Porf-2 Expression in Insulin Receptor (IR) Knock-down FRTL-5 Cells

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Wenjuan Zhang

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This thesis has been approved by

The Honors Tutorial College and the Department of Biological Sciences

_____

Dr. Felicia Nowak
Associate Professor, Biomedical Sciences
Thesis Advisor

_____

Dr. Soichi Tanda
Honors Tutorial College, DOS
Biological Sciences

_____

Dr. Janet Duerr
Honors Tutorial College, DOS
Neuroscience

_____

Jeremy Webster
Dean, Honors Tutorial College

Abstract

Diabetes mellitus is a chronic disease that afflicts 347 million people worldwide. Type 2 diabetes (T2DM) accounts for approximately 90% of diabetes mellitus cases. Insulin insensitivity is a prominent physiological change in patients with T2DM. This is caused by decreased function or dysfunction of the insulin or IGF-1 receptor signaling pathway.

Recent research has indicated that T2DM patients are at increased risk of developing neurodegenerative diseases. Brain shrinkage due to neural cell death is a common feature in neurodegenerative diseases and can be found in specific regions of the brain. Regulatory factors may increase or decrease the rate of neuronal cell death (apoptosis). Pre-optic regulatory factor-2 (Porf-2) is a protein that contains a RhoGAP domain; it has both anti-proliferative and pro-apoptotic properties in neural stem cells.

The goal of this research project was to study potential links between insulin/IGF-1 signaling and Porf-2 expression. We did this by decreasing the levels of insulin receptor (IR). We hypothesized that when the insulin or IGF-1 downstream signaling pathway was reduced, Porf-2 expression would increase.

Fischer's Rat Thyroid cells (FRTL-5) were transfected with small-hairpin RNA (shRNA) constructs to attempt to significantly reduce IR translation. Four IR knock-down constructs were used. Results show that cells transfected with an insKD construct exhibit slower proliferation rates than cell controls. The transfected cells also exhibited different proliferation morphologies. This research project was designed to bring insight

into the broader issue of possible strategies to combat cognitive decline in T2DM patients.

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Introduction

I. Background

Diabetes mellitus is a chronic disease that afflicts 29.1 million people in the United States and 347 million worldwide (American Diabetes Association 2014). It is caused by either a dysfunction of insulin secretion by pancreatic β cells or defects of the body's ability to respond to insulin. Complications and co-morbid conditions include hypo/hyperglycemia, hypertension, diabetic retinopathy, stroke, kidney failure, myocardial ischemia, and lower limb amputations (American Diabetes Association 2014). The three main types of diabetes are diabetes mellitus type 1 (also known as juvenile diabetes or T1DM), diabetes mellitus type 2 (also known as adult-onset diabetes or T2DM), and gestational diabetes.

T1DM is a result of the autoimmune destruction of β cells in the pancreas. β cells are essential for the production of insulin. Absence of these cells will lead to the body's inability to produce insulin. As a result, since insulin is necessary for normal regulation of glucose transport into the cell, the body is defective in transporting glucose into insulin-dependent cells and thereby lowering the blood glucose level. Risk factors include viral exposure that may trigger the destruction of islet cells in the pancreas.

In contrast, lifestyle, epigenetic, and genetic factors are the major predisposing elements to the onset of T2DM. T2DM accounts for approximately 90% of diabetes mellitus cases worldwide. T2DM patients may initially develop insulin resistance, an inability of the insulin receptors or their downstream signaling molecules to respond to

extracellular insulin, followed by the inability of β cells in the pancreas to produce and secrete sufficient insulin. Cells that are usually the most sensitive to insulin (adipose, liver, and skeletal muscle cells) may exhibit compromised metabolism as a result (Kahn et al. 2006). The most direct effect of insulin insensitivity is hyperglycemia, an increase in blood glucose levels. The insulin signaling pathway is impaired, which may result in physical complications that include peripheral neuropathy. Diabetic neuropathy (DN) presents with chronic pain and peripheral insensitivity; it is a major factor leading to lower limb amputations for T2DM patients (Dellon 2004). Current studies are also examining the possibility that T2DM may negatively affect central nervous system function, including cognition.

With 347 million afflicted worldwide, the disease is considered pandemic. Although our understanding of the disease has progressed tremendously since its first characterization by ancient civilizations, there is still a great need for further research. Research needs to elucidate more of the pathways involved with the disease. In particular, as diabetes is associated with increased risk of cognitive impairment (Espeland et al. 2013), there is a need to expand our knowledge of the role that insulin signaling impairment may have on neurodegeneration. This research project addresses a particular aspect of this field; our hypothesis is that neuronal death is increased due to dysfunction of the insulin signaling pathway.

II. Insulin and its receptors

Insulin is a peptide hormone that is produced by pancreatic β cells. The primary function of insulin is to regulate metabolism by promoting glucose uptake

into the cell; as part of this insulin promotes the insertion of glucose transporters such as GLUT4 into the cell membrane. This lowers blood glucose concentration and promotes the conversion of glucose to glycogen for storage. Insulin also plays a role in lipolysis, cell growth, gluconeogenesis, and fatty acid and protein synthesis. Unlike steroid hormones, peptide hormones cannot diffuse through the cell membrane. In order for the hormone to induce signal transduction, it will either bind to an insulin receptor (IR) with high affinity or to an insulin-like growth factor-1 receptor (IGF-1R) with lower affinity. Both IR and IGF-1R are transmembrane receptors (Berg et al. 2007).

The insulin receptor is encoded by the INSR gene; alternative splicing gives two protein isoforms: IR-A and IR-B. Posttranslational proteolytic cleavage of the insulin receptor results in an α and a β subunit. The complete insulin receptor consists of a dimer of two identical units; each unit of the dimer contains two subunits, α and β (Belfiore et al. 2009). The two subunits are linked by a disulfide bond. The α subunit is located in the extracellular region whereas the β subunit lies primarily embedded in the plasma membrane and inside the cell. The two α subunits bind together to form a dimeric unit binding site in the presence of insulin molecules. The β subunit has a receptor tyrosine kinase (RTK) domain that is able to catalyze the transfer of a phosphate from adenosine triphosphate (ATP) to the hydroxyl group of tyrosine. The insulin receptor is in its inactive form when the domain is not phosphorylated. When the two α subunits bind to insulin, the two protein kinase domains inside the cell come together. This allows for the activation loop of one β subunit to fit in the active site of

the other β subunit. In this position, the kinase domains will catalyze phosphorylation of the tyrosine residues in the activation loops using ATP, resulting in a conformation change and thus activating the insulin receptor (Berg et al. 2007).

Once activated, the insulin receptor will initiate a cascade of downstream activity. The phosphorylated sites of the β subunit act as docking sites for substrates such as insulin-receptor substrate (IRS). The adaptor IRS protein contains four sequences that are preferred targets for the RTK domain; the phosphorylated insulin receptor is responsible for phosphorylating the tyrosine residues of IRS (Berg et al. 2007). Phosphorylated IRS will then engage in multiple downstream signaling pathways. The two primary ones are the phosphoinositide-3 kinase (PI3K)/Akt and the Ras/ mitogen-activated protein kinase (MAPK) pathways. The primary function of the PI3K/Akt pathway is to regulate enzyme activity. This pathway begins when PI3K converts PIP2 into PIP3. Once PIP3 is made, PIP3-dependent protein kinase becomes activated and subsequently activates Akt via phosphorylation. Once Akt is phosphorylated, it will phosphorylate and regulate downstream target proteins, mostly those involved in glucose metabolism. One major change is the movement of glucose transporters such as GLUT4 to the cell surface (Berg et al. 2007). The downstream actions of the Ras/MAPK pathway are primarily on transcription (i.e. gene expression) and cell proliferation. Ras is a monomeric GTPase that is regulated by guanine nucleotide-exchange factors (GEFs) and GTPase-activating proteins (GAPs). GEFs act by exchanging GTP and removing the GDP in Ras; GAPs stimulate hydrolysis of GTP, which inactivates Ras (figure 1).

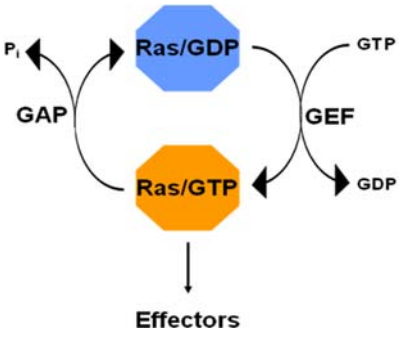

Figure 1. GTPase in/activation through GEFs and GAPs.
(Leonardy et al. 2010)

Ras is activated through the sequential activation of IR, IRS and Src homology 2 domain containing protein (Shc). IRS acts as a docking site for Shc to bind and recruit Sos, a GEF, which promotes the activation of Ras. Activated Ras activates MAPK via a phosphorylation cascade involving Mek. Once MAPK is activated, it will phosphorylate other kinases as well as enter the nucleus to phosphorylate transcription factors to initiate gene expression (figure 2).

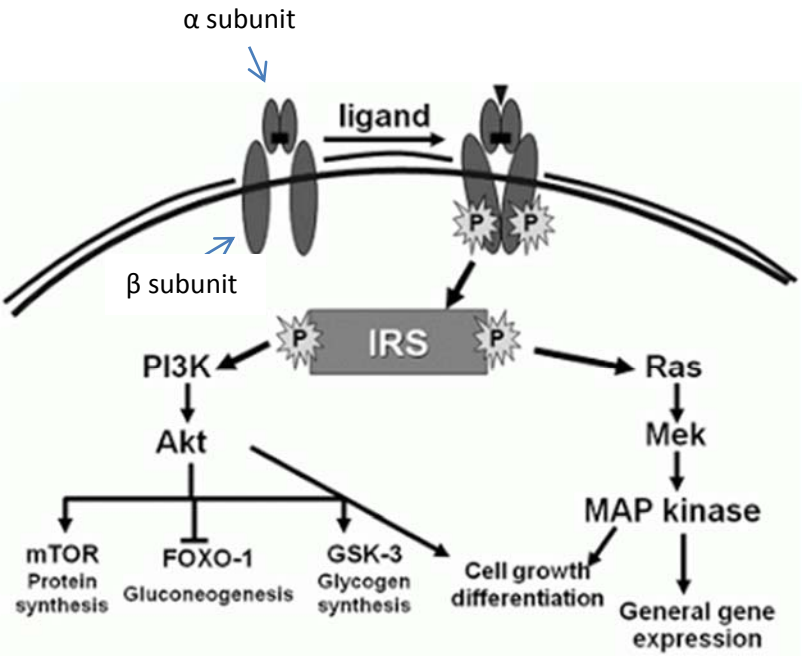

Figure 2. A summary of the downstream actions of insulin binding to either IR or IGF-1R: When a ligand binds to IR, a downstream signaling cascade follows. The PI3K/ Akt pathway leads to stimulation of intermediary metabolism including glycogen synthesis from glucose. The Ras/MAPK pathway signals for cell growth and differentiation. (Eslam et al. 2011).

IGF-1 receptors are similar in structure and function to insulin receptors. Both have RTK domains and share common downstream pathways. Insulin is able to bind to both insulin receptors and IGF-1 receptors (to a lesser extent); IGF-1 has a higher binding affinity for IGF-1R than IR. It should be noted that IGF-1 receptors are more directly linked to mitogenic effects whereas insulin receptors are linked to both metabolic and mitogenic effects (Sciacca et al. 2012).

III. Diabetes and the Nervous System

i) Peripheral Nervous System (PNS)

There has been increased interest in studying the cognitive ramifications of diabetes, particularly T2DM. Diabetic neuropathy occurs as a result of damaged nerves in the peripheral nervous system (PNS). The onset of diabetic neuropathy is thought to be caused by altered insulin signaling in neurons. A recent publication by Grote et al. (2013a) examined insulin signaling in non-diabetic mice administered varying doses of insulin. The study analyzed the phosphorylation of downstream proteins (Akt, GSK3β, mTor, ERK) in tissues that included the dorsal root ganglion (DRG), sciatic nerve (SN), liver, spinal cord, adipose, and muscle. Their primary objective was to determine whether the PNS is insulin responsive. The researchers also compared insulin signaling in the PNS with other peripheral tissues where insulin signaling is better understood (i.e. liver, adipose and muscle tissues). They found the SN had an increased response to insulin compared to the DRG. The downstream

signaling effects of insulin were reduced in both SN and DRG compared to non-nervous tissues.

In a separate experiment, Grote et al. (2013b) investigated the presence of insulin resistance in the peripheral nervous system in *ob/ob* mice. *ob/ob* mutant mice have a mutation on the *obese (ob)* gene. This type of mouse is an obesity model since they eat excessively; they also have a high likelihood of developing T2DM. As described earlier, insulin resistance is a commonly observed symptom in T2DM patients. What remains unclear is whether the nervous system is altered in these patients. For their study, Grote et al. analyzed insulin receptor expression as well as the phosphorylation of kinases involved in the downstream signaling pathway in the DRG and the SN of the PNS. They found *in vivo* insulin resistance in the PNS and a blunted insulin response in ob/ob mice. Insulin receptor levels were significantly decreased in the DRG, and Akt phosphorylation was also significantly reduced in both the DRG and sciatic nerve (Grote et al. 2013b). Further studies are needed to elucidate the role of insulin signaling in the PNS.

ii) Central Nervous System (CNS)

Multiple studies have found that T2DM is linked to Mild Cognitive Impairment (MCI). Diabetes also increases the risk and rate of cognitive decline and dementia in older adults (Williamson et al. 2012). Because dysregulation of glucose metabolism and insulin homeostasis are common features of T2DM, insulin resistance in the CNS is a possible explanation for cognitive impairment.

Both hypoglycemia and hyperglycemia have been implicated as causes of cognitive dysfunction. The pathophysiology of how hypoglycemia may impair memory over time is not well understood. Kodl and Seaquist (2008) reviewed the literature on cognitive dysfunction in diabetes mellitus. They found that T2DM has been associated with decreases in several cognitive functions, including psychomotor speed, frontal lobe function, verbal memory, processing speed, complex motor functioning, working memory, verbal fluency, attention, and immediate and delayed recall. Despite these observed correlations, the degree of impact these cognitive deficits have on patients' daily lives is difficult to quantify. For example, it is observed that T2DM patients are twice as likely to have depression, but this might be attributed to the negative effects of the lack of daily activities. The authors also observe that T2DM patients have an increased incidence of developing Alzheimer's disease and vascular dementia (Kodl and Seaquist 2008).

Some studies on the presence of insulin in the brain suggest that insulin is produced locally in the CNS (Williamson et al. 2012). However, this remains a controversial topic. The current consensus is that insulin reaches the CNS via the blood-brain barrier through specific transporters (which have not been identified). In T2DM patients, hyperinsulinemia in peripheral tissues is commonly seen in the earlier stages; later, the increase in insulin is also found in the CNS. The increase in insulin levels has been shown to sensitize neurons to toxins and stress-induced factors (Williamson et al. 2012).

In further support the idea that T2DM may lead to altered functions in the CNS, it has been well established that insulin receptors are present in multiple areas of the brain. There are quantitative differences in IR expression throughout the CNS; for example, IRs are highly concentrated in areas such as the olfactory bulb, pyriform cortex, amygdala, hippocampus, hypothalamus and various regions of the cerebral cortex (Marks et al. 1990). All of these areas play important roles in cognition. In addition, training mice through the Morris water maze leads to increased IR expression in the dentate gyrus, a region of the brain associated with neurogenesis and memory (Williamson et al. 2012). Clinical studies found that cognitive deficits in T2DM patients tend to be associated with hippocampus-related tasks. Surprisingly, neuron specific insulin receptor knock-out mice do not exhibit cognitive deficits. However, they do become hyperphagic and develop mild insulin resistance. This implies that IRs in the CNS contribute to maintaining homeostasis in the body (Williamson et al. 2012).

The mechanisms by which insulin regulates blood glucose levels in the CNS are still being elucidated. Glucose metabolism is the primary source of energy for neurons; glucose is transported into the cell via GLUT4 and other transporters. Insulin signaling normally increases movement of GLUT4 from intracellular endosomes to the cell membrane. When the uptake of glucose is reduced in T2DM, the supply of glucose in the neurons is diminished. Thus insulin may act as a regulator of neuronal metabolism in the CNS. In the case of insulin resistance, several studies have noted that vasoconstriction increased in the brain (El Khoury et al. 2014). This is predicted

to worsen the symptoms associated with T2DM, as impairment in blood supply delivery will decrease the amount of insulin delivered to the brain. In addition it will cause a decrease supply of nutrients and oxygen and impede the disposal of wastes and harmful metabolites.

Apart from acting as regulators for metabolism, studies suggest that insulin and its receptors are concentrated at neural synapses. This notion is particularly relevant for Alzheimer's disease (AD). The brains of patients with AD exhibit two very well-characterized phenotypes: amyloid plaques (or a soluble form as amyloid-beta oligomers) and neurofibrillary tangles made of tau proteins. T2DM patients have an increased risk of developing Alzheimer's disease (AD) compared to their non-diabetic counterparts (Zhao et al. 2008). Other studies have found that major impairments in insulin and IGF-1 gene expression and signaling occur in the brains of AD patients (Li and Holscher 2007). This impairment could be due to the presence of soluble small protein (Aβ) oligomers (ADDLs), which have been found near synapses. For example Zhao et al. (2008) found that ADDLs were co-localized with a majority of insulin receptors in rats. Furthermore, they found that the presence of ADDLs caused a reduction of phosphorylation of IR, thus reducing its downstream activity. Other studies also suggest altered insulin signaling promotes the formation of neurofibrillary tangles through tau protein hyperphosphorylation (Lesort and Johnson 2000). Tau is a microtubule-associated protein that stabilizes the axon's microtubules. When tau is phosphorylated, it decreases microtubule stability. When tau is hyperphosphorylated,

it may lead to aggregation of tau proteins and a prolonged period of destabilization of microtubules.

IV. Porf-2 and Apoptosis

Cells can die due to injury or due to regulated developmental and functional processes. Cells that die due to injury will swell and burst, releasing factors that can trigger an inflammatory response and damage surrounding cells. This sequence of events is known as cell necrosis or passive cell death. Three common types of regulated of Programmed Cell Death (PCD) are Type 1 (apoptosis), Type 2 (autophagic) and Type 3B (cytoplasmic). PCD is defined as the "spatially and temporally reproducible and species-specific loss of large numbers of individual cells both during development and, in many tissues and organs, throughout life." (Squire 2013). The term 'apoptosis' is derived from a Greek word that means 'falling away from'; it is characterized by shrinkage of cell size and condensation of nuclear chromatin. The cell membrane and organelles initially remain intact. Eventually, as the cell is fragmented, the cellular components will break apart and separate into smaller vesicles; the vesicles' membranes will display phosphatidylserine and other phagocytic stimulating molecules. Phosphatidylserine is usually found on the cytosolic side of the cell membrane, but enzymes known as flippases transfer the phosphatidylserine to the exoplasmic side. Phosphatidylserine acts as a signaling molecule for macrophages to engulf the cell. Unlike necrosis, PCD is a carefully regulated process. Many proteins and their respective regulators act together to ensure

that the death of an individual cell will not interfere with the survival of the others surrounding cells (Squire 2013).

There are two pathways for apoptosis: the intrinsic and extrinsic pathways. Much of our knowledge of the mechanism for apoptosis is derived from studies conducted on *C. elegans*. Because apoptosis is a tightly regulated process, many of the genes involved with the process are conserved across species. Important proteins

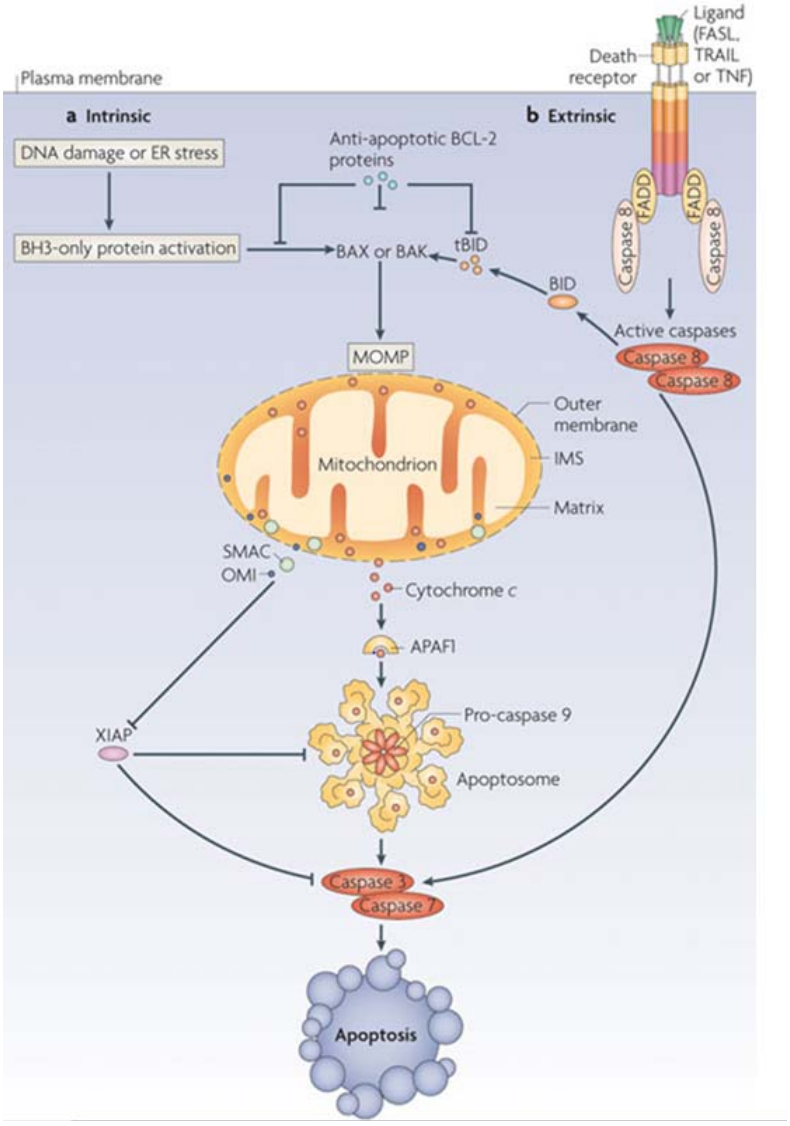

Figure 3. Intrinsic/extrinsic apoptosis pathway: The intrinsic pathway is stimulated by factors inside the cell such as DNA damage or ER stress; the extrinsic pathway is a result of external ligands binding to specific cell-surface pro-apoptotic receptors. It is important to note that Bcl-2 and Bax/Bak may associate with the outer membrane of the mitochondria, which is not illustrated here (Degterev and Yuan 2003).

include the B-cell lymphoma protein-2 family (BCL2) which is divided into three subclasses: anti-apoptotic (e.g. Bcl-2), pro-apoptotic (e.g. Bax, Bak, Bid), and BH3-only (e.g. Bid, Bim, Noxa, PUMA) (Degterev and Yuan 2008). Bcl-2 proteins are present in the outer mitochondrial membrane; their roles include preventing pro-apoptotic proteins, mainly Bax and Bak, from damaging the mitochondria.

The intrinsic pathway is stimulated by factors within the cells such as DNA damage or extreme cell stress. These stimuli activate BH3-only proteins to reverse the effect of anti-apoptotic proteins such as Bcl-2 and induce Bax/Bak activation. Bax is a protein that is usually located in the cytosol. However, when activated, it can be translocated to the outer membrane where Bak resides (Degterev and Yuan 2008). Once the two proteins (Bax/Bak) begin to interact, it is hypothesized that they will form an oligomeric channel that will damage the mitochondria (figure 3). Cytochrome c, an electron transporter, is associated with the inner membrane of the mitochondria. It functions by transporting electrons between Coenzyme Q-cytochrome c reductase (complex III) and cytochrome c oxidase (complex IV) of the electron transport chain. When the mitochondrion becomes damaged by the pro-apoptotic factors Bax/Bak, cytochrome c is released into the cytosol. Cytochrome c will then bind to apoptotic protease-activating factor-1 (APAF-1) proteins, which promote the formation of a heptameric complex known as the apoptosome (figure 3). The apoptosome is composed of APAF-1 and procaspase-9 units. Caspases are a group of cysteine proteases that are present in the cell as zymogens (inactive proteins). They become activated when cleaved at specific sites on the protein. As its name suggests, APAF-1

helps activate procaspase-9 by transforming the zymogen to an active protease through a conformational change of the apoptosome. When caspase-9, known as the 'initiator caspase', is activated, it will act by cleaving other 'executioner' caspases (mainly caspases 3, 6, and 7). The executioner caspases will act downstream to induce apoptosis. For example, caspase 3 is essential for DNA fragmentation and chromatin condensation (Porter and Jänicke 1999).

The extrinsic apoptotic pathway is initiated by pro-apoptotic and pro-inflammatory cytokines such tumor-necrosis factor-α (TNFα). These factors bind to receptors on the plasma membrane known as death receptors. This binding will induce the formation of a complex known as the death-induced signaling complex (DISCs), which will activate an initiator caspase, procaspase 8, by cleavage. Caspase 8 can cleave and activate downstream caspases or may cleave a BH3-only pro-apoptotic protein Bid that will further signal mitochondrial damage (Degterev and Yuan 2008).

The onset of apoptosis and other forms of PCD are tightly regulated. p53 is a tumor-suppressor protein that acts upstream of p21. TP53 (tumor protein 53) is the gene which encodes p53; the protein has transcriptional activation, DNA binding, and oligomerization domains (Macleod et al. 1995). When a cell receives genotoxic or non-genotoxic stress signals such as upstream apoptotic signals, p53 is stabilized by phosphorylation; once stabilized, it will act to increase transcription of p21 (Gartel and Tyner 2002).

Cyclin-dependent kinases (CDKs) are enzymes involved in cell-cycle regulation. When forming a complex with cyclins, CDKs may be activated and

contribute to cell-cycle progression (Gartel and Tyner 2002). In order to regulate cell progression, proteins known as cyclin-dependent kinase inhibitors (CDKIs) may be expressed. CDK inhibitor-1 (p21) is a CDKI that inhibits cyclin bound-CDK1, -CDK2, and –CDK4/6 by binding to the complex; a conserved region at the N-terminus of p21 is believed to be necessary for inhibiting cell cycle progression. p21 downregulates cell proliferation (Xiong et al. 1993). Paradoxically, p21 has varied effects on apoptosis: it may prevent apoptosis, or promote apoptosis through either a p53-dependent and/or independent pathway.

Preoptic regulatory factor 2 (Porf-2) is a protein that was discovered in the preoptic area of the hypothalamus (Nowak et al. 1999). Porf-2 expression is present in other tissues including the cerebral cortex, the anterior pituitary, cerebellum, testes, kidneys, liver, skin, and other peripheral tissues. The porf-2 gene is present in a wide array of animals which include the rat, mouse, chicken, pig, sheep, cow, zebrafish, and human (Hayat 2014). Expression of Porf-2 varies with both age and sex (Nowak 2003). Porf-2 contains a RhoGAP domain, which is an inactivator of RhoGTPases. Abnormal RhoGAPs are linked to neurodevelopmental disorders such as X-linked mental retardation and to neurodegenerative diseases such as Alzheimer's, Parkinson's, Huntington's disease, and amyotrophic lateral sclerosis (ALS) (Etienne-Manneville 2002). RhoGTPases (one of five major types of small G-protein GTPases: Rho, Ras, Rab, Arf, and Ran) have functions including regulating cell polarity, gene transcription, G1 cell cycle progression, cytoskeleton structure maintenance, dendritic spine formation and excitatory synapses, among others (Linseman and Loucks 2008).

Previous studies have indicated that Porf-2 may regulate growth in the developing central nervous system (CNS). Porf-2 knockdown neural stem cells were shown to have a higher percentage of cells in the S phase than in the G1 phase when compared to control cells (Ma and Nowak 2011). The knockdown cells underwent significantly less apoptosis when they were exposed to proapoptotic factors such as bleomycin and staurosporine (STS). STS is derived from the bacterium *Streptomyces staurosporeus*; it is a protein kinase inhibitor. It acts by preventing ATP from binding to the kinase catalytic domain. The exact mechanisms by which it induces apoptosis is not well understood, but it is suggested that it acts through caspase-3 activation. Bleomycin, produced by *Streptomyces verticillus,* is a genotoxin, which initiates apoptosis by damaging the DNA in cells (Ma and Nowak 2011).

The results of these studies indicate that Porf-2 exhibits pro-apoptotic and anti-proliferative properties. It decreases cell metabolism and increases cell apoptosis through p53-dependent and –independent pathways in neural stem cells (figure 4). Increased rates of apoptosis, when not offset by cellular proliferation, may lead to degenerative diseases in the nervous system.

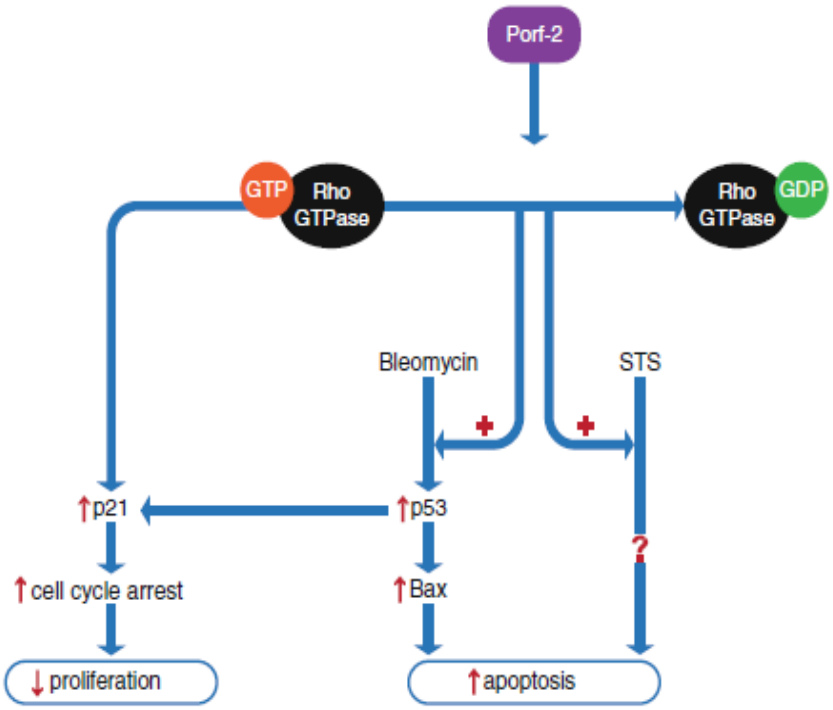

Figure 4. Porf-2 signaling cascade. The downstream effects are decreased proliferation and increased apoptosis (Ma and Nowak 2011).

V. Insulin and Porf-2 Pathways

Insulin may induce cell proliferation and stimulate metabolism when bound to either an insulin or IGF-1 receptor. By activating the PI3K/Akt and/or the Ras/MAPK pathway, the binding of insulin leads to transcription initiation, beginning the process of cellular proliferation. In contrast, studies on Porf-2 show its anti-proliferative properties, most likely through cycle arrest. As shown in figure 4, Porf-2 may act via a p53-dependent and/or –independent pathway. It is apparent that the insulin and Porf-2 effects are opposing. Because of this, it is of interest to us to further understand the interaction between insulin and Porf-2 and its possible role in T2DM. A common characteristic associated with T2DM is that insulin receptors become insensitive. This will lead to reduced downstream insulin/IGF-1 signaling. As described below,

preliminary results indicate that insulin represses expression of Porf-2, which would 'protect' the pro-metabolic role of insulin. As a result, Porf-2 may play a role in promoting signaling for a reduction in cell proliferation as well as an increase in apoptosis when insulin signaling is altered. The link for these two pathway remains to be elucidated.

VI. Fischer's Rat Thyroid Cell Line-5 (FRTL-5)

Fishcer's Rat Thyroid Cell Line (FRTL-5) cells are derived from cloned normal Fischer rat thyroid cells. When cultured *in vitro,* the cells express many thyroid-differentiated markers such as those for epithelial morphology, cytoskeleton organization, and iodine uptake. The cell morphology is polygonal and the cells grow as a monolayer. Thyroid cell function is regulated by thyroid stimulating hormone (TSH), which has a trophic effect on the cells. TSH is needed for FRTL-5 cell growth and differentiation. In the absence of TSH, they will become non-proliferative (Mulcahy et al. 1985).

IR and IGF-1R are both tyrosine kinases composed of two alpha and two beta subunits. Both receptors are homologous and are monomers when inactive; the receptors become functional and signal transduction will occur when the monomers dimerize after binding of insulin or IGF-1. Due to the homology of IR and IGF-1R, one monomer of each can combine and produce a hybrid IR/IGF-1R. As discussed earlier, insulin ligand binds to IR and induces signal transduction; insulin binds with lower affinity to the hybrid IR/IGF-1R, which can also induce a downstream signal.

IGF-1 can also bind to the hybrid receptor with greater affinity than insulin, as well as to its own receptor. FRTL-5 cells were used in this research project because of the presence of both IR and IGF-1R on the cell membrane. This allows for potential hybridization of the two receptors.

VII. Previous Research

Previous research conducted by F. Nowak and Z. Wang investigated the effects of the insulin and IGF-1 signaling pathways on Porf-2 expression in FRTL-5 cells. When FRTL-5 cells are treated with 0-100 ng/mL IGF-1, Porf-2 mRNA expression levels decrease in a dose-dependent manner. Similarly, cells incubated in medium containing insulin have decreased Porf-2 mRNA expression levels with 100ng/mL and 1000ng/mL of insulin present. Maximal Porf-2 expression level suppression was 50% with insulin whereas for IGF-1, it was 60% (Wang 2011).

When FRTL-5 cells were exposed to specific protein inhibitors of the insulin and IGF-1 pathway, the IGF-1 effect on Porf-2 mRNA expression levels was blocked. LY294002 and wortmannin, Akt Inhibitor IV, Raf kinase Inhibitor IV, and PD98095 were used to inhibit PI3K, Akt, Raf kinase, and MAPKK respectively. IGF-1 was used as the ligand to bind to IGF-1Rs and IRs and subsequently activate the downstream signaling. When cells were exposed to Akt inhibitor IV, LY294002 or Raf-kinase inhibitor PD 98095, the effects of IGF-1 on Porf-2 were partially blocked (figures 5 and 6). Wortmannin, which inhibits both PI3-K and MAPKK, blocked the effect of IGF-1 on Porf-2.

Z. Wang also conducted IGF-1 receptor knock-down experiments through the use of small-hairpin RNA (shRNA). shRNA is a gene silencing technique that uses artificially designed RNA sequences to repress the expression of a target mRNA. As predicted, knockdown of the IGF-1R partially suppressed the effects of IGF-1 on expression of Porf-2. Overall, the research indicates that insulin and IGF-1 signaling pathways influence Porf-2 expression levels, thus creating a link between IGF-1 and Porf-2 (Wang 2011).

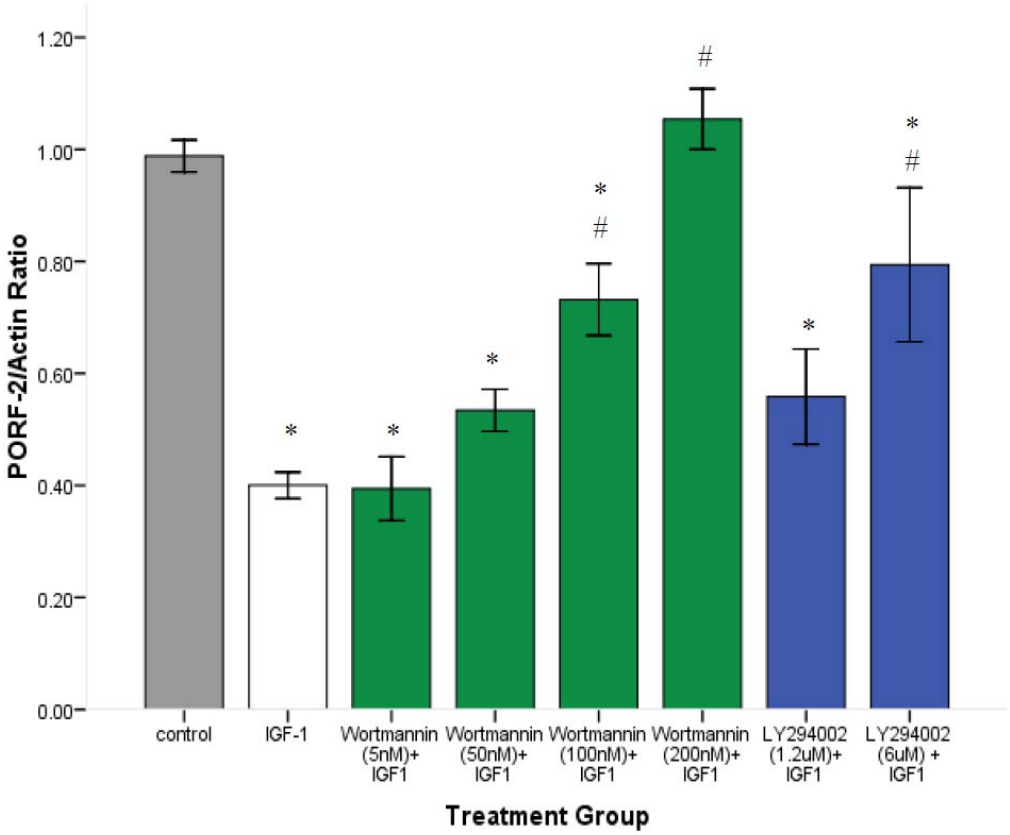

Figure 5: Porf-2 expression levels are inhibited by IGF-1. This inhibition is blocked in a dose-dependent manner when PI-3K (Wormannin and LY294002) inhibitors are added (Wang 2011).

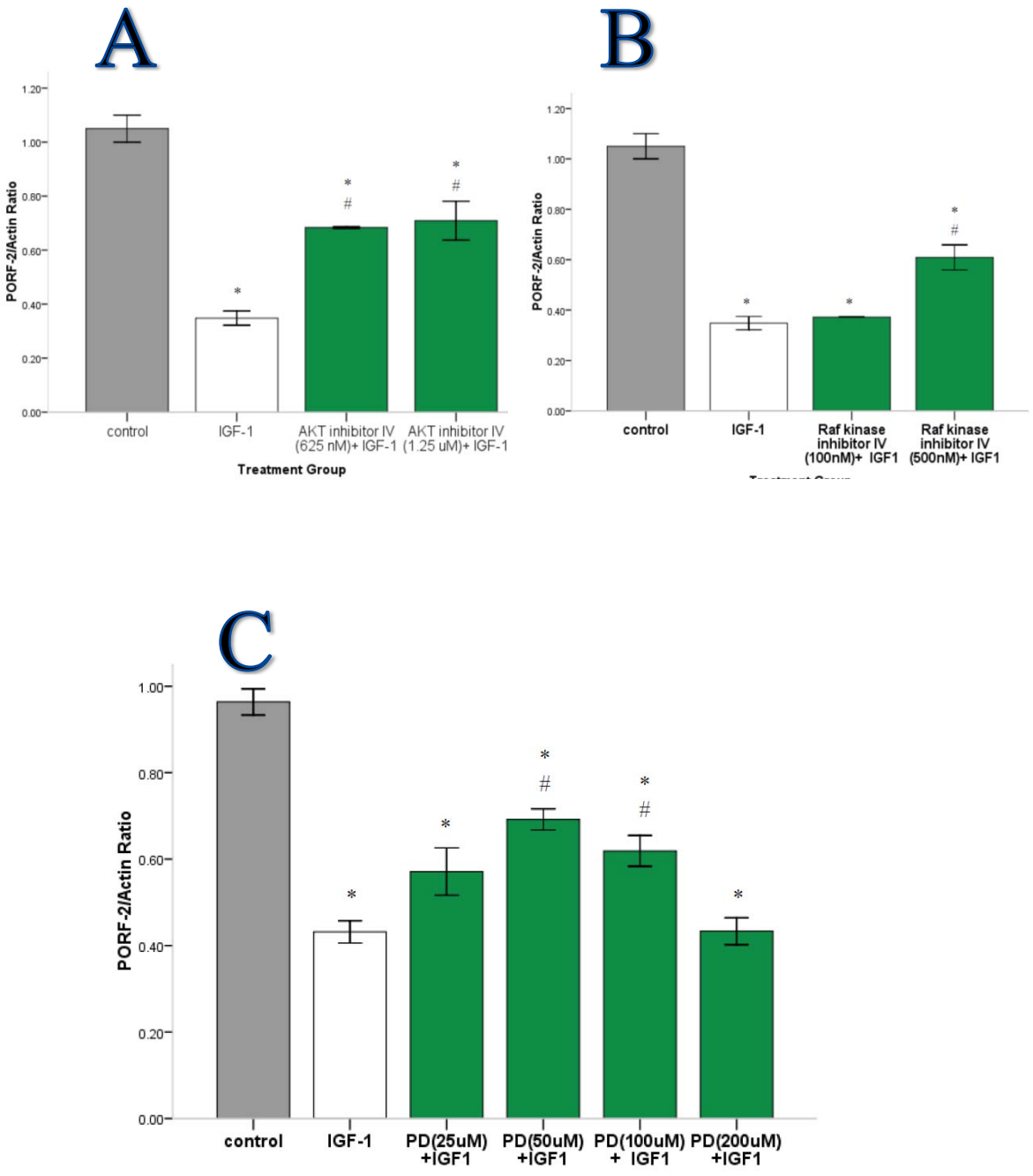

Figure 6: Porf-2 expression levels are decreased after IGF-1 application and increased when various downstream IGF-1 signaling proteins inhibitors are added: (A) Akt inhibitor, (B) Raf kinase inhibitor and (C) MAPKK inhibitor (PD98095) (Wang 2011).

Hypotheses and Significance

I. Hypotheses

The aim of this project is to further characterize signaling pathways that mediates effects of IGF-1 and insulin on Porf-2 expression. We hypothesize that when insulin and IGF-1 receptor functions are significantly decreased, that Porf-2 expression will increase. Furthermore, we hypothesize that the downstream signaling proteins will show altered protein activity levels; this includes a decrease in phosphorylation of essential downstream kinases as compared to controlled cells.

As discussed above, insulin and IGF-1 share signaling pathways. However, there are important distinctions between the two. First, insulin and IGF-1 have different binding affinities for their respective receptors. Insulin has a higher binding affinity for insulin receptor than IGF-1R. Conversely, IGF-1 has higher binding affinity for IGF-1R than insulin receptors. My specific hypothesis is that knocking down the insulin receptor will partially block the effects of insulin and IGF-1 on Porf-2. My specific aim is to knock-down the IR in FRTL-5 cells and measure the effect on Porf-2 mRNA.

II. Significance

Previous studies have demonstrated links between insulin and IGF-1 resistance and cognitive impairment. It is also known that patients with T2DM are more likely to develop neurodegenerative diseases, in particular Alzheimer's disease. It is important to note that many neurodegenerative diseases are associated with decreases in brain

volume, most likely through increased apoptosis. There are proposed pathways by which insulin insensitivity may exacerbate neuronal dysfunction and cognitive impairment. As described above, insulin receptors present at neural synapses co-localize with amyloid beta derived diffusible ligands in Alzheimer's disease patients. Previous research has shown that Porf-2 has anti-proliferative and pro-apoptotic properties. Thus, there may be a link between insulin/IGF-1 deficiency or resistance and neurodegeneration and Porf-2 expression.

By studying FRTL-5 cells, we hope to provide an *in vitro* model to study whether insulin/IGF-1 insensitivity results in an increase of Porf-2 expression. We plan to explore the downstream signaling pathways of insulin/IGF-1 and Porf-2. The ultimate goal of this research project is to establish a deeper mechanistic understanding of how Porf-2 may be involved in the cognitive impairment and possibly other complications seen in T2DM patients.

Experimental Designs and Methods

I. FRTL-5 cells and Cell Culture

The Fischer rat thyroid cell line (FRTL-5) was used for this study. Cells were cultured in cell culture dishes with media composed of 5% heat-inactivated bovine serum, nonessential amino acids, Coon's modified Ham's F-12 (Sigma-Aldrich, St. Louis, MO), and six hormones (TSH, insulin, somatostatin, hydrocortisone, transferrin, and glycyl-histidyl-lysine). Cells were incubated in a Heracell™ CO_2 incubator at 37˚C and 5% CO_2. After reaching 70-80% confluence, the cells were split

into multiwell plates for further growth. Media was changed after 3-4 days by aspiration (Kohn and Valente 1989).

II. Optimum Blasticidin Concentration

Prior to transfection, the optimal blasticidin concentration needed to be determined. Blasticidin is an antibiotic that acts on both prokaryotes and eukaryotes. We used it as a selection agent for transfected cells. Although blasticidin is needed to control for the presence of the vector in transfected cell cultures, a high concentration of the selective agent can impede cellular proliferation and the transfection efficiency. Also, a low concentration of blasticidin will not successfully select against non-transfected cells. Therefore, an optimal blasticidin concentration is needed 21 hr after transfection to select for successfully transfected cells. Previous tests by Z. Wang showed that the ideal length of time for selective killing of FRLT-5 cells in blasticidin

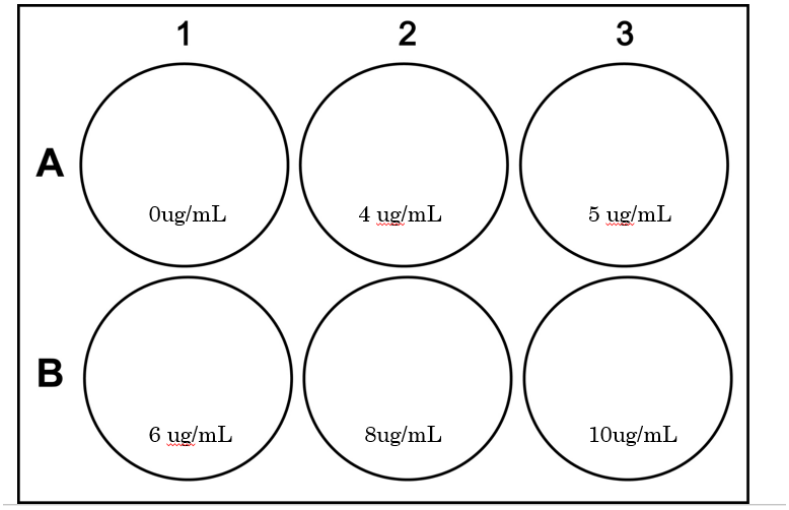

Figure 7. Schematic for determining optimum blasticidin concentration.

was around 2-3 weeks (Wang 2011). To determine the optimal selective concentration, various concentrations of blasticidin were tested on non-transfected FRTL-5 cells. Once the FRTL-5 cells reached 70-80% confluence, the cells were split into two 6-well culture plates with a growth area of 9.5cm^2 for continued proliferation. After the cells reached ~30% confluence, selected wells were given a set concentration of blasticidin. The concentrations of blasticidin that were tested were: 0, 4, 5, 6, 8, and 10 μg/mL. Duplicates of each concentration were performed to establish the optimum blasticidin concentration to use for selection after transfection.

The optimal concentration was determined by measuring the number of days necessary to eliminate the entire population of non-resistant cells in each well while preserving as many resistant cells as possible. Previous testing also showed that blasticidin concentrations lower than 3μg/mL were not effective at killing all nonresistant cells within the selective time frame (data not shown). Therefore, blasticidin concentrations lower than 4ug/mL were not tested.

III. Transfection

After establishing the optimal blasticidin concentration, new FRTL-5 cells were cultured in culture dishes and split into individual plates once they reached 60-70% confluence. Transfection began when the newly split cells reached 60-70% confluence. Insulin receptors were targeted for knocked-down via transfecting the cells with plasmid sequences that contain short hairpin RNA (shRNA). These RNAs

were designed to bind to the transcribed mRNA coding for the insulin receptor and decrease its protein expression.

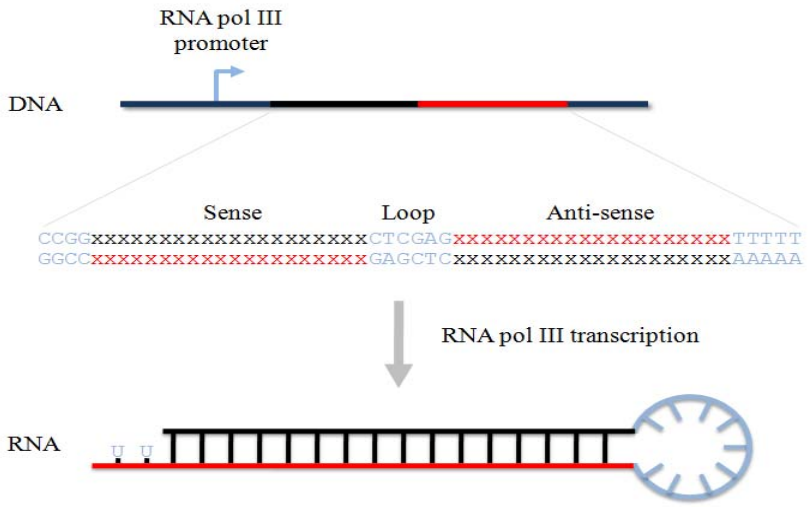

Figure 8. The shRNA plasmid is transcribed from DNA into shRNA; shRNA consists of sense and anti-sense sequences (O'Keefe 2013).

Decreasing insulin receptor expression by using shRNA transfection was preferred over other RNA interference (RNAi) approaches. shRNA uses DNA plasmids as vectors that allows for stable incorporation and selection of cell lines that have reduced expression of a specific gene. Once shRNA plasmids are introduced into the cell, they generally accumulate in the nucleus. In the nucleus, the shRNA are synthesized by either RNA polymerase II or III and form a hairpin structure that consist of a paired stem region and unpaired sense strands (figure 8). The shRNA is then exported out of the nucleus. Dicer protein is an enzyme that is part of the RNase III family that cleaves double stranded RNA (dsRNA) into short, dsRNA fragments. In the cytosol, the shRNA RNA will form a complex with Dicer and will be processed

and cleaved into a small interfering RNA (siRNA). The double stranded siRNA will then bind to the RNA-induced silencing complex (RISC). RISC separates the dsRNA and removes one of the strands. When the single stranded siRNA that remains bound to the RISC binds to the mRNA of interest by complementary base pairing, then RISC cleaves the mRNA in a sequence-specific manner (figure 9) (Taxman et al. 2010).

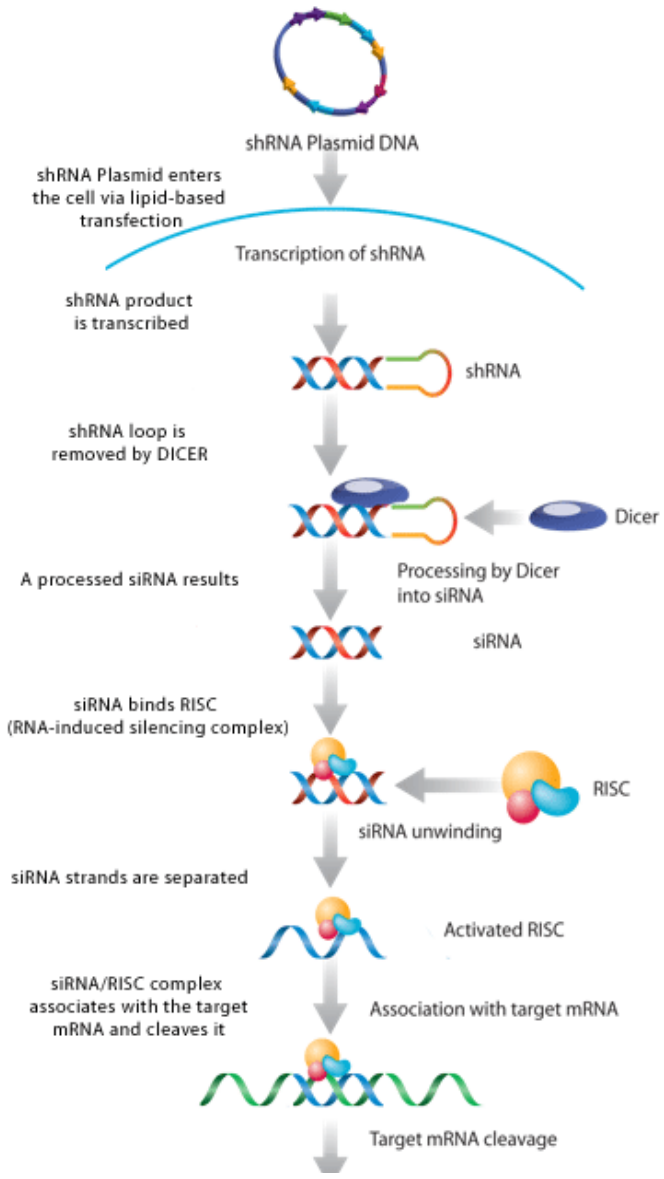

Figure 9. Schematic for shRNA delivery and function in the cell (SantaCruz Biotechnologies 2014)

shRNA should not exceed 30 base pairs (bp); this avoids the double-stranded (ds) RNA of FRTL-5 cells from triggering an antiviral response to the transfection (Origene Technologies 2015). We selected 4 different insulin knock-down (insKD) shRNAs designed by Origene Technologies (Rockville, MD); each insKD construct contains a 29 bp sequence stem designed to bind to a specific portion of the IR mRNA and a 7 bp loop to form the hairpin structure (figure 10). Empty vector (CO) and scrambled vector (CS) inserts were used as controls. The CO plasmid has a sequence that encodes for blasticidin resistance but does not contain a 29 bp construct sequence. The CS plasmid also contains a sequence that encodes for blasticidin resistance and includes a random 29 bp that is not complementary to the insulin receptor gene. CO plasmid was used to show that vector transfection has no effect of the cells, other than providing it with blasticidin resistance. CS plasmid was used to demonstrate that the binding of the insKD constructs was specific. Each plasmid vector also contains a U6 promoter site for shRNA expression and a SV40 promoter site for blasticidin resistance expression.

FuGene HD transfection reagent (Promega, Madison, WI) was used for transfection. Fugene HD is a non-liposomal transfection reagent; the non-liposomal lipids in FuGene HD form complexes with the plasmid vector in the form of micelles. OptiMEM (ThermoFischer Scientific, Grand Island, NY), a reduced serum media, was used during transfection; this media enables the formation of an amphiphilic compound with a micelle core containing the DNA. DNA is delivered into the cell by micelle fusion with the cell membrane.

After newly split FRTL-5 cells reached 60-70% confluence, media was removed by aspiration and replaced with 1mL of OptiMEM in each 9cm^2 plate. A small volume of OptiMEM and DNA (see Table 1) was incubated in a 250µL tube for 5min before adding Fugene HD transfection reagent directly into the mixture. The mixture was then incubated at room temperature for 15 min before adding it into each individual plate. Cells from each plate were given a specific combination of reagent mixture for transfection (table 1). The cells were incubated for 24hr at 37 °C in the mixture. After 24hr, the OptiMEM mixture was removed by aspiration and replaced with regular cell media. Plates 3-9 were also given 4µg/mL of blasticidin. Cells were observed and photographed with a Nikon Eclipse TS100 inverted microscope.

Table 1: Reagents used for transfection. CO=vector only, CS=scrambled insert.

Reagent	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5	Plate 6	Plate 7	Plate 8	Plate 9
OptiMEM	25 µl	22µl	22µl	15.5µl	15.5µl	15.5µl	15.5µl	15.5µl	15.5µl
DNA	-	-	-	7.5µl C0	7.5µl CS	7.5µl insKD-A	7.5µl insKD-B	7.5µl insKD-C	7.5µl insKD-D
Fugene HD	no	2µl	2µl	2µl	2µl	2µl	2µl	2µl	2µl
Blasticidin	no	no	yes	yes	yes	yes	yes	yes	yes

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Figure 10. Nucleotide sequence of IR. insKD A (yellow) and insKD B (pink) are complementary to regions of the alpha chain sequence; insKD C (green) is complementary to the crossing sequence between alpha and beta chains; insKD D (blue) is complementary to the sequence that is located at the very end of the IR beta subunit. Insulin receptor subunits alpha and beta span from 427-2628 and 2641-4500 respectively (NCBI 2014).

IV. Future Experiments: Western Blotting

Western Blot will be used once ~10^7 cells are obtained. Cells will be detached and lysed to extract the appropriate proteins. A 10% gel percentage will be used for polyacrylamide gel electrophoresis. Insulin Rβ Antibody (C-19) (Santa Cruz Biotechnology, Pasa Robles, CA) is a primary rabbit polyclonal antibody that can be used for detection of IR β subunit on mouse, rat, and human cells. Insulin Rβ Antibody will be used in parallel with positive and negative controls. The predicted size for insulin receptor subunit beta is around 95 kDa. For the positive controls, untransfected FRTL-5 cells will be used to compare IR expression levels with FRTL-5 cells transfected with control or experimental constructs. In addition, NIH 3T3 (mouse embryonic fibroblast cell line) whole-cell lysates will be used as a positive control. As our negative control, peptide antagonists (Santa Cruz Biotechnology, Pasa Robles, CA) will be incubated with the insulin receptor before applying the primary antibody; this should block all antibody binding to IR. After incubating the membrane with a secondary antibody, enhanced chemiluminescence (Thermo Fischer Scientific, Grand Island, NY) will be used to visualize protein expression levels.

In addition, Western blots will be run using cells from a previous project that have been transfected with shRNA that knocks-down the IGF-1 receptor. IGF-1Rβ Antibody (C-20) (Santa Cruz Biotechnology, Pasa Robles, CA) is a primary rabbit polyclonal antibody that is used for detection of IGF-1Rβ subunit on mouse, rat, and human cells. Positive and negative controls will be used.

V. Future Experiments: qPCR

Once protein expression levels for each individual insKD construct have been established, knock-down cell lines will be used to measure Porf-2 and IR mRNA expression levels using real-time polymerase chain reaction (RT-PCR). This method of analysis allows us to measure the amount of mRNA present in cells by using reverse transcriptase to transcribe mRNA in the cell to cDNA. SYBR Green (Bio-Rad Laboratories Inc, Hercules, CA) will be used to quantify the cDNA in qPCR. The housekeeping genes beta-actin and histone H3 will be used as a loading control.

Table 2. Primer sequences for qPCR. F- forward primer; R- reverse primer (Wang 2011)

IR	F 5'-TCCTCAAGGAGCTGGAGGAGT-3'
	R 5'-GCTGCTGTCACATTCCCCA-3'
IGF-1R	F 5'-GCCAGAACCCGAGAACCC-3'
	R 5'-ACACATTCCCGCTGATCCT-3'
Porf-2	F 5'-TTCCTCCAGGTGTTCGTC-3'
	R 5'-TGGTATCTAAATGCTGAATGAG-3'
β-actin	F 5'-GGGAAATCGTGCGTGACATT-3'
	R 5'-GCGGCAGTGGCCATCTC-3'

Results

I. Optimum Blasticidin Concentration

Blasticidin was the selective reagent used for selecting transfected cells. It is a nucleoside antibiotic isolated from *Streptomyces griseochrmogenes*. It acts to inhibit protein synthesis. Although transfected cells have a gene whose product provides blasticidin resistance, high concentrations of blasticidin will impede cell proliferation. At optimal blasticidin concentration, the reagent will select for successfully transfected cells while also ensuring that the reagent will not fully disrupt cell proliferation.

To determine optimal blasticidin concentration, non-transfected FRTL-5 cells were cultured in 6-well plates; once all cells reached 30% confluence, varied concentrations of blasticidin were added to determine the optimal concentration to use for selecting transfected cells. After two weeks, all normal cells given a blasticidin concentration of 8μg/mL or 10μg/mL died. After 13 days, all cells given a blasticidin concentration of 5μg/mL or 6μg/mL lost normal cell morphology and only 5-10% of cells remained attached to the well. It was therefore determined that a blasticidin concentration of 4μg/mL is optimal for selecting transfected cells, as all cells died within 17 days, which is within the recommended selection period (data not shown).

Table 3. Cell confluence was observed and estimated with a Nikon Eclipse TS100 inverted microscope.

% confluence	Blasticidin (µg/mL)	Day 1	Day 3	Day 5	Day 7	Day 9	Day 11	Day 13
	0	40-45%	50-60%	50-60%	70%	70-75%	>75%	>75%
	4	35-40%	30-40%	15-20%	15-20% (not healthy, morphological change)	15-20%	10-20%	10-15% (cells not healthy, not proliferating)
	5	35-40%	~40%	15-20% (not healthy, morphology change)	15-20%	15-20%	10-20%	5-10% (cells not healthy, not proliferating)
	6	35-40%	~35-40%	20-25% (not healthy, morphology change)	10-15%	7-10%	7-10%	5-10% (cells not healthy, not proliferating)
	8	40-45%	40-45% (not healthy, morphology change)	30-35%	7-10%	7-10%	5-10%	Very few cells (all died, but few non-morphological cells are attached to plate)
	10	35-40%	35-45% (not healthy, morphology change)	30-35%	20-30%	5-10%<	5-10%<	Very few cells (all died, but few non-morphological cells are attached to plate)

II. Stable Transfected Cell Lines

Based on preliminary results, cell culturing techniques were altered during transfection. After reaching 70-80% confluence, cells were originally split into two multi-well plates (9.5cm^2 /well). However, due to problems with bacterial contamination, possibly as a result of long incubation times in connected wells, it was decided that FRTL-5 cells should be split into individual, 9cm^2 plates and be replated and amplified in 55cm^2 plates.

21 hr after transfection, cells were given 4μg/mL of blasticidin. Although the transfected cells in this study carried the blasticidin resistant gene (BSD^r), the reagent still decreased cell proliferation. Non-transfected, FRTL-5 cells without blasticidin reached confluence in two weeks (figure 11). In contrast, none of the transfected cells reached confluence within two weeks in blasticidin (4μg/mL). FRTL-5 + Blasticidin cells were used as the negative control; all the cells died within 18 days when grown in parallel with transfected cells. To improve growth of transfected cells, the transfected plates were placed in a reduced concentration of blasticidin (2μg/mL) once all the cells from the FRTL-5 + blasticidin (4μg/mL) plate had all died (at day 18).

Empty (CO) and scrambled (CS) insert vector controls had accelerated growth when blasticidin concentration was reduced to 2μg/mL 18 days after transfection (figures 12 and 13). Cells from both CO and CS vector insert plates reached confluence within two and three weeks after reducing blasticidin concentration, respectively. Transfected cells with insKD constructs had much slower growth rates (figures 12 and 13). insKD C and D cells reached confluence after four weeks of

growth in 2µg/mL of blasticidin. insKD B cells reached confluence after nine weeks of growth, and insKD A cells have not yet reached confluence at the conclusion of the research (twelve weeks).

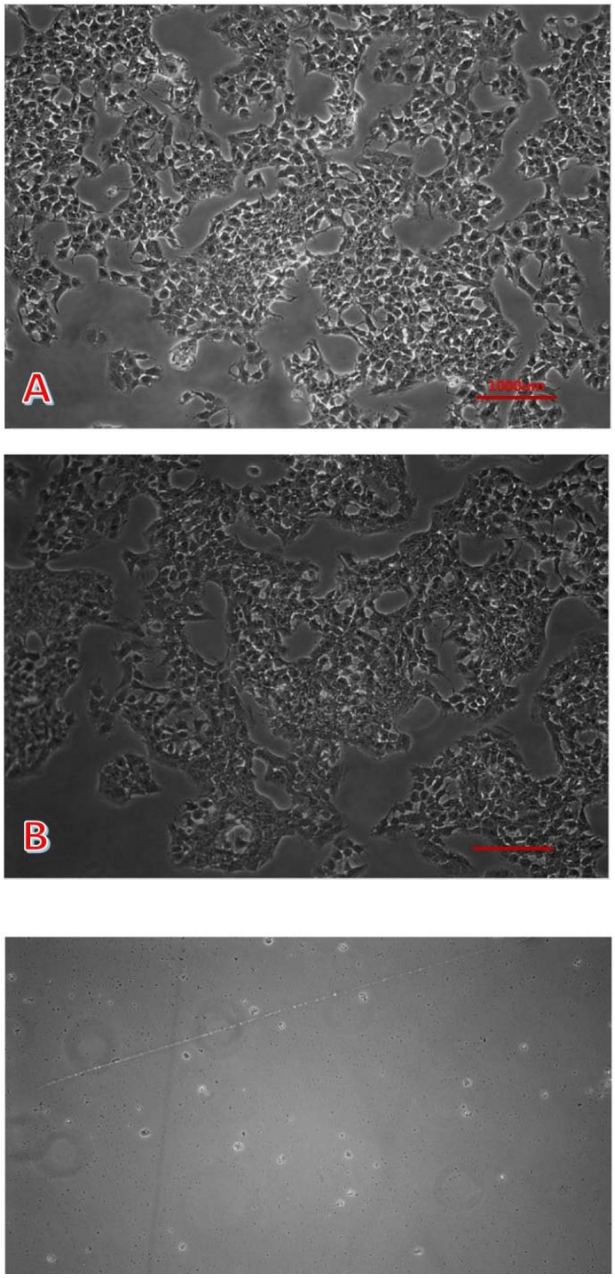

Figure 11. Untransfected FRTL-5 cells (plates 1-3) were grown in parallel with transfected cells (plates 4-9). 18 days after plates 4-9 were transfected (magnification 100x).
(A) FRTL-5 cells; (B) FRTL-5 + FugeneHD; (C) FRTL-5 cells + Blasticidin

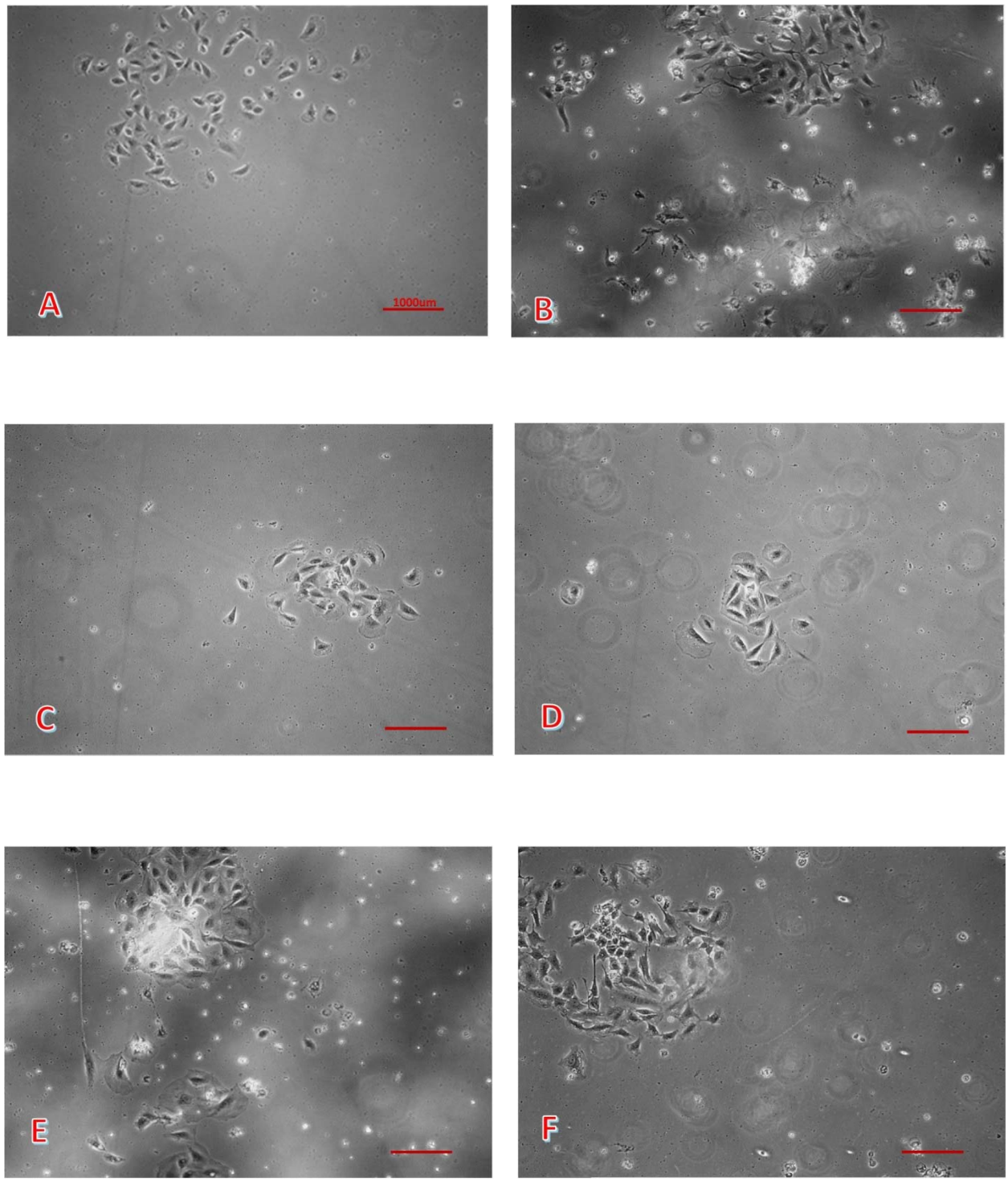

Figure 12. FRTL-5 cells 18 days after cell transfection (magnification 100x)
(A) CO plasmid cells; (B) CS plasmid cells; (C) insKD A, (D) insKD B, (E) insKD C, and (F) insKD D cells. (A-F) All cells received blasticidin.

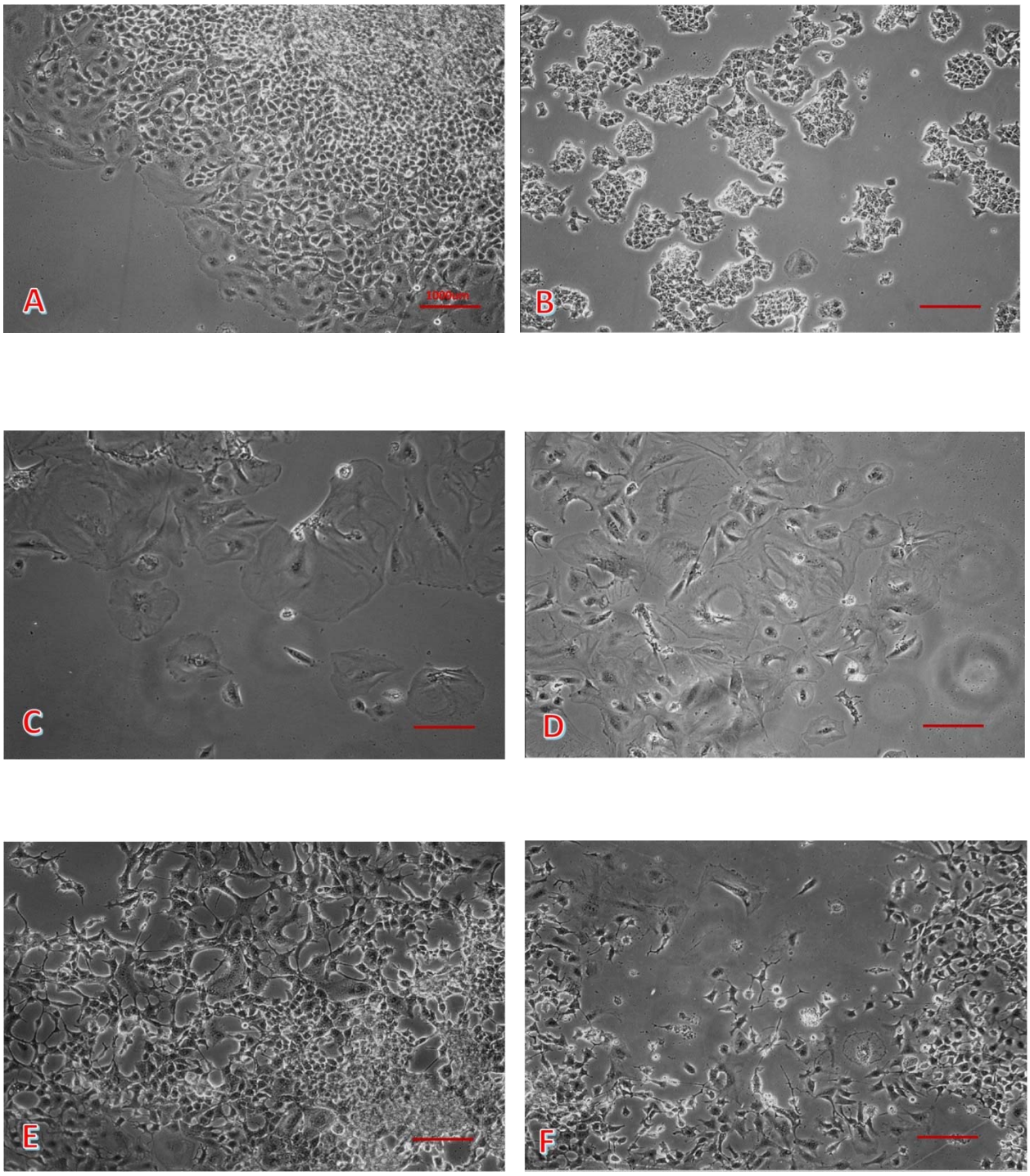

Figure 13. FRTL-5 cells 24 days after cell transfection (magnification 100x)
(A) CO plasmid cells; (B) CS plasmid cells; (C) insKD A, (D) insKD B, (E) insKD C, and (F) insKD D cells. (A-F) All cells received blasticidin.

Slower proliferation rates were observed in CO and CS vector cells (Table 1: plates 4 and 5) when compared to untransfected FRTL-5 cells (Table 1: plates 1 and 2). This is most likely due to the effects of the selective blasticidin concentration present in the media (4μg/mL). After reducing the blasticidin concentration, CO and CS vector cells exhibited faster proliferation rates. CO and CS vector cells also exhibited faster growth rates than insKD construct cells. The observed slower growth rates in insKD construct cells are expected since FRTL-5 cells are thyroid cells. Under normal condition, these cells are stimulated to undergo proliferation in the presence of growth hormone, TSH, insulin and IGF-1. When FRTL-5 cells are successfully transfected with insKD constructs, they should show reduced cell proliferation in the presence of insulin and IGF-1. The differential growth rates in the different insKD constructs could be explained by the effectiveness with which each construct targets and decreases IR expression. This is a positive indication of successful transfection and decreased expression of IR.

Discussion

The results show that insKD constructs cause slower growth rates and morphological changes to the transfected cells. When blasticidin concentration was reduced to 2μg/mL from the initial selective concentration (4μg/mL), CO and CS vector controls exhibited faster growth rates than insKD transfected cells. Also, there were differential growth rates among the four insKD constructs. insKD C and D had faster growth rates than insKD A and B. Cells transfected with insKD A constructs had the

slowest growth rates. These results imply that transfection was successful and that the insKD plasmids successfully targeted the insulin receptor mRNA. The transfected cell lines that were obtained from this research will be used in future research.

Morphological differences were also observed between non-transfected and transfected cells. FRTL-5 cells have polygonal morphology and proliferate as a monolayer. Transfected cells exhibited similar cell morphology to non-transfected cells but grew in isolated, spherical clumps. This observation was most apparent in cells with insKD constructs (figures 11 and 13).

By binding to IR or IGF-1R, insulin may induce cell proliferation and changes in metabolism. A common feature with T2DM is IR insensitivity. Decreased IR or IGF-1R activity may induce Porf-2 activity. Since Porf-2 has both anti-proliferative and pro-apoptotic properties, this decrease in activity might increase cell cycle arrest (figure 4).

Insulin and IGF-1 Receptors in the Brain

Insulin and IGF-1 receptors have been shown to be expressed in the brain through in situ hybridization in rats. Studies show high levels of expression in granule cells, mitral cells, and glomerular layers of olfactory bulbs of the rat brain. Other studies show that IR mRNA is found in both cell bodies and synapses of hippocampal CA1 pyramidal cells, synapses of hypothalamic cells, and dendro-dendritic synapses of olfactory bulb cells. These results indicate that IR and IGF-1Rs are differentially

expressed in the brain and appear to be localized in specific regions involved with cognition, learning and memory (Williamson et al. 2012, Marks et al. 1990).

Influence of T2DM on Cognitive Decline and Brain Volume

In a recent study, Espeland et al. (2013) measured regional brain volumes and ischemic lesion volumes in 1,366 women, aged 72-89 years, using magnetic resonance imaging (MRI). Measurements showed that women with T2DM had smaller total brain volumes and gray matter volumes, but not white matter volumes, compared to those without diabetes. These women also had significant increases in ischemic lesion volumes. The results suggest that diabetes has a negative effect on brain structure (Espeland et al. 2013). Similarly, a systematic review of observational studies by Cukierman et al. showed that a diagnosis of diabetes increased a person's risk of cognitive decline and dementia (Cukierman et al. 2005). These results indicate that individuals with T2DM have increased risk of cognitive decline, dementia, and brain volume shrinkage.

The Roles of RhoGTPases and RhoGAP in Cognition

RhoGTPases are a family of small G-proteins that cycle between an active GTP-bound and inactive GDP-bound forms. They have multiple intracellular functions, including stimulating microtubule stabilization, actin polymerization, and local protein translation. In the nervous system, RhoGTPase activity has shown to have effects on dendritic spine formation, synaptic plasticity and cognitive functions

(Linseman and Loucks 2008). Activation of RhoGTPases and downstream signaling altering actin turnover is important for dendritic spine changes. For example, RhoGAP activation (which decreases RhoGTPase activity) has been shown to decrease spine density and length.

Mutations that affect RhoGTPase associated proteins during pre- and neo-natal development have a negative effect on neural connectivity, which may lead to impaired cognition and mental retardation (Newey et al. 2005). For example, mutations in *ARHGEF6* (a RhoGEF), and *WAVE-1* (a downstream protein), in adults led to a decrease in dendritic synapses, synaptic rearrangement, and learning impairments in mice (Kutsche et al. 2000 and Soderling et al. 2003). Also, oligophrenin-1 is a protein with a RhoGAP domain that is involved in X-linked mental retardation (Billuart et al. 1998). Porf-2 contains a RhoGAP domain and has been shown to have pro-apoptotic and anti-proliferative properties. Further research is needed to show whether increased expression of Porf-2 prolongs inactivation of RhoGTPase, which might lead to decreased reduction of spine density and cognitive impairment in both pre- and neo-natal development and adulthood.

Conclusion

Recent studies have shown that patients with T2DM are more likely to acquire neurodegenerative diseases, in particular Alzheimer's disease, later in their lives. It is important to note that many of the neurodegenerative diseases are associated with brain shrinkage, most likely through increased neuronal cell death. Porf-2, which has anti-proliferative and pro-apoptotic properties, may contribute to the onset of neural

cell death when insulin/IGF-1 signaling is impaired. In this project, we have successfully obtained transfected insKD cell cultures that will be used for future research. Results show that cells transfected with an insKD construct exhibit slower proliferation rates than CO and CS vector controls. The transfected cells also exhibited different proliferation morphologies. Whereas FRTL-5 cells proliferate in a monolayer, transfected cells proliferate and accumulate in isolated clumps (figures 11 and 13). By studying FRTL-5 insKD cells, it is our goal to provide an *in vitro* model of decreased IR/IGF-1R function, which may result in an increase of Porf-2 expression and subsequent cell death. By doing so, our research may provide greater insight into the mechanisms of how insulin resistance in T2DM leads to future cognitive impairment in the CNS.

Future Directions

Confirm IR and IGF-1R Knock-down Models

Further research is needed to confirm that the cell cultures obtained from this project are indeed IR knock-down FRTL-5 cell models. This can be achieved through Western blot, a technique used to measure protein expression levels. Similarly, IGF-1R protein expression levels will be measured in FRTL-5 cells that were transfected with four different IGF-1R knock-down constructs obtained by Z. Wang. Successful IR or IGF-1R knock-down cells should have at least 60% lower IR or IGF-1R protein expression compared to non-transfected FRTL-5 cells. IR and IGF-1R mRNA

expression levels will be measured using qRT-PCR. Their expression is expected to be reduced by at least 60%.

Porf-2 Expression in IR and IGF-1R Knock-down Cells

Previous research on neural stem cells showed that Porf-2 exhibits both anti-proliferative and pro-apoptotic properties (Ma and Nowak 2011). Porf-2 knock-down cells showed increased cell proliferation and underwent lower rates of apoptosis when presented with pro-apoptic compounds such as bleomycin or STS. Also, in the presence of IGF-1, it was shown that Porf-2 mRNA expression decreased. These results imply that Porf-2 is downstream of insulin and IGF-1 in signal transduction. Porf-2 mRNA expression using qRT-PCR technique should be measured in IR and IGF-1R knock-down FRTL-5 cells to test this hypothesis.

Porf-2 Knock-out Mice

The broader goal for this research project is to eventually develop a strategy to combat cognitive decline in T2DM patients. If we can establish that reduced insulin signaling causes increased Porf-2 protein expression, we will have shown that there is a critical link between neurodegenerative pathways and T2DM. Furthermore, the aim is to apply the results from this preliminary study to animal models such as Porf-2 knock-out transgenic mice. We do not know if knock-out (KO) mice will exhibit normal pro-apoptotic and anti-proliferative properties in the CNS. The expected outcome is that a lack of Porf-2 expression in KO mice will decrease

neurodegeneration in T2DM. If this is indeed the case, manipulating Porf-2 expression may lead to development of a therapeutic method to treat T2DM patients who are at risk of developing cognitive decline.

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