SUPPLEMENTAL MATERIALS FOR GIL, ET AL.

Supplemental Methods

Adenovirus vectors

Adenovirus vectors were constructed using the Gateway System (Invitrogen, Carlsbad, CA), with the pENTR shuttle vectors [1] containing each of the reporter genes, recombined into the adenoviral plasmid vector, pAd/CMV/V5-DEST using LR recombination. Vector construction is diagramed in Fig. 1 of the main text. All reporter gene coding regions were amplified using Phusion DNA polymerase (Finnzymes/New England Biolabs), according to the manufacturer's instructions. The luciferase 2 (*Luc2*) as a vector control was amplified from pGL4.13 (Promega, Madison, WI) as previously described [1]. These constructs were then recombined with pAd/CMV/V5-DEST, using LR Clonase II (Invitrogen), according to the manufacturer's protocol, to generate the pAd plasmids.

To generate adenovirus vectors, plasmid DNA for each construct was digested for 2 hours at 37^{o}C with *Pac*I, then purified with PCR Cleanup (Invitrogen, Carlsbad, CA). 1.6 µg DNA was used to transfect HEK293A cells seeded on 12-well cell culture plates, using Lipofectamine 2000 (Invitrogen). After 10 days, cells from wells showing 100% cytopathic effect (CPE) were collected by scraping, the entire well contents (1 mL) were transferred to screw cap tubes, and the cells and debris were subjected to three cycles of freeze/thaws for lysis. The suspension was centrifuged, and the supernatant was used to propagate the adenovirus vectors in fresh HEK293A cells.

Propagation of Adenovirus vectors expressing the PET reporter genes

90% confluent 10cm tissue culture dishes of HEK293A cells were infected with adenovirus vector containing supernatant, incubated for 2 to 3 days until complete CPE

was evident. Cells were collected by scraping, lysed with 3 rounds of freeze/thaw and followed by centrifugation. One more round of infection with the supernatant was performed onto two 500 cm^2 tissue culture plates of 90% confluent HEK293A cells. After 2 to 3 days, with cells showing 100% CPE, plates were collected, and cells were pelleted in 5% original volume, followed by lysis using deoxycholate (0.5% final concentration). After a clarification centrifugation, supernatants were loaded onto a 50 mM Tris·HCl pH 8.0 cesium chloride buoyant density step gradients with 1.35 g/mL and 1.25 g/mL densities. Bands were consolidated, and density and volume were adjusted to 1.33 g/mL for linear gradient ultracentrifugation. Purified adenovirus vector was dialyzed against a 3% sucrose storage buffer and snap frozen in aliquots in liquid nitrogen for storage.

Titration of Adenovirus vectors, using the Infectious Genome protocol [1, 2]

Adherent HeLa cells (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, penicillin (100 U/mL) and streptomycin (100 µg/mL). Cells were seeded at 1:2 dilutions onto 6-well tissue culture plates one day prior to adenovirus vector infection. Wells were infected with the adenovirus vectors by aspirating media, and cells were infected with vector diluted in 0.25 mL DMEM.

HeLa cells were infected for three hours, then washed twice with PBS. Nuclei were isolated using an NP-40 lysis protocol (10 minutes at room temperature) in 0.65% NP-40, 150 mM NaCl, 10 mM Tris·HCl pH 8.0, 1.5 mM $MgCl_2$, followed by pelleting nuclei and removing supernatant). DNA was purified using the DNeasy Blood & Tissue kit (Qiagen, Valencia, CA) and subjected to TaqMan quantitative real-time PCR (Applied Biosystems, Foster City, CA). Primers and probe specific for the adenoviral pX protein

gene were used for absolute quantification, using a plasmid standard with known copy number. The numbers of Infectious Genome Units (IGUs) are determined by comparing the amount of PCR product from the nuclear extracts, normalized to one copy of the amplification region in the plasmid standard.

Measurement of adenovirus DNA and murine genomic DNA in liver extracts

TaqMan PCR based absolute quantification for vector genomes and liver cell genomes was performed on purified DNA, using the same adenovector primers and TaqMan probe used in the cell culture titrations, and primers and probe specific for the mouse *Oct4* gene. Standard curves to measure the number of liver genomes were prepared from pre-weighed liver samples subjected to DNeasy DNA purification, based on an estimation of 1.35×10^8 hepatocytes per gram [3]. Standard curves to measure the number of adenovirus vectors were prepared from purified vector DNA quantified by optical density.

Determination of optimal time for imaging of reporter gene efficacy after adenovirus reporter gene intravenous. injection

Three mice were injected via tail vein with Ad.HL.Luc2 ($5x10^{10}$ IGU/mouse). The mice were then imaged, following luciferin injection [1] with an IVIS instrument (Caliper Life Science, Inc.), at days 1, 2, 3, 4, 5, 6 and 10 after Ad.HL.LUC2 injection. We found a broad peak between days 3 and 5 (Supplemental Fig. 1).

In vivo studies

In vivo studies to compare efficacy of wild-type dCK and the two dCK mutants dCK-R104M/D133A (dCK-DM) and dCK-A100V/R104M/D133A (dCK-A100VTM) were performed as described in the main manuscript, with the modification that two mice per group imaged in one experiment and the experiment was repeated a second time, again

with two mice per group. For the comparison of dCK and the two dCK mutants as PRGs, the data from the two experiments were pooled.

Supplemental Results

To determine the time for optimal imaging after adenovirus injection in our protocol, we injected 3 mice with 5×10^{10} Ad.HL.Luc2 [1], and imaged with luciferase [1] daily for 6 days, then at 10 days. For each optical imaging session mice were anesthetized with 2% isoflurane. For each image, luciferin (3 mg, in 100 □L) was administered intraperitoneally, Mice were imaged in an IVIS® Lumina II imaging system (Caliper Life Sciences, Hopkinton, MA) [1]. Optimal imaging was observed between days 3-5 (Supplemental Figure 1).

Hepatic efficacy of wild type dCK, the double mutant dCK-DM *and the triple mutant dCK-A100VTM as PRGs, with* ^{18}*F-L-FMAU as a PRP*

Four mice per group were injected intravenously with adenovirus vectors (5×10^{10} IGU/mouse); Ad.HL.Luc2, Ad.HL.dCK-WT, Ad.HL.dCK-DM and Ad.HL.dCK-A100VTM. Imaging with ^{18}F-L-FMAU was performed 4 days after vector injection (Supplemental Figure 2a), followed by liver DNA purification. The numbers of vector genomes/liver cell, ± S.E.M, were Ad.HL.Luc2, 86 ± 10; Ad.HL.dCK-WT, 77 ± 14; Ad.HL.dCK-DM, 80 ± 18 and Ad.HL.dCK-A100VTM, 72 ± 19 (Supplemental Figure 2b). Differences in viral genome transductions between groups were not statistically significant (p=0.94), demonstrating consistent transduction efficiency.

PRP-dependent PRG hepatic retention was determined as described in the main manuscript. %ID/g values, corrected for PRP background, but not corrected for differences in the numbers of PRGs present in the liver, were Ad.HL.dCK-WT, 0.05 ±

0.01; Ad.HL.dCK-DM, 5.0 ± 0.56; and Ad.HL.dCK-A100VTM, 5.2 ± 0.58. (Supplemental figure 2c). Values are means ±S.E.M.s. Differences between dCK-WT and each mutant were significant (*** indicates p=0.0001 for both dCK-DM and dCK-A100VTM). However, no statistical difference between dCK-DM and dCK-A100VTM was detected (p=0.80).

To eliminate the variability caused by differences in numbers of PRGs present in livers of each mouse, the [%ID/g-bkg] was normalized to vector transduction. Results for the dCK vectors were Ad.HL.dCK-WT, 7.6 ± 2.6 x 10^{-4}; Ad.HL.dCK-DM, 7.6 ± 2.5 x 10^{-2}; and Ad.HL.dCK-A100VTM, 9.1 ± 2.8 x 10^{-2}. Both Ad.HL.dCK-DM/^{18}F-L-FMAU and Ad.HL.dCK-A100VTM/^{18}F-L-FMAU signals differed significantly from the Ad.HL.cCK-WT/^{18}F-L-FMAU signal (*indicates p=0.02). In contrast no significant difference could be demonstrated between the signals from the Ad.HL.dCK-DM/ ^{18}F-L-FMAU and Ad.HL.dCK-A100VTM ^{18}F-L-FMAU (p=0.70).

Supplemental Figure Legends

Supplemental Fig. 1. Time course for hepatic adenovirus reporter gene expression. Three mice were injected with Ad.HL.Luc2 (5x10^{10} IGU) on day 0, then subjected to luciferin-dependent bioluminescent imaging on days 1, 2, 3, 4, 5, 6 and 10 after virus administration. Signal was normalized to 100% of the maximum for each mouse, and averaged. Data are means ± S.E.M. of triplicate assays for duplicate transductions.

Supplemental Fig. 2. Comparative efficacy of dCK-DM and dCKA100VTM as PRGs, with 18F-L-FMAU as PRP. (Panel a) Four mice per group were injected with

either $5x10^{10}$ IGU of Ad.HL.Luc2, Ad.HL.dCK-WT, Ad.HL.dCK-DM, or Ad.HL.dCK-A100VTM, and imaged 4 days later (1 representative image for Ad.HL.dCK-WT, dCK-DM and dCKA100VTM is shown). (Panel b) One day after imaging the mice were sacrificed and liver samples were processed to determine transduction as measured by qPCR-detected vector genomes. Each point is the mean of three liver samples per mouse. The horizontal lines are the means of all mice in each group, error bars denote ± S.E.M. (panel c) 4 ROIs were drawn within the liver of each animal, and quantified to determine average %ID/g. Each point is the mean of the four liver ROIs with background signal (Ad.HL.Luc2 negative control mouse) subtracted. Error bars indicate means ± S.E.M. Differences between dCK-WT and each mutant were significant (*** indicates p=0.0001 for both dCK-DM and dCK-A100VTM). Differences between dCK-DM and dCK-A100VTM were not statistically significant (p=0.80). (Panel d) Signal was normalized for number of hepatic viral genomes. Differences between dCK-WT and each mutant were significant ((* indicates p=0.02). Differences between dCK-DM and dCK-A100VTM were not statistically significant (p=0.70). Error bars denote ± S.E.M.

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