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Down-regulation of expression of rat pyruvate dehydrogenase E1 α gene by self-complementary adeno-associated virus-mediated small interfering RNA delivery

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Abstract

Mutations in the E1 α subunit gene (PDHA1) of the pyruvate dehydrogenase complex (PDC) are common causes of congenital lactic acidosis. An animal model of E1 α deficiency could provide insight into the pathological consequences of mutations and serve to test potential therapies. Small interfering RNAs (siRNAs) were designed to cleave the messenger RNA (mRNA) of the E1 α subunit and were tested in vitro to assess the feasibility of producing a gene knockdown in rats. HEK 293 cells were co-transfected with a rat PDHA1 expression vector and eight naked siRNAs that specifically targeted rat E1 α mRNA. Quantitative PCR (qPCR) analyses showed that four siRNAs reduced rat PDHA1 RNA levels up to 85% by 24 hours and up to 65% by 56 hours, compared to negative and positive controls. Since oligonucleotide-mediated siRNA delivery provided only transient suppression, we next selected two siRNA candidates and generated self-complementary, double-stranded adeno-associated virus (scAAV) vectors (serotypes 2 and 5) expressing a rat short hairpin siRNA expression cassette (scAAVsi-PDHA1). Rat lung fibroblast (RLF) cultures were infected with scAAVsi-PDHA1 vectors. The RLF PDHA1 mRNA level was reduced 53–80% 72 hours after infection and 54–70% 10 days after infection in RLF cultures. The expression of E1 α and the specific activity of pyruvate dehydrogenase were also decreased at 10 days after infection in RLF cultures. Thus, scAAV siRNA-mediated knockdown of PDHA1 gene expression provides a strategy that may be applied to create a useful animal model of PDC deficiency.

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Keywords

AAV; Gene therapy; RNAi; Pyruvate dehydrogenase deficiency

1. Introduction

Genetic defects of pyruvate dehydrogenase (PDH) complex (PDC) are among the most commonly identified cause of congenital lactic acidosis in humans (Stacpoole and Gilbert., 2006;Cameron et al., 2004;Patel et al., 1995). Most cases of PDH deficiency are caused by mutation of the gene encoding the E1 α subunit on the X chromosome (Brivet et al., 2005;Brown et al., 2004;Lissens et al., 2000). Cardinal characteristics of PDH E1 α deficiency are typically expressed more severely in males and include failure to thrive, progressive neurological and neuromuscular degeneration, structural lesions in the brain, lactic acidosis and early death, usually from multi-organ system failure and/or the underlying acid-base disorder (Stacpoole et al., 2006;Robinson, 2001). There is no proven therapy for the vast majority of PDH E1 α cases and there is no animal model of this condition that is reflected in both viable male and female offspring (Pliss et al., 2004).

To begin to address this problem, we investigated the usefulness of an RNA interference (RNAi) experimental approach in cultured mammalian cells, using small, interfering RNA (siRNA) molecules targeted to the rat PDHA1 gene and self-complementary, double-stranded adeno-associated virus (scAAV) vectors for the delivery of siRNA into cells (Hajitou et al., 2006;Zhong et al., 2004;Wang et al., 2003;McCarty et al., 2003). Recombinant AAV vectors have recently attracted increased attention for the purpose of gene therapy (Asokan et al., 2006;Flotte et al., 2005;Grieger et al., 2005;Srivastava et al., 2005). Studies have shown that AAV vectors based on serotype 2 (AAV2) can introduce a broad range of cell types, including non-dividing cells, *in vitro* and *in vivo*. Additional serotypes, such as AAV5, allow for increased spread of the recombinant vector in the brain (Warrington et al., 2006;Shevtsova et al., 2005;Burger et al., 2004;Peden et al., 2004).

Here, we determined the effect of siRNA targeted to the rat PDHA1 gene, using synthetic siRNA molecules and scAAV vectors for the delivery of siRNA into mammalian cells.

2. Materials and Methods

2.1. Plasmid construction

2.1.1. Rat PDHA1 expression plasmid—A fresh rat liver was obtained and total cellular RNA was isolated by RiboPure kit (Ambion, Austin, Texas). This was followed by reverse transcription, using Omniscript RT kit (Qiagen, Valencia, CA), according to the manufacturer's instructions. A cDNA segment over covering the PDHA1 sequence was amplified, using Pfx DNA polymerase (Invitrogen, Inc.). The PCR product was cloned initially into pCR2.1-TOPO (Invitrogen, Inc.) and then into the mammalian expression vector, pExchange-1 (Stratagene, Inc), at the Not I and BamH I sites to obtain the plasmid, pE-rPDHA1. The sequence was confirmed with the NCBI Accession #: XM_343787.

2.1.2. scAAV based plasmid encoding siRNA—Eight siRNAs that targeted sequences within the rat PDHA1 mRNA were chosen from the PDHA1 genome (Accession #: XM_343787) (data not shown). The oligonucleotide siRNAs were tested by co-transfection human embryonic kidney (HEK) 293 cells with pE-rPDHA1 prior to clone into the H1 polymerase III promoter containing plasmid. Two candidates, si3 and si7, were selected and the short hairpin siRNAs were further cloned into pSilencer3.0-H1 (Ambion, Inc). siRNA target sites were converted into oligonucleotide sequences, according to instructions supplied

with the pSilencer™ kit (Ambion Inc., Austin, TX, USA). The hairpin siRNA vector features a ttaagaga loop situated between the sense and reverse complementary targeting sequences and a TTTT terminator at the 3' primer end. Target and complementary oligonucleotides were heated to 90°C for 3 min, then incubated for 1 hr at 37°C. Duplex DNA was directionally cloned into the pSilencer3.0-H1 driven expression vector (Ambion Inc., Austin, TX, USA). The H1 promoter and RNAi constructs were then introduced into the KpnI site of the scAAV based vector, scAAV-CB-eGFP, to generate a novel plasmid pH1-si-rPDHA1/AAV-eGFP. As shown in Fig. 1, this vector expresses enhanced green fluorescent protein (EGFP) under the control of CB promoter and a hairpin siRNA against rat PDHA1, which is under the control of H1 RNA promoter. The plasmids encoding siRNA targeted cassette were confirmed by sequencing prior to transfection.

2.2. Small interfering RNA and transfection reagents

One day before transfection, 3×10^5 per well of HEK 293 cells were plated on six well plates in complete medium without antibiotics until they reached ~90% confluency. HEK 293 cells were co-transfected with siRNA duplex (final siRNA concentration, 100nM) and 4 µg pE-rPDHA1, using Lipofectamine 2000 (Invitrogen, Inc.), in a final volume of 2 ml Opti-MEM. Transfection medium was replaced by complete medium after 24 hours, and the cells were harvested at variable times for assay. Transfection efficiency was monitored by viewing GFP fluorescence using an inverted fluorescence microscope (Leica DM IRB, Germany). siCONTROL Non-Targeting siRNA#1 (D-001210-01-05, Dharmacon, Inc) was used as a negative control and siCONTROL Cyclophilin B siRNA (D-001136-01-05, Dharmacon, Inc) was used in each experiment as a positive silencing control to ascertain the transfection efficiency.

2.3. Packaging of scAAV encoding siRNA

We used the AAV Helper-Free System from Stratagen (La Jolla, CA) to express the siRNAs using scAAV. Two plasmids, pscAAV2 and pscAAV5, were used to produce the AAV control virus vector (scAAV-CB-eGFP) that also expressed eGFP driven by the CB promoter. HEK 293 cells were cultured with DMEM plus 10% FBS and cotransfected with the pH1-si-rPDHA1/AAV-eGFP along with pAAV-RC (rep and cap genes) and pHelper (adenoviral genes) plasmids by standard calcium phosphate transfection. Cells were harvested 72 hr after transfection, lysed by 2 cycles of freezing and thawing to release the virus and purified, following previously published methods (Zhao et al., 2006). AAV titers were $\sim 1 \times 10^{12}$ vector particles/ml, as determined by DNA slot blot technique.

2.4. Real time quantitative PCR (qPCR)

qPCRs were conducted in a total volume of 20 µL on the DNA Engine Opticon using the DyNAmo Hot Start SYBR Green qPCR kit (MJ Research, Inc., Waltham, MA). cDNA synthesis was carried out using Oligo(dT) primers with the SuperScript III First-Strand Synthesis System (Invitrogen, La Jolla, CA). cDNA synthesized from 50 ng of total RNA was used for each qPCR reaction. Thermal cycle parameters were set according to the manufacturer's instructions. Rat PDHA1 mRNA primers (forward, 5'-CACGGACCATCTCATCTG-3'; reverse, 5'-TAGCACAGCCTCCTCTTCGT-3'; product size: 110bp) were designed for qPCR. The Cyclophilin B (forward, 5'-CTTCCCCGATGAGAACTTCA-3'; reverse, 5'-AGCCAGGCTGTCTTGACTGT-3'; product size 120bp) was used as an internal control. One to three independent qPCR trials were conducted for each template source. In each trial, triplicate samples of template were analyzed. qPCR assays were used to measure rat PDHA1 gene expression relative to cyclophilin B gene expression.

2.5. Trypan blue dye exclusion assay

The percentage of dead cells was calculated by trypan blue dye exclusion. At specified times, the media and trypsinized rat lung fibroblast (RLF) cells from each well were centrifuged, and the pellet was suspended in 1 ml of PBS. A 0.4% trypan blue (300 μ l) and a fibroblast suspension (300 μ l) were mixed, and the number of stained cells and non-stained cells was counted using a hemocytometer under microscope (20 X objective). At least two chambers were counted each time. The calculation of each group was performed in triplicate. The percentage of dead cells was calculated as [stained cells/(stained+unstained cells)] $\times$ 100.

2.6. Western blotting analysis of cultured rat lung fibroblasts

Protein lysates from each cultured cell line were run on a 10% SDS-PAGE gel and transferred to a PVDF membrane. The blot was probed with monoclonal antibodies (mAb; Molecular Probes) to the α subunit of the human E1 enzyme. The membrane was incubated with a horseradish peroxidase-conjugated bovine anti-mouse IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and the secondary antibody was detected using a chemiluminescence luminol reagent (Santa Cruz Biotechnology, Inc.).

2.7. PDH enzyme assay

PDH complex activity was measured by the rate of $^{14}\text{CO}_2$ formation from [$1\text{-}^{14}\text{C}$]-labeled pyruvate, as described previously (Simpson et al., 2006). Six T-75 flasks containing RLF infected with scAAV-siRNA and two vehicle controls infected with scAAV vectors were used at 3000 particles/cell. Cells were harvested at 3 days or 10 days post-infection with trypsin and washed with PBS, respectively, and resuspended in PBS with protease inhibitors (leupeptin and PMSF). A Bradford protein assay was performed to establish protein concentrations. The specific activity of ^{14}C -pyruvate was expressed as nmol $^{14}\text{CO}_2$ produced/min/mg protein.

3. Results

We used eight potential siRNA candidates and both positive and negative controls (Dharmacon, Inc., Dallas, TX). All siRNA sequences were submitted to a BLAST search against the human and rat genome sequences to ensure only one gene per sequence was targeted. The siRNAs were first evaluated for their effects on rat PDHA1 gene expression by co-transfecting HEK 293 cells with pE-rPDHA1 (Fig. 1). qPCR analyses showed that four siRNAs reduced rat PDHA1 RNA levels up to 85% at 24 hrs and up to 65% at 56 hrs, compared to negative and positive controls (Fig. 2). Transfection efficiency, determined by GFP expression, was approximately 90% (data not shown). qPCR also confirmed that the primers designed were specific for the rat PDHA1 gene, and there were no nonspecific targets within the human PDHA1 gene (Fig. 1).

Since oligonucleotide-mediated siRNA delivery provided only transient suppression, we next generated scAAV vectors (serotypes 2 and 5) expressing rat short hairpin siRNA (scAAVsi-PDHA1; Fig. 3) to infect RLF cultures. RLF cells were plated in six-well plates. Cells were infected the next day with scAAV2-si3-rPDHA1, scAAV2-si7-rPDHA1, scAAV5-si3-rPDHA1 or scAAV5-si7-rPDHA1 or one of two control vectors, scAAV2 and scAAV5, at 10,000 particles/cell. qPCR analysis showed up to 80% knockdown of rat PDHA1 mRNA 3 days after infection and up to 70% knockdown 10 days after infection, compared to cells infected with control vectors (Table 1). The number of dead cells, as measured by the Trypan Blue dye-exclusion assay, was also greater in scAAV siRNA-infected cells than that in control cells (Table 2). This indicates the critical importance of a functional PDC in cell growth.

We next determined the effects of siRNA delivery on the expression of PDH E1 α at the protein level. Three $\times 10^5$ RLF cells per well were infected with each vector described above. Western

immunoblotting was carried out 3 and 10 days post-infection. No differences were detected at day 3, but PDH E1 α protein expression decreased 40%–61% from baseline 10 days after infection (Fig. 4). Knockdown of the rat PDHA1 gene was further documented by measuring the specific activity of PDH enzyme, which was reduced by 40%–60% in cultures infected with AAV-siRNA vectors compared with control vectors, 10 days after infection (Table 3).

4. Discussion

Mitochondrial disorders principally affect tissues with high ATP demands, such as muscle and brain. PDC deficiency is one of the most commonly identified genetic causes of primary lactic acidosis in children and is most of the due to mutation in the gene encoding the α subunit. An animal model for PDC deficiency would provide insight into the clinical and biochemical phenotype of this disease. Recently, a gene “knockout” model by inducing a mutation into the PDHA1 gene in progenitor cells of the central nervous system was established; however, the defect is embryonic lethal in males and only female offspring are born (Pliss et al., 2004).

Because of the low transfection efficiency of rat fibroblast cell lines, we used siRNA-mediated knockdown of plasmid-encoded rat PDHA1 mRNA in HEK 293 cells. In designing the rat PDHA1 siRNA, we blasted against the human and the rat databases to exclude the possibility of knockdown of a non-target gene. Our results indicate that siRNA duplexes targeted to the rat PDHA1 region specifically decreased the extent of mRNA expression in co-transfected HEK 293 cells. qPCR analyses showed that four of the eight siRNA duplex candidates achieved gene silencing of rat PDHA1 mRNA up to 85% during the first two days and up to 65% by 56 hours, compared with negative and positive controls. The naked siRNA duplexes as well as the expression cassette in plasmid DNA were short-lived, and their function declined after 72 hours (Fig. 2). We could not detect the rat PDHA1 mRNA expression in HEK 293 cells at day 10 post-transfection by qPCR even in the absence of co-transfection with siRNAs (data not shown).

Since oligonucleotides-mediated siRNA delivery provided only transient suppression, we used two siRNA candidates, si3 and si7, and generated serotype 2 and serotype 5 scAAV vectors expressing rat short hairpin siRNA, scAAVsiPDHA1, and infected RLF cultures. qPCR analysis showed that the RLF PDHA1 mRNA level was reduced by 46–80% 72 hours post-infection and by 54–70% 10 days post-infection (Table 1). Sustained silencing of gene expression of rat PDHA1 was further confirmed at the protein level, both in amount and enzymatic activity (Fig. 4, Table 3).

It is interesting that infection of cells with siRNA directed against PDHA1 caused cell death in some infected RLF cells. We speculate this may reflect cellular energy failure due to inhibition of PDH, as might be expected *in vivo* when the activity of this enzyme is severely impaired. If so, our data suggest a critical role of a functional PDC for survival, even in cells, such as fibroblasts, that are thought to derive much of their ATP from glycolysis, rather than from oxidative metabolism.

In conclusion, our findings indicate that combining RNA interference and recombinant scAAV vector technologies can produce sustained and functionally meaningful knockdown of the PDHA1 gene in transduced mammalian cells *in vitro*. The long-term maintenance of gene silencing using scAAV2 and scAAV5 vectors for delivery of RNAi to knockdown gene expression of rat PDH E1 *in vivo* may lead to the development of an animal model for PDH E1 α deficiency that, in turn, could be useful in developing gene therapy strategies for this devastating condition (Stacpooole et al., 2003).

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Figure 1. qPCR melting curve analysis. A. Positive results were detected by qPCR using the primers designed specific for rat PDHA1 in human HEK 293 cell extracts which were transfected with the pE-rPDHA1. B. Negative results were detected by qPCR using the primers specific for rat PDHA1 in human 293 cell extracts only.

Figure 2.

Kinetics of knockdown of rat PDHA1 gene expression in cell cultures tested by qPCR. Blue bars represent transient suppression of gene expression by oligonucleotide-mediated siRNA co-transfection with an expression plasmid encoding rat PDHA1 gene in HEK 293 cells; red bars represent sustained gene silencing following scAAV-mediated siRNA delivery in RLF cultures.

Figure 3.

Schematic structure of self-complementary AAV-based siRNA vector. First, synthetic double-strand oligodeoxynucleotides coding for shRNA sequences were synthesized (Ambion, inc). Second, shRNA were introduced to pSilencer 3.0-H1 plasmid at the BamH1/HindIII sites. Finally, siRNA cassette encoding the short hairpin siRNA and its H1 promoter were created by PCR (F: 5-GCGGTACCGTTTTCCCAGTCACGAC-3, R: GCGGTACCGAGTTAGCTCACTCATTAGGC-3) flanked with Kpn I restriction on each sites. The siRNA cassettes were then inserted into the Kpn I site of the pscAAV-CB-EGFP to get the pH1-si-rPDHA1/AAV-EGFP. This vector expresses the hairpin form of rat PDHA1 siRNA under the control of H1 RNA Pol III promoter and an eGFP cDNA under the control of CB promoter.

Figure 4.

Suppression of rat PDHA1 expression in RLF cells by AAV-delivered siRNAs. Protein lysates from each cultured cell line were run on a 10% SDS-PAGE gel and transferred to a PVDF membrane. The blot was probed with monoclonal antibodies (mAb) (Molecular Probes) to the α subunit of the human E1 enzyme. The membrane was incubated with a horseradish peroxidase-conjugated bovine anti-mouse IgG (Santa Cruz Biotechnology, Inc.) and the secondary antibody was detected using a chemiluminescence luminol reagent (Santa Cruz Biotechnology, Inc.). RLF cells were infected with scAAV5-eGFP/control (1), scAAV2-eGFP/si3-PDHA1 (2), scAAV2-eGFP/si7-PDHA1 (3), scAAV5-eGFP/si3-PDHA1 (4) and scAAV5-eGFP/si7-PDHA1 (5), respectively. Cellular lysates were prepared 3 day (A) and 10 day (B) after infection. Western blot analysis revealed a 40–60% reduction at 10 days (B), but not at 3 days (A), in the amount of the E1 α subunit in all AAV-siRNAs treated RLF cells compared with that treated with a control AAV vector; (C) the pixel density in each band from panel B was quantitated and subjected to background correction and the internal control (β -

actin) using the ImageJ software, and the levels of E1 α were then compared with the normal control.

Table 1

AAV-mediated siRNAs knockdown of rat PDHA1 gene analyzed by qPCR

Name	Rat-PDHA1RNA Rel. to Cyclophilin B (Internal Control)		% of Control
Control: scAAV2 (n=3)	1.06 ± 0.02 (day 3)	0.54 ± 0.05 (day 10)	100 (day 3)
scAAV5 (n=3)	1.06 ± 0.02 (day 3)	0.54 ± 0.05 (day 10)	100 (day 10)
scAAV2/siRNA3 (n=3)	0.50 ± 0.03 (day 3)	0.21 ± 0.01 (day 10)	38 (day 10)
scAAV2/siRNA7 (n=3)	0.20 ± 0.04 (day 3)	0.16 ± 0.01 (day 10)	29 (day 10)
scAAV5/siRNA3 (n=3)	0.57 ± 0.02 (day 3)	0.25 ± 0.03 (day 10)	46 (day 10)
scAAV5/siRNA7 (n=3)	0.34 ± 0.02 (day 3)	0.35 ± 0.04 (day 10)	65 (day 10)

RLFs were infected with scAAV/rat-PDHA1-siRNA vectors at 10,000 particles/cell, and mRNA was assayed at 3 days and 10 days post-infection by qPCR. scAAV2 and scAAV5-EGFP vectors were used as controls. The cyclophilin B gene was selected as an internal control. The cDNA synthesized from 50 ng of total RNA was used per qPCR reaction. One to three independent qPCR trials were conducted for each template source. In each trial, triplicate samples of template were analyzed.

Table 2

Trypan Blue dye exclusion assay.

Agent	Dead Cells (%)		
	2 days	5 days	8 days
scAAV2	3.33	5.95	9.85
scAAV5	4.35	5.62	8.56
scAAV2-si3	3.62	8.09	17.54
scAAV2-si7	4.90	9.23	16.07
scAAV5-si3	3.77	11	18.41
scAAV5-si7	3.47	7.53	19.88

The percentage of dead rat lung fibroblast (RLF) cells was calculated by Trypan

Blue dye exclusion at various indicated times after infection with scAAV/rat-PDHA1-siRNA vectors at 10,000 particles/cell. At least two chambers were counted each time. The calculation of each group was performed in triplicate. The percentage of dead cells was calculated as [stained cells/(stained +unstained cells)] $\times$ 100.

Table 3

PDH activity in cultured rat lung fibroblasts.

Name	PDH activity*	% of Control
Control: scAAV2 (n=4)	2.06 ± 0.17 (day 3)	100 (day 10)
scAAV5 (n=4)	2.03 ± 0.29 (day 3)	100 (day 10)
scAAV2/siRNA3 (n=4)	1.67 ± 0.16 (day 3)	84 (day 3)
scAAV2/siRNA7 (n=4)	2.21 ± 0.51 (day 3)	110 (day 3)
scAAV5/siRNA3 (n=4)	2.03 ± 0.20 (day 3)	100 (day 3)
scAAV5/siRNA7 (n=4)	1.84 ± 0.44 (day 3)	92 (day 3)

* Expressed as nmoles ¹⁴C CO₂ formed from 1-¹⁴C pyruvate/min/mg protein. Data are means ± SD (replicate determinations).