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REVIEW

Vertically integrated translational studies of PDX1 as a therapeutic target for pancreatic cancer via a novel bifunctional RNAi platform

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RNA interference (RNAi) represents a powerful, new tool for scientific investigation as well as a promising new form of targeted gene therapy, with applications currently in clinical trials. Bifunctional short hairpin RNA (shRNA) are synthetic RNAi molecules, engineered to utilize multiple endogenous RNAi pathways to specifically silence target genes. Pancreatic and duodenal homeobox 1 (PDX1) is a key regulator of pancreatic development, β-cell differentiation, normal β-cell function and pancreatic cancer. Our aim is to review the process of identifying PDX1 as a specific, potential RNAi target in pancreatic cancer, as well as the underlying mechanisms and various forms of RNAi, with subsequent testing and development of PDX1-targeted bifunctional shRNA therapy.

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INTRODUCTION

Pancreatic cancer is well known for its dismal prognosis and resistance to conventional cancer therapies. In 2013, 45 220 patients were diagnosed with pancreatic cancer, and 1 in 67 patients will develop pancreatic cancer in their lifetime.[1] In spite of medical intervention, the current 5-year survival rate remains at a dismal 6%.[1] Thus, development of novel therapies is urgently needed and much attention is being paid to targeted therapies. In hopes of identifying therapeutic targets for pancreatic cancer, recent research has focused on the molecular biology and genetics underpinning pancreatic cancer development. To date, genes implicated in the malignant transformation of pancreatic cancer include Kras, p53, p16, DPC4, BRCA-2 and the genes underlying FAMM (familial atypical multiple mole melanoma), Peutz–Jeghers and HNPCC (hereditary nonpolyposis colorectal cancer) syndromes; however, there are no effective therapies developed to these targets.[2,3] Our previous studies demonstrate that pancreatic duodenal homeobox transcription factor 1 (PDX1) is overexpressed in pancreatic cancer, regulates pancreatic cancer cell lines and is a potential therapeutic target for pancreatic cancer.[4–6]

RNA interference (RNAi) is a promising new form of targeted gene therapy, with applications currently in clinical trials. RNAi has recently been uncovered as a naturally occurring regulatory mechanism in mammals.[7] Endogenous small interfering RNA (siRNA) generated from double-stranded RNA molecules can bind target RNA sequences and interfere with their translation or target them for destruction. By designing synthetic siRNA and taking advantage of existing cellular machinery, researchers are now able to use the same mechanism to target disease-specific genes. This article reviews the identification of PDX1 as a potential molecular target for pancreatic cancer and development of a novel form of RNAi therapy, bifunctional short hairpin RNA targeting PDX1 (bi-shRNA^{PDX1}).

PDX1 REGULATES PANCREATIC DEVELOPMENT, FUNCTION, TRANSDIFFERENTIATION AND REGENERATION

PDX1 is a vital transcription factor involved in pancreatic development, glucose metabolism as well as a number of adult pancreatic functions. PDX1 drives embryonic pancreatic development and PDX1-expressing epithelial cells from the foregut develop into pancreatic buds that later give rise to the entire pancreas.[8] Homozygous mutation of PDX1 is lethal in mice because of pancreatic agenesis, emphasizing the essential role of PDX1 expression.[9,10] In the adult pancreas, PDX1 regulates insulin secretion by binding and stimulating the insulin promoter in β-cells. Defects in PDX1 impair insulin secretion and have a diabetogenic effect. Reversible repression of PDX1 expression in adult mice leads to diabetes within 14 days.[11] In humans, heterozygous mutations of PDX1 cause mature onset diabetes of the young (MODY type IV),[10] and PDX1 missense and insertional mutations are associated with type II diabetes mellitus.[12–14]

In the adult pancreas, PDX1 expression is also associated with pancreatic regeneration and response to injury.[15,16] Elevated PDX1 expression has been noted following pancreatitis, pancreatic resection and hypoglycemic challenge. Following pancreatic injury in mice, PDX1 expands more diffusely in pancreatic ductal epithelium.[17] This expansion of PDX1 likely marks proliferation of immature progenitor cells. Recent studies suggest that immature pancreatic progenitor cells that express developmental genes and have increased proliferation express PDX1. Pancreatic duct glands, identified as possible foci of regeneration, contain cells that express SHH and PDX1.[17] Further supporting PDX1 as a marker of pancreatic progenitor cells, studies of β-cell maturation found two distinct populations of PDX1-expressing cells: those with low to no insulin expression, and those with insulin expression.[18] The PDX1-positive, insulin-negative cells had a greater rate of replication, consistent with

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progenitor cells.[18] Patients with impaired glucose tolerance or newly diagnosed type II diabetes have increased population of dual insulin and glucagon/somatostatin-expressing cells positive for PDX1, suggesting that β-cell neogenesis is regulated by PDX1 as a compensatory mechanism for type II diabetes.[19]

PDX1 expression likely promotes the regenerative process, and can also induce transdifferentiation of mature pancreatic cells. PDX1 overexpression restored euglycemia in mice with streptozosin-induced diabetes.[20] In mature pancreatic cells, PDX1 can also induce transdifferentiation of mature pancreatic cells. When overexpressed in glucagon secreting α-cells, PDX1 induces expression of insulin, glucokinase and islet amyloid polypeptide.[21,22] Also, pancreatic multipotent progenitor cells become insulin-secreting cells with PDX1 and GLP-1 stimulation.[23] These studies demonstrate the plasticity of certain populations of pancreatic cells, and the ability of PDX1 to influence their differentiation.

PDX1 IS OVEREXPRESSED IN PANCREATIC CANCER

PDX1 gained our attention as a regulator of oncogenesis during an investigation of insulinoma gene therapy using a recombinant gene composed of a 508-bp rat insulin promoter fragment(RIP) driving herpes simplex virus thymidine kinase (HSV-TK), followed by ganciclovir (RIP-TK/GCV). Systemic RIP-TK/GCV therapy successfully prevented insulinoma-induced hypoglycemic death in mice.[24] In this study it was determined that PDX1 was the key activator of RIP and mutation of the PDX1-binding site within RIP abrogated the therapeutic effect. We were surprised to find that RIP-lacZ was activated in PANC-1 cells and determined that PANC-1 overexpressed PDX1 as the activator of RIP in the human pancreas cancer cell lines. We subsequently demonstrated that systemic RIP-TK/GCV therapy significantly reduced PDX1-expressing pancreatic cancer tumor volume in SCID (severe combined immunodeficient) mice. These studies spurred the investigation into PDX1 and its possible role in regulating pancreatic cancer and as a therapeutic target.

In the first set of studies, we observed overexpression of PDX1 in insulinoma, neuroendocrine and human pancreatic cancer specimens by immunohistochemistry and western blot analysis with our PDX1 antibody.[5] Further studies using tissue microarrays demonstrated PDX1 overexpression in adult human malignancies originating from the pancreas, breast, colon, prostate, kidney, liver, lung and ovary.[25] Other laboratories demonstrated that PDX1 expression in human pancreatic cancer samples correlated to histologic grade, presence of lymph node metastases, tumor node metastasis (TNM) stage and overall survival.[6,26,27] Although Kras mutations are present in 90% of pancreatic cancers,[3,28,29] transgenic mice harboring the Kras-activating mutation only developed pancreatic cancer when the mutation was targeted to PDX1-expressing cells, emphasizing the importance of PDX1 expression in pancreatic cancer.[30] Together, these studies demonstrate a clear association between PDX1 overexpression and pancreatic cancer.

DEMONSTRATION OF ONCOGENESIS OF PDX1

We began investigating transient and stable expression of PDX1 in benign and human pancreatic cell lines. Human embryonic kidney cell lines (HEK), benign human pancreatic ductal epithelium (HPDE) and human pancreatic cancer cell lines, MIA PaCa2 and PANC-1 cell lines, expressing low and high levels of PDX1, respectively, were selected for these studies. Transient expression of PDX1 was achieved with PDX1 plasmid vector using lipofectamine, whereas PDX1 stably transfected HEK, HPDE and MIA PaCa2 cell lines were created using PDX1 retroviral infection.[4] Successful transfections were verified by western blot and immunostaining with our PDX1 antibody.[4]

Cell line proliferation, *in vitro* invasiveness and anchorage-independent cell growth were assayed and evaluated for tumorigenicity following implantation in SCID mice. Cell proliferation and *in vitro* invasive capability increased significantly with transient and stable PDX1 overexpression in HEK, HDPE and MIA PaCa2 cell lines.[4] With respect to anchorage-independent cell growth, all three cell lines overexpressing PDX1 formed larger and more numerous colonies.[4] Most importantly, PDX1-overexpressing HEK cells developed into large tumors whereas empty-vector HEK 293 cells did not form tumors *in vivo*. Similarly, PDX1-overexpressing MIA PaCa2 cells developed significantly larger tumors compared with empty-vector MIA PaCa2 cells. These observations support the oncogenic properties of PDX1 both *in vitro* and *in vivo*, promoting proliferation, invasion and tumor growth, and could therefore represent a therapeutic target for pancreatic cancer.

RNAi therapy

To antagonize PDX1, a known transcription factor, we selected RNAi as the potential therapeutic platform. RNAi is a naturally occurring mechanism of gene silencing that comes in the form of microRNAs and siRNAs.

MicroRNAs are a natural endogenous form of RNAi, encoded in the genome. An estimated 1% of cellular genes encode microRNAs: RNA molecules that undergo post-transcriptional processing into short double-stranded RNA segments by the microprocessor complex in the nucleus and the dicer in the cytoplasm.[31] The double-stranded sequences are unwound into a passenger strand and effector (guide) strand. The passenger strand is degraded, leaving an effector strand that has an imperfect complementary sequence to their target mRNA. These are incorporated into a RNA-induced silencing complex that bind the 3′ untranslated region of target mRNA, subsequently inhibiting their translation.[32] Several mismatches between the microRNA and target mRNA also lead to decreased specificity. Thus, one microRNA can target several different mRNA sequences.

The siRNAs are short, double-stranded RNA oligonucleotides produced by intracellular processing of exogenous or endogenous double-stranded RNA by dicer, the same mechanism as microRNA. Then the strands are uncoupled, passenger strand is degraded and the effector strand is incorporated into the RNA-induced silencing complex. Unlike microRNAs, siRNAs are perfectly complementary and specific to their target mRNA. Unlike microRNAs, siRNAs bind the open reading frame of the target mRNA and lead to cleavage (Figure 1).[33]

Several forms of exogenous RNAi delivery have been described. The most basic delivery method is naked synthetic siRNA delivered directly by intravenous injection that can be useful for easily injectable target organs. However, the effectiveness of intravenous delivery is limited by degradation and quick clearance of the RNA. Viral vectors or DNA plasmids are also potentially effective means of delivering therapy to target cells but also have limits with repeat systemic dosing related to induction of neutralizing antibodies.

To overcome these limits in delivery of siRNA, studies have described chemical modification or packaging of siRNA into liposomes. Systemically administered siRNA liposomes typically accumulate in hepatocytes and the spleen,[34] and other target organs receive a significantly reduced amount. Thus, applications of liposomal siRNAs are mostly limited to the liver, organs that can be directly injected, or lungs via inhalation. There is additional evidence that siRNAs can be passed cell to cell in the liver partially via exosomes.[35] Alternatively, chemical modifications can stabilize or protect siRNAs. For example, attaching siRNAs to polyethylenimines tightly packages siRNAs until phagocytosed, and are released intracellularly following endosomal disruption.[32]

Several applications of RNA therapy have been described in humans that evolved from proof-of-concept studies to phase II

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clinical trials (Table 1). PANC-1 and MiaPaCa2 human pancreatic cell lines were treated successfully with Kras-targeting shRNA.[36–38] Recently, phase I clinical trials with furin convertase bi-shRNA[39] and protein kinase N3 (PKN3) liposomal siRNA[40] have been published. Patients experienced 'possibly related' adverse effects of abdominal pain and neutropenia; however, there were no treatment-related serious adverse events.[39] PKN3 liposomal siRNA was associated with fatigue, hair loss, abdominal pain, sweating, elevated lipase but no cytokine activation.[40] These studies demonstrate the feasibility of RNAi therapy and its potential as a novel therapy for myriad diseases.

Bifunctional shRNA platform was developed by Gradalis (Carrollton, TX, USA) based upon a miR-30 scaffold.[41] MiR-30 is a commonly employed precursor that is processed by Drosha and Dicer to mature microRNA.[42] The bifunctional design incorporates two structures: one with perfectly complementary RNA strands, and a second with purposefully mismatched passenger strand. Both structures are composed with guide strand perfectly matching the targeting sequence. The two structures mimic the effects of both siRNA and miRNA, causing cleavage of the target mRNA as well as inhibition of translation, respectively. Furthermore, the vector-based expression system allows expression of multiple shRNAs in a single vector. When tested against stathmin1 (STMN1), the bifunctional construct reduced protein levels more so than either cleavage-dependent or cleavage-independent sequences alone.[41] When compared with the equivalent siRNA, bifunctional shRNA resulted in significantly reduced tumor survival. Based upon our initial studies using shRNA PDX1 in pancreas cancer mouse models, we chose to pursue a bi-shRNA platform targeting PDX1.

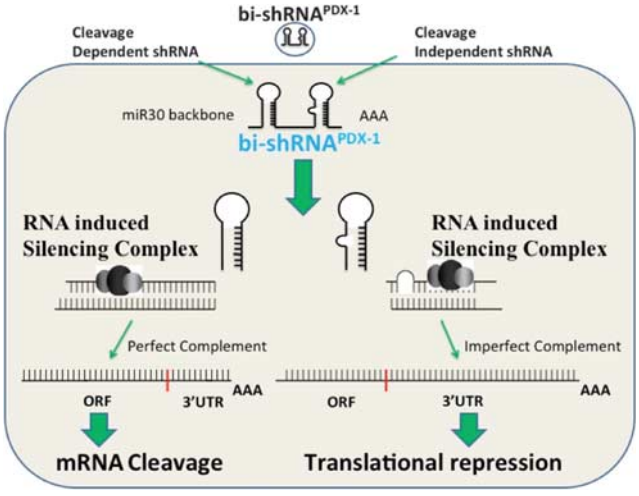

Figure 1. Mechanism of bifunctional short hairpin RNA (shRNA). ORF, open reading frame; UTR, untranslated region.

Development of PDX1 bi-shRNA

The targeting sequence for the initial shRNA expression vectors were designed using Dharmacon siDESIGN Center (www.thermoscientificbio.com/dharmacon) with the murine and human PDX1 gene sequences accessed through GenBank (murine PDX1 gene 'GenBank no. NM_008814' and human PDX1 gene 'GenBank no. NM_000209'). The top four sequences were selected and cloned into a pSUPER vector. The sequence with the greatest knockdown efficiency based upon western blot quantitation of PDX1 protein levels was selected for further studies.[43]

Bi-shRNA^{PDX1} was designed in the same manner as bi-STMN1-shRNA described by Rao *et al.*[41] Bi-shRNA^{PDX1} successfully treated cell lines transfected with PDX1, as well as PDX1-expressing human pancreatic cancer cell lines. The effects of PDX1 over-expression in cell lines, including increased cell proliferation and invasion, were reversed with the administration of bi-shRNA^{PDX1}.[4] This affirmed that oncogenic properties were attributable specifically to PDX1, and that shRNA therapy could effectively silence PDX1 and inhibit oncogenesis in human pancreatic cancer cell lines *in vitro*.

Bi-shRNA^{PDX1} successfully treats pancreatic cancer cell lines and ameliorates insulinoma *in vivo* in mice and effectively knocks down PDX1 in pigs

Bi-shRNA^{PDX1} also successfully treated PDX1-expressing human pancreatic cancer tumors *in vivo* in a xenograft mouse model. Intraperitoneal tumors created by injection of PANC-1 cells into SCID mice formed large tumors in 2 months. Three doses of biweekly intravenous human bi-shRNA^{PDX1} significantly reduced tumor volume in all mice with negligible residual tumor and significantly prolonged survival[4] (Figure 2). Analysis of residual tumors following treatment demonstrated marked apoptosis without PDX1 expression, decreased PCNA and Cyclin E expression and increased expression of p53 compared with untreated

Table 1. Summary of clinical trials using RNAi therapy in humans

Therapy	*Author*	*Disease*	*Target gene(s)*	*Delivery*
siRNA	Coelho *et al.*[48]	Transthyretin amyloidosis	ALN-TTR01, ALN-TTR02	Nanoparticles
siRNA	Tabernero *et al.*[49]	Liver metathesis in endometrial cancer	VEGF, KSP	Nanoparticles
siRNA	Kaiser *et al.*[50]	Choroidal neovascularization, age-related macular degeneration	VEGFR-1	Single dose, intravitreal injection
siRNA	Nguyen *et al.*[51]	Choroidal neovascularization, age-related macular degeneration	RTP801/REDD1	Intravitreal injection
siRNA	Nguyen *et al.*[52]	Choroidal neovascularization, age-related macular degeneration	RTP801/REDD1	Intravitreal injection
siRNA	DeVincenzo *et al.*[53]	Respiratory syncytial virus	RSV nucleocapsid protein	Nasal spray
siRNA	DeVincenzo[54]	Respiratory syncytial virus	RSV nucleocapsid protein	Nasal spray
siRNA	Zamora *et al.*[55]	RSV after lung transplant	RSV nucleocapsid protein	Aerosolized
Plasmid containing shRNA	Gish *et al.*[56]	HBV	HBV polymerase, surface antigen genes	Plasmid
Plasmid containing bi-shRNA	Senzer *et al.*[39]	Cancer	Furin convertase	Intradermal injection, once monthly $\times$ 12 months
dsRNA	Wyszko *et al.*[57]	Glioblastoma multiforme	Tenascin-C	Injected into brain cavity following resection

Abbreviations: bi-shRNA, bifunctional-short hairpin RNA; dsRNA, double-stranded RNA; HBV, hepatitis B virus; RNAi, RNA interference; RSV, respiratory syncytial virus; shRNA, short hairpin RNA; siRNA, small interfering RNA.

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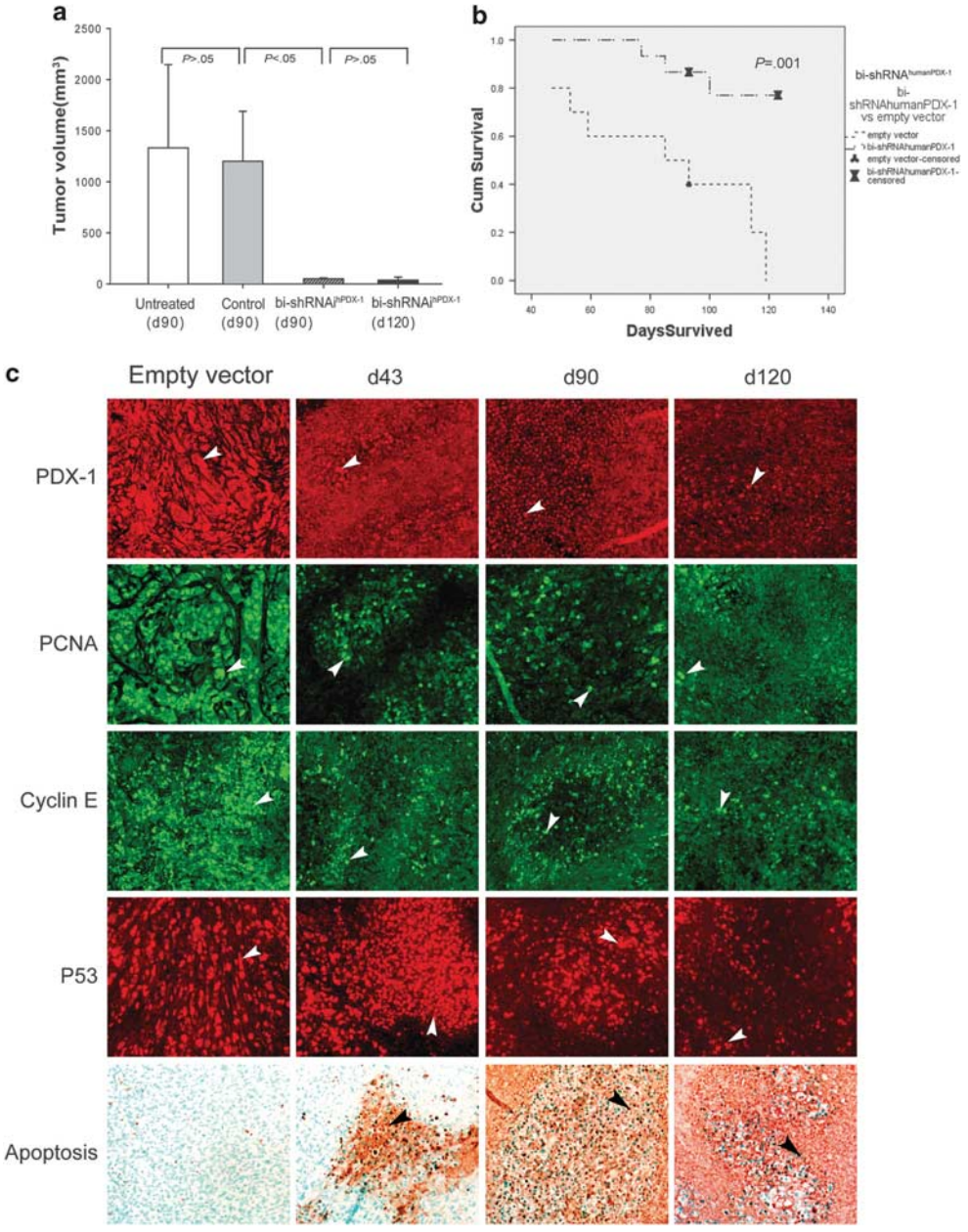

Figure 2. Bi-shRNA^{PDX1} effects in xenograft PANC-1 tumors in severe combined immunodeficient (SCID) mice.[5] PANC-1 tumor volume at 90 and 120 days following short hairpin RNA (shRNA) therapy (**a**). The survival rates of mice receiving treatment and of those receiving the empty vector control were analyzed using Kaplan–Meier in SPSS (IBM, New York, NY, USA) (**b**). Immunostaining for tumor slides with PDX1, PCNA, Cyclin E and P53 as well as TUNEL assay was performed and analyzed (**c**). The positive stain for each marker is indicated by arrow ($\times$ 200). Bi-shRNA^{PDX1}, bifunctional shRNA targeting pancreatic and duodenal homeobox 1; PCNA, proliferating cell nuclear antigen; PDX1, pancreatic and duodenal homeobox 1; TUNEL, terminal deoxynucleotidyl transferase dUTP nick end labeling.

tumors.[5] Interestingly, there were no alterations in glucose and insulin levels, or suppression of PDX1 and insulin expression in the mouse islet, that was attributed to the species specificity of human bi-shRNA^{PDX1}. In a separate series of studies on mouse insulinoma cell lines and a xenograft mouse model of insulinoma, bi-shRNA^{PDX1} therapy was also able to inhibit glucose-mediated insulin expression in insulinoma cells as well as inhibit cell proliferation *in vitro* and prevent death from lethal hypoglycemia *in vivo*. Both these studies demonstrate the ability to treat human pancreatic cancer and insulinoma in mice with bi-shRNA^{PDX1} without overt toxicity.

For a second biorelevant animal model, we chose to use miniature Yucatan pigs and performed a preliminary study to investigate the effectiveness and toxicity of intravenous bi-shRNA^{PDX1}. A single dose of human bi-shRNA^{PDX1} was able to effectively reduce pancreatic levels of PDX1, quantified by western blot of the pig pancreata.[44] Moreover, intravenous bi-shRNA^{PDX1} was well tolerated with premedication and did not affect serum glucose levels (Figure 3).[44] These preliminary results demonstrated that systemic delivery of bi-shRNA^{PDX1} effectively suppressed the target and was well tolerated in a large animal model.

Off-target and adverse effects of RNAi therapy

The main concerns regarding RNAi therapy are hepatotoxicity, inflammatory response due to activation of interferon cascade and

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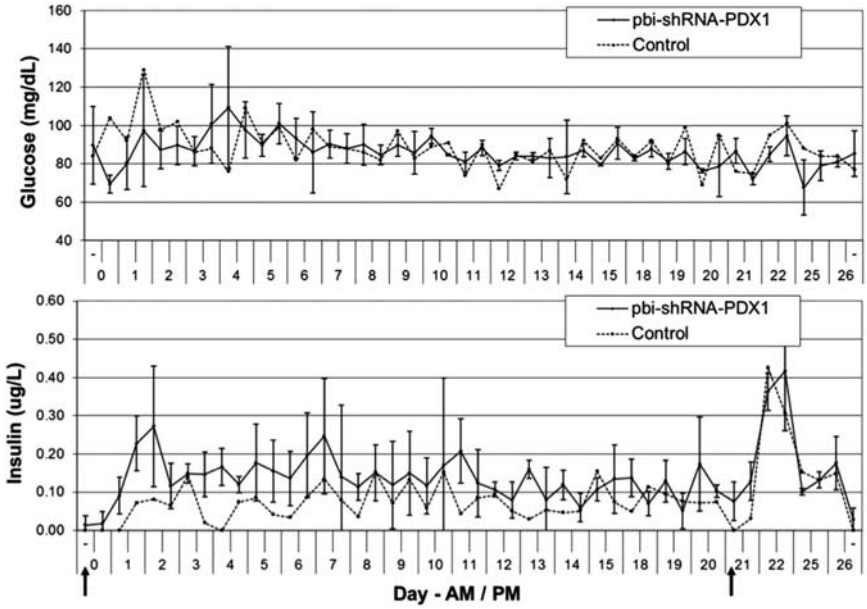

Figure 3. Insulin and glucose levels in yucatan pigs after single bi-shRNA^{PDX1} infusion. Bi-shRNA^{PDX1}, bifunctional short hairpin RNA targeting pancreatic and duodenal homeobox 1.[44]

off-target effects. There has been evidence that shRNA therapy can be fatal to mice, thought to be because of oversaturation of host RNA processing mechanisms.[45] This observed toxicity was resolved by using pol II promoter instead of the pol III promoter that overwhelms the endogenous system.[46] Numerous shRNA-based study mice have been able to tolerate varieties of shRNA expression vectors as well as bi-shRNA^{PDX1} without lethality. With regard to off-target effects, one study demonstrated that a single siRNA caused the expression changes in a significant number of genes.[47] However, the overall and cumulative effect on the organism as a whole still needs to be further studied.

Specific to mouse bi-shRNA^{PDX1}, mice experienced transient hyperglycemia without other signs of toxicity. SCID mice with implanted mouse insulinoma cells routinely experience lethal hypoglycemia and expire at a mean of 60 days. These mice treated with three biweekly treatments of murine bi-shRNA^{PDX1} had significantly prolonged survival. At day 30 following treatment, the mice experienced transient hyperglycemia and hypoinsulinemia associated with suppression of PDX1 and insulin expression within the islets of Langerhans. Circulating insulin and glucose levels, as well as PDX1 and insulin expression in islets, returned to baseline 3 months following therapy, suggesting a regenerative capacity of the murine islet.[5]

Preliminary studies of a single intravenous infusion of human bi-shRNA^{PDX1} in miniature Yucatan pigs demonstrated treatment effects of fever, lethargy and loss of appetite; however, these effects were ameliorated with premedication. Remarkably, there were no discernable effects on serum glucose or insulin levels; however, further biodistribution and toxicity studies using multiple doses of human bi-shRNA^{PDX1} are planned.

FUTURE PERSPECTIVES

The next steps for bi-shRNA^{PDX1} therapy are to complete testing in pigs, a large biorelevant animal model. The goals are to optimize dosing to minimize toxicity while assuring therapeutic effect, as well as to conduct a full investigation of adverse effects. The ultimate aim is to proceed to human clinical trials.

Bi-functional shRNA is a powerful tool for genomic investigation as well as a potential novel therapy for diseases with a discrete genetic abnormality. Future challenges include optimization of delivery to target tissues and determining extent of short-term and long-term off-target effects of shRNA therapy.

CONFLICT OF INTEREST

D Rao, CM Jay, P Kumar, N Senzer and N Templeton are employed by Gradalis. N Senzer, FC Brunicardi, D Rao and J Nemunaitis are shareholders in Gradalis.

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