Enhancing the Utility of Adeno-Associated Virus Gene Transfer through Inducible Tissue-Specific Expression

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Abstract

The ability to regulate both the timing and specificity of gene expression mediated by viral vectors will be important in maximizing its utility. We describe the development of an adeno-associated virus (AAV)-based vector with tissue-specific gene regulation, using the ARGENT dimerizer-inducible system. This two-vector system based on AAV serotype 9 consists of one vector encoding a combination of reporter genes from which expression is directed by a ubiquitous, inducible promoter and a second vector encoding transcription factor domains under the control of either a heart- or liver-specific promoter, which are activated with a small molecule. Administration of the vectors via either systemic or intrapericardial injection demonstrated that the vector system is capable of mediating gene expression that is tissue specific, regulatable, and reproducible over induction cycles. Somatic gene transfer *in vivo* is being considered in therapeutic applications, although its most substantial value will be in basic applications such as target validation and development of animal models.

Introduction

ADENO-ASSOCIATED VIRAL (AAV) VECTORS have proven to be effective vehicles for efficient and stable gene delivery with broad tropism *in vivo* (Gao *et al.*, 2004). The AAV serotype 9 capsid, in particular, has been shown to confer exceptionally broad tropism and to mediate gene delivery to a variety of targets including heart, skeletal muscle, liver, and lung after systemic administration (Inagaki *et al.*, 2006; Zincarelli *et al.*, 2008). Although the AAV9 capsid exhibits a high natural affinity for certain targets, including the liver and heart, strategies aimed at restricting AAV9 vector-mediated gene expression through targeting transduction include the engineering of AAV9 variants with modified tropism (Li *et al.*, 2008; Pulicherla *et al.*, 2011) and the use of localized gene delivery methods such as direct cardiac versus systemic injection (Bish *et al.*, 2008). Further attempts to improve the specificity of AAV9-mediated gene expression have led to the development of AAV9 vectors with microRNA-regulated expression (Geisler *et al.*, 2011; Qiao *et al.*, 2011) and AAV9 vectors with tissue-specific promoters including those for liver, muscle, and heart (Pacak *et al.*, 2008; Wang *et al.*, 2008). The use of these strategies, alone or in combination, can effectively increase the utility of AAV9 vectors for gene therapy applications by limiting gene expression to areas where it is most desirable.

The usefulness of viral vector-mediated gene transfer for both therapeutic and basic research applications would be greatly enhanced if the expression of the transgene could be regulated with respect to both the timing and level of gene expression. Several vector-based inducible gene expression systems have been developed to address this issue by using small-molecule drugs such as tetracycline, ecdysone, or rapamycin to reversibly induce the expression of functional transcription factors (Clackson, 1997; Pulicherla *et al.*, 2011; Vanrell *et al.*, 2011). Although AAV vector systems have been developed on the basis of both the tetracycline- and ARGENT rapamycin-inducible systems, the ARGENT system (previously available from ARIAD Pharmaceuticals [Cambridge, MA]; now available from Clontech [Mountain View, CA] as the iDimerize inducible expression system) combines extremely low basal expression with highly inducible, dose-dependent expression in order to achieve pharmacological control over the levels and kinetics of therapeutic gene expression (Rivera *et al.*, 1999). AAV vectors based on the ARGENT system have been shown to result in regulated gene expression after administration into such diverse targets as muscle (Rivera *et al.*, 1999; Johnston *et al.*, 2003), eye (Auricchio *et al.*, 2002), the CNS (Sanftner *et al.*, 2006; Kim *et al.*, 2012), salivary glands (Wang *et al.*, 2006), and liver (Fang *et al.*, 2007).

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This paper describes the development of AAV vectors that combine tissue specificity with inducible elements from the ARGENT system to achieve stable, tissue-specific, inducible vector-mediated gene expression (Clackson, 1997). In this system, gene regulation is achieved by reconstituting two split domains of a transcription factor, using the dimerizing inducer rapamycin. Our two-vector system based on AAV9 consists of (1) one vector expressing a bicistronic message (encoding the reporters luciferase and enhanced green fluorescent protein [eGFP]) under the control of a ubiquitous, inducible promoter (Z12I) and (2) a second vector encoding transcription factor domains under the control of either a heart-specific promoter (cardiac troponin T, cTnT) or a liver-specific promoter (thyroxine-binding globulin, TBG). AAV9 vectors encoding this system mediate expression that is tissue specific, regulatable, and reproducible over induction cycles. The vectors described here not only enhance the utility of the broadly biodistributed AAV9 but represent a useful system for the delivery of potential therapeutic genes, the expression of which can be limited to tissues of interest and transcriptionally regulated. AAV9 and other serotype AAV vectors based on this system are available through the National Heart, Lung, and Blood Institute (NHLBI, Bethesda, MD) Gene Therapy Resource Program (www.gtrp.org/) as well as through the University of Pennsylvania Vector Core (Penn Vector Core, Philadelphia, PA).

Materials and Methods

Construction of AAV packaging (cis) plasmid for AAV production

AAV plasmids were generated according to standard molecular cloning protocols. The ARGENT gene-regulatory system was originally developed by ARIAD Pharmaceuticals (Pollock and Clackson, 2002). The construct pAAV.CMV.TF1Nc.3.hGH, encodes dual transcription factors (TFs; available from clontech) under the control of the cytomegalovirus (CMV) promoter. To achieve liver- or heart-specific gene regulation, the CMV promoter was removed from pAAV.CMV.TF1Nc.3.hGH by treatment with *Bgl*III and *Mlu*I and replaced with the TBG promoter (Wang *et al.*, 2010) or cTnT promoter (Pacak *et al.*, 2008). Plasmids encoding the dual reporters eGFP and luciferase, placed under the control of the CMV, TBG, or cTnT promoter, or the inducible Z12I promoter, were constructed by SOEing PCR (polymerase chain reaction-splicing by overlap extension) using three sets of primers. In short, the primer set *Mlu*I-GFPfwd (5'-TTCAC GCGTATGGTGAGCAAGGGCGAGGAG-3') and T2A-GFPprev (5'-CGACGTCACCGCATGTTAGTAAGCTTCCGCGGCC TCCTTGACAGCTCGTCCATGCCG-3') was used to PCR-amplify eGFP from the template pAAV.CMV.EGFP2, of which the C terminus was fused with the N terminus of T2A peptide (Szymczak *et al.*, 2004). A second set of primers, T2A-luc2fwd (5'-ACTAACATGCGGTGACGTCGAGGAGAACC CGGGCCCTATGGAAGATGCCAAAACATTAAG-3') and *Kpn*I-luc2rev (5'-AGAGGTACCTTACACGGCGATCTTGC CG-3'), was used for PCR with pAAV.CMV.fluciferase2 as a template to recover the fragment containing the C terminus of T2A fused with luciferase downstream. The thermocycling conditions were 98°C for 5 sec, 55°C for 25 sec, and 72°C for 30 sec, for a total of 25 cycles and the reaction was run with Phusion high-fidelity DNA polymerase (New England

BioLabs, Ipswich, MA). The PCR products from these two reactions served as templates for amplification using primers *Mlu*I-GFPfwd and *Kpn*I-luc2rev. The reaction was run at 98°C for 10 sec, 55°C for 25 sec, and 72°C for 50 sec for a total of 15 cycles. The final SOEing PCR product, *Mlu*I-EGFP-T2A-luciferase-*Kpn*I, was then cloned into pZac2.1, an AAV packaging plasmid, in between the *Mlu*I and *Kpn*I sites, downstream of the CMV promoter and similarly into pAAV.TBG, pAAV.cTnT, and pAAV.Z12I plasmids (available from the Penn Vector Core), so the transgene is put under the control of TBG, cTnT, and Z12I promoter, respectively. All constructs were verified by sequencing analysis before vector production.

Recombinant AAV production

AAV vectors were generated by triple transfection using (1) an AAV *cis* plasmid carrying a transgene expression cassette flanked by the viral inverted terminal repeats (ITRs) (Fig. 1), (2) an AAV *trans* plasmid encoding the AAV2 *rep* and AAV9 capsid genes, and (3) a plasmid encoding adenoviral genes providing helper functions for AAV replication. The plasmids were used to transfect subconfluent HEK293 cells (American Type Culture Collection [ATCC], Manassas, VA) grown in six CellSTACK chambers (Corning Life Sciences, Tewksbury, MA), using PEI-Max (Polysciences, Warrington, PA), and the vectors were purified from cell lysates and supernatant as described previously (Lock *et al.*, 2010). In brief, transfected cells were treated with Benzonase followed by the addition of high-concentration NaCl, and the supernatant was collected after centrifugation. The supernatant was then filtered through a 0.2- μ m filter and concentrated by tangential flow filtration (TFF). The concentrated feedstock was reclarified by centrifugation and the supernatant was loaded onto iodixanol step gradient solutions (OptiPrep; Sigma-Aldrich, St. Louis, MO) in phosphate-buffered saline (PBS) containing 10 mM magnesium chloride and 25 mM potassium. The steps of the gradient were 4 ml of 15%, 9 ml of 25%, 9 ml of 40%, and 5 ml of 54% iodixanol formed in a 40-ml Quick-Seal centrifugation tube (Beckman Coulter, Palo Alto, CA). The tubes were centrifuged for 70 min at 350,000 $\times$ g in a 70 Ti rotor (Beckman Coulter) at 18°C and the gradients were fractionated. Fractions containing AAV were pooled and subjected to concentration and buffer exchange to PBS, using Amicon Ultra-15 centrifugal concentrators (Millipore, Bedford, MA). Glycerol was then added to the diafiltered, concentrated product to a final concentration of 5% and the preparation was aliquoted and stored at -80°C.

Quality control of AAV vectors

Genome titers (i.e., genome copies [GC]/ml) of purified AAV vectors were determined by real-time PCR using a primer-probe set corresponding to the poly(A) region or TF gene of the vector and linearized plasmid standards. The primer sequences for simian virus 40 (SV40) poly(A) TaqMan analysis are as follows: forward primer, 5'-AGCAA TAGCATCACAAATTTACAA-3'; reverse primer, 5'-CCA GACATGATAAGATACATTGATGAGTT-3'; and probe, 5'-6FAM-AGCATTTTTTCTACTGCATTCTAGTTGTGGTTT TC-TAMRA. The primer sequences for the TF assay are as follows: forward primer, 5'-TGC TTG GCA ACA GCA-3'; reverse primer, 5'-TCG GAG TTG TCG ACG GAT-3'; and probe, 5'-6FAM-AGA CCC AGC TGT GTT CAC AGA CCT-TAMRA-3'. Vectors were also subjected to sodium

FIG. 1. Schematic diagrams of (A) transcription factor fusions and (B) reporter gene constructs. CMV, cytomegalovirus promoter; cTnT, cardiac troponin T promoter; FRB p65, the activation domain composed of the FRB (FKBP12-rapamycin binding) domain of human FRAP (FKBP12-rapamycin-associated protein) (amino acids 2021–2113) fused to a portion of human NF- κ B p65; hGH poly A and SV40 poly A, polyadenylation signal derived from human growth hormone and simian virus 40, respectively; IL-2 promoter, minimal promoter from the human interleukin-2 gene; IRES, internal ribosome entry site from encephalomyocarditis virus; ITR, AAV inverted terminal repeat; TBG, thyroxine-binding globulin promoter; reporter gene, bicistronic expression cassette of green fluorescent protein (GFP) and firefly luciferase, regulated by 2A peptide derived from *Thossea asigna*; ZFHD1 3 $\times$ FKBP fusion, DNA-binding fusion of zinc finger homeodomain 1 fused to 3 copies of FKBP; Z12 (12 repeated copies of the binding site for ZFHD1).

dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis for vector purity assessment and *Limulus* amebocyte lysate (LAL) assay for endotoxin detection (Lonza Bio Science, East Rutherford, NJ) before injection into mice.

In vivo gene transfer experiments

All animal studies were conducted according to the *Guide for the Care and Use of Laboratory Animals* (NIH publication No. 85-23, revised 1996) and approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Pennsylvania.

For systemic gene delivery, 8-week-old male C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) were injected with AAV vector resuspended in 100 μ l of PBS via tail vein injection. Before injection, the mice were exposed to a heat lamp to dilate the tail vein and then placed in a restrainer permitting access to the tail vein. The tail was cleansed with 70% ethanol and the injection was made in the lateral vein, using 27-gauge needles. The dose for control groups infused with a single vector were 3×10^{10} , 1×10^{11} , or 3×10^{11} GC of vector. For groups of mice receiving both TF and target vectors of the inducible system, 3×10^{10} , 1×10^{11} , or 3×10^{11} GC of each vector was coadministered.

For gene delivery to the heart, intrapericardial injection was performed in 4- to 5-day old neonatal mice with vectors resuspended in 50 μ l of PBS. A detailed protocol is available (Bish *et al.*, 2008) and is briefly described below. C57BL/6 mouse pups were cryoanesthetized by placement on ice for 2–3 min immediately preceding injection. The vector was drawn with a 25-gauge needle attached to a Hamilton syringe. The needle was then replaced with a 33-gauge Hamilton needle threaded by Tygon tubing with 3 mm of the tip of the needle exposed. The needle was inserted at the left costoxiphoid angle of the pup and advanced superiorly until the Tygon tubing contacted the skin. The vector was slowly

injected into the pericardial space of the pup. After injection, the pups were returned to the cage with the mother and monitored closely until they regained consciousness. For control groups infused with a single vector, mice were administered a low dose (2.5×10^{10} GC) or high dose (2.5×10^{11} GC) of vector. For inducible gene regulation, mice were coadministered TF and target vectors at a 1:1 ratio.

Bioluminescence imaging

For detection of luciferase activity, mice were injected intraperitoneally with D-luciferin (15 mg/ml; Caliper Life Sciences, Hopkinton, MA) in PBS at a dose of 10 ml/g of body weight and then anesthetized by intraperitoneal injection of ketamine and xylazine (70 and 7 mg/kg, respectively). Images were taken at 15 or 20 min for heart and liver, respectively, after luciferin injection, using a Xenogen IVIS Lumina system and quantified with Living Image 3 software (Caliper Life Sciences/PerkinElmer, Hopkinton, MA).

Activation of the inducible system by rapamycin

A rapamycin (LC Laboratories, Woburn, MA) stock solution was prepared by resuspension in *N,N*-dimethylacetamide (DMA; Sigma-Aldrich) at 10 mg/ml. The working solution (2 mg/kg body weight) was made right before injection by diluting the stock solution to 2 mg/ml with DMA and then further diluting with an equal volume of polyethylene glycol 400 (PEG-400; Sigma-Aldrich) and Tween 80 (polyoxyethylenesorbitan monooleate; Sigma-Aldrich) to 1 mg/ml. Mice were injected intraperitoneally with the working solution at 2 μ l/g body weight. Luciferase expression was monitored by Xenogen imaging before and after induction with rapamycin.

eGFP histology

Muscle and liver were harvested at the end of study, fixed immediately in formalin, and embedded in frozen O.T.C.

compound, after which 8- μ m cryosections were prepared. GFP images were captured digitally by fluorescence microscopy (Nikon Instruments, Melville, NY).

Results

Vector design for ARGENT inducible system

A detailed depiction of the components used in the ARGENT inducible system is shown in Fig. 1. In this system, gene regulation is achieved by reconstituting two split domains for DNA binding and transcription activation of a transcription factor using a dimerizing inducer, rapamycin, an orally bioavailable drug. The transcription vector encodes the bicistronic expression cassette encoding the split units. To develop tissue-specific gene regulation, the CMV promoter in the original construct used to drive expression of the transcription factors was replaced with the previously reported liver-specific TBG promoter or heart-specific cTnT promoter. The expression cassette encoded dual reporter genes, that is, firefly luciferase and eGFP (separated by 2A peptide derived from *Thossea asigna*), all under the control of the inducible promoter Z12I. Luciferase is the reporter gene of choice because it allows expression to be monitored over time by bioluminescence imaging; eGFP was included for histological analysis.

Liver-specific gene regulation

We first infused mice systemically with both transcription and target vectors at a 1:1 ratio at a dose of 3×10^{10} , 1×10^{11} , or 3×10^{11} GC/vector/mouse, with TF-coding sequences placed under the control of either the CMV promoter (Fig. 2B) or the TBG promoter (Fig. 2E). As controls, mice received vectors encoding the dual reporters placed under the control of constitutive promoters: either the ubiquitous CMV promoter (Fig. 2A) or liver-specific TBG promoter (Fig. 2C). Three weeks after vector administration, mice were injected with the dimerizing inducer rapamycin to activate the inducible system, and the level of luciferase expression was monitored. In mice infused with AAV9.CMV.luciferase vector, luciferase expression was detected in various tissues including liver, lung, and muscle, with the highest level of expression found in muscle (Fig. 3A). In contrast, significantly elevated levels of luciferase were detected specifically in liver from mice infused with AAV9.TBG.luciferase vectors (Fig. 3B). Expression was unaffected by the infusion of rapamycin when the reporter genes were driven from the constitutive promoters (Fig. 3A and B). In mice coadministered with TF and target vectors, luciferase expression was minimal before induction with rapamycin and was detectable 24 hr postinduction (Fig. 3C and D and Fig. 4). In mice receiving TF vector with expression under the control of the CMV promoter, expression was observed in liver and muscle, among other tissues (Fig. 3C). Alternatively, in mice infused with TF vectors with expression regulated by the TBG promoter together with target vector, expression was restricted to liver and little, if any, expression was detected in tissues outside of liver (Fig. 3D). Furthermore, expression returned back to baseline within 1 week of induction (Figs. 3 and 4), indicating that the inducible system is robust and reversible once the inducer is withdrawn (Fig. 3E).

Reversible, reproducible, and long-term gene regulation in liver

Results from quantification of luciferase activity from the inducible liver-specific vectors over time demonstrate that the peak level of expression was achieved between 24 and 48 hr after rapamycin administration, with levels correlating to vector doses and at approximately 2 logs above baseline (Fig. 3E). The inducible system was activated repeatedly for three more cycles over the duration of the study of 6 months, and the results confirmed that the inducible system is reversible and reproducible and that long-term regulatable gene expression is feasible (Fig. 3E).

When transgene expression derived from constitutive and inducible promoters was compared, expression in liver was found to be significantly higher from the inducible promoter compared with that from the CMV promoter, indicating that the inducible promoter outperforms the CMV promoter in liver (Fig. 3F). Moreover, the level of induction derived from TBG promoter-regulated TF expression was comparable to

FIG. 2. Vectors injected for liver- and heart-specific gene regulation. Mice were injected, via the tail vein for liver or via intrapericardial injection for heart, with AAV9 encoding dual reporter genes under the control of the following regulatory elements. (A) The reporter is under the control of a ubiquitous constitutive CMV promoter. (B) The mice received two vectors. One encodes the split transcription factors, the expression of which is under the control of the CMV promoter and the other encodes reporter genes under the control of an inducible promoter. (C) For liver, the reporter genes are under the control of the liver-specific TBG promoter. (D) For heart, the reporter genes are under the control of the heart-specific cTnT promoter. (E) Mice received two vectors. One encodes the transcription factor expression cassette driven by a liver-specific promoter. The other encodes the reporter construct [the same as in (B)]. (F) Mice received two vectors. One encodes the transcription factor expression cassette driven by the cTnT promoter and the other encodes reporter genes under the control of the inducible promoter.

FIG. 3. Detection of luciferase expression by bioluminescence and induction of luciferase expression in the liver. AAV9 at a dose of 3×10^{11} GC/mouse ($n=5$ per group), carrying a GFP and firefly luciferase dual reporter expression cassette, was delivered systemically by tail vein injection into mice. Three weeks after gene delivery, luciferase expression was detected in mice by bioluminescence, using a Xenogen system and Living Image software. (A) Mice received AAV9.CMV.EGFP.T2A.Luc. (B) Mice were injected with AAV9.TBG.EGFP.T2A.Luc. The images were taken before (pre) or 1 day after rapamycin injection. (C and D) Mice received two vectors (at a ratio of 1:1 and a dose of 3×10^{11} GC/vector/mouse), one encoding the reporter expression cassette for expression from the inducible promoter, and one encoding split transcription factors for expression from the (C) CMV promoter or (D) TBG promoter. Images shown were taken before rapamycin injection as a baseline (pre), and on days 1 and 20 postinduction. (E) Mice coinjected with AAV9.TBG.TF and AAV9.Z121.EGFP.T2A.Luc were intraperitoneally administered rapamycin at 2 mg/kg (arrows) to induce transgene expression. Luciferase activity represented by total flux was determined by bioluminescence imaging after intraperitoneal injection of luciferin. Induction was repeated four times at 3, 4, 10, and 25 weeks after gene delivery in these mice. (F) Mice were injected intravenously with AAV at a dose of 3×10^{11} , 1×10^{11} , or 3×10^{10} GC (columns shaded from dark to medium to light, respectively). Luciferase activity in liver derived from mice treated with a constitutive promoter (CMV) or liver-tissue specific promoter (TBG) is compared with peak-level luciferase activity derived from mice treated with the inducible promoter with TF expression controlled by the CMV promoter (CMVi) or TBG promoter (TBGi) on day 1 after rapamycin injection and on day 21 after vector delivery (* $p < 0.01$, Student t test).

FIG. 4. Induction of eGFP expression in mouse liver. AAV9 carrying a GFP and firefly luciferase dual reporter expression cassette was delivered systemically by tail vein injection into mice. Twelve months after vector injection, the study was terminated and liver was harvested 24 hr after rapamycin treatment, fixed in 4% formalin, and sectioned for eGFP expression. Mice coinjected with AAV9.CMV.TF and AAV9.Z12I.EGFP.T2A.Luc (3×10^{11} GC each) were left uninduced (**A**) or intraperitoneally administered rapamycin at 2 mg/kg (**C**). Mice injected with AAV9.TBG.TF and AAV9.Z12I.EGFP.T2A.Luc (3×10^{11} GC each) were left uninduced (**B**) or induced with rapamycin (**D**) ($n=3$).

CMV promoter-regulated expression and about 10-fold lower compared with expression directed by TBG constitutive expression vectors (Fig. 3F). These results are potentially related to the efficiency of coinfection in liver because induction is achieved only in cells harboring the two-vector system. At the end of the study, eGFP expression was observed in liver (Fig. 4). Apparent eGFP-positive cells were detected in mice receiving either CMV- or TBG-regulated inducible vectors 24 hr after rapamycin injection but not in liver without induction. With the majority of GFP-positive cells surrounding the pericentral area, the results indicate a higher efficiency of coinfection in cells locating in this area of the liver.

We estimate 1–4% GFP-positive hepatocytes (based on the area of GFP-positive signal) fairly evenly dispersed in the liver of mice administered liver-specific regulated vectors with expression induced 1 year after vector injection (Fig. 4D). This level is comparable to that in mice receiving bicistronic reporter vectors with expression driven by the liver constitutive TBG promoter (data not shown). The level of GFP-positive cells is significantly lower than that reported previously from a monocistronic construct with GFP expression directed by a TBG promoter (Wang *et al.*, 2010). We believe the number of cells transduced by the bicistronic vector is underestimated because of poor expression of GFP when placed upstream of a different open reading frame (ORF) separated by the T2A sequence.

Heart-specific gene regulation

Experiments for evaluation of the ARGENT inducible system in the heart were conducted with TF vectors carrying expression cassettes composed of the CMV or cTnT promoter driving TF domain expression (Fig. 2) in combination with target vectors for coadministration by intrapericardial injection into 4- to 5-day-old neonatal mice. For comparison, administration of the control vector, AAV9.CMV.luciferase, in neonatal mice via intrapericardial injection led to high-

level expression in various tissues, notably in heart and muscle 3 weeks after gene delivery (Fig. 5A). In contrast, in mice receiving AAV9.cTnT.luciferase vectors, expression was detected predominantly in the heart but was minimal in other tissues, confirming heart specificity of the promoter in combination with gene delivery via this route of administration targeting the heart, although overall expression was also down (Fig. 5B). Before injection of rapamycin, a minimal baseline level of luciferase expression was detected in mice coinjected with AAV9.CMV.TF and AAV9.Z12I.luciferase (Fig. 5C), and expression was significantly elevated after induction, most noticeably in heart and muscle among other tissues (Fig. 5D). Similarly, in mice receiving both AAV9.cTnT.TF and AAV9.Z12I.luciferase, expression was undetectable before induction (Fig. 5E); however, expression was evident and specific in the heart 24 hr postinduction with rapamycin (Fig. 5F).

Reversible, reproducible, and dose-dependent gene regulation in heart

Figure 5G shows the results of continuously monitored transgene expression over time in mice receiving TF vector, with TF expression under the control of the cTnT promoter together with target vector. Expression reached peak level at 1–2 logs above baseline between 24 to 48 hr after induction and gradually returned to baseline within 2 to 3 weeks. Similar to the situation in liver, the induction could be repeated, indicating the system is reversible and reproducible in the heart. Expression levels derived from the cTnT promoter were slightly higher than those from the cTnT promoter-regulated inducible system, indicating the efficiency of induction is at least partially due to the efficiency of uptake of the two vectors in the same cell. Furthermore, it was demonstrated that the peak level of expression after induction correlated with the doses of the vectors and the inducer (Fig. 5H).

FIG. 5. Detection of luciferase expression by bioluminescence imaging and induction of luciferase expression in the heart. Four weeks after virus administration, mice were intraperitoneally injected with rapamycin at 5 mg/kg, and the bioluminescence images were taken 24 hr later with a Xenogen Lumina system and Living Image software. (A) Mice were injected with reporter construct with luciferase expression coming from the CMV promoter at 2.5×10^{10} GC/mouse. (B) Mice received reporter construct at 2.5×10^{10} GC/mouse with luciferase expression controlled by the cTnT promoter. (C and D) Mice received one construct carrying an inducible promoter driving reporter expression and another construct encoding TF with expression driven by the CMV promoter at 1:1 and a dose of 2.5×10^{10} GC/vector/mouse. (C) Baseline luciferase expression before induction. (D) The mice were then intraperitoneally injected with the dimerizing inducer rapamycin and the induction of luciferase expression was determined 24 hr later. (E and F) Mice received one construct carrying an inducible promoter driving reporter expression and the other construct encoding TF with expression driven by the cTnT promoter at 1:1 and a dose of 2.5×10^{10} GC/vector/mouse. (E) Baseline luciferase expression before induction. (F) Luciferase expression 24 hr postinduction. Neonatal mice were injected intrapericardially with AAV encoding luciferase, the expression of which is controlled by the inducible system. Four weeks after gene delivery, the dimerizing inducer rapamycin at 5 or 2 mg/kg (arrows) was introduced by intraperitoneal injection and the induction of luciferase was determined by Xenogen imaging analysis. (G) Total flux in mouse heart. Mice were injected with high-dose vectors (2.5×10^{11} GC/vector/mouse). Luciferase expression was induced 4 and 10 weeks postinjection with rapamycin at the dose of 5 and 2 mg/kg, respectively, and subsequently monitored by Xenogen imaging. (H) Peak level of luciferase activity 24 hr after rapamycin induction and 4 weeks after gene delivery from mice receiving low-dose (2.5×10^{10} GC) or high-dose AAV (2.5×10^{11} GC) composed of the inducible systems (TF vector:reporter vector, 1:1, $n=8$) was compared with the level of expression derived from the constitutive cTnT promoter ($n=8$). The level of luciferase expression is comparable between the groups receiving vectors carrying the constitutive cTnT promoter or inducible system ($*p=0.27$, Student *t* test).

Discussion

AAV9 was first described as a serologically distinct clade rescued from lumen DNA (Gao *et al.*, 2004). The utility of AAV9 vectors has since been described for applications in liver (Sarkar *et al.*, 2006), lung (Limberis and Wilson, 2006),

heart (Inagaki *et al.*, 2006; Bish *et al.*, 2008; Pleger *et al.*, 2011; Prasad *et al.*, 2011; Pulicherla *et al.*, 2011), muscle (Kornegay *et al.*, 2010; Sun *et al.*, 2010; Pulicherla *et al.*, 2011), eye (Allocca *et al.*, 2007), spinal cord (Foust *et al.*, 2009; Snyder *et al.*, 2011), and CNS (Cearley *et al.*, 2008; Foust *et al.*, 2009; Fu *et al.*, 2011; Masamizu *et al.*, 2011) and for fetal gene delivery

(Tarantal and Lee, 2010). Unique characteristics of AAV9 such as its ability to promote broad genome biodistribution (Zincarelli *et al.*, 2008), cross the blood–brain barrier (Foust *et al.*, 2009; Fu *et al.*, 2011), and undergo retrograde transport (Masamizu *et al.*, 2011) have led to its increased use for a range of basic research and gene therapy applications. However, gene delivery mediated by AAV9 vectors could lead to undesirable toxicity due to nonspecific overexpression of therapeutic protein in nontarget tissues. The utility of AAV9, and potentially other serotype AAV vectors, for gene transfer applications may be enhanced through the use of tissue-specific, regulatable promoters for treating diseases by modulating the expression of growth factors, cytokines, antibodies, or gene knockdown via microRNA or small hairpin RNA (shRNA), for which tight regulation of gene expression is critical for effective therapy.

We report here a strategy to modify the ARGENT inducible system by replacing the CMV promoter with either the TBG or cTnT promoter for controlling TF domain expression, in order to achieve tissue-specific gene regulation in liver and heart. On introduction of the dimerizing inducer rapamycin, the level of tissue-specific expression of the target gene was elevated, up to more than 2 logs from baseline, within 24 hr and peak-level expression was comparable to that derived from a tissue-specific constitutive promoter, for example, TBG or cTnT. Expression subsequently returned to baseline within 1 week for liver and a few weeks for heart once the inducer was withdrawn. Furthermore, we demonstrated that long-term, reproducible, and dose-responsive induction in liver and heart is feasible. Because the efficiency of gene induction by this approach depends primarily on the efficiency of uptake of the TF and target vectors in the same host cells, our results indicate that gene delivery by AAV9 is effective in delivering two vectors simultaneously.

In addition to the promoters used to drive TF expression and the intrinsic stability of the target gene transcript and product, the level and duration of target gene expression on induction are also determined by the following factors: the dose of inducer and the vectors, the accessibility of the inducer, the half-life of the inducer, the half-life of the mRNA and protein product of the TF domains, and finally the stability of the rapamycin–TF complex. Any combination of these factors could explain why we detected higher target gene expression in liver directed by the inducible system with TF expression regulated by the CMV promoter than directly from the CMV promoter (Fig. 3A and C), which instead led to more intense expression in muscle. These factors also contribute to the observation that the kinetics of gene regulation are different between liver and heart, notably, more robust in liver where we detected a greater fold increase over baseline and a shorter duration for each cycle of induction.

With a limitation of the system being the efficiency of delivery of two vectors to a single cell, its utility is expected to be greatest in tissues efficiently targeted by AAV9. Although our results indicate the level of gene expression achieved with the two-vector–inducible tissue-specific ARGENT system may be up to 10-fold lower compared with that achieved with a constitutive tissue-specific single vector (Figs. 3F and 5H), this level may be sufficient to achieve a therapeutic effect for certain applications. Previous studies have demonstrated that a two-vector–inducible ARGENT system directed erythropoietin (Epo) levels that were 1 log lower compared with those

directed by a single constitutively expressing vector; however, the levels were considered biologically relevant (Rivera *et al.*, 2005). For certain applications, the lower expression level achieved by the two-vector system may not be sufficient. However, for applications in which abundant gene expression is not required for a therapeutic effect, the two-vector system might be appropriate and have greater value when combined with tissue specificity.

In conclusion, our results demonstrate that gene delivery using AAV9 vectors encoding the ARGENT regulatory system in combination with liver- or heart-specific promoters can achieve dose-dependent, reversible, reproducible, and tissue-specific regulation that is critical to its use as a research tool and therapeutic delivery platform. The tissue-specific inducible system described in this paper has broad application and can be readily modified by substituting alternative promoters that can direct tissue-specific expression of the TF for targeting lung, muscle, or brain and other tissues.

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Author Disclosure Statement

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