12-2013

Enhanced Pancreatic beta-cells Proliferation and Functionality

Hanan Abdulaziz Alismail
University of Arkansas, Fayetteville

Enhanced Pancreatic β-cells Proliferation and Functionality

Enhanced Pancreatic β-cells Proliferation and Functionality

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Cell and Molecular Biology

by

Hanan Alismail
King Saud University
Bachelor of Science in clinical laboratory, 2008

December 2013

University of Arkansas

This thesis is approved for recommendation to the Graduate Council

_____
Dr. Sha Jin
Thesis Director

_____
Dr. Robert Wideman
Committee Member

_____
Dr. Dan Lessner
Committee Member

ABSTRACT

Biologically functional β-cells proliferate at an extremely low rate with limited turnover capacity. This cellular property hinders cell-based therapy for clinical applications. Many attempts have been made to develop techniques that allow large quantities of production of clinically relevant islet β-cells *in vitro*. A line of studies demonstrates that functional β-cells can proliferate under certain circumstances, providing hope for generating and expanding these cells *in vitro* and transplanting them into the recipient. In this study, we showed that a membrane substrate offers a better niche for beta cell proliferation and insulin secretion. Mouse beta cells were grown on a tissue culture plate (TCP) as a control as well as on polyethylene terephthalate (PET) membrane, and cell numbers were counted four times at 48 hours intervals. The cell doubling time was shortened from 64.7±0.4 h for beta cells grown on TCP, to 38.6±0.5 h (*p*-value 0.03) for those grown on PET membrane substrate with a pore size of 1μm. In addition, there was an increase of approximately ten to thirteen fold in insulin gene expression in cells cultured on PET compared to that on TCP (*p*-value 0.02). Furthermore, to investigate the mechanism of the enhanced proliferation and insulin production using membrane substrate, the expression profile of eighty-four genes that are involved in the apoptotic pathway were measured by quantitative real time polymerase chain reaction (qRT-PCR). Enhancements in Akt and Bcl_2 gene expression were detected. These findings demonstrate that a membrane substrate can offer better physicochemical cues for enhancing β-cells proliferation and function *in vitro*.

ACKNOWLEDGEMENTS

I am heartily thankful to Dr.Jin, thesis director, whose encouragement, guidance, kindness and support from the initial to the final level enabled me to develop an understanding of the subject.

It is a pleasure to thank those who made this thesis possible, especially my thesis committee members: Dr.Wideman and Dr. Lessner. I also thank Dr.Ye lab staff, especially Weiwei Wang, Jon Earls for their help and assisting. Special thanks to Dr.Wen who supported me with her advices in my research work.

I would like to thank my friends and family members, especially my dad, Abdulaziz Alismail, and my friends, Olfat Alaamri and Kholod Alzahrani for supporting and encouraging me during my studies. Without my mom, Fawziyah Aljafari, encouragement, I would not have finished the degree.

Lastly, I offer my regards and blessings to King Saud bin Abdulaziz University for health science who sponsored and supported me to pursue my degree. Special thanks go to Dr. Rhoads, without whose assistance, this degree would not have been successful.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

AKT/PKB	Thymoma viral proto-oncogene 1/ Protein kinase B
AMPK	5' AMP-activated protein kinase
Apaf1	Apoptotic peptidase activating factor 1
APO1	Apoptosis antigen 1
Bad	BCL2-associated agonist of cell death
Bak1	BCL2-antagonist/killer 1
Bax	Bcl2-associated X protein
BCL2	B-cell leukemia/lymphoma 2
Bcl2l1	Bcl2-like 1
Bcl2l2	Bcl2-like 2
Bid	BH3 interacting domain death agonist
Cnb1	Calcineurin b1
CREB	cAMP response element-binding protein
DD	death domain
DMEM	Dulbecco’s modified Eagle’s medium
DPBS	Dulbecco's Phosphate Buffered Saline
EDTA	Ethylenediaminetetraacetic acid
ERK1/2	Extracellular signal-regulated kinase
Fadd	Fas (TNFRSF6)-associated via death domain
FasR	FAS receptor
FBS	Fetal bovine serum
FOXA2	Forkhead box protein A2
FOXO1	Forkhead transcription factor 1
GIP	Glucose-dependent insulinotropic polypeptide
GLP-1	Glucagon-like peptide-1
GSIS	Glucose-stimulated insulin secretion
GSK3	Glycogen synthase kinase-3
HBSS	Hank's Balanced Salt Solution
HGF	Hepatocyte growth factor
HIF	Hypoxia-inducible factor
ICE	Insulin control element
IGF	Insulin growth factor
IRS2	Insulin receptor substrate 2
JAK	Janus kinase
JNK	c-Jun N terminal kinase
Mapk1	Mitogen-activated protein kinase 1
mTOR	Mammalian target of rapamycin
NEAA	nonessential amino acids
NFATc1	Nuclear factor of activated T cell cytoplasmic 1
NFkB	Nuclear factor kB
Pdx-1/IPF1	Pancreatic and duodenal homeobox/ insulin promoter factor 1
PE	Polyester
PET	Polyethylene terephthalate
PI3K	Phosphatidylinositol 3'-kinase

PL	Placental lactogen
PRL	Prolactin
RIP	Receptor interacting protein
ROS	Reactive oxygen species
Smda	Mothers against decapentaplegic homolog
SRF	Serum response factor
STAT	Signal Transducer and Activator of Transcription
STF-1	Somatostatin transcription factor-1
TCP	Tissue culture plate
TGF	Transforming growth factor
TNF	Tumor necrotic factor
Tnfrsf1a	Tumor necrosis factor receptor superfamily, member 1a
TRADD	TNFR-associated death domain
TRAF-2	TNF receptor-associated factor-2
TSC2	Tuberous sclerosis 2
VHL	von Hippel-Lindau

CHAPTER 1

1.0 REVIEW OF LITERATURE

1.1 OVERVIEW:

Pancreatic β-cells are considered one of the most important cells in the islets of Langerhans in the endocrine tissue of the pancreas. These cells are responsible for insulin production in response to blood glucose levels. Diabetes mellitus is a result of either insulin deficiency, or sugar unregulated insulin secretion (Klöppel, Löhr, Habich, Oberholzer, & Heitz, 1985). Type I and II diabetes are two common types of the disease. Type I diabetes (T1D) results from the autoimmune destruction of β-cells, making the body incapable of maintaining normal glycemia in these patients. In Type II diabetes (T2D), either the body does not produce enough insulin due to a decrease in functional β-cell mass, or the insulin secretion does not respond to elevated glucose levels in the blood. Diabetes induces other diseases, including heart disease and stroke, high blood pressure, kidney disease, and blindness. The CDC estimated that 25.8 million people of the U.S. population had this disease in 2011(Centers for Disease Control and Prevention, 2011). The World Health Organization (WHO) suggested that the global number of diabetic patients would reach 300 million as well as become the 7^{th} leading cause of death by the year 2030 (Alwan, 2011). Current treatment for T1D includes insulin supplementation by either tablet or injection (Daneman, 2006), or organ transplantation, which has its own complications including the unavailability of donors and histocompatibility matching issues (Shapiro et al., 2000). Medical treatment for T2D is fairly limited. Driven by the urgent need to treat patients suffering from diabetes, intensive research efforts have been made to create biologically functional islet tissues that can be used to replace diseased islets and regenerate a healthy tissue based on cell therapy.

One of the main challenges that remains in adult pancreatic β-cell therapy is the extremely low proliferation rate which is approximately 0.3% a day in aged adult mice and the minimum replication rate in adult humans (Ribaux et al., 2007). Fortunately, it is now well known that β-cell mass expansion can occur in early postnatal life, pregnancy, and animal models which have been genetically modulated (Buteau et al., 2004; Nir, Melton, & Dor, 2007; Rieck & Kaestner, 2010). For this reason, it is important to fully understand the molecular mechanisms that facilitate enhanced pancreatic β-cell proliferation so that cells can be cultured in an environment suitable for *in vitro* production. β-cell proliferation inducers can be classified into extrinsic and intrinsic categories. Extrinsic mitogens include: glucose, amino acids, insulin and insulin like growth factors (IGFs), prolactin (PRL), placental lactogen (PL), glucagon-like peptide-1 (GLP-1), growth hormone (GH), Hepatocyte growth factor (HGF), epidermal growth factors (EGF), Transforming growth factor (TGF), and extracellular matrix (ECM) (Gasparo, Milner, Norris, & Milner, 1978; Garofalo et al., 2003; SJÖHOLM, 1996). On the other hand, the most prominent intrinsic factors are: cyclins, cyclin dependent kinases (CDKs), and cyclin dependent kinase inhibitors (CKIs) (Heit, Karnik, & Kim, 2006). This review focuses on the most important extrinsic mitogens and associated signaling pathways that are involved in the process of β-cell proliferation. The review also introduces advanced approaches and applications in the field of islet β-cell expansion and biological functionalization.

1.2 NATIVE β-CELLS AND THEIR SRROUNDINGS

Understanding the physiological nature of the native β-cell is a prerequisite for establishing niches required for supporting islet β-cells. Islets of Langerhans are comprised of five types of cells: α, β, δ, ε, and PP-cells. These cells work as a microorgan to maintain glucose homeostasis. The β-cell is the most abundant and important cell in the islets, which sense the circulating

glucose level in the blood and respond to glucose levels by secreting insulin accordingly (Eberhard & Lammert, 2009). β-cells receive their regulation from a pancreatic and non-pancreatic environment promoting their function and proliferation (Eberhard & Lammert, 2009). First, a dense vascular network exists within the islets and facilitates efficient oxygen and insulin transfer. β-cells interact with the endothelial cells of the capillary network through the vascular basement membrane (BM). β-cells secret vascular endothelial growth factor (VEGF) to promote vascular development, whereas the endothelial cells produce a basement membrane rich with laminin to support the insulin gene expression and secretion from β-cells and promot β-cell proliferation (Nobukini & Thomas, 2004). Second, cell to cell contacts between β-cells, through several transmembrane receptors, have a great impact on optimizing the insulin gene expression, and glucose stimulated insulin secretion (GSIS) (Wojtusciszyn, Armanet, Morel, Berney, & Bosco, 2008). Third, β-cells interact with α-cells in reciprocal secretory fashion to maintain glucose homeostasis (Unger & Orci, 2010). Fourth, islets are rich with neurons from the sympathetic and parasympathetic nervous systems. Interaction between β-cells and parasympathetic neurons activate specific receptors to induce GSIS, whereas sympathetic neurons inhibit insulin secretion as a part of the physiological glucose homeostasis (Ahrén, 2000).

In addition, β-cells interact with non-pancreatic tissues such as: liver, bone, fat and gut, and endocrine cells of the intestine (Eberhard & Lammert, 2009). These cells secrete integrins which bind to a G-coupled receptor on the β-cell surface to stimulate insulin secretion and β-cell proliferation (Drucker, 2007). In the process of islet isolation all of the vascular and nerve connections are destroyed by the enzymatic digestion of the pancreas and islet purification through density centrifugation, which could be the major cause of malfunction of the β-cell and

its survival after isolation procedures (Merani & Shapiro, 2006; Wang, Paraskevas, & Rosenberg, 1999). Motivated by the need to create an optimal niche for β-cell transplantation, researchers are exploring the most effective biological materials and accessories for creating a niche that can last long enough after transplantation and would be beneficial for cells to re-communicate with their surroundings. In addition, knowing the signaling pathways and molecular mechanisms would enable manipulating cell proliferation as well as optimizing insulin secretory function. This section is thus discussed in detail in the following section.

1.3 EXTRINSIC MITOGENS

1.3.1 Glucose

Glucose is a well-known physiological regulator. It is one of the important regulators of β-cell proliferation, since the primary function of β-cells is to lower blood glucose levels by insulin secretion. Evidence indicating the role of glucose in β-cell proliferation have been reported in several studies both in *in vitro* and *in vivo*. Glucose has been shown to be an important requirement for cell growth in fetal and neonatal β-cell (Swenne, 1982), insulinoma cell lines (Gahr et al., 2002), primary islets (Hoorens, Van, Klöppel, & Pipeleers, 1996), as well as human β-cells (Tyrberg, Eizirik, Hellerström, Pipeleers, & Andersson, 1996). *In vivo* glucose infusion in diabetic mice and rats ultimately resulted in increasing β-cell mass (Alonso et al., 2007; C. Bernard, Ktorza, Saulnier, & Berthault, 1999; Paris, Bernard-Kargar, Berthault, Bouwens, & Ktorza, 2003).

The signaling pathways which correlate glucose levels with β-cell quantity, proliferation, and apoptosis have not yet been entirely developed. Several pathways thought to be involved are: (1) insulin autocrine effect, (2) calcium signaling, and (3) the TSC2/mTOR inhibitory signaling pathway (Chang-Chen, Mullur, & Bernal-Mizrachi, 2008). *In vitro* studies demonstrated that

glucose induces intracellular signaling molecules such as phosphatidylinositol 3'-kinase (PI3K), protein kinase B (PKB), glycogen synthase kinase-3 (GSK-3), extracellular signal-regulated kinase (ERK)1/2, and mammalian target of rapamycin (mTOR), as well as insulin receptor substrate 2 (IRS2) (Ohsugi et al., 2004; Vaulont, Vasseur-Cognet, & Kahn, 2000). Activation of insulin receptor leads to the activation of the Akt signaling pathway which is considered to be one of the main pathways of β-cell proliferation (Bernal-Mizrachi, Wen, Stahlhut, Welling, & Permutt, 2001; Tuttle et al., 2001). Moreover, this activation is down regulated by mTOR signaling triggered by an increase in ATP production and leads to the subsequent inactivation of AMP kinase (AMPK) (Nobukini & Thomas, 2004). Finally, the calcium signaling pathway also has a significant influence on phosphates level. An investigation of the importance of this pathway has been conducted by deleting the calcineurin regulatory subunit, calcineurin b1 (Cnb1). This study showed reduced β-cell proliferation and the development of age related diabetes. The correction for this defect was made by expressing the active nuclear factor of activated T cell cytoplasmic 1 (NFATc1), which is a downstream transcription factor in this pathway (Heit et al., 2006). Activation of transcription factors cAMP response element-binding protein (CREB) and serum response factor (SRF) improved β-cell growth rate through the glucose/ calcium pathway (Bernal-Mizrachi, Wice, Inoue, & Permutt, 2000; Jhala et al., 2003).

Although the moderate glucose elevation causes an increase in β-cell growth and survival, prolonged exposure to high concentrations of glucose is the main cause of β-cell deterioration and apoptosis. This condition, called glucotoxicity, is caused by several mechanisms that are not fully understood (V. Poitout, Olson, & Robertson, 1996; V. Poitout & Robertson, 2002). A line of studies found a number of transcriptional regulators that are sensitive to the level and duration of glucose and insulin gene expression such as MafA, NFAT (M. C. Lawrence, Bhatt, Watterson,

& Easom, 2001; M. C. Lawrence, Bhatt, & Easom, 2002), somatostatin transcription factor-1 (STF-1), pancreatic and duodenal homeobox 1 (Pdx-1), and the insulin control element (ICE) (Sharma, Olson, Robertson, & Stein, 1995). It was shown that these proteins are expressed in the presence of 0.8 mM glucose as well as a prolonged exposure to a high level of 11.1 mM glucose (V. Poitout et al., 1996). In addition, MafA protein level is able to restore insulin expression in β-cell lines along with the present of prolonged high glucose levels (Harmon, Stein, & Robertson, 2005). High glucose concentrations have also been shown to inactivate AMP protein kinase (AMPK) in β-cells and result in impaired glucose-stimulated insulin secretion (GSIS) due to lipid accumulation which leads to the deterioration of β-cell function (Da et al., 2003; Nyblom, Sargsyan, & Bergsten, 2008; R. H. Unger, Zhou, & Orci, 1999).

Another set of studies revealed that glucose toxicity may lead to oxidative stress in organelles such as in the endoplasmic reticulum and mitochondria (Fridlyand & Philipson, 2004; Nyblom, Thorn, Ahmed, & Bergsten, 2006). Increase in oxidative stress causes a reduction in binding of Pdx1 and MafA to the insulin gene in the pancreas which, in turn, results in defective insulin gene expression and hormone secretion (Harmon et al., 2005; Robertson & Harmon, 2006). It is clear that reactive oxygen species (ROS) activate stress-induced pathways, including nuclear factor kB (NF-kB), c-Jun N terminal kinase (JNK), and hexoanimase pathways (Kaneto et al., 2002; Kawasaki, Abiru, & Eguchi, 2004). The subsequent JNK signaling event leads to the inactivation of IRS-1 by its phosphorylation on Ser307 (Aguirre, Uchida, Yenush, Davis, & White, 2000). A recent study suggested that hypoxia is another cause for the malfunction of β-cells. Expression of transcription factor hypoxia-inducible factor (HIF) plays an adverse role in β-cell function (Gunton et al., 2005). Under exposure to a high glucose level, an alteration in the profile of β-cell gene expression occurs, including a switch from aerobic to anaerobic glycolysis

that leads to impaired GSIS and glucose intolerance. This was investigated through the deletion of the regulatory protein von Hippel-Lindau (VHL) protein for controlling the degradation of HIF which reduces the cellular oxygenation level and causes hypoxia. VHL/HIF oxygen-sensing mechanisms play a critical role in glucose homeostasis through decreasing islet oxygenation level and negatively impacting β-cell function (J. Cantley et al., 2009; Puri, Cano, & Hebrok, 2009).

Furthermore, chronic hyperglycemia leads to excessive accumulation of Ca^{+2} in the cytosol which is a proapoptotic signal that induces β-cell dysfunction and destruction (Grill, 2001). Other potent apoptosis pathways were shown to be related to interleukin-1 β which inhibits β-cell function and promotes Fas-triggered apoptosis in part by activating the transcription factor NF-kB during the autoimmune process of T1D pathogenesis (Maedler et al., 2002). However, knowledge of the signaling pathway involved in chronic hyperglycemia remains elusive. In particularly, the targets and the downstream effects of glucotoxicity have not yet been completely elucidated.

1.3.2 Growth factors and signaling pathways

Insulin growth factor (IGF)

Expressions of insulin growth factor I (IGF-I) and II (IGF-II) and their receptor (IGFR) were found in different stages of pancreatic development. In fact, their expressions act as signal for stimulating β-cell proliferation (R. N. Kulkarni, 2005). IGF-I and IGF-II are able to enhance β-cell proliferation in rat islets and insulinoma cell lines *in vitro* (Hogg, Han, Clemmons, & Hill, 1993). Interestingly, the over-expression of IGF-I results in enhanced proliferation of β-cells in transgenic mice but not the size of islets, whereas the over-expression of IGF-II leads to

abnormal islet morphology with an enlarged irregular shape (George et al., 2002; Petrik et al., 1999).

Studies also demonstrated that insulin receptor (IR) is a stimulator for β-cell proliferation (R. N. Kulkarni et al., 1999), where the Akt and MAPK signaling pathways are involved (Saltiel & Kahn, 2001). Reduction in the IR by up to 80% in MIN6 cells lead to the reduction of growth rate, suggesting that insulin plays a crucial role as a growth factor for this insulinoma cell line (M. Ohsugi et al., 2005). In addition, the effect of IR substrate 1 and 2 (IRS1 & 2) have also been explored as they are modulators in the insulin/IGF signaling cascade. A heterozygous mutation in IRS1/IR in mice showed a 400-fold increase in circulating insulin in parallel to severe insulin resistance with striking hyperplasia in the β-cell mass by 40-50-fold (Bruning et al., 1997). In contrast, animal models deficient in IRS-2 showed abnormalities such as low proliferation, low Pdx-1 expression, small islet size, and an increase in apoptosis (Withers et al., 1998). Moreover, IRS-2 over-expression is associated with glucose stimulation in insulinoma cells. It exhibits a synergistic increment in β-cell proliferation in glucose/IGF-1induced manner *in vitro* (Lingohr et al., 2002). Another study reported that IRS-2 controls other growth promoting mutagens such as exendin-4, which protects β-cells from human islet amyloid polypeptide-induced cell damage (Fan, et al., 2010; Park et al., 2006). All of these studies suggested that the downstream signaling of insulin and IGF receptors are essential for maintaining β-cell mass and proliferation.

Akt/PKB signaling pathway

Akt is also known as protein kinase B (PKB), and is proposed to be a crucial modulator of IRS-2-mediated signals in β-cells. Deficiency in AKT2 has shown a negative impact on β-cell proliferation, which provides an evidence of its importance in the signaling cascade (Garofalo et al., 2003). On the contrary, AKT2 over expression induced β-cell proliferation via enhancing

resistance to apoptosis and improving insulin secretion (Bernal-Mizrachi et al., 2001) , (Tuttle et al., 2001). Some downstream targets such as forkhead transcription factor 1 **(**FOXO1), Glycogen synthase kinase 3 beta (GSK3β), and the mammalian target of rapamycin (mTOR) are down-regulated by the Akt signaling pathway and Akt functions have been described elsewhere (Kaiser et al., 2013; Nakae et al., 2002; Tanabe et al., 2008).

Pdx-1, also known as insulin promoter factor 1 (IPF1) has been investigated as an ultimate transcription factor of Akt signaling, its phosphorylation and nuclear inclusion plays an important role in β-cells proliferation and function (McKinnon & Docherty, 2001). However, it is still unknown how Pdx-1 regulates β-cell proliferation. In addition, Pdx-1 can restore β-cell function in IRS2 knockout mice, suggesting that the dysregulation of Pdx-1 by IRS2 is directly related to the development of T2D (Kushner et al., 2002).

There are multiple evidences for the inverse correlation between the forkhead transcription factor 1 FOXO1and the Pdx-1expression. A group of studies showed several mechanisms in which FOXO1 can antagonize Pdx-1. FOXO1 suppression can be done through the competition binding of forkhead box protein A2 **(**FoxA2) protein to Pdx-1 promoter region and restoring Pdx-1expression level (Kitamura et al., 2002). This was further affirmed by the expression of Pdx-1 in β-cells which contain cytoplasmic FOXO1 but not nuclear FOXO1 (Kitamura et al., 2002). A recent study also confirmed that FOXO1 inhibits β-cell neogenesis but it is required for the maintenance of insulin secretion under metabolic stress (Kobayashi et al., 2012). In addition, FOXO1 can lead to a nuclear exclusion of Pdx-1in oxidatively stressed β-cell as a suppression mechanism (Kawamori et al., 2006). However, it is unclear the type of molecules that are stimulated by Pdx-1 as well as the correlation of the mechanism of these molecules with Pdx-1 to induce β-cell proliferation.

Prolactin, placental lactogen, and HGF

Several other growth factors, such as prolactin (PRL) and placental lactogen (PL), can also enhance β-cell proliferation rate both *in vitro* and *in vivo*. Prolactin as well as PL are highly expressed during pregnancy and have beenshown to be involved in increasing β-cells mass (Vasavada et al., 2000). In rodent studies, injection of PRL or PL leads to higher beta cell growth (Parsons et al., 1992). Moreover, transgenic mice over- expressing PL demonstrated a higher growth rate associated with hyperinsulinemia (Parsons et al., 1992). On the contrary, mice lacking PRL receptors reduced β-cell mass (Freemark et al., 2002). PRL and PL both bind to the PRL receptor. The PRL receptor belongs to the cytokine receptor family in the JAK/STAT signaling pathway (Nielsen, 2001). Thus, these two growth factors trigger β-cell proliferation through signaling pathway JAK2/STA5 as illustrated in Table 1.

Hepatocyte growth factor (HGF) is a mesenchyme-derived growth factor involved in proliferation, migration, and differentiation of several types of tissues (Matsumoto & Nakamura, 1996). Deletion of HGF demonstrated glucose intolerance and impaired glucose stimulated insulin secretion (GSIS). A protective effect against apoptosis and increase of proliferation was exhibited in induced dysfunction mice injected with the exogenous HGF gene (Dai et al., 2003). Thus, HGF seems to be an attractive potential target for therapy. HGF binds to c-met receptor and activates MAPK and PI3K/Akt pathways which are responsible for β-cell proliferation (García-Ocaña et al., 2001). It seems like a mechanism of action regarding the involvement of the signal pathway is complicated and unpredictable. For instance, insulinoma cells (INS-1) treated with HGF exhibit an increase in Protein kinase C expression, advocating the presence of other pathways by which HGF can trigger proliferation (R. C. Vasavada et al., 2007).

Transforming growth factor-beta (TGF-β)

Transforming growth factor (TGF) has two classes of polypeptide growth factors, TGF-α and TGF-β. TGF-β is an important regulator in pancreas development and function. They have been implicated in the pathogenesis of cancer, autoimmune disease, and diabetes. Several studies have reported that impaired TGF level can lead to the onset of both type1 and type 2 diabetes (Rane, Lee, & Lin, 2006). Impaired TGF-β is related to the progression of T1D in non-obese diabetic (NOD) mice. The cause of diabetes in these mice was autoimmune destruction of islets resulting insulin deficiency (Anderson & Bluestone, 2005). An adenoviral expression vector encoding TGF was transfected to the NOD mouse islet cells. The experimental result suggested a protection to the NOD mouse islet cells from apoptosis and immune distraction and delay in diabetes occurrence for 22 days compared to only 7 days in the vector transfected control (Suarez-Pinzon, et al., 2002). Microarray on isolated intact human islets incubated in low and/or high glucose, revealed a highly regulated TGF signaling in the human islets. This suggests that TGF is involved in glucose metabolism and β-cell function as well (Drucker, 2006; Shalev et al., 2002).

Extrinsic factors	**MAPK**	**Akt**	**Ca^{+2} signaling**	**Jak/Stat**	**Smda**	**β- catenin**
Glucose	√		√			
Insulin/IGF-1		√	√			
PRL/PL				√		
HGF	√	√	√			
TGF					√	
GLP-1/GIP	√	√	√			√

Table1.1: Summary of the extrinsic factors and their downstream signaling pathways

1.3.3 Incretins

Incretins are a group of hormones that lead to an increase in the amount of insulin released from the β-cells. Glucagon-like peptide-1 (GLP-1) and glucose-dependent insulinotropic polypeptide (GIP) are the two types of incretin hormones that have been well investigated. They are secreted from the intestine upon glucose ingestion to stimulate insulin secretion. These two hormones have been shown to be involved in increasing β-cell proliferation and decreasing cell apoptosis (Drucker, 2006). GLP-1 infusion into glucose-intolerance rats caused an increase in β-cell mass. Likewise, an increase in β-cell size and neogenesis was observed in mice treated with GLP-1. Moreover, GLP-1 has an anti-apoptotic effect in freshly isolated human islets (Farilla et al., 2003). Buteau *et al.* found that GLP-1 enhances the binding of NFκB transcription factor to two anti-apoptotic genes: protein-2 and Bcl-2, resulting in augmentation of the expression of the anti-apoptotic proteins (J. Buteau et al., 2004). Therefore, GLP-1 has been approved by the FDA for use in T2D treatment (Issa & Azar, 2012). GIP has also shown to be a synergistical mitogen inducer with glucose and a pleiotropic growth factor for insulin production on INS-1 β-cells (Trümper et al., 2001). Indeed, GIP is strictly glucose dependent and it does not show any effect during a low blood glucose level. Thus, GIP seems to act as a blood glucose stabilizer with inverse glucose-dependent effect on pancreatic insulin (Christensen et al., 2011). In addition, the binding of GIP-1 to its G-protein coupled receptor (GLP1R) activates downstream targets including cAMP and PKA, intracellular calcium, and Pdx-1. In fact, many GLP1R consequence events seem to be related to Pdx-1 expression (Holz et al., 1999; Wang & Rosenberg, 1999; Tsuboi et al., 2003). Mouse islet β-cell MIN6 treated with GLP1R can activate the PI3K/Akt signaling pathway and trigger a significant increase in IRS2 (Li et al., 2005). On the contrary, inhibition of both c-SRC and EGFR suppresses GLP1R-mediated PI3K pathway in INS-1 cells

(Buteau, Foisy, Joly, & Prentki, 2003). Recently, GLP-1 was found to be able to induce β-cell proliferation by increasing the β-catenin nuclear content and increasing cyclic D1 expression (Shao et al., 2013).

1.3.4 Extracellular matrix

The extracellular matrix (ECM) is part of the tissue composed of multiple proteins and polysaccharides, and provides structural support to the cells and instructional guidance as well. Adult human islets are surrounded by an incomplete capsule constituted from a single layer of fibroblasts and collagen fibers. Additional matrix protein is attached to this capsule and known as periinsular basement membrane (BM). Mechanical and chemical signaling interactions between cells and ECM are known to regulate several physiological aspects including: survival (Nagata et al., 2002), proliferation (Beattie et al., 1996), and insulin secretion in islets (Kaido et al., 2006). In living tissue, cells synthesize ECM components and deposit them to form a niche. A niche not only affects the tissue composition and mechanical properties, but also determines cellular fate. As aforementioned, islet β-cells can rarely proliferate *in vitro*, thus, many attempts have been made to assess niches required for β-cell expansion. In a recent study, human islet cells were cultured in two ECM environments: rat ECM (804G) and bovine corneal endothelial ECM (BCEC) in the presence of GLP-1 analogue. It was observed that there is approximately 0.082±0.034% proliferation of islet β-cells in a treated/BCEC culture condition. These results indicate that adult human β-cell proliferation can occur *in vitro* but remains an extremely rare event within an environment of certain ECM and signaling molecules (Rutti et al., 2012). In another study, fully differentiated human adult insulin-producing β-cells were unable to proliferate *in vitro* regardless of whether or not the presence of human growth hormone (hGH)

and the GLP-1 analogue liraglutide. However, hGH and GLP-1 enhaced rat β-cell proliferation (Parnaud et al., 2009).

Since an interaction of ECM with integrins triggers an intracellular signaling cascade and modulates the level of gene expressions that control cell behavior (Juliano & Haskill, 1993), the effect of integrin on β-cell proliferation has also been explored. Studies suggest that adult human islets are expressing special types of integrins including α3, α5, αv, α6, β1, β3 and β5 (R. N. Wang & Rosenberg, 1999). Laminin-5 interacting with α6β1 integrin allows rat β-cell proliferation (Bosco, Meda, Halban, & Rouiller, 2000). Among ligands for the α3β1 integrin, including fibronectin, laminin, collagen I, and collagen IV, only collagen I and IV promote rat INS-1 cell viability and proliferation (Krishnamurthy et al.,2008). Collagen type I, IV, and laminin can increase survival rate of islets after isolation procedures (Pinkse et al., 2006). Nikolova and his co-workers identified laminins working as endothelial signals for promoting insulin secretion of β-cells, where this improvement relys on the interaction between β1 integrin and the laminins (Nikolova et al., 2006).

1.4 TISSUE ENGENERING APPROACHES

As mentioned previously, there is considerable interest in understanding the most important regulators and the mechanisms that can stimulate pancreatic islet growth *in vitro*. Tissue engineering approaches have been explored by culturing cells with highly porous scaffold biomaterials to generate a three dimensional (3D) environment for improving islet growth and survival, as well as normal insulin secretion. A line of studies demonstrated that polyethylene glycol (PEG) hydrogel scaffold can mimic cell-cell communication microenvironment required for insulin-secreting β-cells (Lin & Anseth, 2011; Weber, 2008; Weber, 2007). Bernard and his co-workers developed a PEG hydrogel-based microwell cell culture system using

photolithography technique (Bernard, Lin, & Anseth, 2012). Mouse β-cells formed aggregates in PEG hydrogels and demonstrated more than 90% cellular viability in a week long culture. Furthermore, aggregated cells showed considerable increase in insulin secretion compared with single cell culture conditions (Bernard et al., 2012). This study indicates that cell-cell adherent junction is one of the paramount factors required for the function of insulin-secreting β-cells. The importance of cell-cell adherent junction for β-cell survival and function is also verified by Kelly and colleagues (Kelly et al., 2010). PEG hydrogel was also fabricated to contain collagen type I, collagen type IV, fibrinogen, fibronectin, laminin, and vitronectin, and then used to encapsulate β-cells. β-cell survival significantly improved in ECM-containing PEG hydrogels compared to gels without ECM over 10 days. Insulin secretion was also enhanced in cells cultured in ECM-containing hydrogels (Weber et al., 2008). The PEG/ECM-based scaffolds indeed contribute to the re-establishment of the islets-ECM interaction. Hiscox and his co-workers developed a device that allows islets to be cultured in between two layers of prevascularized collagen gels. These islets exhibited a higher level of viability and functionality compared to the free islets control (Hiscox et al., 2008).

While encapsulation of islet cells in a 3D scaffold provides protection against the immune cells and its antibodies, the diffusion of low molecular weight cytokines through the hydrogels remains to be a challenge. To overcome these issue, scaffold surface can be fabricated by coating the PEG-hydrogel with diffusible pro-inflammatory cytokines interleukin (IL)-1β receptor. This modification enabled maintainence of the viability of encapsulated islet cells and function as a glucose-stimulated insulin secretion after the exposure of different cytokines (Su et al., 2010). Another strategy to make a better 3D scaffold is to inhibit TNF receptor 1 activation by scafolding PEG-diacrylate hydrogels coated with TNF receptor 1. As a result, this modified

hydrogel not only preserved islet insulin content, but also reduced mRNA of inducible nitric oxide synthase and IL-6 in pancreases in experimental animals (Wang et al., 2002). Nevertheless, impaired oxygen diffusion within a 3D scaffold hinders the wide use of scaffold for islet cell expansion. In particular normoxia, or higher oxygen tension, promotes islet β-cell development from progenitor cells and increases β-cell viability (Fraker et al., 2007; Pedraza, 2012; Skiles, 2013). As β-cells consume large amounts of oxygen during insulin secretion (Sato et al., 2011), studies have shown that islet aggregates may suffer from hypoxia proportional to the radial distance inward, leading to the cell necrosis and apoptosis as well as activation of anaerobic metabolism (Cantley et al., 2010). This issue may be overcome by culturing islet aggregates in an oxygenated system (Wu et al., 1999). Recently, an oxygenator made from polydimethylsiloxane (PDMS)/calcium peroxide enhanced the mouse MIN6 cell proliferation and insulin secretion for three weeks under hypoxic culture conditions (Pedraza et al., 2012). The oxygenating strategy is particularly promising because islet cells are usually sensitive to chemical compounds such as catalysts or hydrogen peroxide. To overcome the limitation of nutrient transport in a conventional cell culture dish, mouse β-cells were cultured in suspension in a stirred spinner flask. This bioreactor culture helped proliferation and increased sizes of cell aggregates with enhanced responsively to glucose and incretins (Lock, Laychock, & Tzanakakis, 2011).

CHAPTER 2

2. ENHANCED PANCREATIC β-CELL PROLIFERATION AND FUNCTIONALITY

2.1 INTRODUCTION

Pancreatic β-cells are considered as one of the most important cells in the islets of Langerhans in the endocrine tissue of the pancreas. These cells are responsible for insulin production in response to blood glucose level. Driven by the urgent need to treat patients suffering from diabetes, intensive efforts have been made to create biologically functional islet tissues that can be used to replace diseased islets and regenerate a healthy tissue based on cell therapy.

One of the main challenges remaining in adult pancreatic β-cell therapy is the extreme low proliferation rate which is approximately 01.-0.3% a day in aged adult mice and minimum replication in adult human (Ribaux et al., 2007). Fortunately, It is now well known that β-cell mass expansion can occur in early postnatal life, pregnancy, and animal models which have been genetically modulated (Buteau et al., 2004; Rieck & Kaestner, 2010). For this reason, it is important to fully understand the molecular mechanisms that can enhance functional pancreatic β-cell proliferation in a suitable environment for *in vitro* production.

Adult human islets are surrounded by an incomplete capsule composed of a single layer of fibroblasts and collagen fibers. Additional matrix protein is attached to this capsule, which is known as the periinsular basement membrane (BM). Mechanical and chemical signaling interactions between cells and ECM are known to regulate several physiological aspects including: survival (Nagata et al., 2002), proliferation (Beattie et al., 1996), and insulin secretion in islets (Kaido et al., 2006). In living tissue, cells synthesize ECM components and deposit them to form a niche. Basement membrane usually has a mixture of pores (~ 72nm), riges, and fibers (~77nm in diameter) (Abrams et al., 2000). They separate tissue from each other allowing cells

to develop their polarized morphology. Sequentially, the roughness of a substrate and the porous structure are both essential for restoring the *in vivo* niche equilibrium. In addition, the impermeability of the substrate prevents cells from taking or secreting signaling molecules and nutrient from both basal and apical surfaces. Thus, membrane substrate has been used in growing various types of cells in the past (Grobstein, 1953). Nanostructured surfaces are fabricated by dispensing prepolymer (PUA) on a supporting polyethylene terephthalate (PET) film, forming a surface conformal contact with the cells. These nanostructured surfaces found to be able significantly enhance osteogenesis of human mesenchymal stem cells (hMSCs) (You et al., 2010). However, the use of these membrane substrates on promoting the proliferation and insulin secretion of adult pancreatic beta cells has not yet been explored.

Mouse insulinoma 6 (MIN6) cells are cell line originated from a transgenic C57BL/6 mouse insulinoma expressing an insulin-promoter/T-antigen construct, forming an islets like clusters (Skelin, Rupnik, & Cencic, 2010). In addition, these cells express GLUT-2 and glucokinase and respond to glucose within the physiological range in the presence of nicotinamide (Vit B3) (Miyazaki et al., 1990). In this study, we intend to determine whether a porous and permeable membrane substrate provides better tissue niche for pancreatic β-cells proliferation and insulin secretion. In addition, the possible signaling molecules that are responsible in enhancing pancreatic β-cell growth and functionality were examined as well.

2.2 MATERIAL AND METHODS

2.2.1. Cell culture and cell viability assay

Mouse MIN6 cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 25 mmol/L glucose, 2mM L-glutamine, and 2mM sodium pyruvate (Corning). The medium was supplemented with 15% fetal bovine serum (FBS) (ATCC), 10 mmol/mL

nonessential amino acids (NEAA), 50 mg/L streptomycin and 75 mg/L penicillin sulfate, and equilibrated with 5% CO_2 and 95% air at 37ºC. The medium was replaced after each forty eight hours. For passage, MNI6 cells were treated with cold Dulbecco's Phosphate Buffered Saline (DPBS) without calcium and magnesium (Corning) to wash out the serum, and then cells were detached by using 0.05% Trypsin/0.53 mM EDTA in HBSS without Ca^{+2} and Mg^{+2} (Corning) for 5 minutes at 37ºC., Cells were centrifuged at 1400 RPM (300 RCF) for 5 minutes at room temperature. New DMEM was added to the cell pellet to re-suspend cells. Cells were splits at ratio of 1:3-1:5 every 7 days. The cells used in this study were at passage 20- 28 (Cheng et al., 2012).

Polyester (PE) (pore size: 0.4 μm) and polyethylene terephthalate (PET) (pore size: 1 μm) membranes were obtained from Millipore and Corning. The membrane substrates were placed in a six-well plate and seeded with 130,000 cells/ cm^2 MIN6 cells in the DMEM medium. Cells seeded in a six-well tissue culture plate (TCP) was used as control. The viability of the MIN6 was determined at indicated time points by using trypan blue staining assay after detaching the cells from culture plate using trypsin-EDTA. The experiments were performed independently for four times.

2.2.2 RNA extraction, quantification, and cDNA synthesis

At day eight, the cells were harvested to detect gene expression in cells grown on membrane substrates. Total RNA was isolated from the cells using RNA extraction kit (Qiagen). Synergy MX microplate reader (BioTek) was used to measure the RNA concentration. Genomic DNA was eliminated during RNA purification using DNase kit (Promega). 1 μg RNA was synthesized to cDNA in a reverse transcription reaction using RT kit (Applied Biosystems). The thermo cycler was programed as follows: 25°C for 10 minutes, 37°C for 2 hours, 85°C for 5

minutes and 4°C holding temperature. The resultant cDNA was stored at-80°C for further experiments.

2.2.3 Gene expression

Gene expression was carried out by using DNA polymerase (Biolabs) and detected by qRT-PCR using thermal cycling (Eppendorf) after RT reaction. qPCR was performed in 20 ul reaction volume (100 ng of cDNA/ reaction). A control assay was performed to ensure the absence of genomic DNA contamination in the qRT-PCR. The following primer pairs were used for characterization of mRNA expression. Insulin II forward: 5' TAGTGACCAGCTATAATCAGAG 3', Insulin II reverse: 5' ACGCCAAGGTCTGAAGGTCC 3' (288bp), β-actin forward: 5' ACGGCCAAGTCATCACTATTG 3', β-actin reverse: 5' AGCCACCGATCCACACAGA 3' (300bp). The thermo cycler was programed as follows: 95°C for 10 minutes, 40 cycles of denaturation at 95°C for 15 seconds, and annealing/extension at 60°C for 1 minute. The relative expression of insulin gene was normalized against β-actin. Quantification was performed using the comparative threshold cycle $\Delta\Delta C_t$ method to calculate the fold changes between the TCP, PE, and PET samples.

2. 2.4 PCR array for apoptosis receptors gene expression

Total RNA (2µg) was reverse transcribed in a 20µl reaction mixture using RT² First Strand Kit (Qiangen). The cDNA was subjected to gene array plate to examine the expression of eighty-four genes that are involved in the apoptotic pathway. All cDNA was used for qRT-PCR on the MicroAmp™Optical 96-well reaction plate system. The total reaction mix consists of 1339 µl RNase-free water,1350 µl RT^2 SYBR Green Mastermix (2X), and 20 µl cDNA sample. A 25 µl were loaded to each well of the 96-well plate. Gene expression detection was done by

qRT-PCR using thermal cycling. Apoptosis gene expression array plate (Qiangen) is shown in Table 2.1. The thermocycler was programed as follows: 95°C for 10 minutes, 40 cycles of denaturation at 95°C for 15 seconds, and annealing/extension at 60°C for 1 minute. The relative expression of each gene was normalized against β-actin. Quantification was performed using the comparative threshold cycle $\Delta\Delta C_t$ method and calculating the fold changes between the TCP and PET samples.

2. 2. 5 Statistical analysis

All data shown are Mean±SD of three runs. Student's t-test using two-tailed algorithm was calculated and *p*-values≤0.05 were considered statistically significant.

2.3 RESULTS AND DISCUSSION

2.3.1 Enhanced pancreatic β-cells proliferation by porous-structured substrates

To examine whether or not a porous membrane substrate can provide better culture environment for β-cell proliferation, β-cells were seeded on a porous PE and PET membrane substrate, respectively. Cell grown on a conventional tissue culture polystyrene plate (TCP) served as a control for comparison. As shown in Fig. 2.1D, β-cells grew faster on PET membrane compared with cells cultured on TCP. The cell doubling time was 38.6±0.5 h when cultured on PET membrane with pore size of 1μm; while it was 64.7±0.4 h when cell cultured on TCP plate and 62±0.4 h when cultured on PE membrane. Moreover, β-cells tend to form larger colonies on the PET membrane substrate compared to that of TCP plates (Figure 2.1 A-C). These experimental results suggested that the topographical and chemical properties of a membrane substrate considerably influence β-cell proliferation.

2.3.2 Insulin gene expression level in pancreatic β-cells grown on membrane substrates

Having verified β-cell proliferation, we next examined whether or not membrane substrates improve cell functionality. Insulin gene expression level for cells grown on PE and PET was detected through qRT-PCR assay and compared with those grown on TCP cultures. As shown on Fig. 2.2, the insulin gene expression in cells cultured on PET membrane was thirteen-fold higher than those on TCP cultures with *p*-value 0.02. However, the levels of insulin gene expression are similar in cells grown on PE and TCP. The experimental result indicates a significance enhancement in the insulin gene expression when cells are grown on PET membrane substrate.

2.3.3 Regulation of apoptosis pathway in pancreatic β-cells grown on PET membrane substrate

To investigate the mechanism of the enhanced cell proliferation and insulin production using PET membrane substrate, the expression profile of eighty-four genes that are involved in the apoptotic pathway were measured by qRT-PCR.

Apoptosis pathway is well known pathway to explain cell proliferation and programed cell death mechanism. In this process, unwanted cells or any detrimental cells will be disposed to prevent the development of an inflammatory response. There are two distinct pathways for apoptosis, an “extrinsic” and an “intrinsic” pathway (Parrish et al., 2013). In both pathways, signaling result in the activation of a family of Cys (cysteine) proteases, named Caspases, that act in a proteolytic cascade dismantle and remove the dying cell (Parrish et al., 2013). Extrinsic pathway is initiated by the activation of death receptors, such as Fas, Apo2/Apo3 and TNFR1, through its ligands FasL, Apo2L/Apo3L, and TNFα subsequently. These cell surface receptors belong to the superfamily of tumor necrosis factor receptors (TNFR), which are characterized by Cys-rich extracellular domain and a homologues intracellular domain known as death domain

(DD). Adapter molecule containing DD interacts with death receptor and transmits the apoptotic signals to initiator pro-caspases and results in its activation through a proteolytic cleavage.

In this study, the PET substrate is subjected to characterize the signaling pathway involved in enhancing cell proliferation and inhibiting cell apoptosis. Results showed that several genes are up regulated as shown in Figure 2.3, whereas other genes were down-regulated (Fig 2.4). As mentioned previously, Insulin stimulate Akt signaling pathway which are responsible of generating cell signals for cell replication. In addition, several anti-apoptotic genes showed a significant enhancement in PET culture, such as Bcl2, Bcl2l1, and Bcl2l2. These genes prevent apoptosis signaling through competing with Bad and Bax gene in binding to mitochondrial membrane. In addition, TNF plays dual roles in the cell proliferation process. Next to its ability to induce apoptosis, it has the ability to transduce cell survival signals by activating the transcription factor NF-kappa B.It has been observed that when the initial plasma membrane bound complex (complex I) consisting of tumor necrosis factor receptor (TNFR1), the TNFR-associated death domain (TRADD), the receptor interacting protein (RIP1), and TNF receptor-associated factor-2 (TRAF2), activation of NF-kappa B complex I is formed (Figure 2.5) (Micheau & Tschopp, 2003). As a result, complex II consisting of TRADD and RIP1 bind with FADD and caspase-8 failed to assemble by NF-kappa B feedback inhibition (Natoli et al., 1997). Figure 2.4 showed a significant down regulation of caspase 3, caspase 6, caspase 7, caspase 8, caspase 9, Bax, Bad, Bak1, Bid, Fadd, and Apaf1 have occurred in the cells growing on the PET membrane, indicating that the membrane offers better niche by inducing survival signals and suppressing apoptosis through anti-apoptotic modulators signaling feedback inhibition.

2.4 CONCLUSION AND FUTURE WORK

In this work, the down regulation of apoptosis related genes detected in pancreatic β-cells supports our hypothesis of using porous membrane substrates to improve the proliferation of pancreatic β-cells.

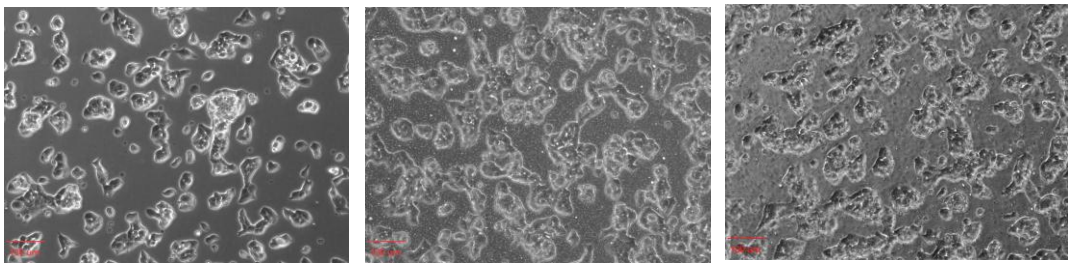
Several experiments can be conducted in the future. mRNA expression level may not correlate to the protein expression level. Thus, it is necessary to confirm protein expression level by Western blotting. In addition, the regulation mechanism remains unclear and need more investigation. Finally, detecting the expression level of adhesion molecules will give more detailed information on the cell adhesion mechanism involved in cell-PET attachment. This could help for further improvement to generate a better biodegradable material to be used in the cell engraftment in the future.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Abl1	Aifm1	Akt1	Anxa5	Apaf1	Api5	Atf5	Bad	Bag1	Bag3	Bak1	Bax
B	Bcl10	Bcl2	Bcl2a1a	Bcl2l1	Bcl2l10	Bcl2l11	Bcl2l2	Bid	Birc2	Birc3	Birc5	Bnip2
C	Bnip3	Bnip3l	Bok	Card10	Casp1	Casp12	Casp14	Casp2	Casp3	Casp4	Casp6	Casp7
D	Casp8	Casp9	Cd40	Cd40lg	Cd70	Cflar	Cidea	Cideb	Cradd	Dad1	Dapk1	Dffa
E	Dffb	Diablo	Fadd	Fas	Fasl	Gadd45a	Igf1r	Il10	Lhx4	Ltbr	Mapk1	Mcl1
F	Naip1	Naip2	Nfkb1	Nme5	Nod1	Nol3	Polb	Prdx2	Pycard	Ripk1	Tnf	Tnfrsf 10b
G	Tnfrsf11b	Tnfrsf1a	Tnfsf10	Tnfsf12	Traf1	Traf2	Traf3	Trp53	Trp53bp2	Trp63	Trp73	Xiap
H	Actb HKG	B2m HKG	Gapdh HKG	Gusb HKG	Hsp90ab1 HKG	MGDC	RTC	RTC	RTC	PPC	PPC	PPC

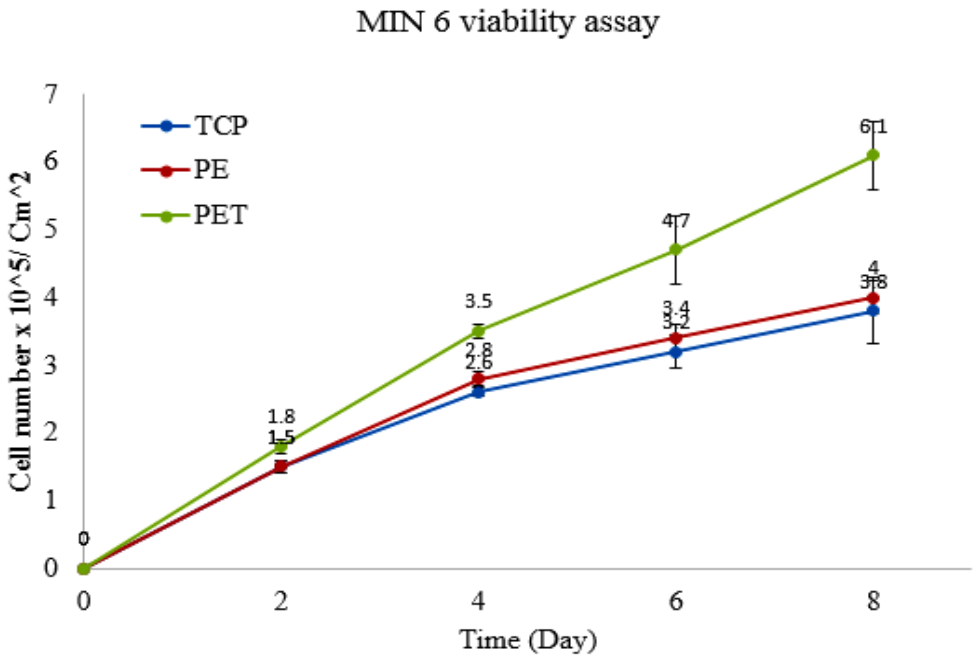
Table 2.1: Apoptosis receptors via 96-well plate.

A **B** **C**

TCP **PE** **PET**



C



D

	TCP	**PE***	**PET***
Doubling Time*[h]	64.7±0.3	62.2±0.3	38.6±0.4
Porosity [pores/cm^2]	0	4×10^6	2×10^6

Figure 2.1 β-cell proliferation on various substrates.β-cell**s** were seeded onto tissue culture polystyrene plate (TCP) **(A)**, 0.4 µm PE **(B)**, and 1µm PET **(C)** membrane substrate in DMEM medium. The micrographic images of cell attachment were taken at day2 after seeding. Scale bar 100 µm. **(D)** Time courses of β-cell proliferation on various substrates. Data were presented as mean± SD. All experiments were carried out three times. * PE *p*-value 0.7, PET *p*-value 0.03.

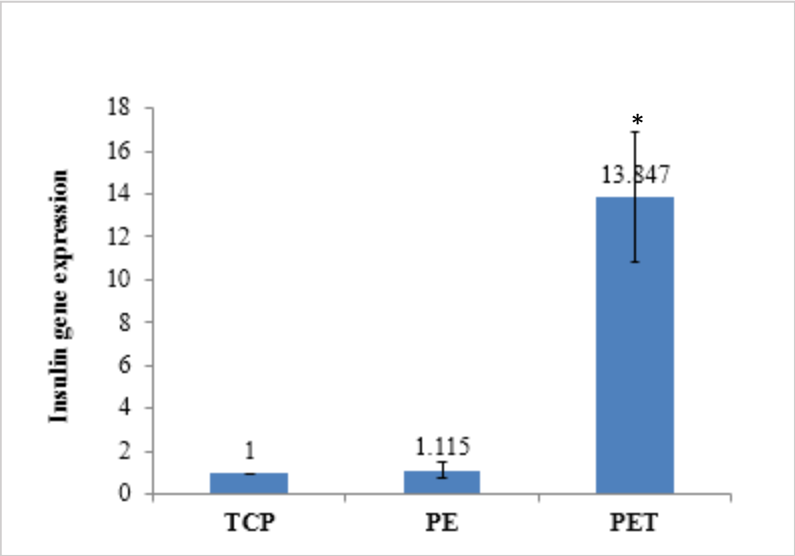
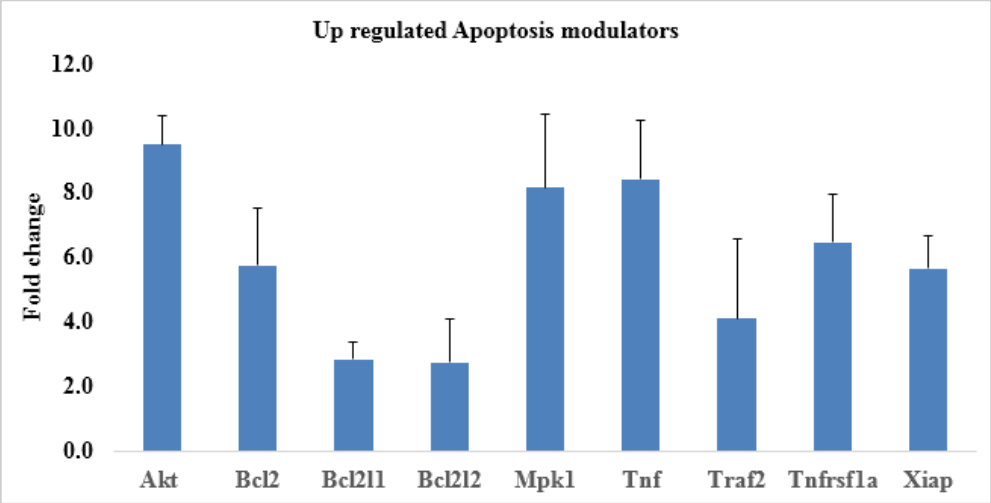
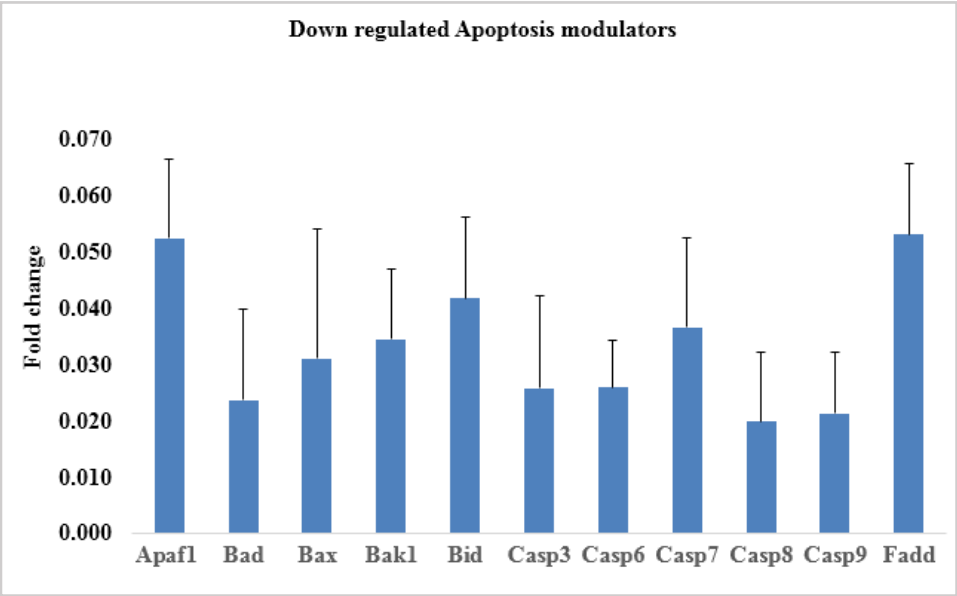


Figure 2.2 Insulin gene expression was detected in cells grown on various membrane substrates at day eight compared to TCP and normalized by β-actin expression. Data were presented as mean±SD. All experiments were carried out three times. * PET (*p*-value 0.02)



Gene ID	Fold change	*p*-value
Akt	9.5±0.9	0.047
BcL2	5.7±1.8	0.012
Bcl2l1	2.8±0.5	0.011
Bcl2l2	2.7±1.4	0.029
Mpk1	8.2±2.3	0.011
Tnf	8.4±1.8	0.034
Traf2	4.1±2.5	0.015
Tnfrsf1a	6.5±1.50	0.028

Figure 2.3 Relative up regulated gene expression levels in cells grown on PET membrane substrate at day eight compared to TCP. Data were presented as mean±SD. All experiments were carried out three times and normalized by β-actin gene expression. Abbreviations: Akt, Thymoma viral proto-oncogene 1; BCL2, B-cell leukemia/lymphoma 2; Bcl2l1, Bcl2-like 1; Bcl2l2, Bcl2-like 2; Mapk1, Mitogen-activated protein kinase 1; Tnf, Tumor necrosis factor; Traf2; Tnf receptor-associated factor 2; Tnfrsf1a, Tumor necrosis factor receptor superfamily, member 1a.



Gene ID	Fold change	*p*-value
Apaf1	0.053±0.014	0.0046
Bad	0.024±0.016	0.024
Bax	0.031±0.023	0.016
Bak1	0.042±0.013	0.0023
Bid	0.026±0.014	0.001
Casp3	0.026±0.017	0.001
Casp6	0.037±0.008	0.01
Casp7	0.020±0.016	0.0001
Casp8	0.021±0.012	0.015
Casp9	0.024±0.011	0.012
Fadd	0.053±0.013	0.011

Figure 2.4 Relative down regulated gene expression levels in cells grown on PET membrane substrate at day eight compared to TCP. Data were presented as mean±SD. All experiments were carried out three times and normalized by β-actin gene expression. Abbreviations: Apaf1, Apoptotic peptidase activating factor 1; Bad, BCL2-associated agonist of cell death; Bax, Bcl2-associated X protein; Bak1, BCL2-antagonist/killer 1; Bid, BH3 interacting domain death agonist; Casp3, Caspase 3; Casp 6, Caspase 6; Casp7, Caspase 7; Casp8, Caspase 8; Casp 9, Caspase 9; Fadd, Fas (TNFRSF6)-associated via death domain.

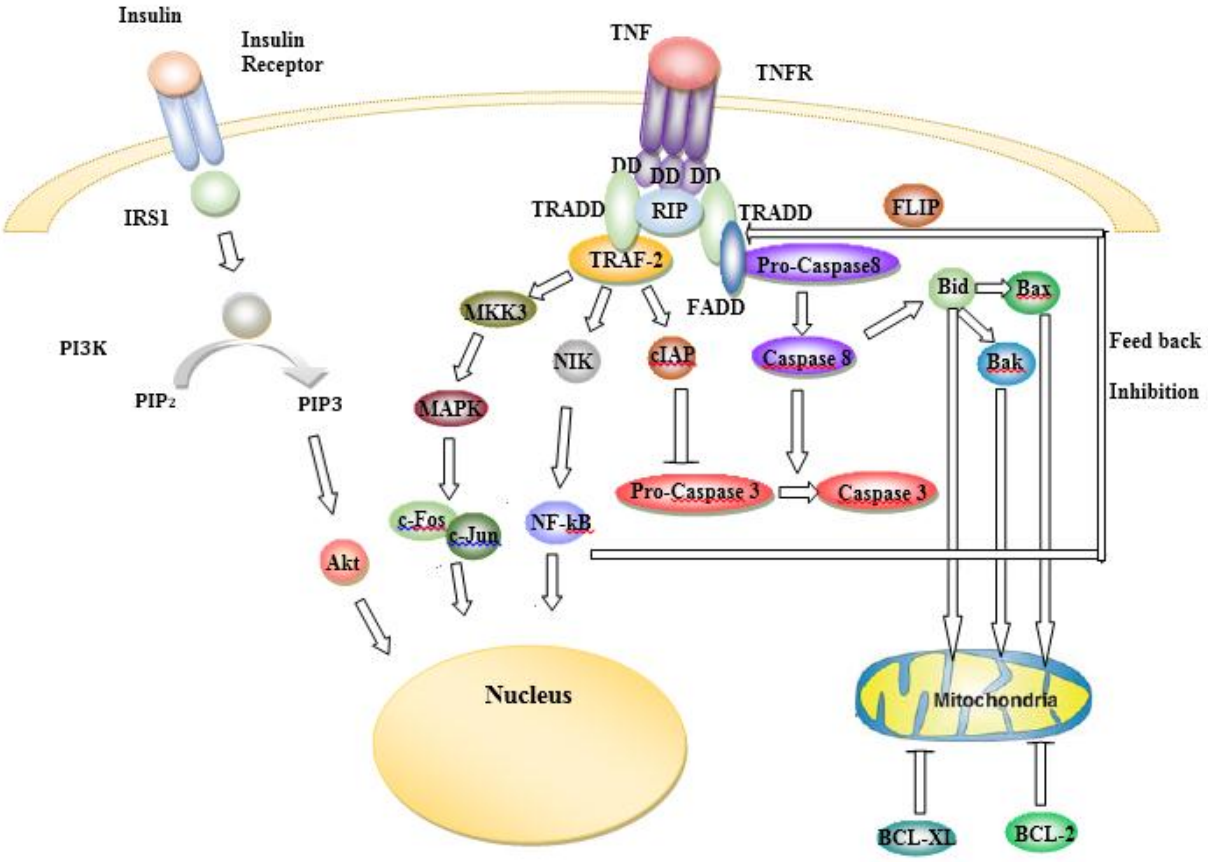


Figure 2.5 Signaling pathways involved in the pancreatic β-cell proliferation.

LITERATURE CITED

Abrams, G. A., Goodman, S. L., Nealey, P. F., Franco, M., & Murphy, C. J. (2000). Nanoscale topography of the basement membrane underlying the corneal epithelium of the rhesus macaque. *Cell and Tissue Research, 299*(1), 39-46.

Abrams, G. A., Schaus, S. S., Goodman, S. L., Nealey, P. F., & Murphy, C. J. (2000). Nanoscale topography of the corneal epithelial basement membrane and descemet's membrane of the human. *Cornea, 19*(1), 57-64.

Aguirre, V., Uchida, T., Yenush, L., Davis, R., & White, M. F. (2000). The c-jun NH(2)-terminal kinase promotes insulin resistance during association with insulin receptor substrate-1 and phosphorylation of ser(307). *The Journal of Biological Chemistry, 275*(12), 9047-9054.

Ahrén, B. (2000). Autonomic regulation of islet hormone secretion--implications for health and disease. *Diabetologia, 43*(4), 393-410.

Alonso, L. C., Yokoe, T., Zhang, P., Scott, D. K., Kim, S. K., O'Donnell, C.,P., & Garcia-Ocaña, A. (2007). Glucose infusion in mice: A new model to induce beta-cell replication. *Diabetes, 56*(7), 1792-1801.

Alwan, A. (2011). In Alwan A. (Ed.), *Global status report on noncommunicable diseases 2010*. Geneva; Switzerland: World Health Organization.

Anderson, M. S., & Bluestone, J. A. (2005). THE NOD MOUSE: A model of immune dysregulation. *Annual Review of Immunology, 23*(1), 447-485.

Beattie, G. M., Rubin, J. S., Mally, M. I., Otonkoski, T., & Hayek, A. (1996). Regulation of proliferation and differentiation of human fetal pancreatic islet cells by extracellular matrix, hepatocyte growth factor, and cell-cell contact. *Diabetes, 45*(9), 1223-1228.

Bernal-Mizrachi, E., Wen, W., Stahlhut, S., Welling, C. M., & Permutt, M. A. (2001). Islet beta cell expression of constitutively active Akt1/PKB alpha induces striking hypertrophy, hyperplasia, and hyperinsulinemia. *The Journal of Clinical Investigation, 108*(11), 1631-1638.

Bernal-Mizrachi, E., Wice, B., Inoue, H., & Permutt, M. A. (2000). Activation of serum response factor in the depolarization induction of egr-1 transcription in pancreatic islet beta-cells. *The Journal of Biological Chemistry, 275*(33), 25681-25689.

Bernard, A. B., Lin, C., & Anseth, K. S. (2012). A microwell cell culture platform for the aggregation of pancreatic beta-cells. *Tissue Engineering Part C-Methods, 18*(8), 583-592. doi:10.1089/ten.tec.2011.0504

Bernard, C., Ktorza, A., Saulnier, C., & Berthault, M. F. (1999). Neogenesis vs. apoptosis as main components of pancreatic beta cell mass changes in glucose-infused normal and mildly diabetic adult rats. *FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology, 13*(10), 1195-1205.

Bosco, D., Meda, P., Halban, P. A., & Rouiller, D. G. (2000). Importance of cell-matrix interactions in rat islet beta-cell secretion in vitro: Role of alpha6beta1 integrin. *Diabetes, 49*(2), 233-243.

Bruning, J., Winnay, J., BonnerWeir, S., Taylor, S., Accili, D., & Kahn, C. (1997). Development of a novel polygenic model of NIDDM in mice heterozygous for IR and IRS-1 null alleles. *Cell, 88*(4), 561-572. doi:10.1016/S0092-8674(00)81896-6

Buteau, J., El-Assaad, W., Rhodes, C. J., Rosenberg, L., Joly, E., & Prentki, M. (2004). Glucagon-like peptide-1 prevents beta cell glucolipotoxicity. *Diabetologia, 47*(5), 806-815.

Buteau, J., Foisy, S., Joly, E., & Prentki, M. (2003). Glucagon-like peptide 1 induces pancreatic beta-cell proliferation via transactivation of the epidermal growth factor receptor. *Diabetes, 52*(1), 124-132.

Cantley, J., Grey, S. T., Maxwell, P. H., & Withers, D. J. (2010). The hypoxia response pathway and ß-cell function. *Diabetes, Obesity & Metabolism, 12 Suppl 2*, 159-167. doi:10.1111/j.1463-1326.2010.01276.x

Cantley, J., Selman, C., Shukla, D., Abramov, A. Y., Forstreuter, F., Esteban, M. A., . . . Withers, D. J. (2009). Deletion of the von hippel-lindau gene in pancreatic beta cells impairs glucose homeostasis in mice. *The Journal of Clinical Investigation, 119*(1), 125-135. doi:10.1172/JCI26934

Centers for Disease Control and Prevention. (2011). *National Diabetes Fact Sheet: National Estimates and General Information on Diabetes and Prediabetes in the United States,*

Chang-Chen, K., Mullur, R., & Bernal-Mizrachi, E. (2008). Beta-cell failure as a complication of diabetes. *Reviews in Endocrine & Metabolic Disorders, 9*(4), 329-343. doi:10.1007/s11154-008-9101-5

Cheng, K., Delghingaro-Augusto, V., Nolan, C. J., Turner, N., Hallahan, N., Andrikopoulos, S., & Gunton, J. E. (2012). High passage MIN6 cells have impaired insulin secretion with impaired glucose and lipid oxidation. *Plos One, 7*(7), 1-12. doi:10.1371/journal.pone.0040868

Christensen, M., Vedtofte, L., Holst, J. J., Vilsbøll, T., & Knop, F. K. (2011). Glucose-dependent insulinotropic polypeptide: A bifunctional glucose-dependent regulator of glucagon and insulin secretion in humans. *Diabetes, 60*(12), 3103-3109. doi:10.2337/db11-0979

Da, S. X., Leclerc, I., Varadi, A., Tsuboi, T., Moule, S. K., & Rutter, G. A. (2003). Role for AMP-activated protein kinase in glucose-stimulated insulin secretion and preproinsulin gene expression. *The Biochemical Journal, 371*, 761-774.

Dai, C., Li, Y., Yang, J., & Liu, Y. (2003). Hepatocyte growth factor preserves beta cell mass and mitigates hyperglycemia in streptozotocin-induced diabetic mice. *The Journal of Biological Chemistry, 278*(29), 27080-27087.

Daneman, D. (2006). Type 1 diabetes. *The Lancet, 367*(9513), 847-858. doi:10.1016/S0140-6736(06)68341-4

Drucker, D. J. (2006). The biology of incretin hormones. *Cell Metabolism, 3*(3), 153-165.

Drucker, D. J. (2007). The role of gut hormones in glucose homeostasis. *The Journal of Clinical Investigation, 117*(1), 24-32.

Eberhard, D., & Lammert, E. (2009). The pancreatic ß-cell in the islet and organ community. *Current Opinion in Genetics & Development, 19*(5), 469-475. doi:10.1016/j.gde.2009.07.003

Fan, R., Li, X., Gu, X., Chan, J. C. N., & Xu, G. (2010). Exendin-4 protects pancreatic beta cells from human islet amyloid polypeptide-induced cell damage: Potential involvement of AKT and mitochondria biogenesis. *Diabetes, Obesity & Metabolism, 12*(9), 815-824. doi:10.1111/j.1463-1326.2010.01238.x

Farilla, L., Bulotta, A., Hirshberg, B., Li Calzi, S., Khoury, N., Noushmehr, H., . . . Perfetti, R. (2003). Glucagon-like peptide 1 inhibits cell apoptosis and improves glucose responsiveness of freshly isolated human islets. *Endocrinology, 144*(12), 5149-5158.

Fraker, C. A., Alvarez, S., Papadopoulos, P., Giraldo, J., Gu, W., Ricordi, C., . . . Domínguez-Bendala, J. (2007). Enhanced oxygenation promotes beta-cell differentiation in vitro. *Stem Cells (Dayton, Ohio), 25*(12), 3155-3164.

Freemark, M., Avril, I., Fleenor, D., Driscoll, P., Petro, A., Opara, E., . . . Kelly, P. A. (2002). Targeted deletion of the PRL receptor: Effects on islet development, insulin production, and glucose tolerance. *Endocrinology, 143*(4), 1378-1385.

Fridlyand, L. E., & Philipson, L. H. (2004). Does the glucose-dependent insulin secretion mechanism itself cause oxidative stress in pancreatic beta-cells? *Diabetes, 53*(8), 1942-1948.

Gahr, S., Merger, M., Bollheimer, L. C., Hammerschmied, C. G., Schölmerich, J., & Hügl, ,S.R. (2002). Hepatocyte growth factor stimulates proliferation of pancreatic beta-cells particularly in the presence of subphysiological glucose concentrations. *Journal of Molecular Endocrinology, 28*(2), 99-110.

García-Ocaña, A., Vasavada, R. C., Cebrian, A., Reddy, V., Takane, K. K., López-Talavera, ,J.C., & Stewart, A. F. (2001). Transgenic overexpression of hepatocyte growth factor in the beta-cell markedly improves islet function and islet transplant outcomes in mice. *Diabetes, 50*(12), 2752-2762.

Garofalo, R. S., Orena, S. J., Rafidi, K., Torchia, A. J., Stock, J. L., Hildebrandt, A. L., . . . Coleman, K. G. (2003). Severe diabetes, age-dependent loss of adipose tissue, and mild growth deficiency in mice lacking Akt2/PKB beta. *The Journal of Clinical Investigation, 112*(2), 197-208.

Gasparo, M. D., Milner, G. R., Norris, P. D., & Milner, R. D. G. (1978). Effect of glucose and amino acids on foetal rat pancreatic growth and insulin secretion in vitro. *Journal of Endocrinology, 77*(2), 241-248.

George, M., Ayuso, E., Casellas, A., Costa, C., Devedjian, J. C., & Bosch, F. (2002). Beta cell expression of IGF-I leads to recovery from type 1 diabetes. *The Journal of Clinical Investigation, 109*(9), 1153-1163.

Grill, V. (2001). Overstimulation and beta-cell function. *Diabetes, 50*, S122.

GROBSTEIN, C. (1953). Morphogenetic interaction between embryonic mouse tissues separated by a membrane filter. *Nature, 172*(4384), 869-870.

Gunton, J. E., Kulkarni, R. N., Yim, S., Okada, T., Hawthorne, W. J., Tseng, Y., . . . Kahn, C. R. (2005). Loss of ARNT/HIF1beta mediates altered gene expression and pancreatic-islet dysfunction in human type 2 diabetes. *Cell, 122*(3), 337-349.

Harmon, J. S., Stein, R., & Robertson, R. P. (2005). Oxidative stress-mediated, post-translational loss of MafA protein as a contributing mechanism to loss of insulin gene expression in glucotoxic beta cells. *The Journal of Biological Chemistry, 280*(12), 11107-11113.

Heit, J. J., Apelqvist, A. A., Gu, X., Winslow, M. M., Neilson, J. R., Crabtree, G. R., & Kim, S. K. (2006). Calcineurin/NFAT signalling regulates pancreatic beta-cell growth and function. *Nature, 443*(7109), 345-349.

Heit, J. J., Karnik, S. K., & Kim, S. K. (2006). Intrinsic regulators of pancreatic β-cell proliferation. *Annual Review of Cell & Developmental Biology, 22*(1), 311-338. doi:10.1146/annurev.cellbio.22.010305.104425

Hiscox, A. M., Stone, A. L., Limesand, S., Hoying, J. B., & Williams, S. K. (2008). An islet-stabilizing implant constructed using a preformed vasculature. *Tissue Engineering.Part A, 14*(3), 433-440. doi:10.1089/tea.2007.0099

Hogg, J., Han, V. K., Clemmons, D. R., & Hill, D. J. (1993). Interactions of nutrients, insulin-like growth factors (IGFs) and IGF-binding proteins in the regulation of DNA synthesis by isolated fetal rat islets of langerhans. *The Journal of Endocrinology, 138*(3), 401-412.

Holz, G. G., Leech, C. A., Heller, R. S., Castonguay, M., & Habener, J. F. (1999). cAMP-dependent mobilization of intracellular Ca2+ stores by activation of ryanodine receptors in pancreatic beta-cells. A Ca2+ signaling system stimulated by the insulinotropic hormone glucagon-like peptide-1-(7-37). *The Journal of Biological Chemistry, 274*(20), 14147-14156.

Hoorens, A., Van, d. C., Klöppel, G., & Pipeleers, D. (1996). Glucose promotes survival of rat pancreatic beta cells by activating synthesis of proteins which suppress a constitutive apoptotic program. *The Journal of Clinical Investigation, 98*(7), 1568-1574.

Huei-Min Lin, Ji-Hyeon Lee, Yadav, H., Kamaraju, A. K., Liu, E., Zhigang, D., . . . Rane, S. G. (2009). Transforming growth factor-β/Smad3 signaling regulates insulin gene transcription and pancreatic islet β-cell function. *Journal of Biological Chemistry, 284*(18), 12246-12257. doi:10.1074/jbc.M805379200

Issa, C. M., & Azar, S. T. (2012). Possible role of GLP-1 and its agonists in the treatment of type 1 diabetes mellitus. *Current Diabetes Reports, 12*(5), 560-567. doi:10.1007/s11892-012-0291-6

Jhala, U. S., Canettieri, G., Screaton, R. A., Kulkarni, R. N., Krajewski, S., Reed, J., . . . Montminy, M. (2003). cAMP promotes pancreatic beta-cell survival via CREB-mediated induction of IRS2. *Genes & Development, 17*(13), 1575-1580.

Juliano, R. L., & Haskill, S. (1993). Signal transduction from the extracellular matrix. *The Journal of Cell Biology, 120*(3), 577-585.

Kaido, T., Yebra, M., Cirulli, V., Rhodes, C., Diaferia, G., & Montgomery, A. M. (2006). Impact of defined matrix interactions on insulin production by cultured human beta-cells: Effect on insulin content, secretion, and gene transcription. *Diabetes, 55*(10), 2723-2729.

Kaiser, G., Gerst, F., Michael, D., Berchtold, S., Friedrich, B., Strutz-Seebohm, N., . . . Ullrich, S. (2013). Regulation of forkhead box O1 (FOXO1) by protein kinase B and glucocorticoids: Different mechanisms of induction of beta cell death in vitro. *Diabetologia,*

Kaneto, H., Xu, G., Fujii, N., Kim, S., Bonner-Weir, S., & Weir, G. C. (2002). Involvement of c-jun N-terminal kinase in oxidative stress-mediated suppression of insulin gene expression. *The Journal of Biological Chemistry, 277*(33), 30010-30018.

Kawamori, D., Kaneto, H., Nakatani, Y., Matsuoka, T., Matsuhisa, M., Hori, M., & Yamasaki, Y. (2006). The forkhead transcription factor Foxo1 bridges the JNK pathway and the transcription factor PDX-1 through its intracellular translocation. *The Journal of Biological Chemistry, 281*(2), 1091-1098.

Kawasaki, E., Abiru, N., & Eguchi, K. (2004). Prevention of type 1 diabetes: From the view point of beta cell damage. *Diabetes Research and Clinical Practice, 66 Suppl 1*, S27-S32.

Kelly, C., Parke, H. G., McCluskey, J. T., Flatt, P. R., & McClenaghan, N. H. (2010). The role of glucagon- and somatostatin-secreting cells in the regulation of insulin release and beta-cell function in heterotypic pseudoislets. *Diabetes/metabolism Research and Reviews, 26*(7), 525-533. doi:10.1002/dmrr.1111

Kitamura, T., Nakae, J., Kitamura, Y., Kido, Y., Biggs, W. H., Wright, C. V. E., . . . Accili, D. (2002). The forkhead transcription factor Foxo1 links insulin signaling to Pdx1 regulation of pancreatic B cell growth. *Journal of Clinical Investigation, 110*(12), 1839.

Klöppel, G., Löhr, M., Habich, K., Oberholzer, M., & Heitz, P. U. (1985). Islet pathology and the pathogenesis of type 1 and type 2 diabetes mellitus revisited. *Survey and Synthesis of Pathology Research, 4*(2), 110-125.

Kobayashi, M., Kikuchi, O., Sasaki, T., Kim, H., Yokota-Hashimoto, H., Lee, Y., . . . Kitamura, T. (2012). FoxO1 as a double-edged sword in the pancreas: Analysis of pancreas- and [beta]-cell-specific FoxO1 knockout mice. *American Journal of Physiology, 302*(5), E603.

Krishnamurthy, M., Li, J., Al-Masri, M., & Wang, R. (2008). Expression and function of alphabeta1 integrins in pancretic beta (INS-1) cells. *Journal of Cell Communication and Signaling, 2*(3-4), 67-79. doi:10.1007/s12079-008-0030-6

Kulkarni, R. N., Brüning, ,J.C., Winnay, J. N., Postic, C., Magnuson, M. A., & Kahn, C. R. (1999). Tissue-specific knockout of the insulin receptor in pancreatic beta cells creates an insulin secretory defect similar to that in type 2 diabetes. *Cell, 96*(3), 329-339.

Kulkarni, R. N. (2005). New insights into the roles of insulin/IGF-I in the development and maintenance of beta-cell mass. *Reviews in Endocrine & Metabolic Disorders, 6*(3), 199-210.

Kushner, J. A., Ye, J., Schubert, M., Burks, D. J., Dow, M. A., Flint, C. L., . . . White, M. F. (2002). Pdx1 restores beta cell function in Irs2 knockout mice. *The Journal of Clinical Investigation, 109*(9), 1193-1201.

Lawrence, M. C., Bhatt, H. S., Watterson, J. M., & Easom, R. A. (2001). Regulation of insulin gene transcription by a ca(2+)-responsive pathway involving calcineurin and nuclear factor of activated T cells. *Molecular Endocrinology (Baltimore, Md.), 15*(10), 1758-1767.

Lawrence, M. C., Bhatt, H. S., & Easom, R. A. (2002). NFAT regulates insulin gene promoter activity in response to synergistic pathways induced by glucose and glucagon-like peptide-1. *Diabetes, 51*(3), 691-698.

Li, Y., Cao, X., Li, L., Brubaker, P. L., Edlund, H., & Drucker, D. J. (2005). Beta-cell Pdx1 expression is essential for the glucoregulatory, proliferative, and cytoprotective actions of glucagon-like peptide-1. *Diabetes, 54*(2), 482-491.

Lin, C., & Anseth, K. S. (2011). Cell-cell communication mimicry with poly(ethylene glycol) hydrogels for enhancing beta-cell function. *Proceedings of the National Academy of Sciences of the United States of America, 108*(16), 6380-6385.

Lingohr, M. K., Dickson, L. M., McCuaig, J. F., Hugl, S. R., Twardzik, D. R., & Rhodes, C. J. (2002). Activation of IRS-2-mediated signal transduction by IGF-1, but not TGF-alpha or EGF, augments pancreatic beta-cell proliferation. *Diabetes, 51*(4), 966-976.

Lock, L. T., Laychock, S. G., & Tzanakakis, E. S. (2011). Pseudoislets in stirred-suspension culture exhibit enhanced cell survival, propagation and insulin secretion. *Journal of Biotechnology, 151*(3), 278-286. doi:10.1016/j.jbiotec.2010.12.015

Maedler, K., Sergeev, P., Ris, F., Oberholzer, J., Joller-Jemelka, H., Spinas, G. A., . . . Donath, M. Y. (2002). Glucose-induced beta cell production of IL-1beta contributes to glucotoxicity in human pancreatic islets. *The Journal of Clinical Investigation, 110*(6), 851-860.

Matsumoto, K., & Nakamura, T. (1996). Emerging multipotent aspects of hepatocyte growth factor. *Journal of Biochemistry, 119*(4), 591-600.

McKinnon, C. M., & Docherty, K. (2001). Pancreatic duodenal homeobox-1, PDX-1, a major regulator of beta cell identity and function. *Diabetologia, 44*(10), 1203-1214.

Merani, S., & Shapiro, A. M. J. (2006). Current status of pancreatic islet transplantation. *Clinical Science (London, England: 1979), 110*(6), 611-625.

Micheau, O., & Tschopp, J. ü. (2003). Induction of TNF receptor I-mediated apoptosis via two sequential signaling complexes. *Cell, 114*(2), 181-190.

Miyazaki, J., Araki, K., Yamato, E., Ikegami, H., Asano, T., Shibasaki, Y., . . . Yamamura, K. (1990). Establishment of a pancreatic beta cell line that retains glucose-inducible insulin secretion: Special reference to expression of glucose transporter isoforms. *Endocrinology, 127*(1), 126-132.

Nagata, N., Iwanaga, A., Inoue, K., & Tabata, Y. (2002). Co-culture of extracellular matrix suppresses the cell death of rat pancreatic islets. *Journal of Biomaterials Science -- Polymer Edition, 13*(5), 579-590. doi:10.1163/15685620260178418

Nakae, J., Biggs,William H.,,3rd, Kitamura, T., Cavenee, W. K., Wright, C. V. E., Arden, K. C., & Accili, D. (2002). Regulation of insulin action and pancreatic beta-cell function by mutated alleles of the gene encoding forkhead transcription factor Foxo1. *Nature Genetics, 32*(2), 245-253.

Natoli, G., Costanzo, A., Ianni, A., Templeton, D. J., Woodgett, J. R., Balsano, C., & Levrero, M. (1997). Activation of SAPK/JNK by TNF receptor 1 through a noncytotoxic TRAF2-dependent pathway. *Science (New York, N.Y.), 275*(5297), 200-203.

Nielsen, J. H. (2001). Regulation of beta-cell mass by hormones and growth factors. *Diabetes, 50,* S25.

Nikolova, G., Jabs, N., Konstantinova, I., Domogatskaya, A., Tryggvason, K., Sorokin, L., . . . Lammert, E. (2006). The vascular basement membrane: A niche for insulin gene expression and ß cell proliferation. *Developmental Cell, 10*(3), 397-405. doi:10.1016/j.devcel.2006.01.015

Nir, T., Melton, D. A., & Dor, Y. (2007). Recovery from diabetes in mice by beta cell regeneration. *The Journal of Clinical Investigation, 117*(9), 2553-2561.

Nobukini, T., & Thomas, G. (2004). The mTOR/S6K signalling pathway: The role of the TSC1/2 tumour suppressor complex and the proto-oncogene rheb. *Novartis Foundation Symposium, 262*, 148.

Nyblom, H. K., Sargsyan, E., & Bergsten, P. (2008). AMP-activated protein kinase agonist dose dependently improves function and reduces apoptosis in glucotoxic beta-cells without

changing triglyceride levels. *Journal of Molecular Endocrinology, 41*(3), 187-194. doi:10.1677/JME-08-0006

Nyblom, H. K., Thorn, K., Ahmed, M., & Bergsten, P. (2006). Mitochondrial protein patterns correlating with impaired insulin secretion from INS-1E cells exposed to elevated glucose concentrations. *Proteomics, 6*(19), 5193-5198.

Ohsugi, M., Cras-Meneur, C., Zhou, Y., Bernal-Mizrachi, E., Johnson, J., Luciani, D., . . . Permutt, M. (2005). Reduced expression of the insulin receptor in mouse insulinoma (MIN6) cells reveals multiple roles of insulin signaling in gene expression, proliferation, insulin content, and secretion. *Journal of Biological Chemistry, 280*(6), 4992-5003. doi:10.1074/jbc.M411727200

Ohsugi, M., Cras-Méneur, C., Zhou, Y., Warren, W., Bernal-Mizrachi, E., & Permutt, M. A. (2004). Glucose and insulin treatment of insulinoma cells results in transcriptional regulation of a common set of genes. *Diabetes, 53*(6), 1496-1508.

Paris, M., Bernard-Kargar, C., Berthault, M., Bouwens, L., & Ktorza, A. (2003). Specific and combined effects of insulin and glucose on functional pancreatic beta-cell mass in vivo in adult rats. *Endocrinology, 144*(6), 2717-2727.

Park, S., Dong, X., Fisher, T. L., Dunn, S., Omer, A. K., Weir, G., & White, M. F. (2006). Exendin-4 uses Irs2 signaling to mediate pancreatic β cell growth and function. *Journal of Biological Chemistry, 281*(2), 1159-1168. doi:10.1074/jbc.M508307200

Parnaud, G., Hammar, E., Ribaux, P., Donath, M. Y., Berney, T., & Halban, P. A. (2009). Signaling pathways implicated in the stimulation of beta-cell proliferation by extracellular matrix. *Molecular Endocrinology (Baltimore, Md.), 23*(8), 1264-1271. doi:10.1210/me.2009-0008

Parrish, A. B., Freel, C. D., & Kornbluth, S. (2013). Cellular mechanisms controlling caspase activation and function. *Cold Spring Harbor Perspectives in Biology, 5*(6) doi:10.1101/cshperspect.a008672

Parsons, J. A., Brelje, T. C., & Sorenson, R. L. (1992). Adaptation of islets of langerhans to pregnancy: Increased islet cell proliferation and insulin secretion correlates with the onset of placental lactogen secretion. *Endocrinology, 130*(3), 1459-1466.

Pedraza, E., Coronel, M. M., Fraker, C. A., Ricordi, C., & Stabler, C. L. (2012). Preventing hypoxia-induced cell death in beta cells and islets via hydrolytically activated, oxygen-generating biomaterials. *Proceedings of the National Academy of Sciences of the United States of America, 109*(11), 4245-4250. doi:10.1073/pnas.1113560109

Petrik, J., Pell, J. M., Arany, E., McDonald, T. J., Dean, W. L., Reik, W., & Hill, D. J. (1999). Overexpression of insulin-like growth factor-II in transgenic mice is associated with pancreatic islet cell hyperplasia. *Endocrinology, 140*(5), 2353-2363.

Pinkse, G. G. M., Bouwman, W. P., Jiawan-Lalai, R., Terpstra, O. T., Bruijn, J. A., & de Heer, E. (2006). Integrin signaling via RGD peptides and anti-beta1 antibodies confers resistance to apoptosis in islets of langerhans. *Diabetes, 55*(2), 312-317.

Poitout, V., Olson, L. K., & Robertson, R. P. (1996). Chronic exposure of betaTC-6 cells to supraphysiologic concentrations of glucose decreases binding of the RIPE3b1 insulin gene transcription activator. *The Journal of Clinical Investigation, 97*(4), 1041-1046.

Poitout, V., & Robertson, R. P. (2002). Minireview: Secondary beta-cell failure in type 2 diabetes--a convergence of glucotoxicity and lipotoxicity. *Endocrinology, 143*(2), 339-342.

Puri, S., Cano, D. A., & Hebrok, M. (2009). A role for von hippel-lindau protein in pancreatic beta-cell function. *Diabetes, 58*(2), 433-441. doi:10.2337/db08-0749

Rane, S. G., Lee, J., & Lin, H. (2006). Transforming growth factor-beta pathway: Role in pancreas development and pancreatic disease. *Cytokine & Growth Factor Reviews, 17*(1-2), 107-119.

Ribaux, P., Ehses, J. A., Lin-Marq, N., Carrozzino, F., Boeni-Schnetzler, M., Hammar, E., . . . Halban, P. A. (2007). Induction of CXCL1 by extracellular matrix and autocrine enhancement by interleukin-1 in rat pancreatic {beta}-cells. *Endocrinology, 148*(11), 5582-5590.

Rieck, S., & Kaestner, K. H. (2010). Expansion of beta-cell mass in response to pregnancy. *Trends in Endocrinology and Metabolism: TEM, 21*(3), 151-158. doi:10.1016/j.tem.2009.11.001

Robertson, R. P., & Harmon, J. S. (2006). Diabetes, glucose toxicity, and oxidative stress: A case of double jeopardy for the pancreatic islet beta cell. *Free Radical Biology & Medicine, 41*(2), 177-184.

Rutti, S., Sauter, N. S., Bouzakri, K., Prazak, R., Halban, P. A., & Donath, M. Y. (2012). In vitro proliferation of adult human beta-cells. *Plos One, 7*(4), 1-4. doi:10.1371/journal.pone.0035801

Saltiel, A. R., & Kahn, C. R. (2001). Insulin signalling and the regulation of glucose and lipid metabolism. *Nature, 414*(6865), 799-806.

Sato, Y., Endo, H., Okuyama, H., Takeda, T., Iwahashi, H., Imagawa, A., . . . Inoue, M. (2011). Cellular hypoxia of pancreatic beta-cells due to high levels of oxygen consumption for insulin secretion in vitro. *The Journal of Biological Chemistry, 286*(14), 12524-12532. doi:10.1074/jbc.M110.194738

Shalev, A., Pise-Masison, C., Radonovich, M., Hoffmann, S. C., Hirshberg, B., Brady, J. N., & Harlan, D. M. (2002). Oligonucleotide microarray analysis of intact human pancreatic islets: Identification of glucose-responsive genes and a highly regulated TGFbeta signaling pathway. *Endocrinology, 143*(9), 3695-3698.

Shapiro, A. J., Lakey, J. R., Ryan, E. A., Korbutt, G. S., Toth, E., Warnock, G. L., . . . Rajotte, R. V. (2000). Islet transplantation in seven patients with type 1 diabetes mellitus using a glucocorticoid-free immunosuppressive regimen. *The New England Journal of Medicine, 343*(4), 230-238.

Sharma, A., Olson, L. K., Robertson, R. P., & Stein, R. (1995). The reduction of insulin gene transcription in HIT-T15 beta cells chronically exposed to high glucose concentration is associated with the loss of RIPE3b1 and STF-1 transcription factor expression. *Molecular Endocrinology (Baltimore, Md.), 9*(9), 1127-1134.

SJÖHOLM, Å. (1996). Diabetes mellitus and impaired pancreatic β-cell proliferation. *Journal of Internal Medicine, 239*(3), 211-220. doi:10.1046/j.1365-2796.1996.377740000.x

Skelin, M., Rupnik, M., & Cencic, A. (2010). Pancreatic beta cell lines and their applications in diabetes mellitus research. *Altex, 27*(2), 105-113.

Skiles, M. L., Wilder, N. B., Sahai, S., & Blanchette, J. O. (2013). *Identifying HIF activity in three-dimensional cultures of islet-like clusters*. Italy: Wichtig Editore. doi:10.5301/IJAO.5000193

Shao, W., Wang, Z., Ip ,W., Chiang, YT., Xiong, X., Chai. T., Xu, C., Wang, Q., & Jin. T . (2013). GLP-1(28-36) improves β-cell mass and glucose disposal in streptozotocin induced diabetes mice and activates PKA-β-catenin signaling in beta-cells in vitro. *Am J Physiol Endocrinol Metab,* doi:10.1152/ajpendo.00600.2012

Su, J., Hu, B., Lowe, W. L., Kaufman, D. B., & Messersmith, P. B. (2010). *Anti-inflammatory peptide-functionalized hydrogels for insulin-secreting cell encapsulation* doi:10.1016/j.biomaterials.2009.09.045

Suarez-Pinzon, W., Marcoux, Y., Ghahary, A., & Rabinovitch, A. (2002). Gene transfection and expression of transforming growth factor-beta1 in nonobese diabetic mouse islets protects beta-cells in syngeneic islet grafts from autoimmune destruction. *Cell Transplantation, 11*(6), 519-528.

Swenne, I. (1982). The role of glucose in the in vitro regulation of cell cycle kinetics and proliferation of fetal pancreatic B-cells. *Diabetes, 31*(9), 754-760.

Tanabe, K., Liu, Z., Patel, S., Doble, B. W., Li, L., Cras-Méneur, C., . . . Permutt, M. A. (2008). Genetic deficiency of glycogen synthase kinase-3beta corrects diabetes in mouse models of insulin resistance. *Plos Biology, 6*(2), e37-e37. doi:10.1371/journal.pbio.0060037

Trümper, A., Trümper, K., Trusheim, H., Arnold, R., Göke, B., & Hörsch, D. (2001). Glucose-dependent insulinotropic polypeptide is a growth factor for beta (INS-1) cells by pleiotropic signaling. *Molecular Endocrinology (Baltimore, Md.), 15*(9), 1559-1570.

Tsuboi, T., da, S. X., Holz, G. G., Jouaville, L. S., Thomas, A. P., & Rutter, G. A. (2003). Glucagon-like peptide-1 mobilizes intracellular Ca2+ and stimulates mitochondrial ATP synthesis in pancreatic MIN6 beta-cells. *The Biochemical Journal, 369*, 287-299.

Tuttle, R. L., Gill, N. S., Pugh, W., Lee, J. P., Koeberlein, B., Furth, E. E., . . . Birnbaum, M. J. (2001). Regulation of pancreatic beta-cell growth and survival by the serine/threonine protein kinase Akt1/PKBalpha. *Nature Medicine, 7*(10), 1133-1137.

Tyrberg, B., Eizirik, D. L., Hellerström, C., Pipeleers, D. G., & Andersson, A. (1996). Human pancreatic beta-cell deoxyribonucleic acid-synthesis in islet grafts decreases with increasing organ donor age but increases in response to glucose stimulation in vitro. *Endocrinology, 137*(12), 5694-5699.

Unger, R. H., Zhou, Y. T., & Orci, L. (1999). Regulation of fatty acid homeostasis in cells: Novel role of leptin. *Proceedings of the National Academy of Sciences of the United States of America, 96*(5), 2327-2332.

Unger, R. H., & Orci, L. (2010). Paracrinology of islets and the paracrinopathy of diabetes. *Proceedings of the National Academy of Sciences of the United States of America, 107*(37), 16009-16012. doi:10.1073/pnas.1006639107

Vasavada, R. C., Garcia-Ocana, A., Zawalich, W. S., Sorenson, R. L., Dann, P., Syed, M., . . . Stewart, A. F. (2000). Targeted expression of placental lactogen in the beta cells of transgenic mice results in beta cell proliferation, islet mass augmentation, and hypoglycemia. *Journal of Biological Chemistry, 275*(20), 15399-15406. doi:http://dx.doi.org/10.1074/jbc.275.20.15399

Vasavada, R. C., Wang, L., Fujinaka, Y., Takane, K. K., Rosa, T. C., Mellado-Gil, J., . . . Garcia-Ocaña, A. (2007). Protein kinase C-zeta activation markedly enhances beta-cell proliferation: An essential role in growth factor mediated beta-cell mitogenesis. *Diabetes, 56*(11), 2732-2743.

Vaulont, S., Vasseur-Cognet, M., & Kahn, A. (2000). Glucose regulation of gene transcription. *The Journal of Biological Chemistry, 275*(41), 31555-31558.

Wang, J., Qian, X., Chinookoswong, N., Lu, J., Chow, G., Theill, L. E., & Shi, Z. (2002). Polyethylene glycolated recombinant TNF receptor I improves insulitis and reduces incidence of spontaneous and cyclophosphamide-accelerated diabetes in nonobese diabetic mice. *Endocrinology, 143*(9), 3490-3497.

Wang, R. N., Paraskevas, S., & Rosenberg, L. (1999). Characterization of integrin expression in islets isolated from hamster, canine, porcine, and human pancreas. *The Journal of Histochemistry and Cytochemistry: Official Journal of the Histochemistry Society, 47*(4), 499-506.

Wang, R. N., & Rosenberg, L. (1999). Maintenance of beta-cell function and survival following islet isolation requires re-establishment of the islet-matrix relationship. *The Journal of Endocrinology, 163*(2), 181-190.

Weber, L. M., Cheung, C. Y., & Anseth, K. S. (2008). Multifunctional pancreatic islet encapsulation barriers achieved via multilayer PEG hydrogels. *Cell Transplantation, 16*(10), 1049-1057.

Weber, L. M., Hayda, K. N., & Anseth, K. S. (2008). Cell-matrix interactions improve beta-cell survival and insulin secretion in three-dimensional culture. *Tissue Engineering.Part A, 14*(12), 1959-1968.

Weber, L. M., Hayda, K. N., Haskins, K., & Anseth, K. S. (2007). The effects of cell-matrix interactions on encapsulated beta-cell function within hydrogels functionalized with matrix-derived adhesive peptides. *Biomaterials, 28*(19), 3004-3011.

Withers, D. J., Julio Sanchez Gutierrez, Towery, H., Burks, D. J., & et al. (1998). Disruption of IRS-2 causes type 2 diabetes in mice. *Nature, 391*(6670), 900-4. doi:http://dx.doi.org/10.1038/36116

Wojtusciszyn, A., Armanet, M., Morel, P., Berney, T., & Bosco, D. (2008). Insulin secretion from human beta cells is heterogeneous and dependent on cell-to-cell contacts. *Diabetologia, 51*(10), 1843-1852. doi:10.1007/s00125-008-1103-z

Wu, H., Avgoustiniatos, E. S., Swette, L., Bonner-Weir, S., Weir, G. C., & Colton, C. K. (1999). In situ electrochemical oxygen generation with an immunoisolation device. *Annals of the New York Academy of Sciences, 875*, 105-125.

You, M., Kwak, M. K., Kim, D., Kim, K., Levchenko, A., Kim, D., & Suh, K. (2010). Synergistically enhanced osteogenic differentiation of human mesenchymal stem cells by culture on nanostructured surfaces with induction media. *Biomacromolecules, 11*(7), 1856-1862. doi:10.1021/bm100374n