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Transfection of pancreatic islets using polyvalent DNA-functionalized gold nanoparticles^{*,1}

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Abstract

Background—Transplantation of pancreatic islets is an effective treatment for select patients with type 1 diabetes. Improved cellular therapy results may be realized by altering the gene expression profile of transplanted islets. Current viral and non-viral vectors used to introduce nucleic acids for gene regulation hold promise, but safety and efficacy shortcomings motivate the development of new transfection strategies. Polyvalent gold nanoparticles (AuNPs) densely functionalized with covalently immobilized DNA oligonucleotides (AuNP-DNA) are new single entity transfection and gene regulating agents (i.e. not requiring lipids, polymers, or viral vectors for cell entry) able to enter cells with high efficiency and no evidence of toxicity. We hypothesize that AuNP-DNA conjugates can efficiently transfect pancreatic islets with no impact on viability or functionality, and can function to regulate targeted gene expression.

Methods—AuNPs were surface-functionalized with control and antisense DNA oligonucleotides. Purified murine and human islets were exposed to AuNP-DNA conjugates for 24 hours. Islet AuNP-DNA uptake, cell viability, and functionality were measured. Furthermore, the ability of antisense AuNP-DNA conjugates to regulate gene expression was measured using murine islets expressing eGFP.

Results—Collectively, fluorescent confocal microscopy, transmission electron microscopy, mass spectrometry, and flow cytometry revealed substantial penetration of the AuNP-DNA conjugates into the inner core of the islets and within islet cells. No change in cellular viability occurred and the insulin stimulation index was unchanged in treated versus untreated islets. Transplantation of

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AuNP-DNA treated islets cured diabetic nude mice. Functionally, antisense eGFP AuNP-DNA conjugates reduced eGFP expression in MIP-eGFP islets.

Conclusions—Polyvalent AuNP-DNA conjugates may represent the next generation of nucleic acid-based therapeutic agents for improving pancreatic islet engraftment, survival, and long-term function.

INTRODUCTION

Type 1 diabetes is an autoimmune disorder where destruction of the insulin producing pancreatic β cells leads to hyperglycemia and long-term health complications, including retinopathy, renal failure, cardiovascular disease, and death. With the development of the Edmonton protocol in 2000, the isolation and transplantation of pancreatic islets became an effective, short-term treatment to restore glycemic control in select patients with type 1 diabetes.¹ Significant advances have been made in the past decade to enhance patient outcomes since that report; however, long-term islet durability still lags behind solid organ pancreas transplantation for diabetes.²⁻⁵ Improving islet engraftment and survival is crucial for achieving the durable outcomes required to advance islet transplantation as a mainstream therapy for type 1 diabetes.

Altering the gene expression profile of the pancreatic β cells is one methodology that may provide enhanced engraftment and survival, improving the lifespan of the islet graft. Heimberg, et. al. reported that viral-mediated transfection with an inhibitor of NF- κ B protected dissociated rat β cells from IL-1 β -mediated dysfunction and death.⁶ In addition, overexpression of human VEGF by mouse islets has been shown to improve islet graft vascularization and functionality.⁷ Finally, apoptosis of human islets was inhibited through caspase-3 shRNA expression via a replication deficient adenovirus.⁸ Despite these successes, robust genetic manipulation of pancreatic islets has proven difficult.

Current methods to deliver genetic material to islets include viral vectors or lipid-based transfection reagents, both of which have major limitations in their application. Cellular transfection with viral vectors may provoke an adaptive immune response through the residual expression of viral proteins.⁹ In addition, toxicity can be observed at the high multiplicities of infection (MOI) needed to achieve adequate transfection efficiencies.⁹ Additionally, delivery of genetic material past peripheral cells to those at the islet center is difficult, if not impossible, using viral vectors.⁹⁻¹⁰ Lipid-based reagents are considered safer than viral vectors; however, transfection efficiencies are low, and delivery of the material to the islet core is not possible. As in the case of viral vectors, increased transfection efficiency is balanced with increasing toxicity.¹¹ Considering the current methods for delivering genetic material to islets, it is clear that new technology is needed. A non-cytotoxic platform capable of transfecting virtually all of the cells in an islet, while maintaining islet function and mediating target-specific protein knockdown, would address important limitations of currently available methods.

Polyvalent AuNP-DNA conjugates have unique properties derived from the dense packing of DNA on the AuNP surface.¹²⁻¹³ These properties significantly enhance the utility of AuNP-DNA conjugates in the context of developing gene specific probes with enhanced sensitivity and specificity.¹²⁻¹⁴⁻¹⁵ Benefits realized for bio-diagnostic applications led to the investigation of AuNP-DNA conjugates for cellular gene regulation,¹⁶ and the discovery that these novel structures act as single entity antisense agents capable of efficiently regulating gene expression.¹⁶ Unlike conventional strategies involving lipids, polymers, and viral vectors, the polyvalent structure acts both as the transfection and antisense agent. Interestingly, these polyvalent structures, when made from duplex RNA instead of DNA, can also act as siRNA knockdown materials with no apparent toxicity and more persistent

knockdown than the same siRNA sequences delivered by commercially available polymer transfection agents.²⁰

We hypothesize that AuNP-DNA conjugates will be non-toxic and effective transfection reagents capable of distributing throughout pancreatic islets including cells in the inner core. The hypothesis was tested by *in vitro* exposure of purified human islets with AuNP-DNA conjugates in a time- and dose-dependent manner. Transfection efficiency was evaluated by confocal microscopy, transmission electron microscopy (TEM), inductively coupled plasma-mass spectrometry (ICP-MS), and flow cytometry analyses. Cellular toxicity was tested by flow cytometric cell viability analysis. Islet β cell endocrine function was assessed by *in vitro* static glucose stimulated insulin secretion assays, and *in vivo* using the nude mouse islet transplant bioassay. The ability of the AuNPs to deliver functional antisense DNA to islet cells was assayed using mouse islets expressing eGFP.

MATERIALS AND METHODS

Human Islet Isolation and culture

Human islet preparations of >99% purity were isolated from donor pancreases by the human islet isolation laboratory at Northwestern University using a modified Ricordi protocol.^{1, 17-19} Briefly, collagenase enzyme and neutral protease from Serva® (Heidelberg, Germany) were perfused through the common bile duct to the pancreas. Pancreatic islets were purified from digested pancreatic tissue using standard methods. The human islets were cultured in supplemented CMRL 1066, with 10U/mL heparin and 0.5% human serum albumin, at 37 °C, 5% CO₂ in a humidified incubator. Islets were cultured for at least 24 hours prior to AuNP-DNA exposure.

Synthesis of DNA Functionalized Gold Nanoparticles

Gold nanoparticles (AuNPs) and AuNPs surface-functionalized with modified DNA oligonucleotides (AuNP-DNA) were prepared according to previously published procedures.¹⁶ Briefly, citrate stabilized colloidal AuNPs were synthesized using a sodium citrate reduction of hydrogen tetrachloroaurate (HAuCl₄) in refluxing water. AuNP size and dispersity were assessed by transmission electron microscopy (TEM, 13 ± 1 nm) and UV-Vis spectroscopy (absorbance maximum = 520 nm), respectively. 3'-alkylthiol modified oligonucleotides [5'-AGGCTCTCGACTTACCCGCT-(A)₁₀-(CH₂)₃-SH-3', 5'-GCAATTTTGTCTTGCTCTT-(A)₁₀-(CH₂)₃-SH-3'] were synthesized using standard phosphoramidite chemistry (Expedite 8909) and purified using reverse-phase (C18) high pressure liquid chromatography (HPLC, Varian). For fluorescent confocal microscopy imaging of AuNP-DNA in cells and for flow cytometry, a molecular fluorophore [cyanine 3 or 5 (Cy3 or Cy5)] was added to the 5' end of the synthetic oligonucleotide (AuNP-DNA-Cy). For antisense experiments, modified antisense-eGFP and control sequences were purchased and used as received [IDT DNA, 5'-GAGCTGCACGCTGCCGTC-(A)₁₀-(CH₂)₃-SH-3' (antisense eGFP), 5'-GAGCTGCACGCTGCCGTC-(A)₁₀-(CH₂)₃-SH-3' (scrambled control)]. Modified oligonucleotides (3 nmol) were added to AuNPs (1 mL, 10 nM) and allowed to shake overnight at room temperature. After 12 hours, sodium dodecylsulfate (SDS) solution (10%) was added to the mixture (0.01% SDS, final) and phosphate buffer (PB, 0.1 M; pH = 7.4) was added (0.01 M PB, final). Next, sodium chloride (NaCl) solution (2.0 M) was added to the mixture to achieve a 0.05 M NaCl concentration, beginning a gradual salt-aging process which ultimately stabilized the AuNP-DNA conjugates in physiologic saline and served to increase the density of AuNP surface-bound oligonucleotides.¹² Three aliquots of NaCl solution were added to the AuNP-DNA mixture over an eight-hour period to achieve a final NaCl concentration of 0.15 M under continuous shaking. To purify AuNP-DNA away from non-reacted oligonucleotides the solution was

centrifuged ($15,871 \times g$, 30 min). Pelleted AuNP-DNA conjugates were re-suspended in sterile phosphate buffered saline (1X PBS). The centrifugation procedure was repeated three times to produce the purified conjugates used in all subsequent experiments and to concentrate the AuNP-DNA particles as needed. The final concentration of AuNP-DNA was determined using UV-vis spectroscopy (molar extinction coefficient 13 nm AuNPs = $2.4 \times 10^8 \text{ M}^{-1}\text{cm}^{-1}$). Solutions of AuNP-DNA ($\pm\text{Cy}$) were stored at 4°C and filter-sterilized ($0.22 \mu\text{m}$) prior to cell culture experiments.

Fluorescent confocal microscopy for cellular uptake of AuNP-DNA-Cy3 in islets

Human islets were cultured with 0 or 10 nM AuNP-DNA-Cy3 or 500 nM DNA-Cy3 for 24 hours, washed twice with sterile 1X PBS, and then transferred to fresh culture media containing no AuNP-DNA-Cy3. Islets were imaged immediately after AuNP-DNA-Cy3 exposure ($T=0$). Also, in order to assess the retention of AuNP-DNA-Cy3 within the islets, similarly treated cells remained in fresh culture media following AuNP-DNA-Cy3 removal and imaged at 24 or 72 hours. In all cases prior to microscopy, islets were washed twice with sterile 1X PBS and then stained with DAPI (1:1000 dilution in 1X PBS) for 10 minutes. Islets were placed on microscope slides under a glass cover slip and sealed with fingernail polish. The islets were visualized using the Nikon C1Si confocal microscope, and imaged under the blue (402 nm, DAPI) and the red (561 nm, Cy3) filter set. Microscope settings used for islets exposed to 10nM AuNP-DNA-Cy3 at $T=0$ were optimized and then held constant for all nanoparticle concentrations and all time points.

Electron microscopy for AuNP-DNA uptake

Human islets were treated with 10 nM AuNP-DNA-Cy for 24 hours, washed twice with 1X PBS and then transferred to fresh media containing no AuNPs for an additional 72 hours. Islets were pelleted and immersed in 2% paraformaldehyde/2.5% glutaraldehyde in 0.1M sodium cacodylate buffer (SCB). The islets were then rinsed with 0.1M SCB and placed in secondary fixative containing 2% osmium tetroxide in 0.1M SCB. Next, 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. 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.

Flow cytometric evaluation of AuNP-DNA-Cy5 incorporation and viability

Human islets treated with 0, 1, 5 or 10 nM AuNP-DNA-Cy5 for 24 or 48 hours were dispersed using Accutase® (Innovative Cell Technologies, San Diego, CA). Briefly, islets were washed twice in 1X PBS, spun at $280 \times g$ for 5 minutes at 4°C and re-suspended in 1 mL Accutase®. The islets were incubated at 37°C for 10 minutes, vortexed briefly, and 1 mL of fetal bovine serum (FBS) was added to stop the digestion. The cell suspension was then passed through a 40 μm filter and spun at $500 \times g$ for 5 minutes at 4°C . The cells were washed with 1X PBS and then re-suspended to 10^3 cells/mL. The cells were then analyzed using flow cytometry to determine the percentage of Cy5 positive cells.

For viability analyses, islets were treated with 0 or 10 nM AuNP-DNA-Cy5 for 24 hours. At 0, 24, and 72 hours post AuNP-DNA-Cy5 exposure, islets were dispersed using Accutase® and stained for live cells using 300 μL of 0.2 nM tetramethylrhodamine (TMRE; Invitrogen, Carlsbad, CA) in 1X PBS for 45 minutes at room temperature. The cells were spun at $500 \times g$ and washed with 1X PBS. 7-aminoactinomycin D (7-AAD; Invitrogen, Carlsbad, CA) in 1X PBS was added 5 minutes prior to flow cytometry to stain for dead cells (300 μL per tube, 5 $\mu\text{g/mL}$).

Glucose stimulated insulin release assay

Human islets were cultured with 0, 5, or 10 nM AuNP-DNA-Cy for 24 hours. Next, the islets were washed twice in 1X PBS and transferred to fresh culture media. Islets were subject to a glucose challenge (T=0), or were cultured in fresh media for 24 and 72 hours prior to glucose challenge using a static incubation method in 5% CO₂ in air at 37 °C. For glucose challenge studies, islets (approximately 25 in each group) were first equilibrated for one hour in Kreb's solution (25 mM HEPES, 115 mM NaCl, 24 mM NaHCO₃, 5 mM KCl, 1 mM MgCl₂, 2.5 mM CaCl₂, 0.1% BSA [pH7.4]) containing 2.8 mM glucose. Next, islets were incubated for one hour in 2.8 mM glucose-containing Kreb's solution, and subsequently for another hour in 28 mM glucose-containing Kreb's solution. The media from each of the one hour incubations was collected for insulin measurement using an insulin EIA kit (Alpco, Salem, NH). Data are presented as a ratio of the amount of insulin released by the islets exposed to high (28 mM) versus low glucose (2.8 mM).

Measurement of intracellular concentration of Au-NP within dispersed islet cells via Inductively Coupled Plasma Mass Spectrometry (ICP-MS)

Human islets were treated with 0, 5, or 10 nM AuNP-DNA-Cy for 24 hours (T=0). Additionally, and following the removal of AuNP-DNA-Cy, cells were transferred to fresh media and cultured for an additional 24 or 72 hours. In all cases, the islets were dissociated using Accutase® and total cell counts were obtained using a hemocytometer. The islet cells were stored at 4 °C in 500 µL of 1X PBS prior to Au measurement via ICP-MS. To prepare samples for ICP-MS, cells were dissolved in 500 µL of trace metal grade nitric acid and incubated at 55 °C for 18 hours. Following incubation, samples were cooled to room temperature, vortexed, and centrifuged at 15,871 × g for 10 seconds. 400 µL of the sample was added to 3.6 mL of matrix buffer containing 2% HCl, 2% HNO₃ and 5 ppb indium internal standard. Gold concentrations (ppb) from each sample were measured using a Thermo VG PG Excell ICP-MS. The background ICP-MS signal from untreated islets was subtracted from the measurements taken from cells exposed to 5 or 10 nM AuNP-DNA-Cy. ICP-MS measurements of gold (ppb) from each sample were converted to number of AuNPs using a calibration curve of AuNPs at known concentration subjected to similar ICP-MS analysis. The number of AuNPs per cell was calculated based on the number of cells in each sample.

Transplantation of human islets into nude mice

Nude mice were made diabetic via a single intraperitoneal injection of streptozotocin (220 mg/kg body weight; Sigma-Aldrich) and were considered diabetic once their blood glucose levels exceeded 300 mg/dL for two consecutive measurements. Diabetic mice were transplanted with 2000 Islet Equivalents (IEQ) of either untreated human islets or islets exposed to 10 nM AuNP-DNA-Cy for 24 hours. Islets were transplanted in the renal subcapsular site. Body weight and blood glucose measurements were obtained at least 3 times per week for 40 days.

Measurement of eGFP knockdown in mouse MIP-eGFP islets

Pancreatic islets from transgenic mice expressing eGFP under the control of the mouse insulin promoter (MIP-eGFP; Jackson Laboratories) were isolated as described previously. 20 The eGFP islets were cultured in RPMI 1640 media containing 10% FBS, 100 U/mL penicillin G and 100 µg/mL streptomycin sulfate. The islets were then treated with 10 nM AuNP-DNA (scrambled), 10 nM AuNP-DNA (anti-GFP), or left untreated and incubated for 24 hours at 37 °C, 5% CO₂. After incubation, the islets were removed from the nanoparticle media and cultured for an additional 72 hours. The islets were then lysed using 1X Cell Lysis Buffer (Cell Signaling Technologies) and the green fluorescence intensity was

measured using a spectrophotometer (excitation: 488 nm; emission: 550 nm; cutoff: 550 nm). All samples were standardized to protein concentration measured using Active Motif's ProStain Protein Quantification kit. Data are presented as a percentage of eGFP fluorescence of untreated MIP-eGFP islets.

Statistical analyses

Analysis of Variance (ANOVA) and t-tests were carried out using the statistical software SPSS, where appropriate. P values of less than 0.05 are considered statistically significantly different.

RESULTS

Incorporation of AuNP-DNA-Cy into human pancreatic islets

Untreated, control islets did not display Cy3 fluorescence, as expected (data not shown). Representative fluorescent confocal images of DNA-Cy3 treated or AuNP-DNA-Cy3 treated islets of different time points are shown in Figure 1. As shown, treatment with 500 nM DNA-Cy3 alone resulted in a low level of Cy3 fluorescence around the periphery of the islet (Figure 1A), suggesting that a relatively small amount of the DNA-Cy3 had been taken up by the peripheral islet cells; however, no Cy3 fluorescence was detected in the center of the islets. No Cy3 fluorescence was seen 24 or 72 hours post DNA-Cy3 exposure (Figures 1B and 1C). Treatment with 10 nM AuNP-DNA-Cy3 resulted in a fluorescent signal spread throughout the islet, including the inner core (Figure 1D). The fluorescence was observed 24 hours after AuNP-DNA-Cy3 exposure (Figure 1E) and began slightly diminishing at 72 hours post exposure (Figure 1F). These data suggest that the AuNP-DNA-Cy3 conjugates were able to transfect the majority of cells throughout the islet and remain in the cells for at least 72 hours post exposure.

Transmission electron microscopy of islets treated with 10 nM AuNP-DNA-Cy for 24 hours and then cultured for an additional 72 hours demonstrates AuNPs in the islet cells (Figure 2). As expected, no AuNPs were observed in untreated islet cells (data not shown).

Flow cytometry was used to measure the percentage of islet cells that had taken up the AuNP-DNA-Cy conjugates. Untreated human islets were used as a control to gate for Cy5 positive cells. Islet treatment with AuNP-DNA-Cy5 at 1 nM, 5 nM, and 10 nM for 24 hours resulted in a dose-dependent increase in Cy5 positive cells of $20.96 \pm 1.33\%$, $50.31 \pm 0.94\%$, and $66.71 \pm 0.63\%$, respectively (Figure 3A). Extending treatment with AuNP-DNA-Cy5 at 1 nM, 5 nM, and 10 nM for 48 hours further increased the percentage of Cy5 positive cells of $50.38 \pm 0.08\%$, $75.19 \pm 0.11\%$ and $79.73 \pm 1.16\%$, respectively (Figure 3B). Taken together, these data demonstrated that AuNP-DNA-Cy5 enter the majority of the islet cells in a dose- and time-dependent manner.

The presence of gold in dissociated islet cells was measured by ICP-MS. Islets treated with 5 and 10 nM AuNP-DNA-Cy showed an increased level of AuNPs in the cells (3.09×10^4 and 3.63×10^5 AuNPs/cell, respectively) following 24 hours of exposure (T=0) (Figure 4). In addition, AuNPs were measured in the islets at both 24 and 72 hours after AuNP-DNA-Cy incubation (Figure 4). These data suggest that the AuNP-DNA-Cy conjugates remain in islet cells for at least 72 hours after nanoparticle exposure.

Impact of AuNP-DNA-Cy5 on islet viability

Islets were treated with 10 nM AuNP-DNA-Cy5 for 24 hours, rinsed with 1X PBS, and then subjected to flow cytometry analysis (T=0) following islet dissociation. In addition, flow cytometry was performed on islets cultured for an additional 24 or 72 hours prior to islet

dissociation. At T=0, the percent viable (TMRE positive) cells did not differ between the untreated and treated groups ($72.72 \pm 3.14\%$ viable for untreated islets vs. $78.32 \pm 2.00\%$ for 10 nM AuNP-DNA-Cy5 treated islets) (Figure 5). Further, there was no difference in viability after 24 hours: $88.36 \pm 5.71\%$ (untreated) vs. $83.92 \pm 11.55\%$ (treated); and 72 hours: $74.64 \pm 2.64\%$ (untreated) vs. $73.06 \pm 1.82\%$ (treated) post AuNP-DNA-Cy5 exposure, demonstrating that treatment of human islets with 10 nM AuNP-DNA-Cy5 did not affect the viability of the islet cells.

Glucose stimulated insulin secretion assay

Human islets were treated with 0, 5, or 10 nM AuNP-DNA-Cy for 24 hours, and the ability of the β cells to secrete insulin in response to glucose was measured at T=0, as well as 24 and 72 hours following AuNP-DNA-Cy exposure. Data are presented as a ratio of the amount of insulin secreted under 28 mM glucose exposure divided by the amount of insulin secreted under 2.8 mM glucose (Table 1). At each time point, there was no difference in the ability of the islets to secrete insulin in response to glucose stimulation.

Transplantation of AuNP-DNA-Cy treated islets into diabetic nude mice

To evaluate islet function *in vivo*, untreated and islets treated with 10 nM AuNP-DNA-Cy for 24 hours were transplanted under the kidney capsule of streptozotocin-induced diabetic (blood sugar >350 mg/dL) nude mice. Post-transplant blood glucose levels and body weight of the mice were followed. Mice transplanted with treated islets were able to successfully achieve euglycemia, as defined by two blood sugar measurements below 200 mg/dL, under the same time frame (within ~5-10 days) as mice transplanted with untreated control islets (Figures 6A and 6B), confirming that treatment with AuNP-DNA-Cy did not negatively affect the function of the islets. In both groups of mice, the body weights increased over time, as expected (Figures 6C and 6D).

Measurement of eGFP knockdown by AuNP-DNA (anti-eGFP) in mouse MIP-eGFP islets

To test the ability of AuNP-DNA conjugates to regulate gene expression, mouse islets expressing eGFP under the control of the mouse insulin promoter were treated with 0 nM, 10 nM AuNP-DNA (scrambled), or 10 nM AuNP-DNA (anti-GFP) for 24 hours. Following incubation, the islets were cultured for an additional 72 hours. Fluorescence of islet lysates was measured using a spectrophotometer and compared as a percentage of untreated MIP-eGFP islets (Figure 7). Treatment with scrambled antisense DNA nanoparticles did not alter eGFP expression, as expected. When the islets were treated with AuNP-DNA (anti-eGFP), the relative fluorescence was reduced to 47% of the untreated control islets ($p=0.01$), indicating that the nanoparticles are able to regulate targeted gene expression in the islet cells.

DISCUSSION

A decade of advances in islet isolation and transplantation since the Edmonton report has improved outcomes for select patients with type 1 diabetes; however, the long-term durability of islet grafts requires improvement before islet transplantation can be considered a mainstream therapy. Altering the gene expression profile to enhance islet graft survival is a promising strategy to further improve results; however, the ability of viral-based and non-viral vectors to deliver genetic material to the islet cells is inefficient and there are concerns related to cellular toxicity.¹⁰ Gold nanoparticles surface-functionalized with oligonucleotides have been used as single-entity *in vitro* transfection reagents for cells grown as tissue culture monolayers.¹⁶⁻²¹ As such, these conjugates regulate gene expression, demonstrate a high transfection efficiency, and are not inherently toxic.¹⁶⁻²¹ However, AuNP-DNA conjugates have not been previously tested on islets that are

comprised of 1000-2000 cells, approximately 150 μm diameter, where cells at the core of the islet have proven difficult to transfect. We set out to determine the utility of AuNP-DNA conjugates in the context of isolated, purified pancreatic islets with regards to transfection efficiency, islet viability, islet function, and the ability of the AuNP-DNA conjugates to regulate targeted gene expression.

The AuNP-DNA conjugates were able to fully penetrate into the cells making up the core of the islets. *In vitro* treatment of islets with an AuNP-DNA conjugate concentration of 10 nM for 24 hours resulted in uptake of the nanoparticles in the majority of the islet cells as measured by fluorescent confocal microscopy, flow cytometry, and ICP-MS. Confocal microscopy and ICP-MS data demonstrate that AuNP-DNA-Cy conjugates remain in the islet cells for at least 72 hours post-exposure, and that a higher dose of nanoparticles (10 nM) results in increased delivery. Electron microscopy of islets at 72 hours post AuNP-DNA-Cy exposure demonstrates the presence of the conjugates in the cytoplasm of islet cells. Interestingly, confocal fluorescent microscopy demonstrates diminishing signal following initial 24 hour transfection; however, ICP-MS data, while consistent with robust and dose-dependent cell internalization of AuNPs, appears somewhat contradictory. The dynamics of internalization and metabolism of the AuNP-DNA-Cy conjugates are areas of current study. One explanation of the ICP-MS data is that AuNPs remain in viable islet cells, and over time islet cell death results in an increased concentration of AuNPs in remaining viable cells. On the other hand, the DNA-Cy component of the conjugates may be slowly degraded, thus, diminishing signal intensity as measured with confocal fluorescent microscopy. The nanoparticles did not alter the viability of the islet cells or the ability of the pancreatic β cells to release insulin in response to glucose. The *in vivo* functionality of the islets was not altered by exposure to AuNP-DNA-Cy in the nude mouse transplant model as a bioassay of islet viability and function. These data demonstrate that AuNPs are promising agents capable of entering all cells within a three-dimensional collection of cells within a purified human islet. It is significant that AuNP-DNA conjugates were able to penetrate to the core of the islets, an area that is generally unreachable by viral vectors or lipid-based transfection reagents.⁹⁻¹¹ The degree of AuNP surface functionalization with oligonucleotides has been shown to dictate their ability to successfully enter cells grown in monolayer culture; in general, the higher the density of AuNP surface bound oligonucleotides the greater the observed cell entry.²² These material properties, as well as their small size, may well be responsible for the observed penetration of AuNP-DNA-Cy conjugates into the core of human pancreatic islets.

Human islet functionality and viability were not detrimentally impacted due to AuNP-DNA-Cy exposure. Typically, with other lipid- and polymer-based transfection reagents, increased transfection efficiency is directly related to increased toxicity. This was not seen with the AuNP-DNA-Cy at the concentrations tested in these investigations. At 10 nM concentration, the AuNP-DNA-Cy did not induce cell death, further supporting the use of AuNP-DNA-Cy as a non-toxic and robust conjugate therapeutic platform. The functionality of the β cells was not impacted by exposure to AuNP-DNA-Cy, as the treated and untreated islets preformed similarly in the glucose stimulated insulin secretion assay.

While the *in vitro* assays are important to determine how well the AuNP-DNA-Cy get into the islet cells and the viability of the islets post AuNP-DNA-Cy exposure, the ability of the islets exposed to AuNP-DNA-Cy to cure diabetes in a transplant model is a crucial component to validate the potential of the platform to safely deliver genetic material to islet cells. Both treated and untreated islets were able to cure streptozotocin-induced diabetic nude mice in the same time frame, suggesting that the AuNP conjugates provide an excellent, non-toxic platform for the transfection of human islets.

All of the experiments performed with human islets utilized AuNPs that had been functionalized with a DNA sequence not intended to target any expressed sequence in the human genome in order to validate the AuNP platform as an efficient and safe method for delivering genetic material to islet cells. To demonstrate the ability of the AuNP-DNA conjugates to regulate gene expression, AuNP-DNA conjugates were fabricated to target eGFP expression in transgenic mouse islets expressing eGFP under the mouse insulin promoter. Treatment with anti-eGFP AuNP-DNA conjugates reduced eGFP expression by approximately 50% at 72 hours post exposure, clearly demonstrating the functionality of AuNP-DNA conjugates in the islet cells.

Collectively, this work points towards the tantalizing prospect of functionalizing AuNPs with therapeutic nucleic acid sequences, either antisense DNA or short interfering RNA, targeted against pro-apoptosis genes (e.g. caspase-3 or 8); cytokine receptors (e.g. IL-1R or TNF-R); second messenger signaling pathway molecules (e.g. IKK β); and/or chemokines (e.g. MCP-1 or IP-10) to protect the islets immediately after transplantation and allow for enhanced islet engraftment, survival, and long-term function. Tailoring the identity of AuNP surface oligonucleotides should provide a methodology to identify the most efficient constructs for enhancing pancreatic β cell survival.

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Figure 1. Fluorescent confocal microscopy for cellular distribution of AuNP-DNA-Cy3 in human islets

Representative overlay fluorescent images of human islets treated with 500 nM DNA-Cy3 (red) or with 10 nM AuNP-DNA-Cy3 (red) for 24 hours (T=0). Following initial incubation, cells were washed and imaged at 24 and 72 hours. Cell nuclei are stained with DAPI (blue). All images were taken at 20X magnification. A. 500 nM DNA-Cy3 labeling at T=0. B. 500 nM DNA-Cy3 labeling 24 hours post T=0. C. 500 nM DNA-Cy3 labeling 72 hours post T=0. D. 10 nM AuNP-DNA-Cy3 labeling at T=0. E. 10 nM AuNP-DNA-Cy3 labeling 24 hours post T=0. F. 10 nM AuNP-DNA-Cy3 labeling 72 hours post T=0.

Figure 2. Electron microscopy of human islets treated with 10 nM AuNP-DNA-Cy
Human islets treated with 10 nM AuNP-DNA-Cy for 24 hours and then cultured for an additional 72 hours. Arrows indicate clusters of AuNP-DNA-Cy in the islet cells. A. 4800X magnification. B. 18500X magnification. C. 18500X magnification. D. 49000X magnification.

Figure 3. AuNP-DNA-Cy5 incorporation into human islet cells

Human islets treated with 0, 1, 5, or 10 nM AuNP-DNA-Cy5 for 24 hours (A) or 48 hours (B) were dissociated and the percentage of Cy5 positive cells was determined using flow cytometry, * $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$.

Figure 4. ICP-MS measurement of Au in islet cells

Human islets treated with 5 or 10 nM AuNP-DNA-Cy were dissociated and the total amount of AuNPs in each cell was quantified. Measurements were taken following initial incubation at 24 hours (T=0), and then 24 and 72 hours post exposure. Exposure to 10 nM AuNP-DNA-Cy lead to the highest concentration of AuNPs in the islet cells, which remained elevated over 72 hours.

Figure 5. Viability measurement

Human islets were left untreated (0 nM) or treated with 10 nM AuNP-DNA-Cy5 for 24 hours (T=0). Cells were subject to flow cytometry or cultured for an additional 24 or 72 hours. In all cases, the cells were dissociated and stained with TMRE/7-AAD prior to flow cytometry analysis. No statistically significant differences in viability were detected between groups.

Figure 6. Blood glucose levels and body weights of diabetic nude mice transplanted with human islets exposed to 0 nM or 10 nM AuNP-DNA-Cy

Diabetic mice were transplanted with 2000 IEQ of either untreated (0 nM), control (A and C) or 10 nM AuNP-DNA-Cy treated islets (B and D). Blood glucose readings for individual mice transplanted with control (A) or AuNP-DNA-Cy (B) islets. Body weights for mice in both treatment groups (C and D). Mouse 5 in the 10 nM AuNP-DNA-Cy treatment group died due to an infection contracted prior to transplantation.

Figure 7. Knockdown of eGFP in mouse MIP-eGFP islets by AuNP-DNA (anti-eGFP)
Islets from mice expressing eGFP under the mouse insulin promoter (MIP-eGFP) were isolated and treated with 0 nM, 10 nM AuNP-DNA (scrambled), or 10 nM AuNP-DNA (anti-eGFP) for 24 hours, cultured for an additional 72 hours and lysed to measure eGFP expression by spectroscopy. Data are presented as a percentage of untreated eGFP fluorescence levels. * $p=0.01$

Table 1
Glucose stimulated insulin secretion assay

Human islets exposed to 0, 5, or 10 nM AuNP-DNA-Cy were assayed for their ability to secrete insulin in response to glucose stimulation following 24 hour nanoparticle incubation (T=0), and at 24 and 72 hours following T=0.

AuNP-DNA-Cy Concentration	Time post exposure		
	0 hours	24 hours	72 hours
0 nM	1.858 $\pm$ 0.227	2.364 $\pm$ 0.399	2.344 $\pm$ 0.585
5 nM	1.538 $\pm$ 0.163	2.115 $\pm$ 0.672	2.187 $\pm$ 0.478
10 nM	1.608 $\pm$ 0.160	2.056 $\pm$ 0.168	2.142 $\pm$ 0.165

Data are presented as a ratio of insulin released at 28 mM glucose divided by insulin released at 2.8 mM glucose $\pm$ SEM. No statistically significant difference was observed between the three treatment groups at each time point.