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Adeno-associated viral vectors for tissue-specific gene delivery in vivo

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Thesis

**ADENO-ASSOCIATED VIRAL VECTORS FOR
TISSUE-SPECIFIC GENE DELIVERY IN VIVO**

by

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DEDICATION

To Ali Boukhobza & My Family

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DELIVERY IN VIVO**

WAJEEHA QURESHI

ABSTRACT

Objective: Clinical gene therapy is increasingly becoming a favorable method for the targeting and treatment of various human diseases. One such gene-transfer method is the use of adeno-associated viruses (AAVs) as a tool for tissue-specific delivery of genes. AAV vectors are attractive vector candidates due to their low pathogenicity, biological safety, and ability to infect both dividing and quiescent cells for sustained gene expression. We sought to develop a protocol for the production, propagation, purification, and characterization of various AAV serotypes to determine their tropism and to observe their integrative and expressive abilities using different reporter genes *in vitro* and *in vivo*.

Methods: We used subcloning techniques to introduce our genes of interest into an AAV expression plasmid and recombined with two other AAV vectors required for packaging, cell-specific targeting, and integration capabilities. Serotypes AAV1/8/9 and 5 which are cross-packaged with the prototype AAV2 were used to individually target cardiac cells/skeletal muscle and neuronal cells respectively in eC57BL/6J mice. Viral particles were propagated *in vitro* and purified using an iodixanol gradient. Purified AAV vectors were introduced into cells in culture and injected into live mice via tail vein injection or using robotic stereotaxic surgery

directly into brain opposite saline controls to determine expression capabilities *in vitro* and *in vivo*, respectively. Live mice were imaged using the IVIS bioluminescent imaging system to measure luciferase luminescent readout. Separately, sections were acquired from the brains of the mice injected with serotype AAV2/5 and imaged using live cell imaging fluorescence microscopy to demonstrate integrative ability of the vectors into neurons based on GFP reporter signal.

Results: A protocol for the generation of neuron-specific AAVs was developed for the successful integration of the reporter gene GFP. The reporter gene was cloned into the AAV expression cassette. Efficient transfection methods were determined along with optimal culture conditions for the propagation of the viral particles *in vitro*. Promoter-driven reporter gene expression was observed in serotype-targeted cell types *in vitro* and *in vivo*. Expression was limited to neuronal cells based on AAV serotype specificity and SYN1 promoter. Integration and expression of the luciferase gene was not observed in the IVIS system.

Conclusion: Here we demonstrate expression of a GFP reporter in specific neuronal cell types which indicates successful integration and expression abilities of AAV vectors. This specific tissue-targeting technique using the AAV vector highlights the potential for the further development of these vectors as a promising gene transfer system.

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LIST OF ABBREVIATIONS

AAV	Adenovirus associated virus
BSA	Bovine Serum Albumin
DA Neuron	Dopaminergic neurons
DNA	Deoxyribonucleic acid
DMEM	Dulbecco's Modified Eagle Medium
FBS	Fetal Bovine Serum
HIV	Human Immunodeficiency Virus
HSPG	Heparin Sulfate Proteoglycan
iPSC	induced Pluripotent Stem Cell
MEF	Mouse Embryonic Fibroblast
PEI	Polyethylenimine
TH	Tyrosine Hydroxylase

INTRODUCTION

Clinical gene therapy is rapidly becoming a favorable tool to combat a multitude of diseases that afflict a variety of organs and cell types. Virtually all cells in the body contain a genetic identification code in the form of deoxyribonucleic acid (DNA) – a signature that we can take advantage of to therapeutically treat a myriad of conditions. Numerous strategies allow for the introduction of new genetic material into cells to replace mutations in patients' genes that can lead to dysfunctional proteins or otherwise defective gene products. One such method is the use of viruses that can act as gene-delivery tools. The use of adeno-associated viruses (AAV) as vectors is especially promising due to their potential for cell-specific gene delivery coupled with tendency to induce little to no immunogenic response and biological safety. Our research group sought to demonstrate AAV transduction capabilities in different cell types using reporter genes in cells in culture and in a live animal model.

Gene Therapy

Clinical gene therapy harnesses the genetic transfer abilities of different technologies to introduce new genetic material into either somatic or germline cells in the body to treat or to prevent a disease. Virtually all cells in patients are susceptible as targets of genetic exchange; however, there is a different consequence of transferring nucleic acid into a somatic, or nonreproductive cell, versus a cell of the germline (eggs or sperm). The latter technique involves the

enhancement of DNA in a postmitotic cell that then passes the change to all subsequent generations. This practice is currently not active and remains largely a theoretical concern (Anderson, 1985). Apart from these ethical considerations, gene therapy promises great potential for the treatment of numerous inherited and acquired diseases such as congenital blindness, hypoxia, and even cancer (Bennet, J. et al., 2012) (Rhim, Lee, & Lee, 2013) (Dachs, Dougherty, Stratford, & Chaplin, 1997).

Gene Therapy Limitations

Although new technologies for safe and effective gene delivery methods are being developed and tested rapidly, there are currently limited methods to ensure that non-specific expression of the therapeutic genes does not occur. Transcriptional control is a necessary component of the application of transgene methods to ensure that no off-target tissues or proteins are affected. Inducible gene expression is a method of regulating transcription and subsequent processes and can occur in a few ways. This is the goal in treating a disease such as cancer, where there is necessity for the selected targeting and killing of tumor cells. Similarly, in cardiovascular or neurological disorders where there is a defect in the function of one protein in a specific cell or tissue type, it would be essential to control for the cell-specific insertion of the corrected gene via a selective vector. To date, both viral and non-viral delivery agents have been used with varying degrees of success. Non-viral methods involving the use of cationic

polymeric and lipid based carriers are attractive due to their safety advantages but have notably low efficiency and no targeting ability compared to some of the viral alternatives available (Brown, Schatzlein, & Uchegbu, 2001).

Viral Vectors

Naturally-evolved properties of viruses include their ability to deliver nucleic acid to a specific host cell type while evading immunosurveillance by that host, which makes them ideal candidates for gene therapy tools. A multitude of viral vehicle types, or vectors, exist and have been modified in the laboratory for therapeutic applications. Retroviruses, adenoviruses, adeno-associated viruses, and herpes simplex viruses all have unique profiles and can be used for a variety of applications.

Viral vectors for gene therapy were first introduced as a revolutionary new treatment method with low risk of side effects for otherwise untreatable diseases. However, early clinical trials brought to light inefficiencies associated with the use of some of these vectors. Early recombinant prototypes did not provide sufficient delivery of the genes into target cells or failed to persist, leading to short-lived expression. Greater understanding of vector biology and of viral vectors-host interactions has led to improved efficiency and some clinical trial successes, however (Thomas, 2003).

Today, there are numerous gene targets linked to diseases that are amenable to the use of viral vectors for gene transfer. No single virus category is

likely suitable for all treatment types, but some have great potential to be effective therapeutic candidates in the long-term. Ideal properties of a viral vector candidate include the ability to propagate and be stably reproduced, incorporate into the host cell type and genetic locus of interest, ability to be purified to high titer, and finally to deliver genetic material with low risk of side effects. In addition to the identification and generation of the vector, a therapeutic gene must be cloned and a comprehensive understanding of the pathogenesis of the diseases is required to ensure targeting and delivery to the appropriate cell type. Retroviruses and adenoviruses are the most oft-used vectors in clinical trials and it is anticipated that the use of adeno-associated viruses and herpes simplex virus will also gain traction in the clinic (Tani, Faustine, & Sufian, 2011).

Viral vectors have been extensively engineered in the laboratory through research and experiments with animal models to minimize any associated health risks and immunogenic responses. Lentiviruses, members of the retrovirus family, were derived from the human immunodeficiency virus (HIV), though they retain less than 5% of the parent genome and less than 25% is incorporated into packaging constructs, thereby almost eliminating the potential for the retro-generation of competent HIV particles. Adenoviruses have also been extensively modified to reduce their induction of inflammatory responses in the host. Helper-dependent adenoviruses that include the deletion of all viral genes (that could cause a cytotoxic response) have been developed and have long term potential due also to their prolonged transgene expression. (Thomas 2003). Adeno-

associated viruses are gaining in popularity because they can affect a variety of cell types selectively, and can do so without cytotoxic effects. In virtually all viral vector systems, non-essential genes are removed and remaining essential genes are spread among different plasmids to ensure safety. Gene components are distinguished by those that are necessary to be provided *in cis* (on the same plasmid) or *in trans* (on a separate plasmid) as the insert-containing plasmid. AAVs require ITR (inverted terminal repeat) elements to be provided *in cis* for efficient multiplication of the AAV genome, and the Rep and Cap genes to be provided *in trans* for packaging and assembly of the final virion.

Adeno-associated Viruses

Adeno-associated viral (AAV) vectors are increasingly becoming more popular as gene delivery tools owing to their safety and efficacy as demonstrated by promising clinical trials and regulatory approvals. AAVs are a human virus type that can infect both dividing and quiescent cells. They can integrate as a provirus to propagate or form a lytic infection. “Adeno-associated” virus derives its name from its inability to initiate successful viral infection without the function of helper adeno- or herpes-viruses. It was first discovered as a contaminant of purified adenovirus preparations and was thought to be either a precursor of the mature adenoviral particle or a residual product of the degradation of the adeno-virion (Atchison, et al., 1965) (Hoggan, Blacklow, & Rowe, 1966). In the absence of a helper virus, AAV cannot produce progeny viral particles, but it can integrate

into host site-specific chromosome to form a latent infection. Host cells carrying an AAV provirus that subsequently become infected with a helper virus allow for the AAV genome to become excised and to proceed to a productive infection cycle (Hoggan, Thomas, Thomas, & Johnson, 1972). AAV can also productively replicate when cells are treated with chemical or physical agents that exert genotoxic activity (Berns & Linden, 1995)

The AAV particle is relatively small virion of 18- to 25-nm and consists of an approximate 5-kilobase single-stranded DNA genome that is packaged into an icosahedral capsid composed of 60 proteins (Berns, 1990). The recombinant vectors contain two 145-basepair inverted terminal repeats that form a T-shaped hairpin at both ends; these are the only *cis*-acting elements that are retained from the wildtype AAV genome (Xiao, Xiao, Li, & Samulski, 1997). Recombinant vectors are straightforward to produce – they consist of a transgene cassette, where the gene of interest is cloned in between the two viral inverted terminal repeats. Once cloned into this plasmid, it is used to co-transfect packaging cells with factors that provide the AAV Rep and Cap proteins required for final virion assembly. Variations in the amino acid sequences of the capsid proteins provide naturally-occurring serotype diversity among AAV vectors; in application this property proves valuable since it can provide the ability to target specific cells based on serotype-receptor specificity. (Zacchigna, Zentilin, Giacca, 2014). To date, 13 distinct AAV serotypes have been identified for application (Mietzsch, et al., 2014), all of which are hybridized with the prototype AAV2.

AAV2 provides the genetic backbone for the complete virus particle and is the most well-characterized. All other serotypes of the recombinant vectors are hybridized with this original archetype and allow for targeting of specific cells based on co-receptor binding. The virus docks primarily by binding to heparin sulfate proteoglycan (HSPG) and then is assisted in internalization by binding to coreceptors. Not all of the AAV serotype receptors are known, but many of their tropisms have been well studied and described. The tissue-targeting ability of AAVs can be further enhanced through genetic regulation by the placement of the gene of interest under the control of a tissue-specific promoter.

AAV Vectors for Gene Therapy

AAV vectors are demonstrating emerging clinical success and are advancing towards the potential to treat some of the most highly prevalent conditions such as neurodegenerative and cardiovascular disorders with limited drawbacks. Viral genes are commonly absent in AAV vectors, and they are unable to integrate into host-genomes, having been engineered as such (Zacchigna, Zentilin, & Giacca, 2014). Patients with a range of diseases from congenital blindness (Bennett, et al., 2012), to hemophilia (Nathwani, et al., 2011), to lipoprotein lipase deficiency (Stroes, et al., 2008) have been targeted for the treatment of these conditions by AAV vectors with universal success. AAV vectors, compared to other viral gene delivery methods, are able to target specific cells of interest without the induction of an inflammatory or otherwise immunological response, even upon readministration of the virus (Bennett et al.,

2012). This heightened ability to target specific cells and reduced risk of systemic dissemination of the virus upon administration, makes this vector type favorable for use in the clinic for the treatment of a myriad of diseases. Though the simple genome limits the size capacity of the therapeutic gene, it is an attractive vector candidate also because it provides sustained expression in the target cell.

Thesis Objectives

The goal of the present study was to describe the tissue-specific expression of AAV vectors by demonstrating their cell-targeting ability in a live animal model. Moreover, we sought to develop and optimize a protocol for the production, propagation, purification, and characterization of various AAV serotypes to determine their tropism and to observe their integrative and expressive abilities using different reporter genes *in vitro* and *in vivo*.

To accomplish this goal, we cloned reporter genes luciferase and GFP into the transgene cassette. Following this, we identified optimal transfection methods and culture conditions, followed by a purification protocol to maximize purified virus preparation titer. Finally, we tested the integrative and expressive abilities of the reporter genes *in vitro* – in differentiated neurons from induced pluripotent stem cells (iPSCs) – and *in vivo* – targeting cardiovascular and neuronal tissue through intravenous delivery.

The long-term goal of these projects is to replace the reporter genes with specific functional proteins for delivery into the organ systems that we tested in

the experiments outlined below. Following successful demonstration of the integration of our reporter genes into cardiovascular and brain tissues, we hope to use AAV vectors for transgene delivery of: fatty acid oxidation rate-limiting protein CPT1B into the heart to rescue the effect of otherwise dysfunctional gene products, and Ca^{2+} -signaling-defective gene *Pla2g6* which is been implicated in familial Parkinson's Disease. Because the impairment of these genes is limited to specific tissue in the disease states, it is necessary to develop a gene transfer method that will deliver and allow integration of the transgene into specific tissue and provide sustained expression of the new DNA.

METHODS

Animal Model

All experimental procedures involving animal use were compliant with ethical regulations and acquired approval from the Institutional Animal Care and Use Committee of Boston University. Animals were maintained in an advanced pathogen-free facility with veterinary service and unlimited access to food and water. Adult male mice were used for *in vivo* studies and females were used for the derivation of mouse embryonic fibroblasts (MEFs) for GFP experiments. Wild type male and female mice both were used for experiments testing for luciferase expression.

Cell Culture

Human embryonic kidney-H cells, cloned from the original 293 cell line, were acquired from Thermo Fisher (Thermo Fisher 11631017) and cultured in serum-free CD 293 suspension medium (Thermo Fisher 11913019) supplemented with GlutaMAX (Thermo Fisher 35050061) and Penicillin-Streptomycin antibiotic (Thermo Fisher 15070063). Cells were first plated as adherent cells in Falcon T25 tissue culture flasks (Thermo Fisher 08772) until recovered from cryostorage; they were passaged and re-plated in T75 tissue culture flasks and were allowed to continue to grow until reaching log phase. Cells were transferred to 250 ml polycarbonate Erlenmeyer flasks (Thermo Fisher 08772257) to be grown in suspension at 30 ml volume at a rotation speed

of 125 rpm at 37 °C and 8% CO₂. Cells were passaged upon reaching 2 million cells/1 ml volume of medium at ratio of 1:2 every third day.

MEFs were isolated from 14.5 day old embryos, and procedure was established and carried out by our collaborators in the laboratory of Dr. Victoria Bolotina at Boston University. Embryos were genotyped. Head, vertebral column, dorsal root ganglia, and internal organs were removed and discarded. Remaining embryonic tissue was mechanically dissociated and incubated in 0.25% trypsin (Gibco) for 15-30 minutes. Cells from each embryo were plated onto a 10-centimeter tissue culture dish in Dulbecco's modified Eagle medium (DMEM; Mediatech Inc.) supplemented with 10% fetal bovine serum (FBS), non-essential amino acids, sodium pyruvate, and Penicillin-Streptomycin (Invitrogen). Upon reaching confluence, primary MEFs from WT embryos were tested and confirmed to be free of mycoplasma contamination, and MEFs from passage 2-3 were studied after 24-48 hr in culture and used for experiments.

iPSC derived DA neurons from MEFs protocol was performed according to procedure previously described by Sommer et. al (2010) by the Bolotina lab at Boston University. Approximately 100,000 MEFs were plated in MEF media and transduced with constitutive STEMCCA vector at an MOI of 2.5 for a 24 hr period. Media was then changed to ESC medium (DMEM with added 15% FBS, GlutaMAX, 350K units of ESGRO leukemia inhibitory factor, and 0.1 mM 2-mercaptoethanol). Medium was changed every second day until appearance of

colonies which were then picked and expanded for STEMCCA excision and characterization. Colonies positive for alkaline phosphatase, OCT3/4, ZFP96, Nanog, and ERas were further expanded and selected for neural differentiation. iPSCs were differentiated into DA neurons first by inducing formation of embryoid bodies in non-adherent conditions for 4 days in knockout serum replacement medium. Cells were then transferred and cultures in ITS medium (DMEM/F12 (Gibco), ITS Supplement (Sigma 113146), and 1 µg/ml Bovine fibronectin (Sigma F1141)) for 6-10 days to induce ectoderm formation. Cells were further expanded and neural identity confirmed by staining against beta-III tubulin and nestin. Differentiation into dopaminergic neurons was performed by incubating cells for 10 days in minimal medium (DMEM/F12 (Gibco), N2 Max + Ascorbic Acid (Sigma, A4403)). DA neuron identity was confirmed by staining cells for TH (Abcam ab 76442), Dopamine transporter (DAT, Abcam ab5990), and vesicular monoamine transporter 2 (VMAT2, Abcam 70808).

Restriction Digests and Gel Electrophoresis

Plasmids were digested with restriction enzymes to confirm DNA identity. Restriction enzymes were acquired from New England Biolabs. 1 µl of enzyme was introduced to reaction tube with 1 µl corresponding salt buffer, up to 10 µg DNA, and PCR-grade H₂O to 50 µl total volume. Sample was incubated in 37 °C water bath for 1-1.5 hr.

One percent agarose gels were prepared by measuring 3.5 g powdered agarose and adding to 1X TAE (Tris, Acetic Acid, and EDTA) with 3.5 μ l GelRed – a nucleic acid stain to intercalate with DNA as an alternative to conventional ethidium bromide. Gel was poured into mold with an 8-lane comb while still in liquid phase. After cooling, 10 μ l of digested DNA product was added to each lane and electric current was applied. Gel was run at 90 V for 30 min to separate DNA bands according to size. Bands were visualized under ultraviolet light and analyzed using Molecular Analyst DNA software.

Terrific Broth Preparation

Added the following to 900 ml deionized H₂O: 12 g bacto-tryptone, 24 g bacto-yeast extract, and 4 ml glycerol with 0.17 M KH₂PO₄ and 0.72 M K₂HPO₄. Added 50 μ g/ml Ampicillin or Neomycin based on specific plasmid resistance gene.

Plasmid Preparation

Plasmid expressing GFP under synuclein promoter control was a kind gift from Dr. Tsuneya Ikezu at Boston University. Plasmid expressing the firefly luciferase gene under the ubiquitous CMV promoter was purchased from Addgene (plasmid #41045). Plasmid DNA for serotype and packaging viral gene expression was acquired from the University of Pennsylvania Vector Core (pAAV2/5, pAAV2/1, pAAV2/8, pAAV2/9, and pAAVH1.shRLuc.CMV.ZsGreen.SV40). Firefly luciferase was cloned into the

AAV expression vector from the donor plasmid PGL3 using restriction digests and blunt-ending technique followed by ligation. Plasmid was sequenced using the primer 5' CTAGCAAATAGGCTGTCCC 3'.

Plasmids arrived in stab cultures that were used to inoculate overnight bacterial cultures in 8 ml terrific broth. Cultures were harvested and spun at 5,000 rpm for 10 minutes for Miniprep plasmid purification using Qiagen protocol (27104). Bacterial cells were pelleted and suspended in 250 μ l resuspension buffer with RNase A. Following this, 250 μ l of alkaline lysis buffer was added for 5 min. Lysis reaction was neutralized with acidic neutralization buffer, followed by centrifugation in 1.5 ml Eppendorf tubes in tabletop centrifuge for 10 min at 17,900 x g. Supernatant was transferred to spin column for DNA capture which was spun at maximum speed for 1 min. Flow-through was discarded. Binding buffer was added and the column spun again with flow-through discarded. Column was washed with 750 μ l ethanol, spun twice, and flow-through discarded. Column was transferred to 1.5 ml Eppendorf collection tube. Ten μ l elution buffer was added, column was incubated for 5 min, and then was spun at maximum speed for 1 min. Final DNA concentration was measured using NanoDrop spectrophotometer.

Maxiprep plasmid purifications were carried out according to Qiagen protocol (12162). Overnight 50 ml cultures were prepared from glycerol stocks of original overnight bacterial culture with addition of 50 μ g/ml Ampicillin. Prior

protocol described for harvest of overnight culture, resuspension, and lysis was followed but with volume of 6 ml for buffers. During first tabletop centrifugation, Maxiprep column was equilibrated with addition of 10 ml Buffer QBT followed by wash with Buffer QC. DNA was eluted and then precipitated with isopropyl alcohol, followed by wash with 70% ethanol. DNA was centrifuged at maximum speed in tabletop centrifuge for 10 minutes and supernatant was decanted. Final DNA pellet was resuspended in Buffer TE and yield measured using NanoDrop spectrophotometer.

Brain Slices: Preparation, Immunostaining

Brains were acquired for analysis by the laboratory of Dr. V. Bolotina at Boston University. Under anesthesia, Paav2/5-SYN-GFP was stereotaxically injected (1 μ l at 0.2 μ l/min) in the left hindbrain (bregma AP -2.8 mm; LM -1.3 mm, DV -4.2 mm). Four weeks following injection, whole brains were sampled for immunohistochemistry and Western blot analyses.

Brains were extracted from WT male mice following 4% paraformaldehyde perfusion and cryopreservation in 30% (w/v) sucrose solution at 4 °C. Brain sections were acquired by following procedure established by Jackson-Lewis and Przedborski (2007). Brains were sectioned in 30-40 μ m sections using a cryostat microtome, collected as free-floating sections in 24-well plate, and frozen at 4 °Celsius. Immunostaining was performed using chicken polyclonal antibody (Abcam ab76642). Goat anti-chicken Alexa594 secondary antibody was applied

at 1:1000. DAPI stain was also used to label nuclei and identify all cells present in the section.

Sections were imaged using the Nikon Ti inverted fluorescence microscope equipped with a Perfect Focus system and environmental chamber (InVivo Scientific). Images were acquired using a X60/1.4 Plan-Apochromat oil immersion Nikon objective lens and filter set for GFP (excitation 465-496, emission 515-555).

Acidified PEI

Acidified polyethylenimine (PEI) was prepared for 293-H transfection experiments as a cost-effective alternative to other commercially-available reagents according to the established protocol by Fukumoto (2010). Linear PEI with (molecular weight 25,000; Polysciences Inc. 23966-2) was dissolved in 0.2 N HCl at 5 mg/ml. Resulting solution had pH 1.0. Aliquot were prepared and frozen at -80 °C; avoid freeze-thaws.

Cell Transfection and Viral Transduction

293-H suspension cells were transfected with plasmid using acidified PEI in an optimized protocol. Cells were grown to ~3-4 million cells/ml prior to transfection. Before transfecting, CD 293 medium was replaced with 25 ml fresh DMEM containing 10% FBS. 30 µg of each plasmid DNA was added to 1.5 ml of serum-free medium. 75 µg (15 µl stock PEI) was diluted in separate tube in 1.5

ml of serum-free medium. The two solutions were combined and gently vortexed to mix. Combined solution was incubated at room temperature for 20-60 min. The solution was then combined with 2 ml fresh DMEM and added drop-wise to flask. Flask was returned to incubator and rotated at 125 rpm at 37 °C and 8% CO₂. DMEM was replaced with CD 293 medium after 3-4 hr. Cells were collected and harvested after 3-4 days, spun at 500 x g for 5 min, and pellets frozen after discarding the supernatant (left ~5 ml of medium in tube, however).

To test for successful AAV transduction in MEFs, 1 ul of purified AAV was introduced into media and cells were incubated for 24-48 hr prior to imaging.

Iodixanol/Optiprep Preparation

The following solutions were prepared to be used in virus purification: 10X PBS with 10 mM MgCl₂ and 20 mM KCl (solution B), PBS with 10 mM MgCl₂ and 20 mM KCl (solution C), 2 M NaCl in solution C (solution D), 54% iodixanol in solution B (solution E). From these, the following solutions were prepared to be layered for the gradient: 15% iodixanol containing 1.5 volumes of solution E with 2.7 volumes of solution D and 1.2 volumes of solution C; 25% iodixanol containing 2.5 volume of solution E and 2.9 volumes of solution C; 40% iodixanol containing 4.0 volumes of solution E and 1.4 volumes of solution C; 54% iodixanol containing 9 volumes of iodixanol/Optiprep and 1 volume of solution B.

AAV Purification

Retrieved frozen pellet of transfected and harvested 293-H cells and subjected to 3-4 freeze-thaw cycles by alternating between -80 °C and room temperature and 37 °C water bath to lyse cells. For the last thaw cycle, prior to the isolation procedure, 1 ul benzonase (E1014 Sigma) per 1 ml medium was added to disrupt cell particles. Pellet was incubated with the nuclease in 37 °C water bath, shaking every 15 min.

Following incubation, resuspended pellet was centrifuged at 1800 rpm for 5 min. The supernatant was transferred to 1.5 ml Eppendorf tube and centrifuged in a tabletop centrifuge and spun at maximum speed for 5 min, ensuring that it was as clarified as possible. Gradient in polypropylene ultracentrifuge tubes was layered as follows: 1 ml of 54% iodixanol, 3 ml of 40% iodixanol, 3 ml of 25% iodixanol, and 1 ml 15% iodixanol. Solutions were introduced into centrifuge tube using sterilized needle, syringe, and pipette in order of decreasing density using gentle overlayer technique. Tubes were sealed atop with heated metal surface. Tubes were balanced, and then were loaded in ultracentrifuge rotor. Tubes were spun 6-16 hours at 100,000 x g. Following spin, tubes were suspended, sterilized with alcohol wipes, and prepared for virus band extraction. Complete AAV particle band was observed between 25%-40% layer, with less distinct, empty AAV-particle band observed higher up. Using sterile technique, one 18-gauge needle was used to pierce top of plastic tube to allow smooth extraction of the

viral band below. A syringe with 18-gauge needle was used to pierce below the viral band and extract it by facing the bevel up. Following extraction and removal, the virus was added to a dialysis cassette (Thermo Fisher 66205) for overnight dialysis in HNE Buffer (50 mM HEPES, 0.15 M NaCl, 25 mM EDTA); buffer was replaced periodically to ensure tonicity required was reached. Final purified AAV product was quantified, aliquoted, and frozen at -80 °C.

AAV Titer Quantitation

Bovine serum albumin (BSA) standards were prepared of the following amounts: 1333 ng BSA, 667 ng BSA, 333 ng BSA, 167 ng BSA, 83 ng BSA, and 42 ng BSA. 12 µl of purified virus were aliquoted in 1.5 ml Eppendorf tube. 2 µl of 6X Sodium dodecyl sulfate Laemmli buffer were added to each tube of 12 µl standard and virus. All samples and standards were boiled at 95 °Celsius for 3 min. Following denaturation, standards and virus were loaded on 12% polyacrylamide gel and run at 200 volts for 30 min. Gel was removed from apparatus and rinsed with deionized H₂O three times for 5 min each. Coomassie G250 stain was added to gel overnight on rotator at 4 °C. The gel was rinsed the following day in deionized H₂O until it was clear with distinct blue bands. The gel was imaged using the Odyssey Infrared Quantitative Imaging System (LI-COR Biosciences) on the 700 channel. Bands were identified and boxed, information was exported in Excel report, and protein quantity per AAV particle determined with linear regression analysis.

Luciferase Detection

Mice were prepared for tail vein injections with purified AAV by anesthesia with isoflurane. Once unconsciousness, up to 250 μ l of purified virus for total of approximately 1×10^{11} AAV particles was injected into tail veins using 27-gauge needles, beveled up. Mice were imaged after a minimum of two weeks using the IVIS bioluminescent imaging system; D-luciferin was first prepared at 15 mg/ml in PBS. This solution was injected into the test and control mice intraperitoneally at 10 μ l per gram of body weight 10-15 min prior to imaging. Mice were then anesthetized and placed in IVIS imager to detect bioluminescent signal.

Following IVIS imaging for the *in vivo* study, mice were anesthetized and sacrificed using cervical dislocation. Mouse organs were extracted including tissue from the heart, skeletal (quadricep) muscle, spleen, lung, and liver. The tissues were homogenized, and 50 μ l of sample were loaded onto a black-bottom 96-well plate. Twenty μ l of luminol substrate was added immediately prior to loading plate into a TECAN bioluminescent plate reader. Samples were loaded and luminescence measure in arbitrary light units to determine relative intensity of signal.

qRT-PCR

A quantitative assessment of firefly luciferase gene expression was performed at the transcript level of tissues extracted from the mice that underwent AAV vector treatment and IVIS imaging. Mice were euthanized and

their heart, lung, liver, quadricep muscle, kidney, and adipose tissues were removed. The tissues were homogenized and RNA was extracted using sterile technique. The mRNA was reverse transcribed to cDNA which was then analyzed by qRT-PCR. Taqman primers for luciferase gene detection were added, and Rsp18 was used as a control (Luciferase TaqMan AF093683_Luc.0 FAM-MGB kit, Catalog #4448892).

RESULTS AND DISCUSSION

Optimization of Virus Purification Protocol

Various transfection reagents and protocols were tested against acidified polyethyleneimine (PEI) to determine greatest transfection efficiency including greatest rate of integration of DNA material into 293-H cells while avoiding using excessive quantities of transfection reagent so as to limit potential cytotoxic response. Transfection efficiency of PEI is dependent on the ratio of PEI to DNA. We tested differing ratios of PEI to DNA, and found greatest virus assembly and production to occur at 75 μg PEI combined with 30 μg per each of three AAV DNA plasmids. The original Fukumoto protocol detailed culture conditions of adherent cells different from the conditions in which we maintained our 293-H cell cultures. Furthermore, they described replacing PEI-containing medium with fresh medium 8-12 hours following transfection. We shortened the incubation time with the PEI-DNA polyplex to limit potential cytotoxic effects of the PEI. Following transfection, we varied days-to-harvest periods to maximize virus particle output; we found we acquired the greatest yield after 4 days, and thus determined to collect cells 4 days post-transfection. A representation of the optimized AAV generation protocol is presented in Figure 1.

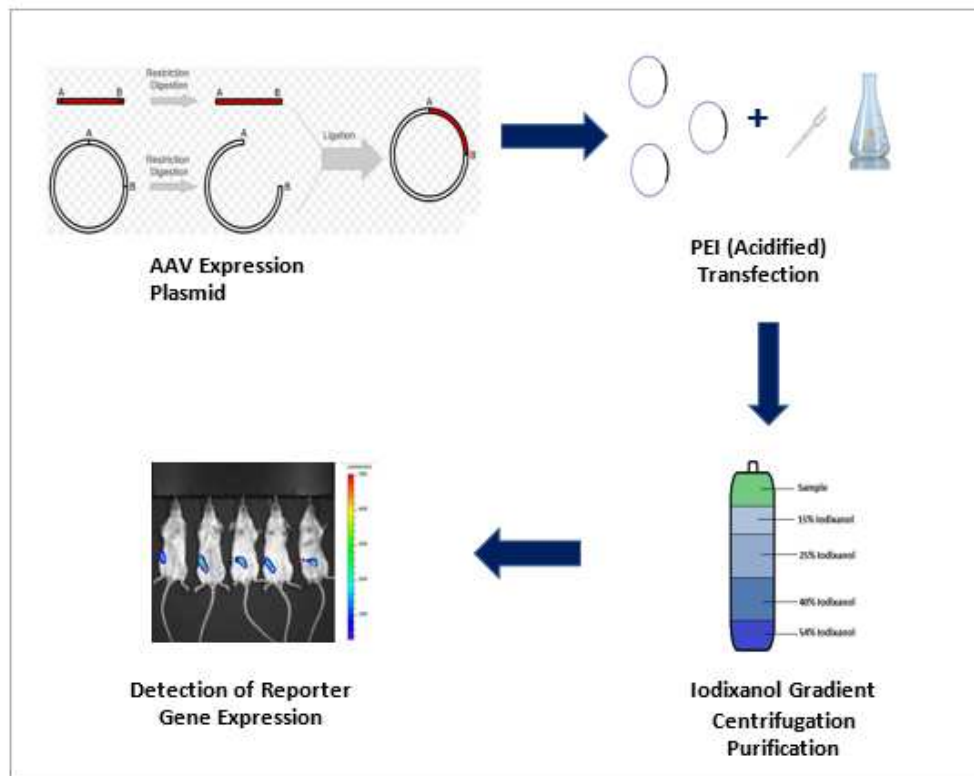


Figure 1. Optimization of Cellular Transfection and AAV Generation Protocol. DNA delivery method into cells using acidified PEI was modified to maximize transfection efficiency. 293-H cells in suspension were transfected with AAV expression, serotype, and packaging vectors. Virus extraction and purification procedure was subsequently carried out using an iodixanol gradient to yield maximum virus particle output.

Determination of Virus Particle Titer

Following optimization of the virus purification process, we then tested various methods for the determination of our purified preparation yield. Figure 2 depicts the result of an analysis using SDS-PAGE followed by treatment with Coomassie Brilliant Blue G250 stain and infrared scanning with the Licor Odyssey scanner. A dilution of a series of bovine serum albumin (BSA) standard was detected along with a single quantity of the AAV2/5-SYN1-eGFP virus. Following the scan, the quantity of BSA nanograms loaded were plotted against the background-corrected fluorescence intensity of the bands. Linear regression analysis was performed and yielded an equation for the calculation of the nanograms of VP3 in the virus sample loaded. The protocol by Kohlbrenner *et al.* (2012) described the determination of the peptide sequence of VP3 and general acceptance of 50 VP3 molecules per viral capsid to calculate that each AAV particle contains 4.97×10^{-9} ng of VP3. Using this, we divided the nanograms of VP3 determined by the gel method by the nanograms of VP3 per virus particle to determine the viral titer.

The final titers for the virus purifications we completed thus far are listed in Table 1. Corresponding target tissues based on the virus serotype are also listed, and were derived from the catalog information made available by the Penn Vector Core. The average AAV titer was 3.82×10^{11} PFU/ μ l. Following successful generation and purification of the vectors described in the table, our next step is

the production of AAV vectors with therapeutic genes for transgene delivery to the brain and cardiac tissue specifically.

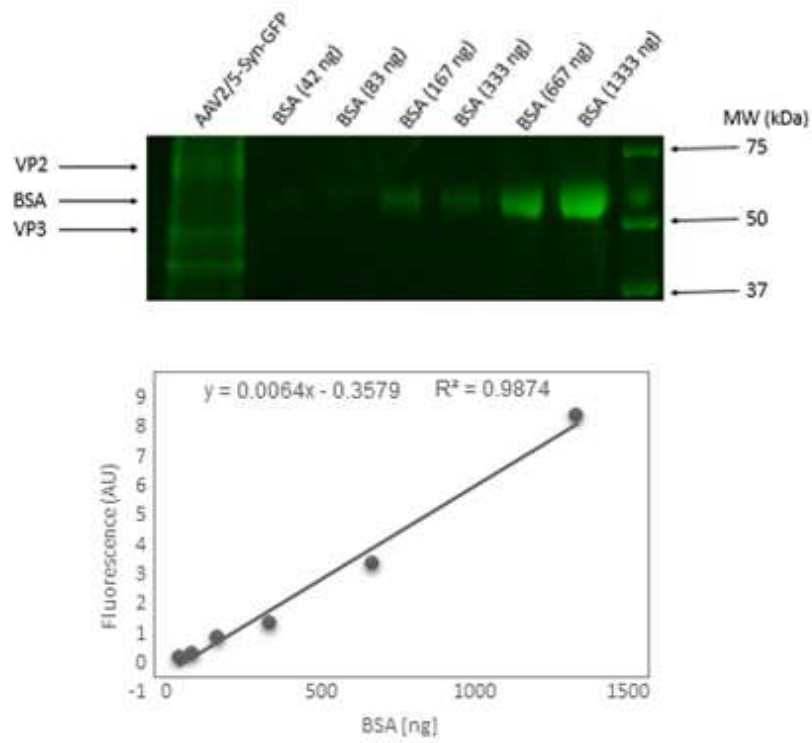


Figure 2. Quantification of AAV2/5-SYN1-eGFP Viral Particle Titters by Coomassie Brilliant Blue-stained SDS-PAGE and Infrared Scanning with a Licor Scanner. Fluorescence intensity is linear over the range of BSA control loaded onto the gel. Positions of the viral capsid proteins (VP2, VP3) with molecular weights are indicated with arrows (*top*). Regression analysis of the standard curve allowed determination of the number of virus particles loaded in the leftmost lane.

AAV Serotypes	Target Tissue	Titer (PFU)
AAV2/1 – Firefly Luciferase	Muscle, Heart, CNS	$3.85 \cdot 10^{11}$ /ul
AAV2/2	<i>In vitro</i> , CNS	Not available
AAV2/5 - SYN1-eGFP	Lung, Eyes, CNS	$1.2 \cdot 10^9$ /ul
AAV2/6	Lung, Heart	Not available
AAV2/7	Muscle, Liver	Not available
AAV2/8 – Firefly Luciferase	Liver, Muscle, CNS	$9.50 \cdot 10^{11}$ /ul
AAV2/9 – Firefly Luciferase	Lung, Liver, Muscle, Heart	$1.94 \cdot 10^{11}$ /ul
AAV2/10	Pleura, CNS	Not available

Table 1. Adeno-associated Viral Vectors with Tropism Defined and Corresponding Titer. Various AAV viral vectors were generated in the laboratory; the target tissue for each Penn Vector Core serotype is given along with the titer for those viruses we successfully generated thus far.

Tissue-Specific Expression of eGFP in Neuronal Cells *In vitro* and *In vivo*

Cell-specific targeting ability of AAV2/5-SYN1-eGFP was demonstrated through treatment of mouse embryonic fibroblast (MEF) cells with purified virus. MEFs were used as a negative control to show the reliance of transgene expression on serotype of the AAV vector (Figure 3).

Dopaminergic (DA) neurons derived from human induced pluripotent stem cells (iPSCs) were maintained in the laboratory and transduced with the reporter gene-containing vector as a positive control. DA cells positively stained for neuronal markers Tuj1 and tyrosine hydroxylase, had apparent expression of the reporter eGFP. This suggests that expression of the gene carried by the AAV vector is specifically driven by serotype and promoter (Figure 4). The same phenomenon was positively observed in iPSCs of mouse origin as well (Figure 5).

Following this, the activity of the virus was then tested *in vivo*. Mice were injected with AAV2/5-SYN1-eGFP using stereotaxic surgery technique to deliver the virus directly into the substantia nigra segment of the brain. Mice were sacrificed four weeks following the virus delivery, and their brains were sectioned and mounted for immunohistochemistry. Here we demonstrated the activity and specificity of the vector through detection of co-localized mouse tyrosine hydroxylase (neuronal marker) and GFP immunofluorescence *in vivo* (Figure 6).

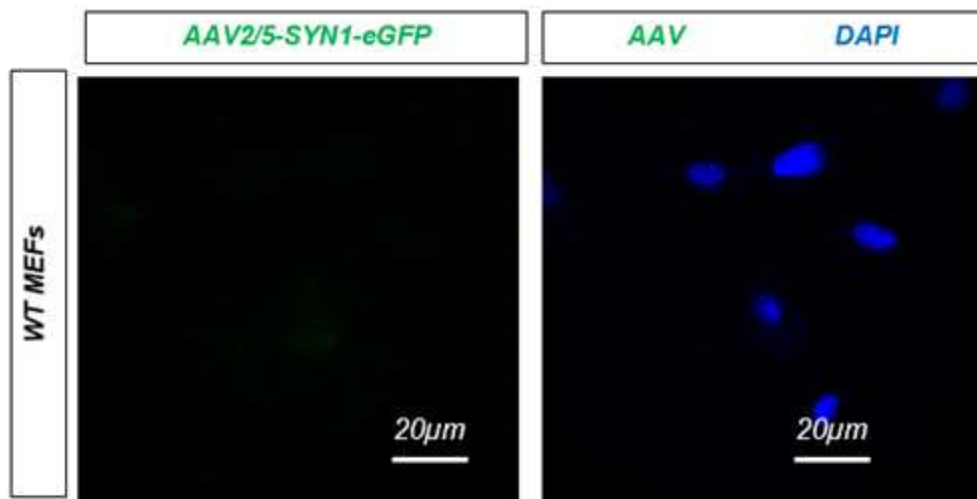


Figure 3. AAV2/5-SYN1-eGFP Does Not Infect MEFs. eGFP expression was only driven in cells of neuronal lineage – here, WT MEFs were used as a negative control to demonstrate that only cells derived from neuronal tissue should positively express reporter eGFP.

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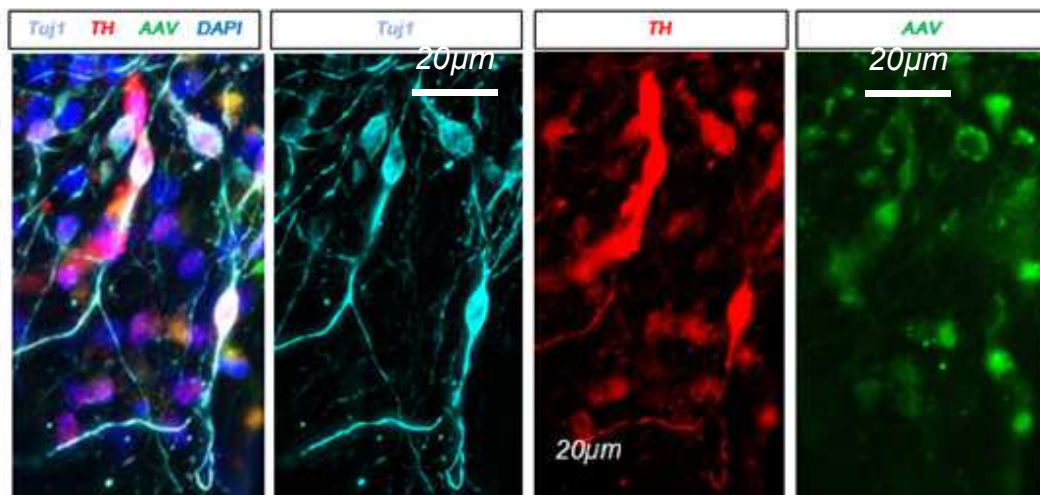


Figure 4. Human iPSC Derived DA Neurons Transduced with AAV2/5-SYN1-eGFP Display Positive Expression. Tuj1 (neuronal marker), tyrosine hydroxylase (TH, DA neuron marker) together confirm that AAV serotype and promoter drive expression of eGFP rather specifically. DAPI was used as stain to identify cell nuclei.

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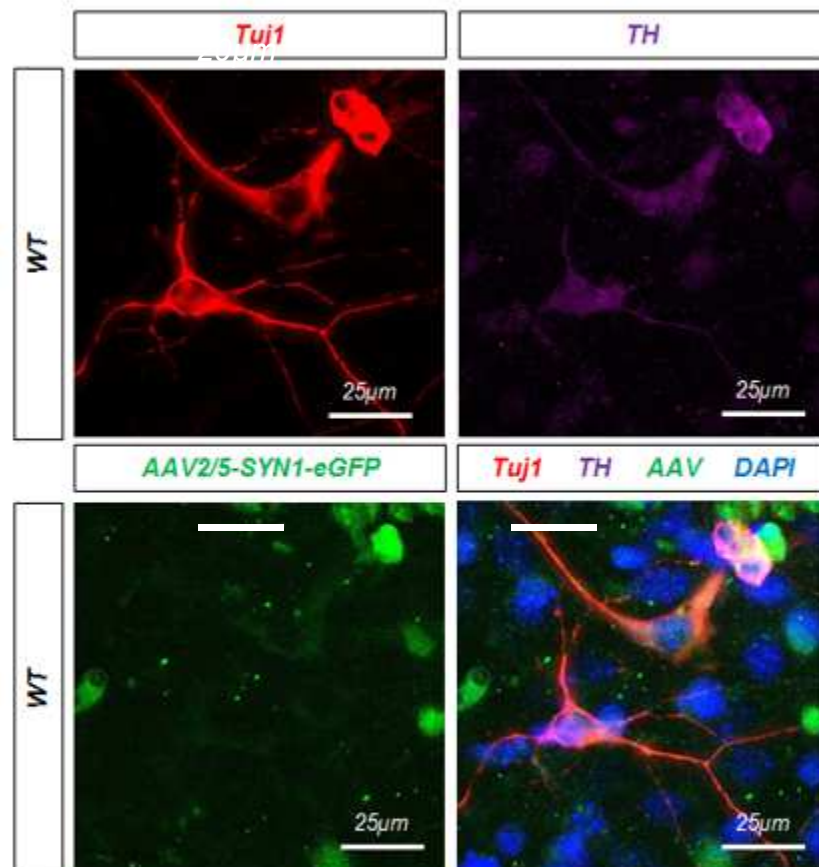


Figure 5. Mouse iPSC Derived DA Neurons Transduced with AAV2/5-SYN1-eGFP Display Positive Expression. Detection of mouse Tuj1 (neuronal marker), tyrosine hydroxylase (TH, DA neuron marker) confirm that AAV serotype and promoter driven expression of eGFP across species. Shim, Joon Simon, laboratory of Victoria Bolotina, Vascular Biology Section at Boston University. Not yet published

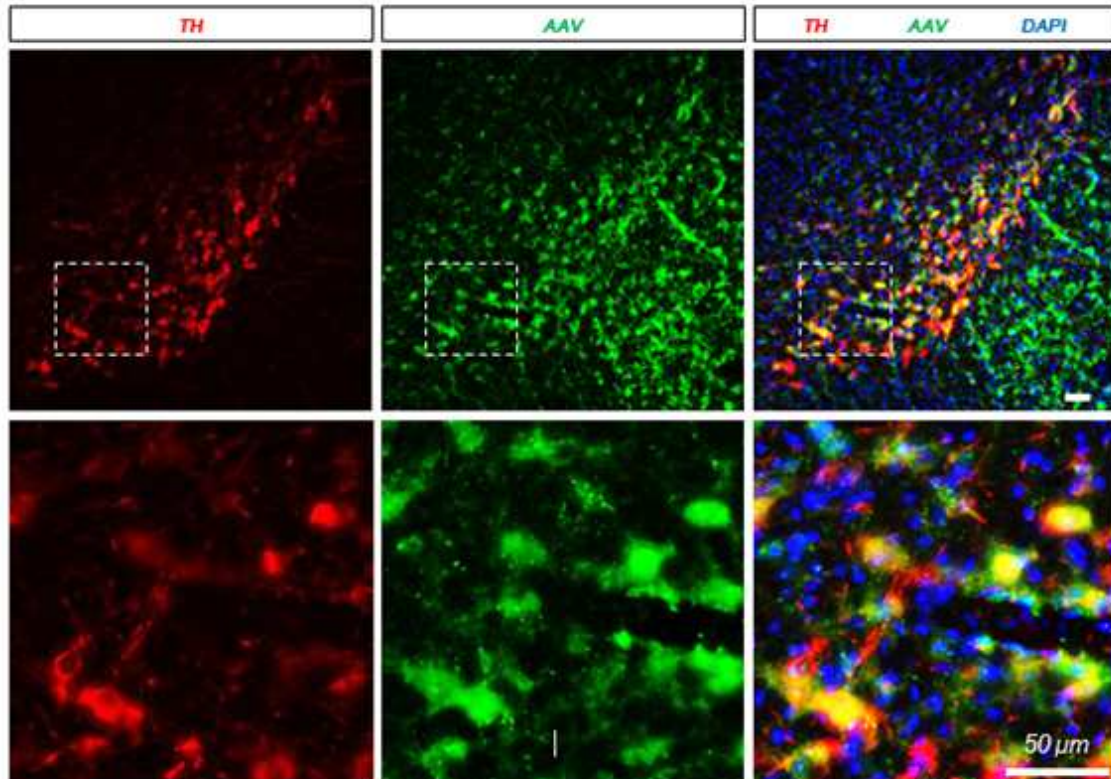


Figure 6. Mouse Brain, 4-weeks Post-AAV Injection Exhibits Reporter Gene Expression. Brain sections from mice treated with AAV2/5-SYN1-eGFP were imaged four weeks following injection. Detection of co-localized mouse tyrosine hydroxylase and GFP immunofluorescence confirmed the activity and specificity of the AAV vector *in vivo*.
Shim, Joon Simon, laboratory of Victoria Bolotina, Vascular Biology Section at Boston University. Not yet published

Tissue-Specific Expression of Firefly Luciferase Gene

Wild type mice were injected with 250 μ l of purified AAV2/1-firefly luciferase, AAV2/9 firefly luciferase, or saline control to determine whether the vector can specifically target and induce reported gene expression in specific tissues according to the virus serotype. No firefly luciferase gene expression was observed, suggesting unsuccessful integration of the gene of interest into the transgene cassette (Figure 7). Virus particle assembly was not affected since a sizeable band was observed and extracted at the final stage of the differential centrifugation step of the virus purification process.

Luminescence is given in arbitrary values (*right*) and corresponds to relative intensity of the signal. The control in this experiment exhibited greatest signal, though it still falls below the recommended 600-count for the lower limit of detection for IVIS. Mice injected with test vector gave an artifactual signal around the abdominal area; this was likely as a result of the substrate D-luciferin injected intraperitoneally into the cavity prior to imaging and is not likely to be a true signal.

Following this experiment, we measured luciferase activity in tissues harvested from the AAV-treated mice. We extracted heart, liver, lung, quadriceps muscle, and spleen tissue from AAV2/1 and AAV2/9 mice and performed a plate assay to look for bioluminescent signal against tissues from control mice. Results demonstrate no discernible pattern of expression in tissues harvested from

vector-treated tissues versus control. The absence of a consistent pattern of luciferase activity suggests there was no expression of the reporter gene (Figure 8).

To better determine whether there was expression of the firefly luciferase gene in the AAV preparations used to inject the mice, we extracted additional tissues from mice injected with AAV2/9 and performed qRT-PCR analysis. Similar to what was demonstrated in the plate assay, there was no corresponding expression in the tissues that were to be targeted by the specific serotype; that is, there was greater intensity of signal in adipose tissue, quadriceps muscle, and kidney tissue than in the heart, lung, and liver; likely this is background signal that was detected. Additionally, there was no significant difference between the level of expression across control and AAV-injected samples. This further compounded the finding of the luciferase plate assay, which suggested insufficient introduction of the reporter gene into the donor AAV vector (Figure 8).

Upon examination of the sequencing results of the firefly luciferase gene into the donor AAV plasmid, we found there was not complete integration of the reporter, thus leading to the unsuccessful detection of the Luciferase gene product in the mice.

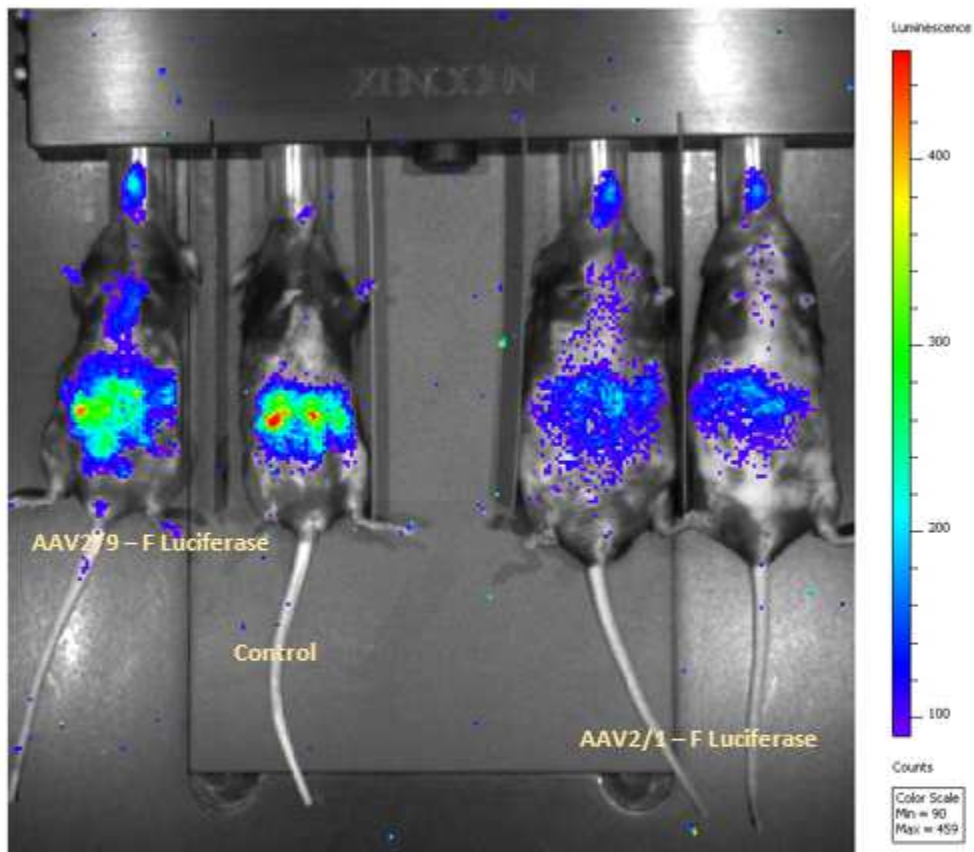


Figure 7. Absence of Expression of Luciferase Reporter Gene in WT Mice. Mice were injected in the tail vein with <math><250\ \mu\text{l}</math> of AAV2/9-FLuc with titer

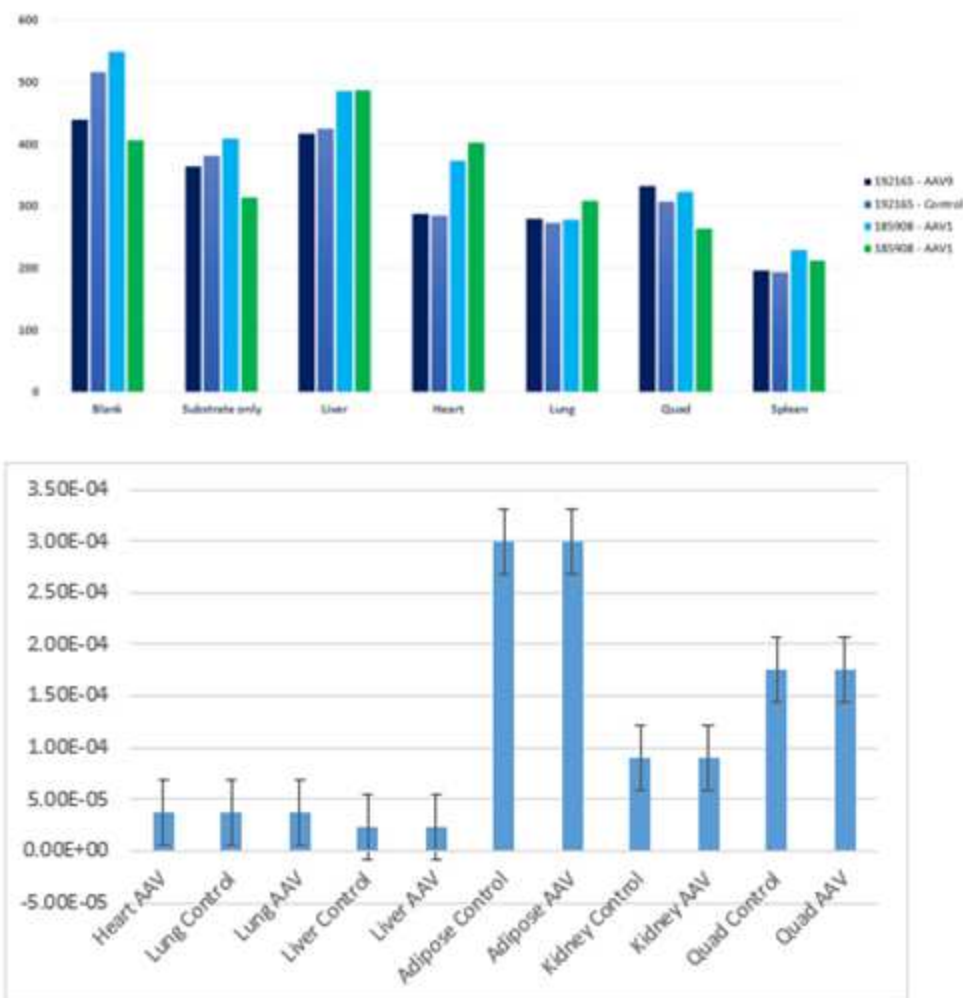


Figure 8. Microplate Luciferase Assay and qRT-PCR Analysis of Luciferase Reporter Gene. (Top) Mice were injected in the tail vein with <250 ul of AAV2/9-FLuc, AAV2/1-FLuc, or vehicle, Heart, lung, liver, quadriceps muscle, and spleen tissue was harvested and analyzed in a plate assay with addition of luminol substrate. There was no difference in pattern of intensity of signal between AAV-treated and control tissues or substrate-only and blank samples. (Bottom) RNA was extracted from tissues harvested from mice injected with AAV2/9-Firefly Luciferase and analyzed using qRT-PCR. There was no significant difference between level of gene expression in tissues harvested from control versus AAV-treated mice.

Conclusion and Future Directions

In the present study, we were successful in establishing a protocol for the generation of functional AAV vectors. We tested the production of the virus particles in adherent versus suspension cells and found the latter to produce greater quantities of mature particles at a faster rate. Cells in suspension were extremely well-suited for bulk virus production as there was less use of materials involved in the maintenance of those cells in culture. Suspension cell growth was only limited by concentration of cells in the medium; this could be controlled by passaging the cells daily or by diluting with addition of extra medium to maintain the cells in log phase. The suspension cells required no enzymatic or mechanical dissociation, and we found them to be robust in the production of AAVs once transfected. We also successfully utilized ultracentrifugation as a purification method for complete virus particles. This was used as an alternative to another common purification technique which involves the use of ion exchange chromatography. We preferred the iodixanol method because the size of AAVs limits the use of chromatographic resins in protein purification (Oksanen et al, 2012), and each serotype would require a different chromatographic method based on their surface properties. In addition, the chromatographic method would not allow distinction between empty and intact virus particles – a difference that is easily discernible when using the iodixanol method. Ultimately, we found ultracentrifugation to be efficient and easily scalable for the potential future production of large quantities of virus particles.

In testing the function of our viral vectors, we were able to successfully demonstrate the ability of neurons and neuronal promoters to drive expression of the reporter eGFP gene delivered via the AAV2/5 serotype. In future, we would seek to incorporate a therapeutic gene, PLA2 – a protein that has been implicated in the pathophysiology of Alzheimer's Disease – to be cloned into the original AAV transgene cassette for delivery into the substantia nigra of the brain where it can potentially rescue the function of the mutated protein.

We were unable to successfully demonstrate integration and expression of the reporter firefly luciferase gene into target tissue with serotypes AAV2/1 and AAV2/9. This is likely due to the inaccuracy in our cloning technique since we were unable to successfully show complete insertion of our transgene into the AAV expression vector through sequencing. It is not likely that transfection or virus assembly was compromised; this is evidenced by the presence of prominent viral bands in the iodixanol centrifugation step of the experiment. In future, we would seek to confirm the appropriate gene sequence cloned into the expression plasmid, and then follow with demonstration of accurate integration and expression of the viral genes.

Upon repeated demonstration of the successful integration of reporter gene-containing AAV vectors into target cells, our next step would be to determine levels and durations of expression of the transgenes. We sought to characterize the functions and capabilities of the AAV vector and to use it as a

gene delivery tool for conditions that cannot tolerate the toxicity or non-specificity of alternative methods. While the present study focused primarily on the characterization of the AAV vector based on its tropism, there is vast potential if we continue to explore its capabilities in a multitude of disease types. Our aim is to continue to hone the AAV generation process and to ultimately use this viral vector as a therapeutic tool.

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