Determination of Methods for Improved Reprogramming of Various Cell Types to Functional Beta-like Cells

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Dedication

This thesis is dedicated to everyone coping with Diabetes.

Abstract

Reprogramming is the process of converting a somatic cell into a different cell type in a controlled manner without an intermediate pluripotent state, usually by expression of exogenous transcription factors. Here, 3 transcription factors, (Pdx1, Ngn3 and MafA) incorporated in a polycistronic adenoviral vector system (Ad-PNM) was used to directly reprogram cells to beta-like cells that produce insulin *in vitro*. In addition, the role of innate immunity activation and open chromatin structure during reprogramming is studied to improve overall reprogramming efficiency while using the Ad-PNM adenoviral vector.

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CHAPTER 1
INTRODUCTION

Diabetes mellitus is an incurable lifelong condition occurring due to high blood glucose levels in the body due to the inability of the body to produce insulin (Alberti & Zimmet 1998). Insulin, produced in Beta cells in the pancreas, is a peptide hormone that permits the entry and usage of glucose into the cells, which is metabolized into energy for cellular processes. Type 1 diabetes or juvenile diabetes is when the pancreas of an individual does not produce insulin because of loss of the endogenous beta cells. This is characterized as an auto-immune disease (Alberti & Zimmet 1998). Various therapies have been developed to mitigate this disease. The current standard of care for Type 1 diabetes is timely delivery of exogenous insulin by injection or automated pump. Although these injections are effective to an extent, they cannot completely mimic the normal secretion pattern of insulin by the beta cells of the pancreas. From a study by the Centers for Disease control and Prevention, 29.1 million (9.3%) of the overall population of United States had diabetes in 2012. In the U.S, a steady rise in the prevalence of diabetes is observed because of diagnosis of ~1.4 million new cases every year and approximately $322 billion was spent last year to treat diabetes.

In well-developed countries, advancements in medical care have led to better availability of supplies for primary care and treatment of diabetes. Whereas, in less-developed countries with lack of appropriate diabetic care, a novel and efficacious therapeutic approach to promote blood glucose control would make a substantial difference for patients. Improper care over time can increase amounts of unutilized glucose causing serious complications that can damage eyes, kidneys, nerves and cause heart failure, stroke, loss of limbs, etc. increasing the risk of premature death (Centers for Disease Control and Prevention 2014). Currently the standard of care to recapitulate endogenous insulin production is limited to insulin injections. For patients with severe diabetic phenotype, islet cell transplantation may be available as an alternative treatment. Most pancreatic islet transplantations are allo-transplants where purified and processed

islets from a deceased organ donor is transplanted into a diabetic person. (Gruessner et al. 2008) Although, this procedure is currently labelled experimental for type I diabetes, it is the most effective way to mitigate diabetes. Each islet allo-tranplantation involves use of specialized enzymes to isolate islets from the whole pancreas. Typically, patients receive two infusions with an average of 800,000 to 10,00,000 islet cells each through a catheter (small plastic tube) to oxygen rich regions that facilitate islets to attach to new blood vessels before secreting insulin. Islets purified from the cadaver pancreas are reintroduced back into the patient into the liver through a catheter. The patients in some cases may require multiple infusions depending on the effectiveness of islet transplantation. Limitations of allo-transplantation include a lack of donor organs and readily available islets for transplantation. (Chhabra & Brayman 2014) This can be overcome by either improving the efficiency of islets transplanted to overall reduce number of islets infused or develop alternative source of insulin-producing cells opening the avenue to treat additional patients. Pancreatic auto-transplantation may also be performed following surgical removal of entire pancreas or total pancreatectomy, in patients suffering from chronic and severe pancreatitis (Witkowski et al. 2014).

More patients may be effectively treated by generating new sources of insulin-expressing cells that function similarly to pancreatic beta cells. Direct reprogramming of somatic cell types into pancreatic beta-like cells may prove to be an efficient therapy for humans exhibiting juvenile diabetes.

Embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSCs) can potentially be reprogrammed into into insulin producing beta-like cells (Pagliuca et al. 2014; Soejitno & Prayudi 2011). This method potentially provides an unlimited supply of cells ready for transplantation and has now reached clinical trial. Reprogramming cells involves introducing specific transcription factors to induce a cell type to stably change its phenotypic identity (Firas et al. 2015). The reprogramming factors can be delivered by viral mediated methods or non-viral mediated methods. Introduction of these factors induce expression of endogenous genes leading to re-specification of cell lineage depending on genes activated. Complete reprogramming is attained when the new phenotype is stably maintained even after exogenous gene expression is lost.

Researchers have attempted to reprogram several cell types into insulin producing cells, including liver cells, skin cells and fibroblasts (Zhu et al. 2016; Zaret 2008; Meivar-Levy & Ferber 2010). An understanding of the embryonic development of the endoderm lineage can inform approaches to the direct reprogramming of liver to pancreatic cells. During development, both the hepatic cells and the pancreas develop from the foregut endoderm epithelium (Zaret 2008). Formation of the pancreatic and liver buds arise due to differential expression of patterning signals from surrounding mesenchyme. These signals include fibroblast growth factors (FGFs) and bone morphogenic protein signaling (BMPs). (Gittes 2009) This distinction occurs around embryonic day 10 (E10) of development in a mouse. Because of the close relationship between the pancreas and liver lineages, arising from common endoderm progenitors, it has been proposed that chromatin accessibility to genes specifying pancreatic fate more accessible in liver cells than distantly related organs. Another common feature in both liver and the pancreas is the presence of Sox9-expressing progenitor cells. Sox9+ cells are present in ductal regions of adult pancreas and in the bile ducts of the liver (Banga et al. 2012). In the pancreas, Sox9 is known to maintain the pancreatic progenitor pool through its regulation of neurogenin3. (Seymour et al. 2007)

Using developmentally necessary transcription factors, cell identity can be re-specified. For successful re-specification towards a ß-cell identity, Pancreatic-duodenal homeobox 1 (Pdx1), Neurogenin 2 (Ngn3) and V-Maf Avian Musculoaponeurotic Fibrosarcoma oncogene homolog A (Maf-A) have been identified as key factors important in the development and maturation of beta-cells *in vivo* (Zhou et al. 2008). Pdx1 is known to drive early pancreatic lineage in pancreatic epithelium, inducing expression of other genes including Insulin, Glut2, Nkx6.1, Nkx2.2. Ngn3 promotes commitment to endocrine lineage of the pancreas (Seymour & Sander 2011) and MafA is identified as a pivotal factor necessary for identity specification of beta-cell and expression of insulin (Artner et al. 2008).

In 2008, Zhou et al. demonstrated effective *in vivo* direct reprogramming of amylase^{+}/CK19^{-}/Ins^{-} mouse pancreatic exocrine cells to Ins^{+}/C-peptide^{+} ß-like cells by infecting with 3 different adenoviral vectors that encoded Pdx1, MafA and Ngn3.(Zhou et

al. 2008) The identification of PNM combination was determined after screening a number of transcription factors that regulate pancreatic cell fate during embryogenesis.

Figure 1. ***Ad-PNM construct*** (Akinci et al. 2012)

In 2012, Akinci *et al,* cloned the PNM genes described in Zhou *et al*, into a poly-cistronic vector, named Ad-PNM (Figure 1). PNM expression was driven by a highly active cytomegalovirus-chicken β-actin (CAGS) promotor allowing high levels of protein expression.(Akinci et al. 2012) The conversion of liver cells into pancreatic beta-cells *in vitro* by overexpression of the PNM (Akinci et al. 2012; Akinci et al. 2013) generated glucose responsive, insulin-secreting cells. Intravenous injection of Ad-PNM through the tail vein in both NOD-SCID and immune-competent, wild type diabetic mice resulted in long-term rescue of the diabetes phenotype. 12-weeks post Ad-PNM introduction, Newport Green dye was used to isolate insulin expressing cells from liver single cell suspensions (Banga et al. 2012). RT-PCR analysis of FACS-isolated Newport Green dye-positive cells displayed approximately 15-fold increase in Ins1, Ins2 and Pdx1 expression. Increase in other transcription factors such as Slc30a8 (beta cell-specific transporter), Sox17 (endoderm marker) and Glut2 demonstrated transcriptional profile alteration as a result of Ad-PNM based reprogramming. Banga *et al*, showed the insulin production was due to the stable formation of duct-like structures expressing pancreatic ß-cell markers. These cells developed from Sox9+ progenitor cells (Banga et al. 2012). These experiments suggested that Ad-PNM could be used as a potential remedy for juvenile diabetes by direct reprogramming cells of non-pancreatic origin (Akinci et al. 2013; Akinci et al. 2012; Banga et al. 2012).

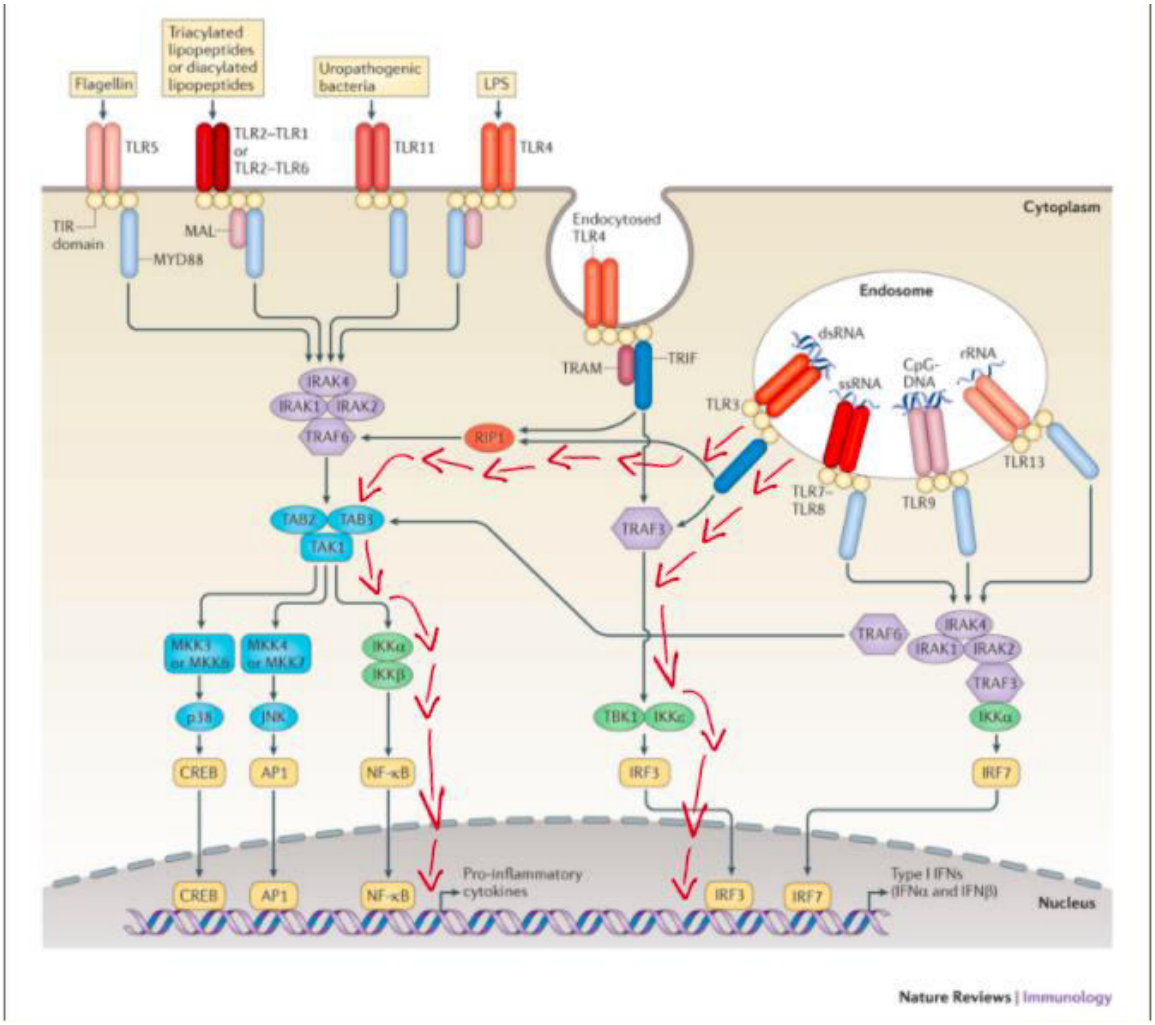


Figure 2. ***Mammalian TLR signaling pathway*** *(Modified from* O'Neill et al. 2013)

Use of adeno-viral constructs for gene delivery is known to also trigger innate immune responses *in vivo*. The innate immune system uses toll-like receptors (TLRs) via structures called pathogen associated molecular patterns (PAMPs) to respond to external stimuli. Drosophila was the first organism in which TLRs were identified. There are ten identified mammalian Toll-like receptors (TLR), of which TLR3 is known to respond to double stranded RNA eliciting innate immune responses (Figure 2) (Matsumoto et al. 2002; Alexopoulou et al. 2001). The stimulation of TLR3 by pathogen leads to the activation of TRIF in turn activating NF-kB and IRF3 through TBK1 (follow red arrows in Figure 2). (Edelmann et al. 2004).

Recently, Lee *et al* showed that activation of innate immunity facilitated in enhancement of epigenetic plasticity leading to increased induction of transcription factors induced pluripotency. It was demonstrated that a combination of innate immune responses and external signals was sufficient to induce direct reprogramming of a particular cell type to another (Sayed et al. 2015). Activation of innate immune responses was performed by addition of polyinosinic:polycytidylic acid (PolyI:C), an immunostimulant due to its double stranded RNA like properties (Fortier et al. 2004). Due to its structural similarity to dsRNA, which is present in some viruses. polyI:C is commonly used for triggering innate immune responses. TLR3 specifically responds to dsRNA, or polyI:C, by activating a cascade of transcriptional pathways mediated by NF-kB causing global epigenetic alterations such as changes in expression levels of histone de-acetylase (HDAC) family and histone acetyltransferases (HATs). Based on these findings, a consensus on the fact that TLR3 plays a pivotal part in triggering innate immunity in response to viral infections (Xagorari & Chlichlia 2008) led to the current study. Our first goal was to determine if activation of innate immunity enhances the reprogramming of mouse small hepatocytes and rat pancreatic exocrine cells into insulin-secreting cells induced by overexpression of Pdx1, Ngn3 and MafA.

Our second goal was to understand open chromatin configuration and its role in promoting direct reprogramming of precursor cells into insulin-secreting cells with administration of Ad-PNM. Early mammalian development involves the differentiation of pluripotent cells into multipotent, ectoderm, endoderm and mesoderm germ layers. Turning on/off genes activated during differentiation involves histone modifications facilitating transcription factors to bind to the chromatin. The differentiation process is driven by cascades of transcription factors promoting conversion to altered cell fates. Cell fate specification is less stable and can be manipulated *in vitro* (Nashun et al. 2015). During early stages of cell proliferation there is a constant change in epigenetic information and structure of chromatin (Gerlitz & Bustin 2011). Gene expression can be altered by external environmental signals by modifying base level chromatin structure. At a molecular level, trimethylation of histone H3 at lysine residue at positions 4 and 27 regulates the levels of gene expression. Transcriptional start sites (TSS) display

trimethylation of histone H3 at lysine residue (Schneider et al. 2004). In undifferentiated embryonic stem cells (ESCs) or induced pluripotent stem cells (iPSc), a major fraction of the development related genes attain a "bivalent inactive state" exhibiting trimethylation of H3K27 and H3K4 (Blomen & Boonstra 2011).

Transcription factors preferably bind to Euchromatin region or a more "open" configuration. In 2011, Xu *et al*, showed distinct patterning of regulatory elements in undifferentiated endodermal cells before specification to either hepatic or pancreatic lineage (Chen & Dent 2014). H3K9 and H3K14 acetylation upregulates expression of hepatic genes driving differentiation into hepatoblasts whereas, H2K27 methylation downregulates expression of pancreatic genes (Figure 3).

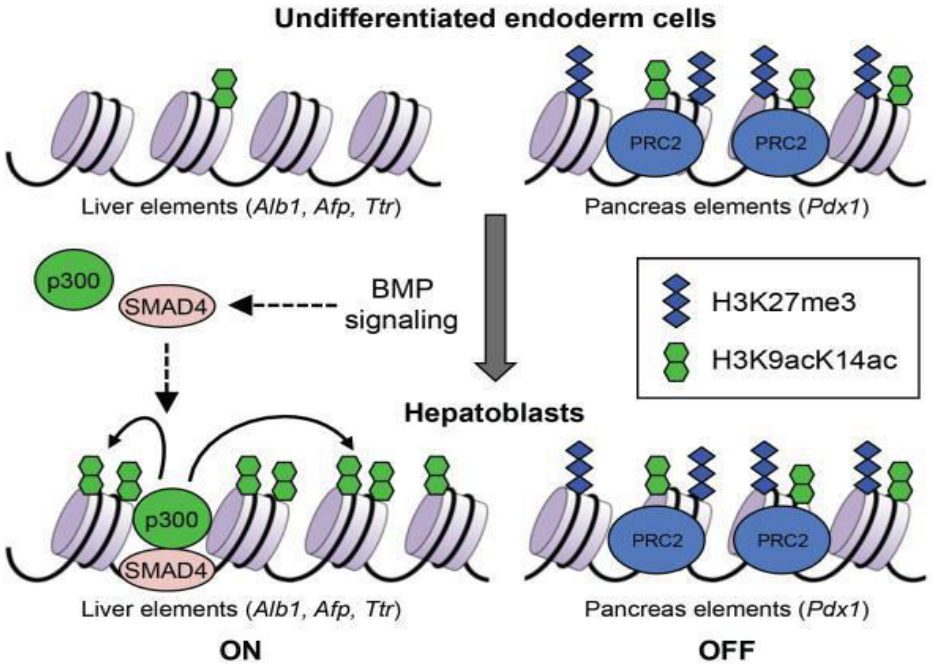


Figure 3. ***Undifferentiated endodermal cells, pre-patterning of chromatin markers*** *upregulate hepatic genes such as Alb1, Afp and Ttr and downregulate pancreatic genes such as Pdx1.*(Chen & Dent 2014) *BMP signaling leads to expression of liver genes and PCR2 complex inhibits expression of pancreatic genes.*

When the ES or iPS cells differentiate into a specific lineage, the genes expressed lose their bivalent state to an active state promoting lineage re-specification (Mikkelsen et al. 2007). Manipulation of chromatin accessibility is an important factor in re-specifying

cell fate of a somatic cell (Nashun et al. 2015). Reprogramming of somatic cells may be more efficient through an undifferentiated progenitor state which presents a more plastic chromatin configuration (Figure 4). Open chromatin state may promote steps of differentiation for cell fate specification (Sha & Boyer 2008).

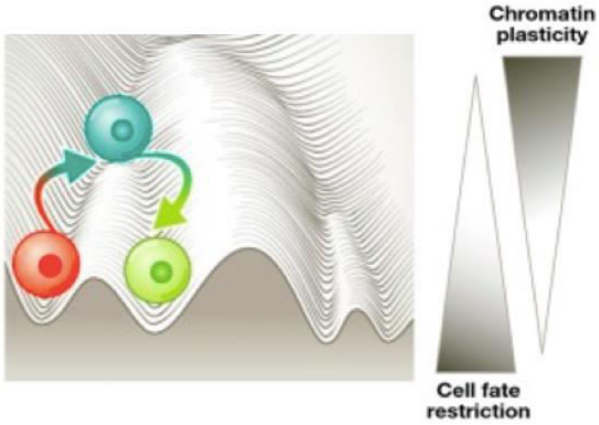


Figure 4. ***Relationship between chromatin plasticity and cell fate restriction****. (Red cell) somatic cell type 1 used to produce induced pluripotent stem cell (Blue cell) directly reprogrammed to new somatic cell type (Green Cell)* (Nashun et al. 2015).

A typical islet transplantation requires about one million islets, however it is difficult to isolate and successfully transplant that quantity of islets (Srinivasan et al. 2007). Direct reprogramming to produce ß-like cells with high efficiency could potentially be used as an alternative instead of autologous transplantation of insulin-producing cells. This study on methods to efficiently reprogram cells to a ß-like cell phenotype would provide an additional source of stable insulin-expressing cells for transplantation for patients living with type I diabetes.

CHAPTER 2
METHODS

The purpose of the current study was to improve overall efficiency of reprogramming and establish a good human *in vitro* model system for Ad-PNM reprogramming. A method to improve reprogramming efficiency was adopted from Sayed et al, and was tested on two different cell lines (rat pancreatic exocrine cell line and mouse primary hepatocyte derived cell line). For developing an in vitro human model system, ductal cells and endodermal progenitor cells were studied. Reprogramming of ductal cells have been carried out earlier in the Dutton Laboratory. In the present study, ductal cell reprogramming was carried out with the addition of small molecules to enable efficient reprogramming as described in Akinci et al. 2013. The combination of small molecules used was 10μM DAPT, 10μM NECA and 2μM BIX (DNB), described in figure 13. Methodology of experiments performed in this study are described below.

Cell culture

Rat pancreatic exocrine-like cells (AR24j-B13; hereafter referred to as "B13 cells") obtained from "chemically induced pancreatic tumors" (Longnecker et al. 1979) was acquired from Dr. David Tosh (University of Bath, UK). AlbCre;R26R, a mouse primary hepatocyte derived cell line, also known as ASH cells ("AlbCre small hepatocytes") was also acquired from the University of Bath (Seglen 1976). Both the cell lines were cultured in low-glucose Dulbecco's Modified Eagles medium (DMEM; LifeTechnologies) supplemented with 10% (v/v) fetal bovine serum (FBS; Coring), Penicillin/streptomycin (1x antibiotic-antimytotic; ThermoFisher Scientific) and 100x Non-essential amino acids (NEAA; LifeTechnologies)

Cell Line	Cell Type	Organism	Source
AR42j-B13	Pancreatic Exocrine Cells	Rat	Dr. David Tosh University of Bath, UK
ASH	Small Hepatocytes	Mouse	University of Bath, UK
HPan	Pancreatic Ductal Cells	Human	Dr. Bernard Herring, Dr. Melena Bellin, Molecular and Cellular Therapeutics, Saint Paul, University of Minnesota, Twin-Cities
vShiPS 9-1*	Induced Pluripotent Stem Cells	Human	Dr. James Dutton, University of Minnesota, Twin-Cities

Table 1. List of Cell lines used
**Appendix (2) for characterization of vShip 9-1 iPS cell line*

Functional assay of batches of Ad-PNM

AR24j-B13 cell line was used to establish the reprogramming efficiency of previously prepared batches of different Ad-PNM adenoviruses stored at –80ºC. Cells were maintained in low-glucose DMEM, 10% FBS in 5%CO_2 at 37ºC. For infections, B13 cells were counted using a hemocytometer and 25,000 cells were plated per 3.8cm^2 in a 12 well tissue-culture plate. The cells were cultured overnight to facilitate cell attachment. The following day, media was changed before viral infection. 1µl aliquot of Ad-PNM adenovirus preparation was diluted 1/1000 in 1ml low-glucose DMEM, 10% FBS (1 in 1000 dilution). 200µl of the different 1/1000 diluted Ad-PNM preparations (namely; A12, C3, C5, C7, C9, C11, C12, C13, C14, C15, C16) were added to each well to infect the cells for a period of 2 or 3 days. Uninfected B13 cells were also plated for uninfected controls and exposed to same conditions excluding viral infection. Cells were fixed three days after infection to perform immunohistochemical analysis. Following immunohistochemistry, cells were imaged in at least 3 distinct fields at 40x magnification to detect cells that express Pdx1 and Ins. Single positive cells for Pdx1 and double positive cells were tallied to determine overall infection and reprogramming efficiency.

Dilution assay of A12 Ad-PNM

To determine the efficiency of infection of A12 batch of Ad-PNM adenovirus, B13 cells were plated as described above. 50µl, 100µl, 200µl and 400µl of 1/1000 diluted A12 virus were added to eight separate wells, 2 wells per calculated amount of diluted virus. One set of wells, 50– 400µl dilutions were cultured for 2 days without media change and second set of wells were cultured 3 days without media change. After infection, the cells were fixed in preparation for immunohistochemistry.

PolyI:C treatment

To induce innate immune response, 100ng/ml, 300ng/ml or 600ng/ml polyinosinic:polycytidylic acid (PolyI:C) complexed with transfection reagent Lipofectamine (Invitrogen) in serum-free low-glucose DMEM was incubated for twenty minutes at room temperature (RT) prior to direct addition to the wells. Cells were fixed in preparation for immunohistochemical analysis 18h after exposure.

When combined with Ad-PNM infection, prior to Ad-PNM infection, cells were treated with calculated amounts of polyI:C. The following day, fresh media without polyI:C was replaced prior to infection. 200µl of 1/1000 diluted Ad-PNM was directly added to the wells. The virus added was left on for 3 days for infection before fixing cells for antibody detection.

Fixation and Immunohistochemistry

Cells were fixed with 10% formalin (Protocol, Fisher-Scientific) at room temperature for 10 minutes. Formalin was aspirated out and the cells were rinsed thrice with Phosphate Buffered Saline 0.1%Tween (PBS-T; PBS; Coring, Tween20; Sigma-Aldrich). Post washing, 1ml of fresh PBS-T was added to the wells and plates were stored at 4ºC. Blocking solution (PBS-T 1% BSA) was added to the cells for 30 minutes to reduce nonspecific antibody binding. For nuclear antibodies, cells were permeabilized with PBS-T 1% BSA 1% Tween for 10 minutes before blocking again for 30 minutes with blocking buffer. Dilution of primary antibody was done by addition of calculated amounts of stock primary antibody in blocking buffer. Primary antibody was diluted and added to the cells

and incubated at room temperature for an hour or at 4ºC overnight. The following day, primary antibody was washed out thoroughly with PBS-T. Corresponding diluted secondary antibody was added to the cells and incubated for one hour at room temperature. Finally, 1/1000 diluted DAPI was added to each well after thorough washing out of secondary antibody.

Antibody	**Manufacturer**	**Catalog**	**Dilution**
Anti-Pdx1 rabbit	Millipore	07-696	1:2000
Anti-Insulin guinea pig	Cell Marque	273A-15	1:500
Anti-p65 rabbit	Abcam	ab-16502	1:500
Anti-IRF3 rabbit	Abcam	ab-25950	1:500
Anti-IRF7 rabbit	Abcam	ab-109255	1:500
Anti-Vimentin chicken	ThermoFisher	PA1-10003	1:3000
Anti-Cytokeratin 19 mouse	Dako	GA61561-2	1:500
Anti-Sox9 rabbit	Millipore	AB5535	1:1000
Anti-Sox17 goat	R & D Biosystems	AF1924	1:1000
Anti-FoxA2	Santa Cruz	sc-9187	1:500

Table 2. List of Primary antibodies used

Secondary Antibody	**Dilution**	**Manufacturer**
Alexa Fluor 488 goat anti-rabbit	1:1000	Life Technologies
Alexa Fluor 488 donkey anti-goat	1:500	Life Technologies
Alexa Fluor 555 donkey anti-rabbit	1:500	Life Technologies
Alexa Fluor 555 donkey anti-mouse	1:500	Life Technologies
Alexa Fluor 555 donkey anti-guinea pig	1:500	Life Technologies
Alexa Fluor 647 donkey anti-mouse	1:1000	Life Technologies

Table 3. List of Secondary antibodies used

Total Pancreatectomy-Islet Auto-Transplantation (TP-IAT) procedure

Multiple publications describe the TP-IAT procedure. Initial islet isolation and purification was performed at Molecular and Cellular Therapeutics (MCT) GMP facility at the University of Minnesota, Twin-cities (Ricordi et al. 1989; Bellin et al. 2015) and provided the residual pancreatic tissue for ductal cell isolation. All tissue donations were conducted with permission from the UMN IRB.

Tissue Digestion

80% of the "UW cold storage" solution from the 50ml conical containing the digested tissue was aspirated and the tissue in the remaining UW solution was moved to a 10cm petri-plate. The tissue was assessed to remove unnecessary contents such as fibrotic tissue, sutures, and residual blood clots. The remaining tissue was manually disintegrated using scalpel and forceps in preparation for digestion. The broken-down tissue was transferred to a 50ml conical. 40 ml of low-glucose serum-free DMEM was added for 10 minutes to wash the tissue. The wash media was removed and 50ml of pre-warmed "EGTA dissociation buffer" (10mM HEPES pH 7.4, 5mM KCl, 50mg/ml Gentamicin, 5mM Glucose, 0.5mM EGTA in PBS to make up the volume to 50ml) was added to the sample. The sample in the EGTA buffer was placed on a shaker inside the incubator at 37ºC for 20 minutes. The tissue was the centrifuged for 5 minutes at 1000rpm and the EGTA buffer was aspirated. 50 ml of digestion buffer (40ml DMEM, 2ml FBS, 1ml collagenase (83.3 mg/ml), 1ml hyaluronidase (66.7 mg/ml), 500µl of 500mM $CaCl_2$ to make up volume to 50ml per 2.5ml of dissociated pancreatic tissue) was added to the tissue and stirred at 37°C inside an incubator for 40 minutes to disintegrate the tissue. 1ml of Dispase was added and the tissue was incubated for 45-60 minutes depending on the degree of digestion of the tissue. Digestion buffer was aspirated post incubation period and the sample was rinsed thrice with 50ml DMEM supplemented with 10% FBS. The digested tissue was passed through a 100µm filter post final wash. The flow-through containing cells were cultured for two days in an ultra-low attachment 6–well plate in DMEM 10% FBS, 1xPen/Strep and 1x NEAA.

Epithelial Aggregation Isolation and Culture

Two days after digestion the sample was observed under the microscope to observe aggregate formation. If only single cells were present, the cells were maintained in culture for an additional night. If aggregates were present, the isolation procedure continued. Cell suspension was collected from the plate to a 15ml conical tube, the wells were washed one additional time to collect any remaining aggregates. The collected cells in suspension were passed through a 37μm reversible strainer/filter to get rid of single cells. The filter was transferred into a fresh 15ml tube and "aggregates" were backwashed into the tube washed with Keratinocyte-serum free media (KSFM). The collected cells were transferred to ultra-low attachment 6-well plates and maintained in culture for 5-7 more days with occasional media change.

Primary Ductal Cell Plating

Aggregates in suspension were collected. An additional wash with KSFM was performed to collect aggregates left behind in the well. A cell pellet was formed by spinning down the collected aggregates for 5 minutes at 1000 rpm. Aggregates were re-suspended in KSFM supplemented with 0.1% gentamycin (v/v) and plated on Bio-coat Collagen I coated plates (CytoOne) and maintained at 37ºC in 2% O_2.

Administration of small molecules with AD-PNM infections

Together with Ad-PNM, a combination of small molecules, DAPT, NECA and BIX (DNB), previously shown to improve Ad-PNM reprogramming efficiency (Akinci et al. 2013) was added to primary ductal cells. Overnight incubation was performed to enable the cells to attach. The following day, calculated amounts of DNB combination was added to the cells (Day 1). After two days Ad-PNM was added to the cells without replacing the media supplemented with the small molecules (Day 3). The following day, fresh media was added (Day 4) and the cells were incubated for two additional days prior to cell fixation in preparation for immunohistochemistry (Refer to Figure 14).

iPS Cell Culture

Induced pluripotent stem cells (iPSC) were maintained in an undifferentiated state by culturing the cells on Vitronectin-coated (Peptrotech) plates in Essential 8 Flex Media (E8 Flex Media Kit; ThermoFisher). Media was changed every day and cells were passaged every 3 days using hypertonic citrate buffer (Nie et al. 2014).

Endodermal Differentiation

For definite endoderm (DE) differentiation, iPSC cells were either seeded as small colonies on vitronectin–coated plates or dissociated as single cells and seeded on 2% Matrigel coated 12-well plates at a density of 150,000-200,000 cells per well. For better survival, as single cells, 5μM ROCKi (ROCK inhibitor; Selleckchem) was added while seeding cells. Single cells were cultured for 2–3 days to 80% confluency. Maintenance media was replaced with Differentiation media containing Essential 6 media (LifeTechnologies) supplemented with 0.5μM Dexamethasone (Dex; Sigma D4902-25MG), 100ng/ml Activin A (ActA; Peprotech) and 3μM CHIR99021 (CHIR; Selleckchem) for 2 days (Day 0 – Day 2) and then, partial replacement of media supplemented with 100ng/ml Activin A is added for an additional two days (Day 2 – Day 4). On the fourth day of differentiation, cells were either fixed in preparation for immunohistochemistry or were infected with Ad-PNM (refer to Figure 17).

CHAPTER 3

RESULTS

Functional assay of different batches of Ad-PNM

Batches of previously prepared Ad-PNM vector were tested for reprogramming efficiency using AR42j-B13 cells. The reprogramming efficiency of individual batch of Ad-PNM (A12-C16) (Table A1; *see appendix (1)*) was determined by measuring Pdx1 and Insulin protein expression visualized by immunohistochemistry. Infection with each batch of Ad-PNM was carried out by addition of 200uL of Ad-PNM adenoviral construct to 25,000 B13 cells. Reprogramming efficiency was calculated after 2 and 3 days respectively (Figure 6). The functional efficiency was calculated by quantifying total number of cells (DAPI stained nuclei), total number of Pdx1+ cells and Insulin+/Pdx1+ cells in at least three different fields with images taken at 40x magnification. Uninfected cells were used as a negative control. Infection efficiency and reprogramming efficiency was calculated as shown in Figure 5. Ad-PNM batches, A12 and C15 displayed infection efficiencies of ~95-98% (Figure 6C) and approximately ~60-62% reprogramming efficiency (Figure 6D). Because of the lack of stored C15 batches of Ad-PNM, A12 was used for future experiments carried out in this study.

$$\text{Infection Efficiency} = \frac{\text{Number of Pdx1}^{+}\text{ cells}}{\text{Number of DAPI-stained nuclei}}$$

$$\text{Reprogramming Efficiency} = \frac{\text{Number of Pdx1}^{+}\text{Ins}^{+}\text{ cells}}{\text{Number of DAPI-stained nuclei}}$$

Figure 5. ***Formulae for calculation of Infection efficiency and Reprogramming efficiency*** *used in the study.*

Figure 6

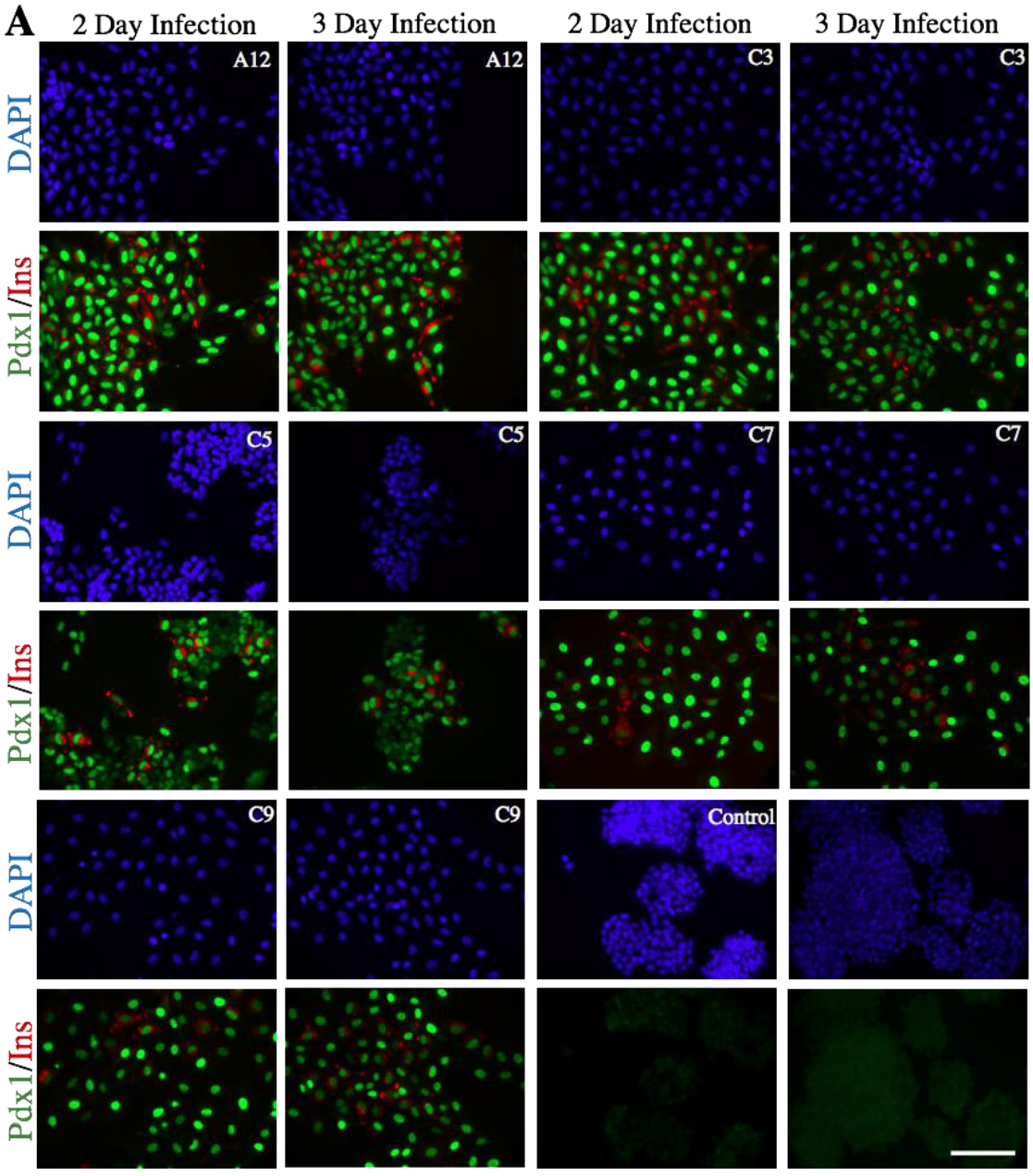
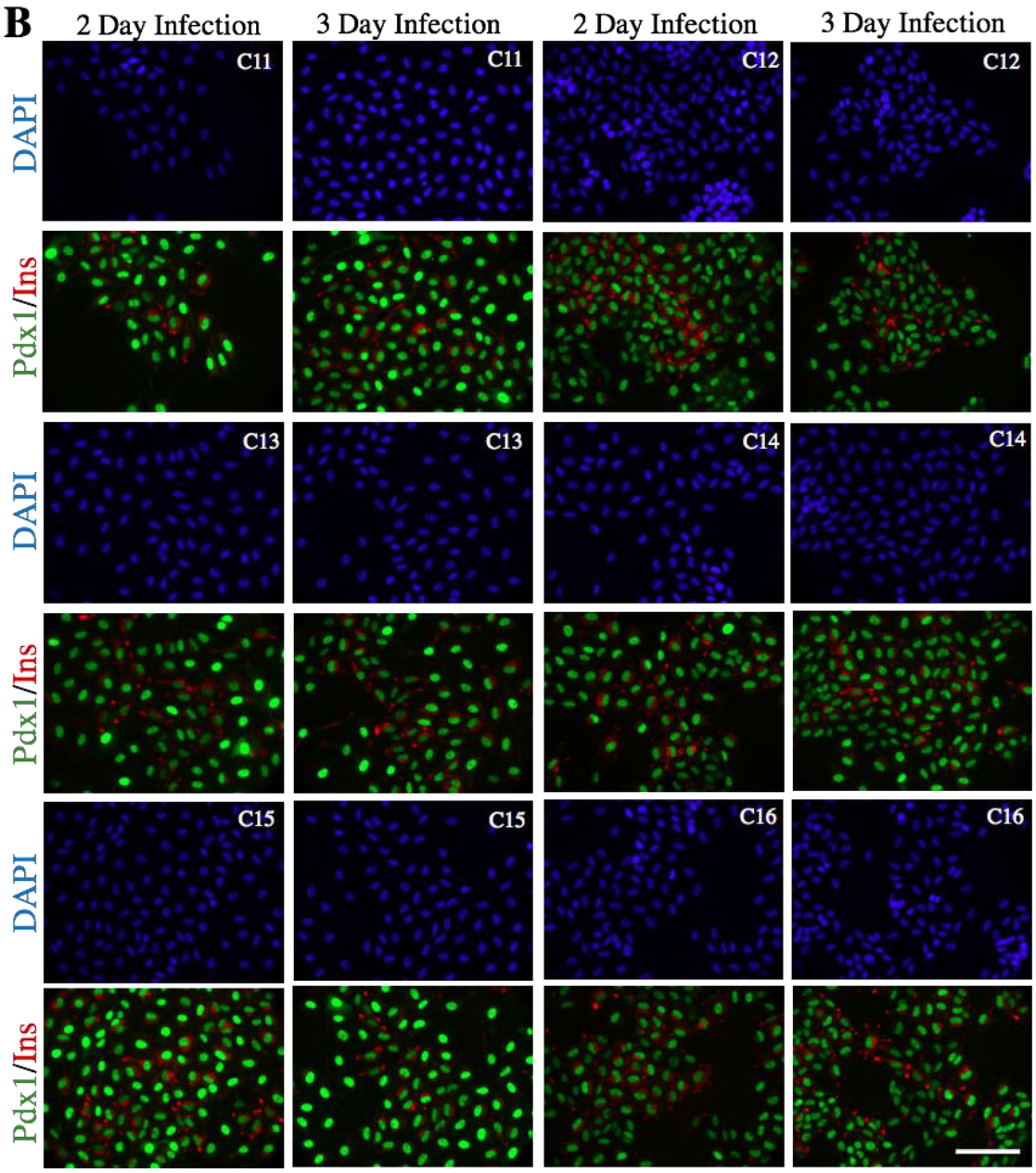


Figure 6


B
2 Day Infection
3 Day Infection
2 Day Infection
3 Day Infection
DAPI
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C11
C11
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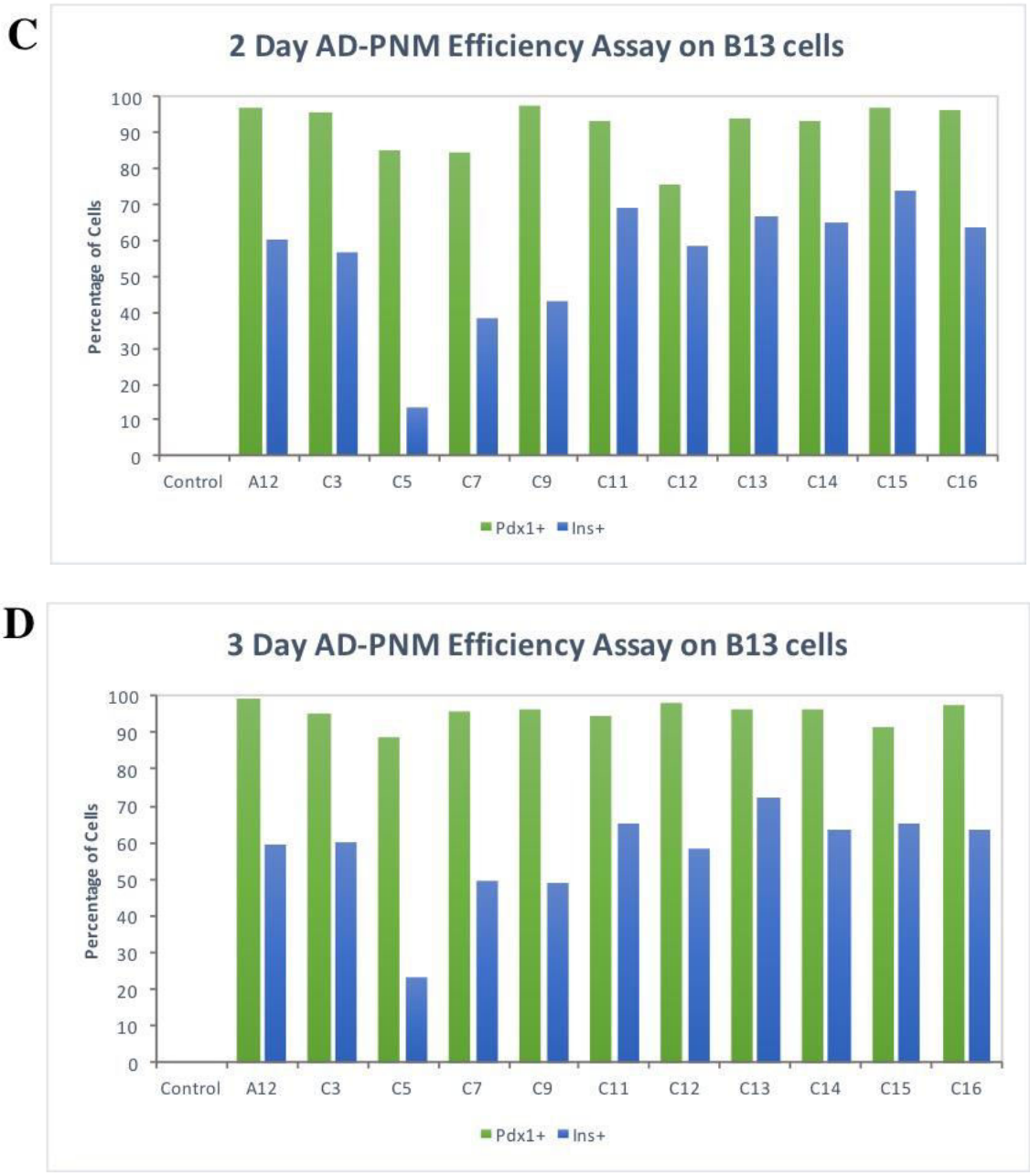


Figure 6. ***Ad-PNM infection efficiency assay.*** *(A) Each batch of Ad-PNM (A12-C9) and (B) batches (C11-C16) was tested for the functionality on B13 cells plated at a density of 25,000 cells per 3.8 cm^2. Each batch was infected and incubated for two and three days respectively. Immunohistochemical analysis was performed on fixed cells for expression of Insulin (red), Pdx1 (green) and DAPI (blue). Uninfected B13 cells were used as the negative control. Imaging was performed using Leica Microsystems at 40x magnification; Scale bar: 100 µm (C) and (D) Bar graph representing IHC data*

displaying percent of cells expressing Pdx1+ and Insulin+ for different Ad-PNM batches (A12-C16) calculated (n=3).

Dilution assay of different batches of Ad-PNM

To estimate the optimal concentration of virus required for efficient reprogramming, A12 Ad-PNM virus was diluted 1 in 1000. Varying amounts of diluted A12 Ad-PNM were added to B13 cells and ASH cells.

- **B13 cells**

A12 Ad-PNM was tested at four different dilutions; 50µL, 100µL, 200µL and 400µL of diluted virus was added to 25,000 B13 cells plated in a 3.8cm^2 12-well culture plate. The cells were in culture for two or three days prior to fixing them for immunohistochemistry (Figure 7A). Percent of Pdx1+ positive cells represented the infection efficiency. Increasing amounts of vector increased the infection efficiency (Figure 7B). However, with the addition of 400µL of diluted vector, we saw cell death leading indicating the vector concentration was too high. Reprogramming efficiency was calculated as described previously (Figure 5).

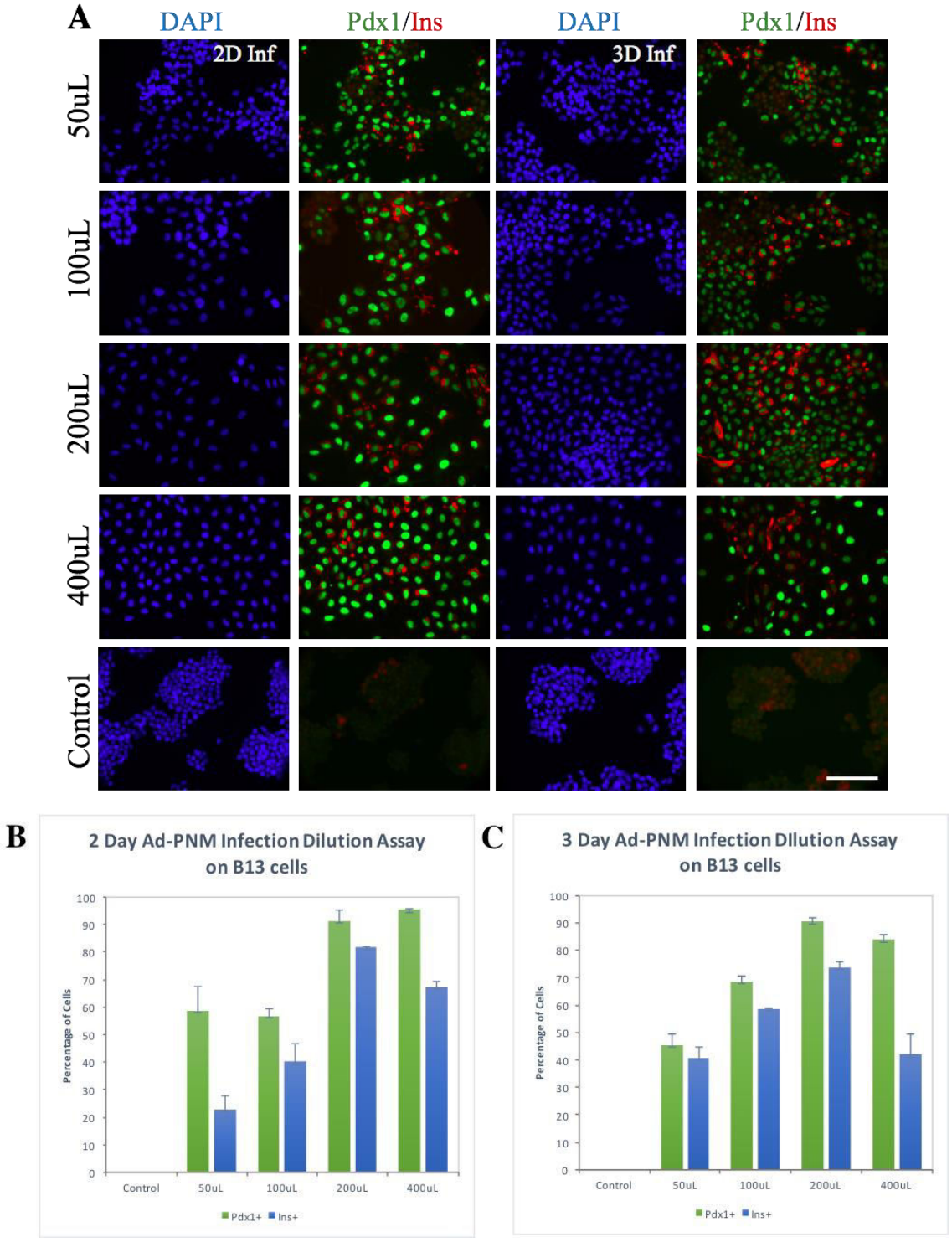


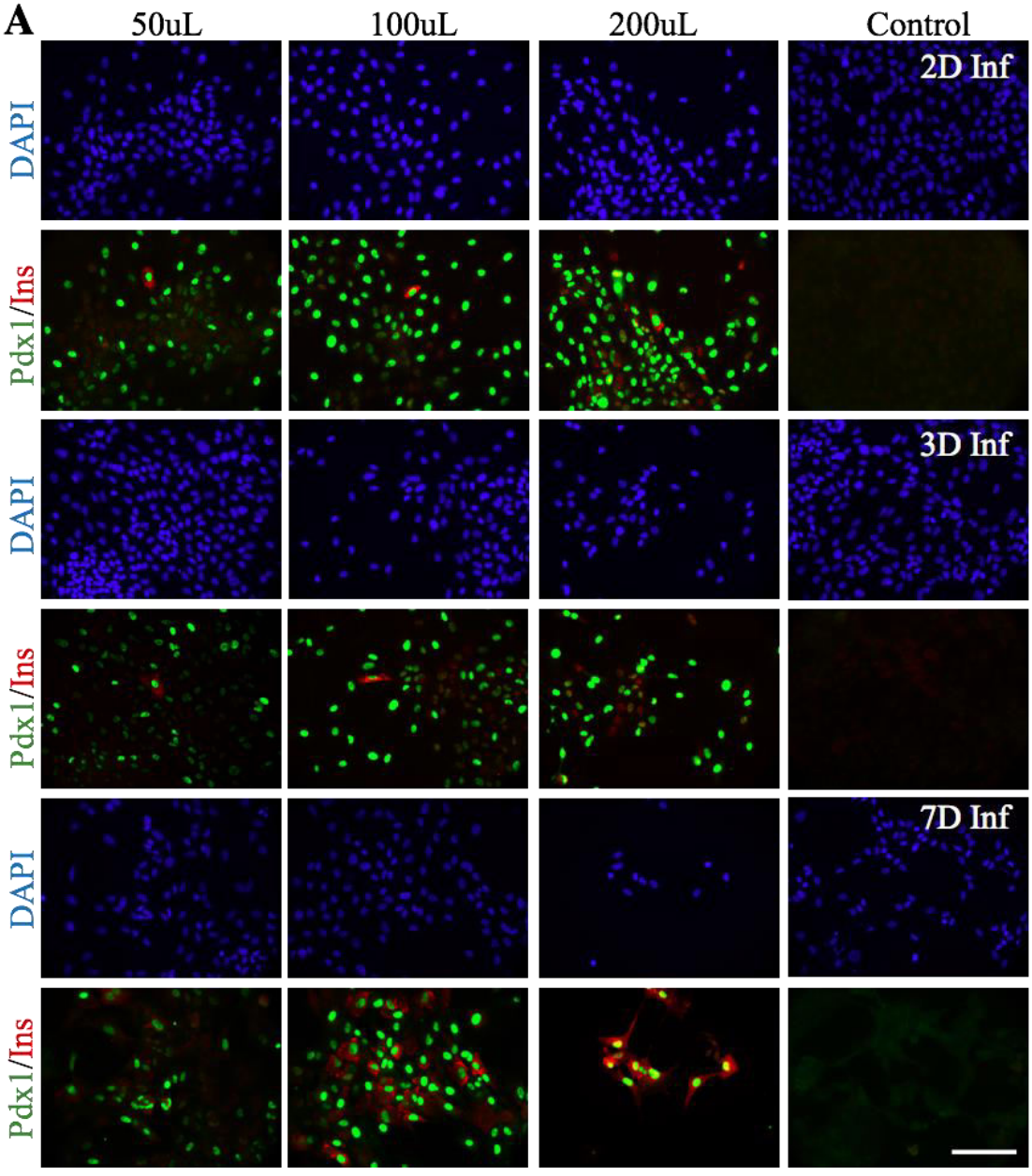
Figure 7. ***A12 Ad-PNM batch dilution assay on B13 cells.*** *(A) A-12 batch Ad-PNM dilution assay was performed by testing four different dilutions; 50µl, 100µl, 200µl and*

400µl on B13 cells for optimal amount required for efficient reprogramming. Scale bar: 100 µm. (B) and (C) Representative quantification of IHC in (A) displaying two and three-day infection. Percentage of cells expressing Pdx1+ and Insulin+ shown (n=3)

- **ASH cells**

A12 Ad-PNM was tested at four different concentrations on ASH cells plated at a density of 50,000 cells per 3.8cm^2 well of a 12-well culture plate. Initially, the cells were in culture for 2 and 3 days. After this period, the cells were stained for Pdx1 and Insulin by Immunohistochemistry (Figure 8A). It was observed that 400 µL concentration of Ad-PNM, killed all the cells in the well. Therefore, we fixed the maximum concentration of virus to 200µL of diluted A12 Ad-PNM virus. Based on the results, an 85% infection efficiency was obtained (Figure 8B). However, reprogramming efficiency of 35% was seen with addition of 200µL of diluted virus. Due to the lack of efficient reprogramming, we cultured the cells for an additional four days after the initial 3–day infection period. Cells were fixed on Day 7. A significant amount of cell death was observed. A similar infection efficiency of 85% was observed and reprogramming efficiency was increased by 15% to an overall efficiency of 50% (Figure 8).

Figure 8



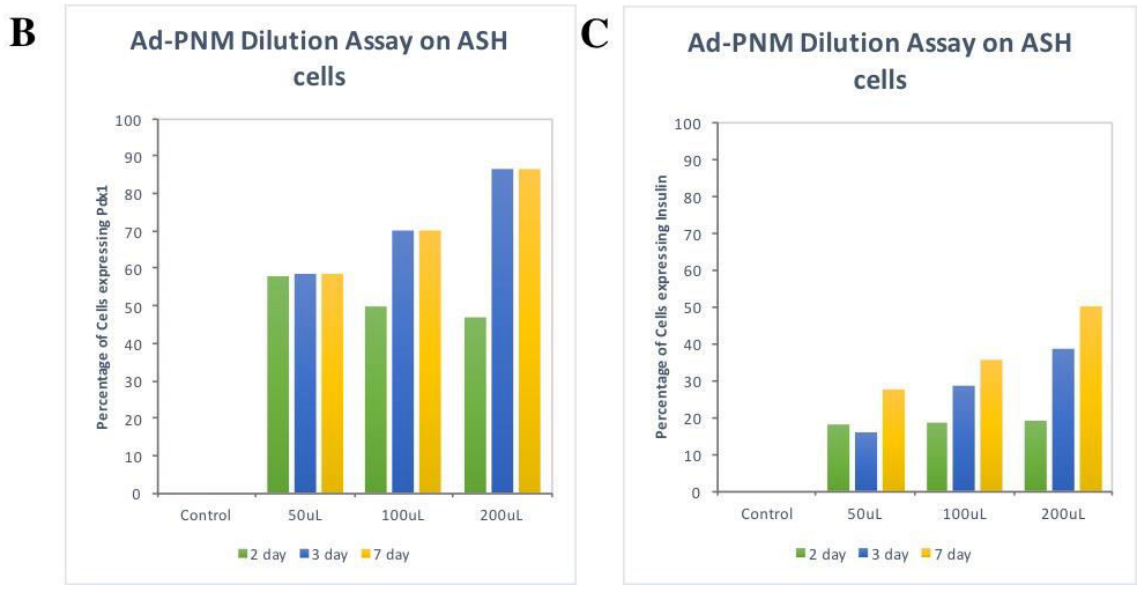


Figure 8. ***A12 Ad-PNM batch dilution assay on ASH cells.*** *(A) A-12 batch Ad-PNM dilution assay was performed by testing three different dilutions; 50ul, 100ul and 200ul on ASH cells plated at a density of 50,000 cells per 3.8 cm^2 to determine optimal amount required for efficient reprogramming. Scale bar: 100 µm. (B) and (C) Representative quantification of IHC in (A) displaying two, three and seven-day infection. Percentage of cells expressing Pdx1+ and Insulin+ shown (n=2)*

PolyI:C addition to determine the role of Innate Immunity

In this study, we used an adenoviral vector delivery system to directly reprogram cells. Activation of innate immunity can potentially trigger a cascade of reactions that change chromatin structure and enhance the possibility of endogenous gene activation following introduction of exogenous genes. To determine the innate immunity activation efficiency, B13 cells and ASH cells were treated with PolyI:C, a synthetic analog of dsRNA, mimicking the innate immunity activation shown previously to enhance iPSC reprogramming (Alexopoulou et al. 2001). A transfection reagent Lipofectamine, was used to introduce the polyIC into the cells. Both ASH and B13 cells were exposed to PolyI:C and lipofectamine for a duration of 18 hours. After 18 hours, the cells were fixed and immunohistochemistry was performed to check for nuclear localization of p65, IRF7 and IRF3, all downstream effectors of TLR3 signaling. Based on the methodology described in Sayed *et al* (Sayed et al. 2015), a concentration 300ng/ml PolyI:C was tested

on B13 cells plated at a density of 25,000 cells per 3.8cm2 of a 12-well plate. Nuclear localization of p65 was observed in 45% of the cells whereas only 3% and 1% of nuclear localization of IRF7 and IRF3 was observed respectively (Figure 9A and 9D). Next, we tested three different concentrations of PolyI:C; 100ng/ml, 300ng/ml and 600ng/ml on ASH cells plated at a density of 50,000 cells per 3.8cm^2 of a 12-well plate. Consistent with the B13 cells, ASH cells displayed responses in p65 only with minimal responses seen in case of IRF7 and IRF3 (Figure 10). p65 nuclear localization consistently increased with addition of increasing amounts of PolyI:C to the cells (Figure 10A and 10D).

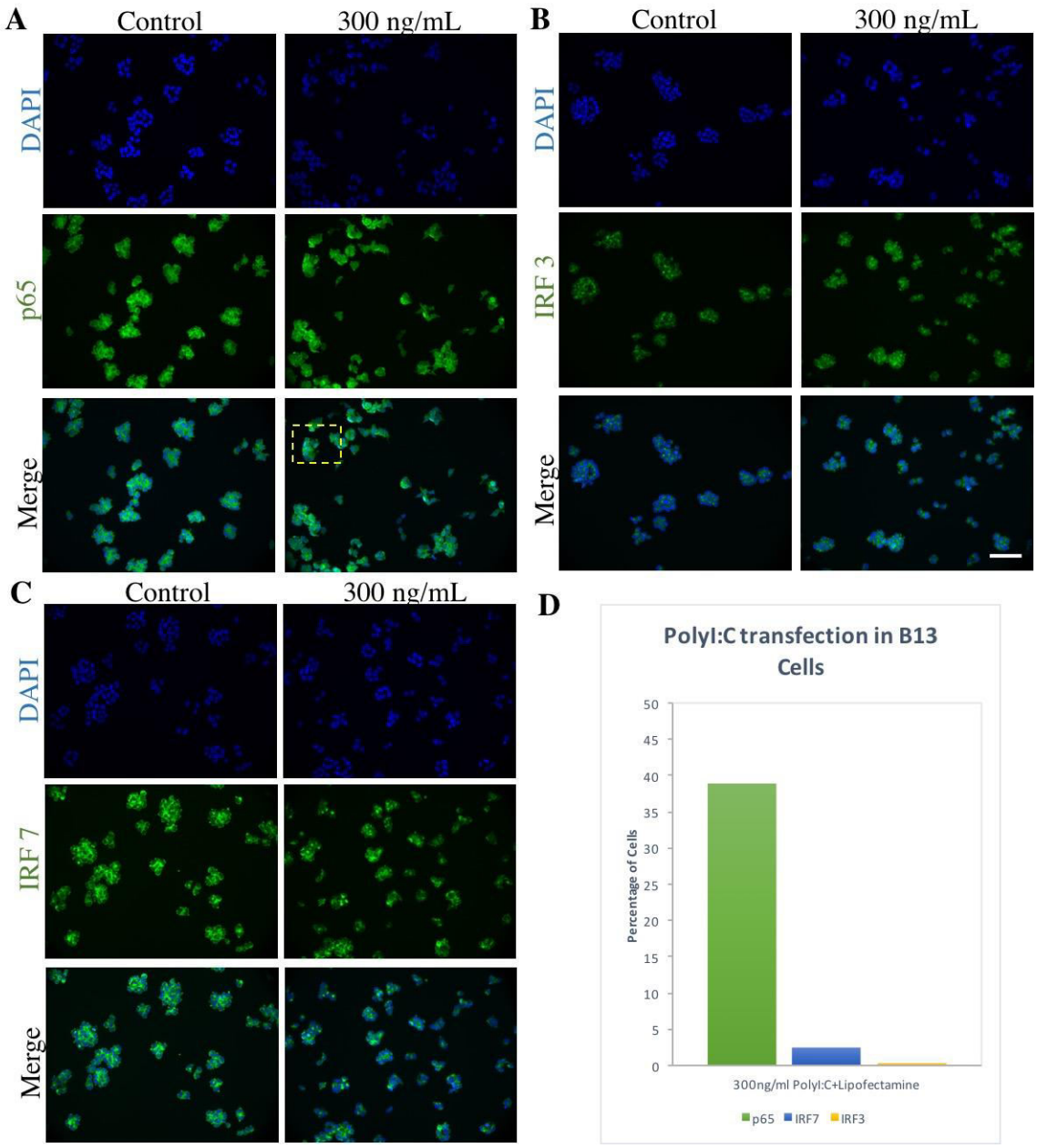


Figure 9. ***PolyI:C treatment on B13 cells to trigger innate immune responses.*** *IHC performed for (A) p65 (B) IRF3 (C) IRF7 to analyze nuclear localization as a measure of activation of innate immune response to 300 ng/ml PolyI:C. (D) Quantification of IHC in (A)(B)(C) represented as percent expression. Scale bar: 100 μm.*

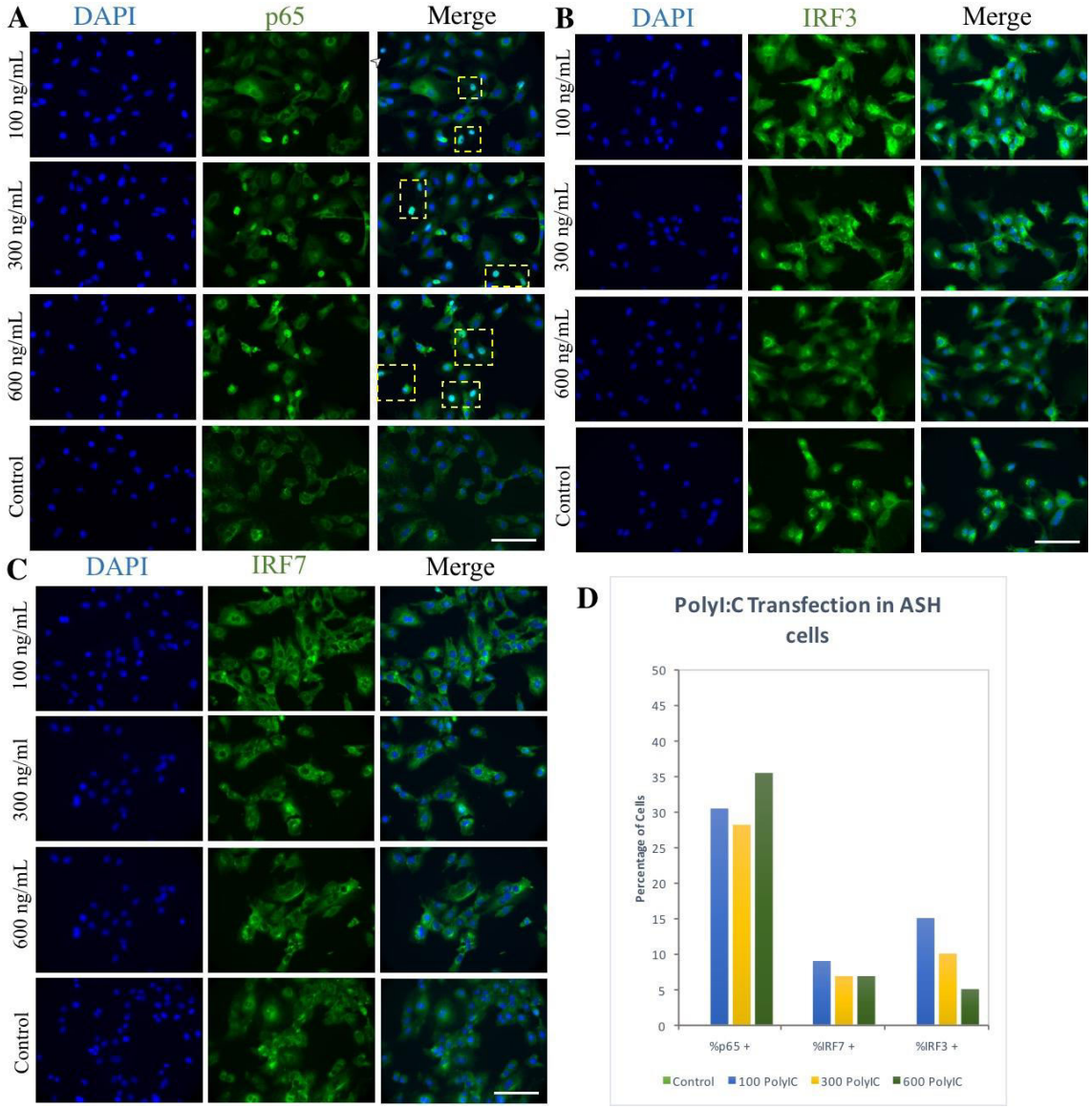


Figure 10. ***PolyI:C treatment on ASH cells to trigger innate immune responses.*** *ASH cells were exposed to differential concentrations of PolyI:C; 100 ng/ml, 300 ng/ml and 600 ng/ml. IHC performed for (A) p65 (B) IRF3 (C) IRF7 to analyze nuclear localization as a measure of activation of innate immune response to concentrations of PolyI:C. (D) Quantification of IHC in (A)(B)(C) represented as percent expression. Scale bar: 100 µm. (n=2)*

PolyI:C treatment prior to Ad-PNM infection of B13 cells to improve overall reprogramming efficiency

From the dilution assay, we found that addition of 50 μL of virus yields 45% infection efficiency after 3 days of infection (Figure 7). This indicated that there is scope for improving the reprogramming efficiency. We treated the B13 cells with polyI:C prior to infecting them with Ad-PNM. 100 ng/ml, 300 ng/ml and 600 ng/ml concentrations were added to B13 cells plated at a density of 25,000 cells per 3.8cm^2 area of 12-well culture plate for a time period of 18h. At 18h, fresh media was added and cells were infected with 50 μL A12 Ad-PNM. An untreated uninfected well was used as double negative control and an untreated infected well was used as a negative control for polyI:C treatment. 3 days post infection, cells were fixed and immunohistochemistry for Ins and Pdx1 was performed (Figure 11). Upon comparison with just 50 μL Ad-PNM infection, it is observed that infection efficiency does not change with 100 ng/ml treatment of polyI:C. However, there is a substantial decrease in infection efficiency in cells treated with 300 ng/ml and 600 ng/ml.

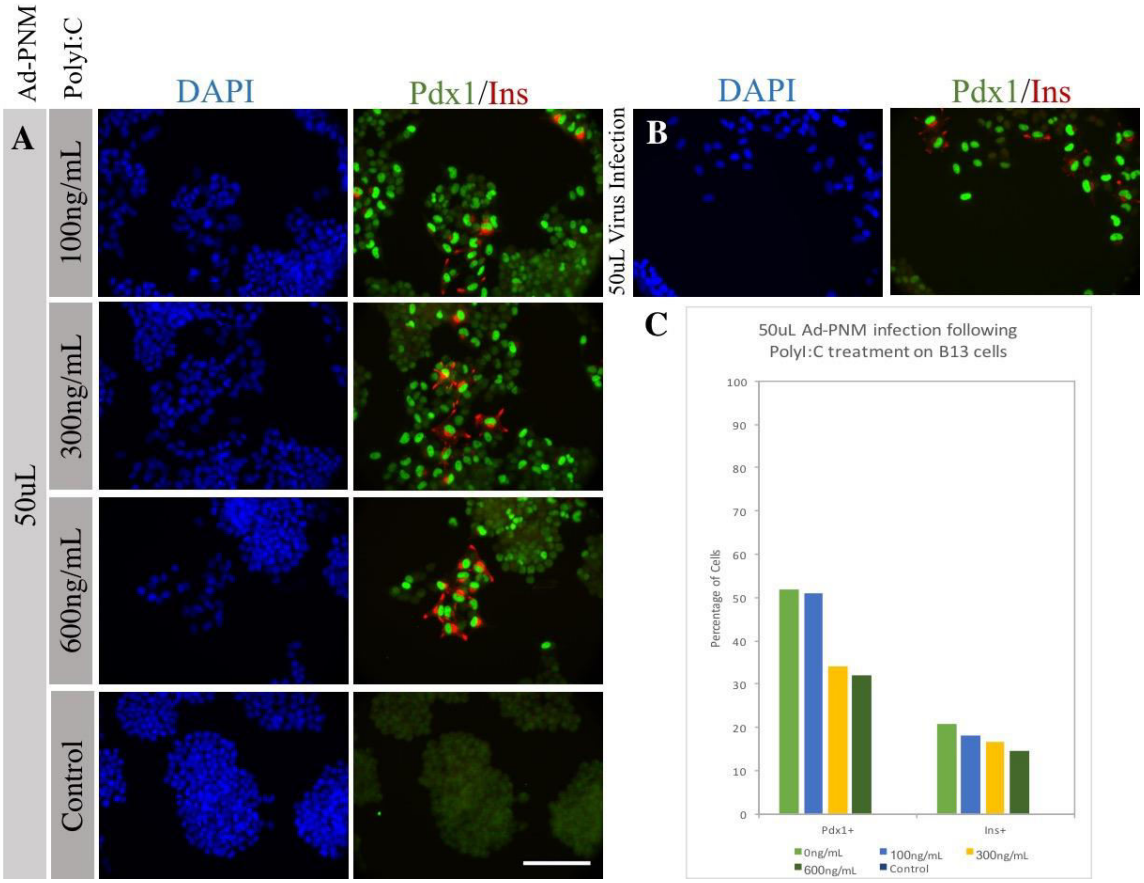


Figure 11. ***A12 Ad-PNM infection on B13 cells post PolyI:C treatment.*** *Cells were exposed to polyI:C for 18h before addition of 50uL Ad-PNM (A) Varying concentrations of PolyI:C added prior to infection with 50 uL of Ad-PNM. (B) 50 uL virus infection without polyI:C treatment as a single negative untreated control. IHC performed to quantify reprogramming efficiency; Insulin (Red), Pdx1 (Green) and DAPI (Blue). Scale bar: 100 µm. (C) Representative quantification of IHC in (A) and (B). Percent expression of Pdx1+ and Insulin+ shown (n=3)*

Isolation of human pancreatic ductal cells

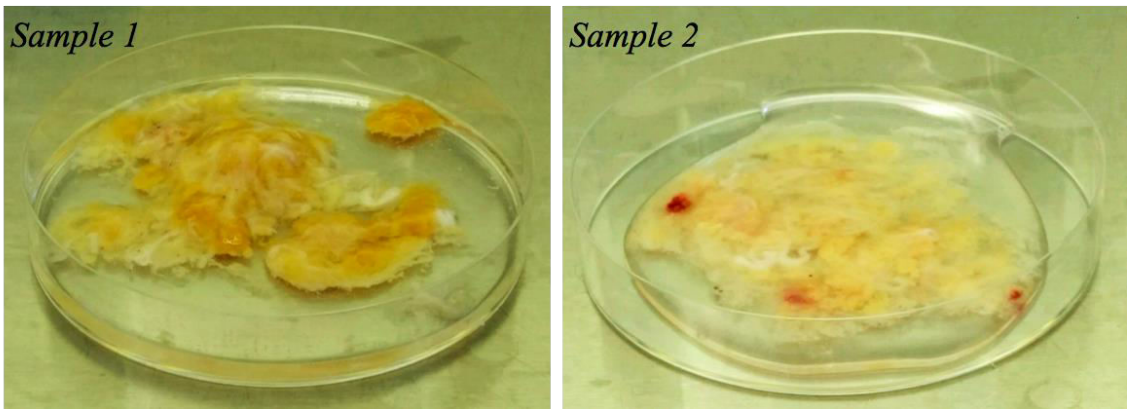


Figure 12. ***Human pancreatic tissue after TP-IAT procedure*** *from two patients in 10 cm petri-dish.*

Pancreatic tissue post islet isolation performed at the Molecular and Cellular Therapeutics Center, University of Minnesota, stored in UW solution, was brought to the Dutton Laboratory. These samples were obtained from patients who underwent TP-IAT procedures. In the lab, the samples were assessed visually and any fibrotic tissue or sutures left behind were removed (Figure 12). The digestion protocol described in the methods section was performed on the sample up to the point where epithelial aggregates were formed. For validation of presence of ductal cells post isolation, the aggregates were collected from suspension and plated on Bio-coat Collagen I plates. IHC for Sox9, Ck19 (ductal cell markers) (Shroff et al. 2014; Bouwens et al. 1994) and vimentin was performed. Co-localization of Sox9+/Ck19+ indicated presence of ductal cells in the sample (Figure 13).

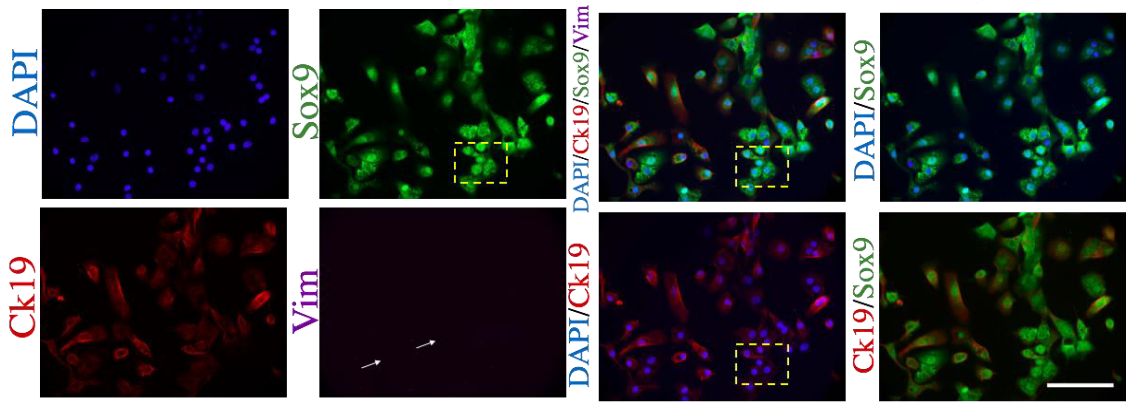


Figure 13: ***Characterization of isolated ductal cells.*** *Pancreatic ductal cells isolated from sample 1 in Figure 11. SOX9+/Ck19+ double positive cells validated presence of ductal cells. Yellow dotted box display co-localization of Sox9/DAPI/Ck19. Scale bar: 100 μm*

Reprogramming human pancreatic ductal cells

After isolating the ductal cells, direct reprogramming using Ad-PNM was performed. The reprogramming was attempted in two ways as described below.

First, the epithelial aggregates were collected from the suspension culture and plated on Biocoat Collagen I plates. The reprogramming was performed as shown in Figure 14. A majority of the cells failed to adhere to the plates. A subpopulation of cells was treated with small molecules (DAPT, NECA and BIX) (Figure 15A) and the second subpopulation was not treated with small molecules. Addition of DAPT; inhibitor of Notch signaling/metabolism, NECA; agonist of adenosine signaling, BIX; chromatin modification (inhibitor of deacetylases) has shown to stimulate an increase in reprogramming.

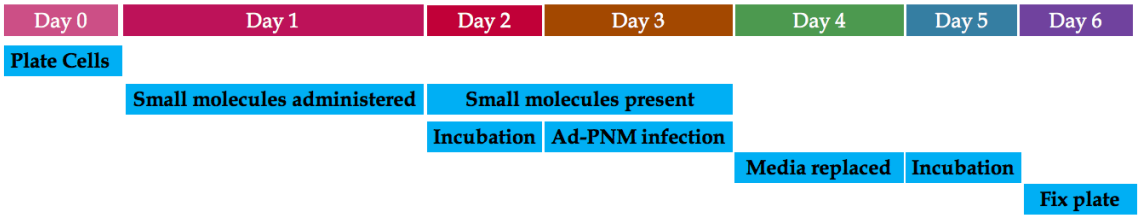


Figure 14. ***A schematic diagram of administration of small molecules prior to Ad-PNM infection****. DAPT (inhibitor of Notch signaling), NECA (agonist of adenosine signaling) and BIX (chromatin modifier; inhibitor of deacetylases) are the small molecules added. Ad-PNM infection was performed for 24 hours.*

An uninfected untreated well was used as the double negative control (Figure 15C). Immunohistochemistry revealed Pdx1 and Insulin expression in both populations of infected cells (Figure 15A and 15B). However, faint insulin expression is seen in (C) (white dotted box) but with no Pdx1 expression, indicating that this expression is likely background. However, infected populations of cells showed Pdx1 and corresponding Insulin (white solid box) demonstrating that two percent of cells infected with Ad-PNM was reprogrammed into cells that expressed insulin.

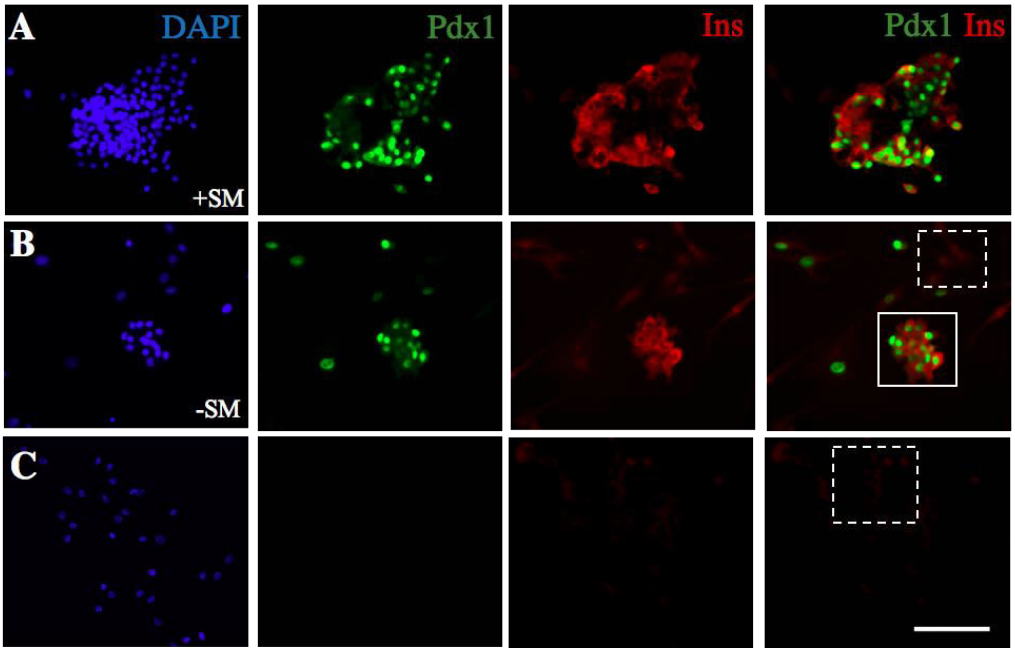


Figure 15. ***Ad-PNM infection of isolated human pancreatic ductal cells.*** *Cells were treated as represented in Figure 13. Insulin (Red), Pdx1 (Green) and DAPI (Blue) expression shown. Scale bar: 100 µm*

Epithelial aggregates were infected as aggregates. 24 hours after Ad-PNM infection, the infected aggregates were collected from suspension and plated on Bio-coat Collagen I plates for 3 days (Figure 16). IHC indicated ~21% infection efficiency calculated as Pdx1+ cells counted from at least 3 different fields of view. Insulin expression was observed in 4% of the infected cells.

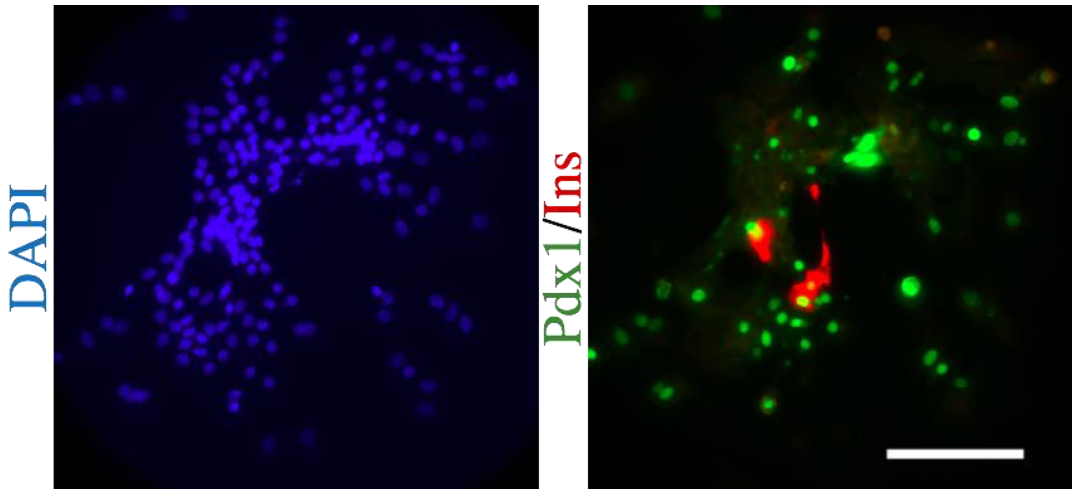


Figure 16. ***A12 Ad-PNM infection of epithelial aggregates.*** *Isolated and purified epithelial aggregates were infected in suspension for 24 hours prior to plating down on Biocoat Collagen I plates. IHC performed after 3 days in culture. DAPI (Blue), Pdx1 (Green) and Insulin (Red). Scale bar: 100 µm*

Differentiation of Induced Pluripotent Stem Cells into Endodermal Progenitors

Progenitor cells derived from iPSCs may have a more open chromatin structure more amenable to reprogramming (Nashun et al. 2015). We sought to initially differentiate induced pluripotent stem cells (iPSC) into endodermal progenitor cells followed by Ad-PNM infection. iPSCs were cultured and differentiated using a protocol previously shown to facilitate definitive endoderm differentiation (Figure 17).

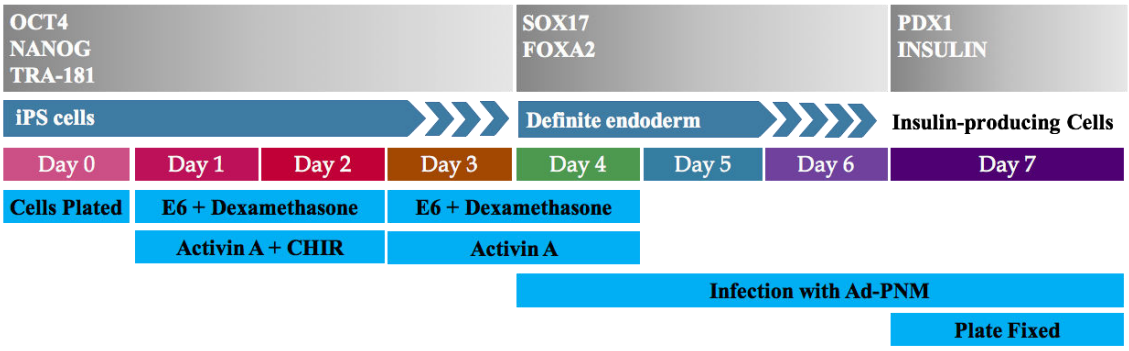


Figure 17. ***Differentiation protocol schematic****. A step-wise protocol was adopted to differentiate iPS cells into definitive endoderm (DE) cells (SOX17+/FOXA2+) and then to insulin-producing beta cells (Insulin+/Pdx1+). Each step is performed by specific treatment as depicted in the diagram above. Dexamethasone (a synthetic adrenocortical steroid); Activin A; CHIR99021 (Wnt signaling activator); Ad-PNM (Adenoviral construct expressing Pdx1, Ngn3 and MafA).*

Screening post induction was performed by analyzing the endoderm markers SOX17 and FOXA2. Co-expression of SOX17 and FOXA2 was observed in 65% of the cells counted in at least three different fields of view (Figure 18).

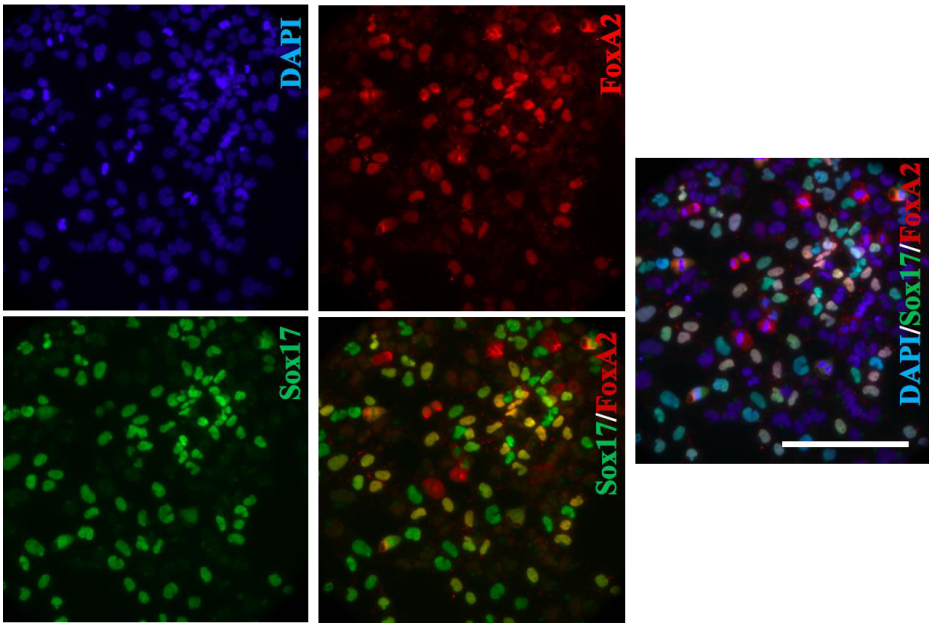


Figure 18: ***Characterization of Day 4 definitive endoderm (DE) cells.*** *IHC performed on day 4 of differentiation to validate differentiation of iPS to endodermal cells. Double positive cells for expression of SOX17 (Green) and FOXA2 (Red) indicate definitive endodermal cells; DAPI (Blue). Scale bar: 100 µm*

Reprogramming iPSC-derived endodermal cells to insulin producing beta-like cells

Differentiated iPSC-derived endodermal cells at day 4 were subjected to A12 Ad-PNM infection. The cells were maintained in culture for 3 days and fixed in preparation for IHC. IHC showed infection efficiency upon addition of just 5µL of diluted virus. The remaining cells express Pdx1 and Insulin which was absent in the control uninfected cells indicating that this is due to Ad-PNM infection and not any endogenous expression by the cells (Figure 19A and 19B).

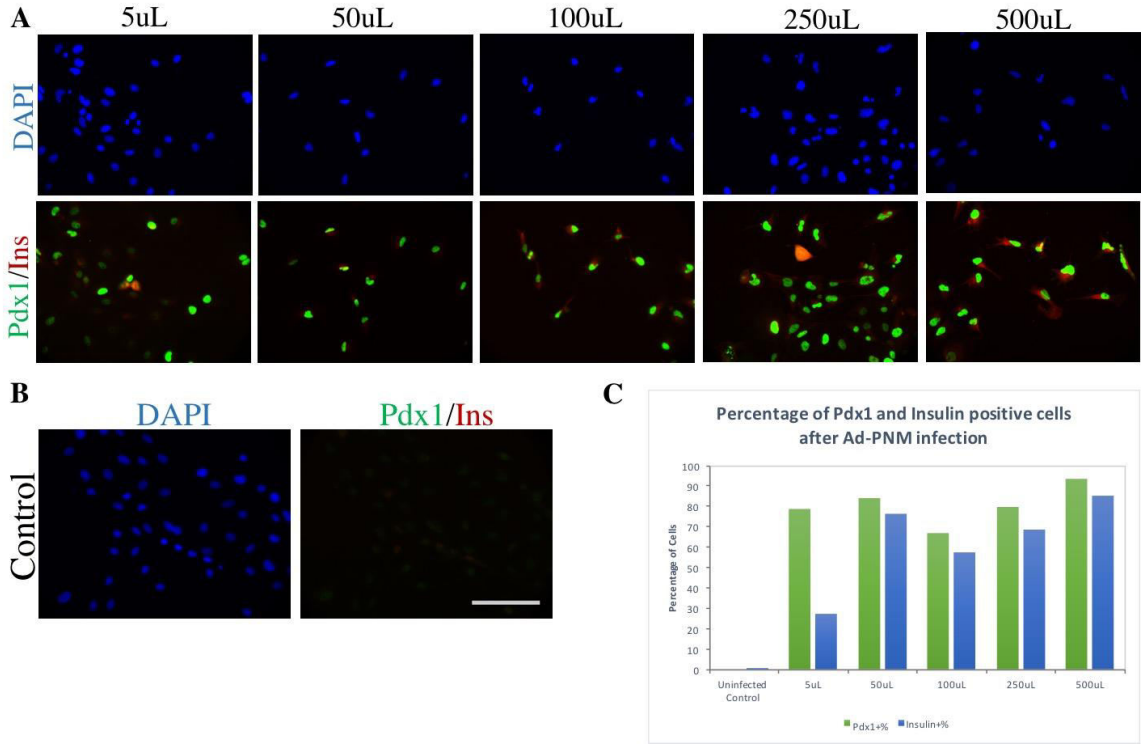


Figure 19. ***Ad-PNM infection of iPSC derived endoderm progenitor cells.*** *Endoderm progenitor cells were infected with Ad-PNM and IHC was performed after 3 days of infection. Insulin (Red), Pdx1 (Green) and DAPI (Blue) expression shown. (A) Increasing concentrations of diluted A12 Ad-PNM added (B) Uninfected cells shown as negative control lack Pdx1 and Insulin expression (C) Quantification of IHC in (A)(B) displaying infection efficiency calculated as Pdx+ percent expression and reprogramming efficiency calculated as Insulin+ percent expression. Scale bar: 100 μm (n=2)*

CHAPTER 4
DISCUSSION

Previously, in studies from the Dutton laboratory, it has been shown that injection of Ad-PNM adenoviral construct encoding the three genes important for pancreatic beta cell development, Pdx1, Ngn3 and MafA, can successfully rescue diabetes in an *in vivo* model (Banga et al. 2012). However, clinical translation of this model requires more *in vitro* and *in vivo* studies showing improved efficiency of production and maintenance of insulin-producing cells. The notable observations of the current study are: (1) Direct reprogramming of cells varies with cell type (2) Use of a TLR3 agonist, PolyI:C, caused nuclear localization of p65 in 40% of treated cells whereas only 1% and 3% nuclear localization of IRF3 and IRF7 was seen. (3) Upon, PolyI:C transfection combined with Ad-PNM infection, no significant difference in reprogramming efficiency was observed. The protocol described by Sayed *et al* (Sayed et al. 2015) may be specific to endothelial cells, therefore requiring more optimization of the current system used in Dutton Laboratory. Some level of nuclear localization of downstream targets of TLR3 signaling was observed with exposure to PolyI:C as opposed to the control (4) Differentiation of iPSC to endodermal progenitor cells was performed using described protocol (5) Ad-PNM infection of endodermal progenitor cells shows Pdx1 and Insulin expression.

To clinically translate reprogramming of cells type to beta-like cells, future work on reprogramming ductal cells as aggregates may help infecting a greater population of cells. Following up this work, the ductal cells could also be cultured as organoids which provide a 3D model to study and optimize culture conditions. In recent times, organoid cultures have achieved greater popularity to study a disease model in vitro (Barker et al. 2010; Boj et al. 2015; Hingorani et al. 2003; Huch et al. 2013; Sato et al. 2011). Once reprogramming efficiency is improved, mouse or higher animal models such as non-human primates would be used for transplantation of the organoids to determine if diabetes phenotype can be rescued. Patient derived iPS cells could also potentially be

used as a source to directly reprogram and make beta cells with a similar protocol used in this study.

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APPENDIX (1)

Ad-PNM Batch	Multiplicity of Infection (MOI) (ifu/ml)
A12	1.97×10^{10}
C3	4.13×10^{9}
C5	4.26×10^{10}
C7	1.6×10^{10}
C9	7.6×10^{10}
C11	1.05×10^{11}
C12	8.55×10^{10}
C13	7.6×10^{10}
C14	6.88×10^{10}
C15	1.3×10^{11}
C16	6.2×10^{10}

Table A1. Multiplicity of Infection of different batches of Ad-PNM batches used in this assay

APPENDIX (2)

UMN PCBC16iPS (Sendai 9-1) human iPS cell line

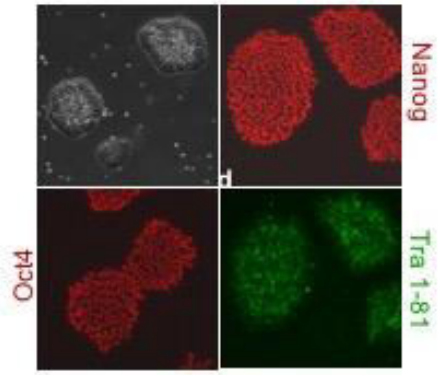
Description

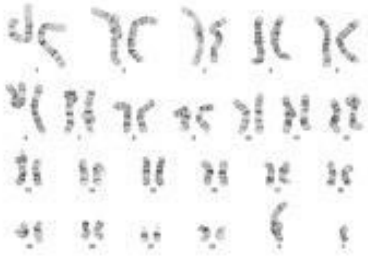
UMN PCBC16iPS (Sendai 9-1) induced pluripotent stem cell line was generated by reprogramming neonatal human dermal fibroblasts with CytoTune® 1.0 Sendai Reprogramming vectors. The iPSCs were derived on irradiated mouse embryonic fibroblasts in hES media containing Knockout serum replacer. Characterized UMN PCBC16iPS (Sendai 9-1) cells were subsequently adapted for culture in *Essential* 8 media on recombinant vitronectin with chelation passaging. (The adapted cell line is labeled UMN PCBC16iPSV or vShiPS 9-1)

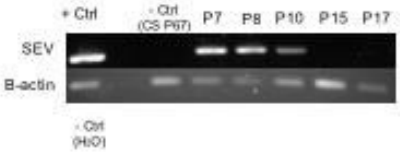
UMN PCBC16iPS (Sendai 9-1) has been extensively characterized by the NHLBI PCBC Cell Characterization Core facility.

iPSC line Specification

Line ID:	UMN PCBC16iPS
Donor ID:	ATCC PCS-201-010
Laboratory ID:	Sendai 9-1 or vShiPS 9-1
Organism:	*Homo sapien* (human)
Cell of Origin:	Neonatal dermal fibroblasts
Gender:	Male
Investigator:	James Dutton, Ph.D. University of Minnesota
Derived by	Lucas Greder (2011)
Number of Cells:	2 million cells per vial
Storage:	Liquid nitrogen







Figures (1a): Phase-contrast morphology of VchIPS XC5 iPS cell colonies at 5X objective 48 hrs after plating. **(1b):** Expression of Tra 1-81 green and Nanog red **(1c):** Oct 4 red and DAPI blue by of VchIPS XC5 iPS cells at 20X objective was confirmed by immunocytochemistry. **(2):** These findings represent a normal male karyotype. No numerical or structural chromosomal abnormality was found. **(3):** The ability of pluripotent VchIPS XC5 iPS cells to differentiate into three germ layers was confirmed by sectioning and HE staining of teratoma formed 6 weeks following injection of 1 million VchIPS XC5 iPS cells into the cranial thigh muscle of a NOD/SCID mouse. Image taken with 10X objective. **(4):** SEV virus **(5):** qPCR **(6):** Bisulfate sequencing

Notes:

The derivation of PCBC16iPS was first described in Ye *et al*., 2013

The GRips iPSC line is PCBC16iPS engineered to constitutively express GFP using a lentiviral construct. GRips were first described in Zhang *et al* 2014 and used in Ye *et al*., 2014

Publications using PCBC16iPS and derivatives:

Ye L, Zhang S, Greder L, Dutton JR, Keirstead SA, Lepley M, Zhang L, Kaufman D, Zhang J. (2013) *Effective Cardiac Myocyte Differentiation of Human Induced Pluripotent Stem Cells Requires VEGF.* ***PLoS ONE*** 8 (1): e53764.

Zhang S, Dutton JR, Su L, Zhang J, Ye L (2014) *The influence of a spatiotemporal 3D environment on endothelial cell differentiation of human induced pluripotent stem cells,* ***Biomaterials*** 35(12):3 786-93.

Ye L, et all., (2014) *Cardiac Repair in a Porcine Model of Acute Myocardial Infarction with Human Induced Pluripotent Stem Cell-Derived Cardiovascular Cells.* ***Cell Stem Cell****, 15, 6, 750–761*