

Brief Report

Robust Hepatic Gene Silencing for Functional Studies Using Helper-Dependent Adenoviral Vectors

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Abstract

RNA interference is currently envisioned as the basis of gene function and drug target validation studies. This novel technology has the advantage of providing a remarkably faster tool for gene silencing than traditional transgenic animal methodologies. *In vivo* administration of short interfering RNA (siRNA) typically results in reduced target gene expression for approximately 1 week. Viral vectors offer the possibility to express constitutive levels of short hairpin RNA (shRNA) so that the effects of knocking down the target gene can be studied for a few weeks, rather than a few days. Helper-dependent vectors have a significant advantage over previous generations of adenoviral vectors because of their much higher cloning capacity, potential for long-term transgene expression, and enhanced safety profiles on administration *in vivo*. Therefore, this advanced type of vector is an excellent tool to carry out *in vivo* studies directed at constitutive expression of shRNA. Here we show it is possible to obtain more than 90% target gene knockdown in an animal model of type 2 diabetes for several weeks, thereby consolidating this technology as an alternative to generating liver-specific knockout animals.

Introduction

RNA INTERFERENCE (RNAi) has become the cornerstone of gene function studies, circumventing the limitations of knockout animals and shortening the otherwise long process of target identification and validation. In some instances, gene ablation is incompatible with survival through the embryo stages, or gene compensatory mechanisms occur. One example is the transcription factor sterol regulatory element-binding protein-1c (SREBP-1), a central mediator of insulin action on lipid and carbohydrate metabolism in the liver (Horton *et al.*, 2003). SREBP-1 activity is enhanced in animal models of sucrose-induced hepatic insulin resistance, obesity, and type 2 diabetes (Shimomura *et al.*, 1999; Nagai *et al.*, 2002). Approximately 85% of SREBP-1 null mice die *in utero*. RNAi has the potential to overcome these limitations because it allows the conduction of experiments in adult animals, thereby bypassing developmental stages.

Helper-dependent or high-capacity adenoviral vectors have emerged as a valuable tool for gene delivery to the liver (Morral *et al.*, 1998, 1999; Schiedner *et al.*, 1998). This advanced

type of vector retains the inverted terminal repeats and packaging signal, but does not contain any viral coding sequences (Mitani *et al.*, 1995; Clemens *et al.*, 1996; Fisher *et al.*, 1996; Haecker *et al.*, 1996; Kochanek *et al.*, 1996; Kumar-Singh and Chamberlain, 1996; Lieber *et al.*, 1996; Parks *et al.*, 1996; Alemany *et al.*, 1997; Chen *et al.*, 1997; Hardy *et al.*, 1997). A single intravenous injection results in lifelong expression of the transgene in mice and rats (Kim *et al.*, 2001; Oka *et al.*, 2001; Toietta *et al.*, 2005). Furthermore, administration of high doses is well tolerated, without inducing major alterations in hepatic function markers (Morral *et al.*, 1998).

To provide the proof of concept that helper-dependent adenoviral vector-mediated short hairpin RNA (shRNA) expression represents an alternative strategy to traditional knockout strategies and allows study of the effects of inhibiting gene activity in liver, we have generated a vector expressing an shRNA to target SREBP-1. A potential problem to generating vectors containing shRNA expression cassettes is the possibility of recombination events that eliminate the hairpin sequence during vector propagation (Narvaiza *et al.*, 2006). In addition to the successful knockdown of SREBP-1,

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we observe that genomes of helper-dependent vectors containing shRNA expression cassettes are not more susceptible to recombination events than transgene-encoding vectors.

Materials and Methods

Expression cassette design and shRNA testing in HEK-293 cells

Plasmid pAlb, containing the albumin promoter/enhancer, was a generous gift from R. Palmiter (Howard Hughes Medical Institute, University of Washington, Seattle, WA) (Pinkert *et al.*, 1987). Plasmid pRES (see Helper-Dependent Adenoviral Vector Production, below) was cut with *Xba*I and *Asc*I, and the resulting fragment was cloned into the *Not*I site of pAlb. Plasmid pRES was then cut with *Xho*I and *Asc*I and the resulting 800-bp fragment was cloned into pRES-Alb. The fatty acid-binding protein-5 (FABP5, a.k.a mall1) cDNA with a bovine growth hormone poly(A) sequence has been previously described (Witting *et al.*, 2008), and was introduced into the *Eco*RV site of pRES-Alb after digestion with *Hind*III and *Xho*I and blunting with Klenow fragment. Plasmid pRES-Alb-FABP5 was used to rescue a helper-dependent adenoviral vector.

To generate constructs containing shRNA hairpins, 19- or 21-bp sequences were selected as described (Witting *et al.*, 2008) and cloned into pENTR of the BLOCK-iT U6 RNAi entry vector kit (Invitrogen, Carlsbad, CA) or pRNA-H1.1/Neo (GenScript, Piscataway, NJ), in accordance with the manufacturer's instructions. The first system (Invitrogen) uses the human U6 promoter to drive shRNA expression. The following sequences were targeted: fatty acid-binding protein-5 (FABP5), 5'-GGA-GAGAAGTTTGATGAAACG-3' (previously described as sh242; Witting *et al.*, 2008); scrambled control (SCR), 5'-GAG-AGTATAAGGAGGTCAAGT-3' (Witting *et al.*, 2008); LacZ (5'-CTACACAAATCAGCGATT; sequence provided in the BLOCK-iT U6 kit); and sterol regulatory element-binding protein-1 (SREBP-1), 5'-GGTTGTGGACACAGACAAACT-3', 5'-GCTTCTAACCTGGCACTAAGT-3', 5'-GGAGGACATCTTGCTGCTTCT-3', and 5'-GCTGGCCAATGGACTACTAGT-3'. The second system (GenScript) uses the H1 promoter. Three constructs were generated to target SREBP-1: 5'-TTGGTTGTGGACACAGACAAA-3', 5'-GATATCTGCAGTTGCTAAATA-3', and 5'-ACCAACTGGCAGTTCATTGA-3', and a scrambled sequence was used as control: 5'-ATAGTTGTCGTTGCACTCGA-3'.

To test the efficacy of the SREBP-1 shRNA constructs, a plasmid expressing the target gene was generated. Mouse SREBP-1 cDNA was obtained from the American Type Culture Collection (Manassas, VA) and cloned into plasmid pEF1 α -SREBP1, containing the elongation factor-1 α promoter and bovine growth hormone polyadenylation signal (Morrall *et al.*, 2002), by blunt-end ligation. HEK-293 cells were obtained from Microbix Biosystems (Toronto, ON, Canada) and cultured in MEM α supplemented with 10% fetal bovine serum. To test shRNA efficacy, HEK-293 cells were seeded in 6-well plates at 1.0×10^6 cells per well and the next day were transfected with 0.75 μ g of pEF1 α -SREBP1 and 1.5 μ g of shRNA-expressing plasmid. METAFECTENE reagent (Biontex Laboratories, Martinsried/Planegg, Germany) was used at a DNA-to-lipid ratio of 1:3. Cells were harvested after 24 hr and lysed in modified radioimmunoassay (RIPA) buffer containing protease inhibitors (50 mM Tris, 150 mM NaCl,

1 mM EDTA, 0.25% [w/v] deoxycholate, 1% Triton X-100, 1 μ M phenylmethylsulfonyl fluoride [PMSF], aprotinin/leupeptin/pepstatin [1 μ g/ml each]; pH 7.5). Western blot analysis was carried out to determine the level of SREBP-1 silencing (data not shown). Sequences 5'-GGTTGTGGACACAGACAAACT-3' (U6 promoter) and 5'-ACCAACTGGCAGTTCATTGA-3' (H1 promoter) gave the highest level of knockdown. The expression cassette was amplified by polymerase chain reaction (PCR), using primers binding the flanking regions, and subsequently cloned into the *Asc*I restriction site of plasmid pRES. Clones were sequenced before being used for rescue of helper-dependent adenoviral vectors (see the next section).

Helper-dependent adenoviral vector production

Helper-dependent adenoviral vectors were generated with a *Cre-loxP* system (Microbix Biosystems) (Chen *et al.*, 1996; Parks *et al.*, 1996; Sandig *et al.*, 2000). Cloning of expression cassettes into plasmid pC4HSU was carried out by homologous recombination in *Escherichia coli* BJ5183 (Youil *et al.*, 2001). Briefly, plasmid pRES was created by cloning a 3.2-kb *Hind*III fragment from pC4HSU into pBluescript II-SK (Stratagene, La Jolla, CA). All expression cassettes were cloned into pRES, as described previously, and the resulting plasmids were digested with *Hind*III. Simultaneously, plasmid pC4HSU (Sandig *et al.*, 2000) was digested with *Asc*I. *Escherichia coli* BJ5183 cells were cotransformed with the linearized plasmid (100 ng) and each of the *Hind*III fragments (300 ng), as described previously (Youil *et al.*, 2001). Helper-dependent adenovirus plasmids containing expression cassettes were generated through recombination between homologous regions.

To rescue the helper-dependent adenoviral vectors, pC4HSU plasmids containing expression cassettes were digested overnight with *Pme*I to remove the bacterial sequence, and DNA was purified with QIAEX II (Qiagen, Valencia, CA). Five micrograms of *Pme*I-digested plasmid DNA was used to transfect 293Cre4 cells, so that approximately 40–50% cells were positive. This minimized the number of passages needed to reach plateau level (Parks *et al.*, 1996). To monitor the amplification process, a pC4HSU construct expressing green fluorescent protein (pC4HSU-GFP) was used in parallel. Twenty-four hours later, helper H14 was added at a multiplicity of infection (MOI) of 3. This MOI was determined in a separate experiment to result in >95% transduction and a low level of helper contamination (< 2.0×10^6 plaque-forming units [PFU]/ml) in cell lysates harvested at the onset of >95% cytopathic effect (CPE). To monitor the percentage of cells producing vector at each passage, we used the vector expressing GFP. We rationalized that maximal vector production occurs when all cells are infected with at least one vector and one helper particle. After three consecutive passages in 6-cm dishes (5×10^6 cells per dish), >95% cells expressed GFP, indicating that maximal vector production was achieved. For large-scale vector amplification 15-cm dishes (2.0×10^7 cells per dish) were used followed by 500-cm² flasks ($\sim 1 \times 10^9$ cells total). A critical point was to use the appropriate dilution factor when escalating to larger dishes, as diluting the lysate excessively resulted in low yields or no vector production (data not shown). To confirm that >95% of 293Cre4 cells produced the vector, separate dishes were used to monitor GFP expression. The virus was purified by one-step gradient

centrifugation (CsCl at 1.25 and 1.40 g/ml; two SW28 tubes) for 2 hr at 22,500 rpm, followed by one isopycnic separation (CsCl at 1.33 g/ml; four SW40 tubes) at 35,000 rpm for 36–42 hr. The helper-dependent adenovirus band (upper) was collected, placed in dialysis cassettes (Pierce Biotechnology, Rockford, IL), and dialyzed in TMNG buffer (10 mM Tris-HCl [pH 7.5], 1 mM MgCl₂, 150 mM NaCl, 10% glycerol) for 4–5 hr at 4°C (dialysis buffer was replaced with fresh cold buffer every hour). Total particle counts were determined spectrophotometrically after particle disruption with 0.1% sodium dodecyl sulfate (SDS) (absorbance at 260 nm [A_{260}] = 1 corresponds to 1.1×10^{12} particles/ml) (Maizel *et al.*, 1968). The level of contamination with helper was determined by plaque assay.

Animals

Male 8-week-old C57BL/6J and *db/db* mice were obtained from The Jackson Laboratory (Bar Harbor, ME). Animal care guidelines set forth by the Indiana University School of Medicine (Indianapolis, IN) were followed. Mice were kept in a BSL2 facility and had free access to a standard chow diet and water at all times. At the time points indicated in text, the animals were killed and tissues were collected and snap-frozen in liquid nitrogen or fixed in 10% buffered formalin for histological analysis.

Western blotting

Lysed HEK-293 cells were centrifuged at 12,000×g and the supernatant was used for Western blot analysis, as previously described (Witting *et al.*, 2008). To generate liver protein extracts, 150–200 mg of frozen liver was homogenized in modified RIPA buffer containing protease inhibitors. Liver extracts were centrifuged at 12,000×g, the fat layer was carefully aspirated, and the supernatant was collected for use in Western blotting. Approximately 40 µg were separated in 4–15% SDS-PAGE Criterion gels (Bio-Rad) and transferred to 0.2-mm polyvinylidene difluoride (PVDF) membranes (Bio-Rad, Hercules, CA). Antibodies to SREBP-1 were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA). Blots were developed with Immun-Star (Bio-Rad) and exposed to enhanced chemiluminescence (ECL) film (GE Healthcare, Piscataway, NJ). Densitometric analysis was carried out with LabWorks 4.6 software (UVP, Upland, CA).

Histology

Four-micron-thick sections were cut from routinely processed paraffin-embedded tissue and stained with hematoxylin and eosin for histological analysis. Processing and staining were performed at the Immunohistochemistry Core (IHC) at Indiana University Medical Center (Indianapolis, IN).

Liver enzymes

Levels of aspartate aminotransferase (AST) in serum were measured with a kit from Pointe Scientific (Canton, MI), in accordance with the manufacturer's instructions.

Real-time PCR

Amplification of helper-dependent vector genomes was carried out by real-time PCR using primer pair pSHL-F

(5'-GCGTGCCAGACAAAAGGAAAG-3') and pSHL-B (5'-CACTCCAGCAGCCCAGAATC-3'). The glucokinase gene was used as loading control, using primer pair GK-F (5'-GTTTCGGAGGGACTGCATGG-3') and GK-B (5'-ACGAGCCTGAGCAGCACAAG-3'). Real-time PCR was performed with an ABI PRISM 7500 instrument (Applied Biosystems, Foster City, CA), and the ABI Power SYBR Green kit, in accordance with the manufacturer's protocol and using a 0.5 mM concentration of each primer. Primer pairs were designed to amplify a fragment of approximately 350–375 bp and were first tested to yield a single PCR product based on the melting curve and confirmation by agarose gel electrophoresis. A standard curve was generated by spiking DNA, isolated from a mouse treated with vehicle, with known amounts of pSHL-GFP plasmid to generate a standard curve with 0.01, 0.1, 1, 10, and 100 copies per cell. DNA levels in standards and test samples were measured by analyzing 30 ng of DNA, in duplicate.

Results and Discussion

Production of shRNA-expressing helper-dependent adenoviral vectors

To generate reproducible yields with each of the vectors rescued, we used the protocol described in Materials and Methods. Using this strategy, titers of the first large-scale preparation were $3\text{--}7 \times 10^{11}$ viral particles (VP)/ml, and overall yields were $7\text{--}28 \times 10^{11}$ VP, similar to those obtained for transgene-expressing vector gAd.Alb-mal1 (Table 1). None of these preparations showed the presence of rearrangements, based on DNA analysis using restriction enzymes (Fig. 1).

To confirm that the shRNA sequence is stable during vector propagation, the first preparation (lot 1) was used as a stock to

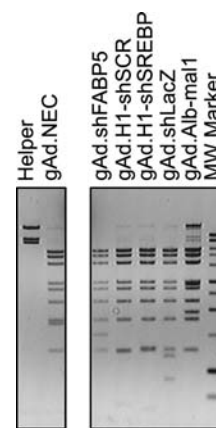


FIG. 1. DNA structure of helper-dependent vectors. To confirm the molecular structure of the viral particles, DNA was isolated and digested with the restriction enzyme *Bam*HI. Bands corresponding to the pattern of helper H14 DNA were barely seen in the vectors, suggesting the level of helper contamination was low. Vector gAd.Alb-mal1 had slightly higher levels of H14 because its expression cassette is approximately 3 kb larger than the shRNA cassette, resulting in a construct with a DNA molecular mass closer to that of the helper (~31 and 36 kb, respectively). After isopycnic centrifugation the vector and helper bands were closer, making collection of the helper-dependent vector band more difficult.

TABLE 1. HELPER-DEPENDENT VECTOR PRODUCTION

Vector	Titer (particles/ml)	Total yield (particles)	Helper Contamination (PFU/ml)	Lot used for infection
gAd.shFABP5-1	2.8×10^{11}	7.7×10^{11}	N/D	
gAd.shFABP5-2	5.2×10^{11}	1.9×10^{12}	N/D	Lot 1
gAd.shFABP5-3	4.1×10^{11}	1.4×10^{12}	7.8×10^6	Lot 1
gAd.shFABP5-4	6.1×10^{11}	1.8×10^{12}	7.8×10^6	Lot 1
gAd.shFABP5-5	3.4×10^{11}	9.4×10^{11}	N/D	Lot 3
gAd.shFABP5-8	7.4×10^{11}	1.3×10^{12}	N/D	Lot 3
gAd.shFABP5-9	1.3×10^{12}	4.1×10^{12}	N/D	Lot 8
gAd.shFABP5-10	7.2×10^{11}	2.2×10^{12}	N/D	Lot 8
gAd.shSCR-1	6.7×10^{11}	2.5×10^{12}	8×10^8	
gAd.shSCR-2	8.6×10^{11}	2.4×10^{12}	8×10^8	Lot 1
gAd.shSCR-3	5.0×10^{11}	1.7×10^{12}	N/D	Lot 1
gAd.shSCR-4	8.8×10^{11}	3.5×10^{12}	N/D	Lot 1
gAd.shSCR-5	9.7×10^{11}	2.4×10^{12}	N/D	Lot 4
gAd.shSCR-6 ^a	5.0×10^{11}	1.3×10^{12}	N/D	Lot 4
gAd.shSCR-12	7.4×10^{11}	2.3×10^{12}	N/D	Lot 11
gAd.shSCR-13	6.9×10^{11}	2.1×10^{12}	N/D	Lot 11
gAd.shSCR-14	5.8×10^{11}	1.3×10^{12}	N/D	Lot 11
gAd.shLacZ-1	3.3×10^{11}	1.3×10^{12}	3.3×10^8	
gAd.shLacZ-2	5.6×10^{11}	1.4×10^{12}	2.6×10^7	Lot 1
gAd.shLacZ-3 ^a	4.7×10^{11}	8.0×10^{11}	N/D	Lot 2
gAd.shLacZ-5	7.4×10^{11}	1.6×10^{12}	4.5×10^7	Lot 2
gAd.shLacZ-6	4.6×10^{11}	7.3×10^{11}	4.5×10^7	Lot 2
gAd.shSREBP-1	6.655×10^{11}	2.86×10^{12}	N/D	
gAd.shSREBP-2	1.265×10^{12}	4.048×10^{12}	N/D	Lot 1
gAd.shSREBP-3	7.205×10^{11}	2.52×10^{12}	N/D	Lot 2
gAd.shSREBP-4	4.29×10^{11}	1.54×10^{12}	N/D	Lot 3
gAd.H1shSREBP-1	3.5×10^{11}	5.3×10^{12}	3.2×10^7	
gAd.H1shSREBP-2	1.0×10^{12}	3.0×10^{12}	1.2×10^8	Lot 1
gAd.H1shSCR-1	3.1×10^{11}	1.7×10^{12}	7.8×10^7	
gAd.H1shSCR-2	1.6×10^{12}	3.5×10^{12}	1.1×10^9	Lot 1
gAd.Albmal1-1	2.5×10^{11}	4.8×10^{11}	N/D	
gAd.Albmal1-2	7.5×10^{11}	2.6×10^{12}	N/D	Lot 1
gAd.Albmal1-3	1.7×10^{12}	6.0×10^{12}	N/D	Lot 2

Abbreviation: N/D, not determined.

^ashRNA expression cassette from this preparation was sequenced.

generate subsequent lots. The yield obtained from lot 1 was similar to or slightly lower than that obtained in preparations generated by infection of cells with purified virus, suggesting that a high yield can be obtained from the first large-scale preparation (Table 1). The stability of the vector genome was maintained in preparations generated with higher lot numbers, such as lots 4 and 8 (gAd.shSCR and gAd.shFABP5, respectively) (Table 1). No rearrangements have been observed in any of these preparations (data not shown). Helper contamination in all preparations tested was 0.1% or lower (PFU:VP). Sequencing of selected vector preparations confirmed that no alterations of the shRNA expression cassette were introduced during vector production (Table 1).

In vivo administration of shRNA-expressing vectors

To determine the efficacy at knocking down the target gene *in vivo*, mice were intravenously injected with 2.0×10^{11} or 1.0×10^{11} VP of gAd.shSREBP1 (to target SREBP-1) or gAd.shSCR (expressing a scrambled sequence). One week after vector administration >90% gene silencing was observed (Fig. 2A), regardless of the dose of gAd.shSREBP1 received. The amount of vector DNA present in the liver was estimated by real-time PCR. Approximately 40 and 20 copies

per cell were present in the liver of mice that received 2.0×10^{11} and 1.0×10^{11} VP, respectively (Fig. 2B). We have previously observed that there is a limit to the level of gene silencing that can be achieved in liver, and that once it is reached, increasing the dose of vector does not translate into a higher level of knockdown (Witting *et al.*, 2008). The current data confirm this observation with an independent gene target. The presence of unnecessarily high shRNA levels may activate the interferon response and induce toxic effects (Witting *et al.*, 2008). Thus, when conducting silencing studies *in vivo*, it is advisable to carry out pilot dose-response experiments to determine the lowest vector dose that results in the highest gene silencing.

shRNA-expressing helper-dependent vectors for drug target validation

To determine whether robust gene silencing can be achieved for several weeks with helper-dependent adenoviral vectors, we tested the gAd.shSREBP1 vector in an animal model of obesity and type 2 diabetes, the *db/db* mouse (leptin receptor deficient). SREBP-1 is transcriptionally regulated by insulin, and compared with control lean animals its levels are increased in *db/db* mice as a result of the hyperinsulinemia

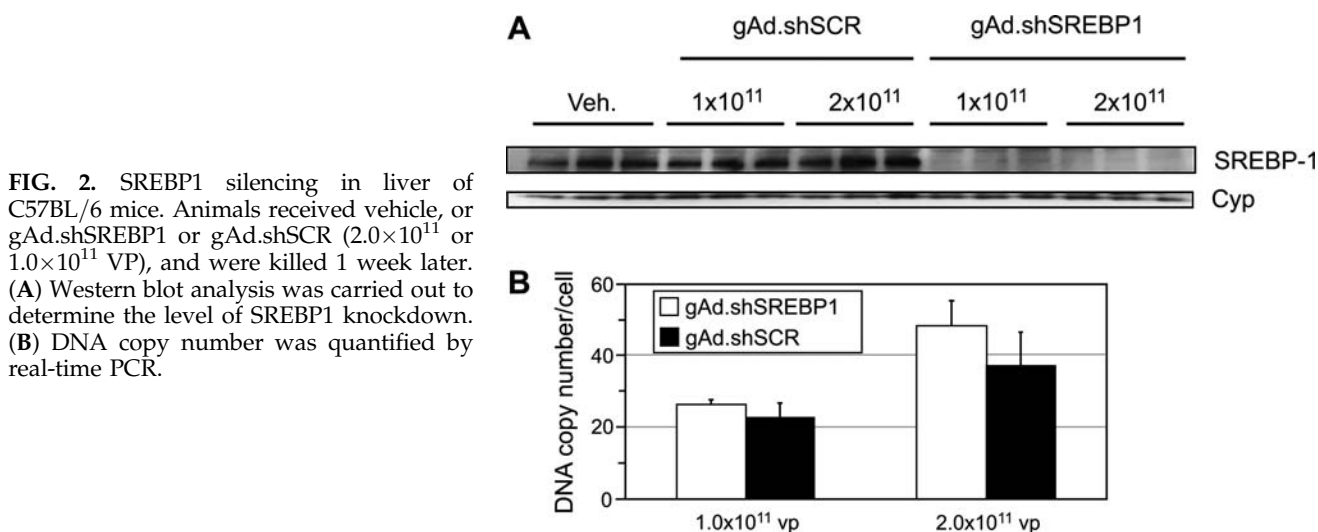


FIG. 2. SREBP1 silencing in liver of C57BL/6 mice. Animals received vehicle, or gAd.shSREBP1 or gAd.shSCR (2.0×10^{11} or 1.0×10^{11} VP), and were killed 1 week later. (A) Western blot analysis was carried out to determine the level of SREBP1 knockdown. (B) DNA copy number was quantified by real-time PCR.

developed in these animals (Shimomura *et al.*, 1999, 2000) (Fig. 3A). Reduction of gene expression in adult mice by RNAi should allow study of the metabolic implications of such treatment and may provide the proof of concept that SREBP-1 is a useful drug target. Groups of eight *db/db* mice received 1.0×10^{11} VP of gAd.shSREBP1 or gAd.shSCR and were killed 3 weeks later. As shown in Fig. 3B, this treatment resulted in > 90% gene silencing, similar to what was observed in C57BL/6 mice after 1 week. Interestingly, despite the difference in body weight between the C57BL/6 mice (21 ± 1 g) and *db/db* mice (36 ± 2.2 g), and the fact that these mice have higher SREBP-1 levels than lean mice, 1.0×10^{11} VP reduced

target gene expression to a similar extent. These data suggest that the increase in body weight in *db/db* mice, which is largely the result of an increase in adipose tissue mass, does not have an impact on the amount of adenoviral vector that transduces the liver. Moreover, the level of shRNA expression is sufficient to knock down SREBP-1 to a degree comparable to that in lean mice, despite the higher level of transcript in the liver. Concomitant with a decrease in SREBP1 levels, liver weight was reduced (1.8 ± 0.2 vs. 2.6 ± 0.2 g; $p < 0.01$) and the gain in body weight over the 3-week period was significantly lower for the gAd.shSREBP1 group compared with control-treated animals (4.3 ± 2.6 vs. 8.9 ± 1.8 g; $p < 0.01$). Metabolic

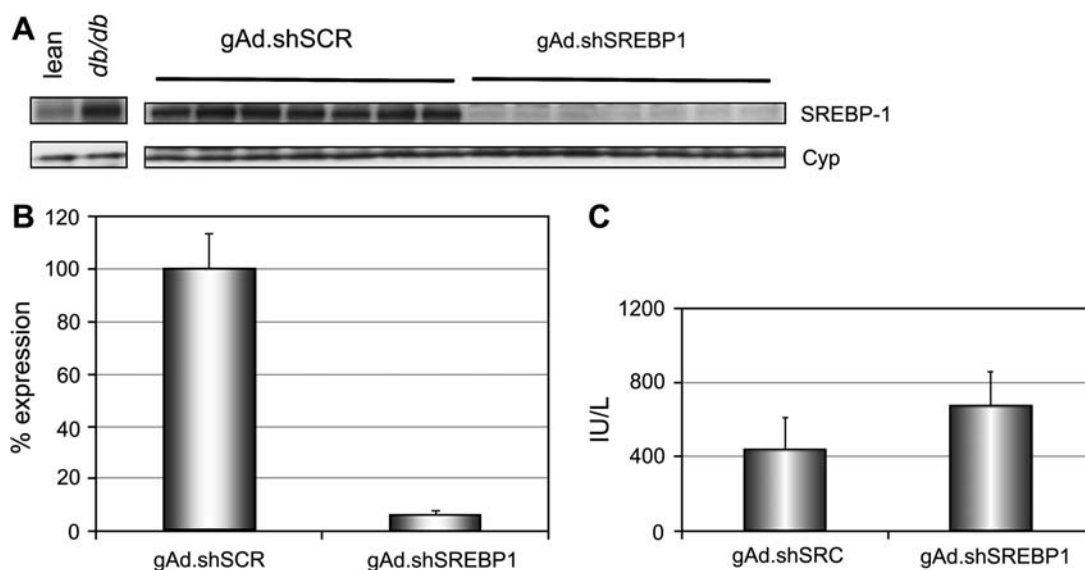


FIG. 3. SREBP1 silencing in liver of *db/db* mice. Mice received 1.0×10^{11} VP of gAd.shSREBP1 or gAd.shSCR, and were killed 3 weeks later. (A) Western blot analysis was used to determine the level of knockdown. *Left:* *db/db* mice have higher levels of SREBP-1 compared with lean (C57BKS) mice. *Right:* *db/db* mice administered gAd.shSREBP1 vector display a 90% reduction in SREBP-1 levels compared with *db/db* animals that received the gAd.shSCR vector. (B) Densitometric analysis of Western blot. (C) Aspartate aminotransferase (AST) levels in the serum of mice 3 weeks after gene transfer. No significant difference was observed between the two groups. SREBP-1, sterol regulatory element-binding protein-1; Cyp, cyclophilin.

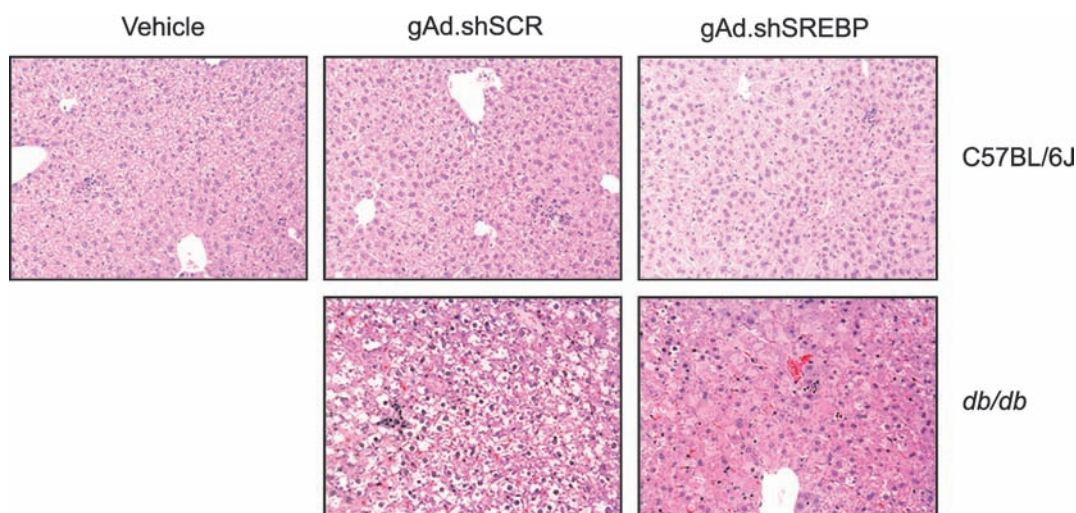


FIG. 4. Liver histology. Mice were treated as described in the captions to Figs. 2 and 3. Four-micron-thick sections were cut from routinely processed paraffin-embedded tissue and stained with hematoxylin and eosin for histological analysis. Livers of mice treated with the gAd.shSREBP1 vector displayed lower levels of glycogen and lipid droplets (R. Ruiz, unpublished data). Processing and staining were performed as described in Materials and Methods. None of the groups had signs of toxicity related to expression of shRNA.

analysis of the impact of reducing hepatic SREBP-1 is reported elsewhere (R. Ruiz, unpublished data).

Maintenance of hepatic gene silencing on day 21 is a remarkable observation given that expression of shRNA using first-generation adenoviral vectors has resulted in complete loss of silencing by day 24 (Narvaiza *et al.*, 2006). This would be anticipated on the basis of the fact that this type of vector is a strong activator of cytotoxic immune responses, leading to loss of liver cells within a few weeks (Yang *et al.*, 1994). In addition, adeno-associated virus (AAV)-mediated expression of shRNA has resulted in the death of animals at high doses of vector (Grimm *et al.*, 2006), which did not occur with our system. None of our animals displayed signs of distress or sickness for the duration of the study. Furthermore, no signs of toxicity were observed on the basis of histochemical analysis of liver sections and AST levels (Figs. 3C and 4). Thus, when using helper-dependent vectors, it is possible to achieve robust gene silencing for at least 3 weeks. Similar results were observed in a study using this type of vector to knock down huntingtin expression in the brain (Huang *et al.*, 2007).

In conclusion, helper-dependent adenoviral vectors containing shRNA expression cassettes can be stably propagated over multiple passages, without genome rearrangements. A critical step in the process to ensure successful rescue and production is to monitor the percentage of cells producing vector by simultaneously amplifying a GFP-expressing vector and infecting >95% cells with vector and helper to ensure efficient production. Developments in helper-dependent vector production will allow for further reductions of helper contamination, minimization of replication-competent adenovirus (RCA) generation, and increases in overall yields (Schiedner *et al.*, 2000; Barjot *et al.*, 2002; Chamberlain *et al.*, 2003; Palmer and Ng, 2003; Sakhuja *et al.*, 2003). Generating transgenic animals for drug development is time-consuming and the breeding process is expensive. In addition, in type 2 diabetes studies silencing gene function in an adult animal (instead of at the stem cell level) provides a model that is more

relevant to the human disease. We have provided evidence that by using helper-dependent adenoviral vectors, a high level of silencing is possible for several weeks in an animal model of obesity and type 2 diabetes. This system offers the possibility to conduct experiments in adult animals, representing an alternative to traditional transgenic animal technologies. Thus, a highly valuable use of helper-dependent vectors is in RNAi-mediated functional genomic and target validation studies.

Acknowledgments

The authors thank Drs. Sheila Connelly and P. Seshidhar Reddy for technical advice on plasmid recombination in *E. coli* BJ5183 cells; the Immunohistochemistry Core (IHC) at Indiana University Medical Center for hematoxylin–eosin staining of liver sections; and Dr. Palmiter for kindly providing the albumin promoter. This work was supported by grants from the NIDDK (DK069432 and DK078595), American Diabetes Foundation, and INGEN (Indiana Genomics Initiative of Indiana University supported in part by Lilly Endowment), and by the American Heart Association (post-doctoral fellowship to S.R.W.).

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Received for publication June 18, 2008;
accepted after revision September 30, 2008.

Published online: January 14, 2009.