The role of decellularized matrix in directing differentiation of pancreatic progenitor cells in pancreatic endocrine cell fate

SUBMITTED TO THE FACULTY OF THE GRADUATE SCHOOL
OF THE UNIVERSITY OF MINNESOTA
BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

James Dutton

November 2011

Abstract:

The direct differentiation of induced pluripotent stem cells derived from somatic cells and pancreatic progenitor cells could generate functional β-cells that secrete insulin, and are also glucose responsive. However, the cellular signals and interactions between the pancreatic epithelium and its surrounding mesenchyme that govern pancreatic specification and differentiation of endoderm into pancreatic progenitor cells and eventually mature beta cells are not fully understood.

In this study, I examined various conditions that would direct the induced pluripotent stem cells (iPSCs) derived from a pdx1: GFP mouse, or normal pancreatic progenitor cells, to predominately β cell fate. It involves the "guided" differentiation of iPSCs to a pancreatic lineage and also the use of a decellularized matrix to induce differentiation into. A decellularized matrix may help mature β-cells since decellularization has been shown to remove all the organ's cells while preserving the composition and biological activity of the extracellular matrix[1]. Thus, decelluarization has several advantages: it removes cells to avoid any immune response post-implantation and maintains the native environment and membrane components that provide cellular growth and maturation of β-cells.

The results showed that the guided differentiation of iPSCs by activin A induced definitive endodermal and pancreatic progenitor cells. Moreover, the decellularized matrix increased exocrine and endocrine gene expression as compared to gelatin and fibronectin, and assisted survival and maturation of all pancreatic cell types. Hence, this novel approach would be useful to produce insulin-expressing β-cells that are also glucose responsive and generate surrogate cells for diabetes therapy.

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Introduction:

Significance:

Diabetes is marked by high levels of blood glucose due to defects in insulin production. There are two types of diabetes: type I diabetes is characterized by the loss of β-cells and develops when the body's immune system destroys pancreatic beta cells. Beta cells make insulin that regulates blood glucose. Thus, in type 1 diabetes, the body cannot make insulin. Type 1 diabetes account for approximately 5% of all diagnosed cases. The risk factors that are associated with type 1 diabetes are: autoimmunity, genetic, or environmental. Type 1 disease is not preventable; moreover, individuals with type 1 diabetes must have insulin by injection or a pump daily. Type 1 diabetes is common in children and young adults; although, onset of this form of diabetes can occur at any age.

In adults, type 2 diabetes accounts for approximately 90-95% of all diagnosed cases. Initially, the onset begins as insulin resistance in which cells fail to use insulin properly and eventually the pancreas loses its ability to produce it as the need for insulin rises. Type 2 diabetes is associated with obesity, family history, impaired glucose metabolism and race/ethnicity. African Americans and Hispanic/Latino Americans have high risk for type 2 diabetes.

In the United States, 8.3% (25.8 million) of the population are diabetic of which 18.8 million are diagnosed while 7.0 million are undiagnosed. Moreover, 79 million people are pre-diabetic with 1.9 million new cases of diabetes were diagnosed in people 20 years and older in 2010[2]. As the incidence of diabetes continues to rise in the United States, cell replacement therapy provides hope for patients with type 1 and 2 diabetes. Currently, there are three major research avenues investigating ways to combat this disease: β cell regeneration, β cell replacement and direct differentiation of stem cells into functional β cells

Regeneration is induced by the profound loss of β cells and experimentally only with extreme loss of β cells approximately 99% did this conversion occur[3]. β cell regeneration involves that conversion of adult α cells into β cells after depletion of all pancreatic β cells. In this case, β cell regeneration is not spontaneous. Moreover, this approach does not account for the autoimmunity that exists in type 1 diabetes.

In adult mouse and humans, β cells replication is a major mechanism by which pancreatic β cells are restored. Therefore, stimulating the replication of β cells is one method in which destroyed β cells could be replenished. Yet, in type 1 diabetes, most if not all of the β cell mass has been destroyed and it is not known if the residual β cells could be stimulated to replicate to a mass adequate to control blood sugar levels. Nonetheless, this would require the blockage of the immune attack on newly replicated β cells, so that they could function. β-cell regeneration could potentially generate insulin-producing β-cells for type 2 diabetes. Thus, for type 2 diabetes therapy, increasing β cell mass by stimulating replication could cure this form of the disease.

Another approach to replacing β cells is to reprogram adult differentiated cells into β cells. For example, adult exocrine cells can be directly reprogrammed into insulin-producing cells by the expression of just three transcription factors. Temporary expression of Pdx1, Ngn3 and MafA, by viral infection of the pancreas *in vivo*, leads to the conversion of exocrine cells to endocrine cells[4]. The direct cell conversion or trans differentiation mechanisms into β cells can be elucidated to other cell types. Overall, it is still unclear whether β cell replication or trans differentiation into β cells could be used for diabetes therapy.

The third approach to generate more β cells is to direct the differentiation of embryonic or pluripotent stem cells into functional β cells. It involves recapitulating normal development *in vitro* from stem cells to fully differentiated β cells, taking developmental cues in attempt to identify the signals required to induce a specific cell type. Other researchers have explored this approach through the application of growth factors and other small molecules as signaling inducers. It has been shown that iPSCs can be guided to differentiate into pancreatic lineages by the addition of growth factors. Even though, they observed cells which were insulin+, they were not glucose responsive suggesting that they were not fully mature β cells[5]. Only partial differentiation into a β-like cell occurred and a fully mature β cell was not generated. Another approach to direct differentiation using a decellularized rat matrix will be discussed in this paper.

Hence, the replacement of destroyed pancreatic β cells or the generation of more β cells could be used as therapy for patients with type 1 and 2 diabetes. However, the cellular signals and interactions that govern differentiation of pancreatic progenitor cells into a "true" β cell are not fully understood. Perhaps, the guided differentiation of iPSCs or pancreatic progenitor cells toward an endocrine fate could provide insight into the cellular interactions that governs pancreatic foregut differentiation into newly formed mature β cells and yield potential therapies for diabetes.

Pancreatic development

The pancreas is highly branched organ, which consist of endocrine and exocrine cells. The endocrine cells secrete hormones (i.e. insulin and glucagon) into the bloodstream while the exocrine cells secrete digestive enzymes (i.e. amylase) into the ducts and are derived from dorsal and ventral pancreatic buds on the primitive gut tube. In the developing embryo, sonic hedgehog (Shh) is expressed in most of the entire gut epithelium. However, the inhibition of Shh expression is required for pancreas formation[6]. The dorsal pancreatic bud forms where the notochord contacts the gut roof. The ventral pancreatic bud does not contact the notochord, and is derived from the ventral endoderm and is formed in the absence of Fibroblast Growth factor (FGFs). Absence of FGFs and cardiogenic mesenchyme, however, will lead to "default" differentiation into ventral pancreas[7].

The developing pancreas undergoes two transition states: the first is called the primary transition and occurs at embryonic day (E) 8.5 in mouse. It involves lineage commitment in which a cell or tissue region indicated that it is programmed to follow a particular development pathway or fate [8]. The secondary transition occurs at E12.5 prior to pancreatic endocrine differentiation while post-secondary transition involves directed differentiation of endocrine cells towards a specific cell type. It occurs at E14.5 when endocrine cells differentiate primarily toward β cells[9].

The development of the pancreas is regulated by various cellular signals and specific transcription factors that regulate the timing of differentiation of pancreatic cell types. Fibroblast Growth Factors (FGFs) are expressed in several epithelial-

mesenchymal interactions and these interactions are important to regulate branching morphogenesis in the development of the pancreas as well as other organs. It has been established that mesenchymal FGF signaling plays a role in the induction of the dorsal pancreatic bud. Moreover, FGF 10 is expressed in the mesenchyme and it is essential for continuous pancreas outgrowth[10].

In addition to FGF, other extracellular signaling pathways are involved in pancreatic development. The TGF-β super-family represents a large signaling family in nearly all known biological process, but most especially, in development processes. There are three major sub-divisions within this superfamily: TGF-β isoforms 1, 2, and 3 in mammals, activin/inhibin/nodal, and BMPs. TGF-β molecules signal through a large family of heterodimeric receptors to activate smads and other intracellular pathways to initiate specific cellular responses.

Activins and Bone Morphogenetic Proteins (BMPs) are two other key sub-families within the TGF-β superfamily. Activins and BMPs share many binding partners, receptors, and inhibitors. They participate in signaling in a large number of developmental processes. Activins have been found to be expressed in early gut endoderm[11]. Activin A is involved in cell growth and differentiation. Activins interact with two types of cell surface transmembrane receptors, which have intrinsic serine/threonine kinase activities in their cytoplasmic domains. Activin A binds to the type II receptor and initiates a cascade reaction that leads to the recruitment, phosphorylation, and activation of type I activin receptors. This activation is followed by phosphorylation of SMADs2/3, two of the cytoplasmic SMAD proteins. SMAD3 then translocates to the nucleus and interacts with SMAD4 through multimerization, resulting in the activation of transcription factor complexes responsible for specific gene expression[12]. Several studies have shown the addition of exogenous TGFβ enhanced endocrine differentiation and increased the formation of endocrine cell types[13].

Transcription factors are essential to initiate specification during pancreas development. Pancreatic duodenal homeobox 1 (Pdx1) is essential in regulating early pancreatic specification. Pdx1 deficiency leads to pancreatic agenesis[14]. In early

pancreatic development, pdx1 is expressed within the entire pancreatic epithelium. Pdx1 expression is then downregulated as pancreatic progenitor cells differentiate to an endocrine cell lineage. However, as endocrine cells begin to differentiate toward the insulin-positive β-cell lineage, pdx1 reappears, and is known to be necessary for proper glucose responsive regulation of insulin synthesis in β-cells[15].

Likewise, pancreas-specific transcription factor 1 (PTF1a or P48) is expressed in the dorsal pancreas. Unlike Pdx1, P48 is not expressed in other parts of the epithelium. P48 is required for exocrine specification and its expression is critical for exocrine development, and PTF1a appears to play an important role in early specification of pancreatic progenitor cells. PTF1a is first expressed slightly later than pdx1, at E9.5, specifically in cells of the foregut endoderm destined to give rise to dorsal and ventral pancreas[16]. Moreover, transcription factor Neurogenin 3 (Ngn3) is important for the specification of endocrine linages and the formation of the four endocrine cell types.

Guided differentiation of pancreatic progenitor cells into an endocrine lineage

Originally, it was believed that a committed cell had no ability to dedifferentiate and be respecified into a new cell type. However, in recent years, it has been established that cells have inherent flexibility and plasticity in that differentiated cells can be respecified into another cell type with a new identity that differ at the molecular, cellular and functional levels. Moreover, recent publications have shown specified cell types possess the ability to change their identity by the introduction of specific transcription factors, environmental stress (i.e. extreme β cell loss) and addition of growth factors[17].

The introduction of specific transcription factors could induce cellular reprogramming. In 2006, induced pluripotent stem cells (iPSCs) were generated from somatic cells (i.e. mouse embryonic or adult fibroblast cells) by a retroviral-mediated transfection of four factors, Oct3/4, Sox2, c-Myc, and Klf4[18]. Thus, a cocktail of transcription factors needed to maintain a pluripotent state can erase a differentiated cell identity and revert it to an embryonic pluripotent stem-like cells. A year later, iPSCs were

generated from adult human dermal fibroblast using the same four transcription factors: Oct3/4, Sox2, Klf4, and c-Myc[19] demonstrating that iPSCs could be derived from adult human fibroblast somatic cells as well. However, iPSCs generated in *vitro* using retroviral vectors with c-Myc, an oncogene that could generate tumorigenic cells from insertional mutagenesis and chromosomal aberrations; therefore limiting iPSCs applications in regenerative medicine. There are new methods now being utilized to prevent the integration of c-Myc vector.

In contrast, direct cell type conversion involves direct reprogramming of a differentiated cell into another cell type and has been induced in many cell lineages. For instance, adult pancreatic α-cells converted into β-cells after extreme β-cell loss[20]. Moreover, three transcription factors: Gata4, Mef2c, and Tbx5 can reprogrammed postnatal dermal fibroblast directly into differentiated cardiomyocyes-like cells that expressed specific cardiac markers[21]. Direct cell conversion has been observed with the trans-differentiation of fibroblast into functional neural stem/progenitor cells (NPCs)[22].

Recently, the induction of hepatocytes-like cells derived from mouse fibroblast with the addition of defined transcription factors was shown. The combination of Hnf4α plus FoxA1, FoxA2 or FoxA3 can convert both mouse embryonic and adult fibroblast into hepatocytes-like cells. Even though, the induced cells were not exactly like liver cells, they had characteristics of hepatocytes. For instance, their global gene expression were cluster closely with hepatocytes, but separated from mouse embryonic fibroblast (MEF). Moreover, the induced hepatocyte-like cells possessed gene expression patterns of liver cells involved in fat, cholesterol, and glucose metabolism; though, some differences existed between the two types of cells. Unlike the generation of iPSCs, direct cell conversion does not generate pluripotent stem cells, but cells with limited plasticity. Though, both processes involve the conversion of somatic cells into another cell type.

Another mechanism utilized in my thesis project was the directed differentiation of iPSC or pancreatic progenitor cell type into a predominately β cell population. The expression of pdx1 pancreatic progenitor cells is essential to generate iPSCs-derived insulin producing cells. However, the spontaneous differentiation of iPSCs when cultured would generate a heterogeneous population of cells. To direct differentiation in a specific

cell fate, growth factors that play an essential in organ development may be used to induce a homogenous population of a specific cell types. Activins are expressed in the early pancreatic rudiment. Activin A and B localize to the developing pancreatic endocrine cells, particularly in glucagon-positive cells[23]. Therefore, growth factors can direct differentiation of one cell type into another.

During pancreas organogenesis, the mesenchyme secretes growth factors to promote development. Previous studies have shown that embryonic stem cells/iPSCs can be guided to differentiate into pdx1-expressing regional-specific definitive endoderm using growth factors. Activin, Fibroblast growth factor (FGF), BMP and retinoic acid (RA) are critical for further differentiation into Pdx1+ expressing cells. The determination of pancreatic fate involves the activation of pdx1 gene[24]. Moreover, when transplanted under kidney capsules, pdx1 positive cells further differentiate into all three pancreatic lineages: endocrine, exocrine, and ducts cells[25].

In addition, it has been shown that mouse embryonic stem cells (ESCs) grown on synthesized basement membrane can differentiate into pancreatic lineages when grafted under mouse kidney capsule; although endocrine and exocrine markers were highly upregulated and 113 ng of insulin per islet was detected from *Ins1*-positive graft, and this approximately correlated with the insulin content of one islet, which is 1000 cell-equivalents. However, glucose-responsive insulin secretion in graft was not observed[26]. Hence, mESCs differentiation in pdx1-expressing definitive endoderm failed to produce glucose responsive β-cells. Unlike ESCs, iPSCs derived from patient's fibroblast do not elicit an immune response.

Like ESCs, iPSCs differentiation in $pdx1^{+}$/$insulin^{+}$ expressing cell failed to produce glucose responsive β-cells. Even though, these two publications provided no evidence that these $insulin^{+}$ cells were also glucose responsive β cells. They presented the possibility that a similar "guided differentiation" event could be induced with growth factors or a decellularized matrix with all the extracellular matrix components.

Another paper showed that the addition of retinoic acid to cultured human embryonic stem cells (hESCs) derived endodermal cells can induce the formation of $Pdx1^+$ cells. Moreover, the presence of retinoic acid can also prevent hepatic differentiation of endodermal cells. The further differentiation of the RA-treated cells generated insulin-expressing cells, whereas no $insulin^+$ cells were found in cells that had not been treated with RA[27]. Hence, retinoic acid directs differentiation of endodermal cells towards a pancreatic fate and plays an important role in altering the fate of the anterior endoderm by promoting the expression of $Pdx1^+$ pancreatic progenitor cells.

The mesenchyme plays an important role in pancreatic development. It provides the extracellular matrix for growth and differentiation by secreting factors to promote pancreatic differentiation. However, efforts to mimic pancreatic differentiation in the presence of these diffusible factors have not been successful. Moreover, the use of synthetic scaffold to promote differentiation of pancreatic progenitor cells towards a β cell fate have been limited because of the failure to mimic the role of factors secreted by the mesenchyme during pancreatic development[28].

One solution to this problem could be a decellularized matrix. Once decellularized, the native pancreatic matrix retains its extracellular matrix, and 3-dimensional (3D) structure. In a paper published in nature, Dr. Taylor's group showed the preservation of the underlying extracellular matrix of decellularized rat hearts. Within the decellularized heart matrix, Collagen I and III, laminin and fibronectin were preserved; even though, cardiac cells were removed.

Perhaps, a decellularized pancreas tissue could be used to support and control the pancreatic progenitor cells fate at different stages during differentiation into a predominantly β cell population since the interaction between cells into their surrounding extracellular matrix is essential to instruct and promote a specific cell phenotype.

In addition, a native pancreas environment may support the maturation of $pdx1^+/insulin^+$ and immature β cells into mature β cells that secretes insulin and also are glucose responsive. Hence, studying the role of decellularized tissue's extracellular matrix on pancreatic progenitor cells behavior in vitro is physiologically relevant because

this approach provides insight into the decellularized matrix involvement in the directed differentiation of a pancreatic progenitor cell into specific pancreatic cell types.

Specific Aim (s)

1. To specify pluripotent iPSCs into definitive endoderm and pancreatic endocrine cell types.

2. Mature pancreatic progenitor cells into insulin producing and glucose responsive cells using a decellularized Rat pancreas matrix.

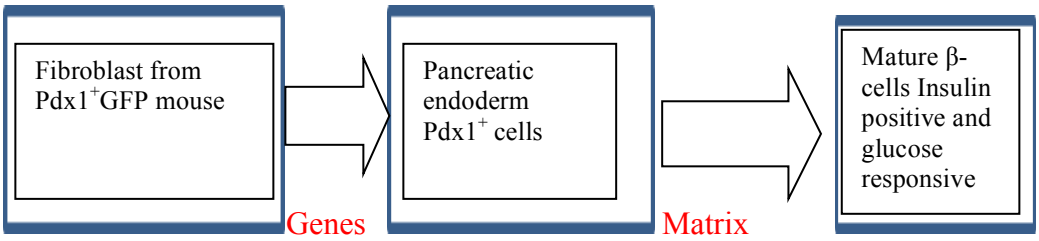

Figure 1: schematic of research overview

Another goal is to analyze the role to growth factors in directing iPSC cells into pancreatic endoderm. In the past decades, there have been multiple studies analyzing the differentiation patterns of both human and mouse embryonic stem cells into pancreatic cell types via spontaneous differentiation, and the formation of multi-lineage progenitors. Yet, none of these approaches led to the formation of a homogenous population of pancreatic progenitor cells and ultimately an increase in the generation of pancreatic endocrine lineage.

Moreover, the spontaneous differentiation of these cells led to the generation of a heterogeneous population of cells and a give rise to a low percentage of definitive endodermal cells. Hence, the use of growth factors may induce directed differentiation of one cell type into another.

In previous experiments, Activin A has been showed to induce differentiation of embryonic stem cells into definitive endoderm and further into pancreatic endocrine lineages and this induction depends on its concentration, culture conditions and time of

exposure to culture cells[29]. Moreover, the serum composition within medium is also essential to generate activin A induced endodermal and pancreatic cell types. Hence, I hypothesized that the addition of activin A would induce "guided" differentiation into definitive endoderm and eventually to a pancreatic cell fate.

Ultimately, the research goal was to investigate the instructive nature of a decellularized pancreatic matrix and its ability to induce and direct foregut endoderm progenitor cells into an endocrine fate. Initially, my project involves two specific aims. First, the direct differentiation of L4-3 iPSCs derived from pdx1: GFP mouse into Embryoid bodies (EBs) and ultimately to an endocrine lineages. Second, seed decellularized pancreatic matrix with partially differentiated iPSCs pdx1 progenitor cells or pancreatic buds from embryonic (E14) mouse embryos. E14 is prior to post-secondary transition during mouse pancreas development.

MATERIALS AND METHODS:

Cell Culture

Plating feeder:

Gelatinized one 6 well tissue cell plate with 2mL gelatin and incubated at 37°C for 30 minutes. Obtained one vial of irradiated MEF feeder cells from liquid nitrogen and de-iced in the water bath, 1mL of feeder cells from feeder was added to 4mL of MEF medium in a 15mL conical tube, centrifuged at 1000 rpm from 5 minutes, removed supernatant, resuspended into 5mL of MEF medium, and then counted cells to get 230,000 feeder cells per well. The gelatin was then removed from each of the 6 well plates and then 1mL of MEF medium was added. Next, 1mL of the feeder cells in MEF medium was then added to each well and incubated at 37°C overnight.

iPSC growth and differentiation

One vial of frozen iPSCs was obtained from nitrogen liquid container and de-iced, and lmL of cells are transferred to a 15mL conical tube containing 4mL of mES medium, centrifuged at 1000 rpm for 5 minutes, the supernatant was removed and then resuspended into 5mL of mES medium. The MEF medium was aspirated off the feeder cells plated the day before, then 1mL of mES containing 5µL of LIF to each of the 6 wells followed by 1mL of iPSCs suspended in mES medium plus LIF and incubated overnight at 37°C. To induce differentiation in embryoid bodies (EBs), the mES medium containing 5µL LIF was replaced by either 10% serum in MEF medium and allowed to differentiate for 3 days and then after 3 days, the MEF medium was removed from 4 out of 6 wells and replaced with either KO serum medium (10% serum knockout replacement) or KO serum + activin (8µg/10mL). The medium were changed every other day and cells were collected using trypsin at different time points: day 2, 4, 6, 8, and 12 for RNA and cDNA synthesis.

iPSCs culture & differentiation medium:

mES medium:

500mL mES medium was made containing 384.5mL knockout DMEM, 50mL FBS, 50mL KO serum, 5 mL L-glutamine, 5 mL non-essential amino acid and 5 mL pen strep, 800µL β-Mercaptoethanol and filtered before usage

Differentiation medium:

Mouse embryonic fibroblast (MEF) medium:

500mL MEF medium was made containing 435mL of DMEM high glucose, 50 mL FBS (10%), 5 mL L-glutamine, 5 mL non-essential amino acid and 5 mL pen strep and filtered before usage.

KO serum medium (10% serum knockout replacement)

500mL Knockout serum medium was made containing 435mL DMEM high glucose, 50 mL knock out serum (10%), 5 mL L-glutamine, 5 mL non-essential amino acid and 5 mL pen strep and filtered before usage.

KO serum medium + activin (8µg/10mL)

Knockout serum medium was made in 500mL containing 435mL DMEM high glucose, 50 mL knock out serum (10%), 5 mL L-glutamine, 5 mL non-essential amino acid and 5 mL pen strep and filtered before usage. Then, 8µL of activin was added to 20mL knock out serum differentiation medium.

Reverse transcription PCR and real-time PCR

Total cellular RNA was extracted according to RNeasy Micro handbook by QIAGEN using 350µL RLT lysis buffer (QIAGEN) containing 5µL β-Mercaptoethanol and RNeasy RNA isolation kit according to the manufacturer's instructions, and then the total RNA isolation was measured using the spectrometer. Then, the amount of RNA needed to yield 2µg was collected and then trace DNA were removed with 2µL DNase and 1µL DNase reaction buffer and diluted to 10µL with dH_2O and then incubated at 37°C for 60

minutes. Subsequently, the DNase was inactivated using 2µL DNase stop solution and further incubated at 65°C for 10 minutes.

cDNA synthesis

12µL of DNase treated RNA along with 1µL of oligo dT primers and 1µL 10nM dNTP were added to a 0.2mL certified DNase/RNase free reaction tube and incubate at 65°C for 5 minutes. The samples were placed on ice for at least 1 minute, then 6µL of the mixture containing (4µL buffer, 1µL DTT, 1µL RNase out) was added only to the negative reverse transcriptase (NRT) control while 7uL of the mixture containing (4µL buffer, 1µL DTT, 1µL RNase out, and 1µL super script III® Out) were added to samples and incubated at 50°C for 60 minutes and 70°C for 10 minutes.

Real-time PCR

After cDNA synthesis, polymerase chain reaction was carried out to analyze gene expression patterns of FoxA2, Sox17, Pdx1, Ins1 and Ins2 in EBs cultured in differentiation media: MEF, KO serum and KO serum + activin. Both forward and reverse mouse primers were ordered from INVITROGEN. PCR reaction was done using 22µL of master mix, 1µL forward primer, 1µL reverse primer for each gene and lµL of cDNA from each sample type.

For Real-time PCR, the cycle number was 35 and ran at a default setting on the eppendorf thermal cycler and the expression of each gene was normalized to β-actin gene expression.

Agrose Gel Electrophoresis

200mL of Tris-acetate-EDTA (TAE) electrophoresis buffer was measured using a 1000mL Erlenmeyer flask, 2.0g of agrose powder was weighed out using a balance. The flask was heated in a microwave until the powder completely dissolved in the TAE

buffer. 2µL of ethidium bromide was added to the gel and mixed by swirling the flask. The solution was then cooled at room temperature for 10 minutes, and then it was poured into the gel tray with a sample comb and allowed to solidify at room temperature. After the gel has solidified, the sample comb was removed and the gel tray was inserted into the gel electrophoresis chamber and covered with TAE buffer. 5µL of DNA ladder was pipetted into the 1st sample well, followed by 20µL of each sample type. The lid of the electrophoresis apparatus was placed on the chamber and 120mV current was applied for 1 hour. After 1hr, the agrose gel was placed on an ultraviolet transillumator to visualize the DNA or RNA and a photo of gel was taken.

qRT-PCR

To minimize cost of IDT primers, qRT-PCR reactions were done using a total volume of 12µL containing 6µL SsoFast probes super mix, 1.2µL of primer pairs, 4.3µL Diethylpyrocarbonate (DEPC) H_2O and 0.5µL cDNA of each sample type. To achieve the final desired stock concentration of 10X, qRT-PCR primers were centrifuged at 750g for 10 seconds. Then, each primer pair was resuspended in 200 µL of IDTE buffer (10nM Tris, 0.1mM EDTA, pH 8.0) and again centrifuged at 750g for 10 seconds and stored in the dark at -20°C.

For qRT-PCR, the cycle number was 40 and ran at a default setting on the eppendorf thermal cycler, the fluorescent dye used was FAM and the expression of each gene was normalized to Gapdh gene expression.

Each gene δ CT value was then subtracted from endogenous gene control (Gapdh) and then δδ CT was calculated by subtracting the δCT of individual gene minus the positive control which is E19 pancreas. After that, the fold change of each sample over the positive control was calculated by taking $2^{(-\delta\delta ct)}$ for each gene.

MICE

Pdx1: GFP matings

Transgenic mouse Pdx1: GFP, GFP expression is driven by the pdx1 promoter (Gu et al, 2004). Timed matings were carried out, with E0.5 as the day of discovery of the vaginal plug. At E14.5, pregnant females were killed and embryos were harvested.
Pregnant Cd1 females were ordered from Charles Rivers Laboratories and E14 embryos were harvested. Using the dissecting microscope, each embryo was carefully dissected and the pancreas was removed in placed in MEF medium containing gentamycin on ice. After pdx1 or cd1 embryos were dissected, some pancreatic buds were dissociated using 1000µL of digestion buffer containing 25X collagenase (40µL), 20µL hyaluronidase, 25µL Dipase diluted in MEF medium. Some of the dissociated E14 pancreatic cells were stored in 350µL lysis buffer and not cultured. The others were plated onto a 6 well tissue culture plate onto a decellularized rat pancreas or gelatin-coated well in MEF medium in gentamicin. The non-dissociated pancreatic buds were placed on fibronectin and grown in MEF medium. Both dissociated on decellularized matrix, or gelatin, and non-dissociated pancreatic buds were cultured for 7 days and then cells were collected for RNA and cDNA synthesis. Gel electrophoresis, RT-PCR and q-PCR were done to analyze pancreatic endocrine and exocrine gene expression.

E19.5 pancreas RNA Isolation for Positive control

Timed matings were carried out, with E0.5 as the day of discovery of the vaginal plug. At E19.5, pregnant females were killed and embryos were harvested. Using the dissecting microscope, each embryo was carefully dissected and the pancreas was removed and placed directly into nitrogen liquid to preserve RNA. Using a sterile mortar and pestle, the pancreatic buds were grinded up in nitrogen liquid and transferred into a 1.5mL eppendorf tube with TRI Reagent and RNA was isolated according to SIGMA TRI REAGENT RNA isolation manual. The homogenized pancreatic cells incubated at room

temperature for 5 minutes in TRI REAGENT. Then 200µL of chloroform was added to TRI reagent and tube was shaken vigorously by hand for 15 seconds**.** Next, the tube was incubated at room temperature for 5 minutes.

After incubation, the tube containing pancreatic RNA was centrifuged at 12,000 x g for 15 minutes at 4°C and then 600µL of the colorless, top layer containing the RNA was transferred to a new RNase-free tube. Then, 0.5mL of isopropanol was added to sample and allowed to stand for 5-10 minutes at room temperature and centrifuged at 12,000 x g for 10 minutes at 4°C. The supernatant was removed and 75% ethanol was added to RNA pellet in the RNase-free tube and was inverted to disperse any visible precipitate. The tube was vortexed and then centrifuged at 7,500 x g for 5 minutes at 4°C. The 75% ethanol was removed using a pipette and the pellet allowed to dry for 5 minutes at room temperature. Then, the RNA was resuspended in 20µL of DEPC treated H_2O and vortex. A spectrometer was used to measure the RNA amount and the tube was stored at -20°C.

Rat pancreas de-cellularization

Protocol adopted from: Ott, H.C, Matthiesen, T.s., Goh., Taylor, D.A et al (2008). Perfusion-decellularized matrix: using nature's platform to engineer a bioartifical heart. Nature Medicine 14, 213-221

Dissect pancreas from perfused Rat and place in scintillation vial with 1% SDS for 48hrs at room temperature on rocker. Initially, 0.1 % SDS was used, but tissue did not decellularized fully. Wash tissue 2 X 20 minutes with dH_2O at room temperature on rocker. Wash tissue 1 X 10 minutes with dH_2O at room temperature on rocker. Wash tissue 2 X 10 minutes in PBS with 1 % Pen/Strep for 24hrs. Incubate at 37°C prior to grinding.

RAT pancreatic matrix

Using a sterile mortar and pestle, the decellularized pancreas was ground in nitrogen liquid, re-suspended in 1 mL MEF medium with gentamicin and then transferred to a 6 well tissue culture plate and was stored at room temperature in the tissue cell hood to partially dry overnight.

RESULTS

The generation of definitive endodermal cells and pdx1^{+} progenitor cells from mouse induced pluripotent stem cells

To induce pancreatic differentiation in mouse iPSCs, L4-3 iPSCs were cultured in MEF medium and allow to form into embryoid bodies. After 6 days in differentiation medium, KO serum plus Activin A, the embryoid bodies were GFP^{+} which is indicative of pdx1 expression. The presence of pdx1 expression within embryoid bodies suggested differentiation of these cells toward pancreatic endoderm. Moreover, the pdx1^{+}GFP^{+} cells are seen on the outer edge of the differentiated iPSCs.

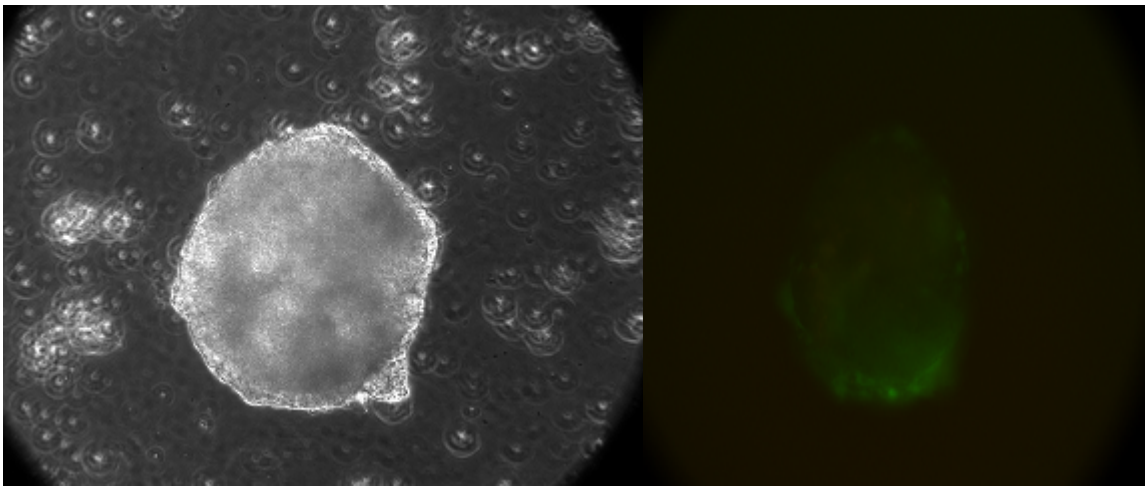

Figure 2: iPSCs differentiation into embryoid bodies and pdx1 gene expression (A) the formation of an embryoid body from iPSCs. (B) The location of pdx1-postive cells within an embryoid body.

To confirm directed differentiation of iPSCs into definitive endoderm, RT-PCR was carried out of differentiated iPSCs at different time points to evaluate expression patterns of two transcription factors: Sry-related HMG-box transcription factor Sox17 and Foxa2 previously known as the hepatocyte nuclear factor (HNF) 3β was used as markers of definitive endoderm. There was continuous expression of both definitive endodermal markers FoxA2 and Sox17 in differentiating embryoid bodies cultured in MEF medium at different time points suggesting that these iPSCs have differentiated to a definitive endoderm fate.

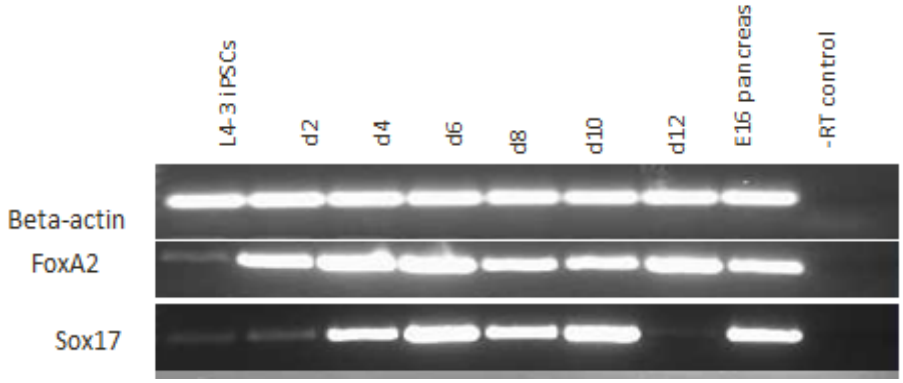

Figure 3: FoxA2 & Sox17 gene expression in differentiating embryoid bodies into definitive endoderm when cultured in MEF medium, each gene expression was normalized to β-actin.

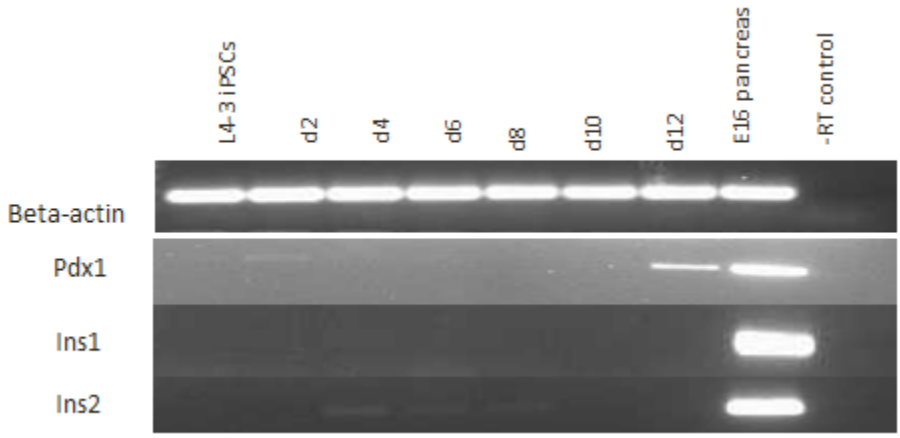

Figure 4: Pancreatic progenitor cells differentiations into pancreatic endocrine lineages, the gene expressions were normalized to endogenous β-actin gene.

However, when RT-PCR analysis was done for pancreatic progenitor cell marker (pdx1) and endocrine cells (Ins1 and Ins2), there was no expression of both Ins1 and Ins2 genes at d2, d4, d6, d8, d10, and d12. Besides, pdx1 expression was only seen at d12 in differentiated iPSCs into pancreatic endodermal cells.

Activin A induced generation of some pancreatic endocrine cells

The addition of exogenous activin A to cultured fetal pancreas did modestly increase the insulin content and insulin-positive cell number[30]. Moreover, other papers have shown that the addition of activin enhances endocrine differentiation. Therefore, I hypothesized that the addition of activin A to partially differentiated iPSCs would direct their differentiation in pancreatic endocrine cells in vitro. To test this hypothesis, activin A in KO serum medium was added every other day to some of the partially differentiated iPSCs to optimize differentiation of definitive endodermal cell into pancreatic lineage while other remained in MEF medium or KO serum without activin A treatment.

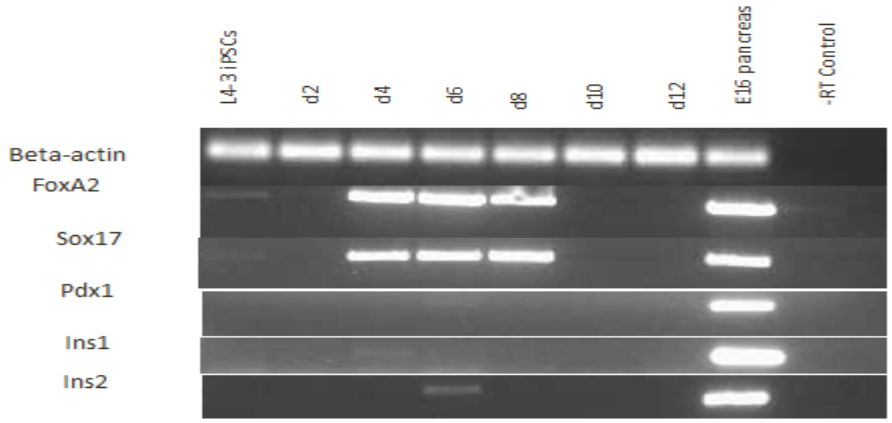

Figure 5: Partially differentiated iPSCs grown in MEF medium for 3 days and MEF was removed and KO serum medium was added and then analyzed for gene expression of FoxA2, Sox17, Pdx1, Ins1 and Ins2 at different time points.

There was a striking difference between differentiated iPSCs cultured in MEF and those grown in KO serum as a d4 without the addition of activin A. In KO serum samples, Foxa2 and Sox17 genes expression are not continuous. However, in partially differentiated iPSCs grown in MEF medium, Foxa2 and Sox17 genes expression were both continuous. Moreover, the addition of activin A to KO serum medium did indeed direct differentiation of iPSCs into definitive endoderm and ultimately towards a pancreatic endocrine fate. Even though, the Pdx1, Ins1 and Ins2 genes expression were minimal, the RT-PCR results showed positive pdx1 gene expression as of d6 confirming

the presence of GFP^+ cells which expression is driven by the pdx1 promoter. Like iPSCs cultured in MEF medium, Foxa2 expression was continuous. However, only in KO serum medium plus activin was Ins2 expression observed. Thus, the data showed that the addition of activin A did induce directed differentiation towards a pancreatic endocrine cell lineage; even though, the endocrine genes expression was minimal.

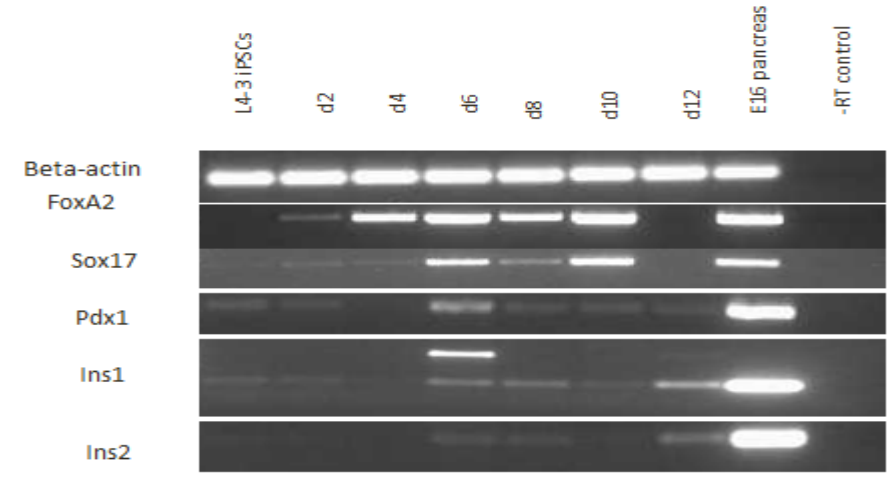

Figure 6: Partially differentiated iPSCs grown in MEF medium for 3 days and MEF was removed and KO serum medium + Activin (8µg/mL) was added and then analyzed for gene expression of FoxA2, Sox17, Pdx1, Ins1 and Ins2 at different time points.

Even though, the initial results were promising, the failure to generate sufficient $Pdx1^+GFP^+$ pancreatic progenitor cells from iPSCs derived from Pdx1: GFP mouse did hinder further experiments. Thus, embryonic pancreatic cells were examined.

Decellularized rat matrix directs embryonic pancreatic cells in endocrine cells in vitro

I examined the differentiation of embryonic pancreas endodermal cells into pancreas lineages. It is thought that the pancreatic mesenchyme harbors key diffusible factors that are both permissive and instructive for the generation of differentiated pancreatic cell types as well as proper pancreatic development. Perhaps, these instructive signals could promote differentiation of pancreatic cells into an endocrine fate.

Thus, I hypothesized that the use a decellularized pancreatic rat matrix with its native extracellular matrix and possibly remaining environmental cues will indeed direct differentiation of E14 pancreatic progenitor cells toward an endocrine lineage.

To test my hypothesis, E14 dissociated and non-dissociated pancreatic buds plated on rat decellularized matrix, gelatin or fibronectin and then analyzed for the expression of $pdx1^{+}$ pancreatic progenitor cells. Lineage-tracing experiments have shown that all the pancreatic endocrine and exocrine cells are derived from $PDX1^{+}$ cells[31].

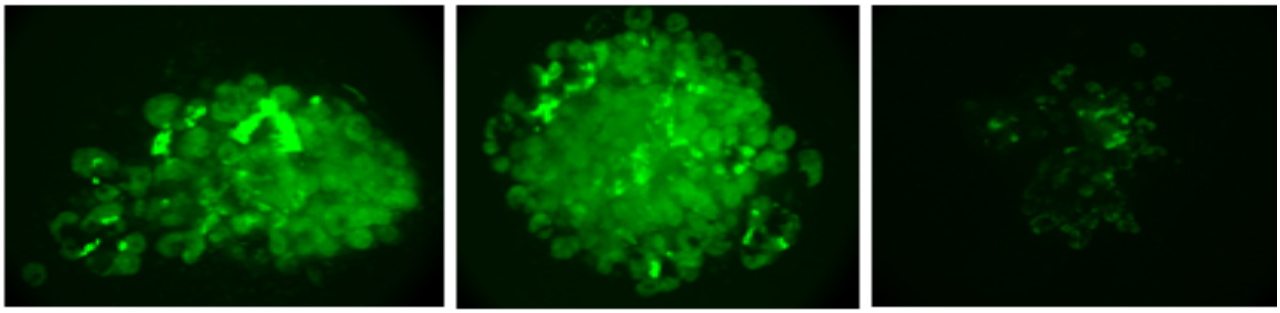

Figure 7: Pdx1 expression in E14 pancreatic buds on fibronectin cultured in MEF at d2. GFP expression is driven by a pdx1 promoter in Pdx1: GFP transgenic mouse. Pdx1 Expression is disperse is pancreatic buds. At d7 in MEF medium, E14 pancreatic buds did express pdx1; however, the pdx1 expression was faint.

As expected, there was a gradual decrease in GFP expression when E14 pancreatic buds were cultured. The data showed that GFP expression was brighter at day2 (left and center) than that d7 (right). These results correlate with pdx1 gene expression in the developing pancreas in vivo.

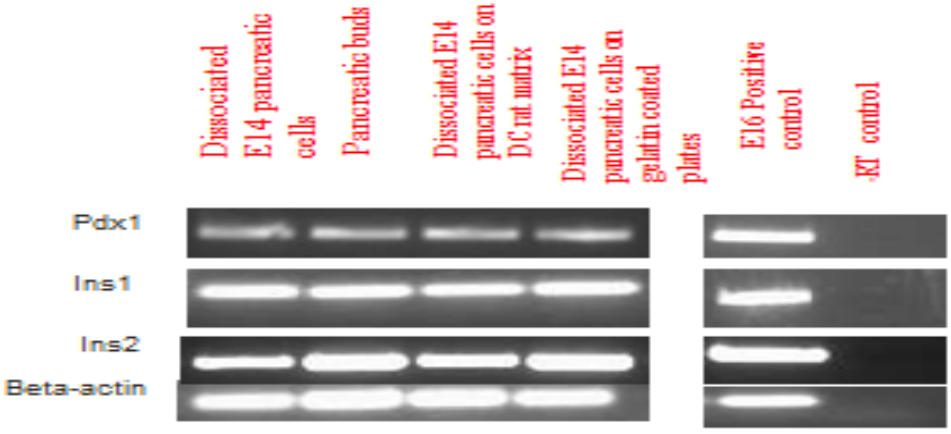

Figure 8: Pdx1, Ins1, & Ins2 gene expression in different cultured conditions. Pdx1, Ins1, and Ins2 expression in dissociated E14 pancreatic cell not cultured, non-dissociated cultured pancreatic buds, dissociated E14 pancreatic cells on decellularized rat matrix, dissociated E14 pancreatic cells on gelatin coated plates cultured for 7 days in MEF medium. Gene expression was normalized to β-actin.

The data showed that Pdx1 is expressed in pancreatic cell types in both dissociated E14 pancreatic cell not cultured, E14 pancreatic buds cultured in MEF medium on fibronectin, dissociated E14 pancreatic cell cultured on decellularized rat matrix, dissociated E14 pancreatic cells on gelatin coated plates. Moreover, both insulin genes were expressed in all experimental groups.

Pancreatic differentiation towards endocrine and exocrine fates

Even though, secondary transition at E12.5 is prior to endocrine differentiation, post-secondary transition at E14.5 involves the differentiation of pancreatic endocrine cells primarily towards a β cell fate. Hence, most pancreatic differentiation towards an endocrine fate occurs between E12.5 and E14; thus, E14 pancreatic cultures were examined for gene expression of endocrine markers (glucagon, somatostatin, and pancreatic polypeptide). In vitro studies of cultured pancreas show that glucagon is necessary for early formation of insulin cells (E11–E13), but not later in the E15 pancreas (Prasadan et al., 2002).

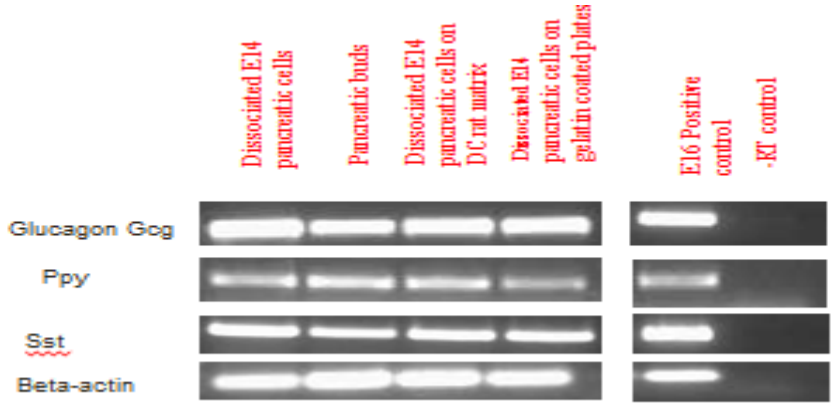

Figure 9: Pancreatic endocrine gene expression of Gcg, Sst, & Ppy. Gcg, Sst, & Ppy expression in dissociated E14 pancreatic cell not cultured, non-dissociated cultured

pancreatic buds, dissociated E14 pancreatic cells on decellularized rat matrix, dissociated E14 pancreatic cells on gelatin coated plates cultured for 7 days in MEF medium. Gene expression was normalized to β-actin.

RT PCR results after 7 days on decellularized rat pancreas matrix, gelatin-coated plate, or fibronectin showed expression of three endocrine gene markers. As compared to the positive control E16 pancreas, glucagon gene expression levels appear to be higher in all cultured and non-cultured pancreatic cell types. The presence of pancreatic cell endocrine markers confirms differentiation of pancreatic progenitor cells into endocrine cell types. To analyze differentiation of pancreatic progenitor cells towards an exocrine fate, RT PCR was done using exocrine markers P48 and amylase. The results showed that P48 gene expression is only present in dissociated E14 pancreatic cells not cultured. However, when cultured in MEF medium, this gene expression is absent after 7days in non-dissociated E14 pancreatic buds, dissociated E14 on gelatin-coated plates and E14 on fibronectin.

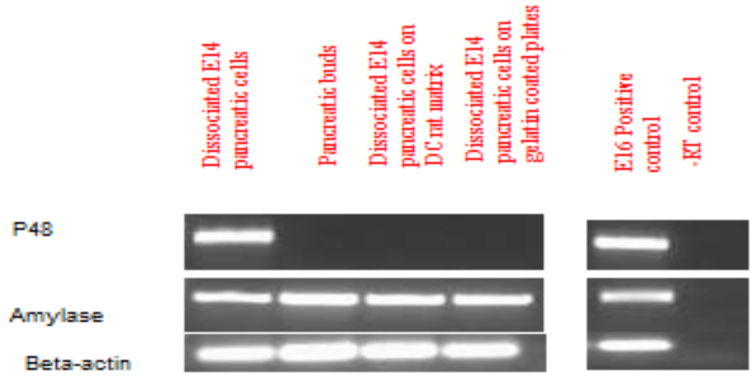

Figure 10: Pancreatic exocrine gene expression of P48 and Amylase. P48 and Amylase gene expressions in dissociated E14 pancreatic cell not cultured, non-dissociated cultured pancreatic buds, dissociated E14 pancreatic cells on decellularized rat matrix, dissociated E14 pancreatic cells on gelatin coated plates cultured for 7 days in MEF medium. Gene expression was normalized to β-actin.

To examine proper mesenchyme development in cultured pancreatic cells, the mesenchyme marker, Vimentin gene expression was analyzed. Interestingly, the presence of mesenchyme, but in the absence of proximity or contact, led to enhanced insulin differentiation over glucagon or other non-insulin cell differentiation[32].

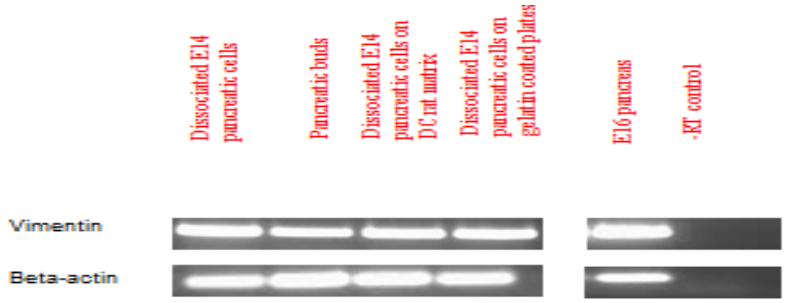

Figure 11: Mesenchyme development in the different pancreatic cell types. Vimentin gene expression in dissociated E14 pancreatic cell not cultured, non-dissociated cultured pancreatic buds, dissociated E14 pancreatic cells on decellularized rat matrix, dissociated E14 pancreatic cells on gelatin coated plates cultured for 7 days in MEF medium. Gene expression was normalized to β-actin.

The result showed mesenchymal development in both cultured and non-cultured pancreatic cell types. Moreover, mesenchymal cells did survive in culture after 1 week in MEF medium. Vimentin found in ventral and dorsal pancreatic rudiments between E12.5 & E13 and peak at birth with a large subset of duct cells containing vimentin[33]
Thus, the survival of pancreatic endocrine, exocrine and mesenchymal cells on decellularized rat matrix showed that the matrix at least maintains growth and maturation of cells. Even though, the RT PCR did not show any significance difference between pancreatic cells growth on gelatin, fibronectin and decellularized rat matrix, it did confirm survival of these pancreatic cell types when cultured on matrix for one week.

qRT-PCR data shows differential gene expression in pancreas culture grown on decellularized rat pancreas matrix compared to culture on gelatin and fibronectin

To evaluate difference between the different culture conditions: decellularized rat pancreas matrix, gelatin, fibronectin, and MEF medium, qRT-PCR analysis of pancreatic progenitor, endocrine and exocrine cell types.

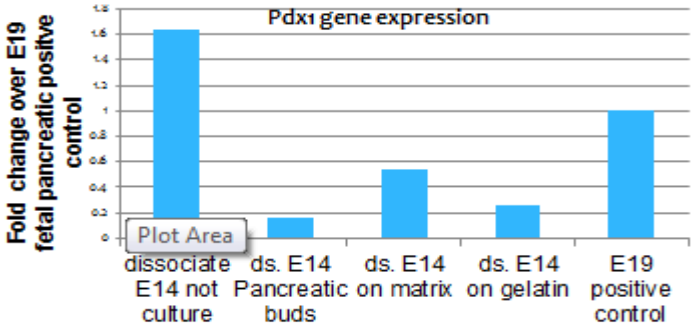

Figure 12: Pdx1 expression in dissociated E14 pancreatic cell not cultured, non-dissociated cultured pancreatic buds, dissociated E14 pancreatic cells on decellularized rat matrix, dissociated E14 pancreatic cells on gelatin coated plates cultured for 7 days in MEF medium.

The results showed that high pdx1 expression in dissociated E14 pancreatic cells not cultured for 7 days in MEF medium. Even though, a decrease in Pdx1 expression was observed in all cultured cells (dissociated and non-dissociated), pdx1 expression in dissociated E14 seeded on decellularized rat matrix was higher than that of dissociated pancreatic cells grown on gelatin and non-dissociated cultured cells grown in MEF medium. Moreover, in all cultured pancreatic cells, Pdx1 expression was lower than E19 pancreas positive control. Thus, the decellularized rat pancreatic matrix did minimize the reduction of pdx1 progenitor cells when cultured for 7 days.

In addition, both Ins1 and Ins2 gene were analyzed to evaluate differential gene expression under the different culture conditions.

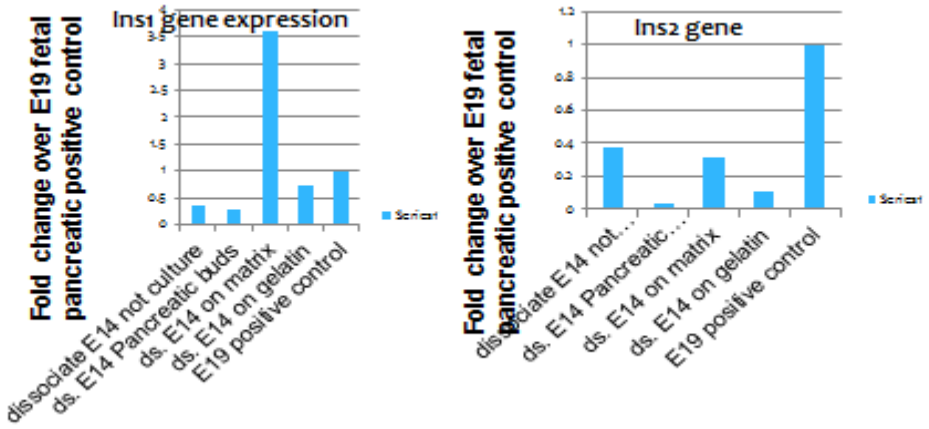

Figure 13: Ins1 & Ins2 expression in dissociated E14 pancreatic cell not cultured, non-dissociated cultured pancreatic buds, dissociated E14 pancreatic cells on decellularized

rat matrix, dissociated E14 pancreatic cells on gelatin coated plates cultured for 7 days in MEF medium.

There was a 3 fold increase in Ins1 gene expression on decellularized rat pancreatic matrix as compared with dissociated E14 pancreatic cells which were not cultured. Moreover, there was more Ins1 gene expression in dissociated pancreatic cells seeded on pancreatic matrix than at E19 mouse pancreas (positive control) and on pancreatic cells cultured on gelatin, fibronectin, and MEF medium.

In addition, Ins2 gene expression was higher in dissociated pancreatic cells cultured on decellularized rat matrix than on gelatin and fibronectin; even though, Ins2 gene expression was less on decellularized matrix than that of dissociated E14 pancreatic cells not cultured and the E19 positive control, the data showed that the pancreatic matrix still maintains Ins2 gene expression and survival of insulin 2 postive pancreatic cells.

Other pancreatic endocrine cell markers were analyzed to assess the decellularized pancreatic rat matrix ability to promote survival, maturation, and direct differentiation into predominately endocrine cell type population. Glucagon, Somatostatin, Pancreatic polypeptide genes expression was analyzed. The result showed a similar trend with these endocrine genes. Like Pdx1, Ins1, and Ins2, there was an increase in glucagon (Gcg), somatostatin (Sst), pancreatic polypeptide (Ppy) gene expression when dissociated E14 pancreatic cells were cultured on decellularized pancreas rat matrix than when cultured on gelatin or fibronectin. Moreover, there was more glucagon, somatostain, pancreatic polypeptide expression on the matrix than dissociated E14 cells that were not cultured for 7 days.

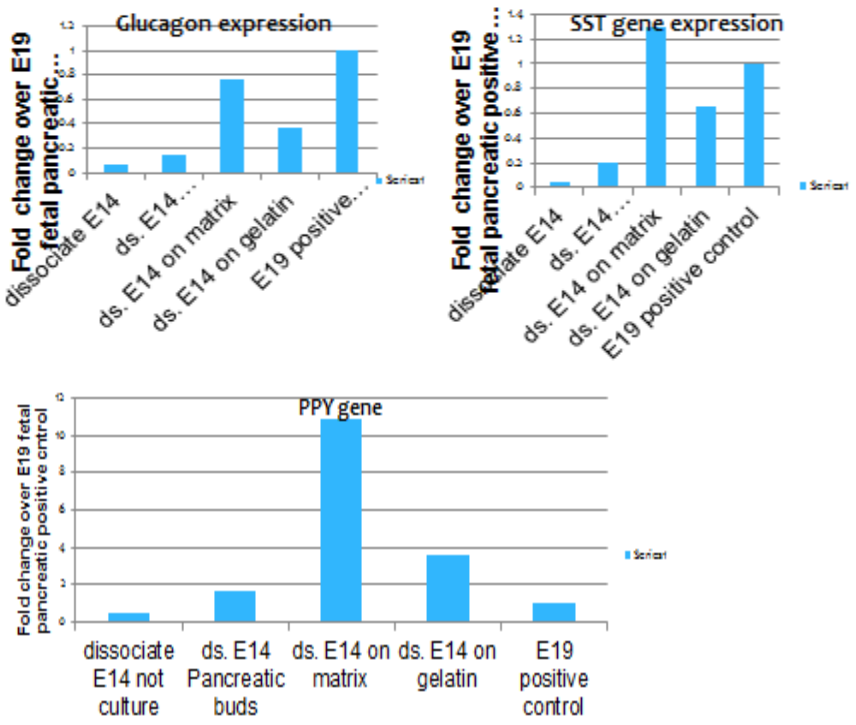

Figure 14: glucagon (Gcg), somatostatin (Sst), pancreatic polypeptide (Ppy) gene expression expression in dissociated E14 pancreatic cell not cultured, non-dissociated cultured pancreatic buds, dissociated E14 pancreatic cells on decellularized rat matrix, dissociated E14 pancreatic cells on gelatin coated plates cultured for 7 days in MEF medium.

Thus, the data showed that the decellularized rat pancreas matrix did support growth, survival, and maturation of cultured pancreatic endocrine cell types. Moreover, dissociated E14 pancreatic cells cultured for 7 days on decellularized rat pancreatic matrix did indeed express more endocrine genes than that of non-cultured E14 pancreatic cells. Also, there was higher gene expression of all the endocrine genes on the pancreatic matrix than gelatin or fibronectin.

To evaluate exocrine pancreatic differentiation by possibly instructive cues remaining on decellularized rat pancreas matrix, PTF1a (P48) and Amylase, two exocrine markers were examined. By lineage tracing analysis, essentially all acinar cells, 95% of ductal cells, 75% of α-cells, and 100% of non-αendocrine cells are derived from PTF1a-positive progenitor cells[34].

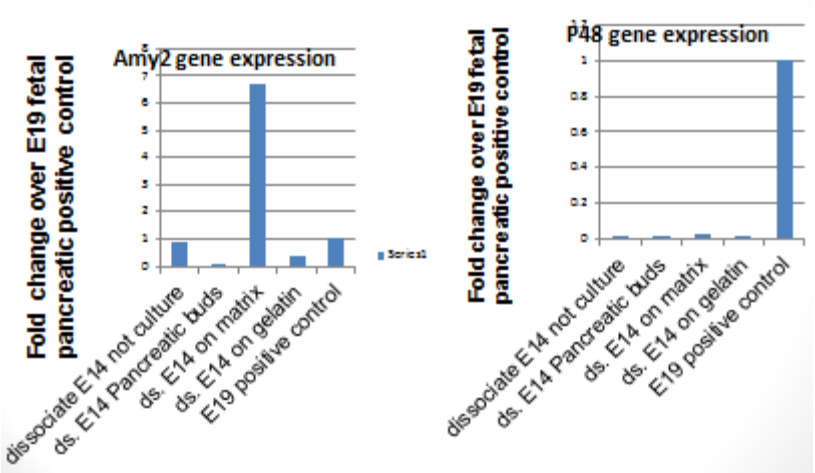

Figure 15: Exocrine gene expression of P48 and Amylase in dissociated E14 pancreatic cell not cultured, non-dissociated cultured pancreatic buds, dissociated E14 pancreatic cells on decellularized rat matrix, dissociated E14 pancreatic cells on gelatin coated plates cultured for 7 days in MEF medium.

As compare to endocrine genes expression, the P48 gene expression was higher on the dissociated E14 pancreatic cells seeded on decellularized rat pancreas matrix; even though, P48 expression was lower in all cultured and non-cultured pancreatic cell types than E19 positive control. Similarly, amylase 2 expression was upregulated on the decellularized matrix than that of the other culture conditions.

The data suggests that both endocrine and exocrine cell markers are upregulated on the decellularized rat matrix. However, the endocrine pancreatic genes were highly unregulated than their exocrine counterparts. Thus, the preliminary data do indicate that the decellularized rat pancreas matrix may play a role in the induction of endocrine and exocrine pancreatic cell type during pancreatic development. In addition, the decellularized matrix retains the developmental cues native of the extracellular matrix; therefore, it enables differentiation of pancreatic progenitor cells into pancreatic endocrine cell types.

Discussion:

Early pancreas development is an intricate cascade of cellular events that program the dorsal and ventral pancreatic buds into the highly organized pancreas organ. The mesenchyme plays an essential role in promoting growth and morphogenesis of the development pancreas. Moreover, it secretes diffusible factors with instructive cue to direct differentiation into specific pancreatic cell types. The differentiation of pancreatic progenitor cells towards a specific cell type is indicative of the involvement to growth factors during pancreatic development.

The addition of activin A induced definitive endodermal cells derived from human embryonic stem cells[35]. Similarly, the results in this study confirmed the instructive role of activin A because the addition of activin A to iPSCs cultured in KO serum medium did express definitive endoderm markers such as Foxa2 and Sox17. Moreover, pdx1 expression was observed sooner in iPSCs cultured in KO serum medium plus activin (d6) than that of iPSCs cultured solely in MEF medium suggesting that the addition of activin A did direct differentiation faster towards a pancreatic endoderm fate. Hence, as demonstrated by other studies, the serum concentration within medium is an essential factor in generating activin A induced definitive endodermal cell and pancreatic progenitor cells.

Even though, all of the different iPSCs culture conditions give rise to definitive endodermal cells because they expressed FoxA and Sox17, only iPSCs cultured in KO serum plus activin A generated cells which were $pdx1^{+}$, $Ins1^{+}$ and $Ins2^{+}$ indicating that the addition of activin A was essential to direct further differentiation of definitive endodermal cells towards a pancreatic fate. Added exogenous activin A to cultured human fetal pancreas and found a modest increase in insulin content and insulin-positive cell numbers[36]. Thus, the addition of activin A to iPSCs in KO serum medium did enhance endocrine differentiation.

However, the addition of exogenous activin A did not generate a homogenous pdx1 expression in embryoid bodies. Thus, it appears that the time of exposure and the

concentration of activin A is crucial in inducing directive differentiation of pancreatic progenitor cells derived from iPSC into endocrine cell fate.

The results in this study showed changes in pdx1 expression during pancreatic development. Initially, pdx1 expression was high in E14 dissociated pancreatic cells not cultured (Fig. 12). Moreover, a decrease in pdx1 expression was seen in all cultured pancreatic cells in MEF medium for 7 days as they differentiated towards an endocrine cell fate. However, E19 post-secondary stage, pdx1 expression was higher than that of all cultured pancreatic cells suggesting that pdx1 is again expressed as endocrine cells differentiate predominately towards a β cell fate.

Even though, P48 was expressed in all cultured E14 pancreatic cells (both dissociated and non-dissociated), it expression was lower on the decellularized rat pancreas than that of the positive E19 control. This data suggested that the pancreatic cells seeded on the decellularized rat pancreas may differentiate primarily towards an endocrine cell fate due to the lower p48 expression since it is crucial in exocrine specification.

Moreover, the decellularized pancreatic matrix did support reseeding and appeared to maintain survival and growth of pancreatic cells. The high expression of the pancreatic endocrine cell markers on decellularized rat matrix as compared to the other cultured condition suggested that the matrix did promote differentiation towards an endocrine fate.

To attempt to fully understand the possibly inductive nature of the decellularized rat matrix, diabetic patient derived iPSCs would be seeded on this rat matrix and analyzed for endocrine cell markers as well as β cell markers. Moreover, the rat pancreatic extracellular matrix composition will be analyzed to determine the factors needed to enhance growth and differentiation is truly preserved. Perhaps, these iPSCs could be directed to differentiate towards a population of predominately β cells in vitro.

Together, these results demonstrated that the addition of activin A is essential in generating pancreatic progenitor cells from fibroblast derived iPSCs and that the decellularized rat pancreatic matrix does support growth and survival of these pancreatic cells. But most importantly, the preliminary data showed that the decellularized rat

pancreatic matrix directed differentiation of pancreatic progenitor cells into different endocrine cell types.

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