Copackaging of Multiple Adeno-Associated Viral Vectors in a Single Production Step

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Abstract

Limiting factors in large preclinical and clinical studies utilizing adeno-associated virus (AAV) for gene therapy are focused on the restrictive packaging capacity, the overall yields, and the versatility of the production methods for single AAV vector production. Furthermore, applications where multiple vectors are needed to provide long expression cassettes, whether because of long cDNA sequences or the need of different regulatory elements, require that each vector be packaged and characterized separately, directly affecting labor and cost associated with such manufacturing strategies. To overcome these limitations, we propose a novel method of vector production that allows for the packaging of multiple expression cassettes in a single transfection step. Here we combined two expression cassettes in predetermined ratios before transfection and empirically demonstrate that the output vector recapitulates the predicted ratios. Titration by quantitative polymerase chain reaction of AAV vector genome copies using shared or unique genetic elements allowed for delineation of the individual vector contribution to the total preparation that showed the predicted differential packaging outcomes. By copackaging green fluorescent protein (GFP) and mCherry constructs, we demonstrate that both vector genome and infectious titers reiterated the ratios utilized to produce the constructs by transfection. Copackaged therapeutic constructs that only differ in transcriptional elements produced a heterogeneous vector population of both constructs in the predefined ratios. This study shows feasibility and reproducibility of a method that allows for two constructs, differing in either transgene or transcription elements, to be efficiently copackaged and characterized simultaneously, reducing cost of manufacturing and release testing.

Introduction

TO DATE, ADENO-ASSOCIATED VIRUS (AAV) has been used in over 100 gene therapy clinical trials. The widespread tropism, sustained gene expression, and excellent safety data that exist for AAV are only a few of the reasons it has reached such popularity. As a nonpathogenic shuttle for therapeutic genes, capable of delivering its payload to many cell types, the basic biological processes governing the behavior of the many AAV serotypes have been an extensive area of research for many years (Zincarelli *et al.*, 2008; Asokan *et al.*, 2012; Gurda *et al.*, 2012; Aschauer *et al.*, 2013; Asokan and Samulski, 2013; Rayaprolu *et al.*, 2013). With its success in correcting the pathology associated with diseases such as seen in the multitude of metabolic myopathies and hematological disorders, AAV is quickly becoming the gene therapy vector of choice for initiating large animal studies and clinical trials (Markusic and Herzog, 2012; Mah *et al.*, 2013).

However, among its drawbacks are host immune responses against the capsid and/or transgene (Boutin *et al.*,

2010; Rogers *et al.*, 2011; Faust *et al.*, 2013; Mingozi and High, 2013); appropriate transduction of the target tissue (Zincarelli *et al.*, 2008; Pulicherla *et al.*, 2011; Aschauer *et al.*, 2013); size limitation, with an optimal packaging size of ~4.7 kb (Dong *et al.*, 1996); and the challenges to produce high-titer vectors in a cost- and time-effective manner (Clément *et al.*, 2009; Doria *et al.*, 2013). Implementation toward large-scale manufacturing of AAV using infection-based systems (herpes simplex virus type 1 and baculovirus systems) rather than transfection will certainly become inescapable to address the large quantities of virus needed for FDA-required extensive preclinical studies, as well as clinical studies. Yet, transfection remains the current standard of vector production in most laboratories and manufacturing cores. Furthermore, some indications may require the use of two or more vector constructs. To mitigate the inability of AAV genomes to carry long therapeutic cDNA, the packaging capacity may be expanded by splitting the genome and rely on what has been referred to as the fragment AAV reassembly model (Rabinowitz *et al.*, 2002;

Hirsch *et al.*, 2013). Gene expression using fragmented vectors relies on the host recombination machinery to splice together one expression cassette containing a splice donor site to another encoding a compatible splice acceptor region (Ghosh *et al.*, 2011). Encouraging results using this strategy have been reported for Duchenne's muscular dystrophy (Lai *et al.*, 2005; Zhang and Duan, 2012; Zhang *et al.*, 2013; Koo *et al.*, 2014) and Usher 1 (Lopes *et al.*, 2013; Dyka *et al.*, 2014).

However, there are many other instances where the simultaneous delivery of more than one AAV vector may be required. Such as for indications where two or more subunits are needed (e.g., hexosaminidase A and B for Tay–Sachs disease) or indications where the expression of the therapeutic gene needs to be elevated in specific tissues, which could be mediated by the use of different promoters upstream of the same therapeutic transgene (Pacak *et al.*, 2009; Palfi *et al.*, 2012; Fagoe *et al.*, 2014). For instance, targeting gene expression to the liver for the purposes of immune tolerance induction while providing an additional vector to correct systemic pathology would allow for the simultaneous treatment of many congenital metabolic myopathies wherein immune responses have proven deleterious to the efficacy of gene therapy.

Clinical applications using two or more AAV constructs would be time and cost prohibitive if each construct was produced separately. To facilitate the use and production of multiple vectors, we investigated a novel production method that exploited the stoichiometric properties of AAV in that only one expression plasmid is packaged per encapsidated virus. Based on this knowledge, we empirically developed a method that allows for the production of multiple vectors in a single transfection step. Combining reporter expression cassettes to be packaged at a known input ratio, we show through quantitative polymerase chain reaction (qPCR) and *in vitro* infectivity assays that the output vector preparation closely recapitulated the input ratios. Additionally, we show that therapeutic constructs containing unique promoter elements could be copackaged and were able to be differentially titrated. Our results indicate that, at minimum, two vectors containing either separate transgenes or regulatory elements can be copackaged while characterized independently.

Materials and Methods

Construction of recombinant AAV vector plasmids

Recombinant vectors containing GFP (pTRUF11) and mCherry (pTRUF11-mCherry) were assembled using the pTR-UF backbone previously described (Zolotukhin *et al.*, 1996). The red fluorescent protein mCherry was cloned in lieu of GFP in the pTRUF11 construct using standard techniques. mCherry was amplified from pRSETB-mCherry (obtained from Dr. R. Tsien, University of California, San Diego) using primers mcherry*NotI*-F 3'ATAAGAATGCGG CCGCCACCATGGTGAG and mcherry*NotI*-R 3' ATAAG AATGCGGCCCGCCACGATGGTGTAGTCC to introduce two *NotI* sites flanking the amplicon. The amplicon was digested with *NotI* and ligated into pTRUF11 *NotI*. A human codon-optimized acid α -glucosidase cDNA (coGAA) (GeneArt; Life Technologies) was cloned into a desmin promoter construct (pTR-DES) previously described (Pacak

et al., 2009; Falk *et al.*, 2013). The liver-specific promoter (LSP) (GeneArt; Life Technologies) contains the apolipoprotein E-hepatocyte control region (Miao *et al.*, 2000; Manno *et al.*, 2006; Cao *et al.*, 2007), the human α_1 -antitrypsin promoter (Cresawn *et al.*, 2005), and 5' untranslated region (UTR) and was subcloned into pTR-DES-coGAA in lieu of the DES promoter (*Bgl*II and *Sall*).

AAV9 production

Recombinant AAV vectors were produced and purified as previously described (Zolotukhin *et al.*, 2002). HEK293 cells were cultured in 5% fetal bovine serum (FBS) and antibiotic-supplemented Dulbecco's modified Eagle's medium. Plasmid DNA was propagated in SURE2 cells (Agilent Technologies, Inc., Santa Clara, CA) and isolated using Qiagen plasmid purification reagents or obtained from Aldevron (Fargo, ND). Cells were seeded in 150 mm dishes at 5.0×10^6 cells 24 hr before transfection. The calcium phosphate precipitate was formed by combining the total amount of expression plasmids, with the equivalent concentration of the capsid plasmid rep2/cap9 (courtesy of Dr. James Wilson of University of Pennsylvania) and twice the concentration of the Ad helper plasmid pXX6-80 (obtained from Dr. Xiao Xiao, University of North Carolina) in 2.5 M CaCl_2 followed by the addition of $2 \times \text{HBS}$, pH 7.05.

For copackaging experiments, ratios of the two expression constructs were varied between 1:9, 1:1, and 9:1 ratios and determined off of the total amount of expression plasmid DNA necessary and the total base pair size of the individual constructs to retain equimolar ratio with the helpers; at the surface area of 148 cm^2 , the final amounts of plasmids were $16 \mu\text{g}$ of expression plasmids, $16 \mu\text{g}$ of rep2/cap9, and $38 \mu\text{g}$ of pXX6-80. Cells were incubated at 37°C at 5% CO_2 for 60 hr, washed in phosphate buffered saline (PBS), harvested in PBS–5 mM EDTA, and centrifuged at $1000 g$ for 10 min at 4°C . Cells were resuspended in lysis buffer (150 mM NaCl, 50 mM Tris, pH 8.4) and subjected to three freeze–thaw cycles between a -80°C freezer and 37°C water bath. Benzonase (50 U/mL) and MgCl_2 were added to the cell lysate and incubated for 30 min at 37°C . The crude lysate was clarified by centrifugation at $3400 g$ for 20 min at 4°C . The vector-containing supernatant was used for qPCR or further purified by iodixanol step gradients (Zolotukhin *et al.*, 2002). Final formulations of iodixanol purified vectors were concentrated in PBS (Apollo Concentrators; Orbital Biosciences, Topsfield, MA). Each ratio of copackaged vectors was performed independently in triplicate.

Titration of AAV vectors

DNA from all AAV vectors, both from crude lysate or iodixanol-purified preparations, was extracted using Qiagen reagents. An amount of $100 \mu\text{L}$ of vector from clarified lysate or $10 \mu\text{L}$ of iodixanol purified vector was treated with proteinase K (Qiagen; 0.2 mg/mL, 55°C , 30 min) followed by DNA extraction following manufacturer's instructions.

For AAV9-GFP and AAV9-mCherry, primers designed for the cytomegalovirus enhancer were used to determine total titer (primer set 1; see Table 1 for primer sequences). Additional forward and reverse primers designed uniquely to the GFP and mCherry transgenes (primer sets 2 and 3,

TABLE 1. PRIMER SEQUENCES FOR POLYMERASE CHAIN REACTION

Set	Primer	Sequence
1	CB2-F	5'-TCCCATAGTAACGCCAATAGG-3'
	CB2-R	5'-CTTGGCATATGATACACTTGATG-3'
2	GFP-F	5'-ATGGAAACATTCTCGGCCACAAGC-3'
	GFP-R	5'-TCGCCGATTGGAGTGTCTGTTG-3'
3	mCherry-F	5'-GGACGGCGAGTTCATCTACA-3'
	mCherry-R	5'-TTGACCTCAGCGTCGTAGTG-3'
4	DES-F	5'-GGCTGATGTCAGGAGGGATA-3'
	LSP-F	5'-GGGACAGTGAATCCGGAAAG-3'
	coGAA-R	5'-AAGTCGTGCAGCAGGATATG-3'

CB, CMV enhancer/chicken β -actin promoter; coGAA, human codon-optimized acid α -glucosidase; DES, desmin promoter; LSP, liver-specific promoter.

respectively) were also used to determine the individual contribution of each construct to the total vector preparation. Endpoint PCR was optimized by amplifying 0.5 ng of extracted DNA on a three-step cycling protocol across a temperature gradient (30 cycles: 94°C for 15 sec, 46–50°C for 15 sec, and 72°C for 30 sec) preceded by a 2 min 94°C incubation and followed by a 1 min 72°C elongation. qPCR titration was optimized by amplifying 1 ng of extracted DNA on a two-step cycling protocol across a temperature gradient (50 cycles: 95°C for 10 sec and 57–63°C for 1 min) preceded by a 10 min 95°C incubation and followed with a melt curve protocol (95°C for 1 min, 63°C for 1 min, and 65–95°C for 5 sec in 0.5°C increments). An annealing temperature of 50°C was used for all primer sets and combinations for endpoint PCR experiments. For qPCR, cytomegalovirus (CMV)-targeted primers were annealed at 63°C, and GFP and mCherry primers were annealed at 50°C.

To titrate copackaged AAV9-LSP-coGAA and AAV9-DES-coGAA, forward primers unique to the promoter sequences were used in conjunction with a reverse primer anchored within the transgene shared by both constructs (primer set 4). Endpoint PCR and qPCR performed on copackaged AAV9-LSP-coGAA and AAV9-DES-coGAA were optimized and performed identically as with copackaged AAV9-GFP and AAV9-mCherry. For qPCR, LSP, DES, and coGAA primers were annealed at 60°C.

Standard curves were generated by using 10^9 – 10^5 total copies, as well as the inclusion of a nontemplate control, of the relevant expression plasmids either singly or in combination with the additional copackaged construct. For each endpoint PCR or qPCR of single or combined expression plasmids, the corresponding primers were also used individually or in combination. For example, four standard curves of pTRUF11 were amplified individually with primers targeting the CMV enhancer (primer set 1), GFP (primer set 2), mCherry (primer set 3), or a combination of GFP and mCherry (primer sets 2 and 3). Likewise, four standard curves of combined pTRUF11 and pTRUF11-mCherry were amplified individually with primers targeting the CMV enhancer (primer set 1), GFP (primer set 2), mCherry (primer set 3), or a combination of GFP and mCherry (primer sets 2 and 3). Each combination of primer sets and plasmids was investigated to ensure the specificity of amplification.

Endpoint PCR was conducted using Illustra PuReTaq Ready-To-Go PCR beads (GE Healthcare, Buckinghamshire, UK). An amount of 0.5 ng of DNA was amplified from each preparation and ran on a 2% agarose gel at 100 V for 90 min for GFP and mCherry vectors or on a 1.5% agarose gel at 110 V for 50 min for LSP and DES vectors. qPCR was performed with iTaq Universal SYBR Green Supermix using 1 ng of DNA on a Bio-Rad CFX96 Real-Time PCR Detection System and analyzed using Bio-Rad CFX Manager v. 3.1 software (Bio-Rad Laboratories, Inc., Hercules, CA). A multiplication factor of two was included when determining vector genomes per milliliter (vg/mL) to account for the packaging of positive- and negative-sense viral genomes.

Single-cell fluorescence assay

The infectious titer of AAV9-GFP and AAV9-mCherry was determined essentially as described previously (Zolotukhin *et al.*, 2002). C12 cells were seeded at 2×10^4 cells in a 96-well plate and infected 18 hr later with the copackaged vectors in a serial 10-fold dilution series. Because of the low *in vitro* transduction efficiency of AAV9, coinfection with Ad5 (MOI of 20) was implemented. Forty hours later, red and green cells were counted using a fluorescent microscope and the infectious titer was calculated based on dilution. Each ratio, packaged in triplicate, was assayed in duplicate. The particle-to-infectivity ratio was then determined by the qPCR titer divided by the infectious titer.

Statistical analysis

Figures were drawn and statistical analysis was performed using GraphPad Prism v. 5.0 (GraphPad Software, La Jolla, CA).

Results

AAV packages expression plasmids in a defined stoichiometry

To facilitate the use and production of multiple vectors, we investigated a novel copackaging method that would allow for the generation of a heterogeneous population of AAV vectors in a single manufacturing step. Our hypothesis was that combining plasmids to be packaged at a known input ratio would result in an output vector preparation containing the equivalent ratio. To demonstrate this hypothesis, we first copackaged two vectors into AAV serotype 9 (AAV9) that only differed by the reporter gene, GFP or mCherry. The vectors were copackaged at 1:9, 1:1, and 9:1 molar ratios, respectively. Vector DNA extracted either from crude lysates or from purified vectors was first analyzed by endpoint PCR (Fig. 1A). Semi-quantitative endpoint PCR revealed that each vector preparation differentially packaged each transgene, recapitulating the ratios they were transfected. Each dual-vector preparation was then subject to qPCR analysis for a more robust quantification assessment. To determine the overall vector titer (two vectors), we used primers targeted toward the CMV enhancer region of the shared promoter to both AAV9-GFP and AAV9-mCherry (see the section Titration of AAV Vectors under Materials and Methods). At a scale of production using 150 mm tissue culture dishes, we determined an

FIG. 1. Differential packaging of expression cassettes combined before transfection. **(A)** The vectors contained different transgenes that were used for delineation. DNA was amplified from each preparation and ran on 2% agarose gel. Green fluorescent protein (GFP) band is 171 bp and mCherry is 191 bp. Lanes 1–3 contain DNA amplified from vectors in crude lysate (postbenzonase treatment); lanes 4–6 contain DNA amplified from vectors purified via iodixanol gradient originating from the crude lysates in lanes 1–3. Vector constructs were copackaged in AAV9 at GFP-to-mCherry ratios of 1:9, 1:1, and 9:1, respectively. Gel is one representative image of three separate copackaging experiments. **(B)** Quantitative PCR using transgene-specific primers on iodixanol-purified GFP or mCherry vectors was performed to determine the respective contribution of each individual vector in the total preparation when copackaged at 1:9, 1:1, and 9:1 ratios, respectively. Each vector yield is expressed as a percentage of total vector genome, with 100% obtained from the summation of the titers determined using transgene-specific primers for either GFP or mCherry. Data represent the average of three separate experiments for each ratio.

overall titer ranging from ~ 1 to 5×10^9 vg/mL in the crude lysate (volume 3 mL) and after iodixanol purification (volume 0.2 mL) (Fig. 2).

The use of a single primer set or a combination of transgene-specific primers simultaneously did not significantly affect the titration outcome (Fig. 2). In addition, we verified that the results obtained from purified vector preparations confirmed those obtained from crude lysates, which excluded potential risks of plasmid carryover or contamination from the transfection precipitate in benzonase-digested crude lysates. Titters determined from the transgene-specific primers revealed that the predicted ratios of 1:9, 1:1, and 9:1 AAV9-GFP to AAV9-mCherry were recapitulated (Fig. 1B). Corroborating what was observed using endpoint PCR, at the 1:9, 1:1, and 9:1 GFP-to-mCherry ratios, the

FIG. 2. Total vector genomes resulting from copackaging. DNA was extracted from postbenzonase-treated crude lysates (Crude) or after iodixanol purification (Purified) of GFP and mCherry vectors copackaged at 1:9, 1:1, or 9:1 ratios, respectively. Total vector genome titer was determined either directly, using a common CMV enhancer (Single Primer Set), or from the summation of each vector titer using transgene-specific primers (Double Primer Set). Data represent the average total titer from crude (final volume of 3 mL) or purified samples (final volume of 0.2 mL) at each ratio assayed in triplicate.

mean percentage of their respective contribution to the total titer was 11.03–88.97%, 64.12–35.88%, and 94.19–5.81% over the three independent packaging experiments. These data strongly support the hypothesis that AAV can package more than one expression plasmid combined at a predetermined ratio in a reproducible and predictable manner.

Ratios of copackaged vectors are maintained in vitro cell transduction

An established method of vector quality control is the infectivity or transduction assay. For marker gene carrying vectors, the assay is based on single-cell fluorescence (Zolotukhin *et al.*, 2002). To determine the infectious titer, we transduced C12 cells in the presence of Ad5 (MOI of 20), with purified AAV9-GFP and AAV9-mCherry copackaged at the above ratios. Two days postinfection, green and red cells were visually counted independently. The infectious titer ranged from 8.5×10^3 to 1.25×10^4 IU/mL closely mirroring the vector genome titers. Furthermore, the average particle-to-infectivity ratios ranged from 2.1 to 4.9×10^5 , which are consistent with ratios observed for AAV9 preparations, and more importantly, the particle-to-infectivity ratios were not significantly different between the two marker constructs or at the different packaging ratios (data not shown). The mean respective contribution of green and red cells to the total infectious titer was 10.85–89.15%, 59.34–40.66%, and 91.22–8.78% (Fig. 3). These results indicate that copackaged vectors also display transduction profiles in the ratios at which they were copackaged.

Therapeutic constructs can be differentially packaged

It may prove efficacious in some instances to use multiple vectors that target transgene expression to specific tissues.

FIG. 3. *In vitro* characterization of copackaged reporter vectors. The percent contribution of either AAV9-GFP or AAV9-mCherry to the total infectious titer (GFP + mCherry) was determined via single-cell fluorescence assay in C12 cells. Data represent the average of two separate experiments for each ratio.

Therefore, we applied this method to the production of therapeutically relevant constructs differing in transcription elements. Vectors containing human coGAA with different promoter elements that target expression to the liver (ApoE-HCR-hAAT promoter [LSP]) or cardiac, skeletal, and neuronal tissue (desmin promoter [DES]) were copackaged and purified at the above ratios in AAV9 in 150 mm tissue culture dishes. Dual-vector preparations were analyzed in a similar manner as the marker containing vectors. Titers were assessed using forward primers within the individual promoters (LSP or DES) and a shared, reverse primer anchored in the transgene (GAA; Table 1). Endpoint PCR confirmed the differential contribution of the two constructs to the total vector production (Fig. 4A). Resulting overall titers ranged from 1 to 3×10^9 vg/mL. Similarly, qPCR revealed that the output ratio recapitulated our predicted ratios as was observed when we copackaged AAV9-GFP and AAV9-mCherry (Fig. 4B). The mean percentage of the total titer consisting of AAV9-LSP-coGAA or AAV9-DES-coGAA was 9.09–90.91%, 57.10–42.90%, and 93.83–6.17%. The data presented support our hypothesis that packaging predetermined ratios of input plasmid containing different transcriptional elements results in a heterogeneous population containing multiple vectors with the potential of expressing in discrete tissues. These results also indicate that production of multiple vectors in a single transfection step can produce dual, or potentially more, vectors at a predetermined ratio reproducibly and at the proportion of the investigator's choice.

Discussion

The widespread use of AAV for gene therapy applications emphasizes its utility and diverse capabilities, but the limitations of manufacturing and packaging size have dampened the rapid successes observed in preclinical models to translation in the clinic. Many groups have investigated manners in which greater quantities of vector can be made quickly and efficiently with varying degrees of success. The most standard protocol to produce recombinant AAV, both at research and clinical grade, is a transfection method using

FIG. 4. Copackaging of therapeutic constructs. (A) AAV9-LSP-coGAA and AAV9-DES-coGAA were copackaged and purified at 1:9 (lanes 1, 4, and 7), 1:1 (lanes 2, 5, and 8), and 9:1 ratios (lanes 3, 6, and 9), respectively. AAV9-LSP-coGAA band is 288 bp and AAV9-DES-coGAA is 453 bp. DNA was amplified from each preparation and ran on 1.5% agarose gel. (B) DNA extracted from copackaged AAV9-LSP-coGAA and AAV9-DES-coGAA was subjected to quantitative PCR to determine the respective contribution of each individual vector in the total preparation when copackaged at 1:9, 1:1, and 9:1 ratios, respectively. Each vector yield is expressed as a percentage of total vector genome, with 100% obtained from the summation of the titers determined using promoter-specific primers for either LSP or DES. Data represent the average of three separate experiments for each ratio. LSP, liver-specific promoter; DES, desmin promoter; coGAA, human codon-optimized acid α -glucosidase.

two or sometimes three plasmids to provide all the *cis* and *trans* functions necessary to package AAV (Zolotukhin *et al.*, 2002). However, transfection-based methods are inherently difficult to adapt to large-scale platforms, and methods using baculovirus (Kotin, 2011; Mietzsch *et al.*, 2014) or herpes simplex virus 1 systems (Clément *et al.*, 2009), together with producer cells grown in suspension, are rapidly improving and paving the way to future manufacturing campaigns. Despite these quick and impressive advancements toward infection-based methods, transfection remains the most versatile and cost-effective method at small- and medium-scale preparations enabling researchers to develop proof-of-principle concepts.

Current methods of AAV production are directed toward the manufacturing of a single vector at a purity and titer conducive for preclinical studies or early phase clinical trials. In some instances, the use of more than one AAV may be beneficial or even required as a therapeutic approach. For some diseases, the production of multiple vectors containing fragmented genomes is required when the constructs exceed the carrying capacity of the vector. Duchenne's muscular dystrophy, hemophilia A, Tay-Sachs disease, and Usher 1 are only a few of the diseases that would rely on multiple gene products or *trans*-splicing vectors to provide for

therapeutic benefit (Mah *et al.*, 2003; Cachón-González *et al.*, 2012; Lopes *et al.*, 2013; Dyka *et al.*, 2014; Koo *et al.*, 2014; Lostal *et al.*, 2014; Wang *et al.*, 2014).

In other cases, it may prove necessary to coordinate and differentially control transgene expression to different target regions with tissue-restricted promoters, such as the central nervous system, eye, or systemically, while avoiding expression in antigen presenting cells and provoking a deleterious immune response (Zhang *et al.*, 2012; Palfi *et al.*, 2012; Bosch *et al.*, 2014; Fagoe *et al.*, 2014). In these lines of examples, a copackaging strategy was recently employed by Xiao's group to deliver a functional factor VIII in a model of hemophilia A (Wang *et al.*, 2014). The study showed that two AAV cassettes, carrying the heavy and the light chain of factor VIII downstream of the same promoter, can be packaged at a 1:1 ratio and that *in vivo* delivery resulted in expression of factor VIII. Our study further demonstrates the possibility of producing cassettes varying in different regions of the viral construct, and that the ratio between the different constructs can be tailored to the need of the study, by testing three different ratios.

The benefit of altering the construct and not the capsid lies in that the coordination of expression may be contingent upon the tropism of a particular serotype, and it has already been shown that much of the population is already seropositive for many of the serotypes in clinical trial (Boutin *et al.*, 2010). It would behoove an investigator then to ensure that all cell and tissue types are transduced at a minimal degree of exposure of the animal or individual to multiple serotypes. This immunization against the various serotypes would preclude any subsequent attempts using different capsid variants without substantial immunomodulation as well as potentially prime innate and adaptive responses against viral components, all of which have been shown to be detrimental to long-term efficacy (Cresawn *et al.*, 2005; Jayandharan *et al.*, 2011; Wang *et al.*, 2011; Sudres *et al.*, 2012; Mingozzi and High, 2013). Based on these considerations, we are pursuing the method described here to develop a single gene therapy product that will allow for the simultaneous induction of immune tolerance and physiologic correction of Pompe disease that may prove beneficial for other metabolic myopathies characterized by systemic pathology and are prone to immune responses to the therapeutic protein.

When more than one vector is necessary to the therapeutic approach, investigators have the sole choice of producing and testing each vector preparation independently, followed by coadministration of the two vectors at time of dosing. As an obvious consequence, processing times are often increased and cost doubled—aspects all the more relevant for clinical manufacturing. Clinical manufacturing and release testing of AAV in compliance with FDA-regulated good manufacturing practices is extremely costly and time-consuming, a nontrivial aspect of designing an AAV gene therapy trial. Furthermore, preclinical toxicology studies would need to integrate additional animals and controls to evaluate safety of each single vector separately, as well as in combination, again resulting in dramatic increases in cost and time toward protocol validation.

The necessity of novel production methods to provide for multiple constructs in an efficient and reliable manner currently stands as an unmet need in the field. This study fo-

cused on the development of such a method. Here it was revealed that vectors containing either different transgenes or transcriptional elements could be combined in predetermined ratios and produce an output of vector that recapitulated that prediction. Although a method for developing mosaic capsids by cotransfection has been previously attempted (Gigout *et al.*, 2005), our study is the first instance of constructing a heterogeneous population of AAV9 vectors containing different payloads in a single manufacturing step. Although not tested in this work, the method would be conducive to any other AAV serotype. In fact, the recent work by Wang *et al.* (2014) shows successful dual-packaging in AAV2 and AAV8.

Here we demonstrated that disparate ratios (1:9 or 9:1) most accurately recapitulated our predicted proportions, at least *in vitro*. Our results also suggested that the smallest vector genome construct tended to yield slightly higher yields than predicted. Variability of packaging efficiency based on vector size has previously been documented (Dong *et al.*, 1996; Wang *et al.*, 2014). The current and previous studies therefore emphasize that careful titration of the vectors must be conducted and warrants the need for pilot copackaging experiments to determine that the desired outcome ratios are achieved. In summary, with minor upstream proof-of-concept experiments to establish the ratios for the specific treatment modality, the copackaging method stands as an alternative method of vector production where more than one gene product is necessary, and as a novel platform for treating diverse congenital disorders for which AAV-mediated gene therapy is applicable. Moreover, this technique could theoretically expand to infection-based systems as the expression cassettes to be packaged could be provided at varying multiplicities of infection to produce a heterogeneous population of vectors similar to our results here using transfection.

With respect to regulatory aspects of AAV clinical manufacturing, the main advantage of this strategy is related to being extremely cost- and time-effective, as developed earlier. The dual-vector preparation should be considered as one single new investigational drug for each given ratio. This advantage may also be a challenge, as precise methods to characterize each vector contribution must be developed and well controlled, and reproducibility of the production method established.

To facilitate FDA review and approval, the chosen dual vector at the therapeutic ratio, similar to a single AAV drug, will undergo extensive toxicology and dose assessment studies. The ratio must remain unchanged throughout the protocol validation, at least within the margin of errors of the methods used to produce and characterize the vector preparation. Identity testing, including whole genome sequencing, will be a challenge. However, new next-generation sequencers allow for massive parallel sequencing (MPS) to provide full sequencing of multiple species in one given sample, which would also confirm the ratio of each vector construct. A potential risk, inherent to the method, is homologous DNA recombination during packaging of constructs sharing large coding or regulatory region sequences, potentially resulting in modified regulatory regions or uncharacterized open reading frames. The consistency of our results suggests that, had recombination events occurred during our experimental protocol, it was at an extremely low

frequency, as it did not affect ratios calculated from PCR titration or marker gene expression *in vitro*. Further analysis would be warranted for each specific copackaging production, including, but not limited to, full sequencing by MPS. From our study, and for the constructs tested here, we believe that with appropriate characterization tools, both vectors can be accurately titrated and that predicted ratios are consistent across several production attempts.

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Author Disclosure Statement

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