Functional silencing of hepatic microsomal glucose-6-phosphatase gene expression in vivo by adenovirus-mediated delivery of short hairpin RNA

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Abstract An expression cassette containing mouse U6 polymerase III promoter directing expression of short hairpin RNA (shRNA) targeting murine microsomal glucose-6-phosphatase (G6P) transcript was generated. This construct was packaged into an adenoviral (AdV) backbone and viral stocks generated. Mice injected intravenously with AdV-G6PshRNA exhibited a significant reduction in postprandial glucose levels and had significantly elevated steady-state hepatic glycogen stores. Target gene silencing was confirmed by measurements demonstrating a significant reduction in both hepatic G6P transcript level and phosphohydrolase activity. These findings provide evidence that AdV delivery of expressed shRNA can be a productive tool to explore gene function in vivo.

Key words: RNA interference; Short hairpin RNA; Gene silencing; Glucose-6-phosphatase; Adenovirus

1. Introduction

The RNA interference (RNAi) pathway is an evolutionarily conserved gene silencing mechanism elicited by double-stranded RNA [1,2]. There is great current interest in the application of RNAi as a research tool in studies of gene function. In mammalian cell lines and mouse model systems, efficient gene silencing has been achieved by the direct introduction of short interfering RNAs (siRNAs) or via the transcription of short hairpin RNAs (shRNAs) from expression vectors or viral delivery systems [3–9]. Recent advances using this technology in vivo include the silencing of reporter and endogenous genes as well as the generation of transgenic animals with silencing of gene expression in adult mice [10–16]. However, exploitation of the RNAi pathway to induce changes in either whole body mammalian physiology or broad metabolic parameters has not been reported to date.

The liver plays a central role in the homeostatic regulation of blood glucose. When circulating blood glucose levels decline, the liver can rapidly release glucose to fuel the energy demands of the body. This response is achieved via two distinct and well characterized biochemical pathways, gluconeogenesis and glycogenolysis. A single enzyme, glucose-6-phosphatase (G6P) (GenBank accession number NM_008061), is responsible for catalyzing the final step in each of these pathways [17,18]. Inhibition of glucose-6-phosphate conversion to free glucose by either genetic ablation of the G6P gene or pharmacological inhibition of the G6P catalytic site or translocase functionality results in alterations to multiple metabolic parameters including the development of hypoglycemia and an elevation of hepatic glycogen stores [19–25].

In this study, we have employed an adenoviral (AdV) vector system to deliver expressed shRNA complementary to G6P to normal mice intravenously. G6P gene silencing in vivo was confirmed by direct measurements of hepatic transcript level and encoded enzymatic activity and results in alterations to whole animal glucose homeostasis and hepatic glycogen content. Our findings provide the first report of quantitative physiological alterations achieved by viral delivery of shRNA in vivo. These results highlight the potential to use this technology for the study of gene function in the context of whole animal physiology.

2. Materials and methods

2.1. shRNA and green fluorescent protein (GFP) template expression vector construction

shRNA expression vector, pShuttle-U6, was constructed by replacing the CMV promoter from vector pShuttle (Becton Dickinson) with a polymerase chain reaction (PCR)-amplified mouse U6 promoter fragment from C57BL/6J genomic DNA (primer sequences: 5′-gatccgacgccgccatctcta-3′ and 5′-cacaaacaaggcttttctccaa-3′). For generation of G6PshRNA, a pair of oligonucleotides 55 bp in length (5′-tttggagtcgtgtcaggcattgttcaagagacaatgcctgacacgactccttttg-3′) and (5′-gatccaaaaaggagtcgtgtcaggcattgtctcttgaacaatgcctgacacgactc-3′) were synthesized, annealed and ligated between the *Bsa*I and *Bam*HI sites of pShuttle-U6. The expressed hairpin is complementary to nucleotides 550–568 in the mRNA sequence. Generation of the 2 bp mutant G6PshRNA was as described as above but with the use of a two nucleotide substitution (large font) in the sequences (5′-tttggaTtcgtgtcaggcattgttcaagagacaatgcctgacaAgactccttttg-3′) and (5′-gatccaaaaaggagtcTtgtcaggcattgtctcttgaacaatgcctgacaAgactc-3′). For construction of control GFPshRNA, a pair of oligonucleotides 55 bp (5′-tttgaagcagcacgacttcttcttcaagagagaagaagtcgtgctgcttcttttt-3′) and 56 bp (5′-gatcaaaaagaagcagcacgacttcttctctcttgaagaagaagtcgtgctgcttc-3′) were synthesized, annealed and ligated as described above. For construction of pG6P-EGFP fusion cDNA, the entire coding sequence of mouse G6P was amplified by PCR from mouse liver cDNA. The PCR fragment was digested and ligated into the *Hin*dIII and *Sac*II sites of p-EGFP-N1 (Becton Dickinson).

2.2. Cell culture, transfections and shRNA screening

Mouse L cells, HEK 293 cells and rat H4IIE cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Clonetics). L cells were seeded at 90% confluence in 12 well cell culture dishes 24 h prior to transfection. shRNA (1.6 μg) and GFP template (0.4 μg) expression vectors were co-transfected and 48 h later cellular GFP signal was either visualized under a fluorescent microscope or quantified using a fluorescent spectrophotometer.

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2.3. Adenovirus packaging and in vitro validation

The shRNA cassettes cloned in the pShuttle-U6 vector was packaged into adenovirus using the Adeno-X viral expression system (Becton Dickinson). High titer virus (1×10^{12} pfu/ml) was generated by large-scale infection and amplification in HEK 293 cells followed by purification using CsCl gradient ultracentrifugation. For in vitro validation, 1×10^{8} pfu of adenovirus were used to infect 1×10^{6} H4IIE rat hepatoma cells.

2.4. Animal studies

All studies were performed with the approval of the Millennium Pharmaceuticals Animal Care and Use Committee. Male C57BL/6J mice (12 weeks of age) were from Jackson Laboratories.

2.5. Adenoviral delivery in vivo

Adenovirus (5×10^{10} pfu) containing expression cassettes for either G6P- or GFP-shRNA or phosphate-buffered saline (PBS) vehicle (100 μl total volume) were injected intravenously into the tail vein of mice. Serum was collected by tail bleeding 1 day prior to and 3, 5, 7 days post injection. Livers were collected on the indicated days, snap frozen in dry ice and stored at −80°C.

2.6. Real-time quantitative PCR

Total RNA was isolated from liver tissue and 2 μg was DNase treated and reverse transcribed. Quantitative gene expression was performed on an ABI Prism 7700 machine (Applied Biosystems). Approximately 20 ng of cDNA was used for each reaction. Commercially available mouse 18S RNA cDNA primer and probe was used as an internal standard. All data analysis was performed as recommended by the manufacturer. G6P mRNA levels were normalized to those of the housekeeping gene and are expressed as relative fold differences.

2.7. Liver G6P assay, serum glucose assay and liver glycogen determination

Liver microsomal fraction was prepared and G6P activity assay performed as described [26]. Serum glucose levels were measured using a glucose color reagent (Raichem) according to the manufacturer's protocol. Liver glycogen levels were determined by using the amyloglucosidase method as described [27].

2.8. Statistics

Where indicated, values are reported as group averages with standard deviations. All statistical analysis was performed pair-wise using a standard Student's two tailed *t*-test.

3. Results

We generated a mammalian expression cassette containing the mouse U6 polymerase III promoter driving expression of shRNA complementary to the transcript encoding mouse and rat G6P. Efficient gene silencing by G6PshRNA was con-

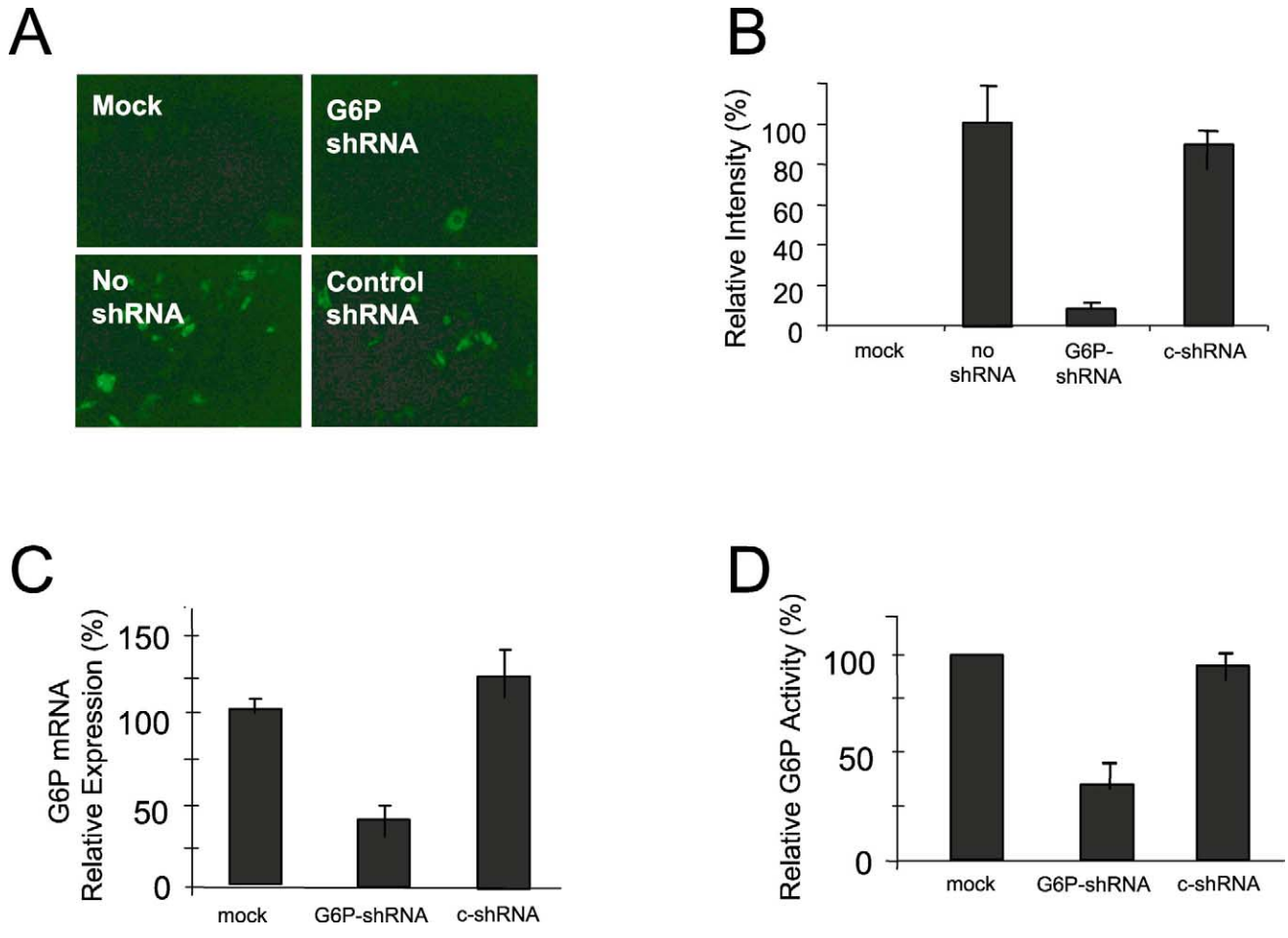

Fig. 1. Generation and testing of expressed microsomal G6P shRNA. A,B: Significant reduction in fluorescence intensity in mouse 3T3 L cells co-transfected with a G6P-EGFP chimeric construct and a vector containing mouse U6 polymerase III promoter directing G6PshRNA expression. A: Fluorescence microscopic images of L cells 48 h post transfection. B: The EGFP signal from A quantified with a fluorescent spectrometer; vector-transfected cell control was set to 100%. C,D: Adenovirus stocks containing the U6 polymerase III promoter driving G6PshRNA efficiently silences endogenous G6P gene expression. 1×10^{8} pfu of adenovirus were used to infect 1×10^{6} H4IIE cells (multiplicity of infection of 100). Cells were harvested 96 h after infection. C: Real-time quantitative PCR analysis demonstrates that G6P mRNA is reduced to approximately 40% of control values. D: G6P enzymatic activity is similarly reduced in cell lysates from the H4IIE cells.

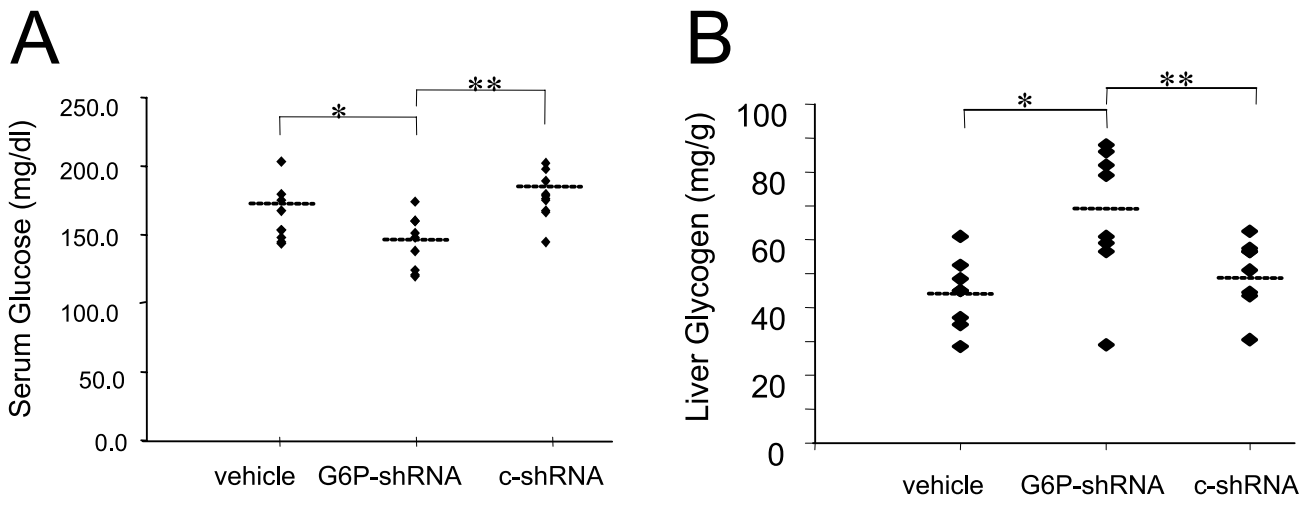

Fig. 2. AdV-G6PshRNA decreases postprandial serum glucose and liver glycogen levels in mice. A,B: Male C57BL/6J mice were intravenously injected with 100 μl PBS (vehicle) or 5×10^{10} pfu. AdV-G6PshRNA or AdV-GFPshRNA (control). A: Serum glucose levels were measured 5 days post injection at 10.00 h. Glucose levels for each animal are shown, with each symbol representing one individual animal; group averages are illustrated with the hatched line ($n=9$/group). Mice that received AdV-G6PshRNA had significantly reduced serum glucose levels (148 mg/dl) compared to the vehicle (170 mg/dl) and control (c-shRNA; 182 mg/dl) groups ($^*P<0.05$, $^{**}P<0.005$). B: Livers were harvested and glycogen levels were measured. Each symbol represents the glycogen value of one individual mouse; group averages are illustrated with the hatched line ($n=8$/group). Groups infected with AdV-G6PshRNA have significantly higher glycogen levels (67.4 mg/g) compared to the vehicle (44.1 mg/g) and control groups (49.1 mg/g) ($^*P<0.01$, $^{**}P<0.05$).

firmed by studies in mouse 3T3 L cells that had been co-transfected with a mouse G6P-EGFP fusion cDNA and the U6-G6PshRNA construct. As shown in Fig. 1A,B, we observed a dramatic reduction of EGFP fluorescence intensity in cells that co-expressed the G6PshRNA. Similar results were found when G6P-EGFP transcript levels were measured by real-time PCR or protein levels were measured by Western blotting (data not shown). Mutation of the G6PshRNA hairpin by a single bp substitution in the G6P complementary sequence dramatically reduces the gene silencing effect (Fig. 1A,B). The U6-G6PshRNA expression construct was then inserted by homologous recombination into an adenovirus backbone vector and low titer viral stocks were generated. Initial characterization experiments using rat H4IIE cells indicated that we could generate infectious AdV particles capable of efficiently silencing G6P gene expression to approximately 40% of control values as evidenced by reduction of G6P mRNA and enzymatic activity (Fig. 1C,D).

Systemic infusion of recombinant adenovirus results in transgene delivery predominantly to hepatic tissue [28]. High titer AdV stocks were therefore generated and experiments performed to determine whether virally delivered shRNA could silence hepatic G6P gene expression in vivo. Groups of C57BL/6J mice were intravenously injected with vehicle, AdV-G6PshRNA or AdV-GFPshRNA control. We observed that circulating levels of serum glucose were unchanged in the three groups of mice at both 24 h and 72 h post infection. However, on day 5 we detected a dramatic and significant reduction in the postprandial serum glucose levels in the AdV-G6PshRNA-injected animals (148 mg/dl) compared to the vehicle (167 mg/dl) and control (182 mg/dl) groups (Fig. 2A). In addition to the impact on circulating postprandial glucose on day 5, mice infected with AdV-G6PshRNA also exhibited a significant increase in steady-state hepatic glycogen levels (67.4 mg/g) versus the vehicle (44.1 mg/g) and control (49.1 mg/g) groups (Fig. 2B). These phenotypic alterations were independent of changes in food intake (data not shown) and are consistent with a model whereby a reduction in liver G6P activity gives rise to alterations in glucose homeostasis.

In order to meet energy demands, the mammalian liver can modulate circulating glucose levels via either an increased rate of gluconeogenesis or the breakdown of stored liver glycogen. Hepatic G6P catalyzes the final and essential enzymatic step in either process [17–19]. Consequently, to determine whether the AdV-G6PshRNA-infected mice would respond normally to lowered circulating glucose by increasing output from the liver, mice were metabolically challenged by an overnight (16 h) fast on day 7 post injection with AdV-G6PshRNA. Livers were then isolated and glycogen levels were quantified.

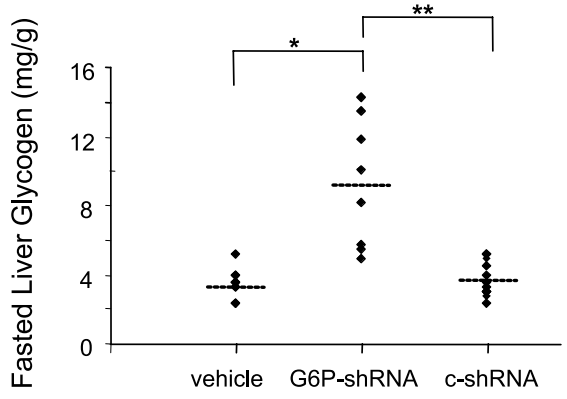

Fig. 3. Retained liver glycogen stores are elevated in AdV-G6PshRNA-treated mice following overnight fast. Male C57BL/6J mice were intravenously injected with 100 μl PBS (vehicle) or 5×10^{10} pfu AdV-G6PshRNA or AdV-GFPshRNA (control). Mice were fasted overnight on day 7 following virus injection. Livers were harvested and glycogen levels measured. Each symbol represents the glycogen value of one individual mouse; group averages are illustrated with the hatched line ($n=9$/group). Groups treated with AdV-GFPshRNA retain significantly higher glycogen levels (8.38 mg/g) compared to the vehicle (3.36 mg/g) and control (c-shRNA; 3.55 mg/g) groups ($^*P<0.05$, $^{**}P<0.005$).

We detected a significant increase in retained hepatic glycogen stores in the fasted AdV-G6PshRNA-infected mice (8.38 mg/g) compared to the vehicle (3.36 mg/g) and control (3.55 mg/g) groups (Fig. 3).

To confirm directly that we had successfully silenced liver G6P gene expression in the AdV-G6PshRNA-injected mice, we assessed both hepatic G6P transcript levels and the encoded G6P enzymatic activity on day 8 post infection. We measured a significant reduction of both G6P mRNA (approximately 35%) and the encoded phosphohydrolase activity (approximately 40%) in the AdV-G6PshRNA groups (Fig. 4A,B). Thus, our data provide clear evidence that we have efficiently silenced hepatic G6P gene expression that has resulted in physiological changes manifested as alterations to whole body glucose homeostasis and the mechanism of fasting-induced glycogenolysis.

4. Discussion

Exploitation of the RNAi pathway has recently emerged as a productive means to assess mammalian gene function both in vitro and in vivo (reviewed in [1,2]). Efficient gene silencing in mice has been successfully achieved by the direct hydrodynamic delivery of siRNAs or via the transcription of shRNAs from expression vectors or viral delivery systems [10–16]. Although exciting progress in application of this technology has been reported, the use of virally delivered shRNA to induce changes in either whole body mammalian physiological or metabolic parameters has not been reported to date.

In this study, we have used adenovirus to deliver shRNA complementary to G6P to livers of wild type mice. Successful silencing of hepatic gene expression by G6PshRNA was confirmed by direct measurements of both G6P mRNA and the encoded enzymatic activity. We measured an approximate 30–40% reduction in hepatic G6P transcript and enzymatic activity levels on day 8 post infection (Fig. 4A,B). Interestingly, although G6P heterozygous knock-out mice have a 50% reduction in measured liver G6P enzymatic activity, these mice exhibit no changes in circulating postprandial glucose levels or glycogen stores compared to wild type litter mates [21]. The discrepancy in observed phenotypes could be due to compensatory changes in the heterozygous knock-out mice. Alternatively, these results could imply that shRNA-induced G6P silencing may have potentially exceeded 50% efficiency during time points of this study when serum glucose levels were significantly decreased following AdV-shRNA infection (for example on day 5 post infection). However, measured effects on circulating serum glucose levels under these conditions would not become apparent until turnover of existing hepatic G6P enzymatic activity was substantial. Clearly, additional experimentation will be required to define the peak, duration and viral load for optimal AdV-mediated G6PshRNA gene silencing.

Hepatic G6P gene silencing results in a significant reduction of normal circulating glucose levels and a significant increase in liver glycogen stores (Fig. 2A,B). In addition, infection with AdV-G6PshRNA results in alterations to normal hepatic glycogenolysis in response to overnight fast (Fig. 3). Our findings are in agreement with previous observations describing the use of synthetic small molecule inhibitors of G6P function (catalytic site or translocase activity) to lower circulating blood glucose concentrations in rats and mice [22–26]. Moreover, these findings agree with the pathophysiology of glycogen storage disease type 1a (GSD-1a) in humans, a homozygous recessive disorder resulting from loss of G6P activity [20]. Patients with GSD-1a exhibit severe hypoglycemia and increased glycogen storage in the liver. Engineered G6P knock-out mice closely mimic the pathophysiology of human GSD-1a patients [19]. However, at present we cannot exclude the possibility that the observed effects of G6PshRNA on hepatic glucose homeostasis could be due in part to shRNA-induced non-specific responses as previously reported by Bridge and colleagues [29].

In summary, our study provides the first example of quantitative physiological and metabolic perturbations achieved by viral delivery of expressed shRNA in vivo and clearly high-

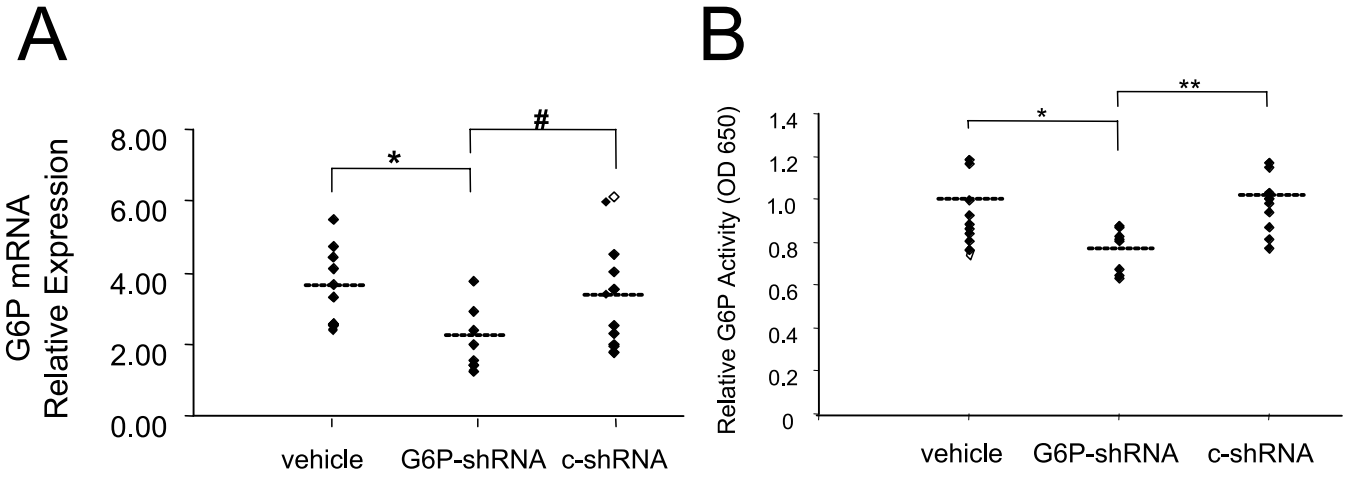

Fig. 4. AdV-G6PshRNA treatment efficiently silences hepatic microsomal G6P expression in mice. A,B: Male C57BL/6J mice were intravenously injected with 100 μl PBS (vehicle) or 5×10^{10} pfu of AdV-G6PshRNA or AdV-GFPshRNA (control). Livers were collected 8 days post virus infection and both mRNA levels and hepatic G6P enzyme activity were evaluated. Each dot represents the glycogen value of one individual mouse; group averages are illustrated with the hatched line ($n=9$/group). Infection with AdV-G6PshRNA results in a significant reduction in both (A) G6P transcript level and (B) phosphohydrolase activity compared to the vehicle and control (c-shRNA) groups ($^{*}P<0.05$, $^{**}P<0.005$, $^{\#}P<0.06$).

lights the potential use of this technology for the comprehensive study of gene function in the context of whole animal physiology. Although the use of adenovirus to deliver shRNA to the liver can induce a strong immune response via the interferon pathway, we believe this mechanism of acute gene silencing may provide a viable alternative to the use of hydrodynamic injection to deliver siRNA [15,16]. This is due in part to the large volume requirements for hydrodynamic injections, the consequential impact on whole body physiology and the stability and degradation kinetics of siRNA compared to shRNA. However, additional comparative studies between these two methodologies will be necessary to gauge both physiological impact, cellular transduction efficiencies and the length and magnitude of gene suppression that can be achieved in vivo.

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