Targeting Visceral Fat by Intraperitoneal Delivery of Novel AAV Serotype Vector Restricting Off-Target Transduction in Liver

Wei Huang,1,2 Xianglan Liu,1,2 Nicholas J. Queen,1,2 and Lei Cao1,2

1Department of Cancer Biology and Genetics, The Ohio State University, Columbus, OH 43210, USA; 2The Comprehensive Cancer Center, The Ohio State University, Columbus, OH 43210, USA

It is challenging to genetically manipulate fat in adults. We demonstrate that intraperitoneal (i.p.) injection of an engineered adeno-associated virus (AAV) serotype Rec2 leads to high transduction of multiple visceral fat depots at a dose of 1 to 2 orders lower than commonly used doses for systemic gene delivery. To target adipose tissue, we develop a single AAV vector harboring two expression cassettes: one using the CBA promoter to drive transgene expression and one using the liver-specific albumin promoter to drive a microRNA-targeting WPRE sequence that only exists in this AAV vector. This dual-cassette vector achieves highly selective transduction of visceral fat while severely restricting off-target transduction of liver. As proof of efficacy, i.p. administration of an adipose-targeting Rec2 vector harboring the leptin gene corrects leptin deficiency, obesity, and metabolic syndromes of ob/ob mice. This study provides a powerful tool to genetically manipulate fat for basic research and gene therapies of genetic and acquired diseases.

INTRODUCTION

Adipose tissue is a multifunctional organ that modulates whole body metabolic homeostasis. Germline genetic manipulation based on transgenic techniques is often used for experimental studies of adipose tissue, but some research and therapeutic applications require adipose manipulation with gene delivery at a certain age. In this regard, viral vectors have become an attractive alternative delivery vehicle, in particular, recombinant adeno-associated virus (rAAV) vectors, because they can transduce both dividing and postmitotic tissues, with low immunogenicity and long-lasting transgene expression without stimulating a cell-mediated immune response.1 rAAVs have gained more attention for their successful applications in clinical studies.3–5 Modifications of the capsid or expression cassette are employed to improve efficiency of gene delivery and transgene tropism with either local or systematic delivery. For example, selecting more efficient naturally occurring serotypes plus the use of an adipose-tissue-specific promoter or microRNA-targeting sequence enable specific transduction of adipose tissue but require a high dose to achieve therapeutic effects.3–5 We recently demonstrated that a novel engineered hybrid serotype Rec2 vector leads to high transduction of adipose tissue that is superior to naturally occurring serotypes (AAV1 and AAV8) and other engineered serotypes (Rec1, Rec3, and Rec4) when the vectors are injected directly to the fat pads at a dose 1 to 2 orders lower than those used in previously reported studies.7–10 Interestingly, the administration route substantially influences the tropism and efficacy of the Rec2 vector. Intravenous (i.v.) administration of the Rec2 vector primarily transduces liver at doses ranging from 2 × 1010 to 2 × 1011 vg per mouse.11 In contrast, oral administration of the Rec2 vector leads to preferential transduction of brown fat, with absence of transduction in the gastrointestinal tract at doses lower than 2 × 1010 vg per mouse.12

Obesity and body fat distribution are important risk factors for type II diabetes and other metabolic syndromes. Visceral adipose tissue (VAT) plays distinctive roles in metabolic homeostasis and disturbance. The type of obesity characterized by increased VAT is strongly associated with adverse metabolic outcomes, whereas accumulation of subcutaneous fat is thought to have neutral or even beneficial effects on metabolism.13–15 This relates to intrinsic functional differences of adipocytes in different depots, including insulin sensitivity, glucose uptake, rate of lipolysis, and adipokine and cytokine secretion.14,15 Thus, a gene delivery tool targeting visceral fat has great potentials for applications in basic research and gene therapy. In this study, we examined a new administration route of Rec2 vector, intraperitoneal (i.p.) administration, and developed a novel strategy to achieve adipocyte-preferential transgene expression by restricting off-target transgene expression in the liver. Furthermore, we conducted a proof-of-concept study in an animal model of human disease: congenital leptin deficiencies. The adipocyte-derived hormone leptin...
is a key factor in energy homeostasis. Mutations in leptin or its receptor cause massive obesity in mice and humans, and leptin is an approved treatment for leptin-deficient obesity patients as well as generalized lipodystrophy.16,17 The primary model for leptin therapy development is the \textit{ob/ob} mouse, a homozygous mutant in the leptin (Lep or \textit{ob} gene) gene. The \textit{ob/ob} mouse in the absence of leptin rapidly develops severe obesity and metabolic syndromes.18,19 In fact leptin-deficient \textit{ob/ob} mice show abnormalities in almost all physiologic systems.17,20 We used \textit{ob/ob} mice to assess the therapeutic potential of this new adipose-preferential vector system.

RESULTS

Efficient Transduction of VAT via i.p. Administration of Rec2 Vector

To improve gene delivery to VAT, we first determined whether the Rec2 vector, through i.p. administration, a simple and less invasive procedure, could efficiently transduce VAT. Rec2 vector carrying a luciferase reporter gene driven by the CMV enhancer and chicken \(\beta\)-actin (CBA) promoter was i.p. injected to male BALB/c mice at a dose of \(2 \times 10^{10}\) vg per mouse, which is similar to the doses previously used in i.v. injection, oral administration, or direct fat pad injection.7,12 2 weeks post-Rec2 administration, mice were subjected to in vivo bioluminescence measurement, and strong luciferase activity was observed primarily in the abdomen (Figure 1A). To confirm the efficacy of the Rec2 vector via i.p. administration, we generated a Rec2 vector to deliver another reporter gene GFP and tested three doses (5 \(\times\) \(10^8\), \(2 \times 10^9\), and \(1 \times 10^{10}\) vg per mouse). 2 weeks post Rec2 injection, tissues were harvested and GFP fluorescence was measured in fresh tissue lysates. Rec2-GFP at the dose of \(1 \times 10^{10}\) vg per mouse resulted in robust GFP expression in all visceral fat depots examined, including epididymal (eWAT), mesenteric (mWAT), and retroperitoneal (rWAT) (Figure 1B). GFP fluorescence was also detected in the liver but not in the intestine (Figure 1B). Immunohistochemistry confirmed the transgene expression in VAT and liver (Figure 1C). No GFP fluorescence was observed in any tissues from mice receiving a very low dose of \(5 \times 10^8\) vg per mouse.

Figure 1. i.p. Administration of Rec2 Vector Transduces Multiple Visceral Fat Depots

(A) Representative in vivo bioluminescence imaging of luciferase 2 weeks after i.p. Rec2-luciferase vector injection (2 \(\times\) \(10^{10}\) vg per mouse). (B) GFP content in tissue lysates of mice receiving Rec2-GFP (1 \(\times\) \(10^{10}\) vg per mouse) 2 weeks after i.p. injection. (C) GFP immunohistochemistry in mice receiving Rec2-GFP (1 \(\times\) \(10^{10}\) vg per mouse). Scale bar, 100 \(\mu m\).

Preferential Transduction of VAT Using a Dual-Cassette Rec2 Vector Restricting Off-Target Transgene Expression in Liver

To prevent transgene expression in the liver, we generated a new AAV expression plasmid that contained two expression cassettes: one used the CBA promoter to drive the transgene, and the other used the liver-specific albumin promoter21–24 to drive a specific microRNA-targeting woodchuck posttranscriptional regulatory element (WPRE) sequence, which only exists in the transgene expression cassette (Figure 2A). WPRE enhances transgene expression5,26 and is incorporated in all of our AAV expression plasmids. The microRNA-targeting WPRE (miR-WPRE) knocked down transgene expression more than 90% in vitro, as measured by qRT-PCR and ELISA. The efficacy of this dual-cassettes vector (named AS/Rec2) was assessed...
by i.p. injection of AS/Rec2-GFP to wild-type (WT) mice. Incorporation of the liver-restricting cassette severely decreased transgene expression in liver at the higher dose of $2 \times 10^{10}$ vg per mouse (Figure 2B). At the lower dose of $1 \times 10^{10}$ vg per mouse, GFP fluorescence was undetectable in the liver, whereas substantial GFP fluorescence was maintained in eWAT (Figure 2B). To further characterize the AS/Rec2 vector, we performed a dose-response experiment with four doses: $1 \times 10^{10}$, $2 \times 10^{10}$, $3 \times 10^{10}$, and $4 \times 10^{10}$ vg per mouse. Across the range of doses, hepatic transgene expression was severely suppressed, even though a large amount of viral vector DNA was detected in the liver (Figures 2C and 2D), confirming the efficacy of the dual-cassette vector. GFP fluorescence was undetectable in the small
Molecular Therapy of Congenital Leptin Deficiency via i.p. Administration of AS/Rec2-Leptin Vector

To investigate the therapeutic potential of the AS/Rec2 vector, we generated the AS/Rec2-leptin vector to treat a leptin-deficient obesity model of ob/ob mice with AS/Rec2-GFP as control. Male ob/ob mice, 6 weeks of age, were randomized to receive an i.p. injection of AS/Rec2-leptin or AS/Rec2-GFP, $4 \times 10^{10}$ vg per mouse. ob/ob mice at 6 weeks of age were obese compared to age-matched WT mice. AS/Rec2-GFP-treated ob/ob mice continued to gain weight rapidly and become morbidly obese (Figures 3A–3C). In contrast, AS/Rec2-leptin treatment completely prevented weight gain and normalized body weight close to age-matched WT mice 4 weeks post AS/Rec2-leptin injection (Figures 3A–3C and 4C). The weight loss was stable and sustained throughout the entire 9-week duration of the study. A sharp drop of food intake was observed in AS/Rec2-leptin-treated mice as early as 1-week post AAV administration, and the reversal of hyperphagia of ob/ob mice was maintained similarly to the weight loss (Figure 3D). AS/Rec2-leptin treatment also corrected the abnormal low body temperature of ob/ob mice and impaired thermogenesis in response to fast (Figure 3E). 4 weeks post-Rec2 administration, body composition was determined using EchoMRI. Adiposity was
decreased by 65%, whereas lean mass was increased by 75% in AS/Rec2-leptin-treated mice compared to GFP mice (Figure 3F). Serum leptin level was measured using ELISA 4-weeks post AAV administration. AS/Rec2-leptin treatment resulted in a circulating leptin level slightly lower than that in age-matched WT mice, with no statistical significance (Figure 4A), whereas leptin was undetectable in GFP-treated \( \text{ob/ob} \) mice. AS/Rec2-leptin treatment completely rescued the impaired glycemic control of \( \text{ob/ob} \) mice (Figure 4B). In fact, blood glucose level in AS/Rec2-leptin-treated \( \text{ob/ob} \) mice was even lower than that in WT mice at a few time points of the glucose tolerance test (Figure 4B). Indirect calorimetry was performed 5 weeks after Rec2 administration. AS/Rec2-leptin-treated mice showed increased oxygen consumption and locomotor activity in both the dark and light phases (Figures 4E and 4F), consistent with elevated energy expenditure. In contrast, food intake was lower in AS/Rec2-leptin-treated mice (Figure 4D), similar to the findings in the home cage (Figure 3D).

At sacrifice 9 weeks after AAV injection, eWAT of AS/Rec2-leptin-treated mice was decreased by 82.5% compared to GFP-treated
mice when calibrated to body weight (Figure 5A). Liver mass was also decreased (Figure 5A), and the liver steatosis was completely reversed by AS/Rec2-leptin treatment (Figure 5C). Moreover, AS/Rec2-leptin treatment completely corrected the hyperinsulinemia of ob/ob mice (Figure 5B). Transgene expression in visceral fat depots was confirmed in AS/Rec2-GFP mice, whereas minimal GFP fluorescence was detected in the livers (Figure 5D).

In a separate experiment, unrestricted single cassette Rec2-leptin vector was i.p. injected to ob/ob mice at the dose of $2 \times 10^{10}$ vg per mouse, and tissues were collected at the same time point of the dual cassette AS/Rec2-leptin experiment. Both unrestricted and liver-restricted Rec2-leptin vectors resulted in high-level leptin in eWAT (Figure 6A) and circulating leptin levels equivalent to that of age-matched WT mice (Figure 6C). In contrast, leptin level in the liver was robustly decreased in mice receiving liver-restricted dual cassette vector, even at a 2-fold higher dose ($4 \times 10^{10}$ vg per mouse) compared to the unrestricted single cassette group (Figure 6B).

**DISCUSSION**

It is challenging to adapt traditional techniques to selectively modulate adipose functions in vivo for experimental and therapeutic applications. A few groups have targeted adipose tissue with naturally occurring AAV serotypes by direct injection to fat depots often requiring high doses.3-5 We previously report that an engineered hybrid serotype Rec2 displays superior transduction of both brown adipose tissue (BAT) and subcutaneous WAT (SAT) compared to AAV1 or AAV8.7 Direct injection of Rec2 vector at a moderate dose ($1 \times 10^{9}$ to $1 \times 10^{10}$ vg per fat depot) is sufficient to modulate the function of the targeted fat depot (BAT or SAT) and result in systemic metabolic changes.7-10 Rec2 vector is efficient for both overexpression and knockdown applications.7-10,12,27 However, up to date, scarce studies report systemic administration of AAV to transduce multiple adipose depots. One study reports that i.v. administration of the AAV8 vector transduces adipose tissues, liver, and other tissues.6 Replacing the CMV promoter with the human adiponectin enhancer/promoter enhances specificity to adipose tissue, whereas transgene expression is not suppressed in liver. Incorporation of the miR-122 target sequence (miR-122T) into the 3’ UTR of the AAV vector can eliminate hepatic transgene expression, but often at the expense of lower transgene expression in adipose tissue.6 In addition, the dose of $1 \times 10^{12}$ vg per mouse used in this study is approximately 100-fold higher than the dose of Rec2 vector direct fat injection.6,7 In this work, we designed a novel dual-cassette AAV expression plasmid and combined this liver-restricting strategy with the adipo-trophic Rec2 serotype and i.p. administration to achieve, for the first time, selective transduction of multiple visceral fat depots. More encouraging is the low dose for i.p. administration of this new AAV vector system that is equivalent to direct fat injection and 2 orders lower than the reported i.v. administration of the AAV8-based adipose-targeting system.6

To enhance tissue-preferential transduction, particularly when using systemic administration, miR-122T is often used to suppress off-target transgene expression in the liver because miR-122 is highly abundant in the liver.6,28-31 However, this approach might cause toxicities, at least theoretically, because exogenous miR-122T inside a highly expressed hepatocyte may compete with endogenous miR-122T for miR-122. Liver toxicities, including hepatic steatosis, hepatitis, or hepatocellular carcinoma, have been reported in miR-122 knockout mice.32 Although this risk is very low,28,30 an alternative approach to eliminate the risk improves safety for clinical applications. We used a liver-specific albumin promoter to drive a microRNA-targeting WPRE that only existed in the same AAV vector harboring the transgene and therefore would not interfere with any
endogenous genes or microRNAs. The miR-WPRE was highly efficient to knock down the transgene expression, even being driven by a strong CBA promoter, and in combination with a proper dose, sufficient transgene expression in VAT, with minimal hepatic expression, could be achieved.

An interesting characteristic of the Rec2 serotype is that its tissue tropism is influenced by the administration route. Oral administration leads to preferential transduction of distal interscapular BAT, with minimal transduction of the stomach or intestine, which could be possibly due to Rec2 transcytoses and circulation via the lymphatic system. i.v. injection results in transduction primarily in the liver. Here, we showed that i.p. injection of Rec2 robustly transduced VAT, whereas minimal transgene expression was observed in SAT or BAT. In the case of i.p. delivery, the majority of vectors is likely encountered in the liver and VAT depots. Due to tropism of Rec2 serotype, both liver and VATs are efficiently transduced, which may limit the quantity of vectors entering blood circulation, therefore leading to minimal transduction of SAT and BAT. However, this feature allows preferential modulation of VAT, the fat depots closely associated with the risk of metabolic syndromes and heart disease. Furthermore, i.p. administration of the Rec2 vector enables genetic manipulation of mWAT that is otherwise difficult to access even via invasive surgical approaches.

Obesity has a substantial genetic component. Congenital leptin deficiencies in humans cause severe obesity, hyperphagia, and hyperinsulinemia. Leptin replacement therapy is necessary in patients with homozygous Lep mutations or acquired leptin deficiency derived from congenital or acquired lipodystrophy. Treatment with recombinant leptin therapy through subcutaneous injection produces short-lasting effects and therefore requires repetitive doses. The supra-physiological increase in circulating leptin level following regular injection is associated with serious side effects. Gene therapies with AAV vectors have been attempted in ob/ob mice, an animal model best recapitulating human congenital leptin deficiencies. AAV-mediated intramuscular leptin gene transfer (1 × 10^{11} vg per injection) alleviates obesity and diabetes. Up to date, only one gene therapy study is reported to reintroduce the leptin gene in its native tissue. i.v. injection of the AAV2/8-based adipose-targeting vector to ob/ob mice at the dose of 1 × 10^{12} vg per mouse results in a peak leptin level that is approximately 7% of the circulating leptin level in the age-matched WT mice and partially attenuates the metabolic syndromes. In contrast, in the same ob/ob mouse model, i.p. injection of our novel adipose-targeting Rec2 vector at 4 × 10^{15} vg per mouse, 25-fold lower than the reported dose, normalized the leptin level close to that of the age-matched WT mice (Figure 4A). Moreover, AS/Rec2-leptin treatment completely reversed hyperinsulinemia and impaired glucose tolerance and robustly corrected other metabolic symptoms, including obesity, hyperphagia, low-energy expenditure, impaired thermogenesis, and low physical activity, much closer to the age-matched WT animals, indicating advances compared to the limited efficacy with the previously reported AAV system.

Both animal and human studies have demonstrated that restoring the circulating leptin level to 10% of normal levels is sufficient to alleviate metabolic syndromes associated with congenital leptin deficiency.
Thus, it is likely that the dose of AS/Rec-leptin could be lowered further to \(1 \times 10^{10}\) vg per mouse, a dose based on body mass near the \(1 \times 10^{12}\) vg per kg dose of alipogene tiparvovec (Glybeta), which has been demonstrated to be safe and effective in patients.\(^{40}\) Moreover, clinical studies of genetic deficiencies in factor VIII, factor IX hemophilia, and lipoprotein lipase (LPL) have shown that AAV gene therapy resulting in very small corrections of physiological levels (5%–10% of normal levels) is able to profoundly reverse physiologic aberrancies, including the first approved gene therapy drug in Europe.\(^{41–43}\) Adipose tissue is a secretory organ and mature adipocytes are terminally differentiated and non-dividing and therefore an attractive target for non-integrating gene expression vectors such as AAV.\(^{44}\) The high efficiency of our new adipose-targeting AAV vector suggests potentially wider applications, for example, replacing a defective gene in adipose tissues,\(^{33,45,46}\) inducing adipose phenotypic changes to achieve systemic benefits, or producing therapeutic proteins for diseases not associated with adipose dysfunction.

In summary, our data demonstrate for the first time that AAV vector selectively transduces visceral fat depots, with minimal off-target transgene expression in the liver. One administration of AS/Rec2-leptin at a dose much lower than previously reported led to sustained and near-complete reversal of metabolic abnormalities in a mouse model representing a human genetic disease, congenital leptin deficiency. Further characterization and refinement of adipose-targeting AAV vectors will provide tools to study adipose function and regulation as well as to facilitate therapeutic applications possibly beyond adipose-related disorders.

MATERIALS AND METHODS

Mice

WT C57/BL6 mice, 8–12 weeks of age, were purchased from Charles River Laboratories. ob/ob mice on a C57/BL6 background were purchased from Jackson Laboratory. All mice were housed in a temperature-controlled room (22°C–23°C) with a 12 hr light-12 hr dark cycle, and maintained on a standard rodent diet (7912 rodent chow, Teklad), with free access to food and water ad libitum. Male mice were used in all studies. All use of animals was approved by and in accordance with the Ohio State University (OSU) Animal Care and Use Committee.

AAV Vector Construction and Package

The rAAV plasmid contains a vector expression cassette consisting of the CMV enhancer and CBA promoter, WPRE, and bovine growth hormone (bGH) poly-A flanked by AAV2 inverted terminal repeats. Transgenes, e.g., GFP, luciferase, and leptin, were inserted into the multiple cloning sites between the CBA promoter and WPRE. Rec2 vectors were packaged and purified as described previously.\(^{7,37,47}\)

MicroRNA Targeting WPRE

Two targeting sequences in the WPRE sequence were cloned into the Block-IT PolII miR RNAi expression vector (pcDNA6.2-Gw/miR, Invitrogen). The knockdown efficiency was determined by co-transfecting HEK293 cells with a standard AAV expression plasmid containing brain-derived neurotrophic factor (BDNF) as the transgene. In in vitro experiments, both miR constructs inhibited BDNF expression by at least 90%, confirmed by qRT-PCR and BDNF ELISA (Promega). The miR-WPRE (mature miR seq: CTATGTG GACGCTGCTTTTA) was chosen for the construction of the dual-cassette plasmid.

Dual-Cassette Adipose-Specific Vector Construction and Package

To construct the dual-cassette adipose-specific expression plasmid, the proximal region of the mouse albumin promoter (−317 to +18, the cap site is designated as 1) was generated by using pALB-GFP as a template, with XbaI and BamHI flanking at each side. pALB-GFP is a gift from Snorri Thorgeirsson (Addgene plasmid #5579).\(^{48}\) The liver-specific albumin promoter length is based on the previous publication.\(^{21}\) Mouse leptin cDNA was amplified from mouse adipose tissue cDNA, sequenced, and then subcloned into the multiple cloning sites between the CBA promoter and WPRE. The cassette containing miR-WPRE driven by the albumin promoter was then cloned to the AAV expression plasmid containing transgenes (leptin or GFP) to generate dual-cassette plasmids (Figure 2). Rec2 serotype vectors were packaged as described above.

Administration of Rec2 Vector

Rec2 vectors were administered into mice through i.p. injection in 150 µL of AAV dilution buffer. For WT mice experiments, doses ranging from \(5 \times 10^9\) to \(4 \times 10^{10}\) vg per mouse were used. For the ob/ob mice experiment, AS/Rec2-leptin vector at a dose of \(4 \times 10^{10}\) vg per mouse was i.p. injected.

Luciferase Imaging

The luciferase imaging was carried out 2 weeks after delivery of Rec2-luciferase via i.p. administration \((2 \times 10^{10}\) vg per mouse). The hairs over the abdomen were partially removed. D-luciferin and potassium salt (Gold Biotechnology) was i.p. injected into each mouse at a dose of 150 µg/g body weight. After 10 min, the mice were anesthetized in an isoflurane chamber and then placed in a warm- and light-tight scan chamber. The bioluminescence imaging was generated using IVIS Lumina II, Caliper (Small Animal Imaging Core Facility of OSU) and presented by radiance unit of photons/s/cm²/steradian (sr), which is the number of photons per second that lead a square centimeter of tissue and radiate into a solid angle of 1 sr.

GFP Content Measurement

Fat pads were dissected.\(^{39}\) GFP content was measured according to a previously published method with modifications.\(^{7}\) In brief, tissues were homogenized in ice-cold RIPA buffer (25 mM Tris-HCl, pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, and 0.1% SDS) supplemented with a proteinase inhibitors cocktail, followed by briefly sonication. The mixture was then spun at 13,000 rpm for 15 min at 4°C. The supernatant was collected. GFP fluorescence in 100 µL of supernatant was measured with a
microreader (Texas Instruments) using a 488-nm excitation wavelength and cut-off 515-nm/525-nm emission wavelength. GFP content was represented as readings after subtracting auto-fluorescence from GFP-null corresponding tissue and corrected by protein content in the samples.

**ob/ob Mice Experiment**

6-week-old ob/ob mice were randomly assigned to receive AS/Rec2-leptin or AS/Rec2-GFP (4 × 10^10 vg per mouse, i.p.). Body weight and food intake were monitored weekly over the period of 9 weeks. In another experiment, unrestricted single cassette Rec2-leptin vector was injected i.p. (2 × 10^10 vg per mouse) to ob/ob mice. Tissue collection was conducted similarly to the AS/Rec2-leptin (dual cassette) experiment.

**Glucose Tolerance Test**

4 weeks after i.p. administration of Rec2 vectors, mice were i.p. injected with glucose solution (1 mg glucose per g body weight) after overnight fast. Blood was drawn from the tail at various time points and the blood glucose concentrations were measured with a portable glucose meter (Bayer Contour Next).

**Rectal Temperature Measurement**

A temperature probe (Physitemp, model BAT-12) topped with lubricant (white petroleum jelly) was used to measure the rectal temperature of ob/ob mice at various time points after i.p. administration of Rec2 vectors.

**Metabolic Studies**

5 weeks after i.p. administration of Rec2 vectors, ob/ob mice were subjected to indirect calorimetry using the Oxymax Lab Animal Monitoring System (Columbus Instruments). An individual mouse was habituated to the instrument for 24 hr, and the physiological and behavioral parameters were monitored for 48 hr (activity, food and water consumption, metabolic performance, and temperature). Oxygen consumption, carbon dioxide production, and methane production were normalized to the body weight and corrected to an effective mass value according to the manufacturer’s software.

**Body Composition by EchoMRI**

EchoMRI was used to measure body composition of fat, lean, free water, and total water masses in live mice without anesthesia. EchoMRI imaging was performed with the EchoMRI Analyzer at the Small Animal Imaging Core of The Dorothy M. Davis Heart & Lung Research Institute, Ohio State University.

**Histology and Immunohistochemistry**

Liver tissues were frozen in OCT and cryo-sectioned at 8 µm. Lipids in liver were stained on frozen sections with an oil red O solution (Sigma). Adipose tissues were fixed in 10% formalin, and then embedded in paraffin and sectioned at 5 µm at the Core Facility of the OSU Comprehensive Cancer Center. Paraffin-embedded sections were subjected to citrate-based antigen retrieval followed by incubation with antibody against GFP (Abcam). The sections were visualized with 3,3 diaminobenzidine (DAB) and counterstained with hematoxylin.

**Metabolic Parameters**

Serum insulin was determined with mouse insulin ELISA (ALPCO). Serum leptin and tissue leptin levels were measured using a DuoSet ELISA Development System (R&D Systems). Leptin levels in tissue lysate were calibrated to total protein levels.

**Viral Vector Copy Number Measurement**

Total DNA from tissues was isolated using the DNeasy Blood and Tissue kit (QiAGEN). WPRE fragment was amplified to determine the copy number of the viral particle. 50 ng of DNA from each sample was used for real-time PCR. Mouse nucleic genomic fragment of the GAPDH gene was used as control for mouse genetic DNA. The standard curve for copy number was generated from a known plasmid DNA. Viral vectors were isolated and purified from blood using the High Pure Viral Nucleic Acid Kit (Roche).

**Statistical Analysis**

Data are expressed as mean ± SEM. We used JMP software to analyze the following: two-way ANOVA for body weight, food intake, and glucose tolerance; and Student’s t test for adiposity, body temperature, and serum biomarkers data.

**CONFLICTS OF INTEREST**

L.C. and W.H. are inventors of a provisional patent application related to the liver-restricting AAV vector. All other authors declare no conflicts of interest.

ACKNOWLEDGMENTS

This work was supported by NIH grants CA163640, CA166590, and AG041250.

REFERENCES

1. Mingozzi, F., and High, K.A. (2011). Therapeutic in vivo gene transfer for genetic disease using AAV: progress and challenges. Nat. Rev. Genet. 12, 341–355.
2. Kotterman, M.A., and Schaffer, D.V. (2014). Engineering adeno-associated viruses for clinical gene therapy. Nat. Rev. Genet. 15, 445–451.
3. Mizukami, H., Mimuro, J., Ogura, T., Okada, T., Urabe, M., Kume, A., Sakata, Y., and Ozawa, K. (2006). Adipose tissue as a novel target for in vivo gene transfer by adeno-associated viral vectors. Hum. Gene Ther. 17, 921–928.
4. Zhang, F.L., Jia, S.Q., Zheng, S.P., and Ding, W. (2011). Celastrol enhances AAV1-mediated gene expression in mice adipose tissues. Gene Ther. 18, 128–134.
5. Jimenez, V., Muñoz, S., Casana, E., Mallo, C., Elias, I., Jambrina, C., Ribera, A., Ferre, T., Franckhauser, S., and Bosch, F. (2013). In vivo adeno-associated viral vector-mediated genetic engineering of white and brown adipose tissue in adult mice. Diabetes 62, 4012–4022.
25. Zufferey, R., Donello, J.E., Trono, D., and Hope, T.J. (1999). Woodchuck hepatitis virus posttranscriptional regulatory element: implications for systemic gene therapy. Gene Ther. 21, 653–661.

26. Liu, X., Magee, D., Wang, C., McMurphy, T., Slater, A., During, M., and Cao, L. (2014). Adipose tissue insulin receptor knockdown via a new primate-derived hybrid recombinant AAV serotype. Mol. Ther. Methods Clin. Dev., Published online February 5, 2014. http://dx.doi.org/10.1038/mtm.2013.8.

27. During, M.J., Liu, X., Huang, W., Magee, D., Slater, A., McMurphy, T., Wang, C., and Cao, L. (2015). Adipose VEGF links the white-to-brown fat switch with environmental, genetic, and pharmacological stimuli in male mice. Endocrinology 156, 2059–2073.

28. Gesler, A., Jungmann, A., Kurecek, J., Poller, W., Katus, H.A., Vetter, R., Fechner, H., and Müller, O.J. (2011). microRNA122-regulated transgene expression increases specificity of cardiac gene transfer upon intravenous delivery of AAV9 vectors. Gene Ther. 18, 199–209.

29. Brown, B.D., Gentner, B., Cantore, A., Colleoni, S., Amendola, M., Zingale, A., Baccarin, A., Lazzari, G., Galli, C., and Naldini, L. (2007). Endogenous microRNA can be broadly exploited to regulate transgene expression according to tissue, lineage and differentiation state. Nat. Biotechnol. 25, 1457–1467.

30. Esau, C., Davis, S., Murray, S.F., Yu, X.X., Pandey, S.K., Pear, M., Watts, L., Boote, S.L., Graham, M., McKay, R., et al. (2006). miR-122 regulation of lipid metabolism revealed by in vivo antisense targeting. Cell Metab. 3, 87–98.

31. Qiao, C., Yuan, Z., Li, J., He, B., Zheng, H., Mayer, C., Li, J., and Xiao, X. (2011). Liver-specific microRNA-122 target sequences incorporated in AAV vectors efficiently inhibits transgene expression in the liver. Gene Ther. 18, 403–410.

32. Wen, J., and Friedman, J.R. (2012). miR-122 regulates hepatic lipid metabolism and tumor suppression. J. Clin. Invest. 122, 2773–2776.

33. Montague, C.T., Farooqi, I.S., Whitehead, J.P., Soo, M.A., Rau, H., Wareham, N.J., Seyter, C.P., Digby, J.E., Mohammed, S.N., Hurst, J.A., et al. (1997). Congenital leptin deficiency is associated with severe early-onset obesity in humans. Nature 387, 903–908.

34. Fidler, T., Marthaler, B., Klingelhutz, A., et al. (2017). SWELL1 is a regulator of adipocyte size, insulin signalling and glucose homeostasis. Nat. Cell Biol. 19, 504–517.

35. McMurphy, T.B., Huang, W., Xiao, R., Liu, X., Dhurandhar, N.V., and Cao, L. (2017). Hepatic expression of adenovirus 36 E4ORF1 improves glycemic control and promotes glucose metabolism through Akt activation. Diabetes 66, 358–371.

36. Huang, W., McMurphy, T., Liu, X., Wang, C., and Cao, L. (2016). Genetic manipulation of brown fat via oral administration of an engineered recombinant adeno-associated viral serotype vector. Mol. Ther. 24, 1062–1069.

37. Chau, Y.Y., Bandiera, R., Serrels, A., Martinez-Estrada, O.M., Qing, W., Lee, M., Slight, J., Thornburn, A., Berry, R., McHaffie, S., et al. (2014).Visceral and subcutaneous fat have different origins and evidence supports a mesothelial source. Nat. Cell Biol. 16, 367–375.

38. Lafontan, M., and Berlan, M. (2003). Do regional differences in adipocyte biology provide new pathophysiological insights? Trends Pharmacol. Sci. 24, 276–283.

39. Ibrahim, M.M. (2010). Subcutaneous and visceral adipose tissue: structural and functional differences. Obes. Rev. 11, 11–18.

40. Banh, G.S., Farooqi, I.S., and O’Rahilly, S. (2000). Genetics of body-weight regulation. Nature 404, 644–651.

41. Friedman, J. (2016). The long road to leptin. J. Clin. Invest. 126, 4727–4734.

42. Maffei, M., Halaas, J., Ravussin, E., Pratley, R.E., Lee, G.H., Zhang, Y., Fei, H., Kim, S., Lallone, R., Ranganathan, S., et al. (1995). Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. Nat. Med. 1, 1155–1161.

43. Ingalls, A.M., Dickie, M.M., and Snell, G.D. (1950). Obese, a new mutation in the house mouse. J. Hered. 41, 317–318.

44. Bray, G.A. (1991). Obesity, a disorder of nutrient partitioning: the MONA LISA hypothesis. J. Nutr. 121, 1146–1162.

45. Ott, M.O., Werle, L., Herbomel, P., Yaniv, M., and Weiss, M.C. (1984). Tissue-specific expression is conferred by a sequence from the S' end of the rat albumin gene. EMBO J. 3, 2505–2510.

46. Wooddel, C.L., Reppe, T., Wolf, J.A., and Herweijer, H. (2008). Sustained liver-specific transgene expression from the albumin promoter in mice following hydrodynamic plasmid DNA delivery. J. Gene Med. 10, 551–563.

47. Gorski, K., Carneiro, M., and Schibler, U. (1986). Tissue-specific in vitro transcription from the mouse albumin promoter. Cell 47, 767–776.

48. Trochne, F., Rollier, A., Bach, I., Weiss, M.C., and Yaniv, M. (1989). The rat albumin promoter: cooperation with upstream elements is required when binding of AFF/ HNF1 to the proximal element is partially impaired by mutation or bacterial methylation. Mol. Cell. Biol. 9, 4759–4766.

49. Zafferey, R., Donello, J.E., Trono, D., and Hope, T.J. (1999). Woodchuck hepatitis virus posttranscriptional regulatory element enhances expression of transgenes delivered by retroviral vectors. J. Virol. 73, 2886–2892.

50. Loeb, J.E., Cordier, W.S., Harris, M.E., Weitzman, M.D., and Hope, T.J. (1999). Enhanced expression of transgenes from adeno-associated virus vectors with the woodchuck hepatitis virus posttranscriptional regulatory element: implications for gene therapy. Hum. Gene Ther. 10, 2295–2305.
45. Agostini, M., Schoenmakers, E., Mitchell, C., Statmari, I., Savage, D., Smith, A., Rajanayagam, O., Semple, R., Luan, J., Bath, L., et al. (2006). Non-DNA binding, dominant-negative, human PPARgamma mutations cause lipodystrophic insulin resistance. Cell Metab. 4, 303–311.

46. Cortés, V.A., Curtis, D.E., Sukumaran, S., Shao, X., Parameswara, V., Rashid, S., Smith, A.R., Ren, J., Esser, V., Hammer, R.E., et al. (2009). Molecular mechanisms of hepatic steatosis and insulin resistance in the AGPAT2-deficient mouse model of congenital generalized lipodystrophy. Cell Metab. 9, 165–176.

47. Cao, L., Jiao, X., Zuzga, D.S., Liu, Y., Fong, D.M., Young, D., and During, M.J. (2004). VEGF links hippocampal activity with neurogenesis, learning and memory. Nat. Genet. 36, 827–835.

48. Heo, J., Factor, V.M., Uren, T., Takahama, Y., Lee, J.S., Major, M., Feinstone, S.M., and Thorgeirsson, S.S. (2006). Hepatic precursors derived from murine embryonic stem cells contribute to regeneration of injured liver. Hepatology 44, 1478–1486.

49. Mann, A., Thompson, A., Robbins, N., and Blomkalns, A.L. (2014). Localization, identification, and excision of murine adipose depots. J. Vis. Exp.