npg

ORIGINAL ARTICLE

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

CM Jay[1], C Ruoff[2], P Kumar[1], H Maass[2], B Spanhel[2], M Miller[2], A Arrington[2], N Montalvo[2], V Gresham[2], DD Rao[1], C Evans[1], Z Wang[1], FC Brunicardi[3], S-H Liu[3], G Zhou[3], N Senzer[1,4], J Nemunaitis[1,4], L King[2], B Weeks[2], FJ Clubb[5], TW Fossum[2] and PB Maples[1]

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.

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).

[1]Gradalis, Dallas, TX, USA; [2]Texas A&M Institute for Preclinical Studies, College Station, TX, USA; [3]Department of Surgery, David Geffen School of Medicine at UCLA Medical Center, Los Angeles, CA, USA; [4]Mary Crowley Cancer Research Centers, Dallas, TX, USA and [5]Department of Veterinary Pathobiology, Texas A&M University, College Station, TX, USA. Correspondence: Dr J Nemunaitis, Mary Crowley Cancer Research Centers, 1700 Pacific, Suite 1100, Dallas, TX 75201, USA.
E-mail: jnemunaitis@marycrowley.org
Received 26 September 2013; accepted 17 October 2013; published online 29 November 2013

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 D5W (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 $8\,^{\circ}C$ 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 D5W and mixed spontaneously to create the final product under current good manufacturing practice conditions at Gradalis. The final concentration of plasmid DNA is $0.5\,mg\,ml^{-1}$. The product is stored at $+2$ to $8\,^{\circ}C$ 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 $50\,\mu g$ plasmid DNA in a 0.4-cm cuvette (Bio-Rad, Hercules, CA, USA) and plated in 6-well plates. Cells were harvested 24 h post electroporation by trypsin digest and pelleted 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 ($50\,\mu g$) of each 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 (ab84987, 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 were performed as described before[18] 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 $2\,\mu g$ DNA per well. Forty-eight hours after transfection, cells were harvested and lysed for western blotting. Twenty micrograms ($20\,\mu g$) 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 venous access port (VAP). Three animals received a single IV infusion (50 ml) of OFHIRNA-PDX1 ($0.156\,mg$ plasmid DNA kg^{-1}—equivalent to $0.09\,mg$ plasmid DNA kg^{-1} in human) and one animal received an equivalent dose of empty nanoparticles in 50 ml D5W over a 30-min period, while under anesthesia. The animals were observed for 14 days, collecting blood twice per day for analysis. Twenty-one days after the first IV infusion, all four animals received $0.156\,mg\,kg^{-1}$ OFHIRNA-PDX1. With this second IV infusion, three animals were premedicated with dexamethasone, diphenhydramine, Indocin, acetaminophen and ranitidine 1 h before test article delivery and medicated again every 4 h up to 20 h post-IV infusion. The fourth animal received OFHIRNA-PDX1 alone, without medication. All four animals were observed for 6 days, and blood was drawn twice daily for analysis. At the end of the study, all animals were sedated, killed for necropsy and the pancreata were collected in a sterile specimen cup containing saline and gentamicin and shipped to Gradalis on wet ice for in-house development/testing.

In a second animal study (pathology and bioactivity), six different, immature female Yucatan swine (116–137-days old) were implanted with a VAP and then administered the same premedication regimen as above. Five animals received OFHIRNA-PDX1 ($0.156\,mg$ plasmid DNA kg^{-1}) and

one animal received D5W only. Animals receiving test OFHIRNA-PDX1 were killed at days 1, 2, 3, 4 and 7 post infusion. The D5W control animal was killed at day 2. At the time of killing, necropsy was performed and organs were collected for histopathological and molecular analysis.

Analysis of animal samples

For the first pig study (analysis of acute toxicity), 3–6 ml of blood were drawn twice daily before feeding to assess insulin and glucose levels. Blood samples were also used for hematology (complete blood count, leukocyte count, erythrocyte count, hemoglobin, hemacrit, mean corpuscular hemoglobin, mean corpuscular volume, mean corpuscular hemoglobin concentration, red blood cell distribution and mean platelet volume), chemistry (albumin, alanine aminotransferase, aspartate aminotransferase, blood urea nitrogen, calcium, creatinine, electrolytes, gamma-glutamyltransferase, phosphorus, bilirubin and protein) and coagulation (activated partial thromboplastin time and prothrombin time) assays.

For the second pig study (pathology and bioactivity), tissue samples from the lung, heart, liver, kidney, spleen, quadriceps and diaphragm were collected and formalin fixed. The fixed tissues were paraffin embedded, sectioned and stained with hematoxylin and eosin (H&E) for analysis. At the time of necropsy, separate tissue samples from the head, mid-body and tail of the pancreas were fixed in 3% glutaraldehyde and prepared for transmission electron microscopy (TEM) analysis. H&E and TEM samples were analyzed by two certified pathologists in a blinded manor. Additional tissue samples from the head, mid-body and tail of the pancreas were flash frozen for molecular analysis. Samples were processed and analyzed for PDX1 protein expression via western blotting by the same procedure as listed above for in vitro testing.

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.[19] Other animal species, such as cow, mini 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 biorelevance 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 14-fold higher PDX1 at 31 kDa (PDX1[31]) as compared with that at 46 kDa (PDX1[46]). In Mia PaCa-2, PDX1[31] was fourfold higher than PDX1[46] as shown in Figure 3a. pbi-shRNA PDX1 significantly

knocked down PDX1[31] 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[46] 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 over-express 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 $55 \pm 2\%$ 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 OF HIRNA-PDX1 kg^{-1} (equivalent to 0.09 mg kg^{-1} in humans) and one animal received an equivalent dose of empty liposomes in D5W 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 $102 \pm 1\,^\circ$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.
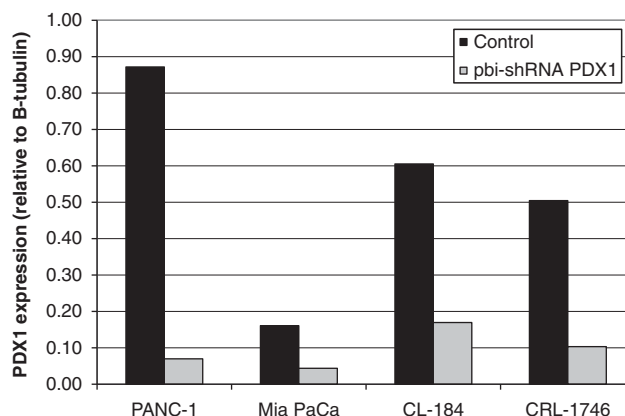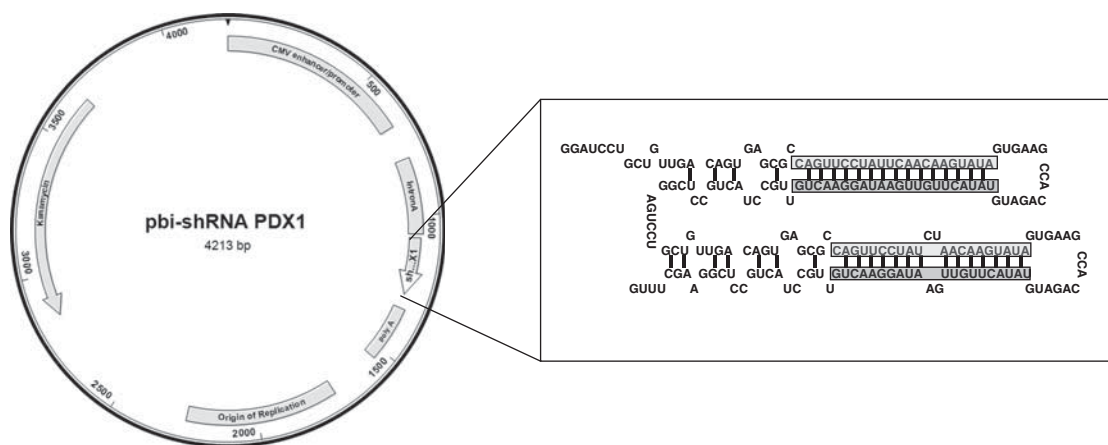
Figure 2. Endogenous expression of PDX1 (pancreatic and duodenal homeobox 1) 24 h after treatment with pbi-shRNA PDX1 plasmid. Three of the four cell lines had medium-to-high levels of endogenous PDX1 expression, and all the four cell lines demonstrated significant PDX1 reduction following treatment with pbi-shRNA PDX1 plasmid.

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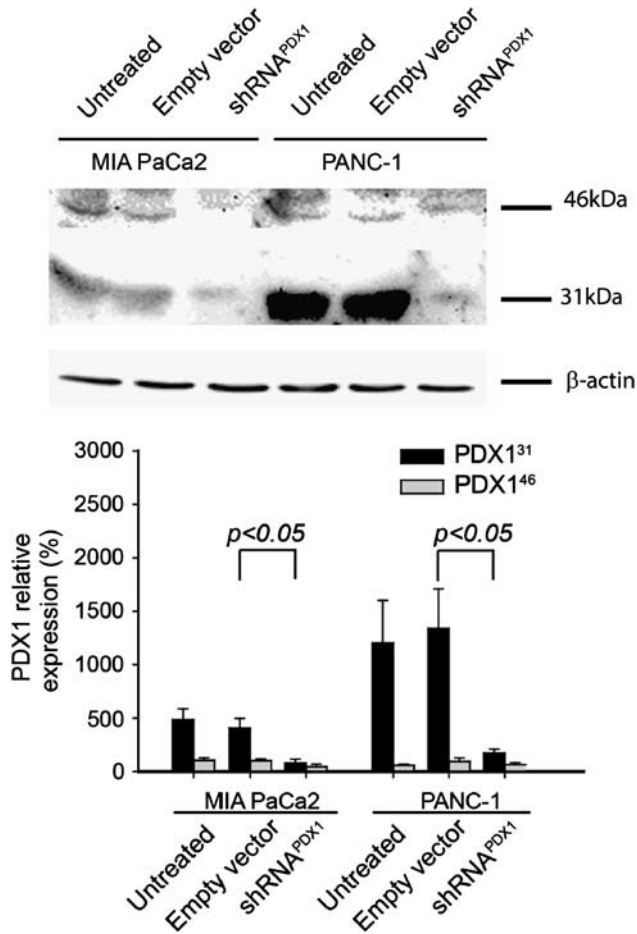
Figure 3. A pattern of endogenous expression of PDX1 (pancreatic and duodenal homeobox 1) isoforms in human pancreatic cancer cell lines. PANC-1 and Mia PaCa2 express predominantly PDX1[31] with reversed ratios of PDX1[31/46]. Forty-eight hours after treatment with pbi-shRNA PDX1, significant knockdown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

Figure 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.
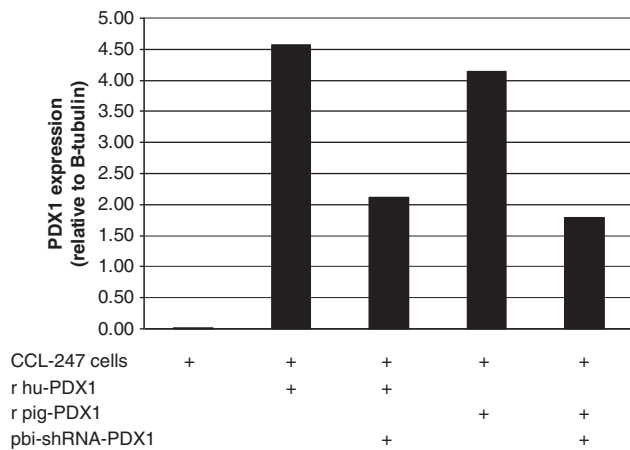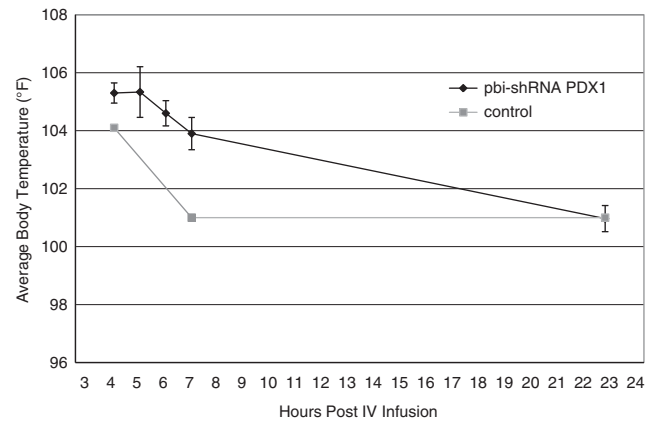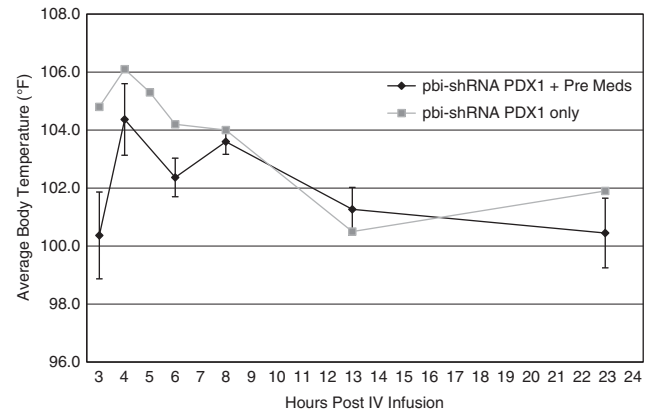

Figure 4. Recombinant PDX1 (pancreatic and duodenal homeobox 1) expression 24 h after pbi-shRNA PDX1 treatment. Recombinant PDX1 (human or pig) was expressed in CCL-247 cells. Co-transfection with pbi-shRNA PDX1 vector resulted in an average $55 \pm 2\%$ reduction of recombinant PDX1 expression.

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
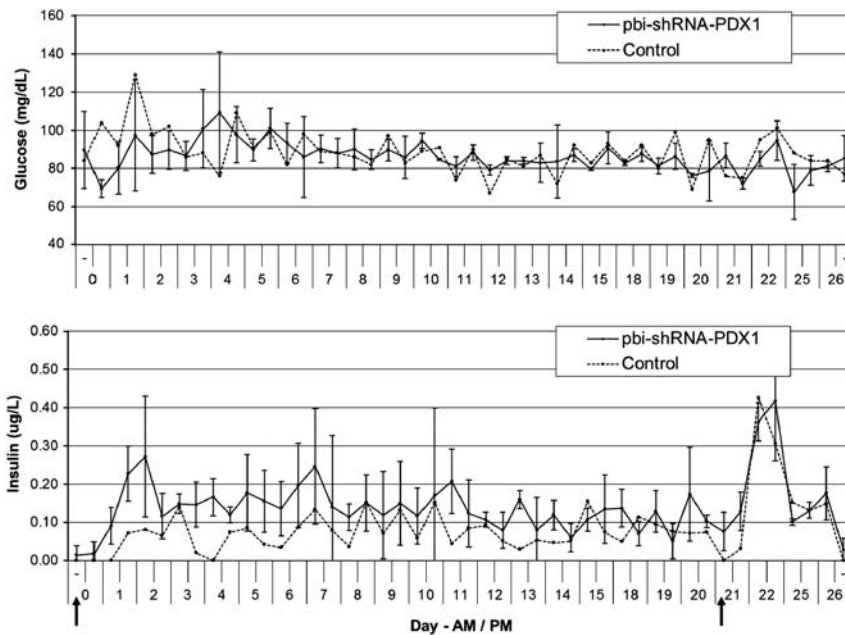
Figure 7. Average glucose and insulin levels in healthy Yucatan swine following intravenous (IV) infusion of OFHIRNA-PDX1 (pancreatic and duodenal homeobox 1). Arrows indicate the time of IV infusion. For the first infusion, three animals received 0.156 mg kg^{-1} OFHIRNA-PDX1 and the control animal received empty nanoparticles. For the second infusion, all animals received 0.156 mg kg^{-1} OFHIRNA-PDX1 plus a medication regimen, with the exception of the control animal, which did not receive medication. The reference glucose level in pigs is 47–104 mg dl^{-1}, and the reference insulin level is 0–0.28 µg l^{-1}.

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. 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 D5W 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 immunohistochemistry for the expression of insulin at days 2 and 7 after
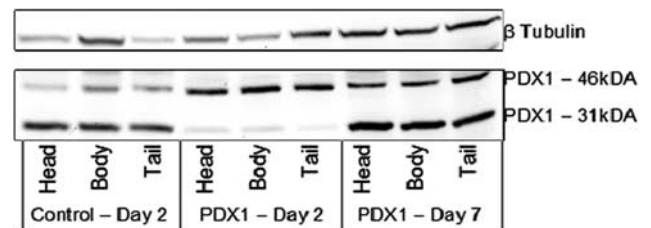

Figure 8. Western blotting of pig pancreata following treatment with OFHIRNA-PDX1 (pancreatic and duodenal homeobox 1). The 31-kDa PDX1 protein was significantly reduced at day 2 (*P* = 0.05) and returned to normal expression by day 7. The 46-kDa PDX1 protein appeared unchanged relative to control.

OFHIRNA-PDX1 treatment. The pancreas of the D5W 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 knocked down (88% average reduction for head, body and tail, *P* = 0.05) in the day-2 OFHIRNA-PDX1-treated animal relative to the D5W control and the day-7 OFHIRNA-PDX1-treated animal displayed 31-kDa PDX1 protein expression similar to the control animal. The 46-kDa PDX1 protein band was unchanged in both the treated animals, relative to control (*P* = 0.23).

To confirm the protein expression data shown in Figure 8, a second set of frozen pancreatic samples (head, mid-body and
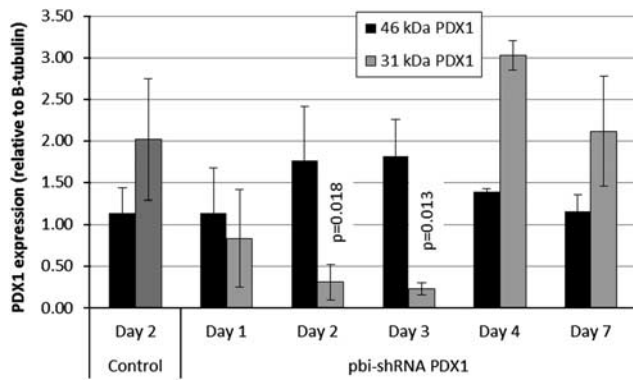
Figure 9. Average PDX1 (pancreatic and duodenal homeobox 1) protein expression (head, mid-body and tail) in pig pancreata following OFHIRNA-PDX1 treatment. The 31-kDa PDX1 protein expression is significantly reduced by day 3 (relative to control), with a recovery to normal expression by day 7. No significant change in the 46-kDa PDX1 protein was observed over the 7-day time period. P-values relative to control.

tail—at all time points) were analyzed via western blotting and quantified for the expression levels of PDX1 (Figure 9). The results demonstrated that the 31-kDa PDX1 protein expression was significantly reduced on day 2 as well as on day 3 relative to control (reduction of 85%, $P = 0.018$ and 88%, $P = 0.013$; respectively) but recovered to normal expression levels by day 7. The changes in the 31-kDa PDX1 expression at days 1 and 4 were not statistically different versus the control ($P = 0.09$ and 0.08, respectively). The expression pattern of the 46-kDa PDX1 again remained unchanged throughout the time course.

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.[6–17] It also has a role in insulin expression and islet maintenance in the adult pancreas. PDX1 is as an essential regulator of many pancreatic endocrine genes such as insulin glucokinase, islet amyloid polypeptide, 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 islet 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[31] 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 periribosomal 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 and 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] Thus, it is possible that PDX1 has two transcripts: one is for the 31-kDa form, and the other is for the 46-kDa form. In this scenario, the 46-kDa PDX1 could be full-length PDX1 or truncated PDX1 with post-translational modification(s), phosphorylation or sumoylation. We are presently investigating these two isoforms of PDX1 to better understand their expression profile and regulatory roles in both islets and pancreatic cancer.

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 predominantly 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 OFHIRNA-PDX1 safely and successfully knocked down the PDX1 target in a biorelevant large animal model. It was remarkable that only PDX1[31] 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 reasonable tolerance of single and multiple IV doses of OFHIRNA-PDX1 given at a dose equivalent maximally tolerated in previously published murine models.[33] Formal good laboratory practice toxicology and biodistribution studies involving multiple doses of OFHIRNA-PDX1 are now designed and ready for initiation as a final step towards clinical IND (Investigational New Drug) submission and Phase I trial initiation.

CONFLICT OF INTEREST

ACKNOWLEDGEMENTS

REFERENCES

1 Hariharan D, Saied A, Kocher HM. Analysis of mortality rates for pancreatic cancer across the world. *HPB (Oxford)* 2008; **10**: 58–62.
2 Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. Estimates of worldwide burden of cancer in 2008: GLOBOCAN 2008. *Int J Cancer* 2010; **127**: 2893–2917.
3 Ferlay J, Shin HR, Bray F, Forman D, Mathers C, Parkin DM. *GLOBOCAN 2008 v1.2, Cancer Incidence and Mortality Worldwide: IARC CancerBase No. 10 [Internet]*. International Agency for Research on Cancer: Lyon, France, 2010, Available from http://globocan.iarc.fr[cited May 2011].
4 American Cancer Society. *Cancer Facts and Figures 2010*. American Cancer Society: Atlanta, GA, USA, 2010.
5 Benson AB, Myerson RJ, Sasson AR. Pancreatic, neuroendocrine GI, and adrenal cancers. *Cancer Management: A Multidisciplinary Approach 13th edn*. UBM Medica LLC: New York, 2010.
6 Koizumi M, Doi R, Toyoda E, Masui T, Tulachan SS, Kawaguchi Y et al. Increased PDX-1 expression is associated with outcome in patients with pancreatic cancer. *Surgery* 2003; **134**: 260–266.
7 Liu T, Gou SM, Wang CY, Wu HS, Xiong JX, Zhou F. Pancreas duodenal homeobox-1 expression and significance in pancreatic cancer. *World J Gastroenterol* 2007; **13**: 2615–2618.
8 Wang XP, Li ZJ, Magnusson J, Brunicardi FC. Tissue microarray analyses of pancreatic duodenal homeobox-1 in human cancers. *World J Surg* 2005; **29**: 334–338.
9 Offield MF, Jetton TL, Labosky PA, Ray M, Stein RW, Magnuson MA et al. PDX-1 is required for pancreatic outgrowth and differentiation of the rostral duodenum. *Development* 1996; **122**: 983–995.
10 Hui H, Perfetti R. Pancreas duodenum homeobox-1 regulates pancreas development during embryogenesis and islet cell function in adulthood. *Eur J Endocrinol* 2002; **146**: 129–141.
11 McKinnon CM, Docherty K. Pancreatic duodenal homeobox-1, PDX-1, a major regulator of beta cell identity and function. *Diabetologia* 2001; **44**: 1203–1214.
12 Gannon M, Ables ET, Crawford L, Lowe D, Offield MF, Magnuson MA et al. pdx-1 function is specifically required in embryonic beta cells to generate appropriate

13 Li Y, Zhang R, Qiao H, Zhang H, Wang Y, Yuan H et al. Generation of insulin-producing cells from PDX-1 gene-modified human mesenchymal stem cells. *J Cell Physiol* 2007; **211**: 36–44.
14 Koizumi M, Doi R, Fujimoto K, Ito D, Toyoda E, Mori T et al. Pancreatic epithelial cells can be converted into insulin-producing cells by GLP-1 in conjunction with virus-mediated gene transfer of pdx-1. *Surgery* 2005; **138**: 125–133.
15 Wang X, Zhou J, Doyle ME, Egan JM. Glucagon-like peptide-1 causes pancreatic duodenal homeobox-1 protein translocation from the cytoplasm to the nucleus of pancreatic beta-cells by a cyclic adenosine monophosphate/protein kinase A-dependent mechanism. *Endocrinology* 2001; **142**: 1820–1827.
16 Aviv V, Meivar-Levy I, Rachmut IH, Rubinek T, Mor E, Ferber S. Exendin-4 promotes liver cell proliferation and enhances the PDX-1-induced liver to pancreas trans-differentiation process. *J Biol Chem* 2009; **284**: 33509–33520.
17 Zhou J, Pineyro MA, Wang X, Doyle ME, Egan JM. Exendin-4 differentiation of a human pancreatic duct cell line into endocrine cells: involvement of PDX-1 and HNF3beta transcription factors. *J Cell Physiol* 2002; **192**: 304–314.
18 Liu S, Ballian N, Belaguli NS, Patel S, Li M, Templeton NS et al. PDX-1 acts as a potential molecular target for treatment of human pancreatic cancer. *Pancreas* 2008; **37**: 210–220.
19 Liu SH, Rao DD, Nemunaitis J, Senzer N, Zhou G, Dawson D et al. PDX-1 is a therapeutic target for pancreatic cancer, insulinoma and islet neoplasia using a novel RNA interference platform. *PLoS One* 2012; **7**: e40452.
20 Rao DD, Maples PB, Senzer N, Kumar P, Wang Z, Pappen BO et al. Enhanced target gene knockdown by a bifunctional shRNA: a novel approach of RNA interference. *Cancer Gene Ther* 2010; **17**: 780–791.
21 Templeton NS, Lasic DD, Frederik PM, Strey HH, Roberts DD, Pavlakis GN. Improved DNA: liposome complexes for increased systemic delivery and gene expression. *Nat Biotechnol* 1997; **15**: 647–652.
22 Bode G, Clausing P, Gervais F, Loegsted J, Luft J, Nogues V et al. The utility of the minipig as an animal model in regulatory toxicology. 2010*J Pharmacol Toxicol Methods* **62**: 196–220.
23 Swindle MM, Smith AC, Laber KL, Goodrich JA, Bingel SA. *Biology and Medicine of Swine*. International Veterinary Information Service: Ithaca, NY, USA, 2003.
24 Senzer N, Nemunaitis J, Nemunaitis D, Bedell C, Edelman G, Barve M et al. Phase I study of a systemically delivered p53 nanoparticle in advanced solid tumors. *Mol Ther* 2013; **21**: 1096–1103.
25 Penninks AH, van Mierlo GJ. Immunotoxicity studies in minipigs. In: McAnulty PA, Dayan AD, Ganderup NC, Hastings KL (eds). *The Minipig in Biomedical Research*. CRC Press/Taylor and Francis: New York, NY, USA, 2012 397–411.
26 Lu C, Stewart DJ, Lee JJ, Ji L, Ramesh R, Jayachandran G et al. Phase I clinical trial of systemically administered TUSC2(FUS1)-nanoparticles mediating functional gene transfer in humans. *PLoS One* 2012; **7**: e34833.
27 Jay C, Nemunaitis G, Nemunaitis J, Senzer N, Hinderlich S, Darvish D et al. Preclinical assessment of wt GNE gene plasmid for management of hereditary inclusion body myopathy 2 (HIBM2). *Gene Regul Syst Bio* 2008; **2**: 243–252.
28 Eager RM, Nemunaitis J. Clinical development directions in oncolytic viral therapy. *Cancer Gene Ther* 2011; **18**: 305–317.
29 Macfarlane WM, McKinnon CM, Felton-Edkins ZA, Cragg H, James RF, Docherty K. Glucose stimulates translocation of the homeodomain transcription factor PDX1 from the cytoplasm to the nucleus in pancreatic beta-cells. *J Biol Chem* 1999; **274**: 1011–1016.
30 Kishi A, Nakamura T, Nishio Y, Maegawa H, Kashiwagi A. Sumoylation of Pdx1 is associated with its nuclear localization and insulin gene activation. *Am J Physiol Endocrinol Metab* 2003; **284**: E830–E840.
31 Berezhna SY, Supekova L, Supek F, Schultz PG, Deniz AA. siRNA in human cells selectively localizes to target RNA sites. *Proc Natl Acad Sci USA* 2006; **103**: 7682–7687.
32 Carlotti F, Zaldumbide A, Charif H, de Koning EJ, Luider TM, Hoeben RC. The 45-kDa form of Pdx-1 does not result from post-translational modifications. *Biochem Biophys Res Commun* 2008; **370**: 225–229.
33 Liu SH, Patel S, Gingras MC, Nemunaitis J, Zhou G, Chen C et al. PDX-1: demonstration of oncogenic properties in pancreatic cancer. *Cancer* 2011; **117**: 723–733.