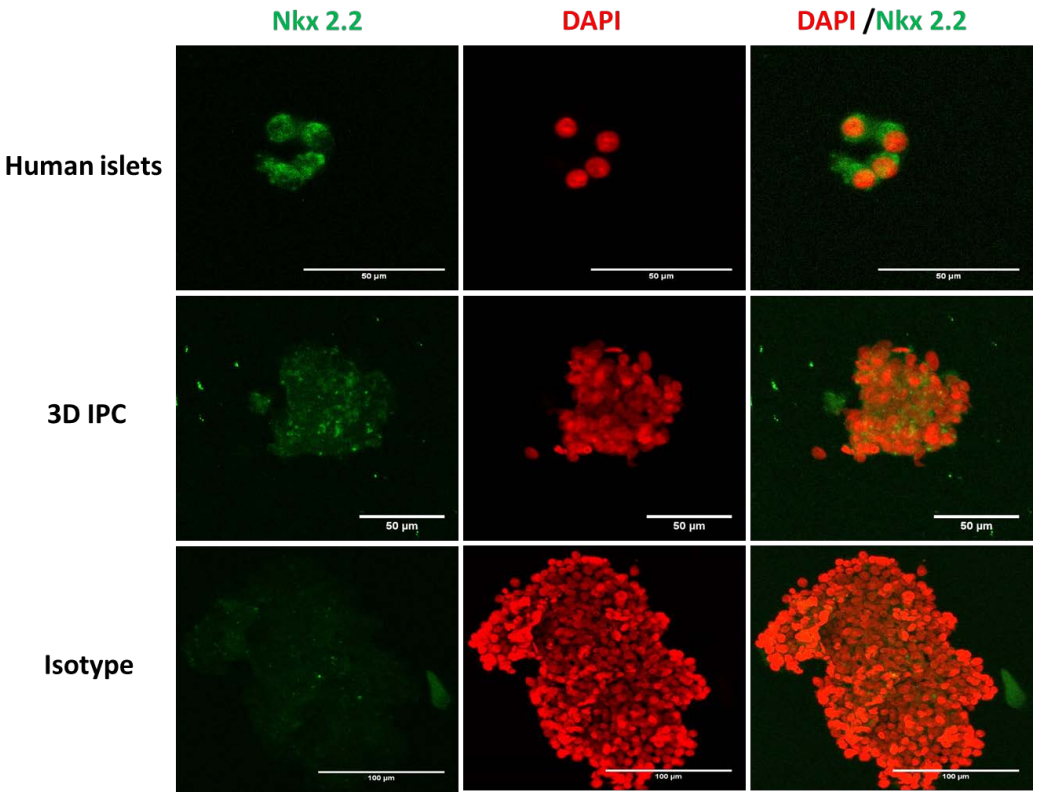


Figure 9. Analysis of Nkx2.2 expression in 3D differentiated IPCs.
Nkx 2.2 is a transcription factor critical for β-cell differentiation and specification. Its presence indicates the cells have been specified to a β-cell fate or have the potential to differentiate to β-cells.

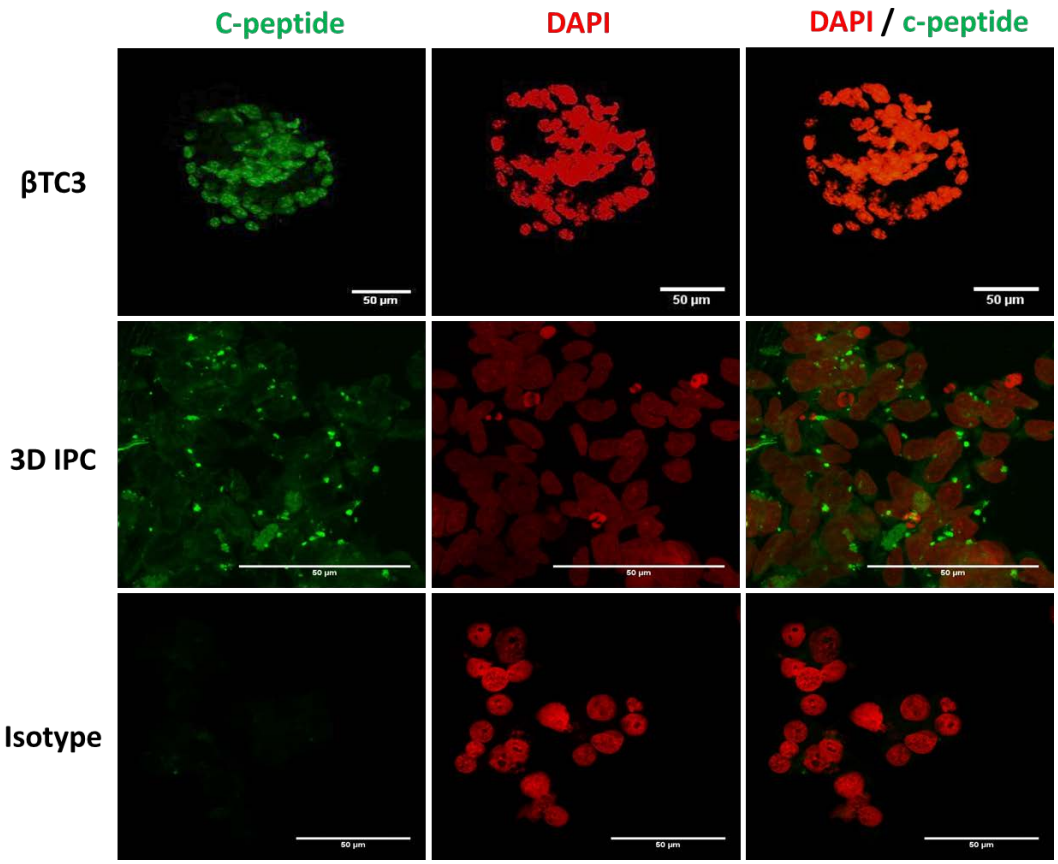


Figure 10. Analysis of c-peptide expression in 3D differentiated IPCs

C peptide is a peptide that binds the insulin chain A to chain B in the proinsulin molecule. The presence of C peptide indicates the presence of proinsulin and hence endogenous production of insulin in cells.

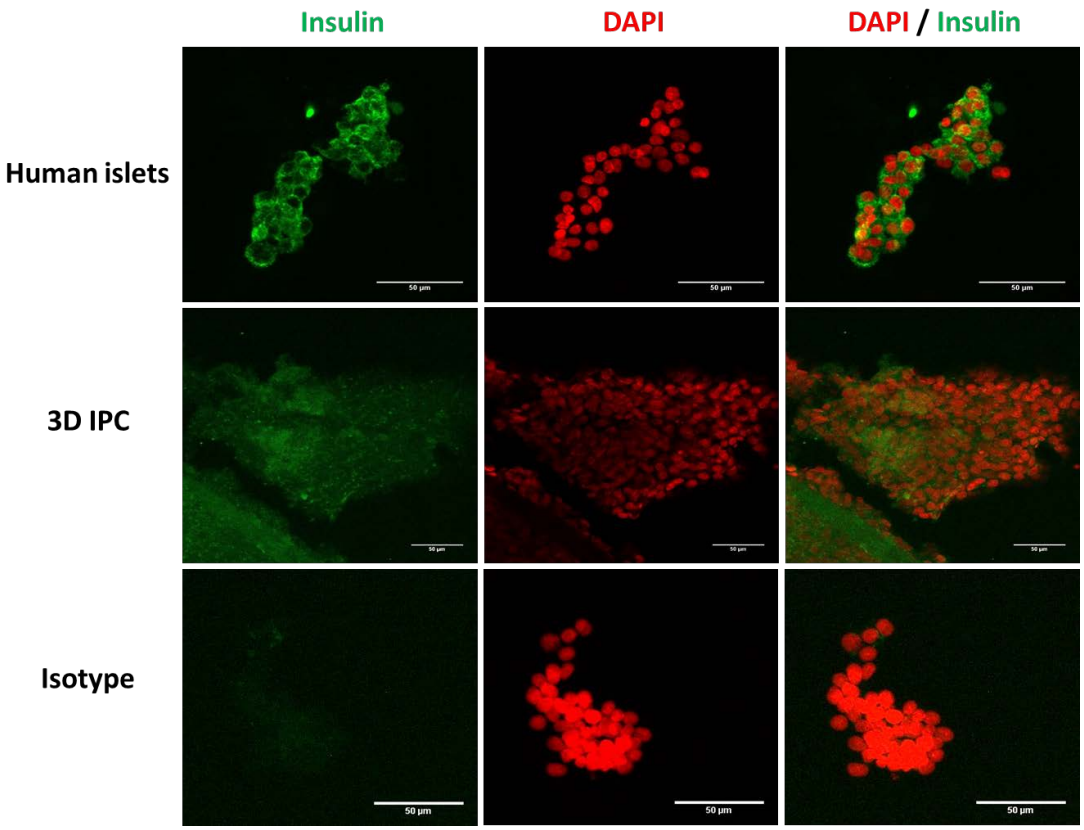


Figure 11. Analysis of insulin expression in 3D differentiated IPCs
The 3D differentiated clusters expressed insulin. This indicated successful differentiation to IPCs. The expression of both insulin and C-peptide showed that the cells were producing insulin.

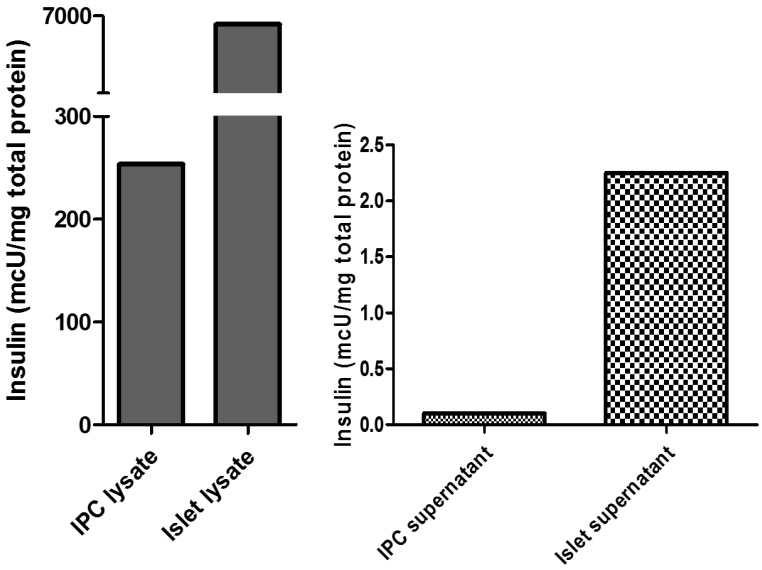


Figure 12. Analysis of insulin production in 3D differentiated IPCs in the presence of high glucose levels

The insulin produced by the 3D differentiated cells and human islets in high glucose environment was quantified using ELISA. This indicated the difference in the insulin production between human islets and the IPCs generated by 3D differentiation.

3.8 Summary

Our experiments to establish a 3D culture system proved that matrigel served as the best polymer for this purpose.

When used for the differentiation of iPS cells, matrigel successfully established a 3D culture environment and maintained the cells through the process of differentiation.

The 3D culture model also successfully differentiated the human iPS cells to fully functional IPCs. The IPCs were positive for the pancreatic transcription factors like Nkx6.1, Nkx2.2, Glut2, and for pancreatic hormones like insulin, c-peptide. They were also able to produce insulin when exposed to a high glucose environment. This proved that a more physiological system like the 3D culture model has the potential to generate functional IPCs.

CHAPTER 4. DISCUSSION

Matrigel is a protein mixture of secretions by mouse sarcoma cells [85]. It is a biopolymer that contains a mixture of many growth factors including bFGF, PDGF, EGF, IGF-1, NGF, TGF-β and tissue plasminogen activator [86, 87]. The biological constituents of matrigel help with cell survival and proliferation. The mixture of growth factors are known to help with maintenance of stem cell pluripotency when used in very diluted amounts and are known to promote stem cell proliferation and differentiation when used undiluted.

Matrigel polymerizes rapidly at room temperature and depolymerizes at 4 degrees Celsius. This property of matrigel was used to establish a cell culture system that supports a three dimensional culture. A 1:1 mixture of matrigel and cell culture medium (without serum) polymerized on cell culture dishes to form a three dimensional environment. The medium used was RPMI 1640, which provided, L-Glutamine, the sugars and the vitamins that are essential for the growth and proliferation of cells. The polymerization of matrigel with the medium ensured the formation of a scaffold with access to the growth factors and the essential nutrients from the medium. Incomplete RPMI was used to form the three dimensional culture systems to prevent the proteases in culture serum from degrading the protein mixture.

In the presence of the polymerized scaffold, the cells clustered and formed the more physiologically similar stem cell cultures than the conventional 2D cultures. The clustering of the cells was initiated naturally by the cells in an attempt to form colonies and support the maintenance of signaling crosstalk. We speculated that the physiologically similar structure thus formed will aid the process of differentiation.

To establish 3D cultures using the βTC3 cells, around 2.5 million cells were seeded to form the cell clusters in the required density. The same number of cells was seeded when this model was implemented for human iPS cell differentiation. The density of

clusters could be varied by increasing the cell number used and by decreasing the surface area used to form the 3D cell culture environment. 2.5 million cells in a 24 well plate was the optimum cell number used for differentiation. This provided the right cell cluster density while providing the space required for cell proliferation through the process of differentiation process.

Pilot experiments when implementing 3D cultures for differentiation included comparison of 2D and 3D differentiation. The 2D cultures were established using a 1:30 dilution of matrigel in RPMI 1640 medium. At room temperature, this mixture polymerized to form a layer on the cell culture dish. Here, the cells did not cluster to form three dimensional spheroids, but adhered to the surface of the culture dish as seen in the conventional 2D cultures.

2D and 3D differentiation were compared at the first stage of conversion to endodermal cells. The low percentage of cells expressing CXCR4 in 2D cultures was an indication of insufficient stimulus to differentiate. After the completion of differentiation, the 2D and 3D cells were analyzed by immunofluorescence and confocal microscopy to investigate the expression of the pancreatic β-cell transcription factors Nkx6.1 and C-peptide. The absence of Nkx6.1 and C-peptide expression in the 2D differentiated cells and their strong expression in the 3D differentiated cells showed that the 2D differentiation was not an optimal method for generating IPCs. This method was therefore discontinued while methods to optimize 3D differentiation were pursued.

The use of Wnt3a in the first step of the differentiation was aimed at enhancing the 3D differentiation process. The use of Wnt3a only for 24 hours with subsequent use of high concentration of Activin A has been reported to lead to endodermal differentiation with the capacity to differentiate into pancreatic lineage. Some reports speculate that Wnt3a induces a mesendodermal state. The cells from this state have the potential to differentiate into both types of germ cells—the mesodermal and endodermal cells. High concentration of Activin A is then speculated to activate the Nodal pathway to induce high percentage conversion to

endodermal cells while suppressing the differentiation to mesodermal cells [88] [89]. Hence, we decided to use Wnt3a for 24 hours before exposing the cells to Activin A only.

The process of differentiation is marked with the expression/downregulation of certain transcription factors. The differentiation of iPS cells is marked by loss of Nanog and Oct4 expression. Although the expression of Oct4 and Nanog was not analyzed for every differentiation, the cells were sorted for CD326 to ensure pluripotency.

The cells then begin to express CXCR4, marking their endodermal cell lineage. Expression of CXCR4 was quantified to make sure the cells were differentiating. Emphasis was on attaining high percentage conversion to endodermal cells. Although only 40% conversion to endodermal cells was observed, reports of extended exposure to high concentration Activin A leading to loss of pancreatic differentiation capability led us to pursue methods to obtain a higher percentage conversion by manipulating the signaling pathways [89]. A recent paper has demonstrated that use of Nodal instead of Activin A produces mature endodermal cells at a much faster and efficient manner with greater capability to differentiate into pancreatic cells [33]. Future efforts will be aimed at generating higher percentage of endodermal cells so as to ensure better efficiency of differentiation into IPC.

The proof of pancreatic cell fate is the expression of Pdx-1. Since all pancreatic cells express Pdx-1, the distinguishing characteristic of IPCs from the other pancreatic cell fate is the expression of Nkx6.1. Nkx6.1 is a transcription factor required for β-cell maintenance and proliferation. Hence, its expression demonstrates differentiation to β-cells. The presence of Nkx2.2 is important for β-cell specification and differentiation. While the presence of both Nkx6.1 and Nkx2.2 indicates the transcription machinery similar to that of the β-cells, proof of insulin production is required to demonstrate the generation of IPCs. Insulin production from the 3D differentiated IPCs was also compared to the islets after exposing them to a high glucose environment. The level of insulin

detected in the cell lysate of the 3D differentiated IPCs was one fifth that of the islets. This supported the conclusion of low percentage of IPCs obtained from the differentiation.

Dithizone stains the granules in cells that have high zinc content. The insulin vesicles produced by the β-cells are known to have high zinc content. Thus, exposure of β-cells to dithizone stains them red. Although, positive stain in the dithizone assay is indicative of presence of zinc granules, it does not definitively indicate presence of insulin. This stain needs to be supplemented with a more specific assay like immunofluorescence analysis. Less numbers of clusters staining red in the dithizone assay indicated the possibility of low percentage of insulin producing cells obtained.

While it was indicated by multiple assays that the number of IPCs obtained was low, it was also indicative of definite presence of IPCs. The 3D differentiation protocol was able to produce IPCs *in vitro* unlike the 2D differentiation protocol. We speculate that the transplantation of these IPCs into diabetic mice will normalize the glucose levels much faster than the IPCs generated from 2D differentiation. With a faster reduction in glucose levels and reduced possibility of immune rejection after transplantation, this culture model has the potential to provide a long awaited cure for Type 1 Diabetes.

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