The Study of Non-Viral Nanoscale Delivery Systems for Islet Transplantation

ΒY

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List of Abbreviations

- AuNPs Gold Nanoparticles
- DNA Deoxyribonucleic Acid
- mRNA Messenger Ribonucleic Acid
- TEM Transmission Electron Microscopy
- T1DM Type I Diabetes Mellitus
- T2DM Type II Diabetes Mellitus
- VEGF Vascular Endothelial Growth Factor
- PP cells Pancreatic Polypeptide Cells
- ATP Adenosine Triphosphate
- GLUT2 Glucose Transporter 2
- HIF 1a Hypoxia Inducible Factor 1 Alpha
- NKT cells Natural Killer Cells
- IBMIR Instant Blood Mediated Inflammatory Response
- PERV Pig Endogenous Virus
- DOX Doxorubicin
- MTX Methotrexate
- AFM Atomic Force Microscopy
- EDA Ethylenediamine

- SPR Surface Plasmon Resonance
- PEG Polyethylene Glycol
- $TNF \alpha Tumor$ Necrosis Factor Alpha
- R Point Restriction Point
- Cdks Cyclin Dependent Kinases
- Rb Retinoblastoma Protein
- NA Nucleic Acid
- HeLa Henrietta Lacks
- stDNA Strong Deoxyribonucleic Acid
- wkDNA Weak Deoxyribonucleic Acid
- GSH Glutathione
- TGF- β Transforming Growth Factor Beta
- MEF Mouse Embryonic Fibroblasts
- NLS Nuclear Localization Signals
- NES Nuclear Export Signals
- BPS bis (p-sulfonatophenyl) phenylphophine)
- mPEG methoxypolyethylene glycol
- EdU 5-ethynyl-2´-deoxyuridine

ELISA – Enzyme Linked Immunosorbent Assay

- SDS sodium dodecyl sulfate
- CTCF Corrected Total Cell Fluorescence
- ANOVA Analysis of Variance

Abstract

Our laboratory described that human islet cells can be driven to proliferate by expressing specific cell cycle proteins via adenoviral vectors [1]. Adenoviral vectors have several limitations including poor penetration into intact islets; only the most superficial cell layers get infected. Due to safety concerns associated with using viral systems clinically to expand islet cells and make them available to many more patients, significant emphasis has been placed on producing a safe and effective non-viral delivery system for biological research and gene therapy. To obtain this goal, we propose the use of an innovative technology that utilizes gold nanoparticles (AuNPs) as a non-viral method of delivery. Our laboratory was one of the first to describe the use of AuNPs in human islets and observe AuNPs can penetrate into the core of islets to deliver a gene to the vast majority of the cells, without damaging the cell [2]. Gold nanoparticles proved to be a biocompatible delivery system both in vitro and in vivo [2].

Thus far, gene therapy and molecular biology have focused primarily on delivering DNA of a specific gene into cells. The risk of this approach is that the DNA can be permanently incorporated into the genome and lead to damages in the cell that could result in overexpression of cancerous tumor cells. This risk does not exist with the use of mRNA. Many researchers believe mRNA is too unstable to be used as a molecular tool to overexpress specific proteins. With advances in nanotechnology, and better understanding of the translation process, methods have been developed that allow for expression of specific proteins by intracellular delivery of protein-encoding mRNA [3].

We used AuNPs conjugated to mCherry mRNA to establish a proof of concept of the feasibility of using AuNP-mRNA to achieve increased expression of a specific protein within cells. To do this, we conjugated mCherry mRNA to AuNPs and tested the feasibility for increasing delivery efficacy and preserve functionality of human pancreatic islets. We believe that with this novel

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technology we can create AuNPs that allow specific mRNA to enter islets and lead to the production of a specific protein within the cell, with the aim to induce beta cell proliferation.

In a previous experiment with single cells, the highest amount of protein expression was observed after 24 hours incubation with mCherry conjugated AuNPs. Based on this, human islets were treated with 12 nm, 7 nm and 2 nm mCherry AuNPs for 24 hours. The expression of of mCherry protein in human islets was analyzed by 3D image reconstruction of *z*-stack images acquired by confocal microscopy. A minimal amount of mCherry protein was expressed in human islets when treated with mCherry mRNA coupled to the 12 nm size AuNP. Decreasing the size of the AuNPs to 7 nm or 2 nm resulted in substantial increase in mCherry protein expression throughout human pancreatic islets when treated at concentrations of 20 nM and 50 nM with mCherry mRNA AuNPs for 24 hours.

We used measurements of calcium influx, KCL and mitochondrial potential to determine the effect of AuNP-mCherry mRNA treatment on islet cell function. The area under the curve was computed for intracellular calcium influx of three different islet preparations. There was no statistically significance difference between (2 nm) 20 nM versus (7 nm) 20 nM, (2 nm) 20 nM versus (7 nm) 50 nM, (2 nm) 50 nM versus (7 nm) 20 nM, (2 nm) 50 nM. For the area under the curve for the KCL there was no significant statistical difference between the groups. In addition, mitochondrial potential indices demonstrated similarity between the control group and mCherry mRNA AuNPs treated human pancreatic islets, there was no statistical difference between the three different sizes and concentrations when compared to the non-treated group. Taken together, AuNP did not impair islet function when concentration was increased.

Although, the optimal size of AuNP that was easily seen to express mCherry protein was 7 nm, when human islet cells were treated with AuNP coupled to mRNA for E2F3 (the β -cell

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proliferation inducing protein), to observe whether there was any sign of enhanced β -cell proliferation, the 12 nm sized AuNP seemed to give a slight increase in β -cell proliferation. Transmission electron microscopy (TEM) was used to determine where within the islets the AuNPs were localized. This validated that both the 12 nm and 7 nm size AuNPs crossed the cell membrane and were found within vesicles, mitochondria and in one case the insulin granules of the islets. A notable difference that was detected under TEM for the two size of AuNPs was that the 12nm appeared predominantly in clusters where as the 7nm AuNP was more evenly distributed within the cell. Further analysis with TEM may provide provide insight on how the size, concentration and kinetics of the AuNPs will influence protein expression and β -cell expansion within human pancreatic islets.

I. Introduction:

Diabetes Mellitus

Diabetes Mellitus, often known as diabetes, describes a group of metabolic diseases in which the individual diagnosed with such a condition has high blood glucose, or increased blood sugar levels. There are two major reasons that will cause an individual to be diagnosed with such a condition including defects in secretion of insulin from pancreatic β-cells, or because the body's cells do not respond overall to insulin and at some point reach insulin resistance, or both [4]. Patients diagnosed with Diabetes suffer a variety of symptoms including frequent urination, increased levels of hunger and thirst, are more likely to increase in weight, sudden fatigue and often times numbness experienced within the hands and feet. Diabetes is a disease that is affecting people at a large scale, in 2013 it was estimated that 382 million people had diabetes world-wide [5]. Diabetes has many secondary complications including increased cardiovascular risks, kidney failure and blindness. Consequently, more research and studies are being conducted in order to provide many individuals with a promising cure. Diabetes is subdivided into two major classifications including Type I Diabetes, to which 10% of the diabetes cases belong, and Type 2 Diabetes which make up 90% of diabetes cases.

Different Types of Diabetes

Type I Diabetes Mellitus (T1DM) affects children, also known as juvenile diabetes. An individual with this type suffers from the destruction of β -cells responsible for producing insulin within the islets of the pancreas. This results in a collapse in pancreatic insulin generation for the maintenance of controlled glucose levels within the body [6]. Insulin production becomes inadequate for the control of blood glucose levels due to the self-destruction of beta cells in the pancreas. The self-destruction occurs over time until the mass of these cells decreases; as a result the amount of insulin being secreted is very limited [4].

When it develops later in life, during adulthood, Type 1 Diabetes can be mistaken with a diagnosis for Type 2 Diabetes. Type 2 Diabetes Mellitus (T2DM) most commonly occurs in people overtime; this usually involves the inability of the body to lower blood glucose levels. Over time, glucose levels increases out of proportion and this is known as hyperglycemia. To compensate for the increase in glucose levels the body attempts to produce sufficient insulin to maintain proper glucose levels, however the body is unable to produce the insulin levels required and overtime this has a negative effect on remaining functioning beta cells and eventually leads to insulin resistance [7]. These increases in blood glucose levels can be life threatening for T2DM patients. In addition, individuals diagnosed with T2DM have been known to have only 50% of their islets to be fully functioning [8]. In addition, a decline in β -cell mass of individuals diagnosed with T2DM can be clearly observed [9]. T2DM can be controlled through changes in diet, daily exercise and lifestyle of the individual diagnosed with such a disease.

A diagnosis of diabetes mellitus comes with many secondary complications. Some of these complications include the risk of developing cardiovascular diseases, potential kidney failure, and even loss of eyesight. Individuals diagnosed with Type 1 Diabetes require daily insulin injections for proper glucose control. These include children whose daily life is interrupted by the need to take insulin on a daily basis, impeding the ability to enjoy their daily activities because they have to worry about injecting the appropriate levels of insulin before they reach a state of hyperglycemia. As a result, individuals diagnosed with diabetes have to clearly monitor their lifestyle on a daily basis. Diabetes is a disease that not only affects the individual diagnosed but also other family members.

Physiology of β-cells

The pancreas is comprised of two major tissue types, exocrine and endocrine tissue. The islets of Langerhans are the primary endocrine cells within the pancreas. Islets are clusters of five

specific cell types comprised of between1,000-2,000 cells. The five cell types within the islets are the alpha cells, beta cells, pancreatic polypeptide cells, delta cells and epsilon cells. Alpha cells are responsible for secreting glucagon, while β -cells secrete insulin in order to maintain proper glucose levels. The third cell type are the pancreatic polypeptide (PP) responsible for forming pancreatic polypeptide, the fourth cell type are the delta cells responsible for the production of somatostatin, which maintain normalization between the secretion of glucose [10]. The final cell type found within islets are Epsilon cells, often this specific cell type only makes up < 1% of the islet cell mass. Glucose is the primary component that controls the release of insulin within the β -cell, at glucose concentrations greater than 7 mM, the cell membrane depolarizes from an initial resting potential of about -70 mV [10].

Thus the entry of glucose into β -cell and subsequent stimulation of insulin, normalizes glucose levels to appropriate physiological levels. When glucose enters the β -cell via the GLUT2 transporters, it gets broken down by the enzyme glucokinase, which initiate a series of steps leading to the metabolism of glucose to generate ATP. The resulting increase in ATP levels causes the K+/ATP channels to close, which in turn leads to an increase in the K+ ions within the β -cell. This process causes the cell membrane to depolarize. To restore the membrane depolarization, the Ca+2/voltage gated ion channels open, thus creating an influx of Ca+2 ions within cytoplasm of the β -cell. The influx of Ca+2 ions results in the exocytosis of insulin granules, which results in the secretion of insulin. Insulin is released by the β -cells in a two phase (biphasic) manner. The first phase of insulin release is from the exocytosis of the insulin that has been initially stored within the insulin granules. The second phase of insulin release is from newly synthesized insulin [10].

Clinical Islet Transplantation

One of the current promising therapies for T1DM is the ability to transplant isolated islets from a cadaveric donor pancreas into the portal vein of the liver of a living compatible recipient. Islet transplantation emerged as a potential T1DM therapy with the introduction of the Edmonton protocol. This protocol has allowed T1DM patients to become insulin independent for many years. [11]. There are several limitations to clinical islet transplantations, for one, a sufficient number of islets need to be isolated in order to be transplanted into the matching recipient. The process of islet isolation is a very extensive and tedious process involving the following steps, perfusion of the pancreas with collagenase enzyme to break down the pancreatic collagen and free the islets, digestion in order to break down the pancreas tissue using a mechanical force by a Ricordi chamber, a purification procedure where the exocrine tissue gets separated from the endocrine tissue by using discontinuous gradient solutions of a high and low density, and finally the culturing of the islets. However, there are a series of drawbacks to the process of islet transplantation. As mentioned previously a sufficient amount of islets must be isolated in order to be infused into the portal vein of the liver. The exact number of islets required for a transplant to take place is based on the weight of the compatible recipient. Once, the number of islets/kg is known, the loss of islets during culture and after they are infused into the portal vein of the liver have to be taken into consideration. Loss of islets during culture and transplantation is inevitable; there are a series of factors which contribute to this phenomenon.

Islets are exposed to hypoxia during the islet isolation procedure; in response the β -cells release a particular factor known as the hypoxia inducible factor 1 alpha (HIF – 1 α). This results in the dedifferentiation of β -cells to a stage of being an epithelial to a mesenchymal cell type. HIF - 1 α also induces a Twist mechanism, which leads to the misfolding from the Twist gene. This is thought to trigger a response from the innate immune system leading to the development of fibrosis [11].

Another significant loss of islets occurs when the cells are infused into the portal vein of the liver of the living recipient. Human islets range from 50 μ m – 500 μ m in size, while the liver vessels are 10 mm in diameter, therefore, the size of the liver vessel is much larger when compared to the size of the islets. During this process, islets are floating and moving into the blood vessel and the only way they may adhere to the surface of the walls of the liver is by attachment to blood clots which forms a clump that will eventually adhere to the walls of the liver [11]. The islets that are unable to be trapped within blood clots eventually die off, because they will be unable to obtain the sufficient amount of nutrients in order to survive.

Another factor that contributes to the loss of islets over time once transplanted is the immune response of the living donor. The body recognizes the transplanted cells as foreign and release natural killer T cells (NKT cells) that attack and destroy the islets. Therefore, in order to minimize the effects of a sudden immune reaction, individuals receiving islet transplantations need to take a series of immunosuppressive drugs on a daily basis. The high concentration of immunosuppressive drugs might provide a form of protection for the islets and prevent the islets from having an undesired response triggered by the immune system. However, these immunosuppressive drugs are present at concentrations that can also pose negative effects to islets. The exact harmful effects are still unknown and need to be further investigated; recent preclinical studies have demonstrated that one of the immunosuppressive drugs, tacrolimus impedes revascularization of isolated pancreatic islets [12].

Not only are islets affected by the immune response, but also once islets are infused within the portal vein of the liver, there is not a fully vascularized network that will provide the appropriate nutrients to survive. It takes a minimum of 14 days for a complex vascular network to be formed [13]. The process of forming a new vascular network takes longer to develop in humans than

within rodent models. In addition, there is low oxygen tension in the liver. This depletion of oxygen further promotes hypoxia, a major contributor to islet loss as previously mentioned.

The instant blood-mediated inflammatory reaction (IBMIR) involves a thrombotic reaction when islets come into contact with ABO compatible blood [12, 13]. IBMIR is responsible for inducing the coagulation and the complement system. This process further induces platelet formation. The surface of the islets is bounded to the platelets, allowing the islets to adhere to the surface of the liver. The platelets then promote the formation of fibrin, this particular fibrin affects macrophages, initiating an inflammatory reaction [14].

<u>Alternative β-cell Therapies</u>

Many researchers are looking into alternatives for β -cell therapies. One of the major limitations with respect to clinical islet transplantations is the shortage of donor pancreata in order to isolate a sufficient number of islets to be transplanted. As a result, many scientists have investigated the possibility of using an alternative cell sources. One of those strategies is by using stem cells in order to mimic the function of the insulin producing β -cell [15-18].

Another potential source of beta cells may be achieved through xenotransplantation. Present studies involving xenotransplantation focus mainly on porcine islets. Human and porcine insulin differ only by one amino acid, and insulin extracted from pigs has been previously used to treat diabetic patients. However, just as there are positive alternatives in using islets from pigs a for human transplantation there are equally many disadvantages including the risk of transfer of zoonotic viruses such as the pig endogenous virus (PERV) [12]. In addition, one has to consider the amount of pig islets that would have to be isolated in order to be transplanted into a living recipient and the ethical problems of using large numbers of animals for such a purpose.

setting. The critical factor being immune rejection is inevitable. As a result, stronger immunosuppressive drugs would need to be further developed and tested.

Even though the aforementioned cell sources are all potential alternatives for β -cell therapies, the possibility of expanding preexisting β -cells within islets by inducing cell proliferation holds a great promise. Cell proliferation occurs through cell growth and division. However, cell proliferation is tightly regulated, as uncontrolled proliferation can lead to carcinogenesis. Proliferation of β -cells within islets is strictly located within the endogenous adult pancreas, making a condition such as diabetes very difficult to target for treatment.

It is now known that the protein E2F3 from the E2F family of genes has potential proliferative effects on beta cells [1]. The E2F family of proteins is known to act as activators or repressors of transcription within human islets [19, 20]. In particular, the E2F3 protein is known to enhance the proliferation of β -cells within the cell cycle. Currently, many researchers use viral methods of delivery of such proteins, nucleic acids and molecular cargo, but these methods of delivery pose a potential risk to patients due to the use of viruses as there might be a genetic mutation that will result from using a viral method of delivery. Therefore, understanding how the E2F family of transcription factors and transcription repressors influences the fate of a cell within the cell cycle is key in understanding whether a cell will grow, continue growing or come to a complete stop within the cycle.

Cell Cycle

The cell cycle can be broken down into a series of different stages, the G0 phase is known as the resting phase of the cell cycle. At this point the cell gets ready to enter the G1 phase. During the resting phase the cells are neither dividing nor expanding, and the phase can last for days, weeks or even years. Often times when a cell enters the G0, the reason this occurs is because a cell needs to go through the restriction point (R – point) of the cell cycle, at the restriction point

this is where the destination of the cell will be determined [21]. If the cell passes the restriction point, then at that moment the cell will continue to divide within the cell cycle and continue from the G1 phase to the S (synthesis) phase of the cell cycle. However, if the cell is unable to pass the restriction point within the cell cycle then the cell will enter the G0 phase where it will remain there for an undetermined period of time [22]. In the G1 phase of the cell cycle, the cells are getting reading to enter the S phase, the cell begins to grow and prepare for the replication of chromosomes. In the S phase of the cell cycle DNA replication occurs, here the duplication of the centrosome takes place. The synthesis phase may take a very long period of time to occur due to the duplication of the chromosomes. Following the S phase, the next phase of the cell cycle is the G2 (Gap 2) phase, this phase is another restriction point which will determine whether the cell proceeds to the M phase (Mitosis phase or Meiosis phase for the germ cell line) of the cell cycle [22].

The M phase of the cell cycle can be subdivided into different stages, the prophase, metaphase, anaphase and telophase. However, there are a series of factors which influence the entry of the cell into the M phase including mass at the cellular level, the rate of growth that the specific cell is undergoing, the time it takes for the division to take place, as well as the time for the completion of DNA replication. The essential key components that regulate the cell cycle are known as the cyclins. The cyclins are proteins and their levels fluctuate based on the step of the cell cycle. The cyclins play a crucial role in the control of the cyclin-dependent kinases (cdks) [23]. These cdks must be bounded to cyclins in order to be activated. In addition, the

The cdks are the essential switches within the cell cycle. Each cdk forms a complex with a cyclin protein; only by forming this complex with a cyclin protein will a cdk get activated [23]. The kinase attaches a phosphate group to different proteins, further allowing cell progression in the

cell cycle. Furthermore, once these phosphates bind to the protein structure they can either cause activation or inactivation of the protein. Each phase of the cell cycle contains certain cdks based on their function [23].

A key protein in the cell cycle is p53, this protein is a transcription factor and binds to DNA. Once bound this protein activates another essential protein p21. This particular protein, blocks the activation of cdk which is required for a cell to progressively advance to the G1 phase. However, by blocking the activation of the cdk, the cell is able to prepare for the replication of DNA. If there is DNA damage this step will allow for its repair. On the other hand, if DNA damage is irreversible then p53 protein generates a signal to the cell and the cell undergoes apoptosis [24]. There are a series of other proteins that inhibit cdks including p16 and retinoblastoma (Rb) proteins [23]. These proteins, including p53, are all tumor suppressors. Therefore, for cancer cells that do not stop dividing, these essential proteins are not activated. Normal cells stop dividing and there are four major key components that stop a normal cell from dividing.

Normal cells require external growth factors. When there are no more growth factors available, this is the cue for the cell to stop dividing, on the contrary, cancer cells do not require the need of these such growth factors. At this point cancer cells are independent. The second factor which makes normal cells differ is the contact inhibition mechanism. When normal cells come into contact with one another, they induce signals to stop dividing. Cancer cells continue to divide uncontrollably regardless of cell contact, and form layers on top of each other. A healthy cell can only undergo approximately 50 divisions [21]. After the 50 divisions a cell will undergo programmed cell death. Once the cell undergoes cell division, the ends of the DNA shorten, these are known as telomeres. An enzyme known as telomerase replaces these shortened ends, however in normal adult cells this specific enzyme is inactivated, and therefore, the cells

will no longer divide. On the other hand, cancer cells have an activated telomerase enzyme and their telomeres will be replaced. Normal cells will stop dividing if there is any form of damage to the DNA, on the other hand, cancer cells will continue to divide even though there is damage to the DNA. In order to prevent any cell from dividing uncontrollably there is a protein that prevents this and it is the tumor suppressor pRB, cancer cells have a defect in this tumor suppressor protein [25]. This particular protein has been shown to have an impact on the encoding family of E2F proteins. Researchers have studied and further analyzed the function of these two key components together and shown that they go hand in hand within the cell cycle. Many researchers believe the E2F family is a double-edged sword, as some members of the E2F family will have an influence on the proliferation of cell while other members of the E2F family will influence the cell to come to a complete cell cycle arrest or undergo apoptosis.

<u>E2F</u>

E2F is a coding family of genes that plays a fundamental role in the proliferation of cells throughout the cell cycle. E2F was originally discovered as a cellular activity [25-26]. This particular family controls the transcription of genes that are necessary for cellular division to occur. In addition, researchers observed E1A caused a cellular protein to dissociate from E2F [25-27]. This further demonstrated that E2F is inhibited by its association with pRB, a known E1A associated protein. E1A stimulates and promotes cell cycle entry by sequestering pRB and inducing the release of transcriptionally active E2F. Many growth inhibitory signals (TGF β) mediate their effect by blocking the phosphorylation of pRB [51-53]. There are six key members of the E2F family, each playing a different role within the cell cycle. The E2F family members include E2F-1, E2F-2, E2F-3, E2F-4, E2F-5, and E2F-6. E2F-1 through E2F-3 are known as the transcription activators, while the proteins E2F-4 through E2F-6 are known as the transcription repressors [28-30]. Recently researchers have begun to classify the E2F family as having eight genes and these are subdivided into two distinct groups. One of the groups includes E2F1-E2F6

and the other group includes the DP genes, DP1 and DP2 [30-32]. As mentioned for, all these proteins of the E2F family have a crucial role during different stages of the cell cycle. The proteins E2F-1 through E2F-4 have a role during the G0, G1 and S phase of the cell cycle. These four proteins interact with the pRB gene. pRB was the first tumor suppressor to be identified. In cells that have not undergone genetic transformation, pRB binds to E2F by the cell-cycle dependent phosphorylation mechanism. The protein E2F-4 plays a central role in the G0, G1 and S phase of the cell cycle. However the tumor suppressor protein it interacts with is p107. E2F-4 and E2F-5 play a role in the G0 and G1 phase of the cell cycle, but these proteins interact with the p130 tumor suppressor gene [25-27].

Furthermore, E2F/Rb complexes have been found in either differentiated or dormant cells, but these complexes are more predominant during the G1 to S phase transition of the cell cycle. There are essentially three types of E2F complexes that are formed within the cell cycle, the activator E2F complexes, the inhibited E2F complexes and the repressor E2F complexes. The activator E2F complexes the activation domain induces for transcription to occur. During the inhibited E2F complexes, the activation domain is impeded by the binding of the pRb protein [25-27]. For the repressor E2F complex the pRb a protein that is recruited to the DNA assembles together to form a repressor activity and prevents further transcription from occurring within the cell cycle. The reason pRB plays a crucial role in the E2F-mediated repression is because there is a binding site for pRB family of proteins within the transcription activation domains of the E2F proteins. The pRb family proteins inactivate E2F by physically blocking the action of these domains [25-37].

The tumor suppressor genes pRB, p107, and p130 each contain multiple cdk phosphorylation sites and the hyper-phosphorylated forms of pRB and p130 have very low affinities for E2F [27]. Studies have shown that most of the pRb/E2F complexes persist in the S-phase and can even

be found in populations in the G2 phase of the cell cycle [27]. The levels and DNA-binding activity of the E2F-3 member of the E2F family has been analyzed and results show that this protein is regulated by the cell cycle during the entrance of cells in the S-phase [28]. Evidence now suggests that pRB regulates E2F-responsive genes through two different mechanisms; the first mechanism is when the tumor suppressor gene pRB binds to an 18 amino acid motif within the transactivation domain of E2F. As previously mentioned, this procedure is now known as the ability for pRB to inhibit activation. In addition, the second mechanism that has been investigated is where the pRB-E2F complex holds its ability to bind to the promoters of E2F responsive genes and can recruit a series of factors including the factors the influence the chromatin structure of these genes [28]. As the chromatin structure of these genes changes in conformation, the change in structure leads to transcriptional repression.

Researchers have observed when E2F-1, E2F-2, and E2F-3 are overexpressed during the cell cycle; they may be more potent transcriptional activators than E2F-4 and E2F-5. The expression of some E2F target genes decreases as the cell exits the S-phase of the cell cycle. In addition, the expression of constitutively active, mutant forms of E2F-1 or DP-1 have caused an accumulation of cells in the S-phase of the cell cycle and eventually leads to apoptosis [25-27]. E2F uses a series of different mechanisms in order to ensure programmed cell death occurs within a series of different cell lines. In addition, the overexpression of the E2F-1, E2F-2 and E2F-3 has been observed to activate a dormant cell and let it proceed into the cell cycle. However, further studies discovered that programmed cell death is particularly specific to E2F1 and not of E2F2 and E2F3 and previously believed [27]. Several researchers investigated and conducted studies on mouse embryonic fibroblasts (MEFs) lacking E2F1, but not E2F2 or E2F3. This specific cell line revealed a resistance to cMyc induced and activated programmed cell death. As a result, from the studies shown by this specific cell type all three of the activation E2Fs can activate apoptosis [27]. Decrease in E2F activity is required by cells to exit the S-

phase and this step is separate from the pRb regulation. In addition, if there is not an appropriate release of the E2Fs can lead to biological signals and create deficiencies within the pRB that might contribute to the overall progress of a specific cell type during the phases of the cell cycle. Many researchers believe that further studies need to be conducted in order to determine whether activation of E2F is required for the entry of S-phase is still not clearly understood. This concept has not been settled yet because when there was a microinjection of antibodies to target specifically the protein E2F-3, there was a reduction in the amount of REF52 cells that were able to enter the S-phase of the cell cycle [26-28]. In certain cases, E2Fs have been observed to prevent certain signals from occurring and they include growth signals such as transforming growth factor β (TGF β) and the cdk inhibitors. In addition, studies performed on E2f3 deficient mice which are mouse embryo fibroblasts (MEF) are known to be missing key mitogen-activation of all known E2F genes; therefore, this further causes a reduction in the rate of proliferation of these types of cells in both primary and transformed cells [28]. In addition, any mutation that occurs to E2F1, E2F2 and E2F3 has been enough to hinder further proliferation from occurring. Furthermore, the key genes that are responsive to E2F activity include and have been key plays within the regulation of the cell cycle and they are cyclin E, cyclin A, cdc2, and cdk2.

E2F-1 and E2F-3 have been further explored and both of these are regulated independently of one another. E2F-1 and E2F-3 have been known to activate programmed cell death within an in vivo system. However, as a whole the E2F family members play a critical role during transcriptional activity. E2F1 and E2F3 have been known to subdue the formation of pRBdeficient tumors while quickening the progression of others, but is cell type dependent. A key question for many researchers came about how the E2Fs might have specific overlapping functions. Because E2F1, E2F2 and E2F3 have overlapping functions during proliferation/apoptosis might have overlapping functions in development as well.

The second subset of the E2F family includes E2F-4 and E2F-5 and both of these transcription factors are activated differently within an in vivo biological system. Significant amount of levels of E2F-4 and E2F-5 have been detected in dormant cells during the G0 phase of the cell cycle [27]. These two transcription factors are completely different then the activating transcription factors which are primarily found within actively dividing cells. While the activating E2Fs are influenced by pRB activity, E2F5 is controlled by the pocket protein p130 and the pocket proteins in vivo at different time points regulate E2F-4 during the progression of the cell cycle [27]. Furthermore, E2F-4 and E2F-5 could not drive dormant cells located in the G0 phase of the cell cycle is cycle to re-enter the cell cycle and begin to divide. There is another difference between the two subgroups of E2F transcription factors; while the activating E2Fs are primarily subcellular localized within the nucleus; E2F-4 and E2F-5 are predominately cytoplasmic. While the activating E2Fs have a basic nuclear localization signal (NLS), Gaubatz and his team of researchers demonstrated E2F-4 has two leucine/isoleucine hydrophobic nuclear export signals (NES).

Several studies have moreover shown while cells progress within the cell cycle, the levels of E2F-4-DP-p107/p130 complexes that play a key role in the promoters decline over time, from this occurrence these complexes get replaced by the activating E2Fs over time.

In mammalian cells, experiments have shown that the overexpression of cyclin E has been able to transfer cells in the S-phase with E2F being inactive, but the overexpression of E2F-1 was observed to have a key role in the movement of cells into the S-phase, but there did not need to be a significant activation of cyclin E- associated kinases.

The most recent transcription factor of the E2F family that was discovered was the E2F-6 transcription factor. Studies in which the transcription factor E2F-6 was overexpressed

demonstrated this particular transcription factor can repress E2F responsive genes. Further evidence suggests the activating E2Fs (E2F-1, E2F-2, and E2F-3) while E2F-4 and E2F-5 act in opposing functions of one another in order to activate or repress E2F responsive genes critical in the entry or exit of a cell within the cell cycle [27]. Therefore, how E2F-6 influences the activity and regulation of the other E2F family members needs to be further investigated. A better understanding of how E2F-6 regulates all or even a certain subset of E2F genes need to be further studied. Another aspect that much research needs to be focused on is whether the influence of E2F-6 occurs during each stage of the cell cycle or is the process constrained to a certain region within the cell cycle. In addition, research focused on determining the biological consequences behind the role of the transcription factor E2F-6 needs to be further explored. E2F includes many target genes, the specific programming of E2F dependent transcription is essential in order for cells to further progress in the cell cycle and allow DNA synthesis to occur. How each of the activating or repressor E2Fs is controlled by the phosphorylation of the pocket proteins in response to signals that tell the cell to grow or die. The whole mechanism behind the E2F family and their target genes raises the question of whether a single E2F target gene is a rate limiting factor for DNA synthesis and to promote further progression within the cell cycle or whether E2F causes a certain stimulation for the production of DNA synthesis to occur via the stimulation of many genes and can recruit various factors that influence the signals for differentiation to occur. Previous studies conducted in the lab demonstrated that the transcriptional activator E2F3 of the E2F family increased β -cell proliferation [1]. However, the cell cycle protein was delivered via an adenovirus, which is not an ideal method of delivery in terms of clinical application. Therefore, many scientists are looking into non-viral methods of delivery by the introduction of a new technology that evolved known as Nanotechnology.

Nanotechnology

This particular type of technology is defined as being between 1 nm – 100 nm size ranges [38]. Nanotechnology is one of the newest fields of functional systems within a molecular scale. Newly developed drugs each year are not water-soluble, which makes their delivery extremely difficult within a biological system. In the form of nanosized particles, however, these drugs could easily be secreted and released to the desired location, and they can be delivered in the conventional form of pills by using nanostructures [39]. One of those types of nanostructures, which has been studied in terms of clinical application and is a very biocompatible method of delivery are gold nanoparticles (AuNPs) as previously mentioned. AuNPs have been applied within clinical application and they are used in terms of cancer therapeutics, two of the cancer therapeutics being delivered via AuNPs are doxorubicin (DOX) and methotrexate (MTX). A series of different nanostructures currently being used were tested with pancreatic islets including polymeric dendrimers, lipoplexes, and AuNPs, but AuNPs seemed to be the most biocompatible system of delivery within pancreatic islets.

Gold Nanoparticles

AuNPs are one of the few metal based particles that has been thoroughly investigated within clinical application. AuNPs exhibit a small surface to volume ratio, range in size based on the particular application, and have modifiable surface groups on the outer surface which make them ideal for conjugation of particular proteins, genes, or nucleic acids. Michael Faraday was the first scientific researcher to publish the procedure of synthesizing gold nanoparticles by reducing aurochloric acid by phosphorous [40]. In addition, with the advancement of microscopy technologies such as transmission electron microscopy (TEM) and atomic force microscopy (AFM), AuNPs were visually seen. Furthermore, AuNPs enter the cell through non-specific receptor mediated endocytosis [41]. Studies using AuNPs in vivo have demonstrated AuNP immediately travel to tumor sites even in the absence of functionalization, the major concept

behind this phenomenon known as the enhanced permeation and retention effect (EPR). The reason AuNPs travel to the tumor site is because of the leaky blood vessels that form throughout a cancerous cell. Opened fenestrations in tumors allow AuNPs to travel to the cancerous site, in contrast to normal cells, which have well-established vascular network [40].

In terms of delivering specific cancer therapeutics, AuNPs can be easily conjugated to folic acid, because many cancerous cell lines exhibit many folate receptors on their outer surface thus enabling the AuNP to easily travel to the tumor site and deliver an anti-cancer drug. Not only are AuNPs being used as a type of delivery system, but also studies have been performed in order to use AuNPs as a form of thermal therapy. Hyperthermia is a technique known to induce apoptotic cell death. Hyperthermia is one technique used with chemotherapy in order to target cancerous tissues; the drawback to this technique is sometimes the target tissue is very deep and the radiofrequency waves are not be able to penetrate that far. However, with the advancement of AuNPs which immediately target cancerous tissue, thermal therapy could be used. Lasers could be tuned to the surface plasmon resonance (SPR) frequency based on the size, shape and composition of the AuNPs. This type of therapy might enable the capacity to more deeply penetrate the cancerous tissue layer.

In terms of in vivo studies with AuNPs there have been a few studies performed in order to observe the behavior of AuNP in circulation within a biological system. In a study conducted within mice with breast cancer tumors, one group received radiation 30 Gy using 250 –kVp while the other group received radiation but had been injected with AuNPs at a high concentration prior to irradiation. As one month of treatment passed one was able to observe the mice, which had been injected with AuNPs and exposed to radiation. These mice showed a decrease in tumor growth over the one month period while the tumors just exposed to direct radiation only revealed a delay in the growth of the tumor [42]. On the contrary mice just injected

with AuNPs alone did not demonstrate a decrease in the size of the tumor. Furthermore, studies were prolonged over a one year period; mice which were injected to AuNPs and exposed to a degree of radiation were able to survive over the one year period versus the 20% of mice that were only applied with direct radiation [42]. In addition, the mice which were only injected with AuNPs and mice which were not given any form of treatment did not survive at all over the one year period. Additionally, pharmacokinetic studies showed there was an increase in AuNP concentration near the vasculature system of the tumor mass post 7 minutes of being injected intravenously into the mice [41]. This further establishes the concept of the EPR affect where AuNPs are geared toward the leaky vasculature system of a tumor cell mass. This was one of the prolonged in vivo studies that were performed in investigating the behavior of AuNPs when injected into a living biological system. Researchers observed the AuNPs in the tumor cell mass versus the liver. However, further in vivo studies need to determine the exact biodistribution of AuNPs within a living biological system.

The first AuNP-based treatment to reach a phase I clinical trial was CYT-6091, these AuNPs were 27 nm in size and they were attached to polyethylene glycol (PEG) and tumor necrosis factor alpha (TNF – α) [43]. These two components attached to the AuNPs had the potency to target the tumor and cause tumor toxicity. Furthermore, AuNPs were observed in post-treatment tumor biopsies of patients and AuNPs were not observed in normal tissue biopsies, which might have been influenced by the EPR uptake mechanism. AuNPs are a unique structure that has been investigated because of the unique properties including high volume to surface ratio, the ability to conjugate thiol or amine groups to the outer surface and their highly biocompatible nature. AuNPs have played a critical role in cancer therapeutics, however not enough studies have been shown using pancreatic islets, therefore, our lab was one of the few research labs to

investigate the treatment of pancreatic islets with these nanostructures. Studies were done using an oligonucleotide sequence conjugated with a fluorescent marker Cyanine 5 (Cy5) [2].

In order to develop the proof of concept of using AuNPs as a type of delivery system to pancreatic islets, studies demonstrating the ability for AuNPs to enter the core of islets needed to be further investigated. Within our lab previous studies have been conducted with a conjugated Cy5-labeled thiol-modified DNA (5'-Cy5 -CAG CTG CAC GCT GCC CTG AAA AAA AAA A-Thiol-3') onto 13±1 nm citrate-stabilized NPs (~3 nM DNA per 1 mL of 10 nM colloid). In vitro and in vivo transfection efficacy and impact on islet function of AuNPs were investigated [2]. Isolated rodent and human islets were incubated for 48 hrs with Cy5 labeled alkylthiol-modified AuNPs (.012 nmol or 7.2 x 10-12) and then analyzed by confocal microscopy demonstrating very high Cy5 fluorescence uptake throughout the cytoplasm of islet cells. Moreover, the deep layers of the islets, including the islet core, were also penetrated by the Cy5 conjugated. Flow cytometry further provided proof of over 98% uptake by the islet cells (99.5 % ± 0.42 , n=4) [2].

To further support, confocal microscopy images demonstrated the AuNPs were able to penetrate into the core of both human and rodent pancreatic islets. In addition, the transfection rate for islet cells was significantly higher than that of acinar cells in vivo. Ex vivo and in vivo assessments of the transfected islets did not show any compromise in function.

We use AuNPs conjugated to mCherry mRNA as a proof of concept to increase expression of the specific mCherry protein. With AuNPs we conjugated mCherry mRNA and tested the feasibility for increasing delivery efficacy and preserve functionality of single dissociated islets, single cells and human pancreatic islets. We believe that with this novel technology we can create AuNPs that allow specific mRNA to enter islets and lead to the production of a specific

protein within the cell, ultimately to proliferate beta cells. One of the major key players that have a critical role in the assembly of proteins is ribonucleic acid (RNA). Because of the studies conducted by S. Park and et al, we decided to use the AuNP-DNA constructs attached to mCherry mRNA as these studies demonstrated there was an increase in protein expression in vitro within cell lysates, therefore, we decided to test mRNA in single cells dissociated islets and intact human pancreatic islets. We wanted to investigate whether there was an increase in the amount of fluorescent protein as well as the cell cycle protein E2F3.

RNA and mRNA

RNA is the major component that carries out the instructions to transfer the genetic information of DNA into proteins. When each base pair of DNA in transferred into the corresponding base pair of mRNA, this procedure is known as transcription. There are three major roles of RNA for protein synthesis. mRNA is translated into protein by the joint action of transfer RNA (tRNA) and the ribosome [44]. mRNA uses the genetic information found in DNA by 3 base codes also known as a codon. Each particular amino acid requires one codon, but to make a full protein usually requires about 20 amino acids. This process is known as translation and occurs in the cytoplasm [44]. RNA contains ribonucleotides while DNA contains the deoxyribonucleotides [45]. When the synthesis of proteins happens, the major amino acid that each protein begins with is methionine. The methionine amino acid consists of the initiator codon AUG. The vast majority of mRNA can only be read in one reading frame.

In addition, a proper type of gene delivery system is required in order for a successful type of gene delivery system to be developed. Delivery of RNA is preferable to delivery of DNA via viral methods in terms of clinical application. For clinical applications in which short term gene expression is required, the delivery of nucleic acids by AuNPs provides more favorable risk to benefit ratio. The delivery of mRNA via AuNPs offers other advantages over viral vectors including safety and low immunogenicity. The ability to use RNA instead of DNA eliminates the

necessity for nuclear translocation, the potential to enhance the transfection of post-mitotic cells without the need to be taken into the nucleus of the cell. Furthermore, previous studies conducted demonstrate that RNA is two to five times more efficient for transfection into cells compared to DNA [3]. In addition, RNA offers safety advantages over DNA delivery via a viral method of delivery.

DNA is often used as a control for mRNA transfections. DNA is more stable, and gives a higher level of expression but this depends on the cell type being transfected. There are critical reasons why many researchers opt in choosing mRNA as a nucleic method of delivery including the fact mRNA does not contain viral promoters, for example CMV which is known to cause toxicity. A major advantage of using mRNA is that it does not need to integrate itself within the host genome; therefore there is no risk of causing a genetic mutation.

A research group led by (Phua et al.) studied and analyzed the in vitro transfection and kinetics of mRNA attached to nanoparticles and naked mRNA to determine which was the best method of delivery [46]. The research group tested the benefits of using a nanoparticle method of delivery versus naked mRNA by testing both systems on human and mouse dendritic cells (DCs). The research group was able to demonstrate the stability of mRNA nanoparticles in small volumes that were compatible within an in vivo system. From the results obtained, GFP nanoparticles were highly effective within the in vitro system, achieving a high transfection efficiency > 97% [46]. The transfections of the mRNA nanoparticles were the first to be introduced within these particular studies with the delivery of mRNA via an in vitro system. On the contrary, naked mRNA was not able to transfect within the dendritic cell lines. Furthermore, the research group decided to test different methods of delivery in order to determine which methods of delivery were more effective in terms of the nasal cavity, intravenous administration, and subcutaneous administration [46].

When administered intranasally, only mLuc delivered via a nanoparticle system was able to demonstrate a bioluminescence. The expression levels of Luciferase was observed 4 hours post-delivery and started to decay exponentially. The splenic luciferase expression was not detected after 24 hours in all the mice. The short retention of luciferase expression might be indicative of rapid breakdown of luciferase protein and mRNA within the splenic cells [46].

The researchers observed that the naked mRNA transfected at a higher rate than nanoparticle mRNA at a subcutaneous level. The transfection was able to occur significantly within 12 hours. However, over a 24-hour period luciferase expression was still detected. However, the researchers noted that subcutaneous transfection was pH dependent [46]. The researchers noted that subcutaneous transfection efficiency of naked mRNA delivery was significantly reduced. However, when the same acidic environment was exposed to HEPES in order to neutralize the environment, the transfection of naked mRNA was enhanced. In addition, the researchers noted that the use of a solution known as Ringer's lactate in naked with mRNA had an increase in the transfection efficiency, the approximate pH of Ringer's Lactate is around 5.5. But by using this solution, there was also a decrease in the half-lives of mRNA. The most interesting aspect of this study was that the expression of mRNA lasted the longest in the administration of the particle via the base-of-tail subcutaneous method; via this method of expression was observed for more than 6 days [46].

Previous studies have analyzed the delivery of naked mRNA at the subcutaneous level, but no studies have been conducted to determine the delivery of mRNA via nanoparticle format. Uptake mechanisms between the naked mRNA and the particle/mRNA are different with the mechanism behind the particle mRNA. Usually naked mRNA is up taken by nucleic acid specific receptors, while nanoparticle uptake may be dependent on another type of non-specific
endocytic mechanism. The mechanism used by the nanoparticle mRNA uptake might be directed toward a various degree of degradative pathways. The major mechanism of which the nanoparticles enter through the islets is endocytosis.

Over time, the research group did notice that the site of administration of the mRNA did not matter, the expression of luciferase protein decayed exponentially in a consistent manner. Furthermore, luciferase expression can be extrapolated in such a way where the background noise could be eliminated, and the actual duration of gene expression could be calculated [46]. Therefore, mRNA delivery is attractive because unlike DNA there is no risk of causing a genetic mutation as the mRNA does not have to be delivered within the nucleus, mRNA could be delivered to the cytoplasm. As a result, many researchers are choosing to use the delivery of mRNA over DNA because of this minimal risk. Therefore, the research group was able to show the delivery of mRNA could be controlled. The ability to deliver mRNA in a controlled manner will enable the ability to use the delivery of mRNA within a clinical setting and won't pose the same oncogenic risks using DNA will pose. Therefore, this is one of the first studies that were able to use mRNA and test the different methods of delivery.

From this study one could conclude that primary dendritic cells could be efficiently transfected with mRNA nanoparticles and allowing for gene expression to decay within an exponential biphasic manner. Furthermore, from this study the research group concluded that nanoparticle mRNA are delivery more effectively if administered intranasally and intravenously versus subcutaneous administration the most effective delivery came from the naked mRNA, but in order for the naked mRNA to be effectively administered, a solution known as Ringer's Lactate needed to be used. Many researchers do not want to use DNA as a nucleic acid for delivery because of the risk that a genetic mutation might occur; therefore, many researchers are now investigating the ability to use mRNA as a basic nucleic acid to deliver to cells. Another research

group decided to investigate to use mCherry protein conjugated to AuNP – DNA conjugates in order to investigate how these AuNP – DNA complexes would affect the translation enhancement of a specific gene within a mixture [47-48]. This group wanted to investigate how AuNP – DNA conjugates would enhance *in vitro* translation of a protein. Higher translation might occur because of the fact that the DNA on the AuNP forms an incomplete duplex with the mRNA. Currently, one of the biggest barriers for utilizing nanoparticle is non-specific adsorption, where biomolecules are non-covalently adsorbed to nanoparticle. Therefore, this non-specific adsorption is a major drawback to advancing within the field of application of nanotechnology within the field of regenerative medicine.

The research group conducted by Park and et al. decided to use AuNP – DNA conjugated complexes because they are the same size of proteins. The research group noted that the expression enhancement by conjugates of AuNPs and DNA complexes was dependent on the mRNA – DNA interaction and the AuNP surface charge [3]. The group decided to use a strand of strong DNA (stDNA) and weak DNA (wkDNA), but the group noted that (stDNA) attached to the AuNPs that had lower coverage enhanced expression to a lesser extent, which suggested that more DNA on the AuNPs facilitates binding to the mRNA and translated related species. However, the research group noted enhancement did not occur with either free AuNPs or naked DNA. Furthermore, mCherry expression decreased with increasing number of free AuNPs. The specific AuNPs used were coated with bis (p-sulfonatophenyl) phenylphophine) [BPS], these nanoparticles have a negative charge, therefore, the charge of the surface of the AuNPs had an influenced in the enhancement of protein expression [3].

During this study, the researchers observed stDNA inhibited translation, this strand of DNA acted as an antisense DNA and blocked ribosomal activity, therefore, preventing translation from occurring by binding to the mRNA. In addition, the enhancement of protein translation by

AuNP – DNA complexes will vary based on the gene that being studied and wanted to be expressed [3,49]. The research group then wanted to analyze via experimental procedures how the non – specific adsorption exhibited by AuNPs influenced the translation; therefore, AuNPs were conjugated with different amounts of methoxypolyethylene glycol (mPEG). By the addition of the mPEG to the outer surface of the AuNPs, this made the BPS coated AuNPs to become less negative by increasing the amount of mPEG attached.

In order to enhance the translation to the BPS coated AuNPs, the researchers decided to test the AuNPs and compare them with BPS coated AuNPs attached to mPEG at a dilution of 1:200. The difference between both types of AuNPs was there surface charge. However, even though they differed slightly in surface charge, they affected translation in opposite directions. The research group noted that free mPEG did not affect the translation in any form. In order to enhance translation AuNPs need to be charged in order to induce non-specific adsorption. The AuNPs need to be charged at a certain degree, the charge of the surface of the AuNP does not need to be as high, because if the charge is too high, then the translation will be inhibited.

The researchers wanted to focus on a third part of the project was to investigate the DNA and mRNA interactions, they decided to use Ribonuclease H (RNase H). The specific RNAse H recognizes RNA – DNA duplexes in order to cleave the RNA, thus decreasing the levels of expression [3]. Furthermore, eGFP and mCherry expression levels were measured when the AuNPs were treated with and without RNase H. From the results obtained, the amount of eGFP expression decreased with stDNA and further decreased with the addition of RNase H. From these results stDNA binds to eGFP mRNA to form a DNA – mRNA duplex which will inhibit translation via the antisense effect but the formation of this duplex will be recognized by the RNase H. On the other hand, RNase H did not reduce the enhanced mCherry expression of AuNP – stDNA, wk – DNA and AuNP – wkDNA exhibited both weak antisense and RNase H

activity. Antisense by an oligo is demonstrated by its capacity to sterically block the protein synthesis machinery the ribosomes from translating or reading the gene of interest. This process could occur from both non-specific and specific binding of DNA to mRNA [3, 49]. The two different proteins used for this experiment were long in base pairs; the eGFP and the mCherry mRNA used were more than 700 base pairs long. These proteins contain multiple sites for partial or complete binding of the DNA. In addition, AuNP – DNA conjugates did not increase the activity of RNase H. This might occur because the RNase H binding mechanism might not need all the translation factor, amino acids and tRNAs for activity. Furthermore, RNase H binding activity does not benefit from non-specific adsorption.

From this particular study, one could conclude specific translation enhancement occurs via a series of combination of non – specific adsorption for the translation machinery and specific binding to mRNA by AuNP – DNA conjugates. AuNP – DNA may also remove mRNA secondary structure upon binding, having the ability for ribosomes to access and enhance protein expression [3]. Overall, attaching mPEG to the BPS coated AuNPs enhances the translation by protecting the AuNP surface and reducing the overall charge of the surface of the AuNPs. For this reason AuNPs have been investigated for the potential to use this type of technique for delivery of nucleic acids such as RNA in particular mRNA.

AuNPs ideal for nucleic acid delivery

Nucleic acid vehicles are generally divided into two categories, biological or synthetic. Since viral vectors provide efficient delivery, the high risk of immune rejection, carcinogenic risks and inflammation are all issues researchers turn away from viral methods as a potential delivery system for clinical application. As previous mentioned AuNPs offer several advantages relative to traditional lipid-based vectors including scalable production. Because the surface of the

AuNPs allows the ability to conjugate different functional moieties and target agents which could be attached covalently or non-covalently to the outer surface [50]. In addition, their cytotoxicity, bio distribution and in vivo excretion properties which can be modulated by having the ability to control the size of the AuNPs as well as the charge of the particle.

In order to conjugate AuNPs with nucleic acids, there are two primary strategies which include covalent binding or supramolecular assembly, based on the application of the delivery of the nucleic acid from the AuNP will depend on the application. The charge of the AuNPs will make a difference in the uptake within cells, a strong negative charge will prevent uptake of AuNPs within cells. Furthermore, uptake of polynucleic acid AuNPs demonstrated the density of the oligonucleotides on the surface of the AuNPs influenced cell uptake, with a higher density providing a more effective delivery [51]. Furthermore, the shape of the AuNPs makes a difference in the uptake of the delivery within the cells.

One of the important factors that are being studied is how cells will uptake the nucleic acid, but one of the challenges is endosomal escape. Besides nucleic acids being attached to AuNPs via covalent binding, noncovalent binding is another type of manner to deliver nucleic acids. The strong negative charge exhibited by nucleic acids makes the attachment to cationic AuNPs an ideal system for conjugation.

The AuNP – DNA complexes have a shape where the DNA is bent around the AuNPs where the DNA becomes protected from the degradation of nucleases and other chemical agents. Noncovalent AuNP – nucleic acid (NA) conjugates are primarily composed of cationic AuNPs that can generate an easy mode of endosomal escape via a "proton sponge" effect mechanism.

In addition, researchers have observed, the AuNP – NA conjugates which are created by using the layer by layer method of the material on the outer top layer plays a fundamental role in

accomplishing endosomal escape. Furthermore, glutathione - mediated release represents a promising approach for release of nucleic acid within an intracellular level specifically from AuNPs. Glutathione (GSH), an important antioxidant is found within the cytoplasm at concentrations of (1 - 10 mmol/l) than within the extracellular, making this an ideal antioxidant to conjugate to the outer surface of the AuNPs. The antioxidant GSH exhibits an overall negative charge. When a cationic AuNPs is produced containing a photo cleavable ester linkage, the overall net charge of the AuNPs has the ability to be alternatively switched back and forth between positively and negatively charges when exposed to irradiation [52-54]. In this particular situation there was an efficient delivery of DNA and effective release was possible within living cells. AuNPs are being investigated by many researchers because of the unique properties they exhibit including the ability to modulate their surface properties in order to attach specific nucleic acids or proteins via covalent or non covalent binding [55-60]. The high biocompatibility when compared to viral methods of delivery, which make AuNPs ideal for application within the clinical field. As a result, AuNPs for the delivery of nucleic acids makes them an ideal candidate. Further cytotoxicity of AuNPs still need to be conducted in order to determine the effects AuNPs have on the cell membrane, whether the AuNPs have oxidative stress, genotoxicity and so forth. In addition, what specific organs the AuNPs travel to needs to be analyzed in order to measure the specific kinetics of the AuNPs. In addition, targeting of these vehicles to specific organs and tissues needs to further investigated in order to minimize any side effects that might arise. Therefore, making modifications to the surface of the AuNPs with specific antibodies might minimize any minimal toxic effects that might occur. In order to introduce AuNPs within the clinical field, issues involving the immune response need to be fully understood and studied. A research group decided that in order to determine uptake of AuNPs by cells, the specific kinetics needed to be analyzed and investigated.

Mechanism behind the uptake and removal of AuNPs of different sizes and shapes

A research group decided to investigate the kinematics involved with AuNPs. For the majority of the time, AuNPs are released via exocytosis from the cells in a linear relationship in comparison to the size of the AuNP [61-65]. This particular characteristic exhibited by AuNPs is completely different because there is a different relationship between the uptake into cells versus the size of the AuNPs. Therefore, understanding the metabolism of AuNPs is essential in order to determine if the nanoparticles will be trapped in vesicles and leave the cells undisturbed; therefore, they will not induce any form of toxicity. Furthermore, the group wanted to investigate the effects of the nanoparticles physical dimension and geometry on cellular accumulation and removal within the treated cells. In addition, the research group wanted a better understanding of the kinetics behind the uptake of nanoparticles within different types of cells. A study piloted determined that nanoparticles of 50 nM were easily taken up by cells at a faster rate and concentration when compared to other sizes and shapes. The research group led by Chithrani examined the uptake process of the transferrin coated nanoparticles entering cells, in particular to the cancerous cell line the Henrietta Lacks (HeLa) cells [63]. The group monitored the uptake via tracking by electron microscopy, with the technique of electron microscopy; the research group was able to visually see the exocytosis process of the nanoparticles within the cells. The research group wanted to expand the project by further determining how nanoparticles coated with different proteins on the outer surface would affect the uptake within cells based on different sizes and shape. In addition, the researchers formulated a mathematical model to predict the kinematics.

AuNPs were used to create the kinematic model because the nanoparticles can be easily controlled during synthesis. Furthermore, the nanoparticles could be quantified within biological samples by using a plasma atomic emission spectroscopy technique. The research group decided to coat the outer surface of the AuNPs with transferrin. This protein controls the level of

iron within biological fluids; specifically transferrin delivers iron into the cells within our body via the mechanism of receptor mediated endocytosis [63]. The studies performed by the research group involving AuNPs of different sizes and shapes coated with the protein transferrin could be further used as a systematic model for studying and analyzing other proteins that will be coated around the outer surface of AuNPs for future studies. This was one of the first studies to investigate this particular protein coated on the outer surface of AuNPs.

One key point that needs to be understood from this research study is that this was the protein that was coated on the outer surface of the AuNPs and different proteins coated on the outer surface of AuNPs may lead to different results. However, the cellular uptake and removal mechanism behind AuNPs coated with transferrin will provide important insight to the advancement of nanoparticles for their potential application of drug delivery within a clinical setting. The uptake and exit of AuNPs spherical in shape coated with the protein transferrin and rod shaped AuNPs coated with transferrin were tested using three different cell lines including, STO cells, HeLa cells, and SNB19 cells [66-67]. The protein transferrin was labeled with Texas red, an organic fluorophore. The fluorescently labeled protein was then adsorbed on the outer surface of the AuNPs, in addition confocal microscopy was the imaging technique used to analyze the uptake of the AuNPs within the intracellular level. The same group had also published previously; the uptake of AuNPs within cells did not create any form of toxicity to the cells. During the experiment, the cells were incubated in low temperatures of 4°C or within an ATP depleted environment. However, if the uptake of AuNPs took place via the receptormediated endocytosis mechanism, then a decrease in the uptake would be observed. For these particular experiments a decrease of approximately 70% was observed. Another experiment led by Leong and Dai demonstrated that carbon nanotubes coated with certain biomolecules entered cells via endocytosis. Thus far, endocytosis is the biggest mechanism for AuNPs to enter cells. Electron microscopy demonstrated some nanoparticles did not enter cells via the

process of invagination. This process involves the process of the nanoparticle being turned upside down or folded back in order to form a pocket. Furthermore, the researchers wanted to explore how the geometry of the AuNPs would impact uptake within cells, the scientists knew there were three major factors that would influence uptake surrounding the geometry of the AuNPs and those included the ratio of adhesion, how far the membrane of the cell would be able to stretch, and the membrane's binding energy [68-70]. As a result, all these factors will influence the uptake of AuNPs within cells. All these parameters affected what is known as the "wrapping time." The "wrapping time" could be described as the amount of time it takes for the nanoparticles to be wrapped by the cellular membrane.

The thermodynamic force refers to the amount of free energy required to drive the AuNPs into the cell. The receptor diffusion kinetics involves the recruitment of receptors to the specific binding site; in this case it would be in the outer surface of the cells where the receptor diffusion kinetics would take place. These two driving mechanisms determine how fast and how many nanoparticles are taken up by the specific cell. The research group led by Gao et al. suggested that nanoparticles that were approximately 55 nm in size had the fastest wrapping [63]. Therefore, the inability for the nanoparticles to not produce the sufficient amount of free space energy will prevent the uptake of the nanoparticles by endocytosis.

In order for smaller nanoparticles to be taken up, the nanoparticles have to be aggregated together. For 50 nm sized nanoparticles entered the cell with no additional treatment, but the 14 nm sized nanoparticles only entered the cells when six nanoparticles were aggregated together. Once the cluster of nanoparticles was formed, they were then able to cross the membrane into the cells. With nanoparticles that are bigger, greater than 70 nm in size, the overall wrapping time is much longer because you experimenting with a bulky nanoparticle and the recruitment of receptors to the outer surface of the cell membrane takes much longer [63].

Since the research group was characterizing AuNPs coated with the protein and how well their uptake was within different cell lines. Once the nanoparticles travel into the cell, the nanoparticles are removed via the process of exocytosis. In order to determine the level and rate of exocytosis from the nanoparticles, the research formulated a mathematical model based on the fraction of the nanoparticles exocytosed. The research group came up with the following mathematical equation

$$Fexo = \frac{Nout}{No}$$

In the above equation the N_{out} is the nanoparticles exocytosed while N_o is the nanoparticles that were internalized. From the above equation one could observe that smaller nanoparticles will exocytose at a much faster rate than larger nanoparticles. Uptake and removal of nanoparticles from cells was dependent on the size of the nanoparticles. Since smaller sized nanoparticles will have less receptor ligand interactions the smaller size will lead to a lower binding constant. In addition, this might explain why the nanoparticles which coated with transferrin were released more quickly. However, when the protein transferrin was delivered only by itself, the protein underwent exocytosis at a much faster rate than if would have been conjugated to a nanoparticle [63]. From the equation that was formulated by this study one would be able to conclude the number of nanoparticles which underwent exocytosis versus the size of the nanoparticles.

The important key factors that need to be gained from these experiments based on the fraction of nanoparticles which underwent the process of exocytosis, this number depended on the efficiency of dissociation of the receptor-ligand complex which was formed around the nanoparticles. In addition, the research observed the fraction of AuNPs exocytosed is dependent on the number of nanoparticles internalized. Furthermore, the amount of AuNPs

undergoing endocytosis is slow when the concentration of nanoparticles in the medium is slow. Therefore, the previous formula that was mentioned above could be written,

$$F_{exo} = \alpha N_0 / S$$

Where α is a constant that depends on the cell type that will be used. Since the research group decided to use and test three separate cells lines, HeLa, SNB and STO cells, the value α was determined to be 0.3, 0.4, and 1.5. N_o is the number of nanoparticles that were internalized into the cells, and S represents the surface area of each individual nanoparticles [63]. The time point at which the information was collected was at t = 0 which marked the beginning of the exocytosis process.

The research group decided to use the imaging technique of transmission electron microscopy (TEM) and fixed the three different cell lines. Based on the TEM images obtained, the nanoparticles mostly were found within the vesicles of the cells prior to exocytosis. Based on the results obtained, the research group tried to obtain a true quantitation for the endocytosis rates. The group tested AuNPs of size that range in 14, 50 and 74 nm, the uptake and removal of the half-life for nanoparticles was studied but an exact true value still might not be the true value. The reason behind this mechanism is because the uncoated AuNPs can bind to other proteins within the serum.

The last part of the project was to investigate the impact of shape on the uptake and removal of AuNPs within the cells. The research group used the imaging technique of TEM in order to monitor the uptake, where the nanoparticles reside in and the release of the rod-shaped nanoparticles. The rod-shaped nanoparticles such as carbon nanotubes, and nanorods comprised of Au haven been explored. Based on the experimental procedures conducted by the researchers, the uptake of transferrin-coated rod – shaped nanoparticles was much less in

comparison to the transferrin - coated spherical nanoparticles [63]. The uptake data was obtained by subtracting the equilibria data from the exocytosis data. The uptake of the rod-shaped nanoparticles appeared to be more dependent upon the width of the nanoparticle in comparison to the length.

In 2010, our group investigated the use of AuNPs for the delivery of molecular cargos into human islets, showing that AuNPs had the unique ability to penetrate into the core of the human islets, and uniformly deliver molecular cargos in intact human islets without any measurable toxicity. It has been demonstrated that AuNPs are easily absorbed by a number of different cell types. AuNPs have a simple chemical nature and exhibit low cell toxicity [2]. AuNPs proved to be a biocompatible delivery system both in vitro and in vivo. AuNPs can efficiently deliver single gene or multiple genes to the vast majority of the cells, without sacrificing transfected cell functionality [2].

In this study, we hypothesize that AuNP based delivery of mRNA encoding E2F3 could lead to controlled expansion of human islet cells in vitro with a safety profile for clinical application. The experimental focus of this research is to develop AuNPs attached to a mCherry mRNA protein and test the feasibility for increasing delivery efficacy and preserve functionality of human pancreatic islets. We believe that with this novel technology we can create AuNPs that allow specific mRNA to enter islets and lead to the production of a specific protein within the cell, ultimately to proliferate beta cells.

Due to safety concerns associated with using viral systems clinically, significant emphasis has been placed on producing a safe and effective non-viral delivery system to be used in biological research and gene therapy. To obtain this goal, we propose the use of an innovative technology that utilizes AuNPs as a non-viral method of delivery. Herein, we use AuNPs to deliver a gene

that will ultimately increase the amount of E2F3 cell cycle protein within islet cells. Theoretically, this can be accomplished by either binding E2F3 to a DNA sequence or a mRNA to the AuNPs. When delivering DNA, the cell has to first transcribe the genetic code into a corresponding mRNA that then will be translated into the target protein [47]. The risk of this approach is that the DNA can be permanently incorporated into the genome and lead to damages in the cell that could lead into overexpression of cancerous tumor cells [47]. In contrast, when delivering specific mRNA it only has to be translated into the specific protein without risk of genome alternation.

Until recently, it was believed that mRNA was too unstable to be used for gene therapy. With recent advances in techniques using particles that have a size in the nanometer range, mRNA can now be delivered to cells in a way that delays their otherwise rapid degradation and mRNA delivery has currently become a hot topic in molecular therapy [46]. For our application of making human islet cells grow, a short lived stimulus would be desirable to avoid creating uncontrolled expansion of cells that could lead to tumor formation. We believe that with a novel technology such as nanotechnology we can create nanoparticles that will allow specific mRNA to enter islets and lead to the production of a specific protein within the cell that can lead to islet cells dividing and augment the number of cells that is required to be transplanted to each individual diabetic patient. It has been previously shown that the use of short, non-sense DNA sequences between the AuNP and the mRNA improves the stability, the delivery and translational efficiency of AuNP-mRNA [49].

Specific aims

Type I diabetes mellitus (T1DM) is an autoimmune disease where β -cells are not able to produce a sufficient amount of insulin to maintain proper blood glucose levels. Human islet transplantation is a promising therapy for restoring normoglycemia in T1DM patients. However, the therapy is hindered by limited supply of donor islet cells. The ultimate aim of this project is to create a functional and safe method for the successful delivery of specific proteins into isolated human islet cells that may in turn enhance β -cell expansion and increase insulin production. If successful, this would minimize the amount of islets required to transplant a Type I diabetic patient in order to successfully achieve long-term insulin independence. In order to achieve this aim we will explore AuNPs as the transportation vector and further conjugate to mRNA translated into the specific E2F3 protein to expand the growth of pancreatic β -cells [1-2].

Aim 1: To demonstrate efficient delivery and expression of current mRNA and AuNP-DNAmRNA constructs for expression of functional proteins in intact human islets. We will test AuNPs of various sizes and concentrations of nucleotide bound to the gold nanoparticles. To facilitate the measurement of delivery and translational efficiency, we use mCherry, a red fluorescent protein, as the target protein. The mRNA of mCherry is composed of 996 nucleotides. Confocal microscopy imaging will be used to quantify the amount of red fluorescence as a measurement of mCherry-mRNA delivery and translational efficiency.

Aim 2: To investigate the efficacy of AuNP-E2F3mRNA (with or without an oligo-DNA) for induction of controlled human islet cell proliferation. We will test different constructs of E2F3mRNA that can encode for different three-dimensional configurations. The success of transfection will be measured by the percentage of beta-cells that are positive for EdU uptake, a type of assay indicative of cell replication.

Aim 3: To evaluate the function and safety of human islets treated with AuNP-(DNA)-mRNA in vivo and in vitro after islet cell expansion. The functionality of human islet cells after incubation with AuNPs will be tested in vitro using a microfluidic multiparametric functional assay. For the safety concern, we will perform karyotype characterization of transfected islet cells, as well as perform long-term implants in Swiss-nude mice to investigate the propensity for tumor formation.

II. <u>Materials and Methods</u>

Gold Nanoparticle Synthesis

All sizes of spherical AuNPs (2 nm, 7 nm and 12 nm) were purchased from NanoPartz (Loveland, Colorado). Final AuNP structure of mCherry mRNA is shown in Figure 1A. Final AuNP structure of E2F3 mRNA is shown in Figure 2B. Table 1 demonstrates AuNP characteristics including diameter, concentration, molarity, number of oligonucleotides per nanoparticle, and molar extraction.



Figure 1: Structure of AuNPs (A.) Schematic representation of 12 nm sized gold nanoparticles attached to a non-specific oligonucleotide and mCherry mRNA. (B.) Schematic representation of 12 nm sized gold nanoparticles attached to a non-specific oligonucleotide and E2F3 mRNA.

Synthesis of AuNPs was performed based on previous methods from Turkevich and Frens by reduction of citrate at 100°C [71]. Reduction of a gold hydrochlorate solution (Chempur, 99%) initiated by sodium tris-citrate (Merck) by bringing the gold solution to a boiling temperature. Gold Nanoparticle constructs were manufactured by Nanopartz Inc. (Loveland

 Table 1: Characteristics of AuNPs

Gold Nanoparticles (AuNPs)	Diameter (nm)	SPS Abs (OD)	Concentration (nps/mL)	Molarity (µM)	Oligo (#/np)	Molar Extraction (M ⁻ ¹ cm ⁻¹)
C11-1.8-Oligo-50	2	60	5.23 x 10 ¹⁶	83.7195	2	6.88 x 10 ⁵
C11-5-Oligo-50	7	76	1.17 x 10 ¹⁵	1.875501	12	3.89 x 10 ⁷
C11-10-Oligo-50, Au/Oligo/E2F3	7	75	1.16 x 10 ¹⁵	1.850823	12	3.89 x 10 ⁷
C11-10-Oligo-50, Au/Oligo/mCherry	12	85	2.64 x 10 ¹⁴	0.422923	20	1.93 x 10 ⁸
C11-10-Oligo-50, Au/Oligo/E2F3	12	60	1.87 x 10 ¹⁴	0.298534	20	1.93 x 10 ⁸

CO). Oligos with the sequence (5' A AAA AAA AAA CTG CCG TCG CAC GTC GAG 3') were bound via the 5' to a covalent polymer bridge to the gold nanoparticle. The 5' end of the mCherry mRNA (996 nucleotides) were bound to the 3' end of the oligo via a covalent polymer bridge. Approximately 20 oligo/mRNA combinations are bound per gold nanoparticle. The final product was manufactured and analyzed using the NanoPartz audited ISO 9000/2001 quality control system. In addition, characterization techniques such as NIST traceable: UV-VIS (Agilent 8453) for extinction_and concentration measurements, NIR (Cary 500) for NIR extinction and concentration measurements, DLS (Malvern Nano ZS) for zeta potential measurements, ICP-MS (Varian 820-MS) for gold mass measurements and TEM (Phillips CM-100 100KV) for sizing. As shown in (Figure 2), the transmission electron microscopy of the AuNPs in different sizes. Before final characterization was confirmed, images of the AuNPs were as seen in the proceeding page.



Figure 2: TEM images of AuNPs of different sizes (A.) 2 nm mCherry mRNA AuNPs. (B.) 7 nm mCherry mRNA AuNPs (C.) 12 nm mCherry mRNA AuNPs.

The AuNPs were re-suspended in Phosphate Buffered Saline (PBS) solution to maintain a pH of 7 per previous established methods [90]. AuNPs have a shelf life of 6 months and were stored at +4°C away from light exposure. The particles were stored as recommended by Nanopartz until use. Before use the particle solutions were vortexed in order to resuspend the particles after storage.

<u>Islet Isolations</u> – Human islets were extracted based on the following protocol, Human pancreata were obtained from organ procurement organizations (OPO) following formal research consent and transported to the cell isolation facility at the University of Illinois at Chicago (UIC). No donor randomizations were applied. The isolation, purification, and culture procedures were performed as previously described [72-84]. Briefly, the pancreata were first trimmed and distended with collagenase and then digested using a modified Ricordi semi-automatic method. Post-digestion, islets were purified using the UIC-UB gradient in a Cobe 2991 cell separator (Cobe 2991, Cobe, CO) and subsequently cultured in Final Wash culture media (Mediatech, VA) at 37°C supplemented with ITS (Invitrogen, CA), Sodium bicarbonate

(Sigma, MO), Hepes, Human Albumin (Grifols, CA) and Ciprofloxacin (Hospira INC., USA) [72-84]

In vitro AuNP transfection of isolated human pancreatic islets, single cells and dissociated islets A total number of 100 human islets were directly incubated with AuNPs conjugated with the non-specific DNA sequence and mCherry mRNA AuNPs or E2F3 AuNPs at a concentration of 10 nM, 20 nM, 50 nM for 6, 12, 24, and 48 hours. AuNPs of different sizes were tested including, 2 nm, 7 nm and 12 nm for the mCherry mRNA AuNPs and 7 nm and 12 nm for the E2F3 mRNA AuNPs. Islets were washed with phosphate buffered saline (PBS) prior to performing confocal imaging in order to remove background fluorescence of AuNP residues. Simultaneously, single HeLa cells and dissociated islets were incubated with the same conditions.

<u>Fluorescence Image Analysis (Confocal Microscopy)</u> - Islets were imaged using a confocal microscope LSM 510 (Zeiss, Thornwood, NY) at 40 x magnifications with a 633 HeNe excitation laser beam to visually inspect the mCherry mRNA protein expression within the human islets. The exposure time intensity measurements remained the same for the human Z-stack images which were taken to determine how far into the core of the islets the mCherry mRNA protein was able to penetrate through. Z-stack images were taken in order to visually see the penetration of the AuNPs within the core of the islets.

<u>Microfluidic Assay</u> - Islets were incubated with ratiometric fluorescent dye of fura-2/AM (Invitrogen) to determine intracellular calcium levels and fluorescent Rhodamine 123 dye (Sigma) to monitor mitochondrial potential changes as previously reported [85-88]. The islets were incubated with 5 μ M Fura-2 and 2.5 μ M Rh123 for 30 min at 37°C in Krebs-Ringer buffer (KRB) containing 2 mM glucose.

The islets were then loaded into a temperature equilibrated microfluidic device mounted on an inverted epifluorescence microscope (Leica DMI 4000B). The loaded islets were perifused by continuous flow of KRB2 at 37°C (pH 7.4) for 5 min. KRBs containing high glucose (25 mM). A fluorescence ratio was normalized versus a basal intensity. In addition, perifusates were collected at a rate of 1mL/min in order to determine the insulin secretory kinetics via an ELISA kit (Mercodia). Dual-wavelength Fura-2/AM was excited at 340 and 380 nm and fluorescent emission was detected at 510 nm. Glucose induced hyperpolarization of the mitochondrial membrane causes uptake of Rh123 into mitochondria with a subsequent decrease in Rh123 fluorescence via fluorescence quenching. Both fluorescence signals were expressed as 'change-in-percentage' after being normalized against basal intensity levels established before stimulation. The perifusates were collected from device outlets using an automated fraction collector (Gilson, model 203B, WI).

<u>Protein Extraction</u> - Islets were lysed using protein lysis buffer, 1k of human islets were used in order for the three different conditions that were tested. Control (no nanoparticle treatment), 10 nM, and 20 nM were the three conditions tested. After 24 hour treatment, islets were transferred to a 1.5 mL Eppendorf tube. Tubes were spun down for 5 seconds using a mini bench top centrifuge. After pellet of islets submerged to the bottom of the tube, supernatant was removed and pellet of islets was washed 3 times with 1x Phosphate Buffered Saline (PBS). After washing of islets, islets were spun down at 10,000 g for 10 minutes. Following, supernatant was removed and transferred to a new Eppendorf tube. Furthermore, a Biorad DC protein assay was performed in order to determine protein concentration. Once the protein concentration was known, the loading dye volume was calculated and 30 μg was used in order to load the appropriate volume of protein with the corresponding loading dye. The maximum amount of volume loaded was 50 μL. In addition, different incubation time points were tested including 4,

8, 12, 16, 20 and 24 hours. The purpose of the different incubation periods was to test the effect of the AuNPs had on the expression of E2F3 protein.

Western Blot – All the essential solutions were made prior to loading the protein samples into the wells of the gel including transfer buffer, TBS buffer, TBS with 0.1% Tween. Samples containing the protein extraction were placed in a boiling beaker of tap water for 5 minutes. Based on the calculation, the appropriate amount of loading dye was added based on the final mass of protein, which was 35 µg. Protein samples were separated on 4-20% polyacrylamide gels (Biorad), the gel was placed appropriately and 10 µL of the ladder and 40 µL of the sample was loaded into individual wells. Running buffer comprised of 100 mL (10x) Tris Glycine with sodium dodecyl sulfate (SDS) buffer was diluted in 900 mL of diH₂O. The gel was left running at a constant voltage of 100 V for 1 hour. When 15 minutes were left prior to the gel being completed, nitrocellulose membrane was soaked with two pieces of filter paper in a tray filled with transfer buffer. Following, a fiber pad was placed onto the black face of the gel holder cassette. The gel was removed and the wells trimmed and placed onto the filter paper which was then covered with a nitrocellulose membrane. Next, a second filter paper and fiber pad were added to make a sandwich. The gel cassette holder was securely closed, and the gel cassette was placed in the transfer apparatus and ice was placed into the side of the tray with an ice block. Transfer buffer comprised of 100 mL of 10x Tris Glycine Buffer, 700 mL of diH₂O, and 200 mL of methanol was filled. The transfer was ran at a constant voltage of 100 V, the current started at 50 mA and progressively increased to 300 mA for 1.5 hours. Once the transfer was complete, the nitrocellulose membrane was placed into a plastic tray and stained with Ponceau Red, in order to visually observe the protein bands were transferred to the nitrocellulose membrane. The Ponceau Red was removed and TBS was added, TBS comprised of 100 mL of 10x Tris Buffered Saline diluted with 900 mL of diH₂O. Blocking buffer made up of 2 g of Bio-Rad non-fat dry milk and 40 mL of TBS with 0.1% Tween consisting of 100 mL of

(10x) Tris Buffered Saline, 900 mL of diH₂O and 1 mL of Tween-20 was added to the nitrocellulose membrane overnight and placed onto a shaker at 4°C. The following day the blocking buffer was removed and the membrane was rinsed with TBS-0.1% Tween for 5 minutes at room temperature on the shaker. The primary antibody was prepared as a 1:2000 dilution with blocking buffer, 10 mL total volume, membranes were stained with an E2F3 primary antibody (Santa Cruz Biotechnology), 1:1000. The next day, the membrane was washed in TBS-0.1% Tween 4 times, 10 minutes each time and placed on the shaker with a high speed. The secondary antibody, 1:2000 horseradish-peroxidase-coupled secondary antibody (Cell Signaling) was diluted into the blocking solution. A total volume of 10 mL of solution was added to the membrane and incubated for 1 hour at room temperature with constant shaking. With 20 minutes remaining for the secondary antibody, the LumiGlow reagents were placed at room temperature to equilibrate, in order to prepare the LumiGlow, 18 mL of diH₂O was added to a 50 mL with 1.0 mL of Peroxide Reagent B. Once the incubation with the secondary antibody was complete, the membrane was washed with TBS 4 times for 10 minutes each time. When the last wash was to be completed, 1.0 mL of Reagent A was added to the mixture. The LumiGlow mixture was vortexed for 6 seconds, 10 mL of the LumiGlow mixture was added to the membrane and incubated for 1 minute. The membrane was then taken to be developed.

<u>EdU Assay</u> – Once human islets are infected and cultured in AuNPs conjugated to E2F3mRNA with an oligonucleotide sequence at a concentration of 10 nM, 20 nM and 50 nM in parallel with a control group and incubated for 24 hours. A concentration of 20 μM of EdU (5-ethynyl-2'-deoxyuridine) was added to the islets and incubated for an additional four days in fresh new media. Islets are collected and washed in 1 mL of Phosphate Buffered Saline (PBS). Islets are fixed with a Bouin's solution. Islets are suspended in a 2% agarose, dehydrated and embedded into paraffin blocks (See Islet Paraffin Processing Below). Slides are stained for EdU by using a Click-IT EdU labeling kit (Molecular Probes). The Click-iT EdU Assay from Molecular Probes is

a nucleoside analog of thymidine and is incorporated into DNA during active DNA synthesis. In this Click-iT EdU Imaging Kit, the EdU contains the alkyne and the Alexa Fluor dye contains the azide. Slides were further co-stained for insulin detection to.

Islet Paraffin Processing – Once islets were cultured with the mRNA E2F3 AuNPs or mRNA mCherry AuNPs, islets were transferred to individual Eppendorf tubes corresponding to their concentration. Islets were spun down for 5 seconds using a mini centrifuge, supernatant was removed and 1.0 mL of (1x) Phosphate Buffered Saline (PBS) was added, the islets were rinsed twice with (1x) PBS. After, 1.0 mL of Bouin's solution was added and incubated for 10 minutes at room temperature. Islets were then spun down once more, until the pellet of islets clumped to the bottom of the tube, all the Bouin's fixative was removed from the pellet, 400 µL of 2% Low Gelling Temperature Agarose was added and incubated on ice for 30 minutes. After the completion of the incubation, PBS was added to each of the agarose mold and the individual molds were cut down to 0.5 mm and placed into a Nalgene tube. In order for the molds to be embedded in Paraffin all the water in the cells/tissue needs to be completely removed. Therefore, increasing the concentration of alcohol will ensure the water in the cells gets replaced by the alcohol preventing further distortion of the cells. However, alcohol and wax do not mix; therefore, Xylene is used as a clearing agent. Here the molds become transparent and clear, the Xylene starts to replace the alcohol which the paraffin will replace the xylene. Xylene is both miscible in alcohol and paraffin. Therefore, the dehydration process for the molds was started as follows.

- I. 70 % EtOH for (1 hr) with (2x) rinses
- II. 80% EtOH for 30 minutes
- III. 95% EtOH for 30 minutes
- IV. 100% EtOH for 30 minutes
- V. 100% EtOH + Xylene, 50/50 solution for 30 minutes
- VI. Xylene for 30 minutes
- VII. Xylene + Paraffin for 30 minutes
- VIII. Rinse (2x) with Paraffin and add Paraffin I for 30 minutes
- IX. Paraffin II for 30 minutes

X. Embed the molds in paraffin and place the block on ice unit for 1 hour Once the block was left sufficiently on the ice unit, sectioning was the next step of the process. <u>Hematoxylin and Eosin Staining</u> – Human islets were infected and cultured in AuNPs conjugated to E2F3 mRNA with an oligonucleotide sequence at different concentrations including 10 nM, 20 nM in parallel with a control group. After 24 hours in treatment conditions with the gold nanoparticle, a concentration of 20 µM of EdU (5-ethynyl-2'-deoxyuridine) is added to the islets and incubated for 4 more days in normal culture conditions. Islets are collected and washed in 1 mL of Phosphate Buffered Saline (PBS) with 3% Bovine Serum Albumin (BSA). Islets are fixed with a Bouin's solution. Islets are suspended in a 2% agarose, dehydrated and embedded into paraffin blocks. Slides were dewaxed, rehydrated, and stained with hematoxylin and eosin. Slides were mounted and imaged for morphological analysis.

Transmission Electron Microscopy

Electron microscopy was used in order to analyze where AuNP-mCherry mRNA uptake was taken place within the human pancreatic islets. Human islets were treated with 20 nM, 50 nM of either 2, 7 nm or 12 nm mCherry mRNA AuNPs for 24 hours of incubation at 37°C 5% CO₂. Islets were pelleted and transferred to a new Eppendorf tube, islets were then washed three times with fresh PBS, where supernatant was removed in order to remove as much of the AuNP residue. Pelleted islets were then immersed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer (SCB) [109]. The islets were then rinsed with 0.1M SCB. Islets were rinsed with distilled water and stained with 3% uranyl acetate. The fixed islet samples were rinsed with distilled water and then dehydrated in ascending grades of ethanol [109]. Propylene oxide was used as a transitional buffer, and tissues were embedded in Epon 812 and Araldite resin. Samples were placed in a 60 °C oven to cure. The blocks were sectioned using an ultramicrotome and then mounted on grids for TEM imaging. TEM images were obtained using a FEI Tecnai Spirit G2 operating at 120 kV [89].

Statistical Analysis

Two-tailed unpaired t-tests were performed for pairs of data and two-way ANOVA were performed for multiple comparisons with the corresponding control group. P-values <0.05 were considered significant. Standard error bars were used in the appropriate figures. The programming software GraphPad Prism was used to make all statistical calculations. For confocal microscopy images, corrected total cell fluorescence (CTCF) was calculated using the following formula,

CTCF = Integrated Density – (Area of the selected cell x Mean fluorescence of background readings) The integrated density and CTCF were graphed for each individual cell per size of AuNP and concentration of AuNP.

III. <u>Results</u>

<u>Protein expression of mCherry is visually observed in cervical cancer cell line HeLa with</u> <u>prolonged incubation</u>

Three different sized mCherry mRNA AuNPs were tested including 2 nm, 7 nm and 12 nm, in addition different concentrations were tested including 0 nM, 20 nM and 50 nM mCherry mRNA AuNPs. Confocal microscopy was used in order to test the amount of protein expression within single cell lines. The most commonly used cervical cancer cell line HeLa was used for the beginning of these studies. The HeLa cells were incubated for 6, 12 and 24 hours to visually obeserve whether a prolonged amount of incubation increased the amount of protein expression within the cells. Based on the results, the highest amount of protein expression was observed at a concentration of 20 nM and 50 nM after 24 hours of incubation (Figure 5A, 5B). Based on statistical analysis using Analysis of Variance (ANOVA) with multiple comparisons between the treated group and the control group, there was a statistical significance with (p < 0.05) for the control versus the 20 nM mCherry mRNA AuNP treated HeLa cells as well as for the control versus the 50 nM mCherry mRNA AuNP treated HeLa cells with the 2 nm sized AuNPs, (Figure 6A). Based on statistical analysis using ANOVA with multiple comparisons between the treated group and the control group, there was a statistical significance with (P < 0.05) for the control versus the 20 nM mCherry mRNA AuNP treated HeLa cells as well as for the control versus the 50 nM mCherry mRNA AuNP treated HeLa cells with the 7 nm sized AuNPs, (Figure 6B). There was a statistical significance (p < 0.05) between 20 nM mCherry mRNA AuNP treated HeLa cells versus 50 nM mCherry mRNA AuNP treated HeLa cells with 7 nm sized AuNPs (Figure 6B). Using ANOVA with multiple comparisons between the treated group and the control group, there was a statistical significance with (p < 0.05) for the control versus the 20 nM mCherry mRNA AuNP treated HeLa cells as well as for the control versus the 50 nM mCherry mRNA AuNP treated HeLa cells with the 12 nm sized AuNPs, (Figure 6C). Moreover, there was a statistical significance between 20 nM mCherry mRNA AuNP treated HeLa cells versus 50 nM

mCherry mRNA AuNP treated HeLa cells with 12 nm sized AuNPs (p < 0.05) (Figure 6C). In addition, because the initial studies were a proof of concept, the next step was to test the different sizes of AuNPs and concentrations within whole human pancreatic islets. Furthermore, for the initial studies within the single cancer cervical cancer cell line Hela order to increase the confluency of the HeLa cells, gelatin was coated onto the confocal dish one day prior to seeding the HeLa cells. After the HeLa cells reached 90-95% confluency they were treated with mCherry mRNA AuNPs. Once HeLa cells were incubated within the corresponding time point, confocal microscopy images were taken in order to visually observe amount of protein expression.

As visually seen in (Figure 7A), HeLa cells were treated with 20 nM mCherry mRNA AuNPs of size 2 nm, (Figure 7B), HeLa cells were treated with 20 nM mCherry mRNA AuNPs of size 7 nm, and (Figure 7C), HeLa cells were treated with 20 nM mCherry mRNA AuNPs of size 12 nm. Based on visual inspection, there is a significant amount of protein expressed within the HeLa cells. A cocentration of 50 nM was tested within the HeLa cells. (Figure 7D) shows HeLa cells treated with 50 nM mCherry mRNA AuNPs of size 2 nm. (Figure 7E) shows HeLa cells treated with 50 nM mCherry mRNA AuNPs of size 7 nm. (Figure 7F) shows HeLa cells treated with 50 mM mCherry mRNA AuNPs of size 12 nm. Based on the confocal microscopy imaging results, one could conclude that a higher concentration of 50 nM, more protein expression is observed with a prolonged incubation of 24 hours. In addition, the confocal microscopy dishes were precoated with Gelatin, the HeLa cells were then plated until they reached confluency for three days, after the HeLa cells were treated with the corresponding concentration of mCherry mRNA AuNPs and imaged. Because the single cervical cancer cell line worked, the next steps were to test single dissociated islets with same size of AuNPs including the 2 nm, 7 nm and 12 nm with the same concentrations that the HeLa cells were incubated with including 0 nM, 20 nM and 50 nM. In addition, the same incubation periods were tested as before, 0, 6, 12 and 24 hours of incubation. Confocal microscopy imaging was used in order to visually observe the amount of

mCherry protein present. The overall goal of this experimental procedure was to determine whether single dissociated islets were easy to penetrate with the AuNPs such as the HeLa cells within the same amount of incubation time. Islets were dissociated by using an enzymatic reaction with Accutase solution.



Figure 3: Increased protein expression with time within HeLa cells (A.) HeLa cells were incubated with different sizes of mCherry mRNA AuNPs, 2 nm, 7 nm and 12 nm at a concentration of 20 nM for different time periods including 6 hours, 12 hours and 24 hours, as visually observed at 24 hours the most amount of protein expression was seen. (B.) HeLa cells were incubated with different sizes of mCherry mRNA AuNPs 2 nm, 7 nm and 12 nm at a concentration of 50 nM for different time periods including 6 hours, 12 hours and 24 hours, as visually observed at 24 hours and 24 hours, as visually observed.



Figure 4: Increased protein expression with time within HeLa cells (A.) Percentage of positive HELA cells treated with 2 nm mCherry mRNA AuNPs at a concentration of 20 nM and 50 nM for 24 hours when compared to the control, non-treated group of HELA cells. (B.) Percentage of positive HELA cells treated with 7 nm mCherry mRNA AuNPs at a concentration of 20 nM and 50 nM for 24 hours when compared to the control, non-treated group of HELA cells. (C.) Percentage of positive HELA cells treated with 12 nm mCherry mRNA AuNPs at a concentration of 20 nM and 50 nM for 24 hours when compared to the control, non-treated group of HELA cells. (C.) Percentage of positive HELA cells treated with 12 nm mCherry mRNA AuNPs at a concentration of 20 nM for 24 hours when compared to the control, non-treated group of HELA cells.



Figure 5: Increased protein expression with increased concentration within HeLa cells (A.) HELA cells treated with 2 nm mCherry mRNA AuNPs at a concentration of 20 nM for 24 hours. (B.) HELA cells treated with 7 nm mCherry mRNA AuNPs at a concentration of 20 nM for 24 hours. (C.) HELA cells treated with 12 nm mCherry mRNA AuNPs at a concentration of 20 nM for 24 hours. (C.) HELA cells treated with 12 nm mCherry mRNA AuNPs at a concentration of 20 nM for 24 hours. (D.) HELA cells treated with 2 nm mCherry mRNA AuNPs at a concentration of 50 nM for 24 hours. (E.) HELA cells treated with 7 nm mCherry mRNA AuNPs at a concentration of 50 nM for 24 hours. (E.) HELA cells treated with 7 nm mCherry mRNA AuNPs at a concentration of 50 nM for 24 hours. (E.) HELA cells treated with 7 nm mCherry mRNA AuNPs at a concentration of 50 nM for 24 hours. (F.) HELA cells treated with 12 nm mCherry mRNA AuNPs at a concentration of 50 nM for 24 hours. (F.) HELA cells treated with 12 nm mCherry mRNA AuNPs at a concentration of 50 nM for 24 hours. (F.) HELA cells treated with 12 nm mCherry mRNA AuNPs at a concentration of 50 nM for 24 hours. (F.) HELA cells treated with 12 nm mCherry mRNA AuNPs at a concentration of 50 nM for 24 hours. (F.) HELA cells treated with 12 nm mCherry mRNA AuNPs at a concentration of 50 nM for 24 hours. (F.) HELA cells treated with 12 nm mCherry mRNA AuNPs at a concentration of 50 nM for 24 hours. (F.) HELA cells treated with 12 nm mCherry mRNA AuNPs at a concentration of 50 nM for 24 hours.

Protein expression of mCherry is visually observed in islets dissociated into single cells with prolonged incubation

Isolated human pancreatic islets were dissociated by enzymatic digestion with Accutase. Single cells were then treated with different sizes of AuNPs including 2 nm, 7 nm and 12 nm at different concentrations including 0 nM, 20 nM and 50 nM. The amount of positive cells for protein expression were quantified versus the incubation period, the total time of incubation was 24 hours. The ultimate goal was to determine whether the size and concentration of the AuNP had an effect on the amount of protein expression. Quantification was done by counting the number of positive cells for each condition and incubation time. Amount of protein expression increased as the incubation time increased, the incubation time points that were tested were 0, 6, 12 and 24 hours. Based on the results, the highest percentage of positive cells that expressed mCherry protein was observed at a concentration of 20 nM and 50 nM after 24 hours of incubation (Figure 8A, 8B). Based on stastical analysis using Analysis of Variance (ANOVA) with multiple comparisons between the treated group and the control group, there was a statistical significance with (p < 0.05) for the control versus the 20 nM mCherry mRNA AuNP treated dissociated islets as well as for the control versus the 50 nM mCherry mRNA AuNP treated dissociated islets. The highest amount of protein expression for all three different sizes of AuNPs was observed after 24 hours. As seen in (Figure 9A, 9B, and 9C) there was a higly significant difference (p < 0.05) between 20 nM mCherry mRNA AuNP treated dissociated single islets versus 50 nM mCherry mRNA AuNP treated dissociated single islets with the different sized AuNPs including 2 nm, 7 nm and 12 nm. Based on confocal microscopy images, protein expression is visible, as seen in (Figure 10 A-C) for the 20 nM concentration. The expression of the mCherry protein is seen the by the red flouresence. Furthermore, the percentage of positive cells expressing mCherry protein increased with a higher concentration (50 nM) as seen in (Figure 10 D-F). There is a similar amount of protein being expressed at the higher concentration of 50 nM for all three sizes of AuNPs. Because when whole islets are

dissociated, one doesn't know how to distinguish between the alpha, beta, delta, PP, and epsilon cells. As a result, the single cells that are expressing the amount of mCherry protein are all a pool of all these cells combined. When islets are dissociated into single cells, the amount of time single cells are able to survive in culture is much lower than when they are intact in full islets. Therfore, because single cells do not easily survive in culture, the studies were limited to 24 hours as a prolonged incubation decreased the viability of the single cells in culture. As previously mentioned, because the initial studies were a proof of concept, the next step was to test the different sizes of AuNPs and concentrations within whole intact human pancreatic islets over the prolonged incubation time points for all three different sizes of AuNPs.



Figure 6: Increased protein expression with time within dissociated islets (A.) Dissociated islet single cells were incubated with different sizes of mCherry mRNA AuNPs at a concentration of 20 nM for different time periods including 6 hours, 12 hours and 24 hours, as visually observed protein expression increases with time. (B.) Dissociated islet single cells were incubated with different sizes of mCherry mRNA AuNPs at a concentration of 50 nM for different time periods including 6 hours, 12 hours and 24 hours, as visually observed protein expression increases with time expression increases with time.



Figure 7: Increased protein expression with time within dissociated islets (A.) Dissociated islet single cells treated with 2 nm mCherry mRNA AuNPs at a concentration of 20 nM and 50 nM for 24 hours when compared to the control, non-treated group of dissociated islet single cells. (B.) Dissociated islet single cells treated with 7 nm mCherry mRNA AuNPs at a concentration of 20 nM and 50 nM for 24 hours when compared to the control, non-treated group of dissociated islet single cells. (C.) Dissociated islet single cells treated with 12 nm mCherry mRNA AuNPs at a concentration of 20 nM and 50 nM for 24 hours when compared to the control, non-treated group of dissociated islet single cells. (C.) Dissociated islet single cells treated with 12 nm mCherry mRNA AuNPs at a concentration of 20 nM and 50 nM for 24 hours when compared to the control, non-treated group of dissociated islet single cells.



Figure 8: Increased protein expression with increased concentration within dissociated islets (A.) Dissociated islets into single cells treated with 2 nm mCherry mRNA AuNPs at a concentration of 20 nM for 24 hours. (B.) Dissociated islets into single cells treated with 7 nm mCherry mRNA AuNPs at a concentration of 20 nM for 24 hours. (C.) Dissociated islets into single cells treated with 12 nm mCherry mRNA AuNPs at a concentration of 20 nM for 24 hours. (D.) Dissociated islets into single cells treated with 2 nm mCherry mRNA AuNPs at a concentration of 20 nM for 24 hours. (D.) Dissociated islets into single cells treated with 2 nm mCherry mRNA AuNPs at a concentration of 50 nM for 24 hours. (E.) Dissociated islets into single cells treated with 7 nm mCherry mRNA AuNPs at a concentration of 50 nM for 24 hours. (F.) Dissociated islets into single cells treated with 7 nm mCherry mRNA AuNPs at a concentration of 50 nM for 24 hours. (F.) Dissociated islets into single cells treated with 7 nm mCherry mRNA AuNPs at a concentration of 50 nM for 24 hours. (F.) Dissociated islets into single cells treated with 7 nm mCherry mRNA AuNPs at a concentration of 50 nM for 24 hours. (F.) Dissociated islets into single cells treated with 12 nm mCherry mRNA AuNPs at a concentration of 50 nM for 24 hours. (F.) Dissociated islets into single cells treated with 12 nm mCherry mRNA AuNPs at a concentration of 50 nM for 24 hours. (F.) Dissociated islets into single cells treated with 12 nm mCherry mRNA AuNPs at a concentration of 50 nM for 24 hours. (F.) Dissociated islets into single cells treated with 12 nm mCherry mRNA AuNPs at a concentration of 50 nM for 24 hours. (F.) Dissociated islets into single cells treated with 12 nm mCherry mRNA AuNPs at a concentration of 50 nM for 24 hours.
Protein expression of mCherry into intact human pancreatic islets is dependent on the size of the AuNP

Islets were imaged using a confocal microscope LSM 510 (Zeiss, Thornwood, NY) at 40 x magnifications with a 633 HeNe excitation laser beam to visually inspect the mCherry mRNA protein expression within the human islets. The exposure time intensity measurements remained the same for the confocal microscopy images, which were taken to determine how far into the core of the islets the mCherry mRNA protein was able to penetrate through. Confocal images showed increased uptake of AuNPs (7 nm) conjugated with mCherry mRNA at a concentration of 20 nM (Fig 1A) and 50 nM. Just as before with the treatment of single cells with different size of AuNPs, different mCherry mRNA AuNPs were tested within human what size and what concentration was better able to penetrate within the cells and have the greatest amount of protein expression. The different sized AuNPs used were 2 nm, 7 nm and 12 nm with concentrations ranging from 0 nM, 20 nM and 50 nM and incubation times at 24 hours. Previously shown in (Figure 1) is the schematic of the AuNP sequence used to treat the islets. The AuNPs were attached to a non-specific oligonucleotide sequence, which was attached to either mCherry mRNA (Figure 1A) or E2F3 mRNA (Figure 1B). For the set-up of the experimental plan, islets were treated with the AuNPs for 0, 6, 12 or 24 hours and confocal microscopy was used in order to observe whether the protein being expressed went into the core of the islet as 12 nm AuNPs were previously shown to penetrate into the core of the islet when just tagged with a fluorescent molecule Cy5 [2].

In a previous study, adenovirus mediated delivery of E2F3 resulted in increased beta-cell proliferation. However, the protein was delivered via a virus vector that is not an ideal system especially within clinical application. I tested the hypothesis that AuNP-based delivery of mRNA encoding for E2F3 could lead to controlled expansion of human islet cells in vitro with a safety profile suitable for clinical application. AuNPs provide a more biocompatible mechanism of

delivery within human pancreatic islets and single cells as seen beforehand. In addition different sized AuNPs with different characteristics as shown in Table 1 were used in order to determine what size and concentration was most effective within islets, without impairing islet function.

When the human pancreatic islets were treated with mCherry mRNA AuNPs, islets were washed with twice with Phosphate Buffered Saline (PBS) prior to imaging by confocal microscopy in order to remove all of the AuNP solution and culture media in order to eliminate background fluorescence. In addition, because mRNA degrades readily, during experimentation, the incubation times were closely monitored.. However, a complete and full comprehensive understanding of the circulation kinetics of the modified alkylthiol-modified AuNPs system remains to be determined. The functionalized AuNPs have been shown to be resistant to nuclease degradation, so we would expect to see higher blood levels of the AuNPs compared to naked AuNPs within in vivo studies. Previous studies have shown naked mRNA transplanted subcutaneously in vivo to work more effectively when compared to nanoparticle mRNA however, nanoparticle mRNA transplanted intranasally and intravenously in vivo demonstrated an extended half-life [38]. These were major key points taken into account within intact human pancreatic islets.

As shown previously in the TEM images Figure 2A depicts the 2 nm mCherry mRNA AuNPs, Figure 2B shows the 7 nm mCherry mRNA AuNPs, and Figure 2C demonstrates the 12 nm mCherry mRNA AuNPs. As observed, the 2 nm AuNPs because of their size tend to aggregate much quicker when compared to the 7 nm and 12 nm. Furthermore, the 7 nm and 12 nm sized AuNPs do not seem to form as much aggregation which might be because of the number of oligonucleotides attached to them, 12 and 20 versus only 2 oligonucleotides attached to the 2 nm sized AuNPs, therefore, they will attach more easily to one another.

Human islets were treated with 12 nm, 7 nm and 2 nm mCherry AuNPs for different 24 hours. As shown previously (Figure 3 and Figure 6) 24 hours was the incubation time that resulted in the highest percentage of positive cells that expressed mCherry protein within single cells and dissociated islets. As seen in (Figure 11A), human pancreatic islets not treated with mCherry mRNA AuNPs or control group appeared round morphologically. As seen in (Figure 11B) there was a minimal amount mCherry protein being expressed as seen in the confocal microscopy images with a concentration of 20 nM. Consequently, if the concentration was increased to 50 nM concentration (Figure 11C), there was no protein expression visible but via bright field images, the aggregation of AuNPs was evident by the dark colored residues. In addition, the function was tested for the islets treated with mCherry mRNA AuNPs in order to determine whether function was not being impaired as will be seen in the subsequent chapter. Following the treatment of islets with the 12 nm size AuNPs human pancreatic islets were incubated with 7 nm AuNPs. Furthermore, when the size of the AuNPs decreased to 7 nm and human pancreatic islets were treated with 20 nM and 50 nM of mCherry mRNA AuNPs for 24 hours, protein expression was increased as visibly seen in (Figure 12B and 12C). Protein expression was evident when compared to the control as clearly there was no protein expression in the non-treated group (Figure 12A), To further investigate the mCherry expression, a 3D image reconstruction was formulated from a z-stack of the human pancreatic islets treated with as seen in (Figure 14A) for the 20 nM (7 nm) mCherry mRNA AuNPs. (Figure 12B) shows the 3D reconstruction of human pancreatic islets treated with the 50 nM (7 nm) mCherry mRNA AuNPs. As observed, there is clear evidence of protein expression within the islets as observed from the reconstruction from the z-stack in the red of the mCherry protein. Quantification on the total amount of protein was computed based on the corrected total cell fluorescence (CTCF) and the integrated density using ImageJ software. CTCF values were calculated based on each individual concentration from the different sizes of AuNPs. As seen in (Figure 15A) demonstrates human adult islets incubated at a concentration of 20 nM with the 2 nm mCherry

mRNA AuNPs. (Figure 15B) shows the human pancreatic islets infected with 50 nM mCherry mRNA AuNPs at 24 hours. As observed integrated density and the corrected total cell fluorescence is much lower when compared to the other sizes of AuNPs.



Figure 9: Human islets treated with 20 nM and 50 nM (12 nm) sized Cherry mRNA AuNPs for 24 hours compared to non-treated group. (A) Confocal microscopy image of non- treated whole human islets incubated for 24 hours prior to imaging with mCherry mRNA AuNPs (0%). (B) Confocal microscopy image of treated whole human islets incubated for 24 hours at a concentration of 20 nM mCherry mRNA AuNPs (10%). (C) Confocal microscopy image of treated whole human islets incubated for 24 hours prior to imaging at a concentration of 50 nM mCherry mRNA AuNPs (15.0%) (scale bar = 50 μ m).



Figure 10: Human islets treated with 20 nM and 50 nM (7 nm) sized Cherry mRNA AuNPs for 24 hours compared to non-treated group. (A) Confocal microscopy image of non treated whole human islets incubated for 24 hours prior to imaging with mCherry mRNA AuNPs (0%). (B) Confocal microscopy image of treated whole human islets incubated for 24 hours at a concentration of 20 nM mCherry mRNA AuNPs (86.8%). (C) Confocal microscopy image of treated whole human islets incubated for 24 hours prior to imaging at a concentration of 50 nM mCherry mRNA AuNPs (89.9%) (scale bar = 50 μ m).



Figure 11: Human islets treated with 20 nM and 50 nM (2 nm) sized Cherry mRNA AuNPs for 24 hours compared to non-treated group. (A) Confocal microscopy image of non-treated whole human islets incubated for 24 hours prior to imaging with mCherry mRNA AuNPs (0%). (B) Confocal microscopy image of treated whole human islets incubated for 24 hours at a concentration of 20 nM mCherry mRNA AuNPs (60%). (C) Confocal microscopy image of treated whole human islets incubated for 24 hours prior to imaging at a concentration of 50 nM mCherry mRNA AuNPs (78%) (scale bar = 50 μ m).





Figure 12: Human islets treated with 7 nm sized Cherry mRNA AuNPs for 24 hours (A) 3D confocal microscopy one sided image of human pancreatic islets treated with 7 nm sized mCherry mRNA AuNPs at a concentration of 20 nM for 24 hours (B) 3D confocal microscopy one sided image of human pancreatic islets treated with 7 nm sized mCherry mRNA AuNPs at a concentration of 50 nM for 24 hours.



Functional analysis of in vitro transfection of mCherry mRNA AuNPs within human pancreatic islets

The impact of AuNPs on islet function was determined by measurement of intracellular calcium signaling using fura-2/AM and mitochondrial potentials using rhodamine 123 dye as previously described [85-88]. When islets were stimulated with a high glucose concentration of (25 mM), intracellular calcium increase of the human islets treated with mCherry mRNA AuNPs were statistically significant when compared to the control group for (2 nm) at 20 nM during an incubation of 24 hours as seen in (Figure 16A) based on a t-test (p < 0.05), when the control group was compared to human pancreatic islets treated with 20 nM of the 7 nm mCherry mRNA AuNPs a t-test showed there was a statistical significance between the intracellular calcium influx with (p < 0.05) as shown in (Figure 16B). When the control group was compared to the human pancreatic islets treated with 12 nm at 20 nM mCherry mRNA AuNPs as shown in (Figure 16C), there is a difference between the intracellular calcium influx when the islets were stimulated at a high glucose concentration (25 mM) and (30 mM) KCL (p <0.05). Furthermore, when there was an increase in the concentration to 50 nM of the AuNPs between the three different sizes there was a statistical significance in the intracellular calcium influx for the human pancreatic islets treated with 2 nm and 7 nm mCherry mRNA AuNPs for 24 hours (Figure 17A and 17B) versus the non-treated human pancreatic islets (p < 0.05). However, for the human pancreatic islets treated with 12 nm mCherry mRNA AuNPs there was no statistical difference in the calcium influx between the non-treated human pancreatic islets and the human pancreatic islets treated with the 12 nm AuNP (p = 0.2145) shown in (Figure 17C).

Furthermore, the area under the curve was computed using the GraphPad Prism 6 software for intracellular calcium influx of three different islet preparations as seen in (Figure 20A). There was no statistically significance between (2 nm) 20 nM versus (7 nm) 20 nM, (2 nm) 20 nM versus (7 nm) 50 nM, (2 nm) 50 nM versus (7 nm) 20 nM, (2 nm) 50 nM.

For the area under the curve for the KCL there was no significant statistical difference between the groups as shown in (Figure 20B). In addition, mitochondrial potential indices demonstrated similarity between the control group and mCherry mRNA AuNPs treated human pancreatic islets, there was no statistical difference between the three different sizes and concentrations (Figure 20C).



Figure 14: In vitro function of transfected human islets. (A) Calcium influx of control human islets compared to human islets treated with 20 nM of the 2 nm sized AuNPs for 24 hours which were statistically significant based on a t-test (p < 0.05) (B) Calcium influx of control human islets compared to human islets treated with 20 nM of the 7 nm sized AuNPs for 24 hours which were statistically significant based on a t-test (p < 0.05) (C) Calcium influx of control human islets compared to human islets treated with 20 nM of the 7 nm sized AuNPs for 24 hours which were statistically significant based on a t-test (p < 0.05) (C) Calcium influx of control human islets compared to human islets treated with 20 nM of the 12 nm sized AuNPs for 24 hours which were statistically significant based on a t-test (p < 0.05). Human pancreatic islets were stimulated with 25 mM glucose between (5 – 25 minutes) and 30 mM KCL between (45 – 60 minutes).



Figure 15: In vitro function of transfected human islets. (**A**) Calcium influx of control human islets compared to human islets treated with 50 nM of the 2 nm sized AuNPs for 24 hours which were statistically significant based on a t-test (p < 0.05) (**B**) Calcium influx of control human islets compared to human islets treated with 50 nM of the 7 nm sized AuNPs for 24 hours which were statistically significant based on a t-test (p < 0.05) (**B**) Calcium influx of control human islets compared to human islets treated with 50 nM of the 7 nm sized AuNPs for 24 hours which were statistically significant based on a t-test (p < 0.05) (**C**) Calcium influx of control human islets compared to human islets treated with 50 nM of the 12 nm sized AuNPs for 24 hours which were not statistically significant based on a t-test (p = 0.2145). Human pancreatic islets were stimulated with 25 mM glucose between (5 – 25 minutes) and 30 mM KCL between (45 – 60 minutes).



Figure 16: In vitro function of transfected human islets. (A) Mitochondrial potential of control human islets compared to human islets treated with 20 nM of the 2 nm sized AuNPs for 24 hours which were statistically significant based on a t-test (p < 0.05) (B) Mitochondrial potential of human islets compared to human islets treated with 20 nM of the 7 nm sized AuNPs for 24 hours which were statistically significant based on a t-test (p < 0.05) (C) Mitochondrial potential of control human islets treated with 20 nM of the 12 nm sized AuNPs for 24 hours which were statistically significant based on a t-test (p < 0.05) (C) Mitochondrial potential of control human islets treated with 20 nM of the 12 nm sized AuNPs for 24 hours which were statistically significant based on a t-test (p < 0.05). Human pancreatic islets were stimulated with 25 mM glucose between (5 - 25 minutes) and 30 mM KCL between (45 - 60 minutes).



Figure 17: In vitro function of transfected human islets. (A) Mitochondrial potential of control human islets compared to human islets treated with 50 nM of the 2 nm sized AuNPs for 24 hours which were statistically significant based on a t-test (p < 0.05) (B) Mitochondrial potential of control human islets compared to human islets treated with 50 nM of the 7 nm sized AuNPs for 24 hours which were statistically significant based on a t-test (p < 0.05) (C) Mitochondrial potential of control human islets treated with 50 nM of the 7 nm sized AuNPs for 24 hours which were statistically significant based on a t-test (p < 0.05) (C) Mitochondrial potential of control human islets treated with 50 nM of the 12 nm sized AuNPs for 24 hours which were statistically significant based on a t-test (p < 0.05). Human pancreatic islets were stimulated with 25 mM glucose between (5 – 25 minutes) and 30 mM KCL between (45 – 60 minutes).



Figure 18: Area under the curve (A.) Figure demonstrate the area under the curve for calcium influx between the 5-25 minute mark of human islets stimulated with (25 mM glucose) treated with mCherry AuNPs of different sizes and concentration for 24 hours in different islet preparations (n = 3), no differences were observed between the groups (B) Figure demonstrate the area under the curve for KCL between the 45-60 minute mark when human islets stimulated with (30 mM KCL) treated with mCherry AuNPs of different sizes and concentration in different islet preparations for 24 hours (n = 3), no differences were observed between the three groups. (C.) Percentage of mitochondrial indices for human islets treated with mCherry AuNPs of different sizes and concentration for 24 hours in different islet preparations (n=3).

Human adult islets treated with E2F3 mRNA AuNPs show slight protein expression of E2F3 Further studies were conducted in order to determine whether AuNPs attached to mRNA E2F3 would induced proliferation in human pancreatic islets. In order to analyze whether there was any form of E2F3 expression, islets were treated with the highest concentration of AuNPs (50 nM) for 24 hours and protein expression was measured by performing a western blot (Figure 21). As observed there was some form of protein expression when the immunoblots were normalized to the protein β -actin. As seen (Figure 21A) shows the control for the human pancreatic islets, (Figure 21B) is the islets treated with 7 nm E2F3 mRNA AuNPs at 50 nM for 24 hours, (Figure 21C) shows the islets treated with 12 nm E2F3 mRNA AuNPs at 50 nM incubated for 24 hours. However, as visually seen the bands between the E2F3 (49 kda) and βactin (42 kda) are very similar because of the close proximity between molecular weights. As a result a second immunoblot will be stained in order to clearly distinguish the band. Based on the quantification as shown in (Figure 20b), there was an increase in the amount of E2F3 protein expression for the human pancreatic islets treated with 7 nm E2F3 mRNA AuNPs for 24 hours.. Values were quantified by normalizing protein expression levels to β -actin by using densitometry.

Human adult islets treated with E2F3 mRNA AuNPs maintain morphology after 24 hours incubation

As previously mentioned, the function of the human pancreatic islets treated with the different concentration of AuNPs was tested by using a multiparametric microfluidic device and measuring the amount of intracellular calcium, mitochondrial potential demonstrated islet function was not impaired with the treatment of AuNPs. However, whether the morphological features of the islets were intact further analysis needed to be performed. In order to ensure the AuNP treated human pancreatic islets does not impair the overall structure and morphology of the islets; a standard hematoxylin and eosin staining were used to ensure the proper structural features of the islets are maintained. The combination of these two dyes used is very common within the pathology field. The hematoxylin becomes oxidized into hematin, the hematin then

binds to the negatively charged phosphate groups found within the DNA backbone. In addition, a complex conjugation is formed and the result forms a permanent stain within the nucleus. The dye then forms a bluish color in neutral to basic conditions. On the other hand, the anionic Eosin will attach to the positively charged groups within proteins. As an example the amino acid Lysine, can have pKa values that reach a value of 10, therefore, the positive ions will not be disrupted throughout an H and E staining. Consequently, an H and E was performed on human pancreatic islets treated with the 12 nm E2F3 mRNA AuNPs for 24 hours at different concentrations after islets were fixed and placed into an agarose mold and embedded into a paraffin block. The islets were then placed in a 2% Agarose mold and embedded, 5 µm sections were taken and the slides stained. As observed in (Figure 24A) with the non-treated human pancreatic islets. As seen there was no general impairment of the morphology of islets was maintained. As seen in (Figure 23B) the islets treated with 12 nm E2F3 mRNA AuNPs at a concentration of 10 nM, no impaired in morphological features was observed. However, in (Figure 23C) when the islets were treated with 12 nm E2F3 mRNA AuNPs, the residues of the AuNPs could be seen forming on the outer surface. With an increase in the concentration 50 nM as seen in (Figure 23D) there an increase in the formation of AuNP residues observed on the outside surface. The dark residues could be seen on the outside, but via this particular stain it's very difficult to observe where the AuNPs enter within the islets how they are able to impact proliferation within the islets. The standard assay used to determine whether there was β-cell proliferation was an EdU assay and islets were co-stained with insulin to determine function was maintained.

<u>Human pancreatic islets treated with AuNPs attached to mRNA for E2F3 protein induce a slight</u> <u>form of β -cell proliferation</u>

As analyzed in (Figure 22a) demonstrates the non-treated human pancreatic islets. (Figure 22b) shows the islets treated with 20 nM of E2F3 mRNA AuNPs of 12 nm, while (Figure 22c) is the 50 nM concentration. As seen by the images two cells were positive for EdU at the highest

concentration. (Figure 23) is the quantification as observed there was only a slight increase for proliferation. Due to the fact, the 7 nm size AuNP expressed more mCherry protein, the 7 nm was used together with the 12 nm E2F3 mRNA AuNP in order to visualize whether the amount of β -cell proliferation increased. As seen in (Figure 24a) is the non-treated human pancreatic islets and (Figure 24b) is the positive control islets treated with a marker known to cause proliferation within cells. As visually seen the positive control demonstrated one single positive cell for EdU. (Figure 24c) is the 20 nM E2F3 mRNA AuNPs at a size of 12 nm, one positive control was also seen. (Figure 24d) is the 50 nM, and more positive EdU cells are observed. (Figure 24e) and (Figure 24f) demonstrate the 7 nm AuNP at concentration of 20 nM and 50 nM, no EdU positive cells are observed. There is slight β -cell proliferation, but an increase in the proliferation was desired. Consequently, where the AuNPs localize within the islets might give us an idea of the kinetics of the AuNPs within the islets. Therefore, TEM was the method used in order to visually inspect where the AuNPs localized within the islets.

Monitoring the overall location of AuNPs within the adult human pancreatic islets once human pancreatic islets are incubated with mCherry AuNPs seems to be surrounding the vesicles and mitochondria and insulin granules

As observed in (Figures 30-31), these are the non-treated adult human pancreatic islets. As observed, there, one could clearly see the nucleus of the pancreatic islets. However, when the islets treated with the AuNPs are imaged one could visually see the AuNPs within the islets. As seen in (Figures 32-34), the 12 nm size mCherry mRNA AuNPs at a concentration of 50 nM could be visually seen inside the islets. Usually some AuNPs could be visually seen on the outer surface of the islets. However, the population could be seen and were able go through inside the islets. Most of the islets that were found inside the islets could be seen within the regions of the mitochondria and vesicles. As visually seen in the TEM micrographs, there were no AuNPs that reached into the nucleus of the islets. However, there were populations of 12 nm AuNPs found within the islets. On the contrary, the TEM micrographs for the 7 nm AuNPs seen in (Figures 35-38) demonstrate a higher number of AuNPs that could travel into the pancreatic islets as visually seen. A larger population of the 7nm AuNPs at a concentration of 50 nM could be seen within the islets as in (Figure 36A). Some of the AuNPs travel closer to the nucleus but not inside the nucleus as visually seen in (Figure 37A, B). However, at some point there was an observation made in the TEM micrographs where some AuNPs even localized inside an insulin granule as seen in (Figure 38A). Consequently, clearly seeing the TEM micrographs one could conclude the size does matter based on how many AuNPs could travel into the cell membrane. Even though both different sizes of AuNPs at the same concentration of 50 nM were able to cross the cell membrane, the 7 nm AuNPs were more distributed within the islets and even at some point this particular size of AuNPs was able to go into an insulin granule. (Figure 36 and Figure 37) demonstrate the TEM images for the human pancreatic islets treated with 2 nm mCherry mRNA AuNPs. As observed the 2 nm size AuNPs were able to cross the cell membrane and even enter the nucleus of the human pancreatic islets. In addition, in one case

(Figure 37B) there is a group of 2 nm mCherry mRNA AuNPs that have been localized within an insulin granule and are then being exocytosed by the same insulin granule. As a result, at some point, the AuNPs are being released from the human pancreatic islets, but size might play a key role in determining for how long they are able to stay within the islets. Therefore, more studies using TEM need to be further performed in order to experimentally determine whether the same behavior occurs and if the same observation of the islets being distributed within the vesicles, mitochondria and even the insulin granules occurs.



Figure 19: Western blot of E2F3 and actin protein found within human pancreatic islets incubated for 24 hours prior to protein extraction. (A.) Control, non-treated islets stained with E2F3 and β -actin primary antibody. (B.) Human pancreatic islets treated with 50 nM (7 nm) E2F3 mRNA AuNPs (C.) Human pancreatic islets treated with 50 nM (12 nm) E2F3 mRNA AuNPs. Dual detection of E2F3 protein and β -actin are similar in molecular weight, therefore, both amounts of protein need to be ran separately.



Figure 20a: Western blot of E2F3 and actin protein found within human pancreatic islets incubated for 24 hours prior to protein extraction. (A.) Control, non-treated islets stained with E2F3 and β -actin primary antibody. (B.) Human pancreatic islets treated with 50 nM (7 nm) E2F3 mRNA AuNPs (C.) Human pancreatic islets treated with 50 nM (12 nm) E2F3 mRNA AuNPs. Dual detection of E2F3 protein and β -actin are similar in molecular weight, therefore, both amounts of protein need to be ran separately. Left is membrane stained for E2F3, right is same membrane stained for β -actin. E2F3 (49 kDa), β -actin (42 kDa).







Figure 21: H & E images of human pancreatic islets treated with 12 nm sized E2F3 AuNPs at different concentrations for 24 hours. (A.) Control, non-treated human pancreatic islets (B.) 10 nM E2F3 mRNA AuNPs treated human pancreatic islets, incubation was for 24 hours (C.) 20 nM E2F3 mRNA AuNPs treated human pancreatic islets, incubation was for 24 hours (D.) 50 nM E2F3 mRNA AuNPs treated human pancreatic islets, incubation was for 24 hours and the outer surface of the pancreatic islets (white arrows).



Figure 22: E2F3 induced proliferation in human pancreatic islets (A.) Non-treated group of human pancreatic islets co-stained with insulin and EdU. (B.) Human pancreatic islets co-stained with insulin and EdU treated with 20 nM (12 nm) E2F3 mRNA AuNPs for 24 hours and incubated with EdU for 4 days. (C.) Human pancreatic islets co-stained with insulin and EdU treated with 50 nM (12 nm) E2F3 mRNA AuNPs for 24 hours and incubated with EdU for 24 hours and incubated with EdU for 4 days. (C.) Human pancreatic islets co-stained with insulin and EdU treated with 50 nM (12 nm) E2F3 mRNA AuNPs for 24 hours and incubated with EdU for 4 days. DAPI (blue), EdU (red), insulin (green).



Figure 23: Proliferation within human pancreatic islets. Quantification of immunohistochemistry for EdU+/insulin+ cells. % β -cell proliferation represents the number of cells that co-stained for EdU and insulin over the total number of insulin-positive cells, p = ns.



Figure 24: E2F3 induced proliferation in human pancreatic islets (A.) Non-treated group of human pancreatic islets co-stained with insulin and EdU. (B.) Positive control of human pancreatic islets co-stained with insulin and EdU, islets were treated a proliferation compound CC-401 and incubated with EdU for 4 days. (C.) Human pancreatic islets co-stained with insulin and EdU treated with 20 nM (12 nm) E2F3 mRNA AuNPs for 24 hours and incubated with EdU for 4 days. (D.) Human pancreatic islets co-stained with insulin and EdU treated with 20 nM (12 nm) E2F3 mRNA AuNPs for 24 hours and incubated with EdU for 4 days. (D.) Human pancreatic islets co-stained with insulin and EdU treated with 50 nM (12 nm) E2F3 mRNA AuNPs for 24 hours and incubated with EdU for 4 days. (E.) Human pancreatic islets co-stained with insulin and EdU treated with 20 nM (7 nm) E2F3 mRNA AuNPs for 24 hours and incubated with EdU for 4 days. (F.) Human pancreatic islets co-stained with insulin and EdU treated with 50 nM (7 nm) E2F3 mRNA AuNPs for 24 hours and incubated with EdU for 4 days. (F.) Human pancreatic islets co-stained with insulin and EdU treated with 50 nM (7 nm) E2F3 mRNA AuNPs for 24 hours and incubated with EdU for 4 days. (F.) Human pancreatic islets co-stained with insulin and EdU treated with 50 nM (7 nm) E2F3 mRNA AuNPs for 24 hours and incubated with EdU for 4 days. (F.) Human pancreatic islets co-stained with insulin and EdU treated with 50 nM (7 nm) E2F3 mRNA AuNPs for 24 hours and incubated with EdU for 4 days. (DAPI (blue), EdU (red), insulin (green).





Figure 26: Electron microscopy of non-treated human pancreatic islets incubated for 24 hours (A.) 11,000 X magnification (B.) 123,000 X magnification. As seen, there is no evidence of AuNPs within the non-treated human pancreatic islets. N = Nucleus, V = Vacuole.



Figure 27: Electron microscopy of non-treated human pancreatic islets incubated for 24 hours (A.) $6,000 \times M$ magnification (B.) 120,000 X magnification. As seen, there is no evidence of AuNPs within the non-treated human pancreatic islets. N = Nucleus, V = Vacuole.



Figure 28: Electron microscopy of mCherry mRNA AuNP treatment of human pancreatic islets incubated for 24 hours, 12 nm size AuNPs at a concentration of 50 nM (A.) 120,000 X magnification (B.) 12,000 X magnification. As observed, there is clear evidence of AuNPs within the islets. The AuNPs are able to enter through the cell membrane, while others linger around the outer surface of the islets. N = Nucleus.



Figure 29: Electron microscopy of mCherry mRNA AuNP treatment of human pancreatic islets incubated for 24 hours, 12 nm size AuNPs at a concentration of 50 nM (A.) 25,000 X magnification. (B.) 20,000 X magnification. As observed, there is clear evidence of AuNPs within the islets. The AuNPs are able to enter through the cell membrane, while others linger around the outer surface of the islets. N = Nucleus, V = Vacuole.



Figure 30: Electron microscopy of mCherry mRNA AuNP treatment of human pancreatic islets incubated for 24 hours, 12 nm size AuNPs at a concentration of 50 nM (A.) 120,000 X magnification. (B.) 120,000 X magnification. As observed, there is clear evidence of AuNPs within the islets. The AuNPs are observed either around the mitochondrial or near vacuoles. As seen by the images, the AuNPs tend to form strong clusters with one another. IG = Insulin Granule.



Figure 31: Electron microscopy of mCherry mRNA AuNP treatment of human pancreatic islets incubated for 24 hours, 12 nm size AuNPs at a concentration of 50 nM (A.) 10,000 X magnification. (B.) 60,000 X magnification. As observed, there is clear evidence of AuNPs within the islets. The AuNPs are observed either around the mitochondrial or near vacuoles. As seen by the images, the AuNPs tend to form strong clusters and aggregates with one another. N = Nucleus, M = Mitochondria, V = Vacuole.



Figure 32: Electron microscopy of mCherry mRNA AuNP treatment of human pancreatic islets incubated for 24 hours, 7 nm size AuNPs at a concentration of 50 nM (A.) 200,000 X magnification. (B.) 200,000 X magnification. As observed, there is clear evidence of AuNPs within the islets. The AuNPs were observed to be either around the mitochondrial or near vacuoles. As seen by the images, the AuNPs tend to form strong clusters or aggregate with one another. V = Vacuole.



Figure 33: Electron microscopy of mCherry mRNA AuNP treatment of human pancreatic islets incubated for 24 hours, 7 nm size AuNPs at a concentration of 50 nM (A.) 150,000 X magnification. (B.) 120,000 X magnification. As observed, there is clear evidence of AuNPs within the islets. The AuNPs are observed either around the mitochondrial or near vacuoles. As seen by the images, the AuNPs tend to form strong clusters with one another. IG = Insulin Granule, M = Mitochondria.



Figure 34: Electron microscopy of mCherry mRNA AuNP treatment of human pancreatic islets incubated for 24 hours, 7 nm size AuNPs at a concentration of 50 nM (A.) 12,000 X magnification. (B.) 40,000 X magnification. As observed, there is clear evidence of AuNPs within the islets. The AuNPs are observed either around the mitochondrial or near vacuoles. As seen by the images, the AuNPs tend to form strong clusters with one another, if they are alone; they tend to be located outside the cell membrane. N = Nucleus, M = Mitochondria.



Figure 35: Electron microscopy of mCherry mRNA AuNP treatment of human pancreatic islets incubated for 24 hours, 7 nm size AuNPs at a concentration of 50 nM (A.) 120,000 X magnification. (B.) 120,000 X magnification. As observed, there is clear evidence of AuNPs within the islets. The AuNPs are observed either around the mitochondrial or near vacuoles. As seen by the images, the AuNPs tend to form strong clusters with one another. However, when the AuNPs are found in the outside of the cell they are mostly found as single AuNPs. IG = Insulin Granule, ER = Endoplasmic Reticulum.



Figure 36: Electron microscopy of mCherry mRNA AuNP treatment of human pancreatic islets incubated for 24 hours, 2 nm size AuNPs at a concentration of 50 nM (A.) $60,000 \times M$ magnification. (B.) $100,000 \times M$ magnification. As observed, there is clear evidence of AuNPs within the islets. As observed, there is nuclear penetration. N = Nucleus, M = Mitochondria.


Figure 37: Electron microscopy of mCherry mRNA AuNP treatment of human pancreatic islets incubated for 24 hours, 2 nm size AuNPs at a concentration of 50 nM (A.) 100,000 X magnification. (B.) 100,000 X magnification. As observed, there is clear evidence of AuNPs within the islets. IG = Insulin Granule, M = Mitochondria, V = Vacuole.

IV. Discussion

Previous studies performed in our laboratory have shown that 12 nm sized AuNPs conjugated to a non-specific oligonucleotide sequence labeled with a fluorescent probe, of Cy5 was able to transfect islets at a concentration of 10 nM after 24 hours incubation [2]. Islet function was not compromised when treated with this concentration of AuNPs, even after an extended incubation period of up to 48 hours. However, this particular type of AuNP was not attached to a functional nucleotide sequence. The focus of my thesis work was therefore, to determine whether AuNPconjugated functional nucleotide sequences could be used to achieve exogenous expression of either a fluorescent protein or a cell cycle protein, in the case of this project, E2F3. Consequently, in this study different sized AuNPs were tested including 2 nm, 7 nm, and 12 nm AuNPs, all attached to mCherry mRNA oligonucleotide sequences. Different sizes of AuNPs were tested in order to observe whether the size of the AuNPs affected the penetration and efficacy of protein expression within single cervical cancer cell line HeLa, dissociated islets, and more importantly in intact human pancreatic islets. The single cells showed protein expression with all different sizes of AuNPs at both concentrations of 20 nM and 50 nM. In addition, different incubation time points were tested to observe whether there was an increase in the percentage of positive cells over time. The different incubation time points tested were 0, 6, 12 and 24 hours. The amount of protein expression increased with an increase in incubation time, based on quantification of the percentage of positive cells. This clearly demonstrated that the AuNP-nucleotide constructs were able to express fluorescent protein via mRNA. Many researchers have avoided the use of mRNAs because they readily degrade over time. We believe that the addition of a non-specific DNA sequence linking the AuNP and the mRNA of mCherry fluorescent protein, increased the stability of the mRNA to allow for protein expression.

The initial studies using the single cells and dissociated islets were a proof a concept in order to determine whether there was an enhancement in the amount of protein being translated in vitro.

The next step was to test human pancreatic islets. We chose to treat human pancreatic islets with the AuNPs for 24 hours because we noted the highest percentage of mCherry positive cells within islet at this incubation time. Following quantification of relative fluorescence intensity, we noted significant differences in the amount of mCherry protein within intact adult human pancreatic islets for all three different sizes of AuNPs conjugated to mCherry mRNA. The two sizes that expressed the highest amount of protein within intact human pancreatic islets were the 2 nm and 7 nm mCherry mRNA AuNPs, after 24 hours of incubation. Based on these results we conclude that the size of the overall AuNP-DNA construct has an effect on the amount of protein expression. A 3D projection built from confocal z-stacks of the treated human pancreatic islets, including the core. We conclude, based on the quantification of the relative mean intensity that, the highest amount of protein expression was seen when human pancreatic islets were treated with the 50 nM (7 nm) mCherry mRNA AuNPs. Therefore, for delivery into human islets, the size of AuNPs is critical, especially for AuNPs attached to the oligonucleotide sequences such as covalent attachment to a mCherry mRNA sequence.

Following the proof of concept results, the next step was to test whether the AuNP-nucleotide construct would impair islet function, as the major goal of this study is to develop a biocompatible non-viral method of delivery for islets. When the islets were treated with the lower concentration (20 nM) or higher concentration (50 nM), the function of islets was not compromised with an incubation period of 24 hours as seen by measuring the amount of intracellular calcium influx, and mitochondrial potential, when compared to the non-treated control group when stimulated with glucose. Furthermore, the analysis of the area under the turve between the 5 to 25 minute point demonstrated a similar response, therefore, none of the three different sizes of AuNPs posed a toxic effect to the human pancreatic islets. This is highly relevant finding in regards to our quest to develop a biocompatible non-viral method of delivery

for human pancreatic islets. In addition, this is the first study where a fluorescent protein and a cell cycle protein were expressed via AuNP within single cells and intact human pancreatic islets. Many researchers stay away from using mRNA as it degrades very easily when delivered into cells and is exposed to a series of cytoplasmic enzymes. However, the covalent attachment to a non-specific oligonucleotide sequence further allowed the mRNA to remain stable and express protein within a 24-hour incubation period. Previous studies conducted demonstrate that AuNPs conjugated to an oligonucleotide sequence was able to prolong and lower the degradation rate of the mRNA over time. The maximum amount of incubation time islets could be treated with the AuNPs would be for 24 hours. After this incubation time point, residues of the AuNPs could be observed within the culture media where the islets are treated. Even after 24 hours of treatment with a high concentration of 50, the morphology of the pancreatic islets remained intact and the overall structure and morphology was maintained.

Islets can be driven to proliferate by expressing specific cell cycle proteins via adenoviral vectors [1]. Due to safety concerns associated with using viral systems clinically to expand islet cells and make them available to many more patients, significant emphasis has been placed on producing a safe and effective non-viral delivery system for logical research and gene therapy. In the first part of this study we use AuNPs conjugated to mCherry mRNA as a proof of concept to increase expression of the specific protein. Next we wanted to test the feasibility of using AuNPs attached to the E2F3 mRNA to achieve increased expression of E2F3 protein and as a result increase islet cell proliferation.

Due to the fact, the 7 nm mCherry mRNA AuNPs showed the higest expression of mCherry fluorescent protein, we hypothesized that the same size of AuNP would cause an increase in the amount of β -cell proliferation. Consequently, when human pancreatic islets were treated with 7 nm and 12 nm E2F3 mRNA AuNPs, the 12 nm E2F3 mRNA AuNPs showed slightly

higher proliferation rate when compared to the 7 nm size E2F3 mRNA AuNPs. Contrary to our prediction, the 7 nm size E2F3 mRNA AuNPs did not show any sign of β -cell proliferation. As a result, from this phenomenon that was observed, a key experimental procedure that needed to be further investigated was where the AuNPs were localizing within the human pancreatic islets. Where the AuNPs are localized within the pancreatic islets might provide the necessary understanding of how kinetics affects the proliferation within intact human pancreatic islets. TEM showed all three sizes of AuNPs did cross the cell membrane, and the AuNPs seemed to localize around the mitochondria, vesicles and insulin granules of the islets. In the case of the 2 nm mCherry mRNA AuNPs, TEM images demonstrated that a group of AuNPs were able to cross and localized within the nucleus. On the other hand, the 12 nm mCherry mRNA and 7 nm mCherry mRNA AuNPs were localized either in vesicles, mitochondria or insulin granules of the human pancreatic islets. Consequently, when protein translation occurs, this takes place in the ribosome, as observed by the TEM images none of the AuNPs were visually seen in the ribosomes of the islets, but then we need to further investigate how different incubation time points will influence the localization within the human pancreatic islets. Depending on which organelle the AuNPs localize within the human pancreatic islets might help us understand how to control the expression of the cell cycle protein E2F3 known to enhance β -cell proliferation. Furthermore, another critical observation that needs to be further investigated is how the quantity of oligonucleotides will influence the delivery of a cell cycle protein versus a fluorescent protein. In the case of the 7 nm mCherry mRNA AuNP, there was an increase in the amount of fluorescent protein within intact human pancreatic islets, however, in the case of the 7 nm AuNP attached to the cell cycle protein there was no evidence of β -cell proliferation and this size of AuNP had a smaller amount of oligonucleotides attached. On the contrary, the 12 nm E2F3 mRNA AuNP was able to show a slight increase in the amount of β -cell proliferation based on the positive EdU cells co-stained for insulin and this was the AuNP that was attached to the greatest amount of oligonucleotides, in this case 20 when compared to the other sizes. Previous

studies, conducted by other researchers have determined the number of oligonucleotides attached to the AuNPs will influence the efficacy of delivery. In the case of the cell cycle protein, the number of oligonucleotides might have played an essential role in the influence on the amount of β -cell proliferation, in particular in the case of E2F3. Therefore, a next step for this project would be to increase the amount of oligonucleotides attached to the 7 nm E2F3 mRNA AuNPs. Increasing the number of oligonucleotides might have an influence on the amount of E2F3 protein that can be expressed within the human pancreatic islets. Therefore, understanding the exact kinetics of the AuNPs once inside the human pancreatic islets will provide insight on how to effectively control the amount of proliferation and achieve a higher proliferation rate.

V. <u>Conclusion</u>

This is one of the first studies conducted using AuNPs conjugated to mCherry mRNA and E2F3 mRNA for delivery with human pancreatic islets and single dissociated islet cells. Previous work demonstrated AuNPs were able to be penetrated with AuNPs conjugated to a non-specific oligonucleotide sequence labeled with a fluorescent fluorophore within human pancreatic islets, but the size tested during this experimental work was 12 nm and there was no specific functional sequence attached to the AuNP. On the other hand, during this experimental procedure we tested a series of different sized AuNPs conjugated to mCherry mRNA and E2F3 mRNA in order to determine whether the size influenced AuNP uptake within human pancreatic islets and single cells. The optimal size of AuNP that was easily seen to express mCherry protein was 7 nm, however, when studies were done to observe whether there was any sign of enhanced β -cell proliferation, the 12 nm sized AuNP seemed to work the best. In addition. TEM was the imaging technique used in order to observe where within the islets the AuNPs were localized. The 12 nm and 7 nm size AuNPs seemed to be able to cross the cell membrane and be found within vesicle, mitochondrial and in one case the insulin granules of the islets. Until recently, it was believed that mRNA was too unstable to be used for gene therapy. With recent advances in techniques using particles that have a size in the nanometer range, mRNA can now be delivered to cells in a way that delays their otherwise rapid degradation and mRNA delivery has currently become a hot topic in molecular therapy [66]. For our application of making human islet cells grow, a short lived stimulus would be desirable to avoid creating uncontrolled expansion of cells that could lead to tumor formation. We believe that with a novel technology such as nanotechnology we can create nanoparticles that will allow specific mRNA to enter islets and lead to the production of a specific protein within the cell that can lead to islet cells dividing and augment the number of cells that is required to be transplanted to each individual diabetic patient.

VI. Appendix 1: Viral Methods of Delivery

<u>Adenovirus</u> – A DNA virus that was first discovered and isolated during the 1950s. Adenovirus is capable of infecting multiple organ systems [90-94]. Adenovirus can infect various cells, both proliferative and quiescent, and thus can infect many different tissues and diseased cell lines. Adenoviruses are double stranded DNA viruses that measure between 70 – 90 nm [95-98]. Where the virus enters, the cells will determine the site of infection. When the adenovirus is transfected within the cells, a series of different interactions occur.

A Lytic infection is a common infection for adenoviruses once they enter the cell. Another possible infection when an adenovirus transfects a cell, is a chronic or latent infection, however, the exact mechanism of how this infection occurs is not fully known. A third side effect of adenovirus infection is an oncogenic transformation that has been currently observed in rats [95].

<u>Retrovirus</u> – Any family of ribonucleic acid viruses that contain the enzyme reverse transcriptase in the virion. The DNA that is formed is then integrated within the DNA of the host cell. The retroviruses are assembled in the capsids of the cytoplasm within the host cell [99-102].

<u>Lentivirus</u> - This particular classification of viruses are described as having long incubation times from a couple of months to years. These viruses have a very flexible genome and a potential of transducing many forms of non-dividing cells. The family of Lentiviruses is the most widely used type of virus vector by researchers in order to transfer genes [103].

With these three major viral methods of delivery come potential problems including oncogenic risks. Thus far, gene therapy and molecular biology have focused primarily on delivering DNA of

a specific gene into cells. The risk of this approach is that the DNA can be incorporated into the genome and lead to random chromosomal damage with the risk of generation of cancerous cells.

Appendix 2. Types of nanoscale delivery methods available

Polymeric Dendrimers

Dendrimers are highly branched, star-shaped macromolecules characterized by nanometer scale dimensions. Dendrimers have a central core, an interior dendritic structure; there are branches that contain outer surface points, which could be attached to proteins, fluorescent molecule, and nucleic acids [106-107]. Dendrimers are currently used from many different applications including drug delivery, gene transfection, catalysis, nanoscale science and technology. Dendrimers range in size and are synthesized by a step-wise chemical reaction and usually the generation of the dendrimer determines the size of the polymeric nanoparticle. Dendrimers are primarily synthesized through repetitive reactions using branched monomers. The two methods used to synthesize dendrimers via the divergent method, the dendrimer branches grow outwards from the core in the center, to form a "tree-like" structure. Usually the dendrimer core can be made up of either an ammonia core or an Ethylenediamine (EDA), when made through the divergent process [106-107]. During the divergent method, the dendrimer grows outwards from a multifunctional core molecule.

The dendrimer continues to grow, and the dendrimer is built layer-by-layer. The divergent method is often the synthesis method used when researchers want to process polymeric dendrimers in large quantities. Because dendrimers are produced in larger quantities, effects within the production of the polymeric dendrimers will result; therefore, this might influence the

actual size of dendrimer being produced, leading to alteration within the final purification of the polymeric dendrimer. In addition, the cost to produce such a large quantity of dendrimers will increase because a large amount of reagents and solutions will be needed to make such a large quantity.

Because the synthesis of dendrimers during the divergent method creates a series of defects within the final product of the dendrimer, a new synthesis method has been developed known as the convergent method to resolve the issues during the synthesis of dendrimers using the divergent method. The convergent method has many advantages over the divergent method including the ease of dendrimer purification following its generation. In addition, polymeric dendrimers made via the convergent process allow for easy conjugation to functional groups at the periphery. However, there are also limitations to the convergent method including the inability to produce dendrimers of higher generation [106-107]. Because of the limitations of the synthesis process of dendrimers, a second type of nanostructure was investigated which were lipoplexes.

Lipoplexes

Cationic lipid mediated gene transfer is another alternative to using viral methods of delivery. Lipid mediated gene transfer is non-immunogenic, simple to produce and does not have the potential to pose an oncogenic risk. However, one of the major limitations of cationic lipids is their minimal transfection efficiency [108]. Researchers observed that a higher transfection from a lipoplex occurs when the lipoplex complex is larger in size because the larger complex facilitates membrane contact. Lipoplex transfection is highly influenced by the cell type, therefore, a single cell will transfect differently than a cluster of cells. As a result, the current cationic lipids available for gene transfer exhibit low transfection efficiency. Further studies

need to be performed in order to observe how the morphology and structure of the lipoplexes influences transfection efficacy.

Lipoplexes are a difficult structure to work with when it comes to pancreatic islets. Several factors influence the transfection efficiency of cells including DNA ratio, the structure of the liposome, and the incubation time of the lipoplex complex within the cells. The key factor that makes lipoplexes a difficult nanostructure to work with is the fact that they exhibit some form of cytotoxicity. Researchers such as Nguyen et al. discovered in 2012 that there was a similarity between cationic lipids conjugated to pDNA but they exhibited some form of cytotoxicity [109]. However, when cationic lipids were incubated with cells they exhibited a very minimal amount of toxicity. The addition of or absence of serum to the culture media did not influence the cytotoxicity of the lipids, both types, serum and serum-free exhibited a form of cytotoxicity within HeLa cells. Furthermore, the pDNA delivered to the cells with nothing conjugated to the outer surface did not exhibit any form of cytotoxicity. In addition, the researchers increased the amount of cationic lipid content and there was also an increase in the amount of cytotoxicity. Therefore, there are several factors that influence the toxicity of the lipoplex when delivered to cells may affect the aggregates formed.

VII: References

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VIII: Curriculum Vitae

Education	
University of Illinois at Chicago, Chicago IL	Currently Pursuing
Ph.D. in Bioengineering	
<u>Thesis title</u> : "The study of non-viral nanoscale delivery systems for islet transplantation	on"
University of Illinois at Chicago, Chicago, IL	2009
B.S in Bioengineering	
Research Experience	
University of Illinois at Chicago, Chicago IL	09/2009 - Present
Dr. José Oberholzer, MD, Associate Professor of Surgery, Endocrinology and Diabetes, a	and Bioengineering
The UIC pancreatic islet lab focuses on improving pancreatic islet transplantation in o method as a notential treatment for Type I diabetes.	rder to enhance the
Graduate Research Assistant, Department of Surgery, Division of Transplantation	
 Research focuses on developing a novel non-viral method of protein delivery in order to in β-cells via gold nanoparticles within the pancreatic islets (extracted from a donor cadaver ultimate goal of using the technique within clinical islet transplantation into human Type-Contributed to the validation of a microfluidic device as a diagnostic tool using the fol viability, glucose static incubation, apoptosis histology staining, and in vivo mice islet procedure will lead to submission to be reviewed by the FDA for a Biologics License A Presented research at 12 local and international conferences, including 3 oral present Contributed to three peer-reviewed publications and one book chapter Worked in training and mentoring medical, undergraduate and high school students of Demonstrated the ability to supervise, train and motivate future scientists which resustudent obtaining a Craig Fellowship. 	iduce the expansion of ic pancreas) with the 1 diabetes patients. lowing techniques, transplantation. The application (BLA). tations during the summer. ilted in one medical
Northwestern University, Evanston, IL Dr. Samuel I. Stupp, Ph.D., Board of Trustees Professor of Materials Science and Engine Medicine, and Biomedical Engineering NSEC labs use nanoscale based technology for the development of innovative drug deli Undergraduate Research Intern, Nanoscale Science and Engineering Center (NSEC Performed solid phase peptide synthesis (SPPS) to create nanofibers for application in reg Created and characterized innovative nanofibers known as peptide amphiphiles (PAS). PA	Summer 2008 ering, Chemistry, very systems.) generative medicine. As were able to mimic
the extracellular matrix (ECM) to enhance cell signaling properties.Designed a PA with a thrombin site using the software ChemSketch.	

Illinois Institute of Technology, Pritzker Institute for Biomedical Science and Engineering, Chicago, IL Summer 2007

Victor H. Perez-Luna, Ph.D., associate professor of Chemical Engineering

Lab focuses on polymeric delivery methods for the eye in order to enhance drug delivery systems for diabetic patients who suffer eye loss over time.

Undergraduate Research Intern, Center for Diabetes Research and Education

- Collaborated with a graduate mentor to create a series of hydrogels to demonstrate the controlled release
 of a protein whose function is related to the loss of eyesight caused by diabetes.
- Demonstrated that the series of hydrogels could be used in future therapeutic applications.

Northwestern University Robert H. Lurie Cancer Comprehensive Center, Chicago, ILSummer 2006Charles V Clevenger, MD/PhD, Adjunct Professor in Pathology5

Research lab focuses on investigating the role of specific hormones in the metastasis of human breast cancer cells.

Undergraduate Research Intern, Continuing Umbrella for Research Experience (CURE)

- Constructed a vector containing the Cyclin D1 promoter region, which is shown to play a critical role in the
 metastasis of cancerous cells, and has been observed to be overexpressed within breast carcinoma.
- Investigated the role of the hormone, prolactin, on the proliferation and expansion of breast cancer cells.
- Performed vector cloning, gel electrophoresis and polymerase chain reaction techniques.

Skills and techniques

- Cellular Biology: Cell culture, primary cell culture, isolation of human pancreatic islets, proliferation, apoptosis and cell signaling.
- Computer Skills: Microsoft Word, Excel, PowerPoint, GraphPad Prism
- Histology: Immunocytochemistry, immunofluorescent chemistry, analysis
- Imaging Software: Image J, Zeiss microscopy imaging system
- Instruments: DIC/fluorescent microscope, microplate reader, flow cytometry sample preparations
- **Microfluidics:** Device used to stimulate islets with a series of glucose concentrations in order to perform functional analysis and simultaneously measure calcium influx, mitochondrial potential and insulin kinetics from islets including mice and human pancreatic islets.
- Microscopy: Light microscopy, fluorescent/confocal laser microscopy
- Molecular Biology: Western Blotting, RTqPCR, PCR, ELISA, and transfections
- Nanotechnology: Polymeric dendrimers, lipid based nanoparticles, gold nanoparticles.
- Protein Chemistry: Protein expression, isolation and purification.

Memberships in Professional Societies

•	The Transplantation Society (TTS)	2013 - Present
•	Bioengineering Organizational Alliance (BOA) – Engineering World Health (EWH)	2009 - 2011
Leade	rship	
•	Graduate Student Council (GSC) – Graduate Student Representative for the Dept. of Surgery	2012 - 2013
Award	ls	
	Medtech Scientist Mentoring and Diversity Program Award Scholar (Chicago, Illinois)	2014
•	Biotech Scientist Mentoring and Diversity Program Award Scholar (San Diego, California)	2014
•	1st place Poster at the Louis Stokes Alliance for Minority Participation (Chicago, Illinois)	2012
•	Bridge to the Doctorate Fellowship funded by NSF (University of Illinois at Chicago)	2009 - 2011
Prese	ntations	

- Improving Islet Transplant Outcomes via Nanotechnology, College of Medicine Research Forum; 2014 (poster)
- Development and Optimization of a Microfluidic-based Multi-Parametric assay to evaluate the function of human islets prior to Transplantation, Midwest Islet Club Conference; 2014 (poster)
- Human islet cell expansion via nanoparticle mediated intracellular delivery of modified mRNA for targeted cell cycle protein translation, Louis-Stokes Alliance for Minority Participation (LSAMP); 2014 (poster)
- Development and Optimization of a Microfluidic-based Multi-Parametric assay to evaluate the function of human islets prior to Transplantation, University of Illinois at Chicago 1st Annual Diabetes and Obesity Research Day; 2013 (poster)
- Development and Optimization of a Microfluidic-based Multi-Parametric assay to evaluate the function of human islets prior to Transplantation, International Pancreas and Islet Transplantation Association (IPITA); 2013 (oral)
- Development and Optimization of a Microfluidic-based Multi-Parametric assay to evaluate the function of human islets prior to Transplantation, American Diabetes Association (ADA); 2013 (poster)
- Development and Optimization of a Microfluidic-based Multi-Parametric assay to evaluate the function of human islets prior to Transplantation, College of Medicine Research Forum; 2013 (poster)
- Development and Optimization of a Microfluidic-based Multi-Parametric assay to evaluate the function of human islets prior to Transplantation, LSAMP; 2013 (poster)
- Development and Optimization of a Microfluidic-based Multi-Parametric assay to evaluate the function of human islets prior to Transplantation, Diabetes Seminar in the Department of Endocrinology at the University of Illinois at Chicago; 2013 (oral, invited)
- The development of G4 Polyamidoamine (PAMAM) dendrimers as an ideal nanoscale delivery system for islet research, LSAMP; 2012 (poster)

- The development of G4 Polyamidoamine (PAMAM) dendrimers as an ideal nanoscale delivery system for islet research, Central Society for Clinical Research Conference; 2011 (poster)
- The development of G4 Polyamidoamine (PAMAM) as an ideal nanoscale delivery system for islet research, College of Medicine Research Forum; 2010 (**poster**)
- Controlling Bioactivity of Peptide Amphiphile Nanostructures, LSAMP; 2009 (oral)

Peer-Reviewed Publications

- Gutierrez D., Marchese E., Nourmohammadzadeh M., McGarthy B., Williams J., Wang Y., Oberholzer J. A Novel, Non-viral mRNA Delivery System For Targeted Protein Translation In Human Islets, *In process*.
- Qi M, Bochenek M, McGarrigle J, Zeng L, Marchese E, Nourmohammadzadeh M, Gutierrez D, Xing Y, Mendoza-Elias J, Wang L, Khan A, Oberholzer J. Preconditioning with Diazoxide reduces preservation injury to non-human primate islets. <u>Journal of Clinical Investigation</u>. (Reference 75907-RG-1), *In process*
- Wang Y, Mendoza-Elias JE, Qi M, Harvat TA, Ahn SJ, Lee D, Gutierrez D, Jeon H, Paushter D, Oberholzer J. Implication of mitochondrial cytoprotection in human islet isolation and transplantation. <u>Biochem Res Int</u>. 2012;2012:395974. Epub 2012 May 7.
- Lee D., Wang Y., Mendoza-Elias J.E., Adewola A.F., Harvat T.A., Kinzer K., Gutierrez D., Qi M., Eddington D.T., and Oberholzer J. Dual microfluidic perifusion networks for concurrent islet perifusion and optical imaging. <u>Biomed Microdevices</u> 14, 7–16.

Book Publications

 Wang Y, Mendoza-Elias JE, Flo J.F, Harvat TA, Feng F, Li Z, Wang Q, Nourmohammadzadeh M, Gutierrez D, Qi M., Eddington D.T, Oberholzer J. <u>Development of a microfluidic strategy for evaluating human islet</u> <u>potency and viability prior to transplant.</u> Microfluidic Devices for Biomedical Applications. *Woodhead Publishing Series in Biomaterials. Edition 1 Chapter 18, 2013.*