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Differentiation of mouse embryonic stem cells into insulin-producing cells: optimization to enhance the overall differentiation efficiency

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ABSTRACT

DIFFERENTIATION OF MOUSE EMBRYONIC STEM CELLS INTO INSULIN-PRODUCING CELLS: OPTIMIZATION TO ENHANCE THE OVERALL DIFFERENTIATION EFFICIENCY

by

Xiaotang Ma

Embryonic stem cells have the potential to differentiate into multiple cell types including insulin-producing cells (IPCs), which is becoming one of the promising cell sources for treating type 1 diabetes mellitus. However, in order to achieve functional stem cell-derived cells, it is important to generate more mature IPCs and to keep long-term viability post differentiation process. In this study, we varied several factors including different embryonic body culture conditions, digested cells seeding density and various coatings required for differentiation to optimize a previously established protocol to enhance the overall differentiation efficiency. Moreover, a three-dimensional *in vitro* collagen tissue culture system was prepared to provide a more physiological culture environment for stem cell-derived IPCs. Survivability of IPCs was examined under both static and flow conditions and low flow rate of 0.02 ml/min resulted in better survival of IPCs in *in vitro* three-dimensional tissues.

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by
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DIFFERENTIATION OF MOUSE EMBRYONIC STEM CELLS INTO INSULIN-PRODUCING CELLS: OPTIMIZATION TO ENHANCE THE OVERALL DIFFERENTIATION EFFICIENCY

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This thesis is dedicated to my parents Hailong and Wenli. Their love and support gave me the ability to complete this work.

致我亲爱的爸爸妈妈，
马海龙和李文利，
你们的爱和鼓励，
让我走的更远。

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CHAPTER 1

INTRODUCTION

1.1 Overview

Diabetes Mellitus (DM) is a chronic disease that affects about 382 million people in the world, and has resulted in 5.1 million deaths in 2013 [1]. It is a common endocrine disease which is characterized by hyperglycemia, and leads to a series of complications. With the development of living standards, extension of lifespan and reduction of physical activities, the death rate of diabetes has risen, making it the 8th leading cause of death in the world.

DM is classified into two main types. Type 1 diabetes mellitus (DM1) is caused by severe damage of insulin-producing β cells, and results in about 10% of DM cases. Patients with DM1 need injections of insulin for a long-period in life. Type 2 diabetes mellitus (DM2) is a heterogenous metabolic disorder, which results from insulin resistance deficiency caused by cells failing to respond to insulin properly. Approximately 80% of DM cases are DM2. One of the main treatments for DM2 involves proper diet and physical exercise.

Pancreas or islets transplantation have been considered as an effective way to treat DM1 and brittle DM2. However, the number of organ donors is limited for sufficient transplantations. Even if the transplantation is performed, the patients need to take a long-term immune suppression drugs to prevent rejection problems, which can compromise their health and well-being. Exogenetic insulin injection has been an important treatment, while it creates problems such as virus transmission and the risk of acute or chronic complications [2].

Recently, studies have shown the possibility of generating insulin-producing cells

(IPCs) from stem cells, which opens an exciting pathway towards obtaining larger number of IPCs required for transplantations [3, 4]. The use of pluripotent stem cells can ultimately overcome the shortage of the healthy donor tissue by producing β cells and reduce the use of immune suppression drugs [5].

Various types of stem cells including pancreatic stem cells [6], liver stem cells [7], mesenchymal stem cells (MSCs) [8], embryonic stem (ES) cells [9], and induced pluripotent stem cells (iPSCs) [10] have been investigated for their differentiation potential into IPCs.

In this study, mouse embryonic stem cells were used to generate IPCs, by using a previously reported protocol [11]. However, the efficiency of this protocol remained low at 5.7% of IPCs by the study previously performed in our laboratory. Therefore, to enhance the overall differentiation efficiency, we examined and varied several factors involved in the differentiation process. Moreover, the three-dimensional (3D) culture conditions to maintain the viability of stem cell-derived IPCs were also modified and the results were compared to the previous study.

Chapter 1 describes the physiology of pancreatic and islet cells, pathophysiology of diabetes and recent methods for generating pancreatic islet cells. Chapter 2 includes the design and optimization of the differentiation protocol used in this research to improve differentiation efficiency. Various factors influencing the efficiency are studied, such as time required for suspension culture of embryonic bodies, seeding cell density for induction into pancreatic lineage, and different coating conditions. Chapter 3 describes culturing ES cell-derived IPCs in a 3D tissue culture condition to provide more physiological environment for long-term *in*

vivo culture. Chapter 4 summarized the thesis with suggestions for future studies.

1.2 Pancreas and Pancreatic Cells

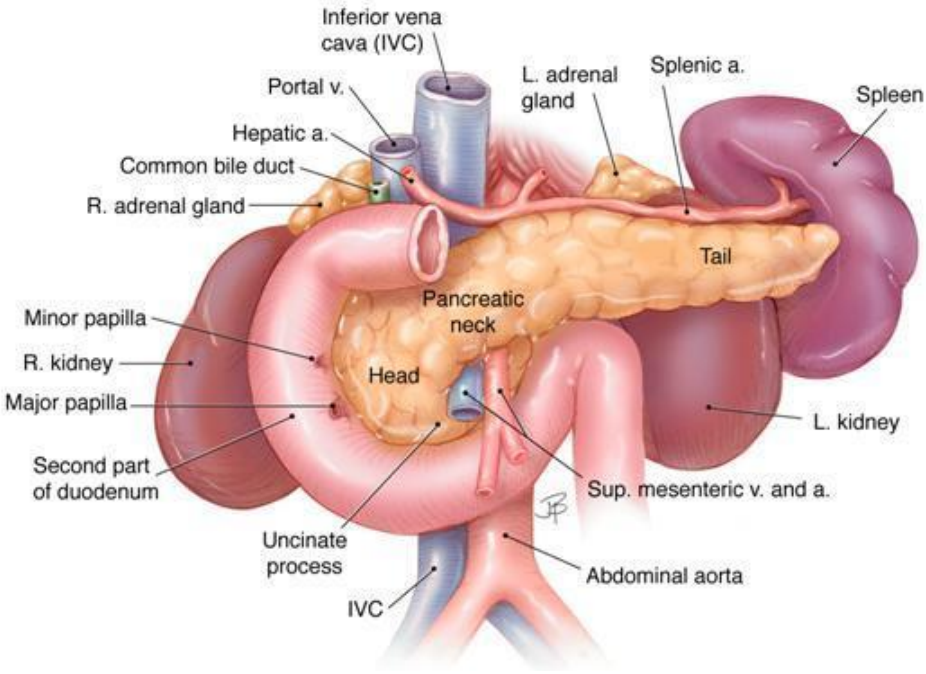

Figure 1.1 Anatomy of the pancreas. The pancreas is shown in relationship of liver, stomach and intestine.
Source: Longnecker, D.S , *et al.* 2014 [12].

The pancreas is a narrow endocrine organ which lies in the upper left abdomen, and is about 6-inch long. It is located behind the stomach with the head surrounded by the duodenum. Figure 1.1 shows the anatomical position of the pancreas, which is composed of a head, neck, body and tail. It starts with a head that rests in the concavity of the duodenum, a body that rests behind the base of the stomach and ends at a tail abutting the spleen. The pancreas is a highly visualized organ with numerous arteries offer fresh blood and nutrients. It develops from the endoderm, which is the innermost layer of three germ layers. The gut tube first

forms in the embryo, and several buds below the section of the stomach grow into the liver, the pancreas rudiments and the gallbladder [12].

The pancreas plays an important role in endocrine and external digestive functions. It is comprised of glandular tissue and a duct system throughout the organ. The glandular tissue is constituted of exocrine cells named acinar cells, which are formed into clusters called acini. Acini produce digestive enzymes and secrete them into ducts. Endocrine cells represent only a small fraction of pancreatic cells. The clusters of these cells are spattered patches called islets of Langerhans, located between acini. Islets of Langerhans carry out endocrine functions, produce important hormones and secrete into the blood.

1.2.1 Pancreas Development

Pancreas development is a complex process composed of several cascades of signaling pathways and transcription factors secreted from the surrounding tissues. The pancreatic primordium is induced into the primitive gut and posterior foregut during embryonic development. In this period, the pancreatic anlage formation is facilitated by retinoid signaling and depends on the inhibition of hedgehog signaling. The epithelial progenitors express PDX1 (lpf1), Hlxb9, Ptf1a, Nkx6-1 and Hnf6 (Onecut) and become endocrine, exocrine and ductal cells. The differentiation of pancreatic epithelium is controlled by the signals such as Fgf10 from the adjacent mesenchyme. The pro-endocrine gene Neurog3 (Ngn3) triggers the expression of some transcription factors, such as Nkx2-2, Neurod1, Nkx6-1, Pax-6, Pax-4 and Isl-1, and these transcription factors regulate the differentiation of endocrine cells. Endocrine cell specification occurs with the inhibition of Notch signaling and

expression of Ngn3 for some of the pancreatic epithelial cells. Further, the islets of Langerhans are formed by the migration of nascent endocrine cells from the epithelium to surrounding mesenchyme. Table 1.1 shows most of the transcription factors in pancreas development [13, 14].

Generation of insulin-producing cells *in vitro* culture requires thorough understanding about how beta cells are formed *in vivo*. Differentiation from stem cells into beta cells *in vitro* requires t a series of transition steps replicating organogenesis in pancreas development. Genetic manipulation is involved in the process as well as epigenetic influences such as differentiating factors *in vitro*. Table 1.2 shows the main differentiating factors required for specific cell type differentiation.

Table 1.1 Transcription Factors Involved in Pancreas Development

Transcription factors	Abbreviation	Function
NK2 transcription factor related, locus 2	Nkx2.2	Pancreatic endocrine development and differentiation into pancreatic β cells
NK6 transcription factor related, locus 6	Nkx6.1	Final differentiation of β cells
Neurogenin 3	Ngn3	Formation of pancreatic endocrine precursors, differentiation of pancreatic precursor cells towards endocrine lineages
Paired box gene 4	Pax4	Formation of β cells and δ cells, represses glucagon transcription
Paired box gene 6	Pax6	Formation of α cells, activates glucagon transcription
Neurogenic differentiation	NeuroD	Differentiation and islet growth, endocrine differentiation in pancreatic progenitors
Avian musculoaponeurotic fibrosarcoma oncogene homolog	MafA	Controls and activates insulin gene expression
Avian musculoaponeurotic fibrosarcoma oncogene homolog	MafB	Formation of α cells and β cells, activates genes involved in mature endocrine function, including those significant to glucose sensing, vesicle maturation, Ca^{2+} signaling, and insulin secretion
Pancreatic and duodenal homeobox	Pdx-1/Ipf-1 (in human)	Early pancreatic development, formation of β cells and δ cells, formation of exocrine tissue, important activator of insulin
Islet-1	Isl-1	Early endocrine cell differentiation
Forkhead box A3	FoxA3	Endodermal marker, differentiation of pancreas

Source: Pokrywczynska, M., *et al.*, 2013[6].

Table 1.2 Differentiation Factors for Specific Cell Types

Cell type	Differentiation factors
Embryonic stem cells	B27, nicotinamide, insulin, transferin, selenium, FN, exendin-4, LY294002, activin-A, LAM, RA, GLP-1, Nodal, IDE1, IDE2, FGF-10, cyclopamine, extendin-4, DAPT
BM-MSCs	Activin-A, conophyline, BTC-δ4, BTC, nicotinamide, L-glutamine, FN, LAM, HGF, extendin-4
Umbilical cord blood derived MSCs	Glucose, RA, nicotinamide, epidermal growth factor, exendin-4, B27
Adipose tissue-derived MSCs	Glucose, nicotinamide, activin-A, exendin-4, HGF, pentagastrin, BTC
Progenitor cells	Glucose, poly-L-ornithine, FN, apo-transferin, L-glutamine, RA, nicotinamide, insulin-like growth factor, matrigel, FGFR2IIIb, HGF, EGF

Source: Pokrywczynska, M., *et al.*, 2013[6].

1.2.2 Cell Types in Islets

The cells comprising islets are derived from both endodermal and neuroectodermal precursor cells. There are four main types of cells, classified by their secretion of hormones. Approximately 75% of islet cells are insulin-producing beta cells, and the other cells are alpha, delta, and pancreatic polyeptide (PP) cells. Alpha cells secrete glucagon, which maintains basic blood glucose concentration by regulating hepatic glucose secretion. Insulin-producing beta cells are clustered centrally in the islets and secrete insulin, decreasing the glucose level. Delta cells and PP cells secrete somatostatin and pancreatic polyeptide respectively, and are located on the periphery of the islets [15].

Beta cells are essential in glucose homeostasis of the body by expressing a set of specific proteins. As the most frequent islet cell type, beta cells detect increased blood glucose levels and secrete insulin, which starts with glucose entering by the Glut2 receptor in the beta cells. Glucose then becomes the glucose-6-phosephate by glucokinase, a main glucose sensor. Cell membrane depolarization results from closure of ATP-sensitive potassium channels on the cell surface, which leads to Ca^{2+} channels opening and boosts the influx of Ca^{2+} inside β cells. The increase of Ca^{2+} levels in the cytoplasm triggers the exocytosis of insulin. The secreted insulin facilitates glucose uptake in three ways: insulin signals the insulin-sensitive peripheral tissue to increase their uptake of glucose, and then insulin increases glycogenesis in the liver, and inhibits glucagon secretion to stop the liver from producing glucose. Insulin also acts to stimulate fat synthesis, promote triglyceride storage in fat cells, cells growth and protein synthesis in the liver and muscle. Beta cells also

secret amylin as a co-secret hormone with insulin, to modify carbohydrate metabolism in skeletal muscle and the liver. Amylin circulates glucose concentration in two mechanisms. It suppresses glucagon secretion after meal, and glucagon-stimulated hepatic glucose output is decreased. In addition, the rate of gastric emptying is weakened by amylin, which slows the delivery rate of absorption from stomach to the small intestine [16].

1.3 Diabetes

Diabetes mellitus is described as a chronic metabolic disease, resulting from failure of insulin secretion. It is characterized by disturbances of carbohydrate, protein and fat metabolism, leading to the symptoms like frequent urination, increased hunger and increased thirst. The acute complications include kidney failure, foot ulcers, cardiovascular disease and damage to the eyes. There are two main types of diabetes, type 1 is due to body failing to produce sufficient insulin and type 2 is called “non- insulin-dependent diabetes mellitus” as cells do not respond to insulin properly. According to etiology, type 1 diabetes is presented in people whose endogenous insulin secretory capacity is damaged. It is classified as type 1A and type 1B. Type 1A results from insulin deficiency, it is caused by immunological destruction of pancreas beta cells. Islet cell antibody (ICA), anti-glutamicacid decarboxylate (anti-GAD), IA-2 or insulin antibodies are present in almost 85-90% of patients with type 1A. These antibodies identify the autoimmune process of ß cells destruction. Type 1B has no evidence of autoimmunity. This disease is more widespread in African and Asian origin [1].

The current treatments for type 1 diabetes include pancreas or islets transplantation.

Unfortunately, there is a shortage of healthy donor organs and tissues. Even though the organ transplantation is performed, the patients need continuous immunosuppression to prevent graft rejection and recurring autoimmune attacks on the islet. It is shown that about 60% of islets are damaged by inflammatory reactions. The release of inflammatory cytokines, such as tumor necrosis factor-α (TNF-α)，interleukin-1 (IL-1) and interferon-γ (IFN-γ) leads to the destruction of the islet graft by the hypoxia condition [2].

Type 2 diabetes mellitus (T2DM) is the most common form of diabetes mellitus, and is often characterized by insulin resistance. It increases the risk of developing macrovascular and microvascular complications. In many cases, approximately 50% of patients are not diagnosed because of the underestimation of the disease. T2DM is often associated with obesity, decreased physical activity and heredity. Women with gestational diabetes are more likely to have T2DM, while people suffering with hypertension and dyslipidemia are also at risk of developing T2DM. Genetic disposition and familial factors are the major causes. T2DM is usually controlled by dietary therapy, hypoglycaemic agents and exercise. The main method to treat T2DM is insulin injections, to keep normal blood glucose level normal. However, insulin injection results in poorer glycaemic control, and has more side effects as the dose increases. Recently, it has been demonstrated that islet and cell transplantation is an effective method for treatment of T2DM [5].

1.4 Stem cells Derived Pancreatic Cells

1.4.1 Embryonic Stem Cells

It has been demonstrated that ES cells from mouse and human have the capacity to differentiate into different cell types such as blood, blood vessels, heart, muscle as well as insulin-producing cells (IPCs). While it is becoming a potential tool for pancreas development and diabetes treatment, it is a yet a challenge to derive fully functional IPCs from pluripotent stem cells [5].

A number of multi-step protocols have been used to differentiate ES cells into pancreatic lineage. The derivation of IPCs from mouse embryonic (MES) cells into IPCs was first described by Soria *et al.* [17]. Assady *et al.* first showed human embryonic stem (hES) cells differentiated into IPCs [18]. Lumelsky *et al.* found that chemical factors have an influence of efficiency of differentiation. The serum free ITSFn (Insulin, Transferrin, Selenium and Fibronectin) medium and fibroblast growth factor (bFGF) were used in some protocols [19]. P13 kinase inhibitors were also involved in some of these studies, but it was demonstrated to trigger apoptotic pathways and facilitate neuronal differentiation [20, 21]. Further, these protocols failed to control insulin secretion from the derived cells, showing that they may not be fully mature or functional *in vitro* conditions [20-22]. Cells were unable to up-regulate their insulin production in response to varying glucose concentrations. The ES cells derived IPCs which were transplanted into mice showed the ability to correct glucose levels, but faded in time, indicating the de-differentiation or cell death. The teratoma formation may also be a problem for the IPCs derived by embryoid methods with mixture of

undifferentiated ES cells [23, 24].

A five-stage protocol was successfully established to differentiate hES cells into IPCs, which mimics the process of pancreatic organogenesis [25]. It involves phases of inducing a definitive endoderm, primitive gut tube, posterior foregut, pancreatic endoderm and cells expressing endocrine hormones. The shortcoming of this protocol was that there were more than one hormones expressed by most of the cells, meaning that the cells were not totally mature. Meanwhile, the C-peptide released in response to a glucose challenge was marginal and the yield of IPCs was low as ~7% [25]. The Wnt and transforming growth factor TGF-β are demonstrated to be essential as two signaling pathways for the formation of definitive endoderm. It has been found that differentiation of human ES cells into definitive endoderm is achieved when cultured in the media with activin-A and Wnt3a. This process was validated by the expression of endodermal markers, such as Sox17, Cxcr4, Gata4, FoxA2 and Cerberus [19, 26]. Also, retinoic acid (RA) is a strong teratogen and it is shown to induce ES cells into different cell types. Based on the time and concentration of RA, it can induce ES cells into neuronal, cardiac and smooth muscle cells [23].

A new protocol was developed based on the five-stage protocol, which consists of only four stages with 12 days of differentiation period [26]. During the first stage, human ES cells were cultured in the media with activin-A and Wnt3a for a single day, then changed by the media with only activin-A for 2 days. In the next stage, cyclopamine was eliminated and keratinocyte growth factor was substituted for FGF10. In the third stage of posterior endoderm formation, the medium was changed with the one supplemented B27, Noggin,

all-trans RA and KAAD-cyclopamine, instead of Fibroblast growth factor (FGF). In final stage, cells were treated without all the factors except B27. The immature pancreatic endoderm was generated and finally grafted into immune deficient mice for maturation step *in vivo*. Human insulin and C-peptide were detected at the level similar to the mice transplanted with about 3000 human islets after stimulation of glucose. The implanted cells upon engraftment appeared to have become more functional cells.

Using nuclear reprogramming method is another interesting way to induce IPCs. Nuclear reprogramming has been used to facilitate the expression of essential transcription factors and guide ES cells to differentiate into IPCs. Shiroi *et al.* studied the capacity of NKX2.2 gene to transfect murine ES cells to differentiate into IPCs *in vitro* [27]. It is known that Nkx2.2 is NK-homeodomain gene expressed in pancreatic progenitor cells, which is needed for pancreatic endocrine development and inducing beta cells. In addition, it was demonstrated that the overexpression of PDX1 and Ngn3 can enhance differentiation for pancreatic cells. PDX1 works in early pancreatic development while Ngn3 is involved in the endocrine precursor formation of pancreatic. Overexpression of PDX1 leads to up-regulate of insulin or other pancreatic genes. This study indicates genetic engineering to be an important tool in differentiation of IPCs [27]. However, if transcription factors are forced to express at non-physiological level on inappropriate time points, it will results in the generation of other cell types [28].

1.4.2 Mesenchymal Stem Cells

Mesenchymal stem cells (MSCs) are self-renewing and multipotent cells which are located in

bone marrow, adipose tissue, umbilical cord blood, or skeletal muscle. MSCs have the potential to be differentiated into endodermal and ectodermal lineages [29], and it has been shown that MSCs can differentiate into insulin producing cells (IPCs) [30]. Chen *et al.* demonstrated that MSCs can be differentiated into insulin-producing islet-like cells, which positively controlled blood glucose level in diabetic rats [8]. Hisanaga *et al.* used a simple method to generate IPCs from murine bone marrow MSCs [31]. The MSCs were cultured in the media supplemented with activin-A, betacellulin-d4 and conophylline. Differentiated cells secreted insulin in response of glucose stimulation *in vitro*. These cells showed the capacity to reduce glucose level when they were grafted into diabetic mice.

A three-stage protocol was reported by Sun *et al.* [32]. Firstly, serum free medium with high glucose supplemented with 2-mercaptoethanol was used. Cells treated with b-FGF, EGF, B27 and NEAA express nestin, PDX1, Ngn3, Pax4, insulin and glucagon. In the next stage, b-cellulin, activin A, B27 and nicotinamide were added to enhance the expression of PDX1 and endocrine hormones of insulin and glucagon. Further, another study used adipose tissue derived MSCs to generate IPCs. The cells expressed transcription factors in induction into a pancreatic endocrine phenotype, including Isl-1, IPF-1, and Ngn3.

1.4.3 Induced Pluripotent Stem (IPS) Cells

IPS cells generated from human skin fibroblast have been demonstrated to be differentiated into insulin producing islet like clusters [33, 34]. The clusters express C-peptide and glucagon by the four-stage protocol. However, the level of insulin secreted by iPS cells derived clusters was lower in response of high glucose, compared with ES cells derived

clusters. Another study by Zhang *et al.* demonstrated a high efficient step wise method [34]. Firstly, the cells were treated by the medium supplemented with basal serum albumin (BSA), N2, wortmannin, activin-A and B27 for 4 days. In the second stage, the medium supplemented with BSA, B27, FGF-7, ITS and NOGGIN was used for another 4 days. In the next stage, the medium was changed to high glucose with ITS, BSA, N2 and EGF for 5 days. In the last stage, the medium supplemented with BMP4, ITS, bFGF, nicotinamide and exendin-4 was used. IPCs generated by the protocol expressed most of transcription factors and markers, including PDX1, MAFA, NEUROD, Isl-1, Glut2 and Nkx6.1. In addition, insulin and C-peptide secretion in response to glucose stimuli was similar to that of adult human islets.

In a current study, Pellegrini *et al.* demonstrated a method for promoting the efficiency of differentiation from iPSCs into insulin-producing cells [35]. Human iPSCs were derived from both fetal and adult fibroblasts, and then iPSCs were differentiated into pancreas committed cells and transplanted into immune deficient mice. After the differentiation, the production of insulin mRNA was increased obviously and 5 ± 2.9 % of total cell population became insulin positive cells. Terminal differentiated cells produced C-peptide in both basal and stimulated conditions, as well as secreting C-peptide in response to glucose stimulus *in vivo* when transplanted into mice.

1.4.4 Progenitor Cells

It was indicated that human neural stem cells also have a broad potential to be differentiated into various cell types including insulin-producing cells [6]. Gao *et al.* reported that the

progenitor cells were generated from human pancreatic ducts during monolayer expansion [36]. The cells were proliferated from cytokeratin 19 (CK19)-positive ductal epithelial cells to nestin-positive fibroblastoid cells. Serum-free medium was used for the differentiation into endocrine cells. Matrigel was used as an essential requirement, while nicotinamide showed potentiating effect. The cells of generated islets buds had response to glucose as efficient as native islets. Furthermore, Feng *et al.* demonstrated that liver progenitor cells could be transdifferentiated into IPCs under specific condition [7]. Moreover, these cells can reduce the non-fasting blood glucose level when transplanted into the mice with alloxan-induced diabetic.

Despite the advancements in stem cell research, a numbers of challenges remain before insulin-producing cell therapy is applied for diabetes treatment. One of the main problems is achieving IPCs that are full mature *in vitro*. Differentiated cells express multiple hormones secret very little insulin and have little response to glucose challenge. It is supposed that the failure of beta cells maturation may be caused by the lack of mesenchyma-epithelium interactions, which occurs through pancreatic embryogenesis. Other problem is that the IPCs grafts do not survive long-term *in vivo and vitro* due to lack of supporting vasculature. Immature IPCs could also dedifferentiate into other cell types. More importantly, large scale of fully functional IPCs are required for clinical application. Further, studies are needed to fully assess the capacity of IPCs for the treatment of diabetes.

1.5 Three-Dimensional System for Pancreatic Islets

Islets cultured in two-dimensional (2D) condition not only exhibit low viability but also transdifferentiation into exocrine cell. 2D tissue surfaces do not provide the cell-matric interactions which are present *in vivo* [37]. Recent studies have demonstrated islet-matrix interactions by culturing beta cells or IPCs on 2D ECM coated tissue surface [38-40]. ECM-derived substrates such as cell-secreted matrices and individual purified ECM proteins improve the survivability of beta cells [41]. It was demonstrated that matrix secreted by endothelial cells from bovine corneal facilitates islet survival, induced adult β cells proliferation and insulin secretion. Rat β cells cultured on matrix produced by rat bladder carcinoma line showed that the integrinα6β1 interacted with laminin and affected β cells function [42]. The purified individual ECM proteins showed better islet survival and function.

Collagen type I, IV and laminin are components of the basement membrane, showing higher insulin release. Collagen type I coated surfaces as well as those treated with fibronectin contributed to less apoptosis and higher insulin secretion from islets [43]. It is also shown that materials such as collagen and PEG hydrogel with matrix proteins entrapped may also be suitable as 3D scaffolds for pancreatic tissue engineering [44, 45]. Zhang *et al.* examined the effects of collagen matrix C and fibroblast-populated collagen matrix on amyloid formation, viability, and function of isolated islets and showed that 3D scaffolds improve viability and function of human islets *in vitro* [46]. Moreover, Matrigel is used for encapsulation of islets to prevent the immune rejection from host animals when implanted *in*

vivo.

To successfully transplant microencapsulated islets, the crucial point is to solve the problems such as deficient nutrient diffusion, microcapsule biocompatibility and local fibrosis [46]. Microfluidics systems have been explored to improve the islet survivability *in vitro* culture by perfusing islets and performing a quick quality assessment after donor isolation. It was shown that the mouse islets cultured in a microfluidic devices resulted in twice the endothelial cell density and have a connection length of capillaries compared with the islets cultured by previous methods. Microfluidic devices allow application of intercellular flow on islets to better deliver of nutrients and gas. However, there are still some limitations such as damage of islet by potential shear and mechanical stresses, low throughput, the difficulty of monitoring the islets real time and the complexity of microfluidic devices [47, 48].

1.6 Significance

This thesis describes strategies to enhance the overall efficiency of obtaining insulin-producing cells (IPCs) differentiated from mouse embryonic stem (ES) cells, by examining the time duration of suspension culture of embryonic bodies (EBs), cell seeding density and different collagen coating conditions. The thesis also includes the pancreatic tissue engineering approach to generate the three-dimensional (3D) collagen tissue for long-term culture *in vitro*.

To begin the thesis, Chapter 1 describes the anatomy and development of the pancreas, diabetes mellitus, and differentiation methods from stem cells into

insulin-producing cells, as well as potential application of pancreatic tissue engineering for islet transplantation. While previous studies have proven the generation of insulin-producing cells (IPCs) from embryonic stem cells by different differentiation methods [24], current challenges in deriving insulin-producing cells include low efficiency and difficulty in maintaining viability and functional phenotype. Thus, Chapter 2 describes strategies to optimize a preexisting three-stage protocol, which includes the formation of EBs, the spontaneous differentiation from EBs into progenitor cells of ectodermal, mesodermal and endodermal lineages, and the induction of differentiation of early progenitors into pancreatic lineage [11].

The influence of culture duration of suspension culture for EBs was first examined. This is the stage of spontaneous differentiation of ES cells into progenitor cells of ectodermal, mesodermal and endodermal lineages. While the existing protocol reports 3 days of suspension culture of EBs, previous work done in our laboratory following the protocol used 2 days of suspension culture. Only 2 days were used for suspension culture as it was found that EBs attach to the bottom in suspension culture after 2 days. However, in this study, this was revisited to examine whether the durations of suspension culture majorly affects the overall differentiated efficiency. The results from 2 and 3 day EB suspension culture were compared.

Secondly, it was initially observed that a higher cell density results in higher number of islet-like clusters. Thus higher differentiation efficiency was expected with higher cell seeding density as previous study also demonstrated that higher initial cell seeding density

enhances pancreatic endocrine formation. Thus, initial cell seeding density was doubled in this study to examine the effects of digested cell seeding density on the differentiation efficiency.

Lastly, various coating methods were explored during the last stage of differentiation. Instead of diluted collagen coating, thin collagen gels as well as thin collagen gels with endothelial cells were used in this study. As islet specific endothelial cells were recently identified in the vicinity of pancreatic islet cells in the differentiation culture [49], it was hypothesized that ECs are important in the development of pancreatic organogenesis and thus may enhance the differentiation of pancreatic progenitor cells into insulin producing cells.

Chapter 3 describes a 3D tissue with IPCs in the collagen type I gels. IPC clusters obtained from the differentiation were much larger in size compared to native islets as they consist of IPCs as well as other cell types. By further isolation and dissociation method, IPCs were purified and the viability of IPCs in 3D tissues were enhanced. Moreover, to increase the viability of IPCs in collagen gels, perfusion flow bioreactor was utilized to apply perfusion flow through the tissue. The survivability of IPC clusters in flow condition was compared to that of static culture. In addition, since previous work using a flow rate of 0.5 ml/min resulted in higher cell death in 3D tissues, a lower flow rate of 0.02 ml/min was used in this study to examine whether the cell death is due to higher shear stress caused by the flow. A summary of the entire work and further directions is discussed in Chapter 4. Appendix sections provide the details of the protocol and medium recipes.

CHAPTER 2

DIFFERENTIATION FROM MOUSE STEM CELLS INTO INSULIN-PRODUCING CELLS

2.1 Introduction

Pancreas or islet transplantation has been one of the therapy options for treating Type 1 diabetes patients. However, this has been limited by lack of donor tissues, immune rejection and associated side effects. Recently, a number of studies have demonstrated the possibility of generating insulin-producing cells (IPCs) from embryonic and adult stem cells that can be used for islet/cell transplantation [19, 50].

So far, studies have used differentiation factors, genetic methods and various approaches to differentiate pluripotent stem cells into insulin-producing beta cells. Some of the differentiation factors include B27, transferrin, selenium, and activin-A. Lumelsky *et al.* used a serum free medium supplemented with ITSFn (insulin, transferrin, selenium and fibronectin) to initially support the proliferation of nestin-positive cell to differentiate mouse ES cells into IPCs [19]. It has been demonstrated that insulin-producing beta cells can be derived from nestin-positive cells [51, 52]. However, this protocol resulted in activating apoptotic pathways and facilitating the differentiation into neuronal lineage at the same time. During embryogenesis, parts of pancreatic and neuroectodermal differentiation are guided by the same factors, including Ngn3, Isl-1 and Pax6. It was found that the selection of nestin-positive cells was not required for successful pancreatic progenitors when it was used for ES cell-derived cells [52].

Other protocols applied retinoic acid (RA), which is a strong teratogen, to induce the differentiation of ES cells into various cell types. It has been reported that cardiac, neuronal and smooth muscle cells can successfully be derived from ES cells using the protocols with RA [53-55]. The application of RA was demonstrated to support the differentiation of ES cells into IPCs, while both pancreatic and neuronal cells were generated at the same time [56]. The generation and selection of endoderm progenitors through ES cells differentiation process could be another strategy to increase pancreatic differentiation based on the spontaneous differentiation. Activin-A is a member of the transforming growth factor-β (TGF-β) superfamily, and it has been shown to guide endodermal differentiation in embryonic bodies (EBs) in serum-free conditions. Activin-A promotes both pancreatic differentiation and neuronal differentiation at the same time [11]. The treatment of activin-A and RA resulted in the increased number of neuronal cells during differentiation of ES cells into IPCs. Thus, the application of RA and activin-A was not used in this study.

During differentiation process, the spontaneous formation of embryoid bodies (EBs) into ecto-, meso- and endodermal lineages is essential for the production of IPCs. Formation process includes suspension culture and plating of EBs culture, although different protocols require different EBs culture conditions. Lumelsky *et al.* cultured EBs from mouse ES cells in suspension culture for 4 days, while Schroeder *et al.* and McKiernan *et al.* used 3 days of suspension culture [11, 19, 57]. As the schematic in Figure 2.1 demonstrates, our studies are based on previously reported protocol with a few variations in an attempt to optimize the process. The differentiation process includes formation of EBs, spontaneous differentiation

from EBs into ectodermal, mesodermal and endodermal lineages and differentiation into insulin-producing cells. A basic culture medium was used for the differentiation into multilineage progenitors, and medium supplemented with insulin, nicotinamide and laminin was used for the induction into pancreatic lineage.

While the original protocol used 3 days of suspension culture, only 2 days of suspension culture was used in a previous study conducted in our laboratory as the EBs started to attach to the petri dish after 2 days culture. In this study, however, efforts were made to keep the suspension culture for 3 days to investigate the influence of time in suspension culture on the overall differentiation efficiency. Results from 2 days and 3 days suspension culture were compared after 33 days of differentiation process.

In addition, during the last of differentiation, EBs that were plated on dishes were digested and seeded on the 10 $\mu g/cm^2$ collagen coated dishes to induce into pancreatic lineage. During this stage, cell seeding density may be an important factor that can affect the efficiency of differentiation. Gage *et al.* demonstrated that the higher initial cell seeding density enhances pancreatic endocrine formation as well as growth of pancreatic progenitor cell populations [58]. Thus, to examine the effects of cell density, the cell seeding density was doubled in this study, and was compared with our previous work which used the same seeding density as the previously reported protocol.

As the last stage is essential for the induction of pancreatic differentiation, different collagen coating conditions were also examined. Recently, it was found that islet-specific endothelial cells are present in the differentiation culture [49]. MES cell-derived endothelial

cells not only expressed normal endothelial markers, but also islet endothelial cells (IECs) specific markers. It was also found that pancreatic β cells are not capable to form a basement membrane without IECs, whereas endothelial cells can secret proteins to promote IPCs proliferation and to regulate insulin [59]. Moreover, islets are vascularized densely to facilitate the fast exchange of glucose and hormones, and vascular endothelial cells are essential to the pancreatic organogenesis. Therefore, it is hypothesized that the presence of endothelial cells may promote differentiation of cells into pancreatic insulin producing cells. Talavera *et al.* developed a co-culture system of mouse EBs and human microvascular endothelial cells, to investigate whether interaction of ECs with EBs enhances the differentiation of IPCs. The expression of some pancreatic markers including PDX1, proinsulin, Nkx6.1, Glut-2, Ngn3 and Ptfl1 was increased at the interface between ECs and EBs, while there was no expression of the markers at the periphery of EBs without ECs [60]. In addition, the activation of the bone morphogenetic protein (BMP) signaling pathway was found by the EB-EC interaction, BMP is an inductive factor for the differentiation of IPCs [60]. Thus we co-cultured IECs with digested EBs in the differentiation dish, by embedding IECs in the thin collagen gel as a specific coating as well as setting up the gel coating without ECs to compare with the normal collagen coating. The differentiation efficiency was evaluated using Dithizone (DTZ) staining, western blotting and insulin ELISA.

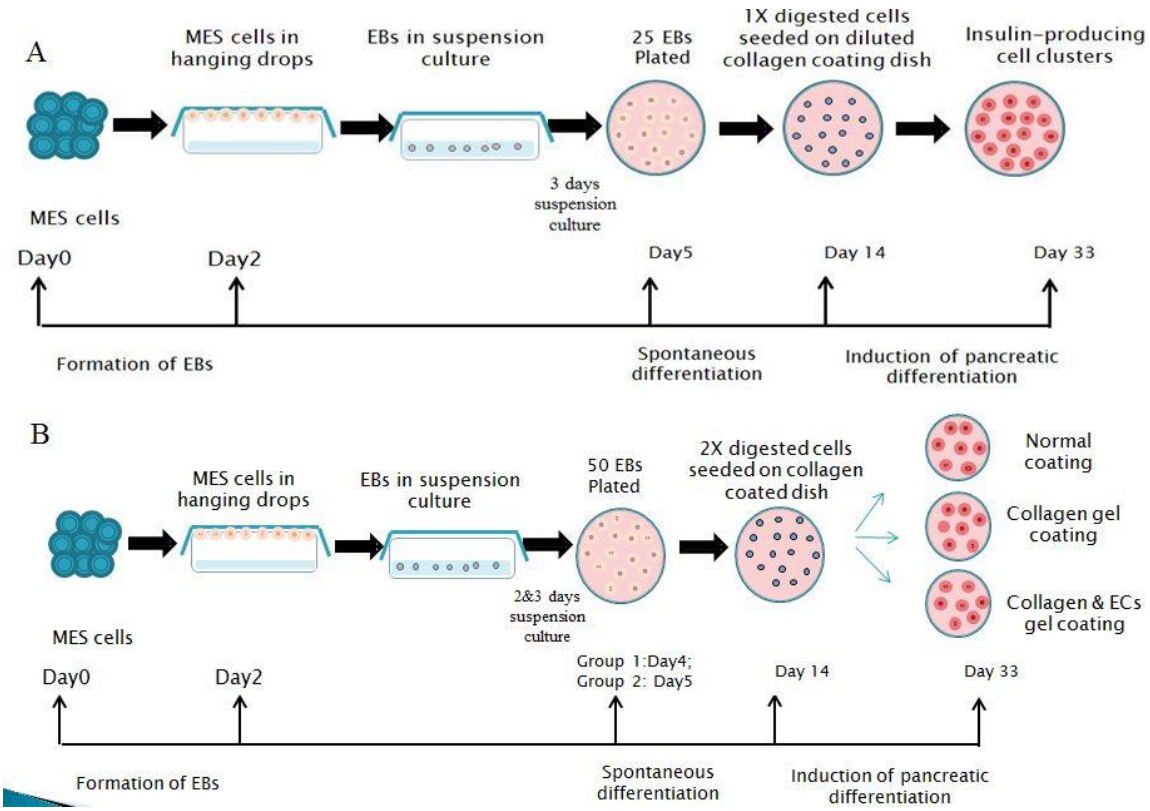

Figure 2.1 A schematic of the IPC differentiation protocol. (A) is the previous established protocol [11], (B) is modified version of (A) which was used in this study.

2.2 Material and Methods

2.2.1 Mouse Embryonic Stem (MES) Cells Culture

The MES cells (a generous gift from Dr. Qyang's lab at Yale University) were cultured on a feeder layer of mouse embryonic fibroblasts (MEF) at 37 ℃ with 5% CO_2. MES medium consisted of high glucose DMEM, 15% knockout serum, 1% sodium pyruvate, 1% L-Glutamine, 1% penicillin-streptomycin and 1% non-essential amino acids, 5 ng/ml Leukemia inhibitory factor (LIF) and β-mercaptoethanol (Appendix A). As ES cells differentiation is strongly depended on the quality of ES cells, it is essential to keep the ES cells in well status. MES cells were passaged every 2-3 days by treating cells with a 1:1 ratio of 0.25% and 0.05% trypsin-EDTA solutions for 3 minutes, and then MES medium was added to

neutralize the trypsin after cells were removed from the plate. Collected cell suspension was centrifuged at 1000 RPM for 5 minutes, and fresh MES medium was used to re-suspend the cells. MEFs were removed by pre-plating for 30 minutes on bare tissue-culture plate, to get the pure MES cells. 0.25 Million cells were passaged onto freshly prepared MEF cell layers. Cells were checked and medium was exchange every day.

2.2.2 Generation of MES-derived Progenitor Cells

MES cells were used for generation of embryonic bodies (EB), to derive IPCs by the previously established protocol [11]. The hanging drop method was used for generating a homogeneous fraction of EBs, which derives reproducible cells and controls the size of EBs. To form hanging drops, 0.3 Million cells were added to10 ml differentiation medium #1, which is composed of IMDM medium, 20% FBS, 1% L-Glutamine, 1% pencillin-streptomycin and 1-thioglycerol (Refer to Appendix A for details). Each drop contained 600 ES cells in 20 μl of differentiation medium #1. Hanging drops were formed on the lids of P100 petri dishes (USA Scientific). 5 ml of 0.01% gelatin was added into P100 petri dishes to keep the drops hydrated. To give 64 EBs per plate, 8 rows of 8 drops were added totally. The hanging drops were cultured for two days before they were collected in 1 ml of differentiation medium #1 and transferred into a P60 Petri dish with 5 ml of medium for suspension culture. EBs were checked with light microscopy to ensure that they have regular round morphology. As shown in Figure 2.1, the suspension culture dishes of EBs were divided into two groups, one group with 2-day suspension culture and the other with 3-day suspension culture, to examine whether any differences were detected. The size of

growing EBs was tracked daily by taking images and calculated by image J.

After either 2 or 3 days for suspension culture, 25 EBs were transferred into a 0.1% gelatin coated P60 tissue culture dish containing 4 ml of differentiation medium #1. The dishes were incubated for at least 36 hours without disturbing, to allow proper attachment of the EBs to the dishes. Cells were cultured for additional 9 days and the medium was changed every 2-3 day. EBs are expected to differentiate into progenitor cells of ecto-, meso- and endodermal lineages.

2.2.3 Induction of Pancreatic Differentiation

The plated EBs on the dishes were digested after 9 days culture. To induce the differentiation of EBs into pancreatic lineage, different coating conditions were investigated before digestion, which provides crucial factors for pancreatic differentiation. As Figure 2.1 shows, three collagen coating conditions were applied by rat tail collagen type 1 (Lab processed). Group 1 was normal coating made by diluting collagen type 1 in the ratio of 1:70 with 0.02N acetic acid to make 10 $\mu g/cm^2$ collagen I-coated dishes, and coat for 1 hour at 37 ℃. Group 2 was collagen gel coating composed of rat tail collagen type I (BD Biosciences), 10X DMEM (Sigma Aldrich) and 10X reconstitution buffer (0.05 N NaOH with 0.16 M HEPES and 0.25 M NaHCO3), mixed on ice in the ratio of 80:10:10 to form collagen solution. The final concentration of collagen was 3 mg/ml. The pH was adjusted to 7.2-7.4 by adding 1N NaOH until the mixture appeared salmon pink in color. Islet endothelial cells (IECs) were used to be mixed with collagen gel. Group 3 was collagen & ECs gel coating by mixing ECs (Details in Appendix C) with collagen, while the density of ECs was 1 Million cells/ ml. the gel coating

was composed of rat tail collagen type I (BD Biosciences), 5X DMEM (Sigma Aldrich), ECs suspension and 10X reconstitution buffer (0.05 N NaOH with 0.16 M HEPES and 0.25 M NaHCO3), in the ratio of 70:10:10:10. The thickness of the coating gel for group 2 and 3 are 0.5 mm. 190 μl collagen gel solution was added into each well with the area of 380 mm^2. The plate was incubated for 30 minutes before adding the digested cell suspension. The cell suspension was 171.2 μl to keep the same cell seeding density in P60 dish.

To digest the plating EBs, the medium was aspirated from dishes of differentiating EB derivatives and the cultures were rinsed 2X with PBS. 0.05% trypsin and 0.25% trypsin were added in 1:1 ratio into the dishes and incubated for 3 minutes at 37 ℃. Trypsin was removed and the cells were gently peeled off by a cell scraper (USA Scientific). The trypsinized cells were mixed well by 2 ml of differentiation medium #2 (Refer to Appendix A for details) supplemented with 10% FBS. 1284 μl of the cell suspension containing single cells and cell aggregates was plated onto P60 normal coating dishes, and 171.2 μl on the 12-well plate for the three different coating conditions. Additional 3 ml and 400 μl of the differentiation medium #2 supplemented with 10% FBS was added and the dishes and 12-well plate, respectively. The digested cells were incubated overnight for cell attachment. FBS was removed the next day to keep serum free conditions, by changing medium to the differentiation medium #2. Medium was exchanged every second or third day and the dishes were incubated for 18 days.

2.2.4 Dithizone Staining

Dithizone (diphenyl thiocarbazone, DTZ) is an organic chemical which chelates zinc in beta

cells of pancreatic islets. It is demonstrated to be able to stain the insulin-producing cells crimson red, which identifies the pancreatic differentiation [61]. To test the positive clusters which have insulin-producing cells, the dishes after 18 days of differentiation were stained with DTZ solution. A DTZ stock solution was prepared with 50 mg DTZ powder (Sigma) in 5mls of dimethyl sulfoxide (DMSO, Sigma), after mixing well, make aliquots in 200 µl tubes and store -20 ℃ for future use. In vitro, 10 µl of DTZ stock solution was added to 1 ml of culture medium. The solution was filtered using a 0.2 µm nylon filter (Nalgene) and placed in the -20 ℃ freezer for 30 seconds. The cells were incubated at 37 ℃ in DTZ solution for 15 minutes, followed by three times rinsing with Hanks balanced salt solution (HBSS). DTZ positive clusters stained crimson red were detected by a light microscope. DTZ positive clusters and total clusters numbers were counted.

2.2.5 Isolation of IPC Clusters

The mixture of undifferentiated ES cells, IPCs and other differentiated cell types may cause teratoma formation. Additionally, after Day33, there were many cell types in the differentiation dish, thus the insulin-producing cells may not be able to get enough nutrients from medium. Isolation of the DTZ positive clusters immediately after the DTZ staining was used in this study, to get purified insulin-producing cells, which has the potential to show higher glucose-response capacity in the further studies. Two 30G needles and 100 µl pipette were used to careful separate DTZ positive clusters, which showed red by DTZ staining, while it was processed under the light microscopy in the hood to keep it sterile. When the cluster was totally separated from the cell sheet and non DTZ positive clusters, it was gently

transferred into a P35 Petri dish with FBS by 100 µl pipette. In this way, the IPCs were purified with minimum disturbance.

2.2.6 Glucose Challenge

Glucose Challenge test is performed on the isolated IPC clusters or on the whole culture dish with IPC clusters. The clusters are suspension cultured in insulin free medium (Refer to Appendix A for details) to get rid of insulin from differentiation medium, so that it does not contribute to the detection of insulin levels. After 24-48 hours incubation, the medium was removed and the clusters were carefully washes 2X with PBS, then incubated with Krebs Ringer Bicarbonate Hepes buffer (KRBH, See Appendix B for details) supplemented with 2.5mM glucose for 90 minutes. The supernatant was collected in 200 µl aliquots and stored at -20 ℃. The cells were immediately incubated with KRBH buffer supplemented with 27.7mM glucose for 2 hours. Then supernatant was collected as 200 µl aliquots and stored at -20 ℃ until ready to use.

2.2.7 Insulin ELISA

The Mouse Insulin ELISA kit (Mercodia) was used to detect insulin from the glucose challenge samples by the manufactures instructions. The micro-plate was read by an Emax plate reader (Molecular devices in Dr. Arinzeh's lab). Softmax pro software was used at the wavelength of 450 nm. A standard curve was drawn using the absorbance values of the calibrators with known insulin concentration and an equation was derived, which was then used to calculate the concentration of insulin in the samples. Experimental values were

verified by a high positive control (Mercodia). The samples were prepared in duplicates.

2.2.8 IPC Yield Calculation.

The yield of IPC cluster was obtained based on DTZ staining. The clusters were associated with cell sheet, thus DTZ positive clusters were count before isolation. The efficiency of differentiation was calculated as DTZ positive clusters/total clusters.

2.2.9 Western Blot for IPC Clusters

Western blot was performed to study the characterization of insulin-producing cell clusters. The isolated clusters were stored at -20 ℃ before making cell lysate. The frozen clusters were washed 2X with ice cold PBS, then incubated with cell lysis buffer (Details in Appendix B) for 20 min at 4 ℃. Tissue (Rat Pancreas) lysates were made by grinding the tissue on dry ice with a hammer pre-colded in -80 ℃, making sure it keeps frozen in whole process. The powdered tissue was transferred to microcentrifuge tube with lysis buffer at 4 ℃ for 20 min. The lysates were centrifuged at 4 ℃ for 30 min at 14,000 rpm in the microcentrifuge. The supernatant was transferred into fresh tube and tested protein quantification by the Bradford assay (Details in Appendix X). The Smart Spec Spectrophotometer (BioRad) was used for reading the results. Cells lysate was diluted in ratio of 1:1 with Laemmli buffer (Bio-Rad, Hercules, CA) containing 5% mercaptoethanol and 2% sodium dodecyl sulfate (SDS). Based on the protein concentration resulted for protein quantification, the volume of aliquots was calculated to make the concentration as 30 μg/μl. The lysates aliquots were stored at -20 ℃ till ready to use.

In this study, insulin/Proinsulin, PDX1 and Glucagon were tested by western blot, the molecular weight of the three proteins are 8.1 kDa, 31 kDa and 17 KDa. Specific protocol was developed to test small proteins. 15% SDS-Polyacrylamide gel was made to separate the bands of small proteins. Dual color precision plus protein standard (BioRad) was used as a protein marker. In addition, 0.2 μm Immunoblot PVDF membrane (BioRad) was used for the transfer process. The transfer condition was 100 V, 45 min for insulin and Glucagon, and 100 V, 60 min for PDX1. One membrane and two membranes conditions were studied to ensure there was no small protein transferring out of membranes. An ice bag was used to keep the process cool. A monoclonal mouse anti-insulin/proinsulin (Abcam) was used as primary antibody for insulin. The PDX1 was tested by monoclonal rabbit anti-PDX1 (Abcam). Different primary antibody dilutions including 1/1000, 1/2000, and 1/3000 were investigated to test the insulin and PDX1. Blots were incubated with secondary antibody for 1 hour at room temperature after three times washes with TBS-Tween buffer. The secondary antibodies are goat anti-mouse IgG HRP for insulin, and goat anti-rabbit IgG HRP for PDX1 (1:2000, Santa Cruz), respectively. After 3X washes by TBS-Tween buffer and one wash with TBS washing buffer, the blots were developed by Supersignal chemiluminescent substrate (Thermoscientific, Rockford, IL). The membrane was incubated with 3 ml of 1:1 ratio of the Supersignal substrate solution and Supersignal enhancer solution in the dark. The membrane was then exposed to UV and developed using a Chemidoc XRS (BioRad). Rat pancreas lysate were used as positive control while ES cells lysate was negative control. The samples were cell lysates of DTZ positive clusters.

2.2.10 Statistical Analysis

Results are shown as mean ± standard deviation. To compare statistical significance between two data sets, a one-way paired student T-test was applied. ANOVA test was used for comparing more than two sets of data. Statistical significance level of $p<0.05$ was used.

2.3 Results

2.3.1 Differentiation of Insulin-Producing Cells

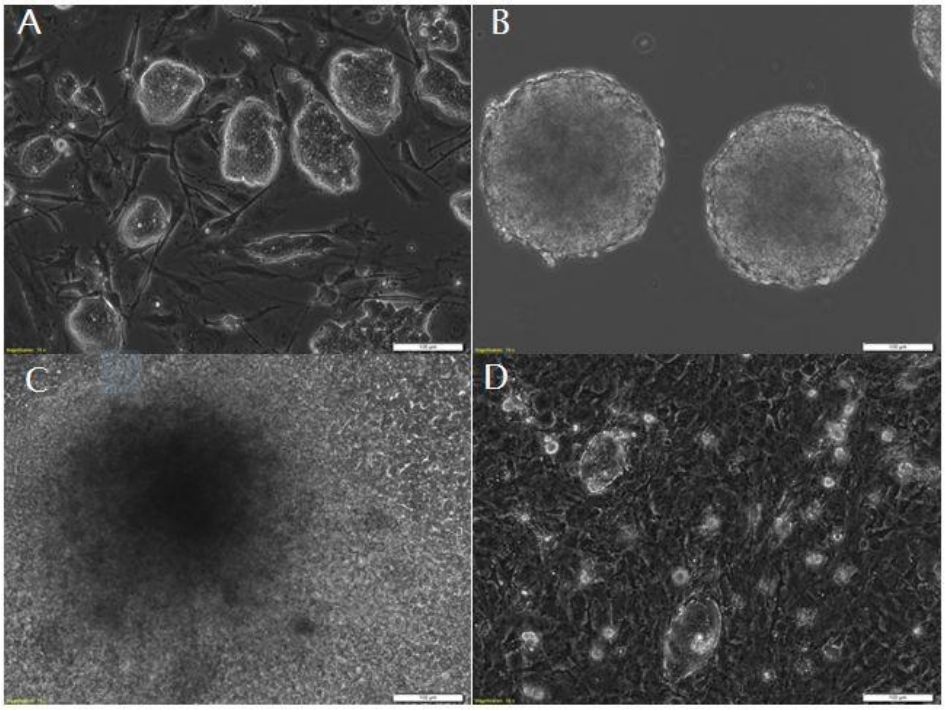

Figure 2.2 A representative microscopic images at varying stages during differentiation. (A) Undifferentiated MES cells on a MEF feeder in culture, (B) EBs on the second day of suspension culture, (C) spreading out EBs after plating for 8 days, and (D) digested cells after overnight incubation. Small clusters indicate committed pancreatic progenitors [11]. Scale bar: 100 μm.

Insulin-producing cells were successfully differentiated from mouse embryonic stem cells using a previously established protocol which comprises of three stages: i) formation of EBs, ii) spontaneous differentiation from EBs into multiple lineages including ectodermal,

mesodermal and endodermal cells, and iii) induction of differentiation into insulin-producing cells [11]. Figure 2.1 illustrates the schematic of a 33 day long differentiation process. EBs were formed by a hanging drop method, resulting in well-rounded shapes with an average diameter of 216 ± 31.8 μm as shown in Figure 2.2. These EBs were then plated and digested on Day 14 in preparation for the last stage of differentiation, which continues for another 18 days. The growth of the clusters was tracked during this period as shown in Figure 2.3

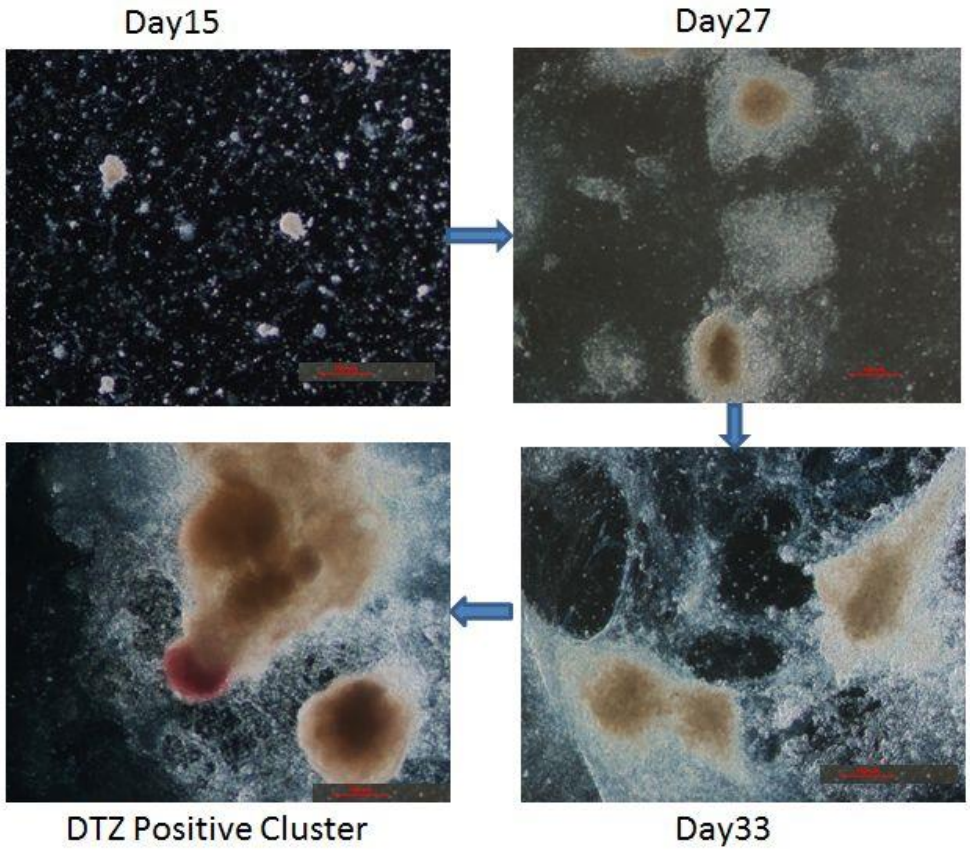

Figure 2.3 Tracking the growth of clusters after digestion in the last stage of differentiation. Many small aggregates were formed in the culture dish after seeding on Day15 of total differentiation process; the aggregates grew into bigger clusters during the differentiation process and some of the clusters become DTZ positive. Scale bar: 100 μm.

After the last stage of differentiation process on day 33, MES cell-derived IPCs were generated and detected by DTZ staining. DTZ positive clusters indicate insulin-producing cells that appear crimson red as shown in Figure 2.3. In the original protocol, the small

aggregates were supposed to be committed progenitor cells, but by tracking of the process, it was found that only some of the aggregates grew into DTZ positive clusters. To determine the yield of IPC clusters, DTZ positive and total clusters were counted right after DTZ staining. On average, about 7 DTZ positive clusters were generated from a 9.5 cm^2 surface area (area corresponding to one well in 6-well plate). The efficiency was calculated by number of IPC clusters/ Total clusters.

2.3.2 Two Days and Three Days of EB Suspension Culture

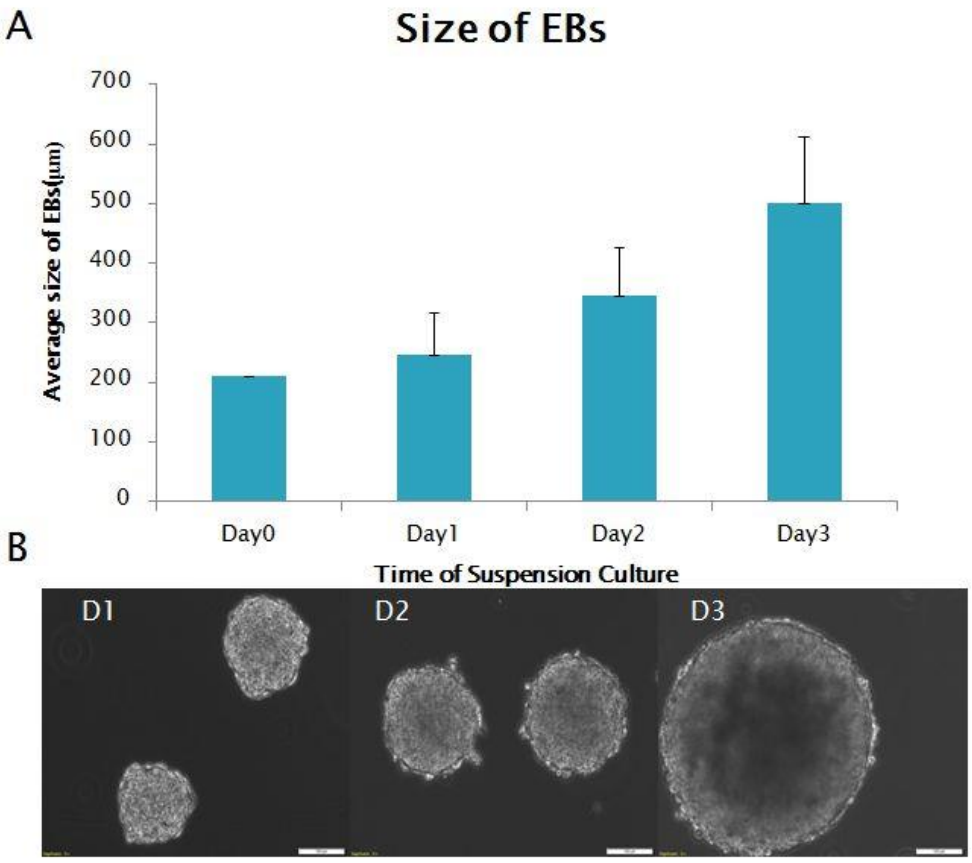

Figure 2.4 Size of EBs in suspension culture. (A) A graph of average size of EBs in suspension culture from Day 0 to Day3. (B) Representative microscopic images of EBs in suspension after 1, 2 and 3 days of culture. Scale bar: 100 μm.

While the original protocol uses 3 days of suspension culture, previous work from our

laboratory used only 2 days suspension as the EBs started to attach to the culture dish after 2 days. As indicated by Figure 2.4, the average size of EBs in suspension culture increases much from Day 2 to Day 3 making the EBs difficult to remain in suspension. However, it was possible to detach EBs by gentle pipetting and agitation, allowing the EBs to remain in suspension culture for 3 days. To compare the 2 day vs. 3 day EB suspension condition, two groups were prepared. In Group 1 EBs were cultured for 2 days and in Group 2, they were cultured for one additional day Three days of suspension culture led to a continuous increase in EB diameter compared to that of 2 day suspension culture. The average diameter of EBs on days 0-3 days were 216 ± 31.8 μm, 259 ± 30.7 μm, 360 ± 50.5 μm and 550 ± 96.6 μm, respectively as shown in Figure 2.4.

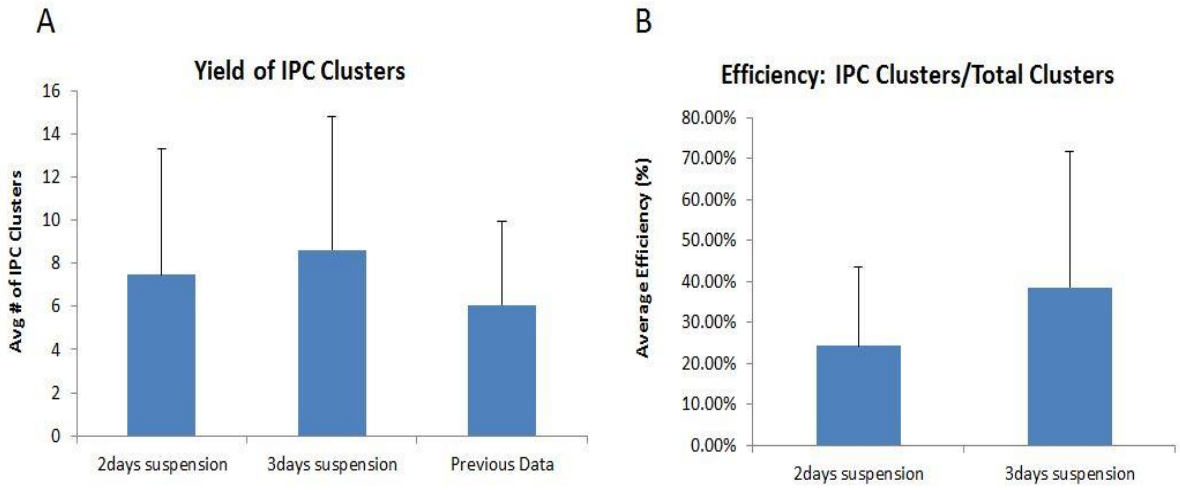

Figure 2.5 Differentiation efficiency from 2 day vs. 3 day EB suspension culture. (A) The average number of IPC clusters were not affected by the suspension culture duration. (B) The average differentiation efficiency of 2 days and 3 days suspension culture was not statistically significant. The results were calculated from the area of 9.5 cm^2 (corresponding to 6-well plate). (Mean ± SD)

The results show that while the number of IPC clusters and the efficiency seems to appear higher for group 2 which had 3 days of EB suspension culture, they were not

statistically different from two day suspension group. It is plausible that the increase of efficiency from 3 days suspension culture was caused by the decrease in the number of total clusters number on the differentiation dish.

2.3.3 Different Digested Cell Seeding Density

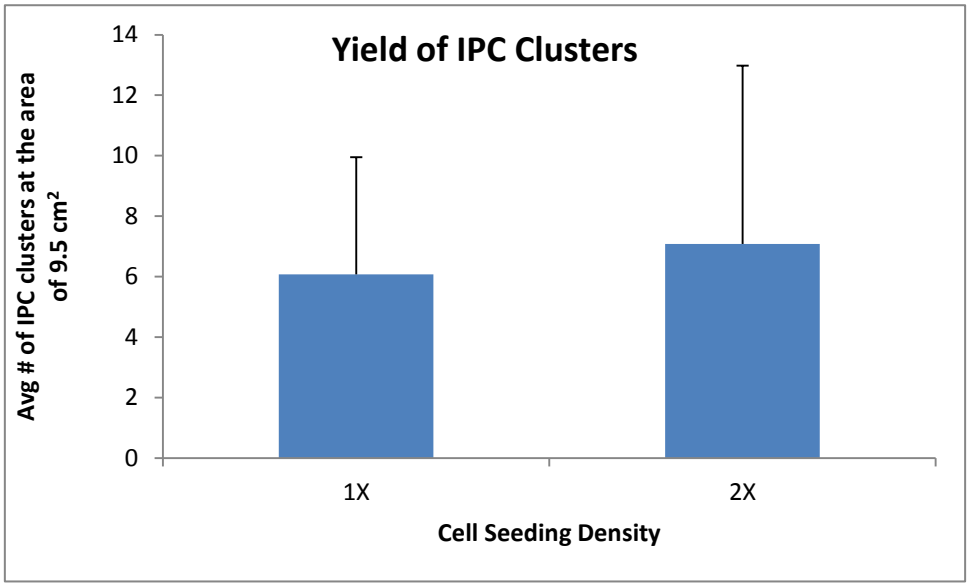

Figure 2.6 The effect of different digested cell seeding density on IPC yield. 1X seeding density indicate the same density as the previously reported protocol [11] and 2X seeding density indicate the doubling of cell density (Mean ± SD).

In this study, 2X digested cell seeding density and 1X digested cell seeding density were investigated in the last stage of differentiation. On Day 14, EBs were trypsinized and digested into a mixture of aggregates and single cells. As it was not applicable to count the cells when there were aggregates in the cell suspension, 2X digested cell seeding density was applied by adding 2 times of digested cell suspension when 1X cell seeding density preserved the same digested cell suspension volume as the original protocol [11]. The 2X digested cell suspension formed more aggregates as well as single cells compared with 1X cell suspension. However, more aggregates did not lead to more DTZ positive IPCs as there was no

significant difference between 1X and 2X cell seeding density on IPC yield (Figure 2.6).

2.3.4 Different Collagen Coating Conditions for Digested Cells

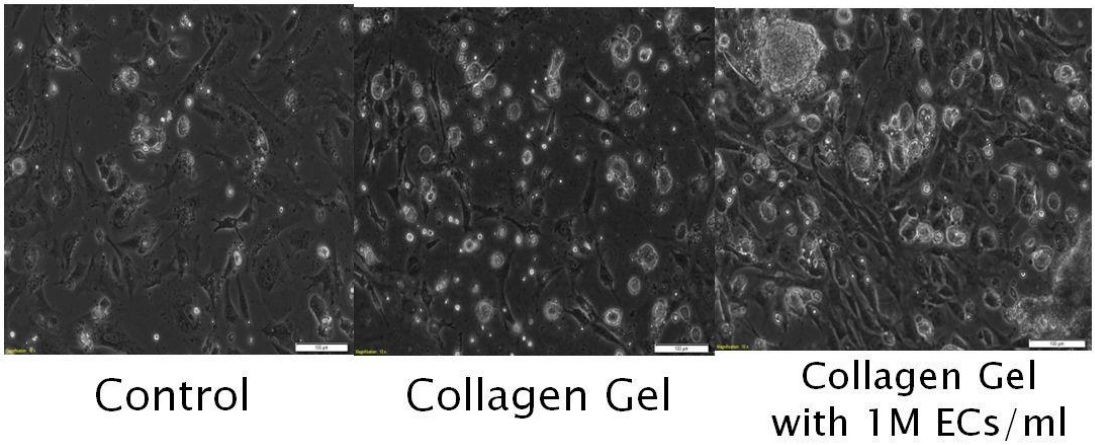

Figure 2.7 Digested cells after overnight incubation. The thickness of collagen gels used was 0.5 mm. Scale bar is 100 μm.

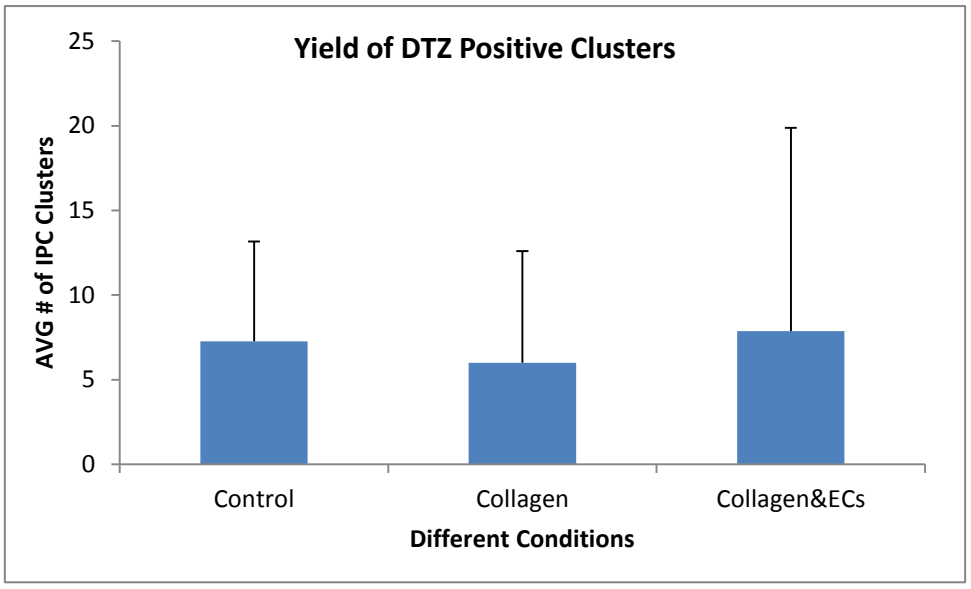

Figure 2.8 Yield of DTZ positive clusters from three coating conditions. The yield of IPC clusters was generated from the area of 9.5 cm^2 (Mean ± SD, $p < 0.05$).

Different coating conditions were set up to investigate whether the co-culture system with endothelial cells would affect the overall differentiation efficiency. Islet endothelial cells (IECs) used for the co-culture system were also MES cell-derived ECs from our laboratory.

The IECs generated near MES cell-derived IPC clusters were supposed to facilitate the differentiation into IPCs [49]. Three conditions including normal diluted collagen coating, a thin collagen gel and a thin collagen gel with ECs (1M cells/ml) coating were explored. The seeded cell density was obviously different after overnight incubation as more aggregates and cells were seeded on the dishes containing collagen gel and collagen gel with ECs (Figure 2.7). The yield of DTZ positive clusters was calculated on Day 33, and the standard deviations were large. Figure 2.8 shows that there was no significant difference among three different coating conditions. The result demonstrates that co-culture system with ECs in the collagen gel coating do not result in a higher production of IPC clusters nor negatively affect the IPC production

2.3.5 Isolation of Insulin-Producing Cell Clusters

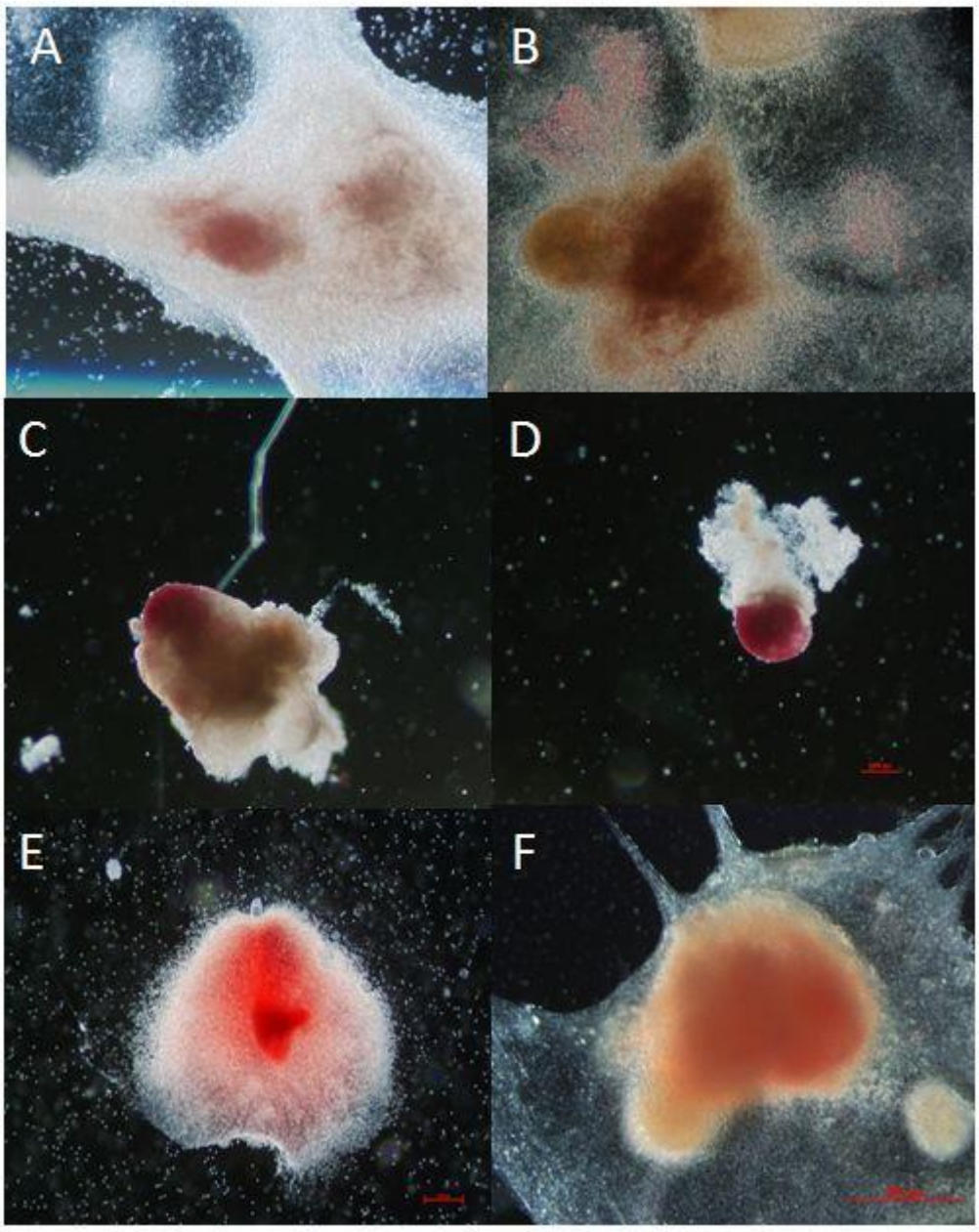

Figure 2.9 DTZ staining of IPC clusters. (A) IPC cluster without isolation, (B) IPCs spread out on the culture dish, (C) shows the whole isolated cluster with only a small DTZ positive part, (D) shows further isolated IPCs and (E) DTZ positive islets from rat pancreas. Scale bar is 100 μm. (F) is an IPC cluster generated from previous work in out laboratory, scale bar is 500 μm.

The IPCs were stained crimson red by DTZ staining at the end of the differentiation. However, the color of DTZ positive clusters changed from pink to amaranth, depending on

the density of IPCs in the clusters. This indicated that whole cluster contains both IPCs and other cell types. When comparing the color intensity of the DTZ positive clusters from the previous work done in our laboratory, it was apparent that the IPC clusters generated from using the optimized protocol have higher density of IPCs. Furthermore, IPCs appeared as spreading out cells and clusters at the same time, indicating that the differentiation process not only results in clusters of IPCs, but also some non-clustered single IPCs. The average size of whole differentiated IPC clusters was about 369 ± 137 µm, while the average size of native islets are much smaller of 150 µm [62], as shown in Figure 2.5. Thus, a further isolation was needed to remove the other cell types to obtain more pure IPCs clusters.

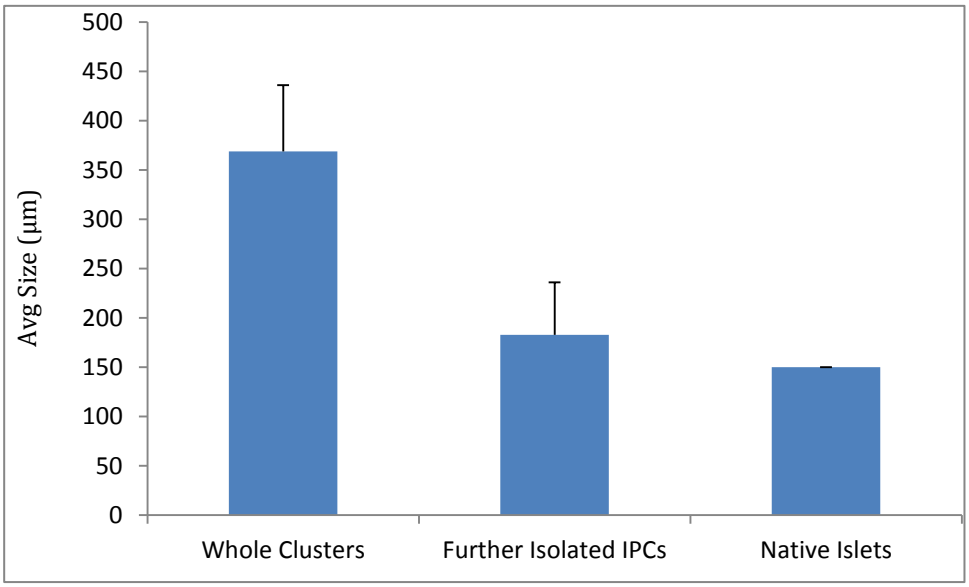

Figure 2.10 Average size of IPC clusters compared with native islets. The average size of generated whole IPC clusters was 369 µm in diameter, the average size of further isolated IPCs was 183µm in diameter (n=25), whereas the native islets are in a size range 50-250 µm [62].

The IPC clusters which showed DTZ positive were then isolated immediately after staining and suspension cultured. The rat pancreas was also isolated and stained by DTZ solution as a

positive control. The IPC clusters derived using a modified protocol exhibited obvious crimson red color compared with the previous derived clusters which were generated by 1X cell seeding density of digested cells,. To generate pure IPCs, a further isolation was processed by using a 30G needle and a 100 µl pipette. The IPCs were well separated from the non DTZ positive cells. As it is shown in Figure 2.10, the average size of further isolated DTZ positive clusters was 183 ± 53 µm in diameter, varying from 100 µm to 300 µm, while the native mouse islets are 50-250 µm in size [62]. The results showed that the isolated IPC clusters were much smaller than the whole DTZ positive clusters after further isolation.

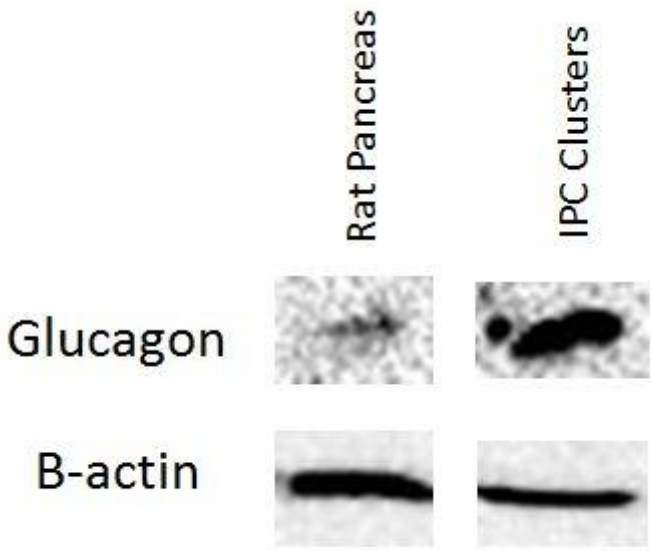

Figure 2.11 Western blot results. Glucagon was detected in both rat pancreas and differentiated IPC clusters. But the insulin and PDX1 expression were not successfully detected by western blotting. Beta-Actin was used as a loading control.

Furthermore, Figure 2.11 shows that Glucagon was detected by western blotting in the isolated clusters and rat pancreas, confirming the presence of alpha cells in the clusters.

However, the insulin/proinsulin and PDX1 were not detected successfully even after several attempts to optimize the western blotting conditions. The reasons for the failure of detection can be predicated by several factors: (i) low level of expression of insulin/proinsulin and PDX1 in cell lysate of isolated IPC clusters, (ii) very low molecular weight of insulin and PDX1 to be detected with western blotting and (iii) unsuccessful extraction of insulin and PDX1 from cell lysate. Ponceau staining was used to detect protein bands but only a few bands appeared without target bands.

2.3.6 Glucose Responsiveness of Generated IPCs

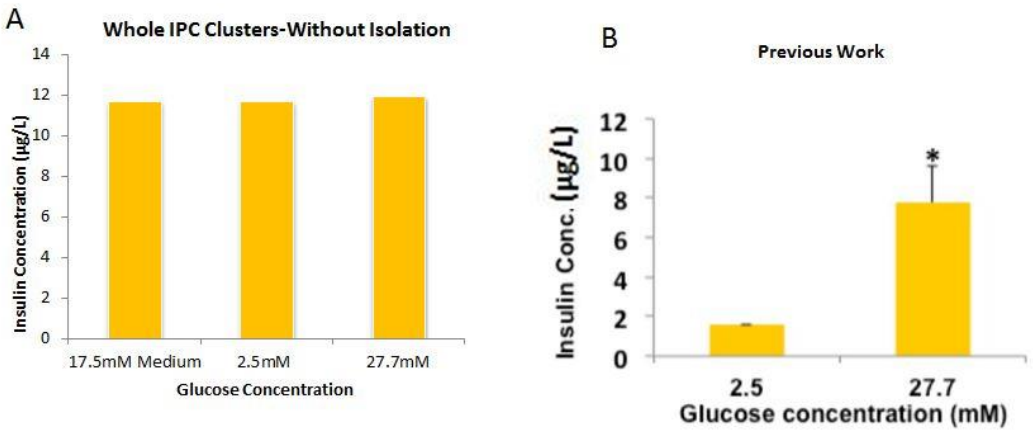

Figure 2.12 Insulin ELISA assay of fresh samples. (A) Glucose challenge results from fresh samples of whole IPC clusters without isolation, (B) Glucose challenge of the whole IPC clusters from the previous work in our lab (n=6, $p<0.005$). 17.5mM medium is the insulin-free medium with high glucose concentration, and it was added before glucose challenge to remove the insulin in the clusters.

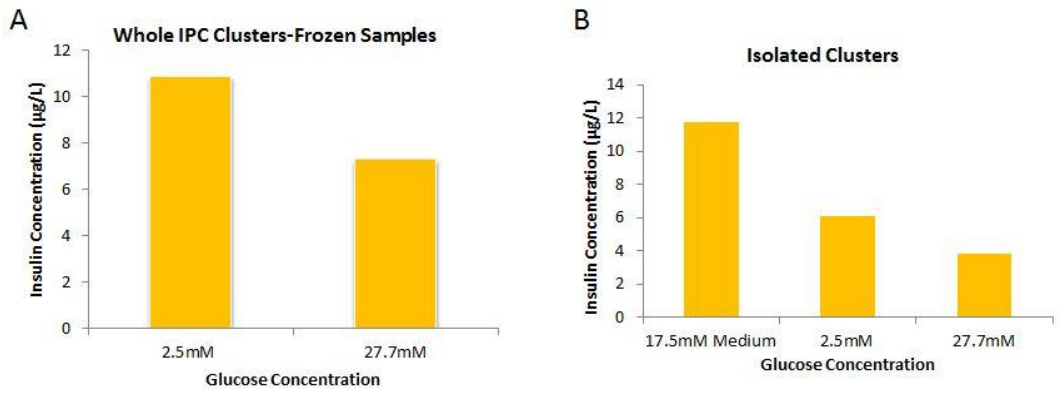

Figure 2.13 Insulin ELISA assay of frozen samples. (A) Glucose challenge from the frozen samples of IPC clusters without isolation. (B) Glucose challenge from isolated clusters.

The amount of insulin secreted by differentiated IPCs was quantified by an insulin ELISA, to test the glucose responsiveness of IPCs. Figure 2.12 and Figure 2.13 show the ELISA results from IPC clusters. The IPC clusters were cultured in the insulin-free medium with a high glucose concentration of 17.5 mM prior to glucose challenge for 24 hours. The results of fresh samples demonstrate that the IPCs secrete slightly higher insulin when exposed to higher glucose concentrations, while there was no glucose response in frozen samples stored 4 months ago, as well as from the isolated clusters. It is suspected that long-term store may affect the quality and stability of the samples. Further, the isolation process may cause harm to the IPCs, leading to failure of glucose responsiveness. While compared with previous work in our laboratory, the IPCS were not as glucose responsive. However, the basal insulin concentration was much higher than the previous results, which demonstrate that the IPCs derived by the optimized protocol led to larger number of IPC cells that have better quality. However, more trials are needed to ensure the glucose responsiveness as this study shows results from one trial.

2.3.7 Other Cell Types on Digestion Dish

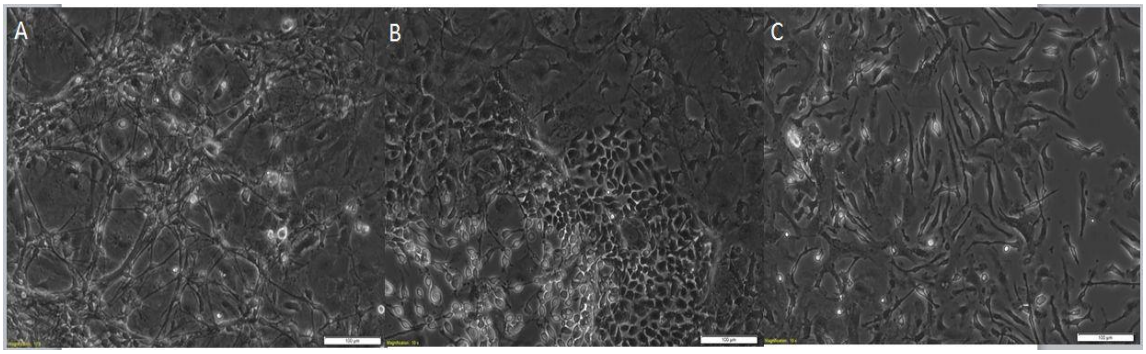

Figure 2.14 Other cell types found on the digestion dish. (A) shows neural cells, (B) shows macrophage cells, (C) shows endothelial cells, by the morphology of the cells. Scale bar is 100 μm.

Different types of cells were found in the differentiation process, cardiac myocytes were discovered on the dishes of plated EBs by continuous beating in the microscope. Figure 2.14 shows several cell types on the culture dish, by the morphology of the cells, there were neural cells, macrophages and ECs on the digestion dish after Day 33. These cell types were less than 10% of total cells in the culture dishes.

2.4 Discussion

Although successful generation of IPC clusters was achieved after 33 days of differentiation from MES cells by using a previously established protocol [11], the efficiency remained low. Therefore, in this study, we investigated the influence of several conditions during differentiation process to enhance the overall differentiation efficiency.

First, the influence of the duration of embryonic bodies (EBs) suspension culture was examined. While the previous established protocol reports 3-day suspension culture for the differentiation into progenitor cells of ectodermal, mesodermal and endodermal lineages

[11], a previous work from our laboratory used only 2-day suspension culture due to attachment of EBs to the dish after 2 days. Therefore, 3-day EB suspension culture was performed in this study and the results were compared to that of 2-day EB suspension. Three-day suspension culture resulted in much larger EBs and it was expected that more clusters are formed after digestion from larger EBs, which can potentially result in higher number of IPC clusters. However, the results demonstrated that the 3-day suspension culture do not lead to improved differentiation efficiency nor negatively affect the number of IPC clusters.

The insulin-producing cells were identified by DTZ staining, which works by forming a chelating complex with zinc ions secreted with insulin from β cells [63, 64]. The differentiation efficiency was calculated based on the ratio of number of DTZ positive clusters to the total number of clusters instead of total number of cells. However, as most of the DTZ positive cells were derived from the clusters, this simple estimation was used. Other studies use flow cytometry to get the efficiency of IPCs in the total cells, and the yield of IPCs were reported in the range of 0.8-7.3% [9, 25].

Secondly, the effect of higher cell seeding density after digestion of EBs on differentiation efficiency was examined. In previous studies, it has been demonstrated that cell seeding density effects the formation of pancreatic cell population [58, 65]. Blair *et al.* examined initial cell seeding density of human embryonic stem (ES) cells from 2.6 to 10.6 × 10^4 cells/cm^2. Efficient definitive endoderm induction was observed above moderate densities of 2.6 × 10^4 cells/cm^2, and PDX1 expression and subsequent hormone positive

populations were increased in cultures seeded at 5.3×10^4 cells/cm^2. The high seeding density cultures resulted in expected temporal expression of maturing pancreatic progenitors that specify endocrine cell fates and finally adopt hormone expression [58]. In this study, 2X digested cell seeding density was investigated based on the original digested cell seeding density from the previously reported protocol [11]. As it was difficult to count cells when there were aggregates in the digested cell suspension, 2X digested cell suspension was added when 1X cell seeding density preserved the same digested cell suspension volume. Digested cells were added to the collagen coated dishes during the third stage of differentiation process to induce the differentiation of pancreatic lineage. However, by comparing with yield of generated IPC clusters from 1X cell seeding density of the previous work in our laboratory, there was no significant difference detected with statistical analysis. Since the effect of cell seeding density was studied at the initial stage of different differentiation in previous studies rather than at the later stages as in this study, it is possible that higher cell seeding density during initial stage of differentiation is more essential to generate more cells in endodermal lineage which can then ultimately become IPCs.

Lastly, different coating conditions such as normal collagen coating, thin collagen gel coating and thin collagen gel coating with ECs were examined. ECs used in this study were islet endothelial cells (IECs) generated beside MES cell-derived IPC clusters in previous work from our laboratory [49]. It was investigated in this study whether IECs help to facilitate the differentiation into IPCs, when thin collagen gel coating was used to compare with the gel coating mixed with ECs. During the developing of pancreas, differentiating

pancreatic cells are in close proximity with endothelial cells from the vasculature. Furthermore, pancreatic islets are densely vascularized to promote rapid exchange of blood glucose and islet hormones. Thus, endothelial cells play an important role in the development of pancreatic organogenesis [66]. IECs were used as feeder layer for seeded cells in previous work from our laboratory, but too many IECs proliferated on the dishes and resulted in low yield of IPCs. In this study, we embedded IECs in the collagen gel to obtain paracrine factors while keeping a low proliferation rate. Much higher cells were initially seeded on the collagen gel and collagen with ECs gel coating dishes, after overnight incubation. However, our results indicated that the islet endothelial cells mixed in the collagen gel coating were not effective for generating more IPCs. No difference was observed when compared to the control condition with normal coating in this study. It indicated that the IECs were either insufficient in the coating gels to produce enough factors, or the high seeded cell density caused cell necrosis and apoptosis during differentiation process. More importantly, for the co-culture system, differentiation medium was used. Lack of endothelial cells medium may have caused endothelial cells to dedifferentiate or not function properly and this awaits further investigation. In addition, other types of ECs such as microvascular ECs need to be investigated in the future work.

Although the factors varied did not result in more production of IPC clusters, the basal amount of insulin secretion was above 11μg/L, it was higher than the previous study with the secreted insulin concentration varying from 2μg/L to 8μg/L. However, the isolated clusters showed no glucose response while the IPC clusters without isolation in the culture

dishes showed minimal glucose response, which indicated that the isolation process may cause damage to the function of IPCs.

In previous studies, it was reported that exposure to high glucose medium impaired function of rat or human islets, and de-sensitized the cells in response to glucose challenge [67-69]. The insulin-free medium used before glucose challenge contained 17.5 mM glucose, which is considerably high. The exposure to the medium may also cause the IPCs to lose the function of glucose responsiveness. It was suggested in previous studies that transferring cells to a low glucose medium in the last stage of differentiation might be capable to recover the function of glucose responsiveness [18]. In addition, to compare glucose challenge with previous work in our laboratory, the results were presented the same way by the concentration of secreted insulin got from ELISA with the unit of μg/L. Whereas insulin secretion was calculated as percentage of total insulin content in the original protocol and some of the current studies [11, 70, 71], it was not capable to compare the results with other studies. The basal insulin concentration of glucose challenge in this study was transformed to 7 ng insulin/mg protein based on the total protein extracted by cell lysate. By comparing with the glucose challenge results in original protocol, it was found that our results were similar to the original protocol [11]. However, the extraction of protein were processed by acid ethanol in original protocol and other studies, thus it was inaccurate to compare our results with the original protocol. More work needs to be done in the future, including extracting intracellular insulin from all the cells by acid ethanol and calculating the insulin secretion by released insulin/total intracellular insulin.

Since the whole cluster is a mix of IPCs and non-IPCs with a large size of 369 μm in diameter, the isolation of IPC clusters was necessary to obtain more pure population of IPCs. Our method of isolation showed the feasible and ensured the IPC clusters to get sufficient nutrients by controlling the size of the cluster. After isolation, the IPCs were purified in a much smaller cluster as big as 183 μm, the further isolated IPCs were similar to the size of native islets of 50~150 μm [62].

Western blotting was applied to test insulin/proinsulin, PDX1 and Glucagon. PDX1 serves as a regulator of β cells function and development by activating genes essential for β cells [72]. The detection of Glucagon demonstrated the presence of alpha cells, but it was difficult to test insulin/proinsulin and PDX1 from the clusters. RT-PCR is the most common method to test the proteins expressed by IPCs, including PDX1, Nkx6.1and Pax4 [8, 11]. The failure of western blot for insulin/proinsulin and PDX1 in this study may be resulted from the low expression of these proteins or the failure to properly extract protein from cells in preparing cell lysates. We also found several cell types on the culture dishes after Day 33 of differentiation, and the cells were speculated to be neural cells, macrophages and endothelial cells by morphology.

In summary, three optimization strategies including different time period for spontaneous formation of suspension culture, different cell seeding density for the induction of pancreatic cells differentiation and different collagen coating conditions for the induction of pancreatic lineage were investigated. The statistical analysis showed that there was no significant difference between each group. However, the insulin secretion concentration by

glucose challenge and DTZ staining results show that the IPCs generated by the optimized protocol were more homogeneous and better in quality, comparing with the IPCs generated by the original protocol.

Further studies are needed, such as examining the influence of a gradient cell seeding density in different differentiation stages, using mixed medium (MCDB ECs medium and Differentiation medium) for the co-culture system, adding more ECs in coating gels, and setting up the co-culture system in initial differentiation stage to generate more endoderm lineage cells. Furthermore, the unit of glucose challenge is ng insulin/mg protein from total cells in some of the current studies including the original protocol, thus further studies are needed to obtain more data with the same calculation method to estimate glucose responsiveness of MES cell-derived IPCs.

CHAPTER 3

FORMATION OF THREE DIMENSIONAL TISSUES USING IPC CLUSTERS

3.1 Introduction

Post-isolation islet survivability is an essential step for the successful islet transplantation, including optimizing islet culture for long-term survival and maintaining functionality *in vitro*. It was found that the IPC clusters lose their insulin secretion and response after several days culturing *in vitro* due to low survivability [45].

Two-dimensional (2D) culture is the most common strategy for culturing islets *in vitro*, however, it does not provides sufficient extracellular matrix (ECM) support as *in vivo*, which is essential in regulating cell adhesion, activating signaling pathways and supplying structure support [73]. However, it was found from previous studies that the three dimensional (3D) culture constructs is more similar to microenvironment than 2D monolayer cultures [74]. While the islets are cultured as monolayer in 2D system, their characteristic and functions are damaged. The cells are not able to maintain their phenotype with the lack of essential microenvironment, and the differentiated cells tend to lose their functions or survivability during long *in vitro* culture. And it is demonstrated that only about 56% islets survive after 48 hour culture in 2D system [75].

Zhang *et al.* tested the effects of two types of 3D scaffold, collagen matrix (CM) and fibroblast-populated collagen matrix (FPCM), on amyloid formation, viability, and function of isolated islets. Amyloid is pathological characteristic of the pancreas in type 2 diabetes mellitus that contributes to progressive-cell death in this disease. The results suggested that

3D scaffolds reduce amyloid formation, and improve viability and function of human islets in vitro, as well as additive effects in enhancing islet function [76]. Therefore, a 3D system providing more physiologically relevant environment may be necessary to maintain the survival and function of islets *in vitro* [77, 78].

Biomaterials are used to strengthen islets survival by making a 3D cellular support and facilitate the delivery of proteins, immunosuppressive agents and growth factors. ECM molecules were demonstrated to enhance cell survival, support differentiation, and improve the function of insulin-producing beta cells. It was found that 3D culture condition composed of collagen type I, IV was useful for the function and survival of IPCs *in vitro* [79]. Collagen type I is a major component of basement membrane, present at the interface of exocrine and endocrine interface, as well as in close proximity to intra-islet endothelial cells. In this study, we used collagen type I to generate 3D tissue with IPC clusters, to enhance the viability of MES cell-derived IPCs after differentiation.

In addition, perifusion system was used to improve the islet survivability *in vitro* culture by providing the cells sufficient nutrient and mimic the physiological environment [80]. Microfluidic device allows application of intercellular flow on islets to better deliver of nutrients and gas. Thus, a flow bioreactor system was used to provide continuous fluid flow through the 3D tissues containing IPCs. Previous study from our laboratory used a flow rate of 0.5 ml/min for the bioreactor of 3D flow gel embedded with IPC clusters with a shear stress of 0.71 dyn/cm^2, which resulted in severe cell death. In this study, a much lower flow rate of 0.02 ml/min was examined for the long-term culture of 5 days, to attenuate the

physical destruction of IPCs caused by the shear stress of flow. By the data from a previous study [77], as the flow rate and shear stress showed linearity, a flow rate at 0.02 ml/min was calculated to be approximately 0.156 dyn/cm^2. This shear stress was much smaller compared with the shear stress of flow rate at 0.5 ml/min.

3.2 Materials and Methods

3.2.1 Suspension Culture of Isolated IPC Clusters *in vitro*

The DTZ positive clusters were directly isolated after the staining, and transferred to a P35 petri dish. Differentiation medium #2 was added to keep the clusters in suspension culture. The clusters were also dissociated to compare IPCs survivability with the whole clusters. Two methods were used for the dissociation: one was processed by using a 3 ml syringe, a 25G needle and a 40 μm cell strainer (BD Falcon); the other was processed by trypsin, a 3 ml syringe, a 25G needle and a 40 μm cell strainer. 0.25% trypsin was used to dissociate the clusters before the physical break. After 3 minutes incubation, the clusters were broken into small pieces by passing through the needle and the cell strainer to be further broken. The clusters were also broken by physical break without trypsin. The dissociated cells were transferred into a microcentrifuge and centrifuged at 1200 RPM for 5 minutes, and then the supernatant was carefully removed.

3.2.2 Preparation of Static Collagen Tissues Containing IPC Clusters

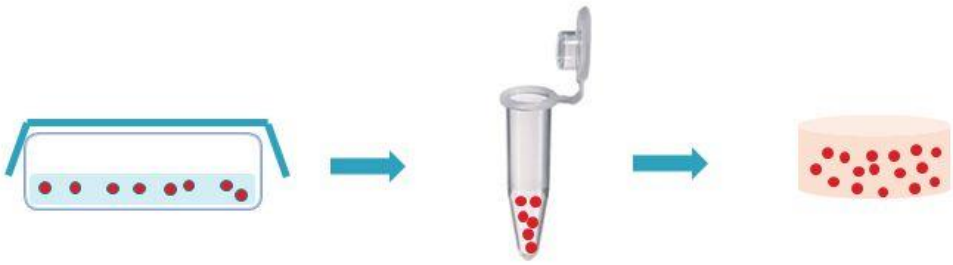

Figure 3.1 Schematic of collagen gel preparation process. IPCs were isolated from differentiation culture (Day 33) into a microcentrifuge tube with FBS. After washing by PBS, IPCs were either kept intact in clusters or dissociated into small pieces. The IPCs were then mixed with collagen type I solution to form the 3D gels.

Figure 3.1 shows a schematic of 3D collagen gel preparation process. Before the process, the IPC clusters were transferred into a microcentrifuge tube, the IPCs were settled down after 5-10 minutes, the supernatant was carefully removed. The collagen solution was composed of rat tail collagen (Lab processed), 10X reconstitution buffer (0.05 N NaOH with 0.16 M HEPES and 0.25 M NaHCO3), and 10X DMEM (Sigma Aldrich) in the ratio of 80:10:10, with the concentration of 3 mg/ml. 1N NaOH was added to adjust the pH to 7.2-7.4 until the solution appeared salmon pink. As shown in Figure 3.1, 25 IPC clusters were suspended in 51.2 µl of collagen solution to make the thickness of 1.6 mm in the 96-well plate. The mixture was added to the well of a 96-well plate. The collagen gel was incubated for 30 min in the incubator to polymerize. Differentiation medium #2 was added after polymerization and changed every other day. After the static gel was set up, the collagen gel was separated from the bottom of dish by fine tip forceps, to keep the clusters from attaching the dish or spreading out.

3.2.3 Application of Fluid Flow Using A 3D Flow Bioreactor System

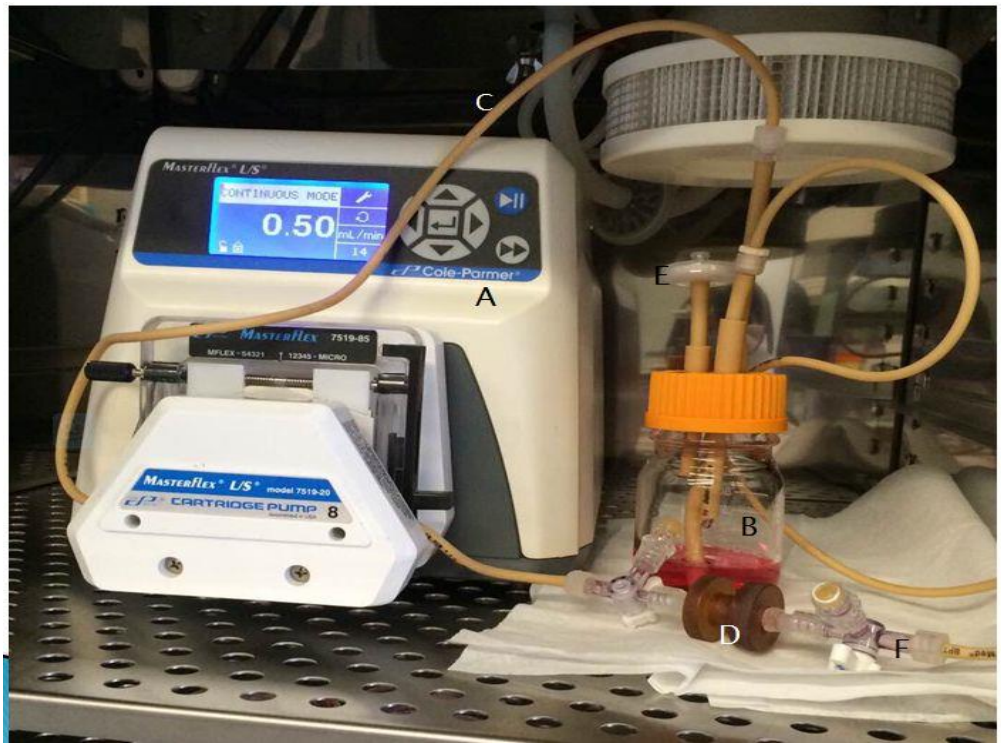

Figure 3.2 Flow bioreactor system. A flow bioreactor system consists of (A) a peristaltic pump, (B) a media reservoir, (C) L13 sized tubing, (D) a bioreactor chamber housing a 3D collagen tissue . (E) a 0.2 μm air filter for gas exchange and (F) a 3-way stop-cock/injection port for medium exchange.

A customized bioreactor was applied for the flow collagen tissue with IPC clusters, which provided constant perfusion to the tissue. The flow bioreactor was used to provide the cells or tissue flow media physiologically. The influence of the flow rate was then examined after 5 days culture, by comparing the tissue of flow condition with tissue in static condition. The volume of collagen solution mixed with IPCs was 152 μl, to keep the same thickness of 1.6 mm with static gel, and the area of the bioreactor is 95 mm^2. The collagen solution with IPCs was added on a polyethylene terephthalate (PET) membrane, which was glued to the PDMS ring on the edge of bottom part of bioreactor. The gel was supported by the PET porous membrane, and to decrease the resistance to flow from the membrane, 15-20 holes were

punched on the membrane using a 30 G needle. Stainless pins were plated in the membrane and the PDMS ring below, to prevent collagen gel compaction in the flow condition culture.

After the gel was added to the membrane, the bioreactor was incubated for 2 hours at 37 ℃ by being placed inside a 50 ml conical tube for polymerization. Then the bioreactor was fully assembled in the flow hood. The top part of bioreactor and 3-way stopcock valves (Smith Medicals) was fastened using connectors (Cole Parmer). The bioreactor assembly was connected to a medium reservoir by two pieces of LS'13 tubing. Syringe ports (Baxter) were used to fill the inlet and outlet tubing by Differentiation medium #2, the ports were attached to stopcock valves. Finally, the bioreactor assembly was connected to the peristaltic pump (Cole Parmer) and the flow was set at 0.02ml/min in continuous mode for 5 days. The shear stress was calculated to be 0.156 dyn/cm^2, based on the previously reported data [77].

3.2.4 Cell Viability

Live/Dead viability/cytotoxicity assay (Invitrogen) was used to test the survivability of IPCs by staining live and dead cells in 3D collagen tissues. Calcein AM (1 μM/ml) and ethidium homodimer-1 (2 μM/ml) were applied to stain live and dead cells, respectively. Staining solution was prepared in sterile PBS. After 5 days culture, collagen tissues in flow condition were transferred from the bioreactor into the 96-well plate by sterile fine tip forceps. Static collagen tissues were stained directly in the 96-well plate. The staining was processed in at 37 ℃ for 30 minutes with foils on the plates, to keep it from light. Samples were washed by PBS, and transferred on a glass coverslip. An inverted fluorescence microscope (IX81 DSU, Olympus) was used for imaging.

3.2.5 Statistical Analysis

Results are shown as mean ± standard deviation. A one way ANOVA test was performed to examine significant difference between the culture conditions. Statistical significance was accepted when $p<0.05$.

3.3 Results

3.3.1 Isolated IPC Clusters Survival in Suspension Culture

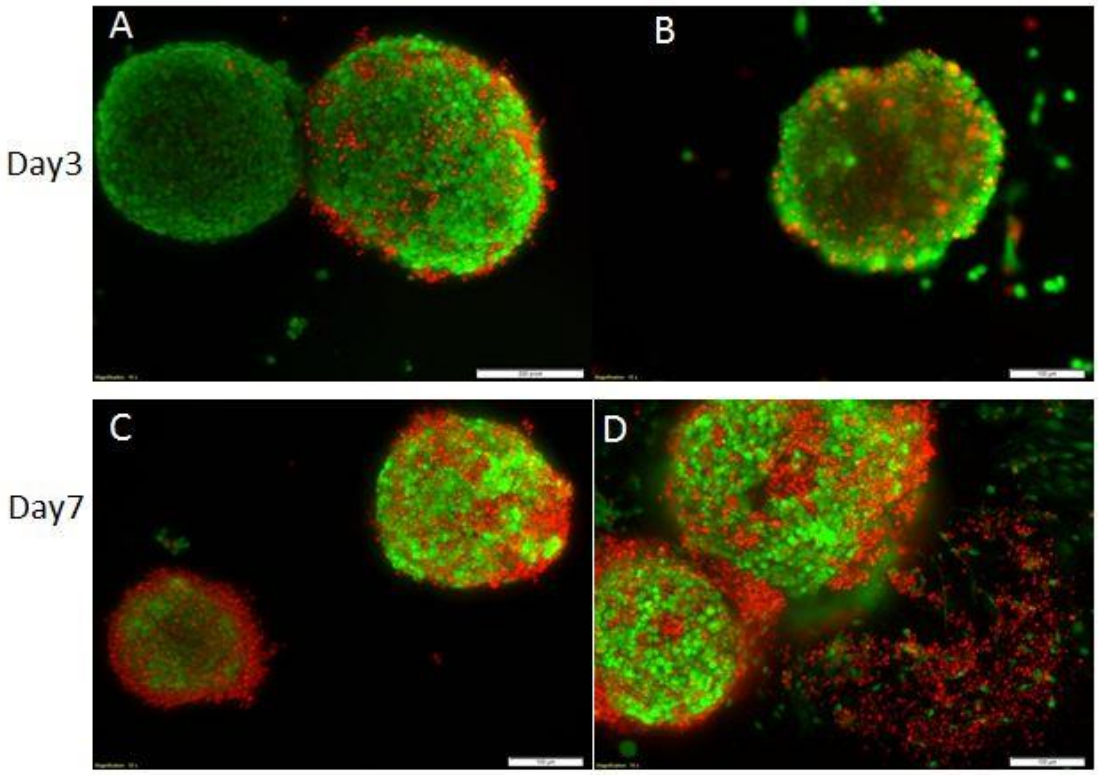

Figure 3.3 Live/Dead staining of suspension cultured IPCs. (A) and (B) show survivability of clusters on day 3, (C) and (D) show survivability of clusters on day 7. Calcein stains the living cells green and ethidium homodimer stains the dead cells in red. Scale bar is 100 μm.

To investigate the viability in suspension culture, IPC clusters were cultured in the differentiation medium for up to 7 days. Live/dead staining on Day3 and Day7 showed more dead cells after 7 days compared to that of 3 days (Figure 3.3). Not only the viability, but the

functionality of the IPC clusters also decreased as the number of DTZ positive clusters decreased tremendously after 7 days of static suspension culture, as shown in Figure 3.4. In addition, there was no significant difference in the sizes of the clusters with a long-term suspension culture for 7 days as shown in Figure 3.5, indicating that not so much proliferation has occurred.

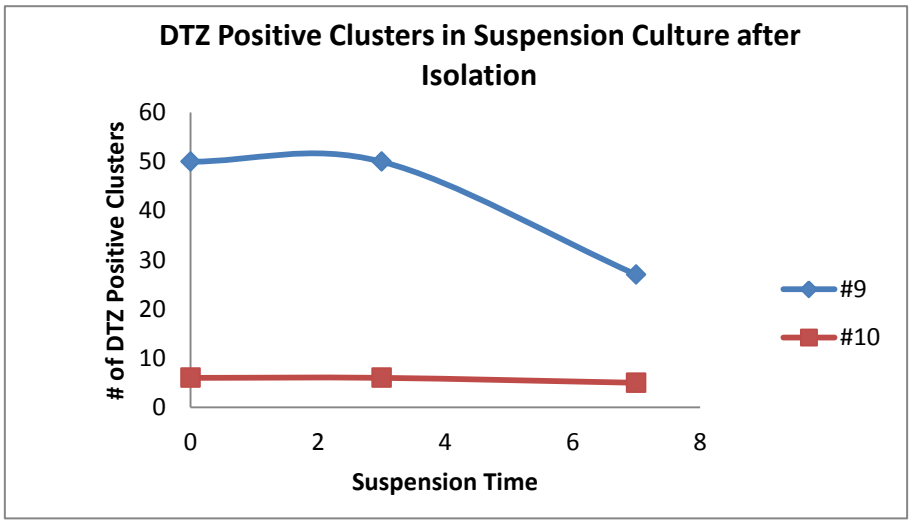

Figure 3.4 DTZ staining of IPC clusters in suspension culture on Day 0, Day 3 and Day 7. DTZ positive clusters in suspension culture showed decrease in the number of positive clusters with longer culture.

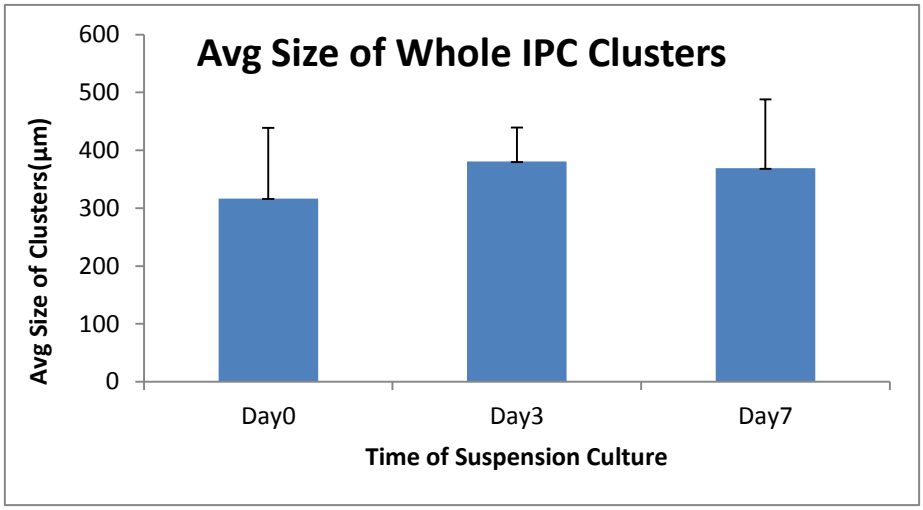

Figure 3.5 Average size of whole IPC clusters on Day0, Day3 and Day7 of suspension culture.

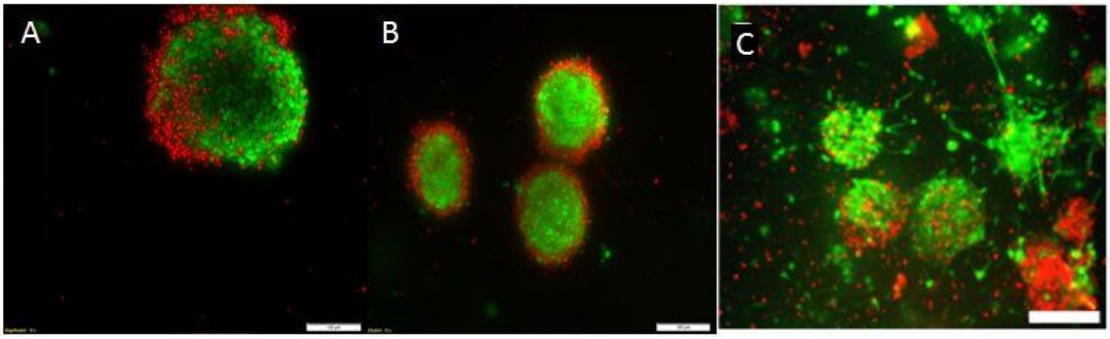

Figure 3.6 Survival of IPCs in suspension culture and collagen tissue on Day 5. (A) IPC cluster in 2D suspension culture, (B) IPC clusters in a free floating 3D collagen gel, and (C) IPC clusters in a 3D collagen gel from the previous work in our laboratory where the gel remained attached to the bottom of the dish. Scale bar is 100 μm.

IPCs survivability was compared in 2D suspension culture with 3D collagen tissue. Live cells were stained green and dead cells showed red. The morphology of IPC clusters was different under different conditions shown in Figure 3.6. Cells were in clusters both in 2D suspension culture and in a free floating 3D collagen gel. However spreading of the clusters was observed from the previous study in which the collagen gels remained attached to the plate (Figure 3.6.C). The difference in the boundary constraint likely caused the difference in IPC spreading behavior. Interestingly, in both 2D and 3D culture conditions, dead cells were found mostly at the edges and not at the core of the clusters. This can be attributed to the manual isolation of the IPC clusters from the differentiation plates, and less from diffusion limitation. However, it is difficult to accurately quantify the number of live/ dead cells in suspension culture condition from these images. Thus in the future, better isolation method is needed to reduce the dead cells at the edges.

3.3.2 Survivability of Whole and Dissociated IPC Clusters in 3D Tissues

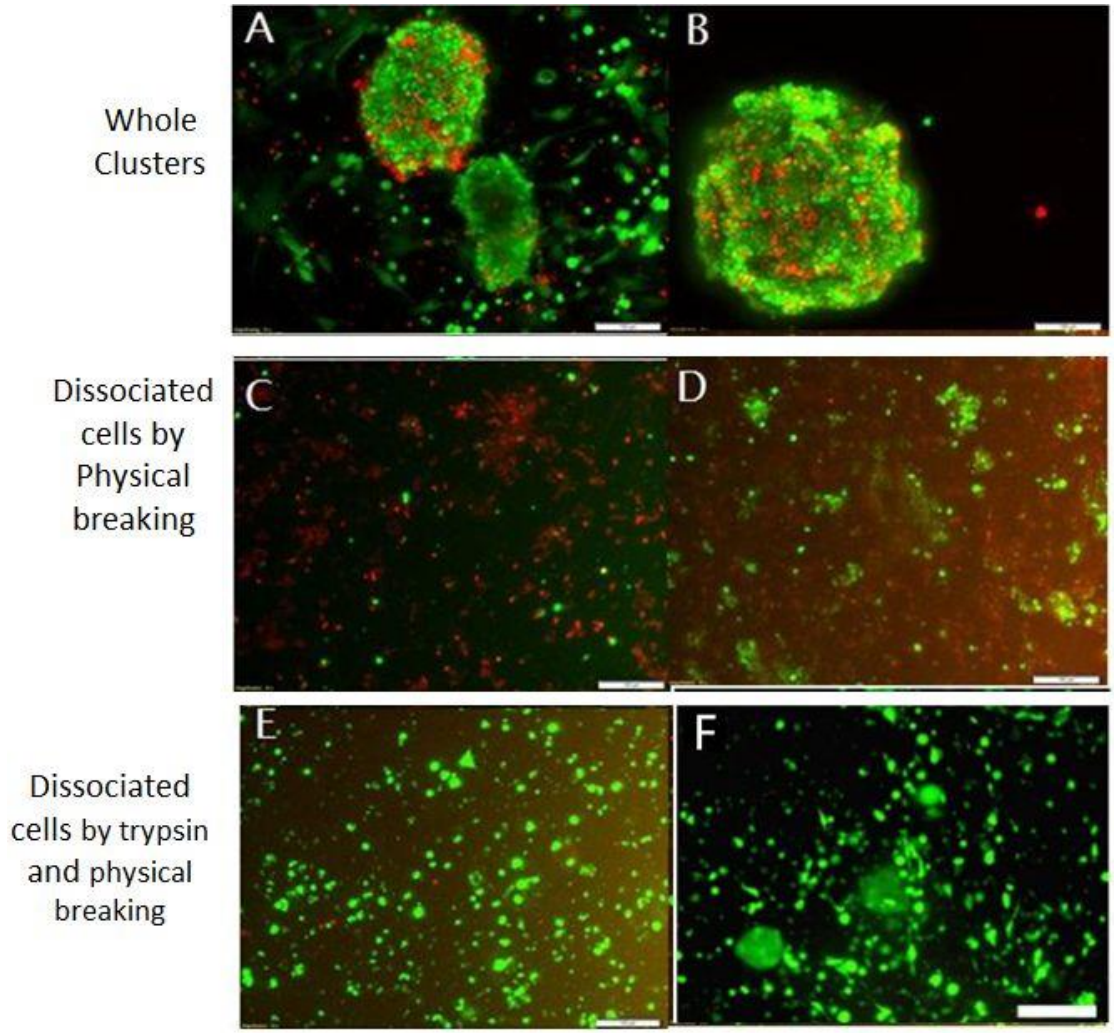

Figure 3.7 Live/Dead staining of whole and dissociated IPC clusters in collagen gels on Day 5. (A) and (B) whole clusters without dissociation, (C) and (D) Cells dissociated from clusters, processed by a 3 ml syringe, 25G needle and a 40 μm strainer. (E) Dissociated cells, generated by trypsin, a 3 ml syringe, 25G needle and a 40 μm strainer, and (F) Dissociated cells generated by trypsin and cell strainer, which was from the previous work in our lab. Live cells were stained green by calcein, dead cells were stained red by ethidium homodimer. Scale bar is 100 μm.

To examine whether the dissociation process is useful to reducing the number of dead cells, different dissociation methods were used. A 25G needle, syringe and 40 μm cell strainer were used to dissociate the whole clusters into small pieces and single cells, and the cell strainer was used to remove the debris and dead cells. It was found that using trypsin before physical

dissociation helped to reduce dead cells, whereas the clusters dissociated without trypsin resulted in a lot of dead cells, as demonstrated in Figure 3.7. Meanwhile, there were much less dead cells in the dissociated cells by trypsin and physical breaking, when compared with the whole clusters. By comparing the dissociated cells generated by trypsin and physical breaking with the previous work using the same dissociation method in our laboratory, it was demonstrated that the well dissociation process helped to reduce dead cells in 3D collagen tissue.

3.3.3 Survival of IPC Clusters in 3D Tissues under Static and Flow Culture Conditions

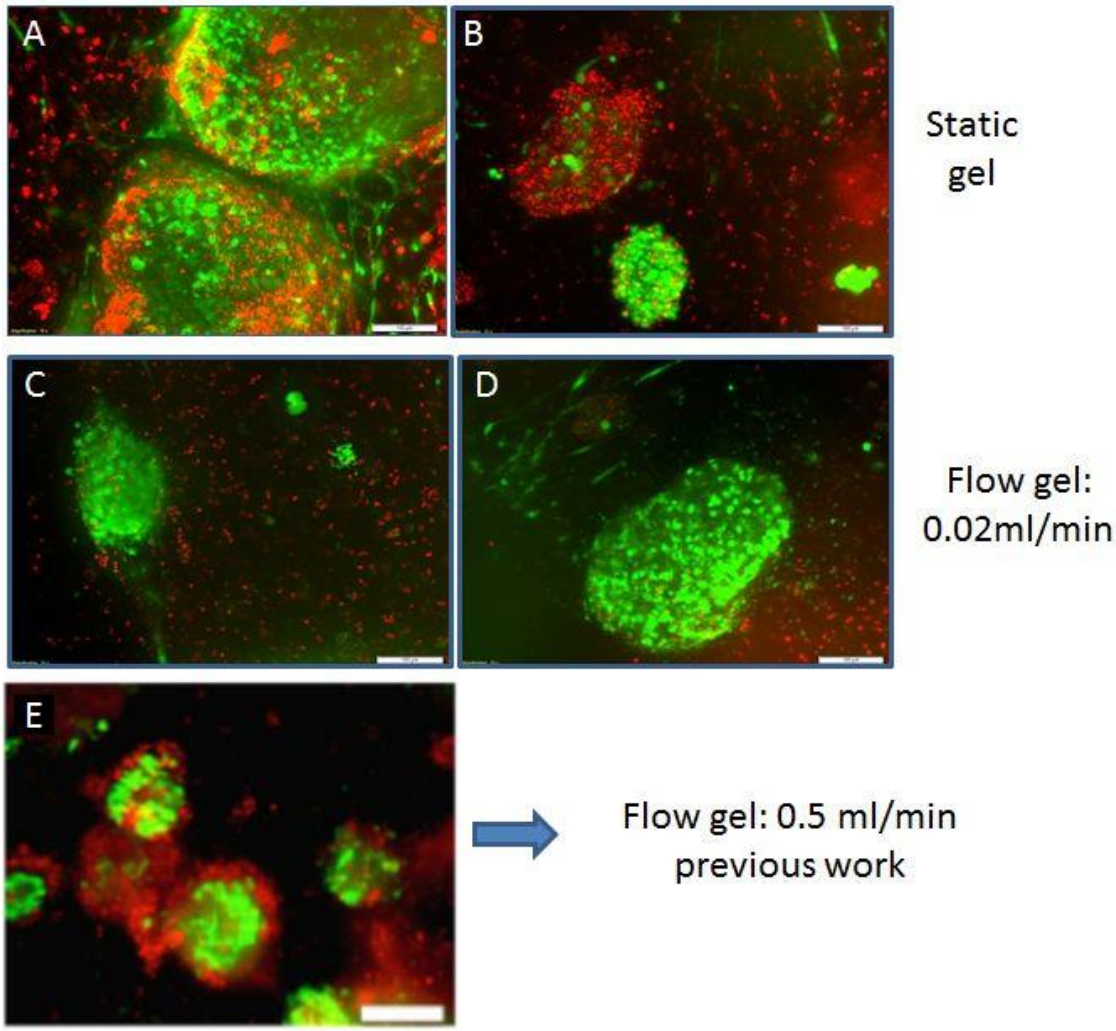

Figure 3.8 Live/Dead staining of collagen gels on Day 5. (A) and (B) show IPC clusters in flow gels with a flow rate of 0.02 ml/min. (C) and (D) show IPC clusters in static gels, (E) shows IPC clusters at a flow rate of 0.5 ml/min, from the previous work in our lab. Scale bar is 100 μm.

The bioreactors were used to provide constant perfusion of differentiation medium through the collagen tissue, cells viability was compared under static and flow conditions after 5 days of culture, as shown in Figure 3.8. Firstly, by comparing viability of IPC clusters in static gels and flow gels, more dead cells were found in the static gels. The survivability of IPC clusters was better in flow gel condition than in static condition. It indicated that constant medium at proper flow rate enhances viability of IPC clusters, and providing sufficient nutrients to the

cells. Secondly, by comparing the survivability of IPC clusters in flow gels at different flow rate, it was found that more dead cells were generated when exposed the cells to the flow rate of 0.5 ml/min. The flow rate used in previous study in our lab was chosen to be 0.5 ml/min, as it was previously proven in previous work in our lab, this value corresponds to a shear stress of approximately 0.71 dyn/cm^2. However, while the individual rat islets *in vivo* are normally exposed to a flow rate of 2 x 10^{-5} ml/min of blood [81], the flow rate was demonstrated to be high and caused the gels compaction. The flow rate at 0.02 ml/min in this study showed that a lower flow rate reduces cell death with a much smaller shear stress of 0.156 dyn/cm^2.

3.4 Discussion

In this study, we successfully further isolated the IPCs in the average size of 183 μm, whereas the average size of former isolated whole clusters was 369 μm, the size of further isolated IPCs was similar to the size of native mouse islets [62]. Survival of IPCs in 2D suspension culture was examined during long-term culture of 7 days, and then the results showed that more dead cells were generated as well as IPCs lost their characteristic of DTZ positive gradually. The survival of IPCs was compared in 2D suspension culture and in 3D static collagen gel, but it was difficult to tell from the staining images. Further studies are needed to compare the survival in 2D and 3D culture conditions, including the expression of PDX1, Pax4 or Nkx6-1 and glucose challenge of IPCs.

Dissociation process was applied to remove the dead cells in the IPCs, the results

demonstrated that using trypsin and physical breaking helped to reduce dead cells in collagen gel tissue, and the dead cells were also much less than the whole clusters condition. Furthermore, the flow condition was used to examine whether the perfusion flow improves viability of IPCs in 3D collagen gel tissue. By comparing the survivability of IPCs at a flow rate of 0.02 ml/min with static gel conditions, it was obvious that IPCs in the flow condition were more viable than the IPCs in static condition. The flow rate was chosen to be 0.02 ml/min based on the previous studies in our laboratory, which a flow rate of 0.5 ml/min was used. The value of 0.5 ml/min was related to a shear stress of approximately 0.71 dyn/cm^2, it has been shown to be in the physiological range in microvessels in *in vivo* conditions [77]. However, the collagen gel underwent a compaction and caused IPCs death when exposed to the flow rate of 0.5 ml/min. In this study, a much lower flow rate of 0.02 ml/min was used to minimize the gel compaction due to the high velocity of flow. The shear stress was much lower as 0.156 dyn/cm^2 based on the data from the research of a novel flow bioreactor [77]. By comparing the survivability with the IPCs in 0.5 ml/min flow rate, it demonstrated that the lower flow rate reduces compaction of the gels and enhances the viability of IPCs. The results demonstrated that reducing the flow rate is capable to enhance IPCs survivability.

In the future work, the shear stress needs to be calculated based on this flow rate, while in this study, the flow rate of 0.02 ml/min was chosen based on the previous study, the shear stress was still unknown. Pressure transducers needs to be applied to get the pressure, thus by the equations of Darcy's law and modified Brinkman equation [77], it is easy to get the accurate shear stress and better study the influences of flow rate. In addition, as the

individual pancreas islet is exposed to the blood at the flow rate of 2 x 10^{-5} ml/min [81], it is still much lower than the flow rate in this study, lower flow rate needs to be studied in the future.

CHAPTER 4

SUMMARY AND FUTURE DIRECTIONS

In summary, three optimization strategies including different time period for spontaneous formation of suspension culture, different cell seeding density for the induction of pancreatic cells differentiation and different collagen coating conditions for the induction of pancreatic lineage were investigated in this study.

4.1 Achievements

More than 7 groups of samples were used for statistical analysis to examine the significant difference of each optimization strategy, which makes the results more reliable. However, the statistical analysis showed that there was no significant difference between each condition. Two days and 3 days suspension culture of EBs for the spontaneous differentiation have no difference, as well as 2X digested cell seeding density was proven to be unable to improve the yield of IPC clusters compared with 1X seeding density by this differentiation protocol.

Though co-culture system with endothelial cells in differentiation process was demonstrated to be able to increase the production of IPCs in some studies [60, 66, 82], the co-culture system failed to increase the yield of IPC clusters in this study. The co-culture system was set up as the collagen coating gel with ECs for digested cells in the last stage of differentiation, to induce pancreatic cells from early progenitors. The co-culture system in other studies was used in the initial stage with embryonic bodies [60]. It is supposed that applying ECs may not be useful in the last stage of differentiation, or the density of ECs was

not high enough to provide the growth factors for the differentiation.

Furthermore, to investigate the MES cell-derived IPCs generated in this study, several trials were attempted to use western blotting to detect insulin, PDX1 and Glucagon, whereas the common methods for the detection are RT-PCR and immunofluorescence. Glucagon was successfully detected by western blotting but insulin and PDX1 were not shown up on the blotting membrane, even insulin was demonstrated by insulin ELISA from the same batch of IPC clusters. Glucose responsiveness of the MES cell-derived IPCs was not obvious by the results of ELISA, thus it is supposed that the high glucose medium used before glucose challenge affects the responsiveness, or the function of IPCs were destructed.

Even though the yield of IPC clusters and efficiency of differentiation were not obviously increased by these optimization strategies in this study, it was found that the generated IPC clusters were better in the quality than the IPC clusters generated by the original protocol in previous work. DTZ positive clusters showed dark red by the dense composition of IPCs while the IPC cluster showed pink in previous study, indicating lower density of IPCs generated in the clusters. In addition, it was found that the secreted insulin concentration was much higher than the previous study. An enhancement in quality was generated by these optimization strategies, though the increase was not obvious in quantity.

Finally, the 3D collagen gel tissue was developed by the isolated IPCs, while the dissociation method using trypsin and physical breaking was shown to be useful to improve the survivability of IPCs, the flow condition at the flow rate of 0.02 ml/min also resulted in a better IPCs survivability than the flow rate of 0.5 ml/min from previous work in our

laboratory.

4.2 Limitations and Future Directions

The calculation method for efficiency of differentiation is important for the research. Other studies used flow cytometry and immunofluorescences are used to count the IPCs and total cells [25, 83]. The efficiency was calculated as DTZ positive clusters/ total clusters in this study. This however, doesn't precisely reflect the overall efficiency as there are many other cell types present in the culture and also not all clusters become DTZ positive clusters. However, relatively good approximation of the efficiency can be obtained from this simple method since most of the DTZ positive IPCs are from clusters.

For the influence of cell seeding density, testing a gradient cell seeding density in different differentiation stages is needed in the future works. The best cell seeding density is supposed to be lower than 2X but higher than 1X. Using a gradient cell seeding density such as 1X, 1.5X, 2X together may help to find out the best cell seeding density based on the original protocol. Moreover, to promote the generation of mature IPCs by co-culturing with ECs, more work needs to be done including using mixed medium, adding more ECs in the coating gels, and investigating the co-culture system in initial differentiation stage of spontaneous differentiation. Different types of ECs also need to be investigated for the co-culture system.

In addition, the results of glucose challenge are shown as secreted insulin/total intracellular protein in some of the current studies. In this study, the results were presented

the same way with the previous work with the secreted insulin concentration directly from ELISA assay. The extraction of intracellular protein can also be done in the future and present glucose challenge as secreted insulin/total intracellular protein. This will allow direct comparison of the results with the other studies. The low glucose medium should also be applied for the glucose challenge in the future, as the glucose responsiveness was weak in this study with the use of high glucose medium before glucose challenge. It was reported that exposure to high glucose medium de-sensitized the cells in response to glucose challenge [67-69]. It might be able to restore the function of glucose responsiveness by switching cells to a low glucose medium in the last stage of differentiation. Thus, future studies are needed to investigate the influence of high glucose culture medium.

Lastly, lower flow rate of 0.02 ml/min was demonstrated to reduce cell death in this study. However, the shear stress of 0.156 dyn/cm^2 in this study was just calculated based on the previously reported data [77], it needs to be accurately estimated. The pressure difference at the inlet and the outlet of the bioreactor can be monitored in the future to accurately estimate the shear stress caused by the low flow rate. In addition, further studies are needed to examine whether there are improvements in functions of the IPCs, including glucose responsiveness and expression of transcription factors such as PDX1 and insulin. In addition, as it was demonstrated that a low flow rate is capable to reduce dead cells in 3D collagen gel for 5 days culture, longer time periods such as 7 days or 10 days are required to be investigated for the survivability of IPCs in 3D collagen tissue in perifusion system. Other assays are also required including RT-PCR and immunofluorescence to investigate the

expression of transcription factors, as well as glucose challenge to test the response to glucose stimulations.

APPENDIX A

CELL CULTURE MEDIUM

The culture medium recipes for all presented studies are shown below. The mediums were stored at 4 ℃ and made in sterile glass bottles. All the mediums were used within two or three months of preparation.

Mouse Embryonic Fibroblast (MEF) Culture Medium:

DMEM (High Glucose)	500 ml, Life Technologies, 11965-084
FBS	58 ml, Life Technologies, 16000044
100 X L-Glutamine	5.8 ml, Life Technologies, 25030
100 X Pen./Strep.	5.8 ml, Life Technologies, 15140
100 X NEAA	5.8 ml, Life Technologies, 11140
100X Na-pyruvate	5.8 ml, Life Technologies, 11360

Mouse Embryonic Stem Cell Culture Medium

DMEM (High Glucose)	500 ml, Life Technologies, 11965-084
Knock Out Serum Replacement	90 ml, Life Technologies, 10828
100 X L-Glutamine	6 ml, Life Technologies, 25030
100 X Pen./Strep.	6 ml, Life Technologies, 15140
100 X NEAA	6 ml, Life Technologies, 11140
100X Na-pyruvate	6 ml, Life Technologies, 11360
2-Mercaptoethanol	4.4ul, Sigma, M6250

LIF is recommended to be thawed out freshly and added to the aliquots of media before use (100 ul/ 50 ml of medium).

Differentiation Medium #1

IMDM	400 ml, Life Technologies, 12440-061
FBS	100 ml, Life Technologies, 10828
100 X L-Glutamine	5 ml, Life Technologies, 25030
100 X Pen/Strep.	5 ml, Life Technologies, 15140
1-Thioglycerol	3.9 ul, Sigma, M6145

Differentiation Medium #2

DMEM/F 12 medium	1000 mls, 12500-062
Progesterone	10 ul [Stock 2mM; Final conc. 20 nM, Sigma]
Putresciene	100 ul [Stock 1M; Final conc. 100 uM, Sigma]
Laminin	1 ml, Sigma L2020
Nicotinamide	1 ml [Stock 1M; final conc. 10mM, Sigma]
Insulin	6.25 ml, Life Technologies, 12585-014
Sodium Selenite	100 ul [Stock: 300 uM; Final conc 30 nM, Sigma]
Apo transferrin	50 mg, Sigma T1147
B 27	20 mls, Life Technologies, 17504-044
Penn Strep	10 mls, Life Technologies, 15140

Endothelial Cell Culture Medium

MCDB 131 Medium	1000 ml, Sigma, M8537
FBS	100 ml, Life Technologies, 10828
Penn Strep	10 ml, Life Technologies, 15140
Endogro	4 ml, VEC Technologies

APPENDIX B

REAGENTS RECIPES

This appendix includes all the recipes for the different reagents used in this study. The reagents were stored at 4 ℃ in the fridge.

Krebs Ringer Bicarbonate Hepes (KRBH) Buffer

Solution A:	
Sodium Chloride	6.92 gm.
Potassium Chloride	0.36 gm.
Monopotassium Phosphate	0.16 gm.
Calcium Chloride	0.38 gm.
Magnesium Sulfate Heptahydrate	0.3 gm.
De-Ionized (DI) Water	200 mls.

Solution B:	
Sodium Bicarbonate	2.08 gm.
DI Water	160 mls.

Solution A	200 mls.
Solution B	160 mls.
HEPES	2.4 gm.
BSA	2 gm.

The contents were mixed and the volume was set to 1 liter by DI water. The pH was adjusted to 7.4 and the solution was filtered through a 0.22 μm filter. The KRBH buffer was stored at 4 ℃.

All the reagents were purchased from Sigma.

Blocking Buffer for Western Blot Applications

A 5% BSA or 5% non-fat dry milk solution was prepared using TBS-Tween buffer (Boston Bioproducts). The solution was placed on a magnetic stir plate till the BSA was completely in solution. The solution was then stored at 4 ℃.

Lysis Buffer Cell Lysate Preparation

RIPA Buffe	500 μl
Protease Inhibitor	5 μl
Triton-X 100	5 μl

APPENDIX C

PROTEIN ASSAY PROTOCOLS

This appendix describes the protocol used for the protein quantification by Bradford assay.

Bradford Protein Assay

Bradford reagent will be needed to perform this assay.

1. Prepare and label the correct number of cuvettes: # of your sample + 1 blank + 7 BSA standards (0.125mg/mL, 0.25 mg/mL, 0.5 mg/mL, 0.75 mg/mL, 1 mg/mL, 1.5 mg/mL, 2 mg/mL).
2. Add 20ul of each sample, standards or RIPA buffer to the cuvettes.
3. Add 1 ml of Quick Start Bradford Dye Reagent, warmed to room temperature, into each cuvette. Pipette well to mix. At this point a color change will be observed.
4. Incubate 5 minutes a room temperature before further processing.
5. Start up the BioRad smart Spec plus Spectrophotometer.
6. Measure the absorption values and the concentration of the sample.

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