**Transcription factors E2F have a role in proliferation of beta-cells and development of type-2 diabetes**

BY

BRIAN RADY
B.S. Loyola University Chicago, 2006

THESIS

Submitted as partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Bioengineering
in the Graduate College of the
University of Illinois at Chicago, 2013

Chicago, Illinois

Defense Committee:

Jose Oberholzer, Chair and Advisor
David Eddington
Yong Wang
Patrick Salmon, University of Geneva Switzerland
Terry Unterman, Department of Endocrinology

For Lily.

**ACKNOWLEDGEMENTS**

I would like to acknowledge the following people and organizations for their assistance. First, I would like to thank José Oberhozler for giving me the opportunity to complete this research in his lab. I realize that 6 years of research is a big drain on the lab, but I appreciate that you stuck it out with me and I hope this research is some small form of repayment for that commitment. I would also like to acknowledge and thank those who served on my PhD committee: José Oberholzer, David Eddington, Yong Wang, Patrick Salmon, and Terry Unterman. Next, I would like to acknowledge Yong Wang for being the one person I could turn to on a daily basis for help. I would also like to thank Enza Marchese for helping me learn everything I know about histology and the rest of the lab for making coming to work every day fun. Finally, I would like to acknowledge the immense and unerring support of my entire family.

**TABLE OF CONTENTS**

## LIST OF TABLES

## LIST OF FIGURES

## LIST OF ABBREVIATIONS

PP cells - Pancreatic Polypeptide Cells

GLUT2 - Glucose Transporter 2

ATP - Adenosine Triphosphate

SUR1 - Sulfonylurea Receptor 1

Kir6.2 - Potassium Inward Rectifier Ion Channel

IP3 - Inositol Triphosphate

ER - Endoplasmic Reticulum

PHHI - Persistent Hyperinsulinemic Hypoglycemia of Infancy

ADP - Adenosine Diphosphate

T1D - Type-1 Diabetes

T2D - Type-2 Diabetes

SNP – Sinlge nucleotide polymorphism

IPEX - Immunodysregulation Polyendocrinopathy Enteropathy X-linked syndrome

APS-1 - Autoimmune Polyendorine Syndrome Type-1

HLA - Human Leukocyte Antigen

VNTR - Variable Number Tandem Repeats

NIH - National Institute of Health

FDA - Food and Drug Administration

MSC - Mesenchymal Stem Cells

NSC - Neural Derived Stem Cells

PMD - Pelizaeus-Merzbacher disease

ESC - Embryonic Stem Cells

iPS - Induced Pluripotent Stem Cells

PERV - Porcine Endogenous Retrovirus

PLHV - Porcine Lymphotropic Herpes Virus

PCMV - Porcine Cytomegalovirus

HGF - Hepatocyte Growth Factor

DNA - Deoxyribonucleic Acid

MOI - Multiplicity of Infection

SV40T - Simian Vacuolating Virus 40 Large T Antigen

hTERT - Human Telomerase Reverse Transcriptase

STZ - Streptozotocin

SCID - Severe Combined Immunodeficiency

BMI - Body Mass Index

R-point - Restriction-Point

BrdU - 5-bromo-2'-deoxyuridine

EdU - 5-ethynyl-2'-deoxyuridine

NLS- Nuclear Localization Signal

ssDNA - Single Stranded Deoxyribonucleic Acid

ChIP - Chromatin Immunoprecipitation

CBD - Common Bile Duct

HBSS - Hank's Balanced Salt Solution

FBS - Fetal Bovine Serum

IEQ - Islet Equivalent

CMRL - Connaught Medical Research Laboratories

UIC - University of Illinois at Chicago

PBS - Phosphate Buffered Saline

BME - β-Mercaptoethanol

SDS - Sodium Dodecyl Sulfate

TBS - Tris-Buffered Saline

HRP - Horseradish Peroxidase

RNA - Ribonucleic Acid

cDNA - Complementary DNA

PCR - Polymerase Chain Reaction

UV - Ultraviolet

ITS - Insulin Transferrin Selenium

RPMI - Roswell Park Memorial Institute

CMV - Cytomegalovirus

GFP - Green Fluorescent Protein

BSD - Blasticidin

Dox/Tet - Doxycycline/Tetracycline

RIP - Rat Insulin Promoter

dGFP - Destabilized Green Fluorescent Protein

KRB - Krebs-Ringer Buffer

Rh123 - Rhodamine 123

FITC - Fluorescein Isothiocyanate

CCD - Charged Coupled Device

ELISA - Enzyme Linked Immunosorbant Assay

P200 - 200uL Pipette

IPGTT - Intraperitoneal Glucose Tolerance Test

DMSO - Dimethyl Sulfoxide

$G_s$ - Serial Resistance

ANOVA - Analysis of Variance

Pre-rc - Pre-Replication Complex

Nfx - Nephrectomy

**SUMMARY**

This work pertains to the search for genetic targets to induce beta-cell proliferation. This proliferation was intended to support the advancement of islet cell transplant as a therapy for type-1 diabetes. Since beta-cells are post-mitotic cells that rarely divide, we initially sought to find anything that would produce more insulin-secreting cells. By comparing proliferative insulinomas and wildtype islets, we identified a transcription factor, E2F3 that was differentially overexpressed in insulinomas. Mimicking this overexpression in wild type islets resulted in the induction of proliferation. These newly formed cells retained the beta-cell phenotype and didn't undergo apoptosis as they were cultured. When transplanted into diabetic mice, these cells were able to reverse diabetes and restore normal blood glucose levels.

We also investigated the role of E2F1 on the loss of beta-cell function that often accompanies type-2 diabetes. We found a link between the BMI of islet donors and E2F1 expression. Additionally, we observed that E2F1 controlled the expression of the potassium channel subunit *Kir6.2*, whose mutational overactivity results in diabetes. E2F1 knockout mice fed a high fat diet did not develop glucose intolerance, while wildtype mice did. Further investigation of the ion channel function in these cells revealed several differences between the E2F1 knockout and wildtype islets. While these differences impacted the over all function of the islets, they don't appear to be responsible for imparting the resistance to glucose intolerance. Rather we suspect a metabolic change in adipocytes from of the global E1F1 knockout provided the protection.

# INTRODUCTION

Tissue Engineering:

Engineering is the process of designing and creating structures. In the subsequently described experiments, the objective is to create a structure that is a human cell, rather than a bridge or building typically associated with an engineer. To design this cell, human beta-cells are used as a starting material, rather than concrete or steel. The expression of genes are increased or decreased to obtain a cell that functions as we want. From this, a brand new cell is created, one that without external manipulation would never exist. This new cell is not the same as a normal human beta-cell, rather it is a genetically engineered cell. It does not express the same genes as a normal cell and does not function exactly like a normal beta-cell, its expression of our inserted genes has fundamentally changed it. This cell ideally functions in a way of the designers choosing - this is the essence of tissue engineering. One of the greatest scientific achievements in the next 50 years will be the creation of whole organs by a process that may be similar to the one described here. The design and creation of these organs is the ultimate goal, and fate, of the field of cell and tissue engineering.

The manufacturing of viable human organs for transplant is something usually reserved to the minds of Phil K. Dick and other science fiction writers. However, with recent advances in our understanding of the human body, we are beginning increase our understanding about some biological processes enough to attempt to make meaningful genetic alterations to these processes. Notably, the cell

cycle and the genes involved in regulating DNA synthesis are coming into focus in such a way that we are able to manipulate specific genes and can make reasonable predictions about the outcome. This knowledge of the cell cycle will fundamentally change the way we treat many diseases. Understanding something so basic and yet so complex like how a cell divides will one day allow us to proliferate any cell in the body and use those new cells to better understand and treat many diseases. Cell based therapies are already a reality in many primitive forms and personalized medicine, while prohibitively expensive and technically awkward in its current state, still holds promise for many diseases that just a decade ago seemed hopelessly incurable. A common limiting step in most of these therapies is the source and amount of the cells used. The field of bioengineering, specifically cell and tissue engineering, is devoted to help eliminate this obstacle.

Islet Physiology and Diabetes:

Islets of Langerhans are clusters of cells located in the pancreas. This endocrine tissue contains several cell types: beta-cells, alpha-cells, delta-cells, pancreatic polypeptide (PP) cells, and epsilon cells. Beta-cells are the most prevalent cell and represent about 60-80% of the islet. These cells secret insulin, a hormone that causes muscle, liver, and adipose tissue to take up glucose from the blood for metabolic use in the cell or conversion into glycogen or triglycerides. Beta-cells also secret amylin, a hormone that compliments insulin in regulating glucose metabolism, slows gastric emptying, and promotes satiety. It may also play a role in the development of Type-2 diabetes [1]. Alpha-cells are the next most prevalent cell

representing about 15-20% of the islet. Alpha-cells secret glucagon, a hormone that works in an opposite manner to insulin by increasing blood glucose levels through conversion of glycogen to glucose in the liver. Delta and PP cells serve to regulate endocrine hormone secretion in the islet itself. As part of the paracrine feedback mechanism, delta-cells produce somatostatin and PP cells secret pancreatic polypeptide, whose endocrine functions are to regulate the release of both insulin and glucagon. Finally, epsilon cells produce ghrelin, which stimulates hunger. While the islet is comprised of many cell-types, the beta-cells and alpha-cells are vital to the islets ability to regulate blood glucose through insulin section.

Insulin is released in two phases from beta-cells. The first phase is a rapid exocytosis of mature insulin granules in response to extracellular glucose levels. The second phase is a slower, constant release of newly matured insulin granules that takes place over a longer time period [2]. During the first phase, extracellular glucose is transported into the cell via the glucose transporter 2 (GLUT2) receptor of beta-cells. Once inside the cell, glucokinase converts glucose into glucose-6-phosphate. Glucose-6-phosphate then enters the glycolysis pathway forming new ATP molecules. $K_{ATP}$ channels close in response to an increase in the intracellular ATP/ADP ratio [2]. The $K_{ATP}$ subunit SUR1 closes the Kir6.2 transmembrane channel and prevents potassium from leaving the cell, depolarizing the membrane [3]. This depolarization opens voltage-gated calcium channels and allows calcium ions to enter the cytoplasm from the extracellular fluid fusing mature insulin vesicles with the cellular membrane, releasing insulin [4].

The ATP-sensitive $K^+$ channel is comprised of two subunits SUR1 and Kir6.2, which help regulate the flow of potassium ions into and out of the cell. $K_{ATP}$ channels will not form from either Kir6.2 or SUR1 alone, but require both subunits to form functional channels. In pancreatic $K_{ATP}$ channels, increases in ATP close the trans-membrane potassium channel by binding to Kir6.2. SUR1 confers a hypersensitivity to the Kir6.2 channel. Hydrolysis of ATP to ADP at the SUR1 subunit results in the loss of SUR1-conferred Kir6.2 ATP hypersensitivity, negatively regulating the SUR1 subunit. In this way, when ATP is low, the Kir6.2 subunit is highly sensitive to ATP causing increases in ATP levels to quickly close the Kir6.2 channel. SUR1 then hydrolyses ATP to ADP, which stabilizes an open $K_{ATP}$ state, allowing the cell to return to the resting membrane potential, ending the first phase of insulin secretion.

$K_{ATP}$ channels are a vital component of insulin secretion. Without the ability to depolarize the membrane by sequestering $K^+$ inside the cell, insulin secretion is not possible. Mutations in either $K_{ATP}$ subunit (Kir6.2 or SUR1) resulting in loss-of-function leads to unregulated insulin secretion and persistent hyperinsulinemic hypoglycemia of infancy (PHHI). Transgenic mice expressing a dominant negative Kir6.2 exhibit PHHI as neonates [5]. The mutated form of the $K_{ATP}$ channel remains closed and is insensitive to changes in ATP/ADP that would normally open them. This permanent closure results in unregulated depolarization of the membrane, continuous insulin secretion, and hypoglycemia in individuals effected. Mutations of the Kir6.2 and SUR1 genes that cause over-activity result in diabetes in children. With these mutations, the efflux of $K^+$ ions out of the cell is increased, hyperpolarizing the membrane. This creates a strong ionic force resisting the influx

of calcium ions and reduces or eliminates the normal insulin secretion response. These pancreatic $K_{ATP}$ channels remain open due to decreased sensitivity to the ATP:ADP ratio [6, 7].

Both Type-1 (T1D) and Type-2 diabetes (T2D) have in common hyperglycemia as a result of a decrease in secretion and/or sensitivity to insulin. Both diseases can be accompanied by serious complications including blindness, stroke, heart disease, kidney failure and ketoacidosis. Type-1 diabetes is sometimes referred to as juvenile onset diabetes, since patients with Type-1 diabetes are often diagnosed early in life. However, Type-1 diabetes can occur at any age depending on genetics and environmental factors [8]. T1D results from an autoimmune attack on the insulin producing beta-cells of the islets of Langerhans in the pancreas. This autoimmune attack destroys or damages beta-cells sufficiently to reduce or eliminate insulin secretion. Type-2 diabetes is usually associated with obesity or increased age and is often the result of insulin resistance in muscle and adipose tissue. These tissues no longer respond to normal levels of insulin produced by beta-cells, so the cells compensate by secreting more insulin to reach the desired effect. This level of secretion is not sustainable however, and the disease is exacerbated as the beta-cells are overworked, eventually losing function. The incidence of both type-1 and type-2 diabetes is on the rise. According the Center for Disease Control, almost 26 million people in the US alone are affected by diabetes, about 8.3% of the population. The incidence of type-1 diabetes has been on the rise during the past decade, increasing as much as 5.3% in the US. If this trend continues, the prevalence of the disease will increase by 70% in children under the age of 15 by 2020 [9].

Determining the causes that predispose individuals and any environmental triggers that may exist are important to curtailing this rapid increase in the incidence of diabetes.

Even with current treatments (insulin injections/pumps) complications and a shorter lifespan (~10 years) are near certainties for diabetics. The origin of this disease lies in both environmental and genetic factors. Genetic predisposition to type-1 diabetes has been known for some time, but given the increased prevalence of this disease aspects beyond genetics may be influencing it. This has led to the idea that environmental factors contribute to the initiation of the disease in genetically predisposed individuals. Given the increasing trend of diabetes, whatever environmental factor it is that is triggering this disease in predisposed individuals is seemingly being encountered more frequently. Efforts to identify exactly what this environmental trigger is have been underway for many years. These investigations implicate such considerations as dietary compounds, bacteria, and viral infections as influencing factors [10-12]. While there is no direct evidence of any of these factors being causative, the idea that various strains of viruses may be a major environmental trigger has gained serious consideration [13, 14]. To illustrate the point of environmental triggers, the rate of monozygotic twins developing diabetes simultaneously is only 50% and only 10% in dizygotic twins [15]. Over an entire lifetime, identical twins usually both develop anti-islet autoantibodies and progress to diabetes. However, one twin may develop autoantibodies more than 30 years after the other develops the disease [16, 17]. Therefore, genetic susceptibility is ever

present for those predisposed, but an environmental trigger seems to be strongly implied.

T1D is rarely caused by mutation of a single gene, or single nucleotide polymorphism (SNP). When this is the case, diabetes is usually not the only autoimmune disease these individuals develop. Some examples of SNPs causing diabetes are IPEX syndrome (*Foxp3* mutation) which leads to dysfunction of regulatory T-cells and APS-1 (*AIRE* mutation) which interferes with thymic deletion of autoreactive T-cells, leading to their escape into the body [18, 19]. Both IPEX and APS-1 lead to multi-organ autoimmune attacks, with diabetes occurring in 80% (IPEX) and 20% (APS-1) of those affected [20, 21]. However, these single mutation diabetics represent only a small percentage of the overall diabetic population. Comparative genetic assays have identified over 40 loci that potentially contribute to the development of T1D [22]. Specific genes, variants and the effect they have on the development of the disease are still being determined. However, one of the most indicative of susceptibility and earliest genetic disparity between non-diabetic and diabetic patients was found in the human leukocyte antigen gene (HLA) [23]. HLA is a class of proteins that present antigens to T-cells. These T-cells then attack and destroy the labeled cells by secreting cytotoxins. There are two classes of HLA proteins, I and II. In over half of type-1 diabetics, these HLA proteins are mutated and allow for T-cell infiltration into the islet [24]. These T-cells recognize and attack the variant HLA expressing beta-cells, destroying them [25]. Another, less significant genetic indicator of a predisposition to Type-1 diabetes is a mutation of the insulin gene itself [26]. Mapping of the insulin gene reveals that the 5'-region contains a

variable number of tandem repeats (VNTR) [27]. The correlation between VNTR and the risk of developing diabetes is related to the length of the VNTR. Shorter VNTR patients are in the highest risk category, while longer VNTRs protect the carrier against T1D [28]. The exact reason for this is not fully understood, but the current hypothesis is that insulin reactive T-cells are more efficiently destroyed in individuals with the longer VNTRs by negative selection in the thymus. Other genetic indicators of a predisposition to T1D include, *PTPN22*, a gene that encodes the lymphoid protein tyrosine kinase; interlukin-2 receptor-alpha; and CTLA-4, cytotoxic T lymphocyte-associated protein 4 [29-31]. Most of these genes are only one part of a complex mechanism that brings about the development of diabetes. No single gene tells the whole story and with rare exception no single gene mutation will cause the disease. Investigations into these genes are beginning to put together pieces of the genetic puzzle that contributes to development of islet dysfunction and diabetes.

Cell Therapy:

Cell therapy is the process of transplanting cells or tissue into a host in order to alleviate a disease. This approach is particularly useful in diseases that involve dysfunction of specific organs or tissues. One of the earliest cases of using a cell therapy was the first successful blood transfusion. Since this time in the early 1800s cell therapy has come a long way. Modern cell therapies address diseases in cardiac, neurological, immunological, genetic blood disease, adipose, endothelial, pancreatic, hepatic, myoblasts and limbal tissues. In 2011 the NIH website listed 123 clinical

trials using stem cells, by far the most common form of cell therapy. Stem cell treatments represent a large portion of current cell therapies, but depending on the application trans-differentiated cells, xenotransplant, or proliferation of adult cells may provide cells for use in a variety of therapies. Most of these trials are in Phase I (safety) or Phase II (efficacy in humans) of FDA approval. Expectations for these trials are high and the future of cell therapy is a promising one.

Many modern cell therapies often utilize differentiated stem cells as the cell source. Both autologous and allogeneic stem cells can be used. Mesenchymal stem cells (MSCs) are stromal-derived multipotent stem cells. They are found in many tissues including umbilical cord blood, bone marrow, adipose tissue, and amniotic fluid. These cells can be collected from fetal and adult tissue, both displaying unique qualities that makes one or the other more appropriate depending on the application.

A patient's MSCs can be used to treat a variety of diseases; even those that involve cell lineages that MSCs do not typically contribute to. In cardiac repair, MSCs from umbilical cords may improve cardiac function following myocardial infarction, but long term recovery has not been clearly demonstrated [32]. Additionally, these MSCs are able to form structures resembling cardiomyocytes *in vitro*, with expression of some genes associated with cardiac muscle cells. However, there is little evidence of myocardial regeneration *in vivo*, despite a 3-4% improvement in left ventricle function following transfusion [33]. Using alternative cell sources, such as cardiac derived stem cells, rather than MSCs, has provided a

more robust differentiation and may help optimize this treatment for future clinical use [34].

Neurological diseases are also benefitting from the development of cell-based therapies. Pre-clinical experiments involving MSCs have resulted in *in vitro* formation of neuron-like cells, but there is little evidence of neuronal functionality [35]. Blood derived stem cells have been shown to be safe for use in spinal cord injuries, but not particularly effective in restoring function [36]. Despite all the data showing little function of MSC-derived neural cells, clinical trials are relatively common representing around 12% of those found on the NIH website (clinicaltrials.gov). However, using neural derived stem cells (NSCs), found in the neonatal and adult brain, several companies have developed treatments in phase 1 and phase 2 clinical trials. NSCs have been used by StemCells, Inc. in California to treat children with Pelizaeus-Merzbacher disease (PMD), a genetic mutation that disrupts myelination of neurons. Transplant of NSCs resulted in the production of oligodendrocytes that re-myelinated the deficient neurons. Similarly, these cells were used to treat spinal cord injuries. As seen with PMD, NSC's were able to re-myelinate neurons damaged by spinal trauma, which may lead to restoration of nerve function. These and many more clinical trials involving NSCs are happening all over the world and they are giving hope to people with debilitating neurological diseases and trauma.

Cardiac and neuronal cells are not the only cell types making significant progress towards productive cell therapies. Current treatment of T1D includes daily blood glucose monitoring and insulin injections. While this life-saving therapy

dramatically improves the life of people with this disease, complications still often arise and their quality of life degrades over time. A more physiological method of controlling blood glucose is an islet transplant, as it is able to dynamically regulate blood glucose and eliminate hypoglycemic episodes sometimes seen in those requiring insulin injections. Islet transplantation is currently indicated for those with unstable T1D and a history of severe hypoglycemic episodes [37]. The first report of an islet transplant being performed was in 1894 by Drs. Watson-Williams and Marshant. They transplanted sections of a sheep's pancreas subcutaneously in a 15 year old end-stage diabetic [38]. The boy's condition improved over the next two days, but this treatment was ultimately unable to reverse his disease for longer periods. A major advancement in 1990 was the first successful islet allograft [39]. Throughout the 1990's the technique was refined until 1999 when it was revealed that glucocorticoid-free immunosuppression greatly improved outcomes in islet-transplanted patients [40]. For a typical allogeneic islet transplant, a cadaver donor pancreas is harvested and islet isolation performed by a combination of chemical dissociation of islets from the surrounding tissue using collagenase and a mechanical digestion chamber [41]. The islets are then purified in a continuous density gradient and cultured. For the transplant, purified islets are slowly infused into the hepatic portal vein by means of a percutaneous transhepatic cannulation. Islet transplant patients receive a course of immunosuppressive drugs, which help prevent rejection and maintain function of the grafted islets. Administration of immunosuppressive drugs is a major drawback for islet transplantation, making it appropriate only for diabetics who cannot maintain their blood glucose through

regular insulin injections or pumps. Most patients become normal glycemic after the transplant, with long term graft survival a reality for about 50-60% of patients, though some require multiple transplants to achieve insulin independence [41]. Islet transplant is an example of a cell therapy helping to successfully manage a complex disease. However, since the source of transplanted islets relies on cadaver donor organs and the number of diabetics in the US is over 25 million, the need for a stable renewable source of transplantable beta-cells is paramount.

Cell therapies have recently reached the point where they are becoming applicable to many diseases. These cell therapies are offering hope to overcome some diseases that have no other treatment options. The future for cell therapies is exciting and the capacity to replace damaged cells with ones grown in a lab has long been a dream of clinicians and researchers everywhere. For islet transplants, the next major obstacle in perfecting the treatment is the generation of a reliable source of transplantable cells.

Sources of Transplantable Beta-cells:

With the success of islet transplant in recent years, the need for a suitable source of transplantable cells has intensified. A variety of biological processes and surgical procedures provide sources of transplantable material. Differentiation of stem and progenitor cells, trans-differentiation of mature cells, xenotransplantation, proliferation of adult beta-cells, and whole pancreas transplant all offer some measure of success. Though they represent different approaches to the same

problem, they all hope to deliver a tissue that can be used to reverse diabetes when transplanted.

Embryonic stem cells (ESCs) are pluripotent cells derived from embryonic tissue, specifically from the inner mass of the blastocyst. These cells can presumably give rise to any type of cell, including beta-cells [42]. They are attractive to use in islet transplants because they readily proliferate in the undifferentiated state making therapeutic numbers of cells obtainable. Part of the difficulty in using ESCs in general is the identification of stages of development *in vitro*. The development of the pancreas involves the stepwise expression of a series of transcription factors [43-45]. Attempts to differentiate ESCs to pancreatic tissue uses knowledge of this step-wise process to mimic *in vitro* what happens *in vivo*. However, expression of a gene or even several genes seen *in vivo* is not necessarily the sole determining factor of a stage in development. Therefore, attempts to mimic this *in vitro* could be missing unknown key factors contributing to the cellular environment of a particular developmental stage. This is evidenced by the fact that *in vitro* expression of genes seen during development does not result in very high efficiency of differentiation to beta-cells *in vivo*. The process of differentiation from endoderm to a beta-cell is complex, involving expression of specific transcription factors in a precise sequence with genes being activated or inactivated at specific times to determine the ultimate fate of a cell. Simply generating cells expressing insulin in a non-differentiated state is not sufficient to create a functional beta-cell. Glucose sensitive insulin secretion, a hallmark of beta-cells, is often absent in differentiation processes that chase insulin expression. In cells differentiated to a beta-cell fate,

insulin mRNA expression can be so low that it represents only 1/100000 cells or 0.00001% of the total population [46]. Recent attempts to drive differentiation of ESCs to insulin producing cells by expression of *Pax4* have resulted in cells that express Isl1, Ngn, insulin, Iapp, and Glut2 [47]. A current method for differentiation of beta-cells from ESCs is a stepwise expression of several genes, among them Sox17, FoxA2, PdX1, Ptf1a, Ngn3, Isl1, Nkx6.1, to produce on average 7.3% of the population as insulin positive cells [48]. However, these cells have limited glucose responsiveness and when transplanted the cells can form teratomas [49-51]. A recent strategy to use stem cells in diabetic patients aimed to generate pluripotent stem cells from a patient's own cells. To do this fibroblasts from the patients were cultured and infected with retroviruses encoding 3 transcription factors: *OCT4*, *SOX2*, and *KFL4*, resulting in the generation of pluripotent stem cells from the fibroblasts [52]. These induced pluripotent stem cells (iPS cells) were then able to differentiate to cells that secreted C-peptide in response to glucose [52]. However, further study is needed to show full maturation and function of these cells. In addition to genetic differentiation, small molecules hold some promise in differentiating functional beta-cells. Two such molecules, called IDE1 and IDE2 are able to induce endoderm differentiation [53]. Another small molecule, indolactam V, is able to induce differentiation of endoderm into PDX1 expressing pancreatic progenitor cells [53]. Concerns over the use of ESCs for transplantation can be assessed by two questions: what percentage of the starting material is differentiated into glucose responsive insulin secretion cells? What percentage of the end population are undifferentiated cells and is there an effective means to eliminate

these potential teratoma-forming cells? These problems of poor efficiency and teratoma formation are currently restricting progress of clinical trials with ESC derived insulin secreting cells, but once this technique is improved it may provide a transplantable source of human insulin secreting cells.

In an effort to obtain a transplantable source of insulin secreting cells, some groups are taking non-endocrine tissue and attempting to trans-differentiate it from one cell type to another. Two examples of this are trans-differentiation of liver cells and pancreatic exocrine cells. Hepatic cells (hepatocytes, biliary and gall-bladder epithelial cells) share a common developmental precursor with the pancreas. In mice lacking the developmental transcription factor *Hes1*, overexpression of *PDX1* changes biliary developing cells to become pancreatic tissue expressing all the hormones normally seen in islets [54]. However, while these cells do secrete insulin in a glucose responsive manner, they contain about 3000-fold less insulin compared to normal islets [55]. *In vivo* hepatic expression of *PDX1* in mice produces cells with expression of both endocrine and exocrine pancreatic hormones [56]. These cells are able to prevent STZ-induced diabetes in rats and demonstrate the feasibility of liver-to-pancreas trans-differentiation. Exocrine cells in the pancreas can also be trans-differentiated into pancreatic endocrine cells. Stem cells derived from mouse pancreatic ductal cells are able to differentiate through an unknown mechanism when transplanted into NOD mice [57]. These cells are able reverse insulin-dependent diabetes in these mice for up to 55 days [57]. However, total insulin content of these cells was not compared to normal islets and the full differentiation and function of these cells remains in question. Human pancreatic ductal stem cells

were also trans-differentiated into beta-cells by adenoviral expression of *Ngn3* [58]. These cells showed weak expression of insulin but did not show expression of *GLUT2*, suggesting the lack of glucose sensitive insulin secretion [58]. These experiments have shown that the trans-differentiation of cell lines completely separate from the pancreas can be directed to a pancreatic fate. While the steps to take a hepatic or exocrine cell to a pancreatic endocrine cell are still be elucidated, the principle has be proven and with further refinement may provide a source of insulin secreting cells for islet transplant.

Pig islets are another option as a source of transplantable islet cells. The problem in proceeding with clinical trials of porcine islets is the possibility of xenozoonosis. Porcine endogenous retrovirus (PERV), porcine lymphotropic herpes virus (PLHV) and porcine cytomegalovirus (PCMV) are viruses that may be transmitted by xenografts into humans [59]. One pre-clinical study used a pig that was specifically designed to be pathogen free. These islets were found to be free of xenotic viruses in pig to primate transplants [60]. When fetal porcine islet-like clusters were transplanted in 10 diabetic patients, 4 of them had measureable porcine c-peptide levels [61]. However, none of the patients were able to stop taking insulin and only one of the grafts stained positive for insulin and glucagon [61]. Graft function and rejection are still issues with porcine islet transplants, even though this data indicates porcine islet transplants are plausible.

Proliferation of mature, functional adult beta-cells may provide a source of transplantable islets. Though it is a debated topic, adult beta-cells seem to respond to adaptive signals for growth, such as pregnancy, by mitotic division of mature

beta-cells rather than differentiation of new beta-cells from a stem origin [62-64]. Genes that control the cell cycle and DNA synthesis have been identified and using this knowledge it is possible to overexpress or inhibit genes to initiate S-phase in an otherwise quiescent cell. Many groups have attempted to drive human beta-cells to divide by exposing cells to growth factors and manipulating genes in various points of the cell cycle. With the maturation of islet transplant, interest in proliferation of human beta-cells has increased. Early attempts in the 1990s to proliferate human beta-cells were typically focused on extracellular signaling and extracellular matrices [65]. Using hepatocyte growth factor (HGF) in the culture media of human islets resulted in proliferation of 17.5% of all cells and a 37% increase in insulin, though recent duplication of this experiments did not achieve similar results [66]. Cells stimulated with HGF have a normal acute insulin secretion, but chronic insulin secretion is significantly impaired [65]. More recently, manipulation of cell cycle proteins has led to robust proliferation in human islets. Using adenoviruses to deliver Cdk-6 and Cyclin D1, human beta-cells were able to divide, increasing the beta-cell mass by 12% [67]. When these cells are transplanted into diabetic SCID mice they are able to reverse diabetes and maintain regular blood glucose levels [67]. Cdk4 and cyclin D1 co-expression, then later only Cdk6, were also found to have a positive effect on functional human islet proliferation [68, 69]. Another cell cycle protein, E2F1, was recent used to proliferate mouse and human beta-cells [70]. E2F1 overexpression induced up to 20% of insulin positive cells to proliferate, but was accompanied by increasing rates of apoptosis as the multiplicity of infection (MOI) was raised. To counteract this, adenoviral *Akt* was added and the apoptosis

was eliminated [70]. In order to generate human beta-cell lines, permanent incorporation of transgenes is required. To do this, adenoviruses must be replaced with lentiviruses or other permanently incorporating virus. Retroviruses are less desirable because they require cell division to transduce a cell, a rare event in human beta-cells. Since manipulations of cell cycle proteins have proven to be a potent stimulus of proliferation, groups have attempted to immortalize human beta-cells. Simian Vacuolating Virus 40 large T antigen (SV40T) is a viral oncogene that inhibits p53 and Rb, thereby inducing proliferation (presumably through E2F expression) [71]. Using a SV40T retrovirus and human telomerase (hTERT) human beta-cells were transfected and cultured. The resulting cell line was able to reverse streptozotocin (STZ)-induced diabetes in SCID mice [71]. Another group established a similar cell line utilizing SV40T lentiviruses to infect human fetal pancreatic buds [72]. These buds were then grafted into SCID mice so the cells could mature. SV40T infected beta-cells proliferated and formed insulinomas, which were then removed, dissociated into single cells and transduced again with hTERT. These cells were again transplanted into SCID mice and insulinomas allowed to form. They were then cultured *in vitro* and were able to reverse STZ-induced diabetic mice when transplanted [72]. It's worth noting that to my knowledge, neither of these cell lines are available commercially or privately and have not been used by the research community or used in clinical islet transplants.

There are several concerns with adult beta-proliferation for use in transplant. First, the difficulty of human beta-cells to respond to proliferative signals. Signals that will proliferate typical cells, like a fibroblast, often have much

more muted effect in islets [73]. Because of this, overexpression of oncogenes is often required, increasing the threat of oncogenic transformation if the tissue is transplanted into a patient. Additionally, efforts to proliferate cells often results in dedifferentiation of beta-cells and loss of insulin expression [74]. Despite all of this, adult beta-cell proliferation is making progress towards supplying cells to be used in islet transplants. However, there are other sources of insulin secreting cells that are currently used to treat diabetics in a manner similar to islet transplant.

The last and perhaps most obvious source of islets is the pancreas itself. Islet transplant is not in competition to whole organ transplant of the pancreas; rather it is something to be considered as an alternative to whole organ transplants. Both have advantages and disadvantages what make one or the other more appropriate for a given donor. BMI, age, and fibrosis all play a role in determining if a donor is better suited for whole organ or islet transplant. Specifically, if an organ were fibrotic, it would be unsuitable for whole organ transplant and would typically be disposed of. However, these organs may still be suitable for islet isolations. Both procedures require long-term immunosuppression, a significant draw-back, but both procedures allow patients to be insulin independent [75]. Whole organ transplants can be done at the same time as transplants of other organs affected by the diabetic condition, such as kidneys, and make the benefits of islet transplant less attractive for certain patients [76]. Rational evaluation of patients is critical to helping them maintain their blood glucose levels. Both whole organ and islet transplants are excellent tools to help them achieve this goal and are not competitive therapies.

The overall goal of obtaining a transplantable source of insulin secreting cells is being approached through many biological processes. Differentiation of stem cells, trans-differentiation of hepatic cells, xenotransplant, proliferation of adult beta-cells, and whole organ transplant all currently provide or are making great progress towards providing tissue for transplant. For adult beta-cell proliferation, understanding the basic mechanism of how a cell divides will allow researchers to make meaningful manipulations of cell cycle genes to give rise to functional beta-cells.

The Cell Cycle:

The cell cycle is divided into several phases: $G_0$, $G_1$, S, $G_2$, and M. $G_0$ is a phase of rest. Cells in this phase are often referred to as quiescent. Quiescent cells are neither dividing nor preparing to divide, but are post-mitotic and retain their function. $G_1$ is the beginning of the cell cycle. Cells enter $G_1$ either from $G_0$ or following mitosis from the previous round of division. $G_1$ is a phase of high activity for the cell as it prepares the necessary proteins and enzymes for DNA synthesis. Once all the necessary preparations have been made, the initiation of DNA synthesis marks the beginning of S-phase. S-phase is the time during which DNA is duplicated, but it is a critical checkpoint for the cell. Following S-phase, $G_2$ is another period of growth and preparation as the cell readies itself for mitosis. Finally, mitosis is the physical division of the cell. Cells proceed with the next phase only once the previous stage has been completed. Each stage is essential to the overall process and is regulated by a complex control mechanism. Since most cells require much more

time to prepare the necessary proteins and machinery to divide than the actual time division takes, many of these phases are simply times of growth and preparation. The gap phases ($G_0$, $G_1$, and $G_2$) not only allow for growth, but also allow the cell to monitor the progress of the overall process of cell division and are used to decide if a cell should begin, continue or end dividing. S-phase is the critical point in the cell cycle as once a cell has duplicated its DNA it will either divide or die via apoptosis since it contains two copies of DNA. This makes the transition from $G_1$ to S a vital point in the cell cycle. This checkpoint, termed the restriction point (r-point), is the point of no return for the cell. If it proceeds with DNA synthesis and the environmental factors that initiated the proliferative signal change or are removed, the cell will still proceed with DNA replication [77]. This major undertaking for the cell is a tightly regulated process with multiple layers of regulation. If a cell does not progress through $G_1$ in anticipation of DNA synthesis, it can enter a state of inactivity called $G_0$. In $G_0$ a cell can remain dormant, but functional for days, weeks or years before it receives a signal to begin the process of proliferation. Once that signal is received, the cell will again enter $G_1$ and prepare for DNA synthesis and cell division.

The $G_1$/S transition is a critical point in mitotic division of a cell. As such, it is a tightly regulated process that ends with DNA synthesis. DNA synthesis is controlled by hundreds of genes. In order to transcribe this DNA machinery transcription factors control large numbers of genes involved in this process. In the case of the $G_1$/S transition, a family of transcription factors control, among other things, the transcription of genes involved in DNA synthesis. One of the

transcription factors involved in this process is called E2F. They are a regulator of DNA synthesis and therefore the cell cycle in general.

The concept of a restriction point in $G_1$ is a point at which a cell commits to cycling. If the proliferative stimulus is removed prior to this point the cell will revert to quiescence. If it remains, the cell will enter S-phase and complete the cycle [77]. There is a high threshold requirement for proliferative signals prior to the r-point. This ensures that the cell has everything it will need to complete the non-reversible process once it begins. When these criteria are met the cell begins dividing. At this point there is a low maintenance mechanism that completes the cell cycle, even in the absence of any proliferative signals [77] This is why the criteria to pass the r-point is so strict.

In order to monitor the progress of the cell cycle, several tools have been developed for researchers to see when a cell synthesizes DNA. S-phase labeling of cells began in the 1960s with the use of radioactive nucleosides to detect DNA synthesis. This was a slow and technical process. It was replaced with an antibody-based system using a nucleoside analog 5-bromo-2'-deoxyuridine (BrdU). This method is still in use today and proven to be a robust tool for measuring S-phase in cells. A recent improvement on this method uses 5-ethynyl-2'-deoxyuridine (EdU) instead of BrdU. It eliminates many harsh treatments, such as DNase, that were necessary when using BrdU. EdU is not an antibody based system like BrdU, rather it relies on a reaction of the ethynyl group of the incorporated EdU with a small fluorescent azide-containing probe to generate the detectable fluorescence. This

chemical reaction method produces much brighter signals than typically seen with BrdU.

The understanding and ability to monitor the cell cycle is vital to the study of proliferation of adult beta-cells. By studying the role of the many regulatory genes involved in mitosis, our attempts to grow beta-cells will become more sophisticated as they begin to match more closely the actual process of mitosis. Transcription factors such as E2F that control the expression of many genes are ideal candidates for engineering a cell that is able to divide since the manipulation of one gene can influence hundreds of others.

E2F Transcription Factors:

E2F was discovered in 1979 as a gene that was expressed during the early replication cycle of adenoviruses [78]. The adenovirus replication cycle is divided into 3 phases: early, delayed early, and late. During the early phase, the genes expressed are simply named early-1, 2, 3 or 4. A factor was found that was induced by early-1 and bound to the early-2 promoter [78]. Its ability to initiate early-2 expression led it to become known as early-2 factor (E2F). E2F was later found to be involved not only in adenovirus replication, but mitotic division as well, leading to extensive study uncovering its role in a myriad of other cellular processes [79].

E2F1 was the first discovered and best-studied of the 8 known members of the E2F family. E2F1 shares homology in both structure and function with E2F2 and E2F3. If either E2F1 or E2F3 is knocked out, the other will compensate for the loss with increased expression, without disruption of function [80]. While E2F1 -/- mice

have a decreased pancreas size, beta-cell mass and impaired insulin secretion, they are not overtly diabetic. However, combined knockout of E2F1 and E2F2 mice are diabetic, indicating that while E2Fs have overlapping function, they also retain specific functions unique to the individual E2Fs [81, 82]. The group of E2F1, 2, and 3 are transcriptional activators of genes involved many cellular process, including apoptosis, mitosis, differentiation and development [83-85].

Regulation of E2F1/2/3 is achieved by formation of a complex with the retinoblastoma protein (Rb) [79, 86]. Retinoblastoma is a member of a family of regulatory proteins called pocket-proteins. So named, because of the pocket seen in their tertiary structure that forms the regulatory domain that confers function. Rb is not the only pocket protein, P130 and P107 are homologous to Rb in both form and function [87]. Hyper-phosphorylation of pocket proteins by cyclins D, A and E and Cdks 1, 2, 4, and 6 release E2F from Rb-E2F complexes and allow it to positively or negative affect target genes. Some E2Fs bind to a dimerization protein (DP) which enhances its transcriptional activity [88]. Expression of E2Fs is cyclic with E2F1-3 highly expressed during $G_1$/S and minimally expressed during the other phases of the cell cycle [89]. E2F1 is only bound to DNA immediately after exiting quiescence. During subsequent rounds of division only E2F3 is required to drive proliferation [80]. Once E2Fs have been activated, through exposure to serum, they stay activated even when the proliferative signal is removed [77]. This suggests E2F is a key regulator of r-point control and once its expression drives the cell past that point, division will proceed without any further proliferative stimulus.

E2F1-3 contains nuclear location signals (NLS) and must be localized in the nucleus to effect transcription of their target genes. The E2F3 gene encodes two isoforms that have opposing functions. E2F3a is the full-length E2F3 protein and induces transcriptional activation of its target genes. E2F3b is a N-termini truncated form of E2F3 that occupies E2F3a target gene promoters, restricting transcription of these genes [90].

In addition to its role in apoptosis, proliferation and differentiation, E2F was recently found to have a link to metabolism through its control over expression of Kir6.2 and therefore insulin secretion [91]. Loss of E2F1 leads to decreased expression of Kir6.2, impaired insulin secretion and glucose intolerance in mice [91]. By over expressing Kir6.2, these mice are able to regain normal insulin secretion and glucose regulation. Additionally, high glucose was found to induce proliferation through an increase in E2F1 expression in both beta-cells and vascular smooth muscle cells [91, 92]. This genetic link between hyperglycemia and insulin secretion may provide new therapeutic targets for type-2 diabetics.

The remaining E2Fs (4 through 8) are normally seen as negative regulators of transcription. E2F4 and 5 regulate expression of their target genes by occupying the promoters of E2F1-3 target genes and preventing activating E2Fs from binding and driving transcription [93]. They are thought to be a major influence on the maintenance of quiescence [86, 94]. E2F4/5 lack a NLS and are excluded from the nucleus during S-phase, but re-localized to the nucleus once complexed with a pocket protein after mitosis is complete [95]. E2F4/5 preferentially bind to p130 and p107, but also have a weak affinity to Rb. They must be coupled with a pocket

protein in order to repress transcription of E2F1-3 target genes [96]. While E2F4/5 maintain quiescence, E2F6/7 help create the cyclic nature of E2F expression. E2F6/7 are believed to decrease expression of the pro-proliferative E2Fs after S-phase, when the expression of E2F1-3 is no longer needed [97, 98]. Though they have a similar function, E2F6 and 7 are unique in that E2F6 binds to DP while E2F7 does not bind to any dimerization protein. Both function independently of any pocket protein [97]. E2F6 has also been reported to be involved in maintenance of quiescence [99]. The overlap of E2F6 with E2F4/5 may depend on the cell type and specific environment that leads the cell to $G_0$. E2F7 only binds to a subset of E2F1-3 target genes, those involved in proliferation, while it does not repress E2F target genes involved in other functions of E2F1-3 such as differentiation [98]. There are two isoforms of E2F7a and b. While they appear to have similar functions they differ in expression patterns, A is continually expressed while B is expressed only in S-phase [87]. The most recently discovered E2F is the 8th in the family. E2F8 does not form a heterodimer with any DP protein, nor is it regulated by any pocket protein [100]. Its shape is similar to that of other E2Fs when they are complexed to DP, presumably eliminating the need for a dimerization protein. Its exact function is still being discovered, but it appears to function like E2F6 and 7 to eliminate E2F1-3 target gene expression following G1/S [101]. The concept of overlapping function again appears with E2F8. It is functionally similar to E2F6/7, but does not require a dimerization partner, similar to E2F6. Instead, the conformation usually obtained by an E2F6-DP complex is achieved with the native E2F8 protein. This may allow for E2F8 to fulfill the necessary repressive role of E2F6 in the absence of both E2F6 and

DP. Given the critical aspect of E2F and the cyclic nature of its expression, redundant fail safes are required to ensure that cells are dividing when they are needed, but do not become oncogenic.

During $G_1$ the necessary protein complexes and kinases are assembled in preparation for DNA synthesis. Once the necessary machinery is formed a transition state is reached where the cell will proceed with DNA synthesis. At the end of $G_1$ there are several checkpoints that ensure DNA is intact and that organelles and the cell as a whole are functioning normally. If the cell passes this point, genes involved in DNA synthesis are transcribed and DNA replication begins. E2F target genes include histone *H2A*, *PCNA*, DNA polymerase, *RPA*, *Cdc6*, and *MCM* [83, 87]. Histone *H2A* is a histone core protein that allows access to supercoiled DNA sequences. *PCNA* acts as a DNA clamp physically holding DNA polymerase on the leading strand of DNA during replication. The rate-limiting step in DNA synthesis is the association/dissociation of DNA polymerase with the template strand. *PCNA* greatly increases the number of nucleotides that DNA polymerase can synthesize on the growing strand before dissociating and associating again [87]. *PCNA* slides down the template strand with DNA polymerase, limiting the occurrence of dissociation events. RPA is a protein that binds to single stranded DNA. This is useful during DNA synthesis because it prevents the newly created DNA strand from fold back on itself and forming secondary structures [83]. It also helps keep template ssDNA unwound during DNA synthesis. MCM and Cdc6 proteins are part of the pre-replication complex. This complex is formed at the origin of replication and once assembled MCMs are phosphorylated, unwinding DNA and initiating DNA replication. MCMs

help form and propagate the replication fork. Cdc6 helps load MCM proteins onto the DNA downstream of the origin sequence. However, Cdc6 also inhibits the function of the MCM proteins, providing another level of genetic regulation. When DNA synthesis is initiated, Cdc6 is phosphorylated (by Cdk1) and no longer represses the MCM proteins it laid in place. The DNA strand is then unwound, allowing DNA polymerase (held in place by PCNA) to begin replication of the ssDNA. These two E2F target genes form over half of the pre-replication complex (pre-rc). In this way, E2F is able to control the proliferation of cells, both positively and negatively. Cdk1 is cyclically expressed in a dividing cell. In early $G_1$ phase it is low, this allows the pre-rc to form and Cdc6 to insert MCMs onto the DNA. Later in $G_1$ when CDK1 expression is increased Cdc6 is phosphorylated and the pre-rc complex is activated. This step-wise procedure ensures that DNA replication only happens once per cell division. Cdc6 overexpression (on its own) does not cause re-replication in cells that have divided, because the high levels of CDK1 prevent it from existing in its pre-phosphorylated state and therefore can't carry out the functions associated with that conformation, essentially resetting the cell cycle clock to $G_1$.

While E2F1 overexpression has been used to immortalize hematopoietic cells and ectopic expression leads to proliferation in human beta-cells, proliferative and repressive E2Fs do not always adhere to their standard classifications [70, 102]. E2Fs behave very differently dependent on cell type, species and developmental stage [103-105]. This dual nature of E2F activators is completely dependent on the cellular environment. A tumor cell's gene profile will be radically different than that

of a fibroblast. Similarly, the E2F repressors are not always repressors. In cardiomyocytes, some of the activating E2Fs increase proliferation, but so does E2F4, a classical repressor [103]. Additionally, it does so with a decreased incident of apoptosis compared to E2F1 [103, 105]. While there is overlap in function of the activating and repressive E2Fs, it is also possible that overlap exists between the two groups such that repressive E2Fs could drive transcription and vise-versa. Factors such as Rb/p107/p130 phosphorylation, in the context of the specific cellular environment, may play a role in deciding how a specific E2F will function.

E2F binding to DNA consensus sites is regulated not by the binding region in the E2F complex alone, but also with other elements surrounding the promoter. Transcription factors such as *YY1* and *TFE3* are found around the E2F binding sites and their presence or absence can influence which E2Fs bind and when they bind [106] [107]. This site-specific priming of the E2F consensus region is what gives E2Fs the ability to regulate specific target genes while still allowing for overlap of function. E2F3 and TFE3 combine to activate the p68 subunit gene of DNA polymerase A. Even though they share a similar sequence, E2F1 does not have the ability to activate this target gene [107]. Additional target genes unique to E2F3 are DHFR, cdc2 and cdc6 among others [108]. Despite the fact that many E2Fs share a similar promoter consensus sequence, specific E2Fs target specific genes depending on co-expression of many yet undetermined factors. Even across the normal grouping of E2F1-3a and 3b-8 E2Fs have similar DNA binding regions in their target genes (accounting for the overlap in function) that are regulated by factors that surround the binding sites (accounting for unique binding of specific E2Fs to

specific target genes) [109]. This may explain how something like E2F4, which would normally inhibit proliferation, could induce proliferation if the cellular environment (i.e. expression of these co-factors) allowed E2F4 to bind to the target genes of E2Fs that would normally drive proliferation [103]. Once we gain a better understanding of the promoter environment, we will be able to better control from the hundreds of genes E2Fs effect what genes and therefore what biological process (apoptosis, proliferation, differentiation) occurs.

E2F6 is an Rb independent regulator of the "activating" E2Fs. That is, it binds to the same target genes as E2F1-3 and regulates their transcription. During the $S/G_2$ transition E2F6 binds to and inhibits transcription of some genes that were activated during $G_1/S$. In this way it acts to repress the genes that were involved in $G_1/S$ as the cell transitions from S to $G_2$ and a new set of genes must be expressed. E2F4 is bound to the promoters of E2F1-3 target genes during quiescence and $G_0$. It is displaced as the cell progress through $G_1/S$ and activating E2Fs bind to the domain. In the case where E2F6 is knocked out, E2F4 will act in its place. During $G_0$ E2F4 is seen to occupy the promoter of E2F target genes, as normal. However, in the absence of E2F6 it is found again in the promoter regions of these genes, as E2F6 would have been if it were present, during $S/G_2$ [97]. In this way, E2F4 is able to compensate for a loss of E2F6. As a cell progresses through the multiple stages of the cell cycle the E2F binding is changed. E2F4 is displaced in $G_1/S$ by the activating E2Fs in preparation for DNA synthesis. As the cell progresses further E2F6 displaces the activating E2Fs as the cell progress into $G_2$ and mitosis. Understanding this

pattern of E2F expression is vital to understanding proliferation of not just beta-cells, but all cells in general.

## MATERIALS AND METHODS

*Islet Isolations* – Male Lewis rats and C57B6 mice, 8-12 weeks old, were used for islet isolations. Rodents were anesthetized with vaporized isoflurane and then euthanized by heart perfusion exsanguination. They were laid supine and the abdominal fur wet with 70% alcohol. The abdomen was opened in a V-shape starting from the pubic region and extending to the lateral portion of the diaphragm in order to expose all organs in the peritoneal cavity. The animal was then turned so that the nose is closest to the surgeon with the tail pointed away. Gauze was used to secure the lobes of the liver. Next, the common bile duct (CBD) is clamped with a hemostat near the junction with the CBD and duodenum. For rats, a 27G needle secured to a 20mL syringe filled with 15mL of 2.2mg/mL collagenase XI (sigma) was used to canulate the CBD at the junction of the cystic duct from the gall bladder and left hepatic duct from the liver. For mice, a 10mL syringe with 0.375mg/mL of collagenase P (Sigma) was used. The pancreas was distended with 15-20mL (rat) or 2mL (mouse) of the enzyme solutions and then carefully removed from the animal. The enzyme filled pancreas was placed in a 50mL conical tube and stored on ice while the remaining rodents were processed. Once all pancreases were infused and removed, the 50mL conical tubes were placed in a 37°C water bath for 14 (rat) or 11 (mouse) minutes. After this time, the tubes willed half way with 4°C HBSS (Cellgro), shaken vigorously for several seconds and the remaining volume filled with 4°C HBSS. Following centrifugation, the pellet was washed 2 more times in 4°C HBSS. Next, the pellet was separated in a discontinuous ficol gradient (1.108, 1.096, 1.069, and 0.570g/mL) (Cellgro). Islets were collected from the interfaces of 1.069/1.096

and 1.096/1.108 g/mL. After purification, islets were cultured in CMRL 1640 (Gibco) with 11.5mM glucose, supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. Typically, to improve purity, islets were handpicked after overnight culture at 37°C before experimental use. One islet equivalent was defined as an islet of 150um diameter. Normal yield for one rat was 800-1000 islet equivalent (IEQ). All experiments performed with animals were done in accordance with protocols approved by the University of Illinois at Chicago Office of Animal Care and Institutional Biosafety.

*Protein Extraction* – Sample human islets were obtained from the UIC Islet Isolation Center and washed once in PBS. Islets where then mixed with a 1:100 solution of Cellytic M (Sigma) and a protease inhibitor at 30ul/$10^6$ cells and left overnight at -80°C. The next day, samples were thawed on ice, vortexed 30 seconds and homogenized by passage through a 27G needle 15 times. The lysate was then centrifuges at 10,000g for 10 minutes and the supernatant collected. To determine the protein concentration, the colorimetric Detergent Compatible (DC) Protein assay (Biorad) was used per manufacturer's instructions.

*Western Blot* – 50ug of protein extracts were mixed with a loading dye consisting of β-mercaptoethanol (BME) (Sigma) and Lamelli Sample Buffer (Biorad) in a 1:20 ratio. The addition of BME helps reduce the occurrence of protein dimers, which are undesirable in electrophoresis, by its ability to cleave disulfide bonds. Once the protein sample is mixed with the loading dye, it is heated in boiling water for 5

minutes to denatured to proteins. Once this is complete, 40uL the sample and 10uL of a protein ladder are loaded into the wells of a 4-20% polyacrylamide gel (Biorad) and run at 100V in a Tris Glycine + SDS buffer until the samples have traveled the length of the gel. Next, the gel is removed from its plastic case, overlaid with a pre-soaked nitrocellulose membrane, and sandwiched between filter paper. The membrane was run at 100V in a Tris Glycine + 20% methanol buffer for 1.5 hours at 4°C to ensure efficient transfer of the protein from the gel to the membrane. Transfer was verified by staining with Ponceau S, followed by 3 washes in TBS to remove the stain. Next, the membrane was blocked with non-fat dry milk, washed in TBS, and probed overnight in a primary antibody solution (Figure X for dilutions) at 4°C. The next day, the membrane was washed in TBS-Tween and incubated with the HRP-linked secondary antibody (1:2000) for 2 hours at room temperature. The addition of chemiluminescent reagents causes a chemical reaction whereby HRP converts the added luminol to an excited state, emitting light on return to its ground state. This light is captured on x-ray film by exposures ranging from a few seconds to ten minutes. In cases where the membrane was re-probed, it was first stripped using Restore Western Blot Stripping Buffer (Thermo) and incubated in primary and secondary antibodies as described above. Exposed films were scanned and analyzed by densitometry using ImageJ (NIH) normalized to beta-actin to determine relative amounts of protein expression.

*RT-PCR* - Islets not immediately processed for RNA were stored in RNALater (Ambion). Whole RNA was extracted with the RNeasy Minikit (Qiagen) per

manufacturer's instructions. RNA samples were combined with oligonucleotide primers by heat shock in a 70°C water bath followed by 5 minutes in ice. cDNA was synthesized by reverse transcription of 1ug of whole islet RNA with the ImProm-II Reverse Transcription System (Promega) following the manufacturer's instructions. Tubes were annealed for 5 minutes at 25°C, extended for 1 hour at 42°C and inactivation of the reverse transcriptase occurred at 70°C for 15 minutes. PCR was performed with 3uL of cDNA from the heat-inactivated PCR tube. Tubes contained a final volume of 25uL of: 12.5uL PCR Master Mix (Promega), 8.7uL nuclease-free water, 3uL cDNA, .4uL (20nM) forward primer, .4uL (20nM) reverse primer. Primers were used for E2F1, 2, 3, 4, 5, 6, 7, 8, and GAPDH (as a housekeeping gene) in human islets. Tubes were run at 95°C for 4 minutes, cycled 30 times (95°C for 30 seconds, 52°C for 30 seconds, 72°C for 1.5 minutes), and finally 72°C for 7 minutes. Next, the PCR products were separated on 2% agarose gels and visualized with SyberSafe at 1:10,000 (Invitrogen), a UV transilluminator, and a digital camera.

*Cell Culture and Viral Infections* – Human islets were isolated from pancreata of organ donors at the University of Illinois at Chicago Islet Isolation Center. All islets were rested overnight after isolation and cultured in CMRL 1066 (Mediatech) with 5.5mM glucose supplanted with 2.5% human albumin, 0.25% sodium bicarbonate, 0.02mg/mL ciprofloxacin, 0.2% ITS, and 1mM HEPES. Rat islets were isolated as described above and cultured in RPMI 1066 with 11mM glucose supplemented with 10% FBS and 1% penicillin and streptomycin. Full-length cDNA for human E2F3 was obtained from Origene and sent to Vector Biolabs for packaging into an

adenovirus – type 5 (dE1/E3) with a CMV promoter and GFP tag. Control adenovirus for Ad-CMV-LacZ was purchased directly from Vector Biolabs as well. Construction of lentiviral plasmids from human cDNA for E2F3 and DP2 (Open Biosystems) were a gift from Dr Salmon. E2F3 and DP2 plasmids were developed with the target genes under a tetracycline/doxycycline gene switch. These customized lentiviruses featured an optimized all-in-one TET-rtTA gene switch, the presence of a selectable marker, Blasticidin (BSD), and a cloner-friendly recombination cloning cassette. This allows dox-switchable control of our genes of interest with the addition of a single lentivirus for E2F3 or DP2. Additionally, he supplied plasmids for rat insulin promoter (RIP) driven destabilized GFP (RIP-dGFP), RIP-Puromycin, and hTERT. The RIP-Puromycin lentivectors will select for cells expressing insulin, independent of BSD, which will be used for cells containing the gene switch. Infection of human and rat islets were performed in ultra-low attachment 24-well plates (Corning) containing 300uL of the appropriate media. For experiments involving the lentivirus with the dox-switch, 1ug/mL of doxycycline (dox) (Sigma) was added to the media. Next, virus was added to the islets. For adenoviruses a MOI of 500 was used and for lentiviruses the MOIs used ranged from 1, 10, 50 and 100. For determination of MOI, 1 IEQ was estimated to contain 1000 cells. The islets were then cultured overnight at 37°C in 5% CO2. The following day 500uL of media supplemented with 20uM of EdU and 1ug/mL of dox (if applicable) was added to the plates. Following 4 days of culture the islets were collected, fixed and embedded as described below.

*Immunohistochemistry* – Following infection and culture, islets were collected, washed in PBS and fixed in Bouin's solution (Thermo) for 15 minutes. During this time, a 2% agarose solution was prepared and left to cool at room temperature. Following removal of the fixative, 400uL of molten agarose was used to disperse the islets and then quickly centrifuged to re-pellet them. The molten agarose was allowed to solidify on ice for 10 minutes, the mold was removed, and the excess agarose trimmed. The agarose molds where then dehydrated through an increasing series of alcohol washes (70%, 80%, 95%, 100%, half xylene/half 100% alcohol) ending with 100% xylene. Next, the molds were washed in molten paraffin for 1 hour, then embedded in paraffin blocks. After solidifying overnight at 4°C, the blocks were serially sectioned with 5um sections taken every 30um. These 5um sections were floated in 37°C water, attached to positively charged microscope slides, and allowed to dry in a 37°C incubator overnight. For EdU incorporation staining, the slides were rehydrated in xylene and a descending series of alcohol for 8 minutes each (Xylene I, xylene II, 100%, 95%, 80%, 70%, 50%, 35%) ending in de-ionized water. Next, the slides were stained with the Click-IT EdU labeling kit (Invitrogen) according to manufacturer's instructions. Following EdU staining, the slides were co-stained for insulin with a polyclonal guinea pig anti-insulin primary antibody (Dako, 1:200) overnight at 4°C. The next day, the slides were washed in PBS and stained with an anti-guinea pig 594 secondary antibody (1:200) for 1 hour at room temperature. Next, the slides were washed and the nuclei were stained with DAPI, a fluorescent stain that binds strongly to A-T rich regions of DNA. The final slides contained fluorescence emitting at 461nm (DAPI), 594nm (insulin), and 647nm

(EdU). Digital photographs were taken using a inverted fluorescence microscope (Leica). Stains for glucagon were conducted in a similar manner, polyclonal rabbit primary antibody (Sigma 1:200) overnight at 4°C followed the next day with anti-rabbit secondary antibody staining (Invitrogen 1:200) for 1 hour at room temperature.

*Microfludic Assays* – For all perifusion and imaging experiments conducted in this study, mouse islets were labeled with ratiometric fluorescent dye Fura-2/AM (Fura-2, Molecular Probes, CA) to determine intracellular calcium levels and fluorescent Rhodamine 123 dye (Rh123, Sigma, MO) to determine changes in mitochondrial potentials [110]. In brief, the islets were incubated with 5 μM Fura-2 and 2.5 μM Rh123 for 30 min at 37°C in Krebs-Ringer buffer (KRB) containing 2 mM glucose. The islets were then introduced into a temperature equilibrated microfluidic device through the loading port with the device mounted on an inverted epifluorescence microscope (Leica DMI 4000B). The loaded islets were perifused by continuous flow of KRB2 at 37°C (pH 7.4) for 10 min. KRBs containing high glucose (14 mM) or tolbutamide (250 μM) were then administered to the islets using a peristaltic pump. Dual-wavelength Fura-2/AM was excited at 340 and 380 nm and fluorescent emission was detected at 510 nm. Intracellular $Ca^{2+}$ was expressed as a ratio of fluorescent emission intensity F340 /F380 (%). Rh123 is a lipophilic cation that partitions selectively into negatively charged mitochondrial membranes. Glucose-induced hyperpolarization of the mitochondrial membrane causes uptake of Rh123 into mitochondria with a subsequent decrease in Rh123 fluorescence via

fluorescence quenching. Rh123 was excited at 490 nm±10, and emission was measured at 530 nm±10. Fura-2 and Rh123 fluorescence emission spectra were filtered using a Fura-2/FITC polychroic beamsplitter and double band emission filter (Chroma Technology. Part number: 73100bs). These images were collected with a CCD (Retiga-SRV, Fast 1394, QImaging). SimplePCI software (Hamamatsu Corp.) was used for image acquisition and analysis. Both fluorescence signals were expressed as 'change-in-percentage' after being normalized against basal intensity levels established before stimulation. The perifusates were collected from device outlets using an automated fraction collector (Gilson, model 203B, WI) at an interval of 1 mL/min to determine β-cell insulin secretory kinetics. Collected microfluidic perifusate samples were frozen at −80°C prior to insulin quantification. All samples were then completely thawed and mixed well at room temperature prior to determination of insulin levels via ELISA kit (Mercodia AB, Uppsala, Sweden) according to manufacturer protocol.

*Gluco-static Incubations* - Following infection and 4 day culture, islets were washed once in cold PBS and 5 islets were handpicked in 3 replicates. Islets were picked based on size with 1 small (<100um), 3 medium (100-200um), and 1 large (>200um) islet per condition. Islets were placed in a 24-well plate containing cell strainers with a 12um mesh-bottom in a low glucose solution of 2.8mM in KRB. Once islets were loaded, the liquid was drained from the cell strainer and transferred to a fresh low glucose solution for the islets to rest for 1 hour at 37°C 5% $CO_2$. Following the rest, islet-containing cell strainers were again drained of

liquid and placed in a new well containing fresh low glucose media (low glucose condition) for 1 hour at 37°C 5% $CO_2$. Next, the liquid was drained from the cell strainers and they were transferred to a new well containing a high glucose solution of 28mM glucose (high glucose condition). Following a 1 hour incubation at 37°C 5% $CO_2$ the strainers were drained and transferred to acid ethanol (1.5% HCl in 70% EtOH) for 15 minutes at 37°C 5% $CO_2$ (total insulin condition). Insulin was analyzed in each sample my ELISA (Mercodia) per manufacture's instruction. Stimulation index was reported as high glucose insulin value/low glucose insulin value.

*Mouse Transplants and In vivo EdU labeling* – All experiments performed with animals were done in accordance with protocols approved by the University of Illinois at Chicago Office of Animal Care and Institutional Biosafety. Islets were infected as described above and cultured for 4 days prior to transplant. 8-12 week old male nude mice were rendered diabetic by a single intraperitoneal injection of streptozotocin (STZ) at a concentration of 220 mg/kg. 11mg/mL of STZ powered was dissolved in a solution comprised of 25mL of 0.478g of citric acid and 25 mL of 0.735g sodium citrate diluted 1:10 in sterile water for a working solution. Following STZ treatment, mice were weighed and their blood glucose measured daily. Diabetes was considered established after two consecutive days of blood glucose readings over 350mg/dL. Mice with weight loss >20% or consistent blood glucose levels >600mg/dL were considered too sick to use and sacrificed. On the day of transplantation, islets were washed in PBS. A sterile gel pipette loading tip was gently bent to form a 'U' shape in the narrow end of the tip and placed into the

opening of a 1.5mL microfuge tube. Washed islets were allowed to settle in a 1.5mL microfuge tube, collected with a P200 in a minimal volume of media and transferred to the bent gel loading tip. After the islet had settled into the bend of the tip, excess media was removed. The narrow end of the gel loading tip was then attached to a length of PE-50 tubing, while the large end of the tip was attached to a P200 pipette. The islets were slowly loaded into the tubing and the tubing removed from the gel loading tip. The PE-50 tubing was then attached to the needle of a 25uL Hamilton syringe with the plunger withdrawn. The plunger was depressed until the islets were near the end of the opening of the PE-50 tube. The syringe was set aside so the islets could collect near the opening of the tube. Next, the mice were anesthetized with vaporized isoflurane and injected with 0.03mg/mL bodyweight of Buprenex subcutaneously. The animals left kidney was identified through the skin and the incision site was alternately scrubbed with beta-dyne and 70% alcohol. An incision was made directly above the kidney, parallel to the spinal cord, about 2 cm long through the dermis and peritoneal wall. The kidney was forced through the peritoneal opening and exposed. Once exposed and intermittently throughout the procedure, the exposed kidney was wet with room temperature sterile HBSS. A small (~2mm) posterior incision was made in the kidney capsule from the left lateral side to the right lateral side with a 27G needle. A flame-pulled glass capillary tube probe was inserted into the incision in the kidney capsule and moved in an anterior direction along the dorsal lateral surface of the kidney. This created a pocket between the kidney capsule and the kidney parenchyma where the islets would be transplanted. The PE-50 tube containing the islets was cut directly above

the aggregated islets in a beveled fashion. The beveled tubing was gently inserted into the sub-capsular pouch and moved to the most anterior end of the capsule. The islets were slowly deposited beneath the renal capsule by depressing the plunger on the Hamilton syringe. After removing the tube the islets were gently packed into the proximal end of the pouch by sliding a glass probe over the surface of the kidney posterior to anterior. The small incision on the kidney capsule was then cauterized and the kidney gently returned to the peritoneal cavity. Finally, the incision in the dermis and peritoneal wall is sutured closed with 5-0 sutures and the wound cleaned with beta-dyne and 70% alcohol. As the mice were removed the anesthesia, they are injected with 500uL of warmed saline subcutaneously to ensure proper post-operative hydration. Mice were then monitored daily for the first 5 days and 3 times weekly thereafter for weight and blood glucose for 21 days. For in vivo labeling of EdU, mice were injected intraperitoneal with 60uL of a 2.5ug/uL stock solution on day 27 post-transplant. On day 21, after an intraperitoneal glucose tolerance test, the grafted kidney was removed, fixed, trimmed of excess tissue, embedded in paraffin and sectioned as described above. For the EdU staining, slides were process as described above.

*IPGTT and Nephrectomy* – After 21 days, mice were assayed for glucose tolerance by intraperitoneal glucose tolerance test. First, the animals were fasted overnight. The next day a 20% glucose solution was prepared by dissolving 1g of glucose in 5mL of sterile water. The animals were injected with microliters of the 20% glucose solution equivalent to 15 times their body weight in grams. The mice were weighed

and their blood glucose collected at the time points: 0, 15, 30, 60, and 120 minutes following injection. After the IPGTT, the grafted kidney was removed from the transplanted mice. The mice were anesthetized and an incision made as previously stated above. The kidney was exposed and the renal blood vessels clamped with a hemocrit. The blood vessels were tightly tied off with a suture string. The kidney is then excised distal to the tied off blood vessels and placed in a fixative for histological processing. The incision is closed and the mice monitored as before for an additional two days.

*Electrophysiology* – Islets from E2F1 knockout (Jackson Labs) and C57B6 mice (Harlan) were isolated as detailed above and rested overnight. The next day, islets were dissociated by 1 minute alternating culture in Accutase (Invitrogen) and a 37°C water bath. Dissociation was stopped with the addition of complete media containing FBS, once a single cell suspension had been microscopically verified. Next the single cells were plated in 35mm tissue culture treated dishes to attach overnight cells were superfused with an external solution of 138mM NaCl, 5.6mM KCl, 1.2mM $MgCl_2$, 2.6mM $CaCl_2$, 5mM HEPES, and pH adjusted to 7.4 with NaOH. 10 and 14mM glucose was added where indicted. Experiments were conducted at 29-31°C. Membrane potential and whole-cell currents were measured using a perforated patch whole cell method with an EPC-7 patch clamp amplifier. A solution of 10mM KCl, 10mM NaCl, 70mM $K_2SO$, 7mM $MgCl_2$, 5mM HEPES, pH adjusted to 7.35 with KOH was used for the pipette solution. The tip of the pipette was filled with the above solution and then back-filled with the same solution with the

addition of β-escin 50 uM in 0.2% DMSO. Perforation of cells was assured by the decrease in serial resistance ($G_s$) and the increase in ability to compensate for the capacitance of the cell. Perforation for Volatage-clamping ($G_s$ < 50 MΩ) could be performed after 15 minutes of perforation and seal formation. For membrane potential, only a few minutes were necessary before recording. Beta-cells were verified by their response to glucose.

*Statistical Analysis* – Two-tailed unpaired *t*-tests were performed for pairs of data and two-way ANOVA was performed for multiple comparisons with a control group. The area under the curve of blood glucose measurements was determined by Mann-Whitney U test. *P*-values <0.05 were considered significant. Standard error bars were used in the appropriate figures.

## RESULTS

*Overexpression of E2F3 promotes proliferation of functional human beta-cells without induction of apoptosis*

Since expression of E2F4-6 is known to inhibit proliferation, we sought to characterize their expression in wild type human islets. Whole cell protein extracts from 4 human islet isolations (H1-H4) were probed for the transcription factors E2F1-6 (Fig. 1A). These immunoblots were densitometrically normalized to beta actin and expressed as relative protein expression (Fig. 1C). These islets contained detectable levels of 6 E2Fs in varying degrees of expression, depending on the isolation. E2F4, an anti-proliferative E2F, was the most highly expressed, with other anti-proliferative E2F 6 and 7 being expressed at detectable levels as well. Proliferative E2F1-3 were all expressed at similar levels, but below the expression of the anti-proliferative E2F4. Since E2Fs are only active when they are located in the nucleus we performed western blots for E2F1-6 on nuclear extracted proteins run alongside positive controls on the same gel, to determine which E2Fs may be currently associated with DNA in the nucleus [95]. While all E2Fs were detectable in the whole cell lysate, when the nuclear fraction of 4 other isolations (H5-H8) was isolated, only the anti-proliferative E2Fs 4-6 were detectable (Fig. 1B). When these are analyzed and normalized to beta actin by densitometry, we found E2F6 is the most highly expressed, with high levels of E2F4 and E2F5 also present (Fig 1D). Pancreatic donor information is described in table 1. The high expression levels of repressive E2Fs, especially in the nucleus, may account for the slow proliferation rate seen in human islets in vivo.

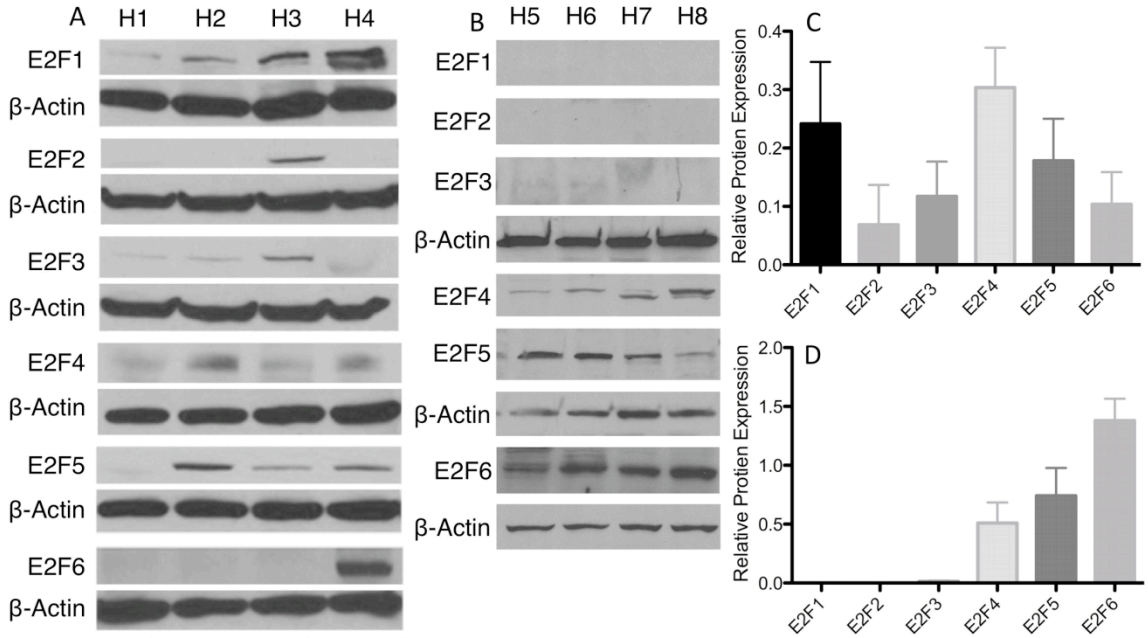


**Figure 1 - Endogenous E2F expression.** (**a and b**)**:** Western blots showing differential E2F expression in whole cell (**b**) and nuclear (**b**) protein samples from eight human islet isolations (H1-H8). (**c and d**) Quantification of whole cell (**c**) and nuclear (**d**) immunoblots for E2F expression in human islets, relative protein expression normalized to beta-actin by densitometry.

| Isolation ID | Age | Sex | BMI (kg/m$^2$) | Purity (%) | Cold Ischemia Time (hh:mm) |
|---|---|---|---|---|---|
| H1 | 58 | F | 25.8 | 95 | 3:54 |
| H2 | 57 | F | 19.7 | 85 | 4:08 |
| H3 | 49 | M | 30 | 90 | 10:21 |
| H4 | 48 | M | 34.9 | 81.50 | 9:35 |
| H5 | 52 | F | 27.9 | 90 | 7:15 |
| H6 | 54 | M | 25.3 | 92.50 | 12:33 |
| H7 | 44 | F | 39.2 | 91 | 5:41 |
| H8 | 70 | M | 25.3 | 75 | 6:36 |

**Table 1: Islet donor information**

To examine the E2F expression in proliferative primary human beta-cells, we repeated the E2F protein screen on whole cell lysates of insulinomas obtained from two patients, one benign (I-1) and the other malignant (I-2), from the University of Illinois at Chicago hospital. These patients were hyperinsulinemic, hypoglycemic and gained a significant amount of weight. E2F1-6 protein expression was again probed by western blot (Fig. 2A) and normalized to beta actin (Figs. 2B and C). In these insulinomas, the pro-proliferative E2Fs 1 and 3 were highly expressed. Unlike wild type islets, none of the anti-proliferative E2F4-6 were observed in either of these samples. Both samples displayed similar levels of proliferative E2Fs and though access was limited to two samples, the results seem comparable between each insulinoma. The high expression levels of proliferative E2Fs may account for the increased proliferation rate seen in these human insulinomas.

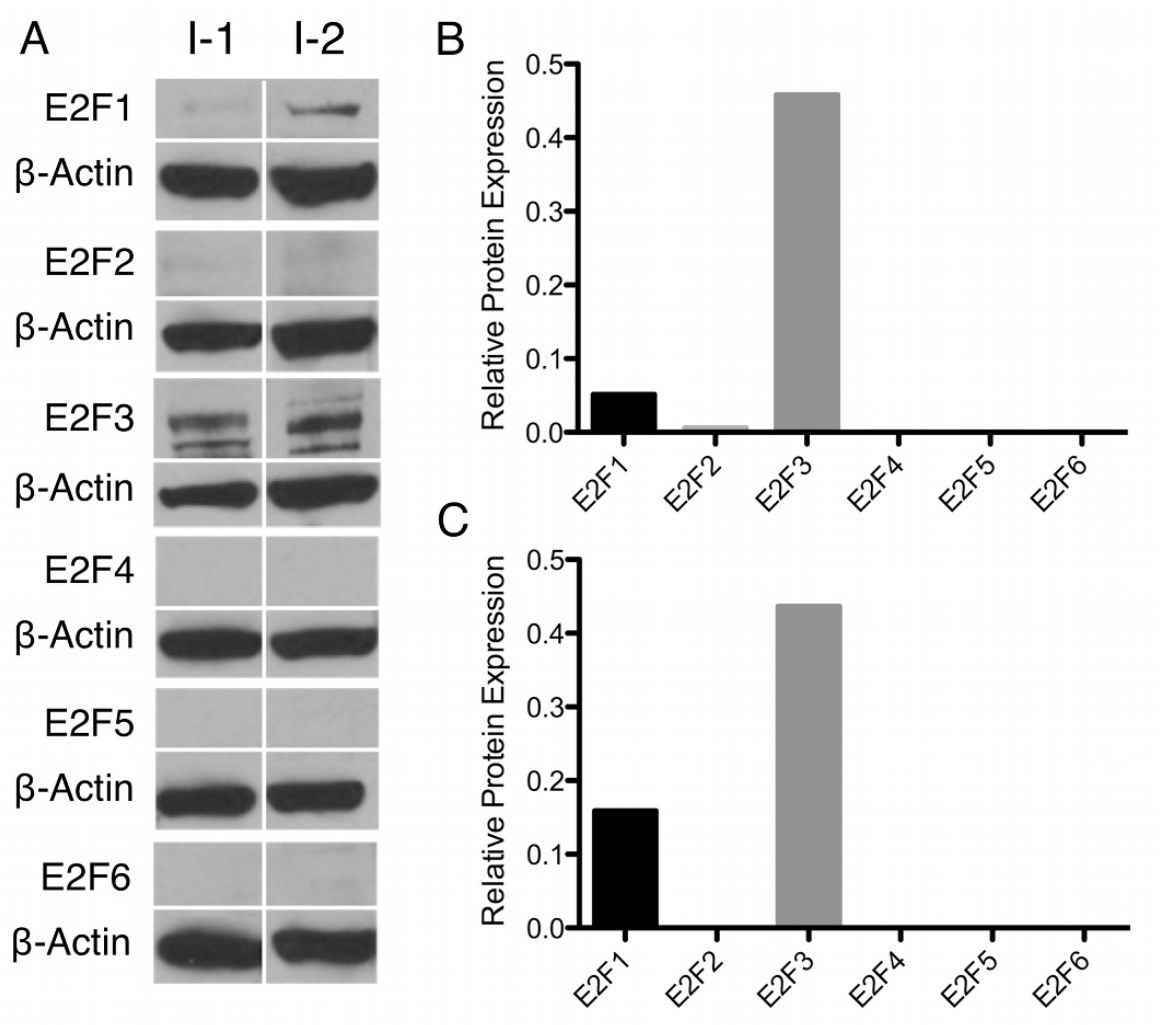


**Figure 2 - Insulinoma E2F expression.** (**a**)**:** Whole cell protein samples from benign (I-1) and malignant (I-2) human insulinomas probed for E2F1-6 by western blot. (**b and c**)*:* Insulinoma protein expression normalized to beta-actin by densitometry.

Since E2F3 was highly expressed in the proliferating insulinomas and wildtype islets had such diminished endogenous expression we wanted to see if ectopic induction of E2F3 in normal islets would lead to similar proliferation as insulinomas. To this end, we infected adult human islets with adenoviruses expressing E2F3-GFP to assess their ability to induce transgene expression in whole islets. First, the viruses were tested for replication competency. If the viruses have become replication competent, transduced cells would propagate the virus and lyse. This would form plaques, large circular areas of lysed cells, which would indicate the presence of viruses that were capable of synthesizing DNA. This DNA synthesis would interfere with our assays for proliferation, so the absence of viruses capable of replication is critical. Figure 3A and B shows no plaque formation in Hela cells infected with E2F3 adenovirus indicating that the virus was not able to reproduce. This ensures any proliferation seen by the addition of this virus is not due the replication of the virus itself. When whole human islets are infected with the ad-E2F3 at a MOI 500 for 4 days robust expression of E2F3 is seen by western blot (Fig. 4A). When normalized to beta actin, a more than 3-fold increase is seen in E2F3 expression (Fig. 4B). Histology of an infected islet stained for E2F3 reveals that the adenovirus penetrates only about 1-5 cell layers deep into the islet, but does not infect the core (Fig. 4C). Visualization of the GFP tag further confirms the viruses' ability to infect whole human islets as GFP is readily seen after ad-E2F3 infection while it is absent from uninfected islets (Fig 4D). E2F3 infected islets appear to have several dead cells surrounding the islet, while uninfected islets have fewer dead cells. Additionally, a necrotic center is noticeable in the E2F3 infected islets,

however this is typical of islets of a certain diameter and we do not attribute it to the viral infection.

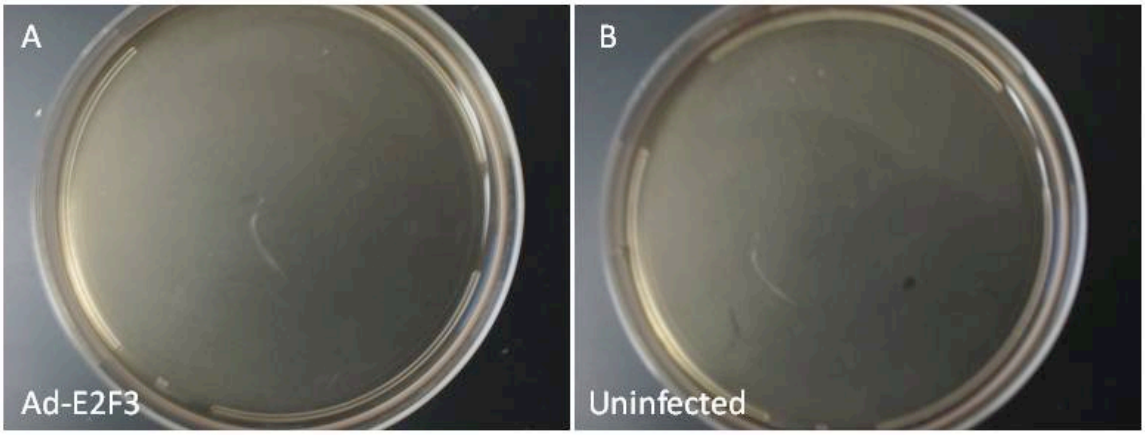


**Figure 3 - Non-replication competent adenovirus.** (**A**) Hela cells were infected with ad-E2F3 at a MOI of 500 and cultured for 7 days (**B**) Uninfected Hela cells (MOI 0) cultured for 7 days as a negative control.

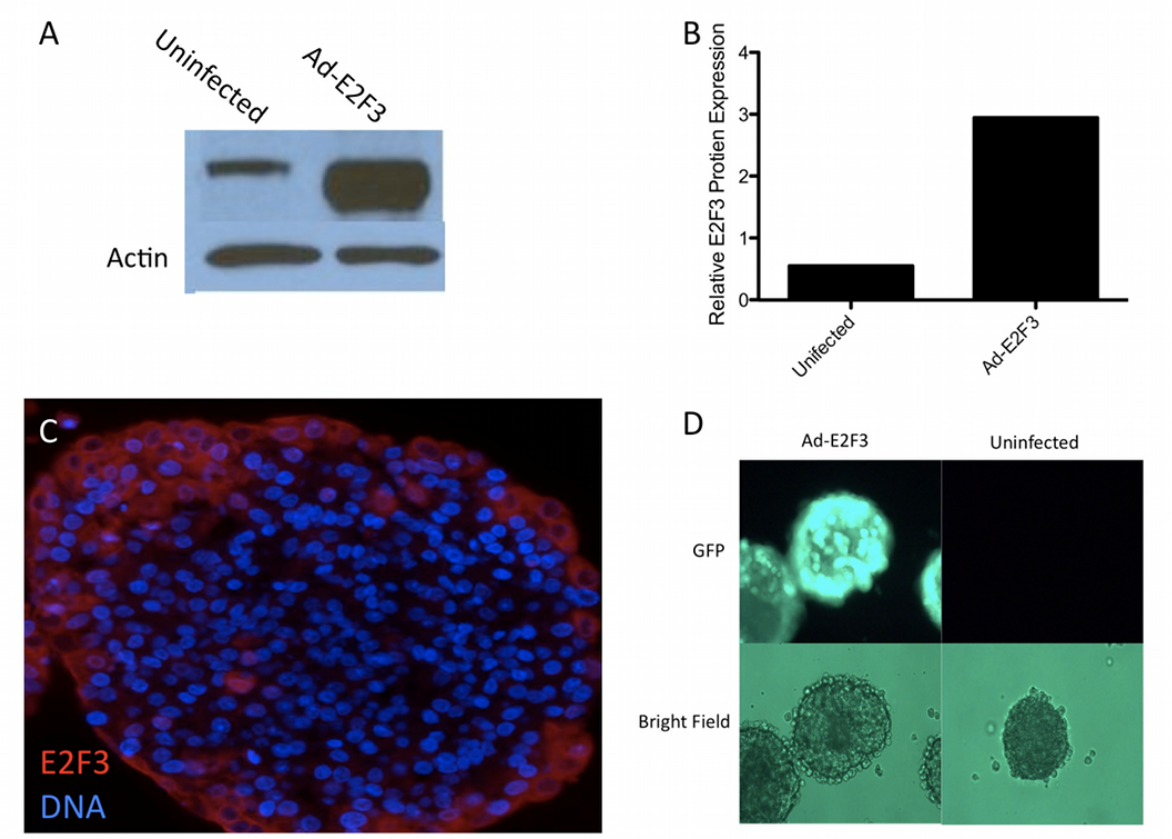


**Figure 4 - Adenovirus function and infection.** (**a**)**:** Western blot for whole cell protein extracts following ad-E2F3 infection at a MOI of 500. (**b**)**:** Quantification of western blots for E2F3 in uninfected and ad-E2F3 infected islets, normalized to beta-actin by densitometry. (**c**)**:** Human islets infected with ad-E2F3 and fluorescently stained for E2F3 showing the level of penetration the adenovirus achieves in whole human islets. (**d**)**:** Expression of GFP-tagged ad-E2F3 in infected and uninfected human islets after 3 days of culture.

To determine if ectopic E2F3 enhanced beta-cell proliferation, S-phase entry was examined by EdU incorporation in rat islets transfected with ad-E2F3. Uninfected (Fig. 5A) and ad-LacZ (Fig. 5B) infected islets showed no significant difference with only 2.638% ± 0.2688 and 2.879% ± 0.2122 proliferation of insulin positive cells. The addition of the E2F3 adenovirus significantly increased beta-cell specific proliferation to 23.83% ± 3.659, $p$=0.004 (Fig. 5C). These results are quantified for 3 rat preparations, with a range of 700-1000 insulin positive cells counted per preparation (Fig. 5D).

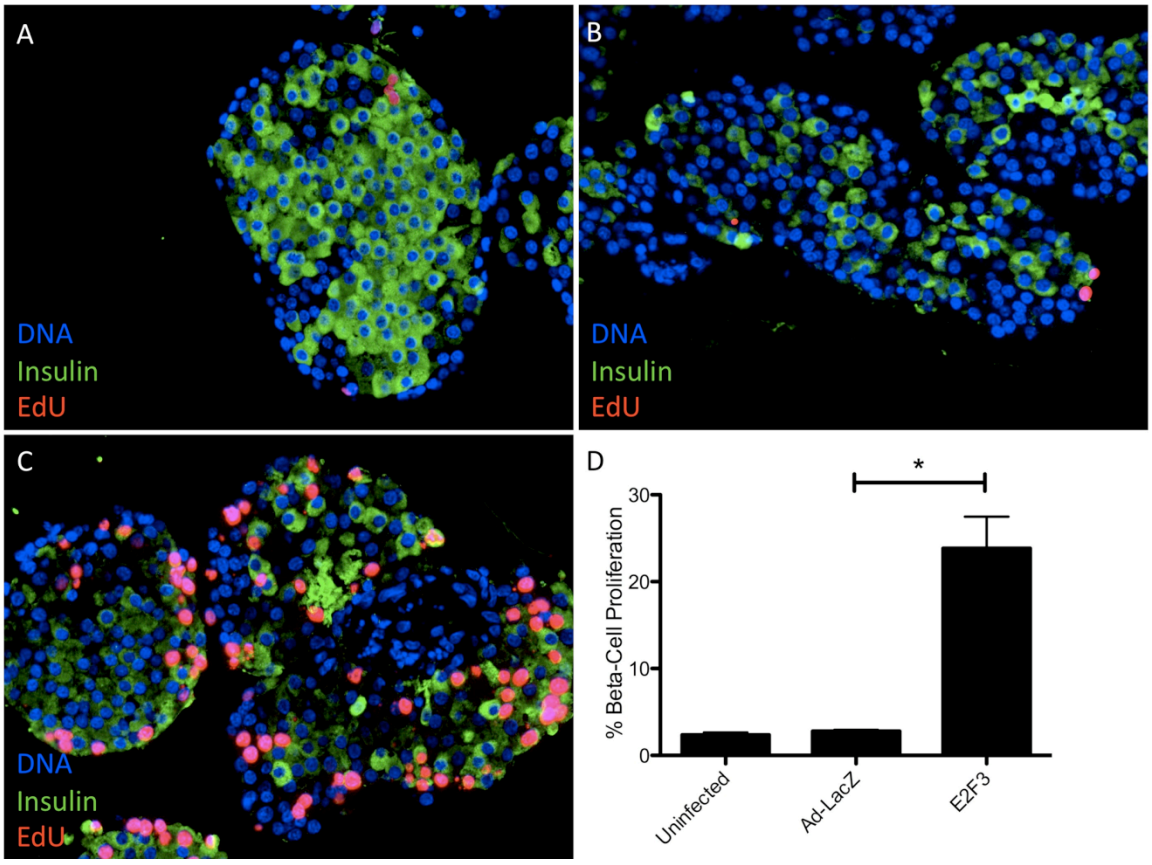


**Figure 5 - E2F3 induced proliferation in rat islets.** (**a, b and c**)**:** Representative pictures of paraffin embedded sections for uninfected (MOI 0) (**a**), infection with ad-LacZ (MOI 500) (**b**) and ad-E2F3 (MOI 500) stained for EdU (red), insulin (green) and DNA stained with DAPI (blue) (**c**). (**d**)**:** Quantification of immunohistochemistry for EdU+/insulin+ cells. % beta-cell proliferation represents the number of cells that co-stained for EdU and insulin over the total number of insulin positive cells, (*) $p$=0.004. n=3 islet preparations

Many methods of proliferating rodent beta-cells do not translate or translate poorly into humans [111]. To confirm the proliferative effect of E2F3 seen in rodents, human islets were infected and assayed for EdU incorporation. Representative pictures of uninfected (Fig. 6A) and ad-LacZ (Fig. 6B) showed little proliferation, 0.5208% ± 0.0579 and 1.504% ± 0.22 respectively, with $p$=0.04. Others have noted that adenovirus infection with transgenes typically used as viral controls can activate *Akt* expression and produce small, but statistically significant levels of proliferation, similar to what we see here[112]. Infection with ad-E2F3 significantly raised the beta-cell proliferation rate to 7.158% ± 0.4776, $p$<0.001 (Fig. 6C). These results were quantified for 3 human islet preparations with 700-1000 insulin positive cells counted per preparation (Fig. 6D).

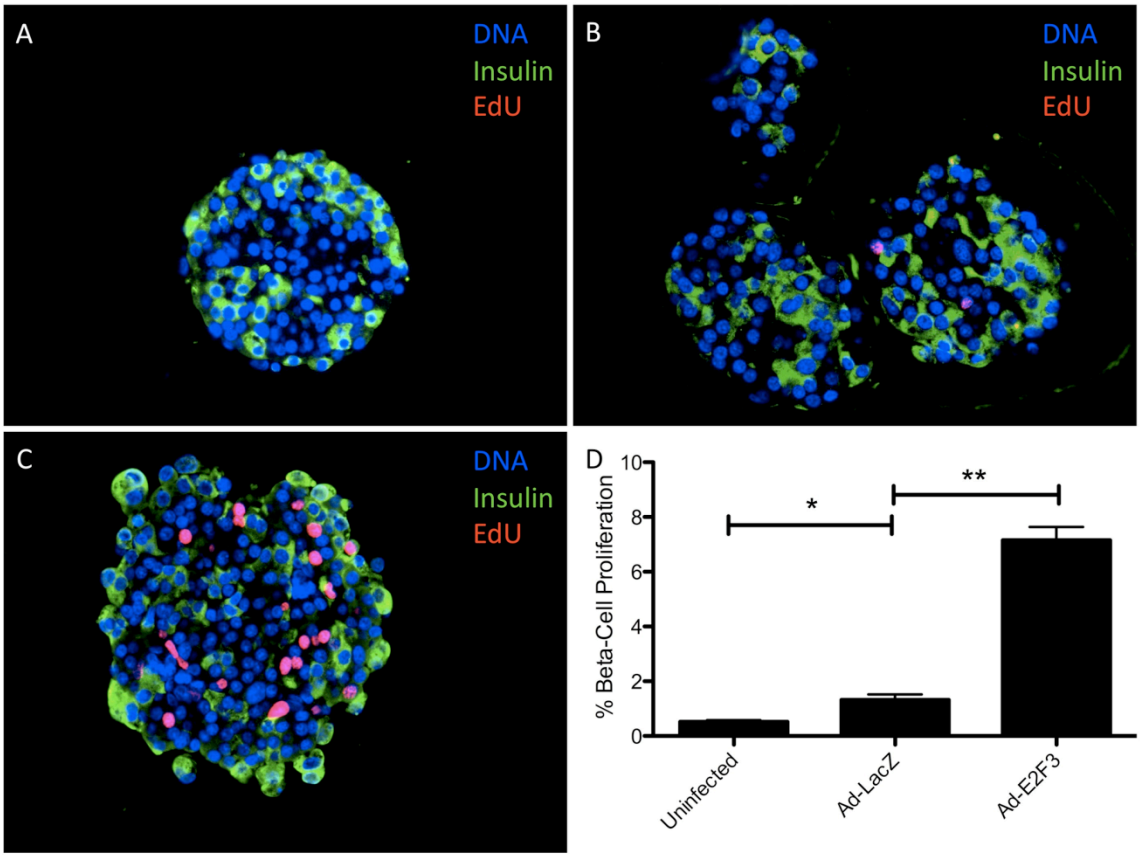


**Figure 6 - E2F3 induced proliferation in human islets.** (**a, b and c**)**:** Representative pictures of uninfected (MOI 0) (**a**) human islets, ad-LacZ infected (MOI 500) (**b**), and ad-E2F3 infected (MOI 500) (**c**) cultured in the presence of EdU for 4 days and stained for EdU (red), insulin (green) and the DNA stained with DAPI (blue). (**d**)**:** Quantification of immunohistochemistry for EdU and insulin positive cells, (*) $p$=0.04 (uninfected to ad-LacZ) *(**)* $p$<0.001 (ad-LacZ to ad-E2F3). % beta-cell proliferation represents the number of cells that co-stained for EdU and insulin over the total number of insulin positive cells. n=3 islet preparations

Efforts to proliferate beta-cells often result in dedifferentiation and apoptosis as they grow[70, 113]. To ensure that E2F3-proliferated islets retained their phenotype and glucose-sensitivity, we performed a static glucose incubation on rat and human islets. Rat (Fig. 7A) and human (Fig. 7B) islets showed that insulin secretion in response to both high (28mM) and low (2.8mM) glucose were normal in E2F3 infected islets. Additionally, overexpression of E2F3 did not affect the total insulin content of the cells (Fig. 7C and D). Previous efforts to proliferate beta-cells using E2F1 resulted in activation of apoptosis and cell death[70]. To investigate the possibility that overexpression of E2F3 induced apoptosis, we assayed infected human islets for caspase-3 activity. Uninfected, ad-LacZ, and ad-E2F3 infected islets all showed similar levels of Caspase-3 activity, indicating that unlike E2F1, E2F3 induced proliferation does not activate apoptosis (Fig. 7E).

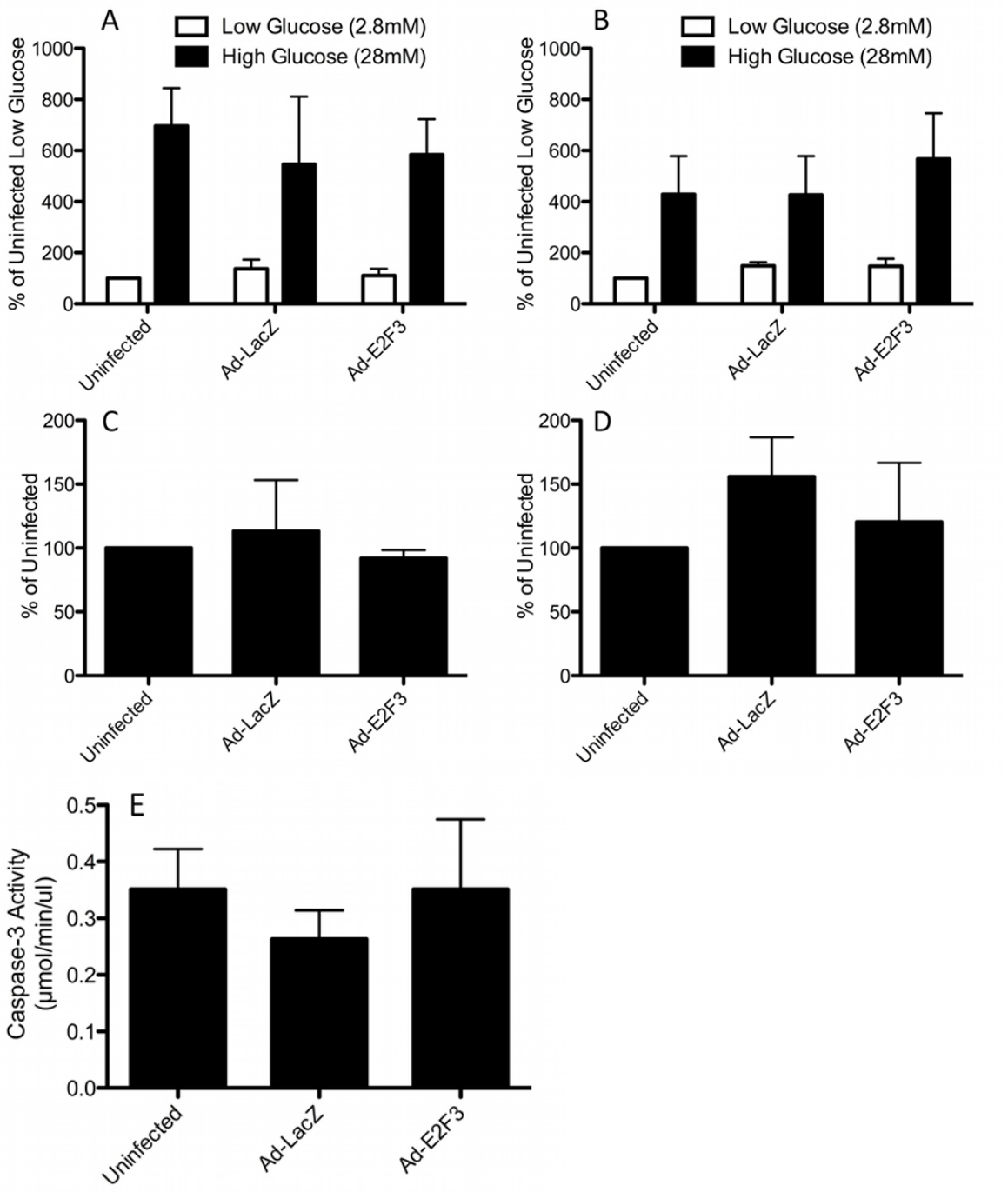


**Figure 7 - Assessing beta-cell function: static glucose incubation and apoptosis.** (**a and b**)**:** Rat (**a**) and human (**b**) islets were incubated in low (2.8mM) and high (28mM) glucose for 1 hour, *p*=ns (**c and d**)**:** Total insulin content of uninfected, ad-LacZ (MOI 500) and ad-E2F3 (MOI 500) islets, *p*=ns, n=3 islet preparations (**e**)**:** Caspase-3 activity in uninfected, ad-LacZ and ad-E2F3 infected human islets, *p*=ns, n=4 islet preparations.

To further characterize the beta-cell function, we assayed E2F3 infected islets for: 1.) intracellular calcium concentrations in response to glucose or KCl, an indicator of the glucose-stimulated insulin secretion kinetics determined mainly by $K_{ATP}$ and calcium ion channels and 2.) hyperpolarization of the mitochondrial membrane, an indicator of glucose sensitivity and ATP production. Despite a trend of decreased calcium flux, the area under the curve for both the entire curve, $p=0.854$ and the glucose stimulated portion only, $p=0.0657$, are not significantly different (Fig. 8A). Uninfected, ad-LacZ and ad-E2F3 infected islets all showed similar intracellular calcium levels (Fig. 8A). Mitochondrial potential and dynamic insulin secretion also revealed no impairment or loss of function (Fig. 8B and C). Overall, no negative effects on insulin secretion, glucose sensitivity, total insulin content, caspase-3 activity or $K_{ATP}$/calcium ion channels were observed with the overexpression of E2F3 and the islets appear to retain their phenotype.

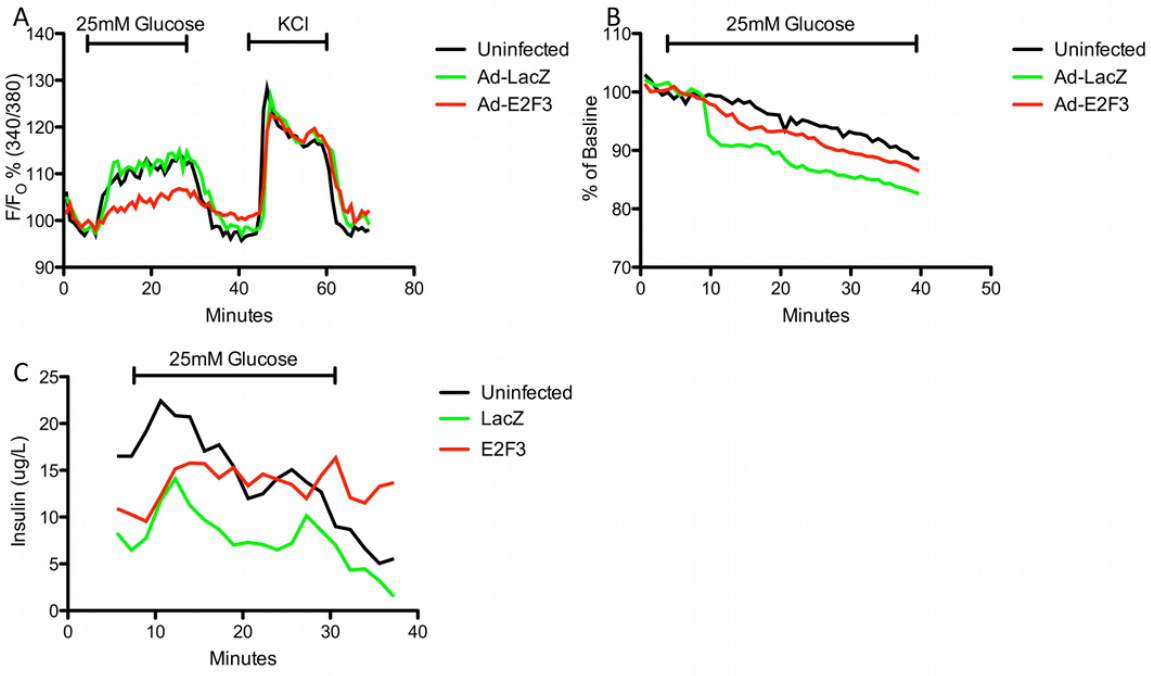


**Figure 8 - Assessing beta-cell function: microfluidic assay**. (**a**)**:** Dynamic measurement of intra-cellular calcium levels in uninfected, ad-LacZ and ad-E2F3 infected human islets. Islets were incubated with Fura-2 then loaded into a microfluidic device mounted on an epifluorescence microscope and perifused by continuous flow of 2 mM glucose and 25 mM glucose in krebs-ringer buffer. KCl was then added to inhibit $K^+$ efflux and depolarize the cells. Fura-2/AM was excited at 340 and 380 nm and emission was detected at 510 nm. Results are presented as percent of baseline. *p*=ns, n=3 (**b**)**:** Rh123 excitation at 490 nm and emission at 530 nm was measured to assess mitochondrial potential during exposure to 25mM glucose. Results are presented as percent of baseline. *p*=ns, n=3 (**c**)**:** Dynamic insulin secretion during the intracellular calcium measurements in **a**, *p*=ns.

To determine if the *in vitro* proliferative effect of E2F3 enhanced islet function due to increased beta-cell mass we used a minimal mass islet transplant model with infected rat and human islets into streptozotocin-induced diabetic nude mice. E2F3 infected rat islets are able to reverse diabetes (reversal defined as blood glucose <210 mg/dL), obtaining an average blood glucose of 184.2 ± 30.4 mg/dL, compared to 280.1 ± 30.4 mg/dL with uninfected islets, $p$=0.009 (Fig. 9A). All mice gained weight throughout the 21-day period (Fig. 8B). After 21 days, to further characterize the graft function we performed IPGTTs on the transplanted mice, revealing the E2F3 infected islets had an enhanced ability to clear glucose, with an AUC of 24390 mg/dL/min ± 2547 compared to 42654 mg/dL/min ± 10684 for uninfected, $p$=0.04 (Fig. 8C and D). Following the IPGTT, a unilateral nephrectomy of the grafted kidney showed the transplanted islets restored normoglycemia, rather than a spontaneous regeneration of the native pancreas (Fig. 9A and B).

To test the *in vivo* function of expanded human islets, we transplanted 2000 IEq, of uninfected islets as a positive control and 1000 IEq of uninfected islets as a negative control. We found mice transplanted with 1500 IEq of E2F3 infected islets did reverse the diabetic condition, but did not obtain superior non-fasting blood glucose levels with an average of 203.2 mg/dL ± 19.2 compared to LacZ, 157.3 mg/dL ± 20.2, and 1500 IEq uninfected islets, 158.1 mg/dL ± 19.8, revealing no significant difference (Fig. 9E). Additionally, all groups showed weight gains during the 21 days (Fig. 9F). Glucose tolerance test further revealed no significant enhancement of the islet graft with an area under the curve of 13868 mg/dL/min ±

4629 for uninfected islets, 12792 mg/dL/min ± 776 for ad-LacZ and, 20138 mg/dL/min ± 3652 for ad-E2F3 infected (Fig. 9G and H).

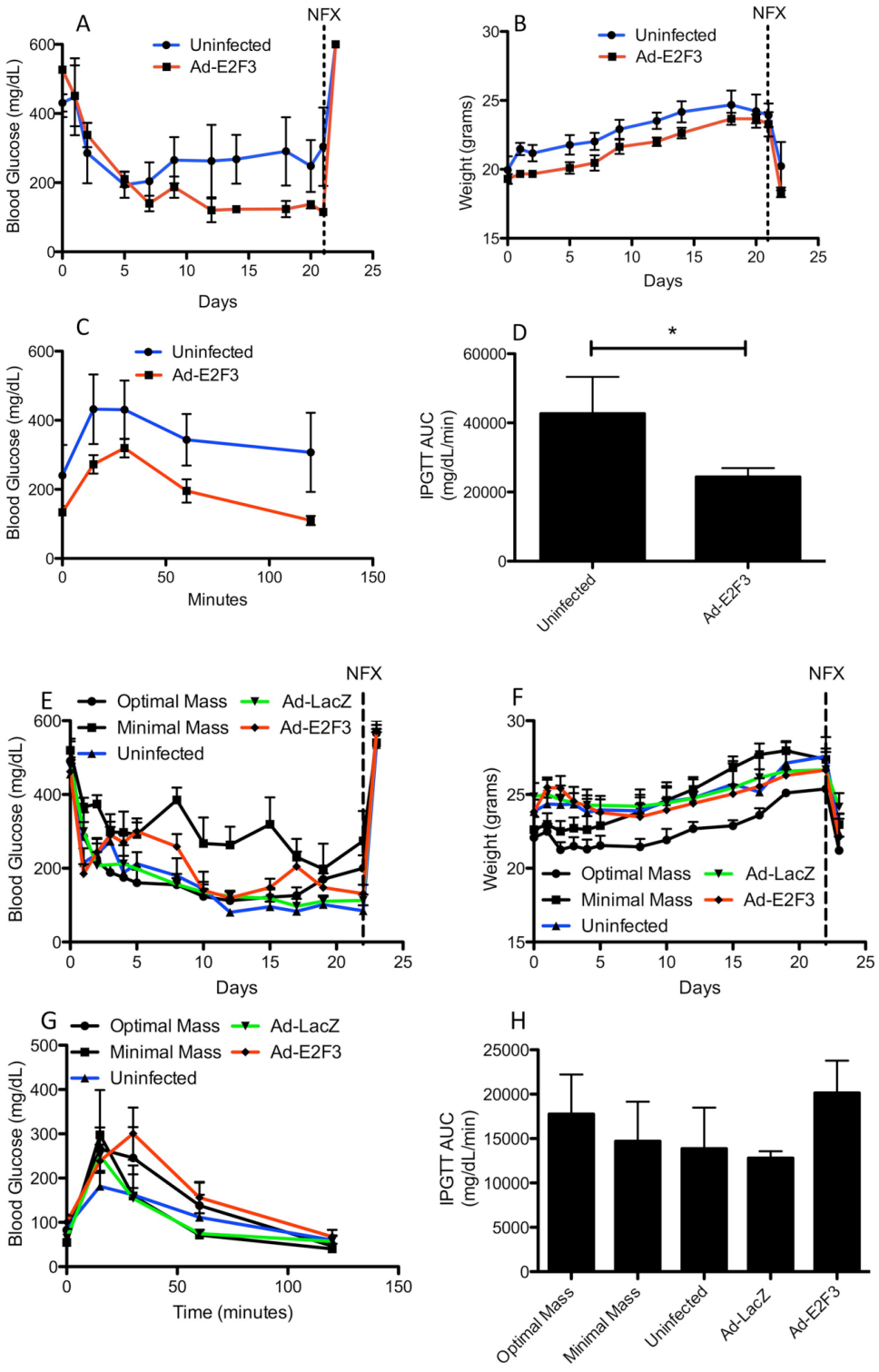


**Figure 9 - In vivo function of E2F3 infected rat and human islets.** (**a**)**:** Blood glucose measurements for mice transplanted with minimal mass (250 IEq) of ad-E2F3 infected (MOI 500) or uninfected (MOI 0) rat islets, AUC $p$=0.009 (**b**): Weight of mice transplanted with rat islets infected with ad-E2F3 and uninfected.

Nephrectomy performed at day 21. (**c and d**)**:** Intraperitoneal glucose tolerance testing in transplanted mice 21 days after minimal rat islet mass transplant and area under the curve of glucose clearance, (*) $p$=0.04, n=4. (**e**)**:** Blood glucose measurements for mice transplanted with 1500 IEq of uninfected (MOI 0), ad-LacZ (MOI 500), and ad-E2F3 (MOI 500) infected human islets, AUC $p$=ns. (**f**)**:** Change in weight over the 21 day transplant follow up. Nephrectomy performed at day 21. **(g and h):** 21-day IPGTT of mice transplanted with human islets and area under the curve of glucose clearance, AUC $p$=ns, n=5. **NFX** - unilateral nephrectomy

To determine if the normalization of blood glucose in the transplanted rat islets was due to continued proliferation of the islets after they were transplanted we injected mice with EdU for the last 3 days prior to IPGTT and nephrectomy. Little or no proliferation was seen in both the uninfected and E2F3 infected islets (Fig 10 A and B) indicating that any proliferation resulting in increased beta-cell mass occurred in vitro or early in vivo, before loss of transgene expression.

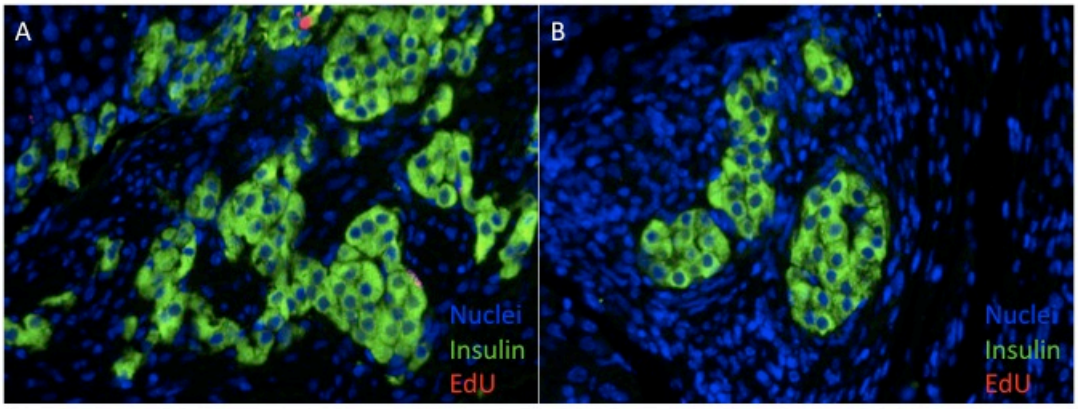


**Figure 10 - In vivo proliferation.** Representative sections from uninfected (**a**) and ad-E2F3 (**b**) transplanted islets exposed to EdU during the last 3-days of the 21-day transplant and stained for EdU (red), insulin (green), and DNA (DAPI).

Islets shown in Fig. 4 C only had approximately 25% of the total cells of the islet infected. In order to establish a method to increase the infection efficiency, islets were dissociated, infected, and re-aggregated as means to allow the virus access to islet cells located in the core of the islet. Islets were mildly dissociated and infected with E2F3. Infection of single cells was verified by robust GFP expression in the majority of cells (Fig 11A and B). Infected islets showed an increased rate of proliferation (Fig. 11C) over LacZ (Fig. 11D) and uninfected islets (Fig. 11E). Given the large number of non-insulin positive cells, we wanted to see if these were alpha-cells or another cell type. Staining of dissociated islets revealed the presence of glucagon positive cells, indicating alpha cells had survived the dissociation and were able to be cultured with beta-cells (Fig 11F). However, there were still a large percentage of cells that did not stain for either glucagon or insulin. These cells are believed to be a mix of other pancreatic endocrine cell types, fibroblasts, and perhaps dedifferentiated alpha and beta-cells. For re-aggregation, dissociated islet cells where then plated in 3 densities, 42k, 84k, and 168k in non-adherent plates to see what effect seeding density had on re-aggregation of islets. All conditions contained islet-like clusters of cells, with increasing densities resulting in more islet-like clusters rather than larger clusters (Fig 12 A, B, and C)

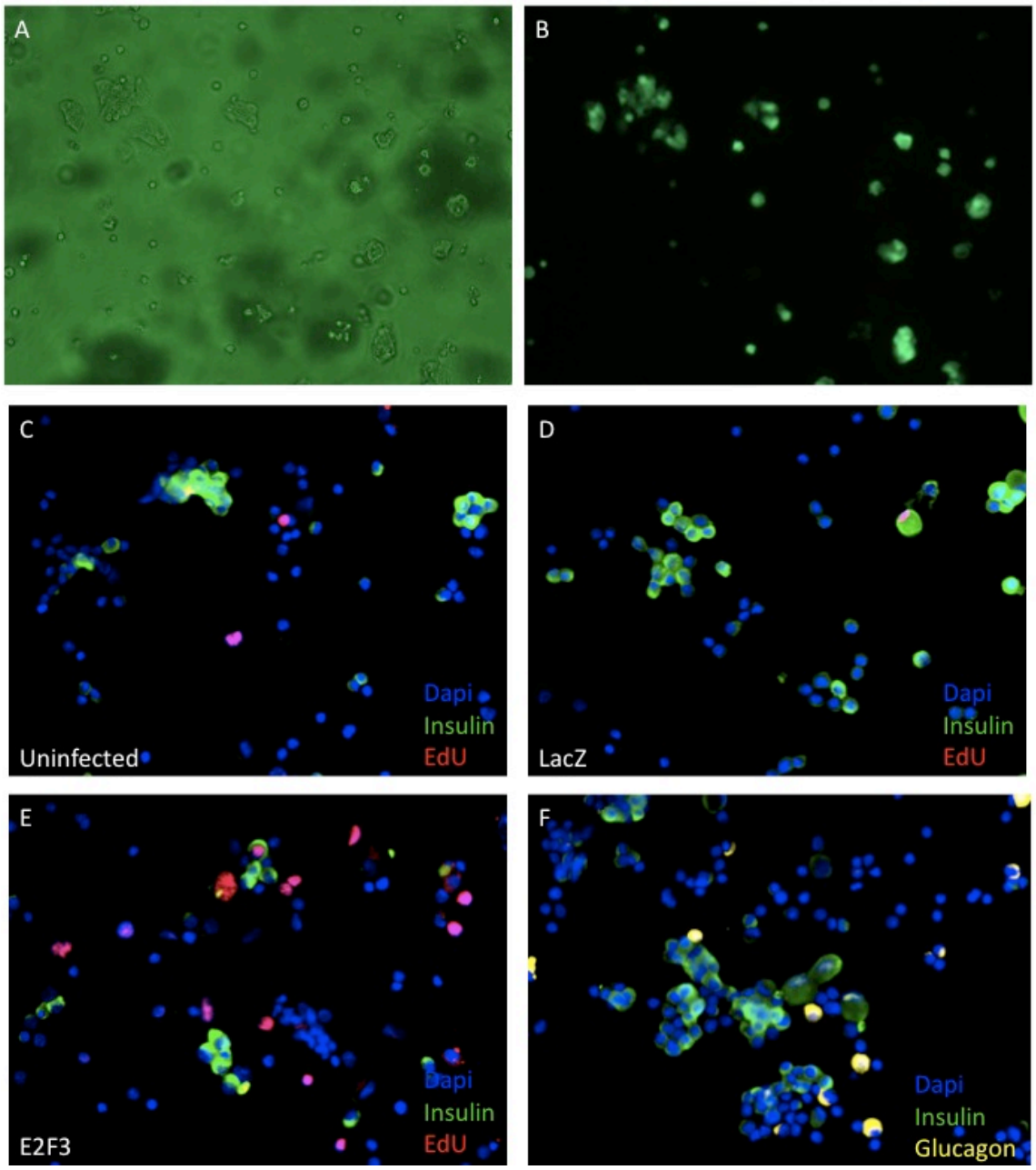


**Figure 11 - Dissociation, and infection of human islets.** Sample GFP (**a**) and bright field images (**b**) of islets that have been dissociated and infected with GFP tagged ad-E2F3. Induction of proliferation in uninfected (MOI 0) (**c**), control virus ad-LacZ (MOI 100) (**d**) and *ad-E2F MOI 100* (**e**). (**f**) Immunohistochemistry for glucagon (yellow), insulin (green), and DNA (blue) in dissociated islets.

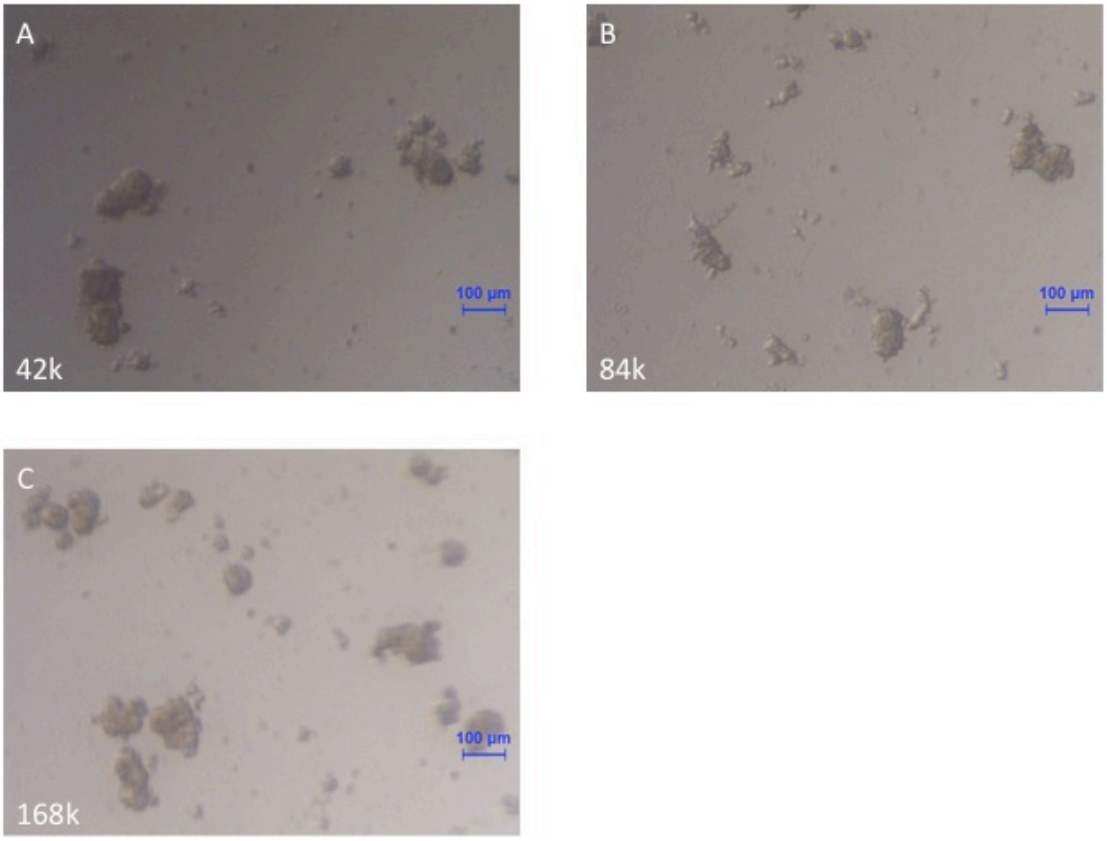


**Figure 12 - Re-aggregation of dissociated islet cells.** Islets were dissociated and then re-aggregated in low attachment dishes for 4 days at 3 seeding densities: (**a**) 42,000 cells, (**b**) 84,000 cells, and (**c**) 168,000 cells.

In order to extend the culture time of infected islets and prolong the proliferative period we infected islets with a genome-incorporating lentivirus to produce cells permanently transuded with E2F3. These viruses contained a doxycycline dependent promoter controlling expression of E2F3. Upon infection with a dox-on lenti-dox-E2F3 at a MOI of 50, in the presence of doxycycline, an increase in E2F3 expression was visible by western blot after 3 days (Fig. 12A). Quantification of the expression revealed an almost 4-fold increase in E2F3 (Fig. 12B). However, infection of RIP-dGFP virus produced no visible GFP expression (Fig. 12 C and D) raising doubts about the viral packaging process.

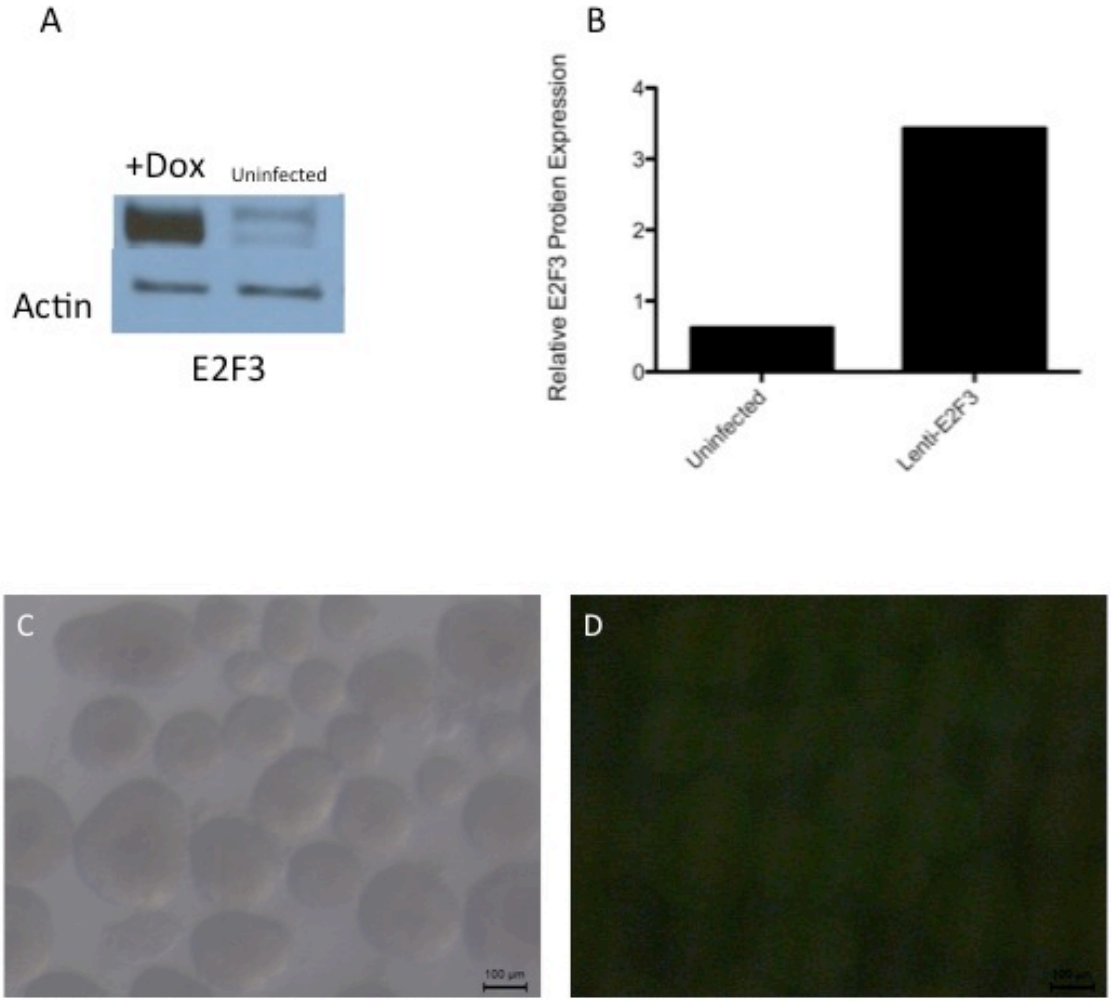


**Figure 13 - Lentivirus function.** (**a**) Western blot for human islets infected with lent-dox-E2F3 in the presence of doxycycline for 3 days. (**b**) Quantification of western blot for E2F3-infected human islets, normalized to beta-actin. Bright field (**c**) and GFP (**d**) in lenti-RIP-GFP infected (MOI 50) rat islets.

When human islets were cultured with the E2F3 lentivirus at MOIs of 1, 50 and 100, then assayed for EdU, no proliferation was seen in any of the conditions (Fig. 13 A and B). To verify the expression of E2F3 in infected cells, we fluorescently probed for E2F3 expression in both lenti-E2F3 human islets and rat islets infected with ad-E2F3 as a positive control. Cytoplasmic and nuclear expression of E2F3 was readily visible in the rat ad-E2F3 islets (Fig 13 C). However, in lenti-dox-E2F3 infected cells, while there is cytoplasmic expression of E2F3, there are fewer nuclei containing E2F3, showing a significantly decreased level of expression and infection using lenti-E2F3. (Fig 13D).

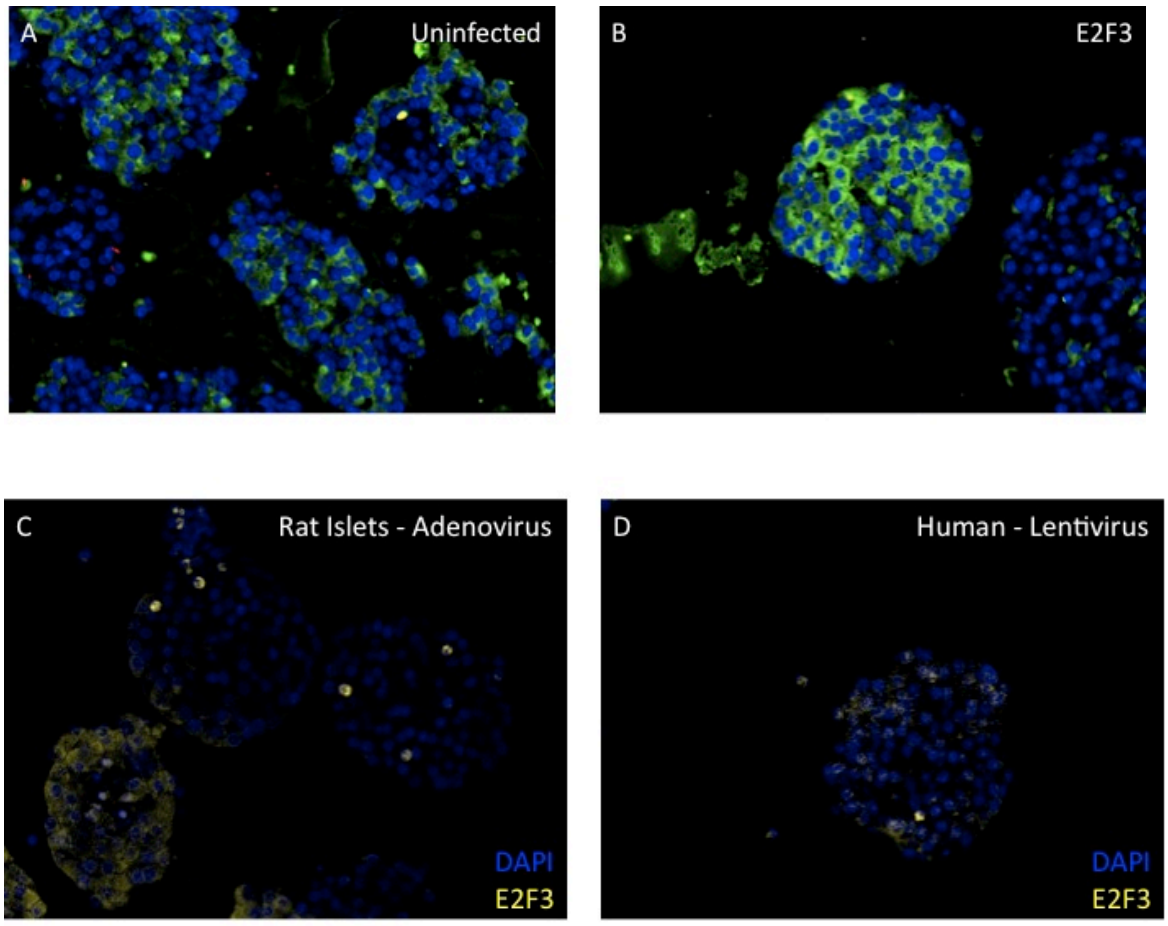


**Figure. 14 – Lentivirus E2F3 induced proliferation and infection.** Representative images from three human islet isolations infected with no virus (**a**) or lenti-dox-E2F3 (**b**) and stained for EdU (red), insulin (green), and DNA (blue). Infection of rat islets with ad-E2F3 (**c**) was compared to infection of human islets with lenti-dox-E2F3 (**d**) for expression of E2F3 (yellow).

Role of E2F1 and Kir6.2 in the development of diet-induced diabetes

We hypothesized that development of type-2 diabetes is influenced by hyperglycemia due to systemic insulin resistance which increases E2F1 expression in beta-cells leading to Kir6.2 overexpression and ultimately impaired insulin secretion. If this were true, we reasoned that hyperglycemia would be correlated to BMI and patients with high BMI would also have high E2F1 expression. In 7 human islets isolations, a positive correlation between E2F1 expression and BMI was discovered (Fig 15A). 53% of the variation in E2F1 expression is explained by BMI, $R^2$=0.53, so while BMI is not the sole determinant in E2F1 expression it is seemingly a contributing factor. If E2F1 expression is related to BMI, we wanted to verify that increased levels of E2F1 led to increased levels of Kir6.2. We infected rat islets with E2F1 adenovirus and cultured them for 3 days, and then collect RNA for PCR. RT-PCR on day 3 revealed a 2.7-fold increase in Kir6.2 RNA upon activation of E2F1 (Fig. 15B).

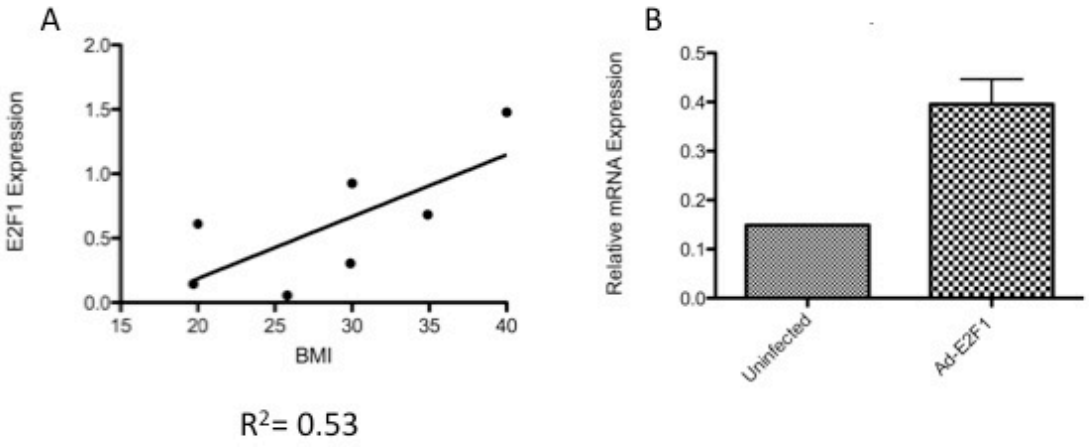


**Figure 15 - E2F1 expression, BMI and Kir6.2.** (**a**) Whole islet protein from 7 human pancreases probed for E2F1 expression and correlated to to BMI of the donor, $R^2$=0.53 (**b**) RT-PCR for Kir6.2 in rat islets infected with ad-E2F1.

Next, we decided to see if E2F1 knockout mice were less susceptible to development of diet-induced diabetes. E2F1 knockout mice fed a 60% fat diet did not gain weight vs. the same knockout mice fed a regular diet. However, control wildtype mice on the same high fat diet did gain weight (Fig. 16A). While blood glucose was variable over the 60-day period, changes in non-fasting glucose levels were not observed in any of the groups (Fig. 16B). IPGTTs on day 0 revealed the ability to clear glucose in all groups (Fig 16C). However, by day 60 the ability of the control mice fed a high fat diet to clear glucose was significantly impaired (Fig 16D). These mice were so impaired that they died following the IPGTT despite multiple insulin injections. This suggested that systemic glucose metabolism is impaired due to decreased sensitivity to insulin, as insulin elicited a muted response. Both high fat and regular diet E2F1 knockout mice were able to clear glucose on day 60 similarly as they were on day 0 (Fig. 16D). Insulin taken during the IPGTT revealed a low level of insulin secretion in E2F1 KO mice in both conditions compared to control mice, furthering the idea that these mice are unaffected by high fat diets due to altered metabolic capacity rather than retaining a normal beta-cell function under the high fat diet (Fig 16E).

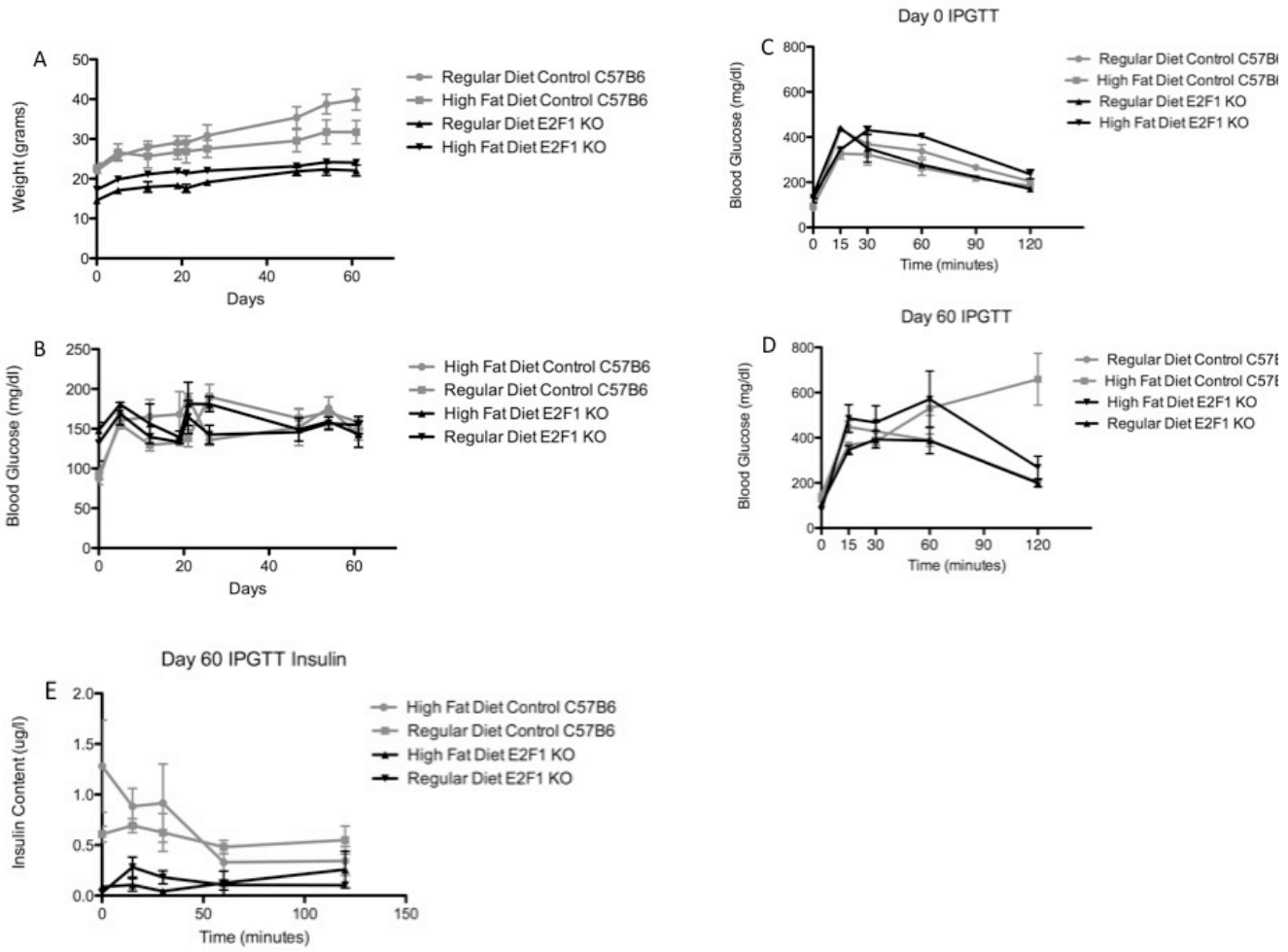


**Figure 16 - *In vivo* data and IPGTT for E2F1 knockout mice.** Weight (**a**) and blood glucose (**b**) measured over 60 days for E2F1 knockout and C57B6 mice fed high fat (60% fat) and regular diet (**c**) IPGTT for all mice on the day (day-0) high fat and regular diet began. (**d**) IPGTT for E2F1 knockout and control mice following 60 days of high fat or regular diet. (**e**) Insulin secretion during 60-day IPGTT. n=3

The decreased insulin secretion found in the E2F1 knockout mice led us to hypothesize that the protective effect of E2F1 knockout was not due to changes in beta-cell insulin secretion dynamics, but to changes in metabolism or hypersensitivity to insulin. To further investigate the function of the islets in all conditions we isolated islets from these mice as described above and performed a microfluidic assay testing intracellular calcium flux, mitochondrial membrane potential and performed electrophysiology on resting membrane potential and $K_{ATP}$ channels. During the isolations it was noted that the E2F1 knockout mice had significantly smaller pancreases than control mice and similarly fewer islets were isolated from these mice, as has been reported previously [81]. Calcium flux assays revealed no difference between the groups when exposed to 10mM and 14mM glucose (Fig 17A). Insulin collection during this assay revealed a decreased insulin secretion in E2F KO mice isolated islets, similar to what was seen *in vivo* at the 60-day IPGTT (Fig. 17B). Mitochondrial potential decreased in all islets in response to both 10mM and 14mM glucose, indicating ATP production via glycolysis (Fig 17C). Proper function of $K_{ATP}$ channels was established by stimulation with the potassium channel closer Tolbutamide and opener Diazoxide. All conditions displayed normal calcium flux (Fig. 17D) and no change in mitochondrial potential, (Fig. 17E) as would be expected since the ATP-dependent potassium channels were closed by Tolbutamide rather than ATP synthesis. In E2F1 knockout mice fed the high fat diet had noticeably greasy fur compared to high fat diet control mice (Fig. 18).

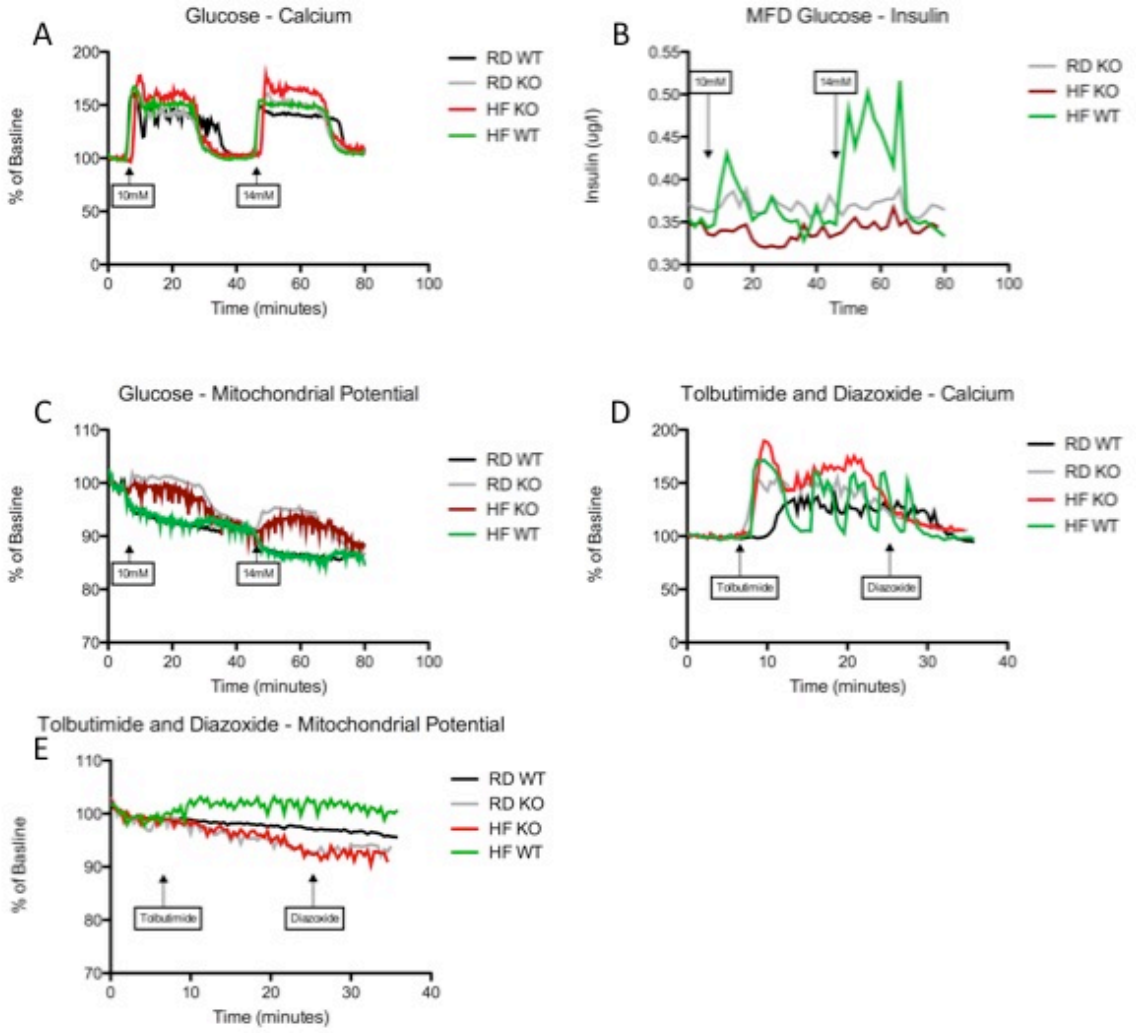


**Figure 17 - Microfluidic assessment of wild-type and E2F1 knockout islets.** Islets from all conditions were assayed for calcium flux (**a**) by exposure to 10mM then 14mM glucose and the resulting insulin secretion (**b**) measured by ELISA. Changes in mitochondrial potential due to 10mM and 14mM glucose (**c**). Function of $K_{ATP}$ channels was assessed by exposure of isolated islets from all conditions to tolbutimide and diazoxide for calcium flux (**d**) and mitochondrial potential (**e**).

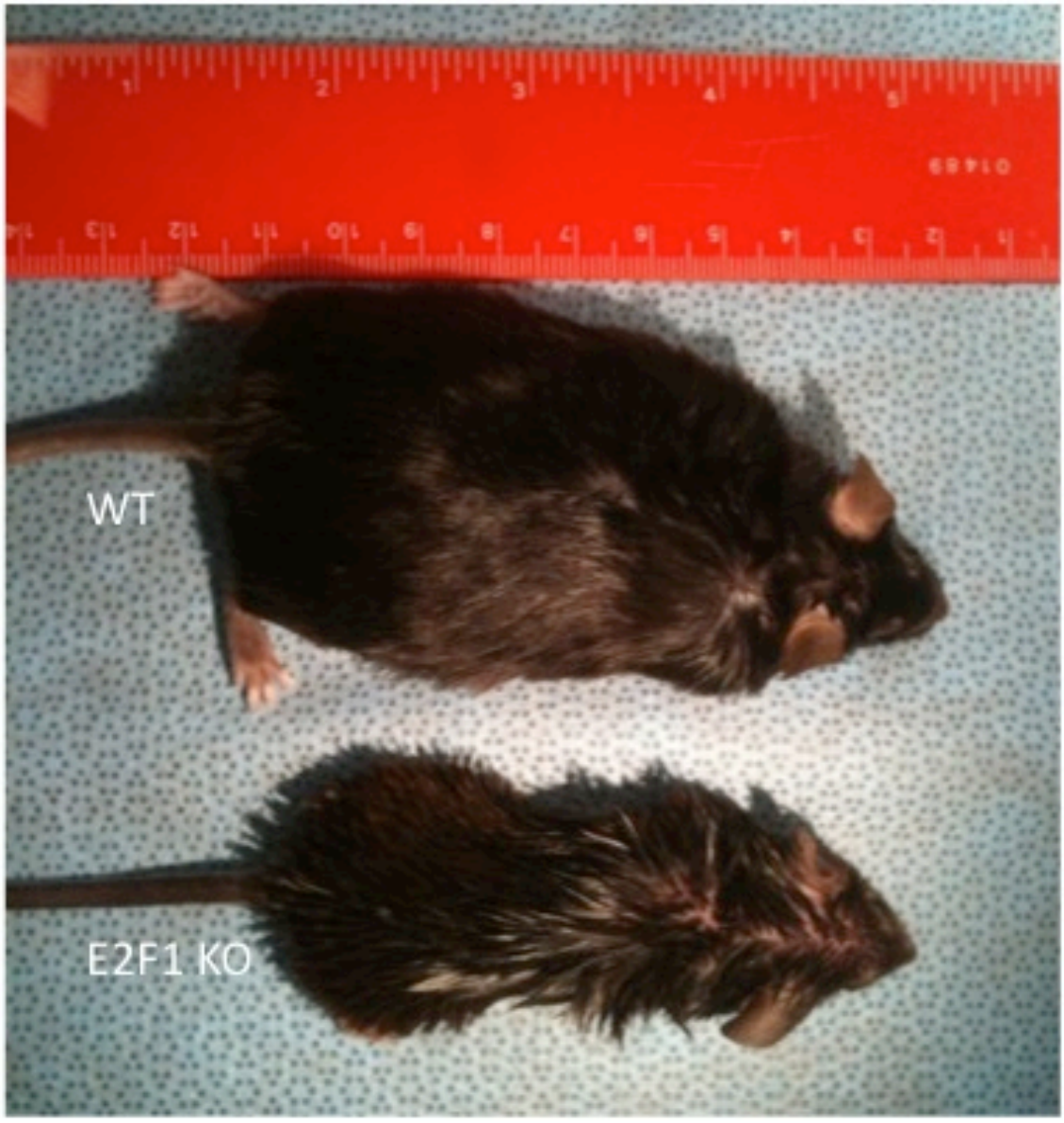


**Figure 18 - Image of WT and E2F1 KO mice after 60 days of high fat diet.** WT mice (top) and E2F1 KO mice (bottom) showing the difference in size and greasy coating of the fur in E2F1 KO mice.

Islets from all conditions of mice were then assayed for plasma membrane action potentials to see if the resistance to high fat diet-induced diabetes came about because of the E2F1 knockout decreasing expression of Kir6.2, which would normally be increased by the hyperglycemia of a high fat-diet. Figure 19 shows stimulation of action potentials in beta-cells by two glucose concentrations, 10mM and 14mM, in both wild-type and E2F1 knockout mice fed the regular diet for 60 days (Fig 19 A-D). Resting membrane potential for WT mice at both glucose concentrations was -68mV+/-3.1 (10mM glucose) and -70.8mV+/-1.2 (14mM glucose) and for KO mice -66.67mV+/-2.9 (10mM glucose) and -67.27mV+/-0.82 (14mM glucose), showing no significant effect of E2F1 knockout on resting membrane potential, p=ns (Fig 19 E). Maximum depolarization potential (MDP), the voltage at which the cell depolarizes, for wild type and E2F1 knockout mice at both glucose concentrations were -35mV +/- 2.7 for WT at 10mM, -45.3mV +/-3.7 for WT at 14mM, -46.33mV +/-3.3 for KO at 10mM, and -39.5mV +/-0.5 for KO at 14mM, which shows no significant difference in maximum depolarization potential p=ns (Fig 19 F). The time to MDP (t-rise) for WT and KO mice was 4 minutes +/- 1.1 for WT at 10mM, 3.4 minutes +/- 0.58 for WT at 14mM, 8.1 minutes +/-0.4 for KO at 10mM, and 7.9 minutes +/-0.81 for KO at 14mM, representing a significant, p=0.0019, delay in the time to maximum depolarization (Fig 19 G). Finally, amplitude and frequency of the spikes resulting from oscillations of calcium flux typical of an action potential were measured. For WT mice, the amplitudes were 22.3mV +/-3.9 at 10mM glucose and 45mV +/-3.6 at 14mM and the frequency of the oscillations was 4.477Hz +/- 4.477 at 10mM and 2.513Hz +/- 0.7589 at 14mM (Fig

19 H and I). The spike amplitudes between cells stimulated with 10mM and 14mM glucose are significantly different for WT mice, p=0.008, while the frequency of the oscillations is not, p=0.2053. For E2F1 knockout mice, the spike amplitude appears to be much lower when stimulated. At 10mM glucose, oscillatory spikes are not above background noise and were not measurable. For 14mM glucose, the spike amplitude was 27.33 +/- 8.19 and the frequency was 3.044 +/-1.18. The frequency of the calcium oscillations is similar to WT, p=0.416, but the amplitude of the spikes is significantly lower in E2F1 knockout islets when stimulated with 14mM glucose, p=0.008 (Fig 19 H and I).

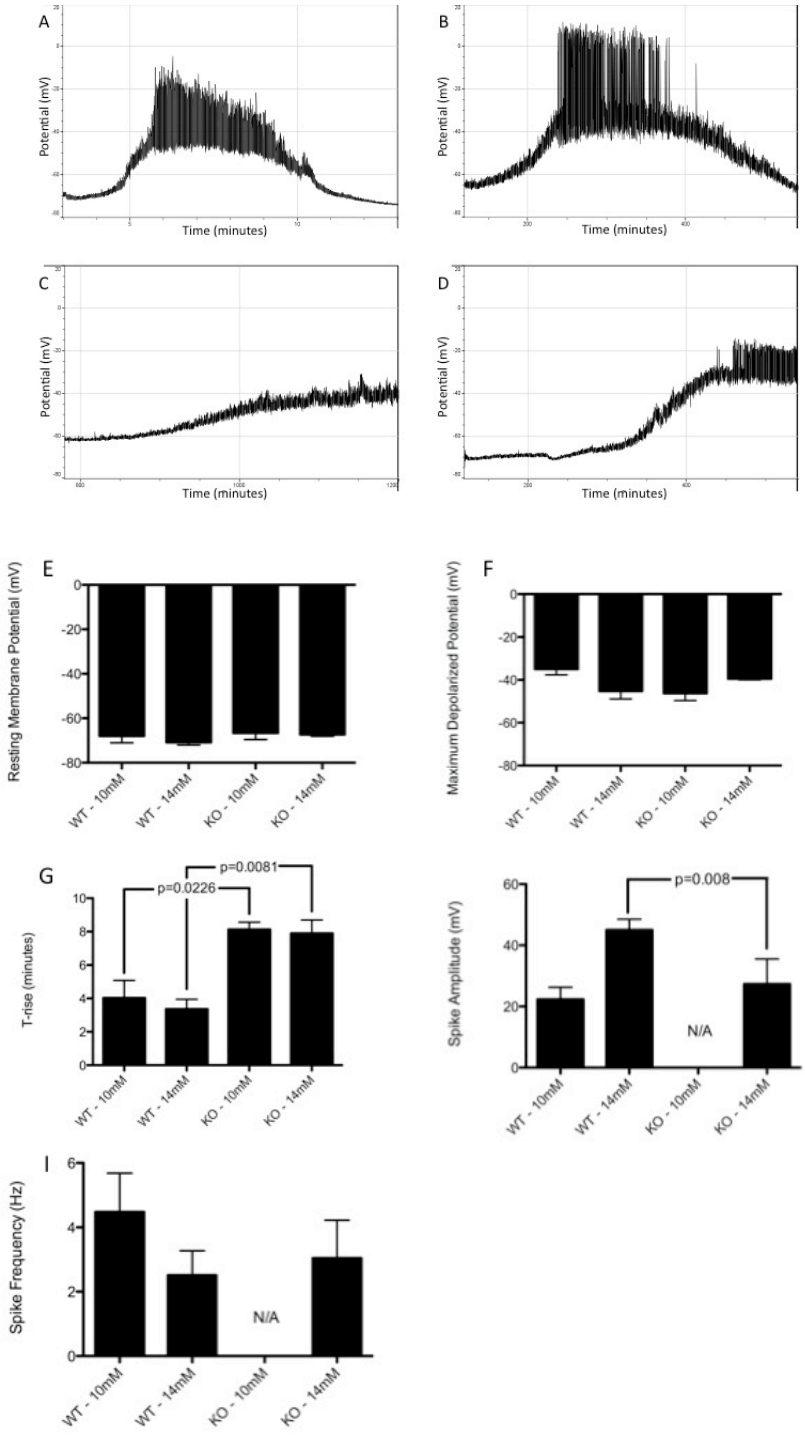


**Figure 19 - Electrophysiology of wild-type and E2F1 knockout mice.** Measurements of resting membrane potential and action potentials by exposure of islets to 10mM (**a** and **c**) and 14mM glucose (**b** and **d**). Quantification of electrophysiology results, significant p-values shown in graphs: resting membrane potential (**e**), maximum depolarized potential (**f**), t-rise (time to MDP) (**g**), spike amplitude (**h**), and spike frequency (**i**).

We hypothesized that the time to depolarization and a shift in glucose sensitivity, such that 10mM glucose did not produce measurable calcium oscillations, but 14mM did, was a result of reduced Kir6.2 expression because of the E2F1 knockout. Therefore, we believed $K_{ATP}$ channel function was impaired. To test this, we performed voltage-clamp electrophysiology on $K_{ATP}$ channels to measure current through $K_{ATP}$ channels in resting (0mM glucose) and stimulated (14mM glucose) conditions. Figure 20 shows $K_{ATP}$ current (normalized to capacitance, pA/pF) of WT and KO islets in their un-stimulated state (0mM glucose) (Fig 20 *A* and *C*) and stimulated (14mM) (Fig 20 *B* and *D*). Upon the addition of 14mM glucose, wild type cells show a decrease in $K_{ATP}$ current, as would be expected as $K_{ATP}$ channels close in response to glucose stimulation, p=0.0379. However, though there is an apparent decrease in $K_{ATP}$ current with the addition of 14mM glucose in E2F1 knockout cells, this observation is not statistically significant, p=0.1559. This suggests E2F1 knockout beta-cells have an impaired ability to close Kir6.2 channels in response to glucose, eliminating the hypothesis that E2F1 knockout confers an enhanced beta-cell function. Though it does not address this under high fat diet, where we hypothesized you would see a difference.

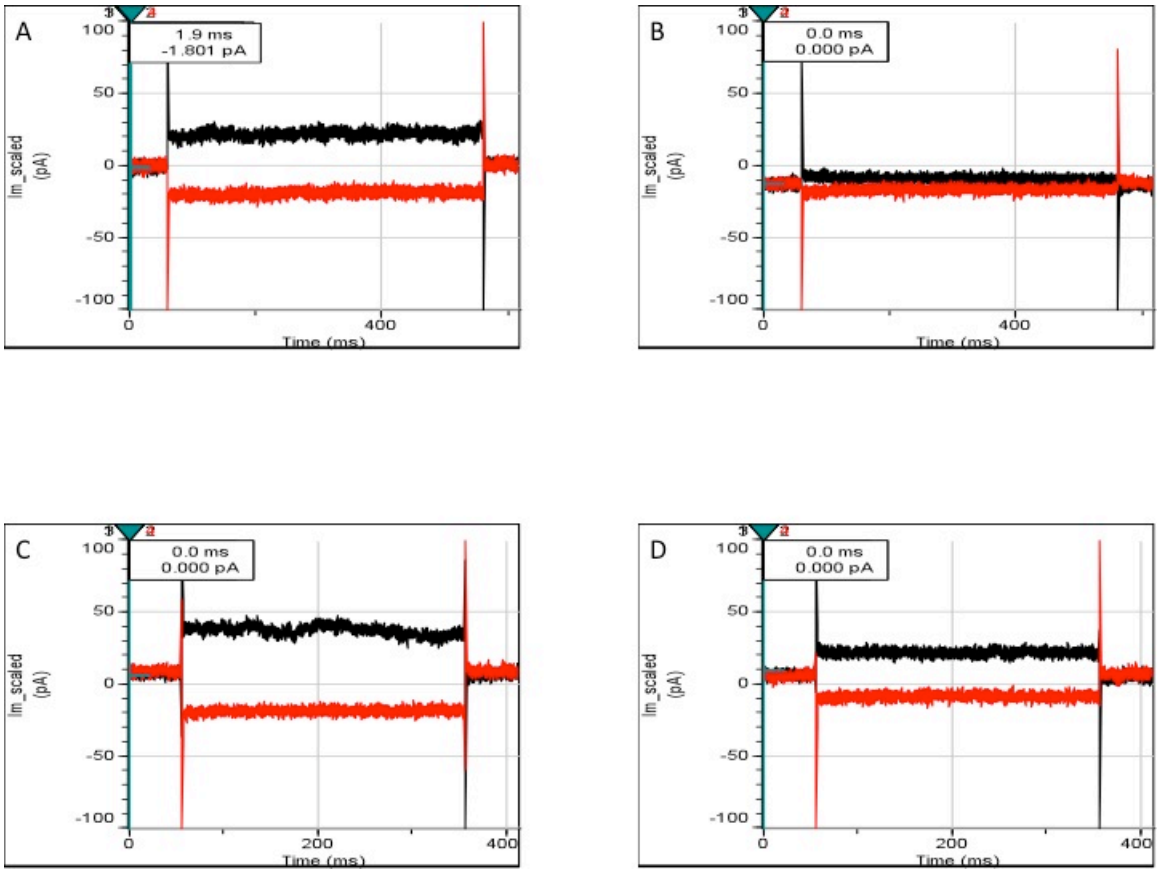


**Figure 20 - $K_{ATP}$ channel currents in WT and KO beta-cells.** Current through potassium channels of WT cells stimulated with 0mM and 14mM glucose, and E2F1 KO cells stimulated with

## DISCUSSION

*Overexpression of E2F3 promotes proliferation of functional human beta-cells without induction of apoptosis*

We report several new observations of human beta-cell proliferation. First, we show that wild type islets contain a high nuclear amount of repressive E2Fs. Second, we show that proliferative human insulinomas have no expression of these repressive E2Fs, but high amounts of the proliferation-inducing E2F3. Third, when E2F3 is overexpressed in wild type rat and human islets it induces significant beta-cell proliferation. Lastly, we show that this expansion does not impair the beta-cell phenotype and the proliferation occurs without an increase in apoptosis. These observations add to the growing picture of the human beta-cell cycle both *in vitro* for cell replacement therapies and *in vivo* for regeneration of islets.

Replication of adult human beta-cells is a rare event, with multiple mechanisms limiting proliferation and maintaining quiescence. Our observation of high expression of E2F4-6 in the nucleus of wild type islets could represent one such mechanism. During $G_0$ of the cell cycle, E2F4/5-p107/130 complexes and pocket protein-independent E2F6 occupy the promoter region of genes responsible for controlling DNA synthesis, inhibiting their transcription and preventing transition from $G_1$ to S-phase[86, 93, 95, 96, 99]. With the expression of these genes repressed, G1 and S progression is impossible. E2F4 and 5 lack a nuclear location signal and are therefore sequestered in the cytoplasm unless bound to a pocket protein[95, 96]. In our immunoblots, we see a high expression of E2F4/5 in whole cell protein extracts. This cytoplasmic E2F4/5 is likely not contributing to repression of cell cycle genes

since its subcellular location implies it is free E2F rather than complexed to a pocket protein. This implies the nuclear expression of E2F4 and 5 we see is p107/130 complexed and functionally repressing E2F1-3 target genes. In addition to the nuclear E2F4, we also detected increased levels of E2F6. E2F6 has been reported as a main E2F that maintains quiescence in cells [99]. The overlap in function with E2F4/5 is likely additive and dependent on cell type and the cellular environment that leads to G0. In our human islet preparations, we find a combination of these E2Fs maintaining quiescence. These strong inhibitory signals may explain why extra-cellular growth factors often fail to induce high levels of beta-cell proliferation (typically less than a fraction of a percent)[114-116]. Previous reports have shown contradicting endogenous nuclear expression of E2F1, 3 and 4, in human beta-cells, which we could not reproduce this in the fresh human islet isolations we analyzed[67].

Debate over whether proliferation or differentiation of new beta-cells maintains the adult beta-cell mass is ongoing[62-64]. In reality, a combination of these two pathways likely maintains and adapts the adult pancreas to increased biological demand. Insulinomas show that cell cycle mutations are capable of proliferating insulin-producing beta-cells and could provide a model for their expansion for research and cell therapies. Rodent insulinomas have been used to reveal D cyclins and cdks, upstream effectors of E2F, as potent inducers of proliferation[67, 117]. For the first time, we describe high levels of pro-proliferative E2F3 and no repressive E2F4-6 expression in human insulinomas. Since E2F lies downstream in the $G_1$/S checkpoint, mutations of the many upstream regulatory

genes can profoundly effect it, resulting in aberrant expression in almost all forms of cancer[118]. E2F3 in particular is highly expressed in lung, bladder and prostate cancers[119-121]. Additionally, E2F3 is required for normal proliferation and is rate limiting in the proliferation of tumor cell lines[108]. Surprisingly, though E2F1 and 3 are necessary to start quiescent cells cycling, only E2F3 is needed for subsequent rounds of division[80]. This may explain why both E2F1 and 3 were expressed in our insulinomas, with E2F3 being the more prevalent one. Given this finding, overexpression of E2F1 and 3 may prove to be a potent "one-two punch" to bring beta-cells out of $G_0$ into $G_1$/S. E2F1-3 are maximally expressed during $G_1$/S and minimally expressed during $G_0$, while E2F4-6 remains relatively constant[89]. The expression of anti-proliferative E2F4-6 was not detectable in these insulinomas, indicating that they had both a loss of the anti-proliferative E2F expression (the brakes of the cell cycle) and overexpression of proliferative E2Fs (driving the cell cycle). This implies a decrease in E2F4-6 is not required for E2F1-3 to drive proliferation as pro-proliferative E2Fs are able to displace the occupying E2F4-6[86]. In fact, E2F1-3 are able to override inhibitory signals when their expression is increased [122]. A decrease in expression of E2F4-8 is not required for E2F1-3 to drive proliferation as they are able is displace the occupying E2F. A reduction of E2F4-6 does not appear necessary to induce mitosis, but could further enhance E2F3 induced proliferation.

Insulinomas represent a sort of positive control for proliferation of adult beta-cells. These cells readily proliferate, secrete insulin, and do not lose function over time. For those who question the plausibility of adult beta-cell proliferation as

a means to generate cells for transplant, these cells prove the principle is sound. There is a manipulation of the cell cycle genes that will allow beta-cells to readily proliferate and retain their function, we just need to find it. This is almost exactly the type of cell that we hope to be able to create *in vitro*, with the addition of the ability to control expression of the genes driving proliferation and increasing the glucose-sensitive insulin response.

Extra-cellular signals of proliferation that have dramatic effects in other cell types often do not elicit a similar response in human beta-cells[114-116]. In contrast, efforts to manipulate genes controlling the G1/S checkpoint have led to robust proliferation of insulin positive cells[67-70]. These signals likely activate DNA synthesis and proliferation through E2F, since they are upstream effectors of E2F[111]. E2F expression has been shown to control proliferation through activation or repression of genes involved in DNA synthesis[83, 87, 122]. However, we do not uncover specific E2F3 target genes involved in either maintaining quiescence or inducing proliferation. Our finding that E2F3 drives proliferation in islets is previously unreported, but recently it has been shown that E2F1 overexpression is capable of inducing proliferation and considerable apoptosis in mouse and human beta-cells[70]. In contrast, here we show that overexpression of E2F3 induces significant proliferation without apoptosis. Induction of apoptosis may be unique to E2F1 as its target genes include *p53*, *p73*, several caspases and *APAF1*, while E2F3 is not an activator of these genes[85, 123]. Similarly, E2F3 is able to positively regulate a unique set of pro-proliferative target genes, such as DNA polymerase A, *DHFR*, and *cdc6* (among others), while E2F1 is not[107, 108].

The detection of replication competent viruses is essential for experiments addressing proliferation. Since one of the assays for this is the incorporation of labeled nucleosides during DNA replication, this technique would not distinguish between viral DNA synthesis and DNA synthesis associated with normal mitotic division of a cell. If replication competent viruses were to infect a cell, it would stain positive for EdU, giving an inaccurate view of proliferation in the cells. This is additionally important with adenoviruses for E2F. Since E2F is a transcription factor involved in both mitotic and viral DNA synthesis, special care must be taken. Typically, adenoviruses are rendered replication incompetent through deletion of the E1 and E3 genes. However, E2F is unique in that it is an E2 activating transcription factor [78]. This means the potential to bypass one of the genetic safeguards of adenoviruses by activation of E2, despite E1 deletion, exists. We are confident that our adenoviruses are not replication competent because of our own plaque forming assays and the assays performed by Vector Biolabs after packaging and expansion of the viruses. Our adenoviruses are able to infect whole islets with great efficiency. Our infection of whole islets results in an infection depth of 1-5 cell layers, or about 25-30% of the islet, similar to what others have seen [124]. In the bright field images of our infected islets, a ring of apparently dead cells is present. While there are similar dead cells in the uninfected islets, they are significantly less. These cells are normally washed away during the subsequent fixation and embedding process, so they do not appear on fluorescently stained images. However, we do suspect that this mild increase in dead cells is related to our viral infections. Since we only infect the outer layers of whole islets, it is reasonable to

assume that a gradient exists with the outer cells being most infected and inners cells exposed to less viral particles. Additionally, since the MOI is calculated for a whole islet assuming all of the cells are infected, the actual MOI of all infected cells is most likely higher than described. This increased effective MOI and the gradient due to the penetration of whole islets, may lead to increased cell death on the outer mantle of the islet. Despite this, we still see robust proliferation and function of these infected islets.

Whereas many attempts to proliferate beta-cells result in the loss of glucose-sensitive insulin section and the beta-cell phenotype, E2F3 proliferated islets retained these abilities[113]. These cells displayed unimpaired intracellular calcium levels, mitochondrial potential and dynamic insulin secretion, confirming the presence and integrity of glucose-stimulated insulin secretion coupling by the presence of functional $K_{ATP}$ and voltage-gated calcium channels. The Cdk4-E2F1 pathway regulates the expression of *Pdx1* and *Ngn3*, two genes expressed in the developing pancreas[125]. Additionally, E2F1 was recently found to regulate expression of the potassium channel subunit *Kir6.2*[91]. Despite significant homology between E2F1 and E2F3, it is not know if E2F3 also regulates *Kir6.2* expression and the secretion of insulin, though our results imply that the pathway allowing for insulin secretion remain intact. Maintenance of *Pdx1* and *Kir6.2* expression could be one mechanism by which E2F3-proliferated islets retain their function during culture. These features make E2F3 a favorable target for increasing human beta-cell mass and lend to its ability to induce proliferation independent of apoptosis while retaining the beta-cell phenotype.

E2F3-proliferated rat islets showed robust function both *in vitro* and *in vivo* through functional increases in beta-cell numbers. In humans, islets infected with E2F3 also showed normal function and apparently retained the beta-cell phenotype *in vitro*, but did not increase the beta-cell mass sufficiently to improve transplant outcomes. One explanation for this is the difference in E2F3-induced proliferation rates between rats (23.8%) and humans (7.2%). The disparity in knowledge between the rodent and human beta-cell cycle has been emphasized recently for this exact reason[111]. Despite this, the overall function of the islets was not seemingly impaired by ectopic delivery of E2F3. We propose that either improving the infection efficiency or concomitant E2F4-6 knockout could increase E2F3-induced proliferation sufficiently to functionally impact islet grafts and reduce the islets required to reverse diabetes in this model. Future strategies for increasing the infection efficiency could include dissociation of islets, infection and expansion, then re-aggregation before transplantation or the use of an alternative, non-viral DNA delivery system, such as gold nanoparticles, that infect the whole islets rather than merely the mantle[126, 127].

Of note for studies using adenoviruses to induce beta-cell proliferation is the finding of the "adenovirus effect". Infection of human islets with adenovirus increases proliferation of beta-cells, simply as a results of viral infection [112]. When freshly isolated human islets are infected with adenovirus type-5 containing a RIP–GFP or Luciferase a small, but statistically significant increase in BrdU incorporation was seen. The exact mechanism of how this happens is not well understood, but an increase in Akt1 and Cyclin D3 has been observed in infected

islets [112]. We see a small adenovirus effect in our human islets between uninfected (0.5208%) and Ad-LacZ infected (1.504%) with a p-value of 0.0417. Though it is just barely significant, it is worth noting as it has been previously described [112].

Once the infected islets had been transplanted into nude mice, they were able to reverse diabetes. This was most likely due to an increased beta-cell mass from *in vitro* and early *in vivo* expansion, since no EdU positive cells were found at the end of the *in vivo* period. While there was no detectable EdU incorporation during days 18-21, there may still have been proliferation in the graft earlier *in vivo*. The lack of proliferation after longer than 2 weeks of culture (*in vivo* or *in vitro*) is not surprising as the adenovirus transgene expression typically begins to decrease after a week and normally is back to baseline expression by week 2. Though it has been shown that islets infected with Cdk6 and cyclin D1 can be cultured and divide in vivo, however this was within 2 days after transplant [67]. Expansion of islets in vivo has been used by others as a means of culture, notably the human beta-cell line endoC-βH1 [72]. However, the transplantation of immature cells into mice and the retrieval of mature cells after several months represents a sort of 'black box'. This blind *in vivo* differentiation is difficult to study and while this is an interesting mechanism for culture, it neither provides a product that would be of direct benefit to human diabetics or furthers the understanding of the processes it have occurred during the transplantation time. It is possible that these cells could be used to understand the differentiation of a beta-cells or the progress of proliferation without the loss of the beta-cell phenotype. However, more often than not this 'back

box' is used when all other currently understood methods of proliferation or differentiation have been used and failed to deliver the desired outcome. It is a last resort to obtain functional beta-cells by any means necessary, even at the expense of understanding the process by which you got there. It can be used to generate cells for further study, but should be avoided as part of a protocol to generate beta-cells for transplantation.

With the success of recent attempts to drive proliferation of adult beta-cells at increasingly high rates, it is worth exploring the potential that current proliferation efforts have on impacting islet transplant. Though infection of whole islets is limited to only about 30% of the islet, it is possible to increase the functional beta-cell mass. The recent success with proliferation of adult beta-cells using E2F1 was not in whole islets, but rather dissociated islets [70]. This has resulted in E2F1/Akt inducing about 25% beta-cell proliferation. When compared with proliferation rates in whole islets infected with adenovirus, E2F3 resulting in 7% proliferation and cyclin D1/Cdk6 inducing about 12% proliferation, these results seem comparable when infection efficiency is taken into account. Using any of these methods, it would be possible to increase the transplantable mass of a human islet isolation by about 12%. If a typical islet isolation transplants 400k islets, then this would increase the transplantable mass by 48k islets. When islet transplant recipients often require multiple transplants in order to become normalglycemic, 48k additional islets may provide an actual benefit. However, this is not a solution to the disparity between the number of organ donors and the number of diabetics who would require this treatment. If the current rates of proliferation do not greatly

benefit graft enhancement, maybe a longer term culture could provide meaningful proliferation. If instead of transplanting 400K islets they were instead infected with a cell cycle gene and induced to proliferate at a rate of 12% every 48 hours, a low estimate, you would end up with a little over 600k islets after a week. This assume no loss in culture, which is not a reasonable assumption, and that beta-cells are able to continually divide without a refractory period [62, 63]. Though transplantation of 600k islets would be of significant benefit compared to 400k, the process by which you would arrive at that number is unreasonable. For proliferation to have a meaningful impact on graft expansion it would require about a 30% increase in transplantable beta-cell mass (400k to 520k islets). If *in vitro* culture of beta-cells is to provide a sustainable source of transplantable cells, then the culture conditions and de-differentiation of beta-cells needs to be addressed. Currently rates of proliferation do not offer a therapeutic advantage in either enhancement of islet grafts or long-term culture of proliferating cells. In order to reach these levels a new method DNA delivery that can penetrate to the core of the islet (such as gold nanoparticles [128]), culture conditions that significantly minimize cell loss and de-differentiation, and finally development of a DNA delivery and culture system that would be approved by the FDA. Until these issues are addressed, beta-cell proliferation is in the same place it has been for the last two decades, finding ways to increase division of beta-cells by furthering our understanding of the cell cycle.

Manipulation of cell cycle genes has been used to create beta-cell lines in rodents, such as BTC [129]. Since the E2F3 infected islets appeared to have an increased functional mass, but did not proliferate for more than, at most, 2 weeks,

due to temporary transduction with an adenovirus, we sought a means to permanently transduce the cells with E2F3. Our lab and others have been able to reversibly immortalize a variety of primary human cells with SV40 Large T antigen and BMI-1 lentiviral vectors. Additionally, those vectors induced limited proliferation in human beta-cells [130, 131]. Currently, we developed lentiviruses with a dox-dependent promoter controlling expression of our transgene, E2F3. The addition of the dox-switch was twofold: to be able to induce short rounds of proliferation and then end the proliferative signal to give the cells time to rest without expression of E2F3 and to provide a way to control expression of a potentially oncogenic gene *in vivo*. The addition of the rest period was to help prevent any loss of beta-cell phenotype associated with the efforts to induce proliferation [74]. By limiting the duration of the proliferative period, we hoped to minimize the loss of phenotype that often is associated with it. Others have shown robust induction of proliferation and periods of rest in human islets using dox-switchable adenoviruses for cyclin D1 and Cdk6 [132]. Using the time points developed by this group as a starting point, 3-days of growth followed by 7-days of rest, we hope to find the ideal pattern of growth and rest to maximize proliferation and minimize dedifferentiation. Control over expression of the transgene is important for transplantation of these cells too. Since E2F3 overexpression could produce cancerous cells, it is important that we be able to 'turn it off' when proliferation is not required. The addition of the dox-switch also leads to an interesting characteristic of cells that have been infected and transplanted. If the cells were grown *in vitro* to a needed mass for a specific patient, then transplanted,

the patient would ideally become insulin independent. However, islet transplants often require two or three grafts before the patient becomes normal glycemic. Without transduced cells, the patient would need only take the antibiotic Doxycycline for a couple weeks, inducing the expansion of their beta-cell graft. Once the graft reached a size that was able to regulate their blood glucose levels, they could stop taking the drug. Though this is only a hypothetical situation and the requirements to show that this is efficient with the dox-switch genes have not been met, the concept is plausible. Our initial attempt to generate dox-switchable E2F3 lentiviruses resulted in non-functional lentiviruses that we suspect are due to manufacturing error when the viruses were packaged. Across various projects, 6 lentiviruses were purchased from the same company and none have been found to be functional, though all plasmids were fully sequenced before shipment for packaging. This may explain our lack of proliferation in human islets transduced with these viruses. It is also possible that the level of transgene expression with the lentiviruses is too low to induce proliferation through E2F3. Expression of transgenes with adenoviruses is much higher than with lentiviruses and we were already using a high MOI with the adenoviruses. It would be possible to reach this MOI with lentiviruses, but would require almost an entire shipment of virus. It is also possible that the 3 human islet isolations were 'bad' for a number of reasons, the end result being that they are not susceptible to proliferation. Age, BMI, and various factors in the isolation process can all influence the viability and therefore the proliferative capacity of an islet. These lentiviruses will be remade and the experiments repeated with the new virus. If they still prove to be unsuccessful, then

the lentivirus delivery system is just not able to reproduce what we see with adenoviruses, possibly due to the level of expression of the transgene, which in the case of E2F3 is very sensitive to the correct level of expression.

E2Fs role in processes outside of the cell cycle also makes it an attractive target for proliferation of beta-cells. The Cdk4-E2F1 pathway also regulates the expression of PDX1 and Ngn3, two genes expressed in the developing pancreas. [125]. This may help E2F-prolfierated islets retain their phenotype. In addition to its role in proliferation, apoptosis and differentiation, E2F1 was recently found to regulate expression of the potassium channel subunit Kir6.2 [91] Though there is much overlap between E2F1 and E2F3, it is not known if E2F3 also regulates Ki6.2 expression and the secretion of insulin, but with homology between the two proteins, it's not impossible. Loss of expression of Kir6.2 may be one mechanism by which islets dedifferentiate in culture. If E2F expression was able to maintain Kir6.2, it could help prevent the loss of beta-cell phenotype in the presence of proliferative stimuli. This is an area that warrants further investigation.

The idea of the work here was simply to try to explain why human beta-cells are particularly resistant to efforts to proliferate them, compare that to a proliferating beta-cell in an insulinoma and see if we could alter expression of the genes controlling G1/S in an effort to make the non-proliferative human islets proliferate like the insulinoma. In order to continue with the exploration of E2F3 in human islets, it may be necessary to increase the infection efficiency with any type of virus. To accomplish this, dissociation of the islets into single cells would allow access to the cells found beyond just the first 1-5 cell layers and would result in a

greater functional beta-cell mass for transplantation. Similar to what has been shown in rat islets, human islets could be dissociated, infected and then re-aggregated to form functional pseudo-islets containing all endocrine cells normally found in an islet [126]. These pseudo-islets would ideally mimic the cellular composition and structure of an islet. This could be accomplished by culture in ultra-low attachment plates as above or utilizing the hanging drop method. Expansion of hang drop to clinically relevant numbers of islets may prove to be a difficult technical task, but could be accomplished if it were the last barrier between a cell line and being used in transplants. For pilot studies such as this, it is not necessary or ideal to further complicate the search for genes that will drive proliferation. Issues such as this, apoptosis, virus titer, culture conditions etc. are minutia of proliferation that best left to a second phase of optimization after discovery of a gene that will drive proliferation.

Further understanding of the beta-cell cycle is critical for the advancement of islet transplantation and the understanding of *in vivo* development, growth and death of insulin secreting cells. Here, we identify expression of a key G1/S checkpoint regulator, E2F in the wild type islets and human insulinomas. Mimicking this expression in human islets induces proliferation of beta-cells without induction of apoptosis. Furthermore, these proliferated beta-cells retain their phenotype with no impairment of glucose-sensitive insulin secretion. This identifies E2F3 as a novel target to induce expansion of insulin-secreting cells *in vitro* and may one day allow for *in vivo* pancreatic regeneration.

Role of E2F1 and Kir6.2 in the development of diet-induced diabetes

Here we describe several new observations of E2F1's role in development of diet-induced glucose intolerance and beta-cell function. First, we show E2F1 is related to BMI, with increasing BMI resulting in increased level of E2F1 in islets. Next, we support the conclusions found by other groups, that the potassium channel subunit Kir6.2 is an E2F1 target gene. Furthermore, we find E2F1 knockout mice do not gain weight when fed a 60% high fat diet compared to control mice. E2F1 knockout confers resistance to the development of diet-induced glucose intolerance compared to wildtype mice. Characterization of E2F1 knockout mice islets revealed they have normal calcium flux, mitochondrial potential, normal response to tolbutimide and diazoxide, but decreased overall insulin secretion. Further electrophysiological studies revealed no change in resting membrane potential, action potential spike frequency, or maximum depolarization threshold, but an increased T-rise, decreased spike amplitude, and an impaired ability to close $K_{ATP}$ channels. Together, these observations further the understanding of the function of human beta-cells and provide a link between the cell cycle and insulin secretion.

In addition to the proliferative effects of E2F, we also describe a role in the development of diet-induced diabetes. We show a strong correlation between E2F1 and BMI and that increases in E2F1 expression result in an increase in potassium channel subunit Kir6.2 mRNA. We also report the ability of E2F1 knockout mice to retain their ability to clear glucose after 60 days of a high fat diet. We show that while these mice have some characteristics that are typical of the E2F1 knockout phenotype, reduced pancreas size and decreased insulin secretion, we find the mice

to be glucose tolerant [81]. In fact, when exposed to a high fat diet for 60 days, we find these knockout mice retain their glucose tolerance where control mice were unable to regulate their blood glucose. However, this diabetes resistance is likely due to their impaired adipogenesis and a hypersensitivity to insulin, rather than a beneficial effect in the islets due to decreased Kir6.2 expression from the E2F1 knockout [81, 133].

Others have reported that E2F1 knockout results in glucose intolerance. Our results showed normal glucose tolerance at day-0 and an improved glucose tolerance compared to wildtype mice when fed a high-fat diet. It is possible that whatever changes are elicited by the high fat diet are beneficial to the function of E2F1 knockout islets, and the E2F1 knockout mice as a whole, whereas these changes negatively effect wildtype mice. This same group has shown that glucose increases CDK4 and thereby E2F1 and kir6.2 expression. We translate this to a model of type-2 diabetes to show that E2F1 confers the ability to resist glucose intolerance.

Decreased expression of Kir6.2 in mouse islets leads to hyperinsulinemia and a slightly increased ability to clear glucose [134]. We anticipated generating cells phenotypically similar to this by knocking down a gene involved in regulating Kir6.2 expression, E2F1, and feeding mice a high-fat diet. However, Kir6.2 knockout mice, show increased resting membrane potential, no insulin secretion in response to glucose or tolbutimide, but hypersensitivity to insulin results in only mild glucose intolerance suggesting that Kat channels in skeletal muscle may be involved in the action of insulin [135]. This is similar to the phenotype we see in our cells,

decreased insulin secretion, but hypersensitivity to insulin. However, we find a normal resting membrane potential, delayed T-rise and decreased sensitivity to glucose. But we also see $K_{ATP}$ current, so we know that the cells are not 100% deficient in Kir6.2. This may be due to confounding aspects of the E2F1 knockout, rather than just Kir6.2 as well as possible development compensation for the loss of E2F1. When fed a high-fat diet, both knockdown and knockout Kir6.2 mice led to a decreased insulin secretion and glucose intolerance, despite initially having increased insulin secretion and excitability [136]. However, this loss of secretion does not occur unless subjected to the increased dietary stress of a high fat diet, further the idea of specific genetic changes in beta-cells that negatively impact insulin secretion, except in E2F1 knockout islets. Other studies have shown that control mice progress to hypersecretion of insulin on high fat diets [136]. Perhaps, our control mice were unable to be rescued after 60-day IPGTT due to hypersecretion, but desensitivity to insulin. This does not seem to be the case as control mice were still able to regulate blood glucose levels, suggesting proper sensitivity to insulin. Though they may not have developed the desensitivity to insulin that accompanies hyperinsulinemia yet. Even still, compared to E2F1 knockout islets, there was significantly more insulin secretion in the control mice fed high fat diet.

We see conflicting results in both our microfluidic assays of calcium flux and mitochondrial potential and electrophysiology. In the microfluidic assay, while the E2F1 KO islets show a decreased insulin secretion, the calcium flux and changes in mitochondrial potential in response to both 10mM and 14mM glucose are similar to

control islets. Electrophysiology of glucose induced action potentials, revealed a prolonged duration of $K_{ATP}$ channels closing (T-rise), resulting in a significant increase in the time to MDP when stimulated with glucose. Additionally, when stimulated with 10mM of glucose calcium oscillations were not seen in individual beta-cells, whereas it was seen in the microfluidic assay of whole islets. Furthermore, 14mM glucose elicited noticeably smaller spike amplitude in KO islets than WT islets. Still, the microfluidic assay showed no impairment of the KO islets to respond to 14mM glucose. These conflicting results are admittedly perplexing. How does an individual beta-cell not respond to 10mM glucose and barely respond to 14mM glucose, while a whole islet responds with a seemingly normal calcium flux? It is possible that the beta-cells chosen for electrophysiology were in fact not beta-cells. Though the presence of a response to 14mM glucose seems to validate that the cells investigate were beta-cells. Further study is need to determine any faults in either of these methods or if there is a hypothesis that could explain the apparent conflicting results.

For beta-cells, MDP is the point where enough $K_{ATP}$ channels have closed to trap $K^+$ ions in the cytoplasm to trigger voltage gated calcium channels to open and increasing cytoplasmic levels of $Ca^{2+}$. The E2F1 knockout islets may have fewer $K_{ATP}$ channels because of less expression of Kir6.2 due to the E2F1 KO [91]. Because of this, at a given concentration of glucose/ATP it will take longer for the cells to reach the threshold for depolarization and calcium influx. By way of example, suppose an E2F1 knockout cell has 2 $K_{ATP}$ channels while a wildtype cell has 6 channels. For 1 molecule of ATP it is more likely to hit a $K_{ATP}$ channel and close it in the WT than in

the KO simply because of receptor-ligand kinetics. Also suppose that every time ATP hits a $K_{ATP}$ channel, it associates and dissociates causing one $K^+$ ion to stay in the cell due to closing of the $K_{ATP}$ channel. If the cells in our example need 5 $K^+$ ions to trigger volatage-gated calcium channels, the WT will reach 5 much faster than the KO, and have faster T-rise (as we see in our electrophysiology data). Once it reaches that point, calcium begins to enter the cell. The amount of calcium that comes into the cell is proportional to the 'strength' of the signal, i.e. 14mM glucose will produce larger calcium oscillations than 10mM. Once the cells reach the point where calcium begins to enter the cytoplasm, they both contain the same amount of potassium ions (5 in our example). After reaching the MDP, potassium channels remain closed until the voltage gated calcium channels close at the "peak" of the first phase of insulin secretion. The closing of $K_{ATP}$ channels at the MDP helps further depolarize the cell and reach this peak. During this brief time, suppose 1 second in our example, $K^+$ is still being sequestered in the cell. In our model, we find 1 ion per $K_{ATP}$ channel remains in the cytoplasm, 6 more in our WT cell and 2 more in out KO cell. Since calcium influx into the cell is proportional to the amount of $K^+$ ions in the cytoplasm, we would get 11 $Ca^{2+}$ in our WT and 7 $Ca^{2+}$ in our KO. This model may account for the difference in spike amplitude in the electrophysiology data between wild type and knockout cells.

Whole cell ion current is dependent on (1) the number of ion channels (n), (2) the current through individual channels (i), and (3) the probability of an open $K_{ATP}$ channel. The impaired ability to close $K_{ATP}$ channels, is likely what leads to the increase in T-rise times we observed. This makes sense when we look at $K_{ATP}$

current, it is not necessarily that the $K_{ATP}$ channels are slower to close or less sensitive to ATP, but rather that there are fewer $K_{ATP}$ channels in the cell due to the E2F1 KO. This decrease in the number of $K_{ATP}$ channels leads to a longer T-rise (as explained above) and therefore an overall impairment of $K_{ATP}$ channel closure. With the higher $K_{ATP}$ current we observed, we would expect that these cells would take longer to reach the MDP and initiate $Ca^{2+}$ influx (an increase in i in the formula above). This extended T-rise is confirmed by our membrane potential studies.

Closure of $K_{ATP}$ channels is a hallmark of insulin secretion in response to glucose. Here, we measure $K_{ATP}$ current at -80 (influx) and -60 (efflux) mV in the E2F1 knockout islets. We see an impaired ability to decrease the $K_{ATP}$ current with the addition of glucose in E2F1 knockout cells, likely due to their impaired ability close $K_{ATP}$ channels. In the unstimulated case, we see an apparent increase in $K_{ATP}$ channel current versus wildtype cells, though this difference is not statistically significant. One possible explanation for increased unstimulated current, is that with fewer $K_{ATP}$ channels, more potassium ions need to leave the cell in order to maintain the same membrane potential. Since the resting membrane potential between the wildtype and kncokout cells is similar, it is likely that this is maintained in the E2F1 KO cells with an increased K ion flux through fewer $K_{ATP}$ channels, which reveals itself as a high $K_{ATP}$ channel current in our electrophysiology experiments.

The hypothesis of the E2F1 knockout experiments has not been sufficiently addressed by the experiments shown here. The knockout mice have developmental repercussions from the lack of E2F1, which adds numerous variables to the model. Additionally, the assays performed on isolated islets reveal no increase in beta-cell

function, though the data is incomplete. Also, though mice were glucose intolerant by IPGTT, they did not develop increased non-fasting blood glucose levels during the experiment. A more ideal model would be one that develops more overt diabetes during this time purely from their diet, without the need to use transgenic mice. A better system would also include a way to limit the *in vivo* expression of E2F1 only during exposure to high-fat diet, avoiding the confounding developmental variables of E2F1 knockout. The experiments performed here only look at the E2F1 KO phenotype, and don't really address the issue of: will limiting E2F1 expression during bouts of hyperglycemia prevent development of type-2 diabetes by limiting overexpression of Kir6.2? Possible ways to decrease *in vivo* expression of E2F1 *in* vivo include the use of a RIP-E2F1 siRNA vector or the use a small molecule to knockdown the expression of E2F1.

The development of type-2 diabetes is complex process involving the loss of insulin sensitivity in peripheral tissues and often is accompanied by the loss of insulin secretion. This loss of insulin secretion may be related to Kir6.2 expression and therefore E2F1. If this link between the cell cycle and glucose metabolism is further investigated, it may reveal a new model for the development of type-2 diabetes. One in which high blood glucose levels, damage insulin secreting beta-cells by overexression of Kir6.2 through E2F1. Should this model be proven correct, it could provide for new therapeutic targets to not only treat the symptoms of type-2 diabetes, but also prevent the disease from fully developing.

## REFERENCES


1. Westermark, P., A. Andersson, and G.T. Westermark, *Islet Amyloid Polypeptide, Islet Amyloid, and Diabetes Mellitus.* Physiological Reviews. **91**(3): p. 795-826.
2. Hedeskov, C.J., *Mechanism of glucose-induced insulin secretion.* Physiological Reviews, 1980. **60**(2): p. 442-509.
3. Zingman, L.V., et al., *Signaling in Channel/Enzyme Multimers: ATPase Transitions in SUR Module Gate ATP-Sensitive K+ Conductance.* Neuron, 2001. **31**(2): p. 233-245.
4. Huopio, H., et al., *KATP channels and insulin secretion disorders.* American Journal of Physiology - Endocrinology And Metabolism, 2002. **283**(2): p. E207-E216.
5. Miki, T., et al., *Abnormalities of pancreatic islets by targeted expression of a dominant-negative KATP,Äâchannel.* Proceedings of the National Academy of Sciences, 1997. **94**(22): p. 11969-11973.
6. Koster, J.C., et al., *Targeted Overactivity of Beta Cell KATP Channels Induces Profound Neonatal Diabetes.* Cell, 2000. **100**(6): p. 645-654.
7. Gloyn, A.L., et al., *Activating Mutations in the Gene Encoding the ATP-Sensitive Potassium-Channel Subunit Kir6.2 and Permanent Neonatal Diabetes.* New England Journal of Medicine, 2004. **350**(18): p. 1838-1849.
8. Atkinson, M.A. and G.S. Eisenbarth, *Type 1 diabetes: new perspectives on disease pathogenesis and treatment.* The Lancet, 2001. **358**(9277): p. 221-229.
9. Patterson, C.C., et al., *Incidence trends for childhood type 1 diabetes in Europe during 1989-2003 and predicted new cases 2005-20: a multicentre prospective registration study.* The Lancet. **373**(9680): p. 2027-2033.
10. Filippi, C.M. and M.G. von Herrath, *Viral Trigger for Type 1 Diabetes.* Diabetes, 2008. **57**(11): p. 2863-2871.
11. Karjalainen, J., et al., *A Bovine Albumin Peptide as a Possible Trigger of Insulin-Dependent Diabetes Mellitus.* New England Journal of Medicine, 1992. **327**(5): p. 302-307.
12. Bach, J.-F.o., *The Effect of Infections on Susceptibility to Autoimmune and Allergic Diseases.* New England Journal of Medicine, 2002. **347**(12): p. 911-920.
13. Gamble, D.R., et al., *Viral Antibodies in Diabetes Mellitus.* BMJ, 1969. **3**(5671): p. 627-630.
14. Banatvala, J.E., et al., *COXSACKIE B, MUMPS, RUBELLA, AND CYTOMEGALOVIRUS SPECIFIC IgM RESPONSES IN PATIENTS WITH JUVENILE-ONSET INSULIN-DEPENDENT DIABETES MELLITUS IN BRITAIN, AUSTRIA, AND AUSTRALIA.* The Lancet, 1985. **325**(8443): p. 1409-1412.
15. Kyvik, K.O., A. Green, and H. Beck-Nielsen, *Concordance rates of insulin dependent diabetes mellitus: a population based study of young Danish twins.* BMJ, 1995. **311**(7010): p. 913-917.
16. Redondo, M.J., et al., *Concordance for Islet Autoimmunity among Monozygotic Twins.* New England Journal of Medicine, 2008. **359**(26): p. 2849-2850.

17. Redondo, M.J., et al., *Heterogeneity of Type I diabetes: analysis of monozygotic twins in Great Britain and the United States.* Diabetologia, 2001. **44**(3): p. 354-362.
18. Bussone, G. and L. Mouthon, *Autoimmune manifestations in primary immune deficiencies.* Autoimmunity Reviews, 2009. **8**(4): p. 332-336.
19. Liston, A., et al., *Aire regulates negative selection of organ-specific T cells.* Nat Immunol, 2003. **4**(4): p. 350-354.
20. Bacchetta, R., et al., *Defective regulatory and effector T cell functions in patients with FOXP3 mutations.* The Journal of Clinical Investigation, 2006. **116**(6): p. 1713-1722.
21. Villaseñor, J., C. Benoist, and D. Mathis, *AIRE and APECED: molecular insights into an autoimmune disease.* Immunological Reviews, 2005. **204**(1): p. 156-164.
22. Polychronakos, C. and Q. Li, *Understanding type 1 diabetes through genetics: advances and prospects.* Nat Rev Genet. **12**(11): p. 781-792.
23. Singal, D.P. and M.A. Blajchman, *Histocompatibility (HL-A) Antigens, Lymphocytotoxic Antibodies and Tissue Antibodies in Patients with Diabetes Mellitus.* Diabetes, 1973. **22**(6): p. 429-432.
24. Aly, T.A., et al., *Extreme genetic risk for type 1A diabetes.* Proceedings of the National Academy of Sciences, 2006. **103**(38): p. 14074-14079.
25. Risch, N., *Assessing the role of HLA-linked and unlinked determinants of disease.* Am J Hum Genet, 1987. **40**(1): p. 1-14.
26. Bell, G.I., S. Horita, and J.H. Karam, *A Polymorphic Locus Near the Human Insulin Gene Is Associated with Insulin-dependent Diabetes Melliitus.* Diabetes, 1984. **33**(2): p. 176-183.
27. Bennett, S.T., et al., *Susceptibility to human type 1 diabetes at IDDM2 is determined by tandem repeat variation at the insulin gene minisatellite locus.* Nat Genet, 1995. **9**(3): p. 284-292.
28. Anderson, M.S., et al., *Projection of an Immunological Self Shadow Within the Thymus by the Aire Protein.* Science, 2002. **298**(5597): p. 1395-1401.
29. Bottini, N., et al., *A functional variant of lymphoid tyrosine phosphatase is associated with type I diabetes.* Nat Genet, 2004. **36**(4): p. 337-338.
30. Lowe, C.E., et al., *Large-scale genetic fine mapping and genotype-phenotype associations implicate polymorphism in the IL2RA region in type 1 diabetes.* Nat Genet, 2007. **39**(9): p. 1074-1082.
31. Nistico, L., et al., *The CTLA-4 Gene Region of Chromosome 2q33 Is Linked to, and Associated with, Type 1 Diabetes.* Human Molecular Genetics, 1996. **5**(7): p. 1075-1080.
32. Copeland, N., D. Harris, and M.A. Gaballa, *Human umbilical cord blood stem cells, myocardial infarction and stroke.* Clinical Medicine, Journal of the Royal College of Physicians, 2009. **9**(4): p. 342-345.
33. Rangappa, S., R. Makkar, and J. Forrester, *Review Article: Current Status of Myocardial Regeneration: New Cell Sources and New Strategies.* Journal of Cardiovascular Pharmacology and Therapeutics. **15**(4): p. 338-343.

34. Koninckx, R., et al., *Mesenchymal stem cells or cardiac progenitors for cardiac repair? A comparative study.* Cellular and Molecular Life Sciences, 2011. **68**(12): p. 2141-2156.
35. Ali H, B.H., *Umbilical cord blood stem cells – potential therapeutic tool for neural injuries and disorders.* Acta Neurobiol Exp (Wars), 2010. **70**(3): p. 316-324.
36. Cristante, A.F., et al., *Stem cells in the treatment of chronic spinal cord injury: evaluation of somatosensitive evoked potentials in 39 patients.* Spinal Cord, 2009. **47**(10): p. 733-738.
37. Ryan, E.A., et al., *Assessment of the Severity of Hypoglycemia and Glycemic Lability in Type 1 Diabetic Subjects Undergoing Islet Transplantation.* Diabetes, 2004. **53**(4): p. 955-962.
38. Williams, P., *Notes on Diabetes Treated With Extract and by Grafts of Sheep's Pancreas.* Br Med J, 1894(2): p. 1303-4.
39. Tzakis, A.G., et al., *Pancreatic islet transplantation after upper abdominal exeriteration and liver replacement.* The Lancet, 1990. **336**(8712): p. 402-405.
40. Shapiro, A.M.J., et al., *Islet Transplantation in Seven Patients with Type 1 Diabetes Mellitus Using a Glucocorticoid-Free Immunosuppressive Regimen.* New England Journal of Medicine, 2000. **343**(4): p. 230-238.
41. Ichii, H. and C. Ricordi, *Current status of islet cell transplantation.* J Hepatobiliary Pancreat Surg, 2009. **16**(2): p. 101-12.
42. Lumelsky, N., et al., *Differentiation of Embryonic Stem Cells to Insulin-Secreting Structures Similar to Pancreatic Islets.* Science, 2001. **292**(5520): p. 1389-1394.
43. Jensen, J., et al., *Control of endodermal endocrine development by Hes-1.* Nat Genet, 2000. **24**(1): p. 36-44.
44. Gradwohl, G.r., et al., *neurogenin3 is required for the development of the four endocrine cell lineages of the pancreas.* Proceedings of the National Academy of Sciences, 2000. **97**(4): p. 1607-1611.
45. Sander, M., et al., *Homeobox gene Nkx6.1 lies downstream of Nkx2.2 in the major pathway of beta-cell formation in the pancreas.* Development, 2000. **127**(24): p. 5533-5540.
46. Rajagopal, J., et al., *Insulin Staining of ES Cell Progeny from Insulin Uptake.* Science, 2003. **299**(5605): p. 363.
47. Blyszczuk, P., et al., *Expression of Pax4 in embryonic stem cells promotes differentiation of nestin-positive progenitor and insulin-producing cells.* Proceedings of the National Academy of Sciences, 2003. **100**(3): p. 998-1003.
48. D'Amour, K.A., et al., *Production of pancreatic hormone-expressing endocrine cells from human embryonic stem cells.* Nat Biotech, 2006. **24**(11): p. 1392-1401.
49. Fujikawa, T., et al., *Teratoma Formation Leads to Failure of Treatment for Type I Diabetes Using Embryonic Stem Cell-Derived Insulin-Producing Cells.* The American Journal of Pathology, 2005. **166**(6): p. 1781-1791.

50. Blum, B., et al., *The anti-apoptotic gene survivin contributes to teratoma formation by human embryonic stem cells.* Nat Biotech, 2009. **27**(3): p. 281-287.
51. Carpenter, M.K., J. Frey-Vasconcells, and M.S. Rao, *Developing safe therapies from human pluripotent stem cells.* Nat Biotech, 2009. **27**(7): p. 606-613.
52. Maehr, R., et al., *Generation of pluripotent stem cells from patients with type 1 diabetes.* Proceedings of the National Academy of Sciences, 2009. **106**(37): p. 15768-15773.
53. Mayhew, C.N. and J.M. Wells, *Converting human pluripotent stem cells into [beta]-cells: recent advances and future challenges.* Current Opinion in Organ Transplantation. **15**(1): p. 54-60 10.1097/MOT.0b013e3283337e1c.
54. Ferber, S., et al., *Pancreatic and duodenal homeobox gene 1 induces expression of insulin genes in liver and ameliorates streptozotocin-induced hyperglycemia.* Nat Med, 2000. **6**(5): p. 568-572.
55. Sahu, S., et al., *Islet-like cell clusters occur naturally in human gall bladder and are retained in diabetic conditions†.* Journal of Cellular and Molecular Medicine, 2009. **13**(5): p. 999-1000.
56. Ber, I., et al., *Functional, Persistent, and Extended Liver to Pancreas Transdifferentiation.* Journal of Biological Chemistry, 2003. **278**(34): p. 31950-31957.
57. Ramiya, V.K., et al., *Reversal of insulin-dependent diabetes using islets generated in vitro from pancreatic stem cells.* Nat Med, 2000. **6**(3): p. 278-282.
58. Heremans, Y., et al., *Recapitulation of embryonic neuroendocrine differentiation in adult human pancreatic duct cells expressing neurogenin 3.* The Journal of Cell Biology, 2002. **159**(2): p. 303-312.
59. Denner, J., H.-J. Schuurman, and C. Patience, *Chapter 5: Strategies to prevent transmission of porcine endogenous retroviruses.* Xenotransplantation, 2009. **16**(4): p. 239-248.
60. Garkavenko, O., et al., *Absence of transmission of potentially xenotic viruses in a prospective pig to primate islet xenotransplantation study.* Journal of Medical Virology, 2008. **80**(11): p. 2046-2052.
61. Groth, C.G., et al., *Transplantation of porcine fetal pancreas to diabetic patients.* The Lancet, 1994. **344**(8934): p. 1402-1404.
62. Teta, M., et al., *Growth and Regeneration of Adult [beta] Cells Does Not Involve Specialized Progenitors.* Developmental Cell, 2007. **12**(5): p. 817-826.
63. Dor, Y., et al., *Adult pancreatic [beta]-cells are formed by self-duplication rather than stem-cell differentiation.* Nature, 2004. **429**(6987): p. 41-46.
64. Bonner-Weir, S., et al., *Beta-Cell Growth and Regeneration: Replication Is Only Part of the Story.* Diabetes, 2010. **59**(10): p. 2340-2348.
65. Beattie, G.M., et al., *Ex Vivo Expansion of Human Pancreatic Endocrine Cells.* Journal of Clinical Endocrinology & Metabolism, 1997. **82**(6): p. 1852-1856.
66. Hayek, A., et al., *Growth factor/matrix-induced proliferation of human adult beta-cells.* Diabetes, 1995. **v44**(n12): p. p1458(3).
67. Fiaschi-Taesch, N., et al., *Survey of the Human Pancreatic Beta-Cell G1/S Proteome Reveals a Potential Therapeutic Role for Cdk-6 and Cyclin D1 in*

*Enhancing Human Œ≤-Cell Replication and Function In Vivo.* Diabetes, 2009. **58**(4): p. 882-893.
68. Cozar-Castellano, I., et al., *Induction of Beta-Cell Proliferation and Retinoblastoma Protein Phosphorylation in Rat and Human Islets Using Adenovirus-Mediated Transfer of Cyclin-Dependent Kinase-4 and Cyclin D1.* Diabetes, 2004. **53**(1): p. 149-159.
69. Fiaschi-Taesch, N.M., et al., *Induction of Human Beta-Cell Proliferation and Engraftment Using a Single G1/S Regulatory Molecule, cdk6.* Diabetes, 2010. **59**(8): p. 1926-1936.
70. Grouwels, G., et al., *Ectopic expression of E2F1 stimulates beta cell proliferation and function.* Diabetes, 2011.
71. Narushima, M., et al., *A human [beta]-cell line for transplantation therapy to control type 1 diabetes.* Nat Biotech, 2005. **23**(10): p. 1274-1282.
72. Ravassard, P., et al., *A genetically engineered human pancreatic beta cell line exhibiting glucose-inducible insulin secretion.* The Journal of Clinical Investigation, 2011. **121**(9): p. 3589-3597.
73. Hoglund, E., et al., *Growth hormone increases beta-cell proliferation in transplanted human and fetal rat islets.* JOP, 2009. **10**(3): p. 242-8.
74. Kayali, A.G., et al., *Limited Capacity of Human Adult Islets Expanded In Vitro to Redifferentiate Into Insulin-Producing Œ≤-Cells.* Diabetes, 2007. **56**(3): p. 703-708.
75. Ryan, E.A., D. Bigam, and A.M.J. Shapiro, *Current indications for pancreas or islet transplant.* Diabetes, Obesity and Metabolism, 2006. **8**(1): p. 1-7.
76. Ludwig, B., et al., *Islet Versus Pancreas Transplantation in Type 1 Diabetes: Competitive or Complementary?* Current Diabetes Reports. **10**(6): p. 506-511.
77. Yao, G., et al., *A bistable Rb-E2F switch underlies the restriction point.* Nat Cell Biol, 2008. **10**(4): p. 476-482.
78. Berk, A.J., et al., *Pre-early adenovirus 5 gene product regulates synthesis of early viral messenger RNAs.* Cell, 1979. **17**(4): p. 935-944.
79. Nevins, J.R., *E2F: A Link Between the Rb Tumor Suppressor Protein and Viral Oncoproteins.* Science, 1992. **258**(5081): p. 424-429.
80. Kong, L.J., et al., *Compensation and specificity of function within the E2F family.* Oncogene, 2006. **26**(3): p. 321-327.
81. Fajas, L., et al., *Impaired pancreatic growth, beta-cell mass, and beta-cell function in E2F1 -/- mice.* The Journal of Clinical Investigation, 2004. **113**(9): p. 1288-1295.
82. Iglesias, A., et al., *Diabetes and exocrine pancreatic insufficiency in E2F1/E2F2 double-mutant mice.* The Journal of Clinical Investigation, 2004. **113**(10): p. 1398-1407.
83. Bracken, A.P., et al., *E2F target genes: unraveling the biology.* Trends in Biochemical Sciences, 2004. **29**(8): p. 409-417.
84. Dimova, D.K. and N.J. Dyson, *The E2F transcriptional network: old acquaintances with new faces.* 2005. **24**(17): p. 2810-2826.
85. DeGregori, J. and D.G. Johnson, *Distinct and Overlapping Roles for E2F Family Members in Transcription, Proliferation and Apoptosis.* Current Molecular Medicine, 2006. **6**(7): p. 739-748.

86. Takahashi, Y., J.B. Rayman, and B.D. Dynlacht, *Analysis of promoter binding by the E2F and pRB families in vivo: distinct E2F proteins mediate activation and repression.* Genes Dev., 2000. **14**(7): p. 804-816.
87. Blais, A. and B.D. Dynlacht, *Hitting their targets: an emerging picture of E2F and cell cycle control.* Current Opinion in Genetics & Development, 2004. **14**(5): p. 527-532.
88. Helin, K., et al., *Heterodimerization of the transcription factors E2F-1 and DP-1 leads to cooperative trans-activation.* Genes & Development, 1993. **7**(10): p. 1850-1861.
89. Zhu, W.W., et al., *Temporal Control of Cell Cycle Gene Expression Mediated by E2F Transcription Factors.* Cell Cycle, 2005. **4**(5): p. 633-636.
90. Danielian, P.S., et al., *E2f3a and E2f3b make overlapping but different contributions to total E2f3 activity.* Oncogene, 2008. **27**(51): p. 6561-6570.
91. Annicotte, J.-S., et al., *The CDK4-pRB-E2F1 pathway controls insulin secretion.* Nat Cell Biol, 2009. **11**(8): p. 1017-1023.
92. Fujita, N., et al., *Hyperglycemia enhances VSMC proliferation with NF-[kappa]B activation by angiotensin II and E2F-1 augmentation by growth factors.* Molecular and Cellular Endocrinology, 2002. **192**(1-2): p. 75-84.
93. Stevens, C. and N.B. La Thangue, *E2F and cell cycle control: a double-edged sword.* Archives of Biochemistry and Biophysics, 2003. **412**(2): p. 157-169.
94. Litovchick, L., et al., *Evolutionarily Conserved Multisubunit RBL2/p130 and E2F4 Protein Complex Represses Human Cell Cycle-Dependent Genes in Quiescence.* Molecular cell, 2007. **26**(4): p. 539-551.
95. Muller, H., et al., *Induction of S-phase entry by E2F transcription factors depends on their nuclear localization.* Molecular and Cellular Biology, 1997. **17**(9): p. 5508-20.
96. Dyson, N., *The regulation of E2F by pRB-family proteins.* Genes & Development, 1998. **12**(15): p. 2245-2262.
97. Giangrande, P.H., et al., *A role for E2F6 in distinguishing G1/S- and G2/M-specific transcription.* Genes Dev., 2004. **18**(23): p. 2941-2951.
98. Di Stefano, L., M.R. Jensen, and K. Helin, *E2F7, a novel E2F featuring DP-independent repression of a subset of E2F-regulated genes.* EMBO J, 2003. **22**(23): p. 6289-6298.
99. Ogawa, H., et al., *A Complex with Chromatin Modifiers That Occupies E2F- and Myc-Responsive Genes in G0 Cells.* Science, 2002. **296**(5570): p. 1132-1136.
100. Christensen, J., et al., *Characterization of E2F8, a novel E2F-like cell-cycle regulated repressor of E2F-activated transcription.* Nucl. Acids Res., 2005. **33**(17): p. 5458-5470.
101. Maiti, B., et al., *Cloning and Characterization of Mouse E2F8, a Novel Mammalian E2F Family Member Capable of Blocking Cellular Proliferation.* J. Biol. Chem., 2005. **280**(18): p. 18211-18220.
102. Gala, S., et al., *Overexpression of E2F-1 leads to cytokine-independent proliferation and survival in the hematopoietic cell line BaF-B03.* Blood, 2001. **97**(1): p. 227-234.

103. Ebelt, H., et al., *Divergent Siblings: E2F2 and E2F4 but not E2F1 and E2F3 Induce DNA Synthesis in Cardiomyocytes Without Activation of Apoptosis.* Circ Res, 2005. **96**(5): p. 509-517.
104. Wang, L., R. Wang, and K. Herrup, *E2F1 Works as a Cell Cycle Suppressor in Mature Neurons.* J. Neurosci., 2007. **27**(46): p. 12555-12564.
105. Wang, D., J.L. Russell, and D.G. Johnson, *E2F4 and E2F1 Have Similar Proliferative Properties but Different Apoptotic and Oncogenic Properties In Vivo.* Molecular and Cellular Biology, 2000. **20**(10): p. 3417-3424.
106. Attwooll, C., E.L. Denchi, and K. Helin, *The E2F family: specific functions and overlapping interests.* EMBO J, 2004. **23**(24): p. 4709-4716.
107. Schlisio, S., et al., *Interaction of YY1 with E2Fs, mediated by RYBP, provides a mechanism for specificity of E2F function.* EMBO J, 2002. **21**(21): p. 5775-5786.
108. Humbert, P.O., et al., *E2f3 is critical for normal cellular proliferation.* Genes & Development, 2000. **14**(6): p. 690-703.
109. Freedman, J.A., et al., *A combinatorial mechanism for determining the specificity of E2F activation and repression.* Oncogene, 2009. **28**(32): p. 2873-2881.
110. Adewola, A., et al., *Microfluidic perifusion and imaging device for multi-parametric islet function assessment.* Biomedical Microdevices, 2010. **12**(3): p. 409-417.
111. Kulkarni, R.N., et al., *Human Beta-Cell Proliferation and Intracellular Signaling.* Diabetes, 2012.
112. Icyuz, M., et al., *Adenovirus infection activates akt1 and induces cell proliferation in pancreatic islets1.* Transplantation, 2009. **87**(6): p. 821-4.
113. Weinberg, N., et al., *Lineage Tracing Evidence for In Vitro Dedifferentiation but Rare Proliferation of Mouse Pancreatic Beta-Cells.* Diabetes, 2007. **56**(5): p. 1299-1304.
114. Rutti, S., et al., *In Vitro Proliferation of Adult Human Beta-Cells.* PLoS One, 2012. **7**(4): p. e35801.
115. Cozar-Castellano, I., et al., *Evaluation of Beta-Cell Replication in Mice Transgenic for Hepatocyte Growth Factor and Placental Lactogen.* Diabetes, 2006. **55**(1): p. 70-77.
116. Guney, M.A., et al., *Connective tissue growth factor acts within both endothelial cells and Œ≤ cells to promote proliferation of developing Œ≤ cells.* Proceedings of the National Academy of Sciences, 2011.
117. Cozar-Castellano, I., et al., *Lessons From the First Comprehensive Molecular Characterization of Cell Cycle Control in Rodent Insulinoma Cell Lines.* Diabetes, 2008. **57**(11): p. 3056-3068.
118. Sherr, C.J. and F. McCormick, *The RB and p53 pathways in cancer.* Cancer Cell, 2002. **2**(2): p. 103-112.
119. Cooper, C.S., et al., *Nuclear overexpression of the E2F3 transcription factor in human lung cancer.* Lung Cancer, 2006. **54**(2): p. 155-162.
120. Olsson, A.Y., et al., *Role of E2F3 expression in modulating cellular proliferation rate in human bladder and prostate cancer cells.* Oncogene, 2006. **26**(7): p. 1028-1037.

121. Foster, C.S., et al., *Transcription factor E2F3 overexpressed in prostate cancer independently predicts clinical outcome.* Oncogene, 2004. **23**(35): p. 5871-5879.
122. Johnson, D.G., et al., *Expression of transcription factor E2F1 induces quiescent cells to enter S phase.* Nature, 1993. **365**(6444): p. 349-352.
123. DeGregori, J., et al., *Distinct roles for E2F proteins in cell growth control and,Äâapoptosis.* Proceedings of the National Academy of Sciences, 1997. **94**(14): p. 7245-7250.
124. Barbu, A., et al., *A perfusion protocol for highly efficient transduction of intact pancreatic islets of Langerhans.* Diabetologia, 2006. **49**(10): p. 2388-2391.
125. Kim, S.Y. and S.G. Rane, *The Cdk4-E2f1 pathway regulates early pancreas development by targeting Pdx1+ progenitors and Ngn3+ endocrine precursors.* Development, 2011. **138**(10): p. 1903-1912.
126. Jeong, J.H., et al., *Proliferation of Pancreatic Endocrine Cells Using Disaggregation-Expansion-Reaggregation Technology in Isolated Rat Islets.* Transplantation Proceedings, 2010. **42**(3): p. 907-910.
127. Vega, R.A., et al., *Modified gold nanoparticle vectors: A biocompatible intracellular delivery system for pancreatic islet cell transplantation.* Surgery, 2010. **148**(4): p. 858-866.
128. Rink, J.S., et al., *Transfection of pancreatic islets using polyvalent DNA-functionalized gold nanoparticles.* Surgery, 2010. **148**(2): p. 335-345.
129. Efrat, S., et al., *Beta-cell lines derived from transgenic mice expressing a hybrid insulin gene-oncogene.* Proceedings of the National Academy of Sciences, 1988. **85**(23): p. 9037-9041.
130. Salmon, P., et al., *Reversible Immortalization of Human Primary Cells by Lentivector-Mediated Transfer of Specific Genes.* Mol Ther, 2000. **2**(4): p. 404-414.
131. Christophe Cudré-Mauroux, T.O., Stéphane König, Patrick Salmon, Laurent Bernheim, and Didier Trono, *Lentivector-Mediated Transfer of Bmi-1 and Telomerase in Muscle Satellite Cells Yields a Duchenne Myoblast Cell Line with Long-Term Genotypic and Phenotypic Stability.* Human Gene Therapy 2003. **14**(16): p. 1525-1533.
132. Takane, K.K., et al., *Regulated and Reversible Induction of Adult Human Beta-Cell Replication.* Diabetes, 2012. **61**(2): p. 418-424.
133. Fajas, L., et al., *E2Fs Regulate Adipocyte Differentiation.* Developmental Cell, 2002. **3**(1): p. 39-49.
134. Koster, J.C., et al., *Hyperinsulinism induced by targeted suppression of beta cell KATP channels.* Proceedings of the National Academy of Sciences, 2002. **99**(26): p. 16992-16997.
135. Miki, T., et al., *Defective insulin secretion and enhanced insulin action in KATP channel-deficient mice.* Proceedings of the National Academy of Sciences, 1998. **95**(18): p. 10402-10406.
136. Remedi, M.S., et al., *Diet-Induced Glucose Intolerance in Mice With Decreased Œ≤-Cell ATP-Sensitive K+ Channels.* Diabetes, 2004. **53**(12): p. 3159-3167.

**VITA**

| | |
|---|---|
| NAME: | Brian Rady |
| EDUCATION: | B.S., Biology, Loyola University Chicago, Chicago, IL 2006<br>PhD., Bioengineering, University of Illinois at Chicago, Chicago, IL 2013 |
| TEACHING: | Department of Bioengineering, University of Illinois at Chicago: Cell and Tissue Engineering Lab, 2008 |
| AWARDS: | Richard Lounsberry Award, Scientific Exchange.<br>Lille, France, 2010 |
| ABSTRACTS: | **Rady B,** and Oberholzer, J. Islet genesis project update. Verbal presentation, Chicago Diabetes Project annual meeting, University of Illinois at Chicago, Chicago, IL. October 2007-2012<br><br>**Rady B**, Vaca-Sanchez P, Wang Y, and Oberholzer J. Co-Expression of the transcription factors E2F3:DP2 increases the transplantable mass of rodent islets. Poster presentation, 7th annual Chicago Diabetes Day, University of Chicago, Chicago, IL. May 2012<br><br>**Rady B**, Vaca-Sanchez P, Wang Y, and Oberholzer J. Human insulinoma expression of E2F3 reveals a role in beta-cell proliferation. Poster presentation, College of Medicine Research Forum, University of Illinois at Chicago, Chicago, Awarded – 4th place. IL. November 2012 |
| PUBLICATIONS: | **Rady B**, Vaca-Sanchez P, Wang Y, and Oberholzer J. "Overexpression of E2F3 promotes proliferation of functional human beta-cells without induction of apoptosis." (in submission) |