**ORIGINAL ARTICLE**

**Assessment of intravenous pbi-shRNA PDX1 nanoparticle (OFHIRNA-PDX1) in yucatan swine**

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PDX1 (pancreatic and duodenal homeobox 1) is overexpressed in pancreatic cancer, and its reduction results in tumor regression. Bi-functional pbi-shRNA PDX1 nanoparticle (OFHIRNA-PDX1) utilizes the endogenous micro-RNA biogenesis pathway to effect cleavage- and non-cleavage-dependent degradation of PDX1 mRNA. We have shown that OFHIRNA-PDX1 reduces pancreatic tumor volume in xenograft models. Thus, we are now exploring biorelevant large animal safety of OFHIRNA-PDX1. Mini pigs were chosen as the biorelevant species based on the similarity of human and pig PDX1 target sequence. In the initial study, animals developed fever, lethargy, hyporexia and cutaneous hyperemia following administration of OFHIRNA-PDX1. Twenty-one days later, the same animals demonstrated less toxicity with a second OFHIRNA-PDX1 infusion in conjunction with a prophylactic regimen involving dexamethasone, diphenhydramine, Indocin and ranitidine. In a new group of animals, PDX1 protein (31 kDa) expression in the pancreas was significantly repressed at 48 and 72 h (85%, P = 0.018 and 88%, P = 0.013; respectively) following a single infusion of OFHIRNA-PDX1 but recovered to normal state within 7 days. In conclusion, a single intravenous infusion of OFHIRNA-PDX1 in conjunction with premedication in pigs was well tolerated and demonstrated significant PDX1 knockdown.

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**INTRODUCTION**

Pancreatic cancer is the fourth most (in United States) and ninth most (worldwide) cause of cancer-related deaths.1–3 The prognosis with any stage is poor, with a 25% survival rate at 1 year and only 6% survival at 5 years.4 Specifically, 80% of individuals present with metastatic or advanced disease and have a median survival of 6–10 months.5 Recent studies have demonstrated PDX1 (pancreatic and duodenal homeobox 1) overexpression in pancreatic cancer.6–8 PDX1 is critical to embryological pancreas development and insulin expression in adult islet cells.9–12 Overexpression of PDX1 in benign and malignant cell lines increased tumor formation and proliferation in mouse xenograft models.13–17 Treatment with OFHIRNA-PDX1 resulted in a significant reduction of tumor volume and prolonged survival in mice; however, it also resulted in temporal and mild hyperglycemia owing to knockdown of PDX1 in murine islets.18,19 The bifunctional shRNA PDX1 platform is designed to express two shRNA products, both targeted against huPDX1 mRNA.20 The shRNA products are processed by the host RNAi machinery and utilize the small interfering RNA (siRNA; RNA-induced silencing complex (RISC)-cleavage dependent) and micro-RNA (miRNA)-like (RISC-cleavage independent) pathways; despite differential functionality, both are complementary to the same target mRNA sequence.

Based on the promising in vitro and in vivo efficacy data, biorelevant animal studies were designed to evaluate the safety of OFHIRNA-PDX1 following a single intravenous (IV) infusion. Yucatan mini pigs were chosen for this study based on the target sequence (PDX1 mRNA) and the similarity to human PDX1. The results presented here demonstrate that infusion of OFHIRNA-PDX1 at dose levels sufficient for knockdown of expressed pancreatic PDX1 result in a moderate inflammatory response that can be mitigated with appropriate anti-inflammatory premedication.

**MATERIALS AND METHODS**

**Vector design**

The expression unit for the bifunctional shRNA to human PDX1 (bi-shRNA PDX1) is inserted in the multi-cloning site of the pUMVC3 vector (Aldevron, Fargo, ND, USA) and is driven from an enhanced cytomegalovirus-immediate early promoter (RNA polymerase II promoter). It contains two stem-loop structures in a miR-30a backbone, one with complete matching passenger and guide strands (RISC-cleavage dependent; siRNA-like component) and the other with two base-pair mismatches between passenger and guide strands (RISC-cleavage independent; miRNA-like component). The AG to CU switches are at positions 11 and 12, creating mismatches at the central location similar to most miRNAs but on the passenger rather than the guide strand, thereby allowing for persistence of effector-target mRNA complementarity. Gradalis (Dallas, TX, USA) manufactured the pbi-shRNA PDX1 plasmid under current good manufacturing practice conditions.

Two separate reporter vectors were created, using the pUMVC3 backbone. Human PDX1 (Genbank no. NM_000209.3) and pig PDX1 (Genbank no. NM_001141984.1) were PCR amplified and inserted into the pUMVC3 multi-cloning site. The cDNA inserts were confirmed by sequence analysis (SeqWright, Houston, TX, USA). These reporter vectors were used in vitro to demonstrate the bioactivity of the bi-shRNA PDX1 platform against human and pig PDX1. These two plasmid vectors were manufactured by Aldevron (Fargo, ND, USA).

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Delivery vehicle

The BIV (bilamellar invaginated vesicles) nanoparticles consist of a 10:9 molar ratio of DOTAP cationic lipid and plant-derived cholesterol (Avanti Polar Lipids, Alabaster, AL, USA). The lipids are resuspended in chloroform, mixed and dried down to a thin film using a rotary evaporator. The lipid film is freeze dried to remove residual chloroform and then resuspended in DSW (Baxter, Deerfield, IL, USA), sonicated and passed through a series of decreasing pore-size filters incorporating a manual extrusion process.21 The stock BIV nanoparticles, manufactured by Gradalis under current good manufacturing practice conditions, were stored at +2 to 8 °C until ready for manufacturing of the final OFHIRNA-PDX1.

OFHIRNA-PDX1

GMP BIV DOTAP:Cholesterol nanoparticles and GMP pbi-shRNA PDX1 plasmid DNA are diluted in DSW and mixed spontaneously to create the final product under current good manufacturing practice conditions at Gradalis. The final concentration of plasmid DNA is 0.5 mg/mL. The product is stored at +2 to 8 °C until ready for use. Each batch of OFHIRNA-PDX1 is tested for sterility, endotoxin, particle distribution, identity and potency.

In vitro testing

Human cancer cell lines (CCL-247, Panc-1 and Mia PaCa-2), mouse insulinoma cell line β-TC-6 and pig embryonic cell lines (CL-184 and CRL-1746) were purchased from ATCC (Manassas, VA, USA). All cell lines were grown according to the propagation recommendations from ATCC. Cell cultures were electroporated with 50 μg purified pDNA in a 0.6-cm cuvette (Bio-Rad, Hercules, CA, USA) and plated in 6-well plates. Cells were harvested 24 h post electroporation by trypsin digestion and pelleted at 1.500 × g, 5 min, at 4 °C, for analysis. The cell pellets were dissociated using Cellytic (Sigma, St Louis, MO, USA) and total protein concentration was estimated by BCA protein assay (Thermo Scientific, Rockford, IL, USA). Fifty micrograms (50 μg) of cell lysate was separated by polyacrylamide gel electrophoresis (PAGE) and analyzed via western blotting (Bio-Rad, Hercules, CA, USA). Monoclonal antibodies were used to detect PDX1 (ab94897, Abcam, Cambridge, MA, USA) and β-tubulin (sc-55529, Santa Cruz Biotechnology, Dallas, TX, USA). The bands were visualized using the G-box system (Syngene, Frederick, MD, USA) and the PDX1 protein levels were quantified and normalized relative to β-tubulin expression. Western blotting studies of PANC-1 and Mia PaCa-2 cell lines were described before18 using anti-PDX1 C-terminal polyclonal antibody (FCB laboratory, Los Angeles, CA, USA). The human pancreatic cancer cells were plated in six-well plates. Transfection with lipofectamine (Invitrogen, Grand Island, NY, USA) was performed using empty vector or pbi-shRNA PDX1, respectively, at a dose of 2 μg DNA per well. Forty-eight hours after transfection, cells were harvested and lysed for western blotting. Twenty micrograms (20 μg) of lysate protein were applied for each lane.

In vivo testing

Large animal studies were performed at the Texas A&M Institute for Preclinical Studies (College Station, TX, USA). In the first study (analysis of acute toxicity), four immature female Yucatan pigs (110–114-days old, from Sinclair Bio Resources, Auxvasse, MO, USA) were surgically implanted with a specimen cup containing saline and gentamicin and shipped to Gradalis (Syngene, Frederick, MD, USA) and the PDX1 protein levels were quantified by western blotting (Bio-Rad, Hercules, CA, USA) and normalized relative to (Syngene, Frederick, MD, USA) and the PDX1 protein levels were quantified by western blotting (Bio-Rad, Hercules, CA, USA) and normalized relative to (Syngene, Frederick, MD, USA) and the PDX1 protein levels were quantified by western blotting (Bio-Rad, Hercules, CA, USA). The human pancreatic cancer cells were plated in six-well plates. Cells were harvested 24 h post electroporation by trypsin digestion and pelleted at 1.500 × g, 5 min, at 4 °C, for analysis. The cell pellets were dissociated using Cellytic (Sigma, St Louis, MO, USA) and total protein concentration was estimated by BCA protein assay (Thermo Scientific, Rockford, IL, USA). Fifty micrograms (50 μg) of cell lysate was separated by polyacrylamide gel electrophoresis (PAGE) and analyzed via western blotting (Bio-Rad, Hercules, CA, USA). Monoclonal antibodies were used to detect PDX1 (ab94897, Abcam, Cambridge, MA, USA) and β-tubulin (sc-55529, Santa Cruz Biotechnology, Dallas, TX, USA). The bands were visualized using the G-box system (Syngene, Frederick, MD, USA) and the PDX1 protein levels were quantified and normalized relative to β-tubulin expression. Western blotting studies of PANC-1 and Mia PaCa-2 cell lines were described before18 using anti-PDX1 C-terminal polyclonal antibody (FCB laboratory, Los Angeles, CA, USA). The human pancreatic cancer cells were plated in six-well plates. Transfection with lipofectamine (Invitrogen, Grand Island, NY, USA) was performed using empty vector or pbi-shRNA PDX1, respectively, at a dose of 2 μg DNA per well. Forty-eight hours after transfection, cells were harvested and lysed for western blotting. Twenty micrograms (20 μg) of lysate protein were applied for each lane.

RESULTS

PDX1 sequence comparison

The pbi-shRNA-PDX1 vector was designed to target the 3' region of human PDX1 mRNA. The shRNA target sequence was assessed against PDX1 mRNA from multiple species (Figure 1). Within the target site sequences, mouse and rat sequences contain four mismatches out of the 19 nucleotides between human and rodents. Our initial comparative knockdown data have shown the human sequence-directed OFHIRNA-PDX1 was not effective in mouse PDX1 knockdown. Other animal species, such as cow, pig, cat and dog, have two mismatches at positions 3 and 12 of the guide strand (Figure 1). All could be biological relevant species for human OFHIRNA-PDX1; the mini pig was chosen because of close similarities with human pancreas and previous veterinary experience with this species.22

In vitro analysis of pbi-shRNA PDX1 against human and pig PDX1 targets

Human cancer cell lines (PANC-1 and Mia PaCa-2) and pig embryonic cells lines (CL-184 and CRL-1746) were grown according to the vendor's recommendations and electroporated with pbi-shRNA PDX1 plasmid. Cell cultures were harvested 24 h post electroporation and analyzed via western blotting for the expression of PDX1 protein (Figure 2). In vitro results demonstrated persistent medium-to-high expression of PDX1 protein in three of four control (non-treated) cell lines, whereas all cultures treated with pbi-shRNA PDX1 had a significant reduction (72–92%) of endogenous PDX1 protein. This preliminary screening of cell lines demonstrates the ability of the pbi-shRNA PDX1 vector to effectively knock down endogenous PDX1 expression in multiple cell lines. It also demonstrates the bio relevance of pig PDX1 mRNA sequence to human.

The endogenous PDX1 expression pattern was investigated by western blotting analysis of human pancreatic cancer cell lines PANC-1 and Mia PaCa-2 following treatment with empty vector or pbi-shRNA PDX1. The results showed that PANC-1 cells expressed four-fold higher PDX1 at 31 kDa (PDX146) compared with that at 46 kDa (PDX146). In Mia PaCa-2, PDX146 was four-fold higher than PDX146 as shown in Figure 3a. pbi-shRNA PDX1 significantly
knocked down PDX1 expression in both PANC-1 and Mia PaCa-2 cells, compared with empty vector controls (P<0.05). There was no significant effect on knockdown of PDX1 expression in either human pancreatic cancer cell line. These data reveal interesting differences in PDX1 isoform expression patterns and response to PDX1 knockdown in human pancreatic cancer cell lines versus that seen in pig islets.

In a separate experiment, CCL-247 cells were used to overexpress recombinant human PDX1 or recombinant pig PDX1. The cells were electroporated with recombinant PDX1 expression plasmid plus empty vector or pbi-shRNA PDX1 vector. Cells were harvested 24 h post electroporation and analyzed by western blotting (Figure 4). These results demonstrate forced expression of recombinant PDX1 (human and pig) and an average 55 ± 2% knockdown of recombinant PDX1 protein when treated with pbi-shRNA PDX1.

The combined results of these in vitro tests demonstrate that pbi-shRNA PDX1 is able to knock down endogenous human and pig PDX1 protein. In addition, recombinant human and pig PDX1 was overexpressed in the colon cancer cell line, CCL-247 and subsequently knocked down with pbi-shRNA PDX1. These data also demonstrate that pig PDX1 mRNA is similar to human PDX1 at the targeted region. Based on these results and the data from previous mouse xenograft efficacy results, safety studies in a biorelevant animal model (Yucatan mini pigs) were designed to further evaluate OFHIRNA-PDX1 as a clinical product.

In vivo study no.1 (analysis of acute toxicity)

Four immature female pigs (112 ± 2-days old and 14.9 ± 0.6 kg) underwent surgical implant of a VAP. After implantation, but while still under anesthesia, three animals received 0.156 mg OFHIRNA-PDX1 kg 1 (equivalent to 0.09 mg kg 1 in humans) and one animal received an equivalent dose of empty liposomes in DSW via IV infusion (50 ml per 30 min). The animals were observed for 14 days, and blood was collected twice daily. The three pigs receiving OFHIRNA-PDX1 demonstrated elevated body temperatures within the first few hours post infusion and resolved within 24 h (Figure 5). (Normal body temperature for a healthy mini pig is 102 ± 1 °C).23 These three animals were also lethargic, had a loss of appetite and displayed cutaneous hyperemia, which resolved within 3 days. The empty liposome animal (control) behaved normally and displayed no adverse effects following the infusion. This animal also had elevated body temperature 4 h post infusion, but it is unknown if this was a result from the activity in the room or a reaction to the surgery/anesthesia and treatment.

Figure 2. Endogenous expression of PDX1 (pancreatic and duodenal homebox 1) 24 h after treatment with pbi-shRNA PDX1 plasmid. Three of the four cell lines had medium-to-high levels of endogenous PDX1 expression, and all the four cell lines demonstrated significant PDX1 reduction following treatment with pbi-shRNA PDX1 plasmid.

Figure 1. Illustration of pbi-shRNA PDX1 (pancreatic and duodenal homebox 1) vector and comparison of target sequence in different species. The figure above illustrates the bifunctional shRNA design with both the cleavage-dependent and cleavage-independent hairpin loops. The sequence alignment below highlights the shRNA target sequence in multiple species. Underlined bases are different from the human PDX1 target.
Twenty-one days following the first IV infusion, all four pigs received a second dose (0.156 mg OFHIRNA-PDX1 kg\(^{-1}\)) of the test article. The three animals that received test article in the first infusion received a premedication regimen consisting of dexamethasone, diphenhydramine, Indocin, acetaminophen, and ranitidine 1 h before test article delivery and repeated every four hours up to 20 h post IV infusion. This premedication regimen is similar to the schema used in previous clinical trials with similar DNA-nanoparticle formulations.\(^24\) The fourth animal (empty liposome control from the first IV infusion) did not receive any premedication. The animal receiving OFHIRNA-PDX1 only (no premedication) demonstrated the same acute effects (fever, lethargy, loss of appetite and cutaneous hyperemia) until day 3. However, the three animals receiving premedication had an attenuated reaction and recovered sooner (bright, alert and normal activity 1 day following infusion) (Figure 6). All animals were observed for 6 days following the second treatment, blood was collected twice per day and then the pigs were killed for necropsy and pancreata collection. The necropsy found no significant changes/abnormalities. The pancreas samples were used for cell culture/molecular analysis (data not shown).

The analysis of blood samples from both premedicated and non-premedicated animals revealed no significant changes in hematology, clinical chemistry or coagulation parameters (data not shown). Additionally, the three pigs receiving a second dose of

**Figure 3.** A pattern of endogenous expression of PDX1 (pancreatic and duodenal homeobox 1) isoforms in human pancreatic cancer cell lines. PANC-1 and Mia PaCa2 express predominantly PDX1\(^{31}\) with reversed ratios of PDX1\(^{12/46}\). Forty-eight hours after treatment with pbi-shRNA PDX1, significant knockdown of only PDX1\(^{31}\) was observed in both PANC1 and Mia PaCa2 cells.

**Figure 4.** Recombinant PDX1 (pancreatic and duodenal homeobox 1) expression 24 h after pbi-shRNA PDX1 treatment. Recombinant PDX1 (human or pig) was expressed in CCL-247 cells. Co-transfection with pbi-shRNA PDX1 vector resulted in an average 55 ± 2% reduction of recombinant PDX1 expression.

**Figure 5.** Average body temperature of healthy Yucatan swine following single intravenous (IV) infusion of OFHIRNA-PDX1 (pancreatic and duodenal homeobox 1).

**Figure 6.** Average body temperature of healthy Yucatan swine following a second intravenous (IV) infusion of OFHIRNA-PDX1 (pancreatic and duodenal homeobox 1) with or without medication.
OFHIRNA-PDX1 did not demonstrate any added toxicity relative to the results after the first infusion. Blood glucose levels were unchanged throughout the study (Figure 7). Animals receiving OFHIRNA-PDX1 demonstrated a slight increase in insulin levels 24–36 h following IV infusion (Figure 7). However, insulin levels were not significantly different from the reference level.25

**In vivo study no.2 (pathology and bioactivity)**

In a second study, a VAP was implanted into six immature female pigs (116–137-days old and 17.5 ± 1.1 kg), and then all animals were given the same premedication regimen 1 h before IV infusion. Five animals were administered 0.156 mg of OFHIRNA-PDX1 kg⁻¹ in 50 ml D5W per 30 min and one animal received control treatment in an equivalent volume of D5W only, while under anesthesia. All animals received additional premedication every 4 h up to 20 h post-IV infusion. Animals were observed and killed at days 1, 2, 3, 4 and 7 (OFHIRNA-PDX1 treated) or day 2 (D5W control). Tissue samples were collected from major organs for pathology and molecular analysis. Similar to the first pig study, the animals receiving OFHIRNA-PDX1 with premedication displayed transient fever, lethargy and loss of appetite with symptoms abating within 24 h. The animal receiving D5W was bright and alert and showed no signs of distress at any time.

After the animals were killed, tissue samples were prepared and analyzed via H&E staining and TEM. Two certified pathologists analyzed the tissue samples of each animal in a blinded approach. Similar to the first pig study, the animals receiving OFHIRNA-PDX1 demonstrated a slight increase in insulin levels 24–36 h following IV infusion (Figure 7). However, insulin levels were not significantly different from the reference level.25

After the animals were killed, tissue samples were prepared and analyzed via H&E staining and TEM. Two certified pathologists analyzed the tissue samples of each animal in a blinded approach. The analyses (H&E and TEM) determined that all changes observed were minimal to mild, transient, reversible and within normal limits. Both pathologists ascribed the observed changes to the handling, fixation and background of the sample and not related to the test article. Comparison of the pancreas of the DSW control with the OFHIRNA-PDX1-treated animals (killed at days 2 and 7) illustrated no visible histological difference.

In addition, fixed pancreatic tissue was analyzed by immuno-histochemistry for the expression of insulin at days 2 and 7 after OFHIRNA-PDX1 treatment. The pancreas of the DSW control animal killed at day 2 was used as a reference. Immunohistochemical staining demonstrated no significant change in insulin expression.

To further evaluate the changes in PDX1 levels in the pancreas, frozen tissue samples were analyzed via western blotting for protein expression. Tissue samples from the head, mid-body and tail of the pancreata from the bi-shRNA PDX1 nanoparticle-treated (days 2 and 7) and D5W control (day 2) animals were compared side by side (Figure 8). Western blotting showed two different PDX1 protein bands, at 46 and 31 kDa. The 31-kDa PDX1 protein was significantly reduced at day 2 \( (P = 0.05) \) and returned to normal expression by day 7. The 46-kDa PDX1 protein appeared unchanged relative to control.
several tumor xenograft mouse models in response to multiple treatments in the mice model resulting in accumulative effect of knockdown of both of PDX1\(^{17}\) and PDX1\(^{46}\) forms in islets, whereas one treatment in the pig was mostly working on the PDX1\(^{31}\) form as discussed below. Molecular analyses of pig pancreatic tissue from normal animals treated with OFHIRNA-PDX1 demonstrated a significant, transient reduction of endogenous PDX1\(^{31}\) expression. Western blotting analysis demonstrated a differential effect on the two isoforms of PDX1 (46 and 31 kDa) with downregulation of only the 31-kDa protein. This difference in protein expression may be related to the in vitro cell culture conditions in comparison to the native in vivo conditions. Alternatively, the difference in RNAi-mediated downregulation of the two isoform may be due to either spatial or temporal rate-related effects on, purportedly, cytoplasmic (31 kDa) versus nuclear (46 kDa) PDX1\(^{29,30}\). Despite evidence of the potential for nuclear RISC targeting,\(^{31}\) published literature suggests the involvement of post-translational phosphorylation and/or sumoylation of the 31 kDa resulting in the 46 kDa to allow for nuclear translocation. If this process does have such a role, then the bi-shRNA, both in its direct mRNA cleavage/degradation as well as in its interference with transcription, acting in the perinuclear region would effectively silence the transcription/translation processes producing the 31 kDa, which our data would then reflect, but would not substantively affect the nuclear-translocated post-translationally modified 46 kDa, the temporal duration of which would depend on its half-life. Only a handful of publications have addressed the two isoforms of PDX1 in islets and have not been consistent in delineating structural or defining functional differences. PDX1 is a protein of 283 amino acids with predicted molecular mass of 31 kDa. Mouse PDX1 expressed in bacteria does have the expected molecular mass of 31 kDa.\(^{30}\) However, endogenous PDX1, which predominates in the nuclei of β-TC-6 cells, has a molecular mass of 46 kDa.\(^{30}\) Overexpressed PDX1 in COS-7 cells also had a molecular mass of 46 kDa.\(^{30}\) Both phosphorylation\(^{29}\) and sumoylation\(^{30}\) have been reported to contribute to the increase in molecular mass of PDX1 from 31 to 46 kDa. However, one study using mass spectrometry analysis showed that PDX1 migrates at 45 kDa position without any post-translational modifications, while the PDX1 running at 31 kDa position is a truncated form of PDX1 missing amino acids 1–63 at its N-terminus in which transactivation domain exists.\(^{32}\) It also has a role in insulin expression and islet maintenance in the adult pancreas. PDX1 is an essential regulator of many pancreatic endocrine genes such as insulin glucokinase, islet amyloid peptide, glucose transporter type 2, pancreatic polypeptide and somatostatin and therefore has a critical role in maintaining glucose homeostasis. Previously, we have demonstrated significant knockdown of PDX1 in several tumor xenograft mouse models in response to multiple doses of IV-infused OFHIRNA-PDX1.\(^{19}\) In SCID (severe combined immunodeficiency) mice, three IV doses of human OFHIRNA-PDX1 (used against a human tumor xenograft) had no effect on islet PDX1, insulin and glucose levels in mice; however, three IV doses of mouse shRNA PDX1 nanoparticles did result in the knockdown of PDX1 and insulin expression associated with mild hyperglycemia with near doubling of normal glucose levels during treatment, which was reversible; 60 days after discontinuation of treatment, islet PDX1 and insulin, as well as glucose and insulin levels, returned to normal. No other toxicity was seen. The difference likely could be attributed to a species difference. These results verify the safety of systemically delivered OFHIRNA-PDX1 in a mouse model and support evidence of activity in relation to PDX1 knockdown.

In the pig studies, a single high-dose IV infusion of human OFHIRNA-PDX1 (0.156 mg kg\(^{-1}\), equivalent to 0.09 mg kg\(^{-1}\) in humans or 6.3 mg in a 70-kg adult human) elicited a non-specific acute, inflammatory response that is effectively palliated with ‘premedication’. This response is similar to previous findings in clinical studies using similar DNA nanoparticle formulations\(^{24,26,27}\) and viral therapeutics.\(^{28}\) A regimen of dexamethasone, diphenhydramine, Indocin, acetaminophen and ranitidine reduced the severity and duration of the inflammatory response. Interestingly, there were no significant changes in insulin or glucose levels following treatment with OFHIRNA-PDX1 insofar as disruption of PDX1 expression, even transiently, could be postulated to effect insulin expression, thereby causing perturbations in blood glucose levels. The discrepancy between current results and previous data could be partially explained as the result of consecutive multiple treatments in the mice model resulting in accumulative effect of knockdown of both of PDX1\(^{17}\) and PDX1\(^{46}\) forms in islets, whereas one treatment in the pig was mostly working on the PDX1\(^{31}\) form as discussed below. Molecular analyses of pig pancreatic tissue from normal animals treated with OFHIRNA-PDX1 demonstrated a significant, transient reduction of endogenous PDX1\(^{31}\) expression.

**DISCUSSION**

PDX1 is a transcription factor that has a critical role in regulating embryological pancreas development and is well described as a pro-cancer signal effector.\(^5,17\) It also has a role in insulin expression and islet maintenance in the adult pancreas. PDX1 is an essential regulator of many pancreatic endocrine genes such as insulin glucokinase, islet amyloid peptide, glucose transporter type 2, pancreatic polypeptide and somatostatin and therefore has a critical role in maintaining glucose homeostasis. Previously, we have demonstrated significant knockdown of PDX1 in several tumor xenograft mouse models in response to multiple doses of IV-infused OFHIRNA-PDX1.\(^{19}\) In SCID (severe combined immunodeficiency) mice, three IV doses of human OFHIRNA-PDX1 (used against a human tumor xenograft) had no effect on islet PDX1, insulin and glucose levels in mice; however, three IV doses of mouse shRNA PDX1 nanoparticles did result in the knockdown of PDX1 and insulin expression associated with mild hyperglycemia with near doubling of normal glucose levels during treatment, which was reversible; 60 days after discontinuation of treatment, islet PDX1 and insulin, as well as glucose and insulin levels, returned to normal. No other toxicity was seen. The difference likely could be attributed to a species difference. These results verify the safety of systemically delivered OFHIRNA-PDX1 in a mouse model and support evidence of activity in relation to PDX1 knockdown.

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Interestingly, our data on western blotting analysis of human pancreatic cancer cell lines and mouse insulinoma cells also exhibited different PDX1 isoform expression patterns. PDAC cells expressed predominantly PDX1\(^{31}\), whereas the mouse insulinoma cells line expressed predominantly PDX1\(^{46}\). The knockdown effect of PDX1 RNAi in PDAC cell lines was predominately on PDX1\(^{31}\), which is consistent with the effect seen in pig islets. This is the first study to demonstrate a difference in PDX1 isoform expression ratios in human pancreatic cancer cell lines versus a mouse insulinoma cell line, suggesting that there could be a differential role of the isoforms in regulating cancer versus insulin, which could influence the response to PDX1 RNAi therapy.
These encouraging results demonstrate that systemically delivered OFFHIRNA-PDX1 safely and successfully knocked down the PDX1 target in a biorelevant large animal model. It was remarkable that only PDX1 isoform was temporally knocked down in pig islets with no changes in insulin or glucose levels, which could have positive implications regarding timing of this targeted therapy for patients with pancreatic cancer to avoid potential hyperglycemic off-target effect seen in mouse models. The use of an anti-inflammatory premedication regimen results in potential hyperglycemic off-target effect seen in mouse models.

PHASE I trial initiation.

**CONFLICT OF INTEREST**

CJ, PK, DR, CE, ZW, FCB, NS, JN and PM are shareholders in Gradalis, Inc. All the other authors declare no conflict of interest.

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**REFERENCES**