Recombinant adeno-associated viral (rAAV) vectors have been widely used in human gene therapy. One major impediment to its broad application is the inability to produce high-quality vectors in mass quantity. Here, an efficient and scalable suspension cell culture system for the production of rAAV vectors is described. In this system, the AAV trans factors, Rep78, Rep52, VP1, VP2, and VP3, were stably integrated into a single vaccinia virus carrier by maximizing the use of alternative codons between genes with identical amino acids, and the cis rAAV genome was carried by an E1/E3 gene-deleted adenovirus. Infection of improved, E1 integrated, suspension-cultured cells with these two viral vectors resulted in the robust production of rAAV vectors. The newly enhanced system can consistently produce ~1 × 10^15 genome containing rAAV vectors per liter of suspension cells. Moreover, the capsid composition of rAAV vectors produced by this system is markedly different from those produced using the traditional system in that the VP1 protein is more abundant than the VP2 protein (19:1 versus 1:1). The unique VP1 superabundant rAAV vectors produced in this new system exhibited improved transduction in vivo after intravitreal injection.

INTRODUCTION

Recombination adeno-associated viral (rAAV) vectors have been widely used as gene-delivery vehicles for both basic scientific research and human gene therapy.1–3 They have been successfully used in clinical trials and are approved as a drug for human use.4–6 Although a variety of systems have been proposed to produce rAAV vectors,7–13 large-scale manufacturing of high-quality rAAV vectors that meet the needs of basic research in large animal models and clinical uses remains a major challenge.14

The common method used for producing rAAV requires co-transfection of adherent HEK293 cells with two or three plasmids carrying the necessary cis and trans components.8,14–16 The two inverted terminal repeats (ITRs) are the only cis elements required for rAAV replication and packaging. Although four non-structural proteins are encoded in the AAV genome, Rep78, Rep68, Rep52, and Rep40, only Rep78 and Rep52 are required as trans elements for rAAV production.17 The capsid proteins VP1, VP2, and VP3, are essential for AAV capsid assembly and are therefore required trans elements. Besides coding VP1, VP2, and VP3, the cap gene also encodes an assembly-activating protein (AAP), which may further support AAV assembly.18 The AAV helper viruses, such as adenovirus (Ad), and herpes simplex virus (HSV), provide the additional trans-acting factors required for rAAV production.19 The rAAV vectors produced by the traditional transient transfection have been validated in clinical trial for their safety, convenience, and effectiveness. However, the method itself has limited scalability potential.20

As an alternative to the transfection method, both baculovirus and HSV-based carriers have been developed to produce rAAV vectors using a suspension culture system.20–22 These viral-based systems have been reported to generate 0.5–1 × 10^9 vector genomes (vg)/cell of rAAV vector, and when scaled-up to a 100–200 L volume, can produce 1 × 10^16 vg of rAAV vector.14,19 However, there remains concern regarding the potency of the vectors produced, the possibility of generating replication competent AAV (rcAAV) particles, and the difficulties associated with engineering the carrier viruses, considering their limited use. Recently, a novel rAAV production method using a vaccinia carrier from our group has been described, which can eliminate the generation of rcAAV particles while retaining similar scalability.23 However, this method requires the use of two vaccinia viral (VV) vectors, an Ad-AAV hybrid vector (Ad/AAV), and a wild-type Ad (wtAd), making this system rather complicated.

Traditionally, the AAV capsid is comprised of 60 subunits of VP1, VP2, and VP3 capsid proteins in a ratio of ~1:1:8. To control the protein ratio, wild-type AAV relies on the use of overlapping, and alternative mRNA splicing combined with the use of a non-canonical start
are essential for virus trafficking and cell-specific transduction. It is well documented that insufficient VP1 levels will result in reduced rAAV transduction rates, and recovery of VP1 expression boosts rAAV5 per-particle infectivity rates.

In the present study, a novel VV-Ad system was developed that efficiently produces VP1-superabundant rAAV vectors. This system uses a single VV vector to provide the necessary Rep and Cap proteins and a single Ad/AAV carrying the rAAV genome. Results demonstrated that approximately $2 \times 10^7$ vg/cell or $1 \times 10^{13}$ vg/L of rAAV vector can be produced by simply infecting the E1a/E1b-expressing QW158-7 suspension cells with these two viral vectors. The resulting rAAV vectors carried more VP1 and less VP2 compared with traditional rAAV vectors and displayed higher transduction efficiency after intravitreal injection.

**RESULTS**

**Generation of a Single VV Carrier That Stably Expresses All AAV Packaging Genes**

In the previously described VV-Ad system for rAAV production, Rep78 and Rep52, and VP1 and VP2, were allocated into two different constructs (Figure 1A; W5 and W8), because the identical nucleotides at their 3' ends destabilized the VV carrier. Therefore, one VV carrier expressing all four genes was not generated (Figure 1A; W5/W8). Here, a novel strategy was employed that allowed the formation of a single, stable VV carrier, capable of expressing all four genes simultaneously. Through maximization of alternative coding, novel Rep52 (Rep52*) and VP1 (VP1*) genes were designed to express the Rep52 and VP1 proteins (Figures S1 and S2). Although Rep52 and Rep52* express identical polypeptides, the homology of Rep52 and Rep52* was demonstrated that approximately $2 \times 10^7$ vg/cell or $1 \times 10^{13}$ vg/L of rAAV vector can be produced by simply infecting the E1a/E1b-expressing QW158-7 suspension cells with these two viral vectors. The resulting rAAV vectors carried more VP1 and less VP2 compared with traditional rAAV vectors and displayed higher transduction efficiency after intravitreal injection.

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Rep52* at the nucleotide level is only 61.2%. Similarly, VP1 and VP1* share a homology of 59.2% at the nucleotide level, despite coding for identical amino acids. Rep52* and VP1* exhibited similar expression profiles compared with unmodified Rep52 and VP1, controlled either by cytomegalovirus (CMV) promoter or vaccinia P7.5 promoter (Figure S3). As expected, these four genes could be successfully built into a single VV construct (VV22) in which each gene was driven by a P7.5 promoter (Figure 1A).

Infection of HeLa-S3 cells with VW22 resulted in the production of Rep and Cap proteins to similar levels as HeLa-S3 cells co-infected with W5 and W8 (Figures 1B and 1C). Since VP2 is expressed by VP2 is less robust compared to VP3 in this system. When VW22 (MOI = 1) was introduced to cells 16 hr after co-infection with wtAd (MOI = 1) and Ad/AAV hybrid was observed (Figure 1D). The rAAV vector yield is proportional to the MOI of VW22 (Figure 1E). About 3.2 × 10⁴ vg/cell of rAAV vector was generated when MOI was 4, approximately two-fold higher than that of W5 and W8 co-infection. VW22 is stable due to the increased toxicity to the host cells and interactions between the two types of viruses (Figure 1F). Taken together, all the AAV packaging genes are stably and functionally expressed from a single VV carrier.

Eliminating wtAd from the New rAAV Production System

wtAd has been demonstrated to increase rAAV packaging in HeLa-S3 cells when using the previously described VV-Ad system for rAAV production. We compared the helper functions of wtAd and an E1 deleted Ad, Ad375, in this system (Figure S4A). Ad375 failed to increase rAAV genome replication and was unable to improve rAAV production, indicating that the E1a/E1b genes, but not the E3 genes of wtAd, are primarily responsible for enhanced replication of rAAV genomes. Therefore, the E1a/E1b genes were introduced into HeLa-S3 cells to eliminate the use of wtAd. The resulting E1a- and E1b-expressing cell line was named QW158-7, which can be easily propagated in suspension and have similar cell morphology as the original HeLa-S3 cells. Not surprisingly, they complemented rAAV vector production quite efficiently in adherent culture conditions (Figure 2A). Results demonstrated that even in the absence of wtAd infection, over 3 × 10⁵ vg/cell of rAAV vector can be produced using suspension-cultured QW158-7 cells when the VW22 helper virus was added at an MOI of 4 (Figure 2C). The expression profile of Cap and Rep proteins from VW22 was similar between QW158-7 and HeLa-S3 cells (Figure 2B). QW158-7 cells provide earlier and more abundant replicated rAAV genomes for AAV packaging (Figure 2D), which are generally positively correlated to rAAV yield.

Increasing MOI of the Ad/AAV hybrid from 1 to 50 significantly promoted total rAAV genome accumulation and improved rAAV yield (Figures S8E and S5F). However, a further increase of the Ad/AAV vector failed to substantially enhance rAAV production, presumably due to the increased toxicity to the host cells and interactions between the two types of viruses (Figure S5H). We have systematically evaluated whether individual Ad genes, E1a-13s, E1a-12s, E1b-55k, E1b-19k, E2a, VA I RNA, and E4orf6, would increase rAAV packaging or not. Each gene was expressed by a VV vector that was controlled by a P7.5 promoter. As demonstrated in Figure S6A, adding E1a-13s,
Modulating VP2/VP3 Expression Can Improve rAAV Production Yield

An increase in MOI of VW22 significantly improved rAAV vector production (Figure S5A). However, a high VW22 MOI was toxic to the host cells. It inhibited the replication of the Ad/AAV hybrid and reduced rAAV genome accumulation (Figures S5B and S5C). In an effort to further improve this system without increasing the MOI of VW22, the effect of individual AAV genes (Rep78, Rep68, Rep52, Rep40, VP1, VP2, VP3, AAP, and X-gene) on rAAV packaging was evaluated. The expression levels of VP2 and VP3 were found to be proportional to rAAV vector yield (Figures S6B and S6C). For example, enhancing VP2 expression improved vector yield by 5-fold, and the optimized expression of VP3 lead to a 3-fold increase in vector yield. In contrast, increasing VP1 expression decreased rAAV yield in the VV-Ad system. This result suggested that insufficient VP2 and VP3 might have been the primary cause of inefficient rAAV packaging in the new system.

To address the issue of inefficient VP2/VP3 expression, it was hypothesized that the universal adoption of the P7.5 promoter might lead to the unbalanced expression of AAV genes. Since there are a variety of VV promoters available with different expression profiles and potency, eight VV promoters (Leo160, Pr4LS5E, pHyb, PrS5E, PrMVA13.5-long, mH5, pSyn, and ATI; Table S1) were systematically assessed for their effect on rAAV production. To accomplish this, either individual promoter or various combinations of these promoters were used to drive the VP2 gene (Figure 3A). As presented in Figure 3A, the VW22 carriers containing a Pr4LS5E or an mH5 promoter consistently resulted in higher rAAV yield. In fact, using these promoters resulted in the production of 3–6-fold more rAAV vectors compared with that of original VW22 carrier in adherent cultured QW158-7 cells. Moreover, the VW22 carrier with a Pr4LS5E-mH5 dual promoter (VV22-PM) led to the highest rAAV yield, $3.5 \times 10^5$ vg/cells, which is approximately an 8-fold increase over the VW22 (Figure 3A). The rAAV yield with the VW22-PM carrier in suspension QW158-7 cells produced approximately $3 \times 10^5$ vg/cell, which is about seven times higher than that of VW22 (Figures 3B and S7A). Earlier expression and accumulation of VP2/VP3 was considered as the likely reason for more efficient replication and packaging of rAAV vectors (Figures 3C and S7B). In contrast, the replication of Ad/AAV and VW in the system was not affected when the Pr4LS5E-mH5 dual promoter was used to control VP2/VP3 expression (Figures S7C and S7D). Based on this data, it was concluded that the use of a strong and early VV promoter improved expression of VP2/VP3 and enhanced rAAV packaging in the new vector production system.

An Optimized System for Robust rAAV Production

The combination of all the discussed enhancements and optimization resulted in a new VV-Ad system for rAAV vector production using suspension cells without transfection procedures (Figure 4). This new system includes three major components: a single, cytoplasmic VV carrier (VV22-PM) providing AAV helper functions; one Ad/AAV hybrid carrier containing rAAV genomes and providing Ad helper functions; and suspension host QW158-7 cells containing the E1a/E1b functions (Figure 4, right). This is in contrast to the original VV-Ad system that required five components: two VVs (W5 and W8), two Ads (Ad/AAV hybrid and wtAd), and used HeLa-S3 as host cells (Figure 4, left). The new system eliminates excessive procedures and cost for additional VV carrier preparation, provides a more direct method, and importantly, removes wtAd from the production system.

To further confirm that this new production system is competent for general rAAV vector production, it was used to produce the following rAAV vector-based AAV serotype 2: ssAAV-CMV-LacZ, ssAAV-CB-AAT, dsAAV-CB-EGFP, and dsAAV-CB-Gaussia luciferase (Gluc). Ad/AAV hybrids carrying the corresponding transgene cassettes and AAV ITRs were generated. The AAV genome size for these vectors ranged from 2.6 to 4.7 kb. As presented in Table 1, both single-strand rAAV vectors (ssAAV-CMV-EGFP, ssAAV-CMVC-MV-LacZ, and ssAAV-CB-AAT) and self-complementary rAAV vectors (scAAV-CB-EGFP and scAAV-CB-Gluc) were produced successfully. The yield ranged from $0.59 \times 10^5$ to $2.76 \times 10^5$ vg/cell (Table 1). Moreover, increasing the cell density as high as $5 \times 10^6$ cells/mL still allowed for a high yield of rAAV vector per cell. Under this condition, over $1 \times 10^{15}$ vg rAAV vectors could be produced from 1 L of cell culture.

Since the new system uses a VV carrier and an Ad/AAV hybrid to produce rAAV vectors, the chance of residual carrier viruses in the final rAAV preparations is a safety concern. However, no residual VV or wtAd contamination could be detected using a plaque assay (the sensitive of our assay was 1 VV or wtAd out of $10^{10}$ AAV particles). The Ad/AAV hybrid has a peak density of 1.344 g/cm$^3$, which is close to the density of empty AAV particles and is easy to inactivate by heating treatment at 56°C for 1 hr (Figures S8F and S8G). Moreover, there were less rAAV genomes detected in rAAV vectors produced by the VV-Ad system compared with that of the triple-plasmid transfection system (Figures 5B and S8E), which confirms previous results.

VP1 Superabundant rAAV Vectors Efficiently Express Transgene In Vivo

It is notable that rAAV vectors produced by the new system displayed a high abundance of VP1 (VP1 superabundant AAV). For these rAAV vectors, the ratio of VP1:VP2:VP3 is close to 1.90:1.8, which differs from the traditional AAV capsid with a ratio of 1:1:8.
Those VP1 superabundant rAAV vectors demonstrated similar genome status, morphology, thermal stability, and CsCl gradient buoyant density as the canonical rAAV vectors (Figures S8A–S8D). As the abundance of VP1 is critical for infectivity of rAAV,23,24 the transduction efficacy of VP1 superabundant rAAV vectors were compared to canonical vectors through injection into the vitreous chamber of mice to target the retina cells. At 2 weeks post-injection, it was observed that the VP1-enhanced vectors produce higher expression in retina ganglion cells and optic nerve cells than canonical rAAV vectors (Figure 5C).

**DISCUSSION**

In the current study, a robust and scalable manufacturing system was described that contained significant improvements over the previous VV-Ad system for rAAV production (Figure 4). In fact, all components of the system have been substantially improved and optimized.
A single VV carrier, VW22-PM, was engineered to consolidate all the necessary AAV trans factors. The wtAd was completely eliminated, and its essential functions for rAAV production have been incorporated into a HeLa-S3 derivative cell line, QW158-7 cells, which can be grown in suspension. This new rAAV vector production system performed significantly better than other rAAV production systems that have been previously described. The main advantages of the new VV-Ad system include the following: (1) rAAV vectors can be generated in a yield greater than $2 \times 10^9$ vg/cell or greater than $1 \times 10^{15}$ vg/L; (2) the scalable system can be readily implemented, since production cells can be grown in suspension and the helper carrier virus can be propagated easily; (3) it completely avoids rAAV generation by sequestering rep and cap genes and the rAAV genomes into two separate compartments of the host cell; (4) the presence of different VV promoters (early, intermediate, late) and sequential infection of two helper viral vectors enables temporal regulation of Rep and Cap expression and genome replication, which can closely mimic wtAAV production and increase rAAV production; (5) it avoids the use of wtAd; (6) it generated a VP1 superabundant rAAV vector, which has high in vivo transduction; (7) it is especially flexible for producing chimeric AAV vectors that contain a VP1 from one serotype and VP2 or VP3 from other sources.

One main obstacle of using a single VV carrier to express Rep78 and Rep52, VP1, VP2, and VP3 simultaneously is that these proteins have identical nucleotide sequences in the AAV virus. It was initially determined that viruses harboring large homologous repeats were unstable and limited their expansion for large-scale rAAV production. This problem was solved by maximizing the use of alternative codons for Rep52 and VP1 that allowed VP1, Rep52, VP2, and Rep78 proteins to be expressed in a single VV without changing either the amino acid sequences or the expression profile of these proteins (Figure 1). The elimination of the extra VV carrier not only reduces the labor and cost for VV carrier preparation, but also improves the rAAV yield (Figure 1E), presumably by reducing cell cytotoxicity. The VV carrier was able to support rAAV production without losing its effectiveness even after 12 consecutive passages. This is significantly better than systems using
Table 1. rAAV Yield of the Optimized VV-Ad System

| Vector Name   | Size (kb) | Ad/AAV Name   | vg/Cell ± ssAAV2-CMV-EGFP | vg/L ± ssAAV2-CMV-LacZ | vg/Cell ± dsAAV2-CB-EGFP | vg/L ± dsAAV2-CB-Gluc | vg/Cell ± ssAAV2-CB-hAAT | vg/L ± ssAAV2-CB-hAAT |
|---------------|-----------|---------------|---------------------------|-------------------------|--------------------------|-------------------------|--------------------------|-------------------------|
| ssAAV2-CMV-EGFP | 4.0       | Ad/AAV-CMV-EGFP | 2.37 ± 0.07 × 10^5         | 0.96 ± 0.14 × 10^5       | 1.54 ± 0.07 × 10^5       | 4.04 ± 0.09 × 10^5       | 4.04 ± 0.09 × 10^5       | 4.04 ± 0.09 × 10^5       |
| ssAAV2-CMV-LacZ | 4.7       | Ad/AAV-CMV-LacZ | 2.01 ± 0.42 × 10^5         | 0.59 ± 0.04 × 10^5       | 1.94 ± 0.04 × 10^5       | 2.76 ± 0.11 × 10^5       | 2.76 ± 0.11 × 10^5       | 2.76 ± 0.11 × 10^5       |
| ssAAV2-CB-EGFP | 4.2       | Ad/AAV-CBV-EGFP | 1.75 ± 0.14 × 10^5         | 0.59 ± 0.04 × 10^5       | 1.60 ± 0.04 × 10^5       | 2.76 ± 0.11 × 10^5       | 2.76 ± 0.11 × 10^5       | 2.76 ± 0.11 × 10^5       |
| ssAAV2-CB-Gluc | 4.1       | Ad/AAV-CBV-Gluc | 4.48 ± 1.08 × 10^5         | 1.95 ± 0.22 × 10^5       | 4.48 ± 1.08 × 10^5       | 2.76 ± 0.11 × 10^5       | 2.76 ± 0.11 × 10^5       | 2.76 ± 0.11 × 10^5       |
| ssAAV2-CB-hAAT | 2.6       | Ad/AAV-CBV-hAAT | 1.20 ± 0.42 × 10^5         | 0.59 ± 0.04 × 10^5       | 1.17 ± 0.04 × 10^5       | 2.76 ± 0.11 × 10^5       | 2.76 ± 0.11 × 10^5       | 2.76 ± 0.11 × 10^5       |

Suspension QW158-7 cells were infected with the Ad/AAV hybrid (MOI = 50) and VV22-PM (MOI = 2). The titer of rAAV vectors was measured by qPCR at 48 hr post-VV22-PM infection. A complete lack of VP2 did not affect packaging efficiency. The VP1 capsid may have a pH-sensitive protease that can catalyze autolytic cleavage of the capsid and lower the production yield. The smaller amount of VP2 may not be a critical limitation for rAAV production in this system, as additional supplementation of VP3 only also dramatically improved rAAV package efficiency. Consistently, a complete lack of VP2 did not affect packaging efficiency. As the ratio of VP1:VP2:VP3 affects rAAV production, it is important to balance the expression of three capsid proteins. In the current study, it was demonstrated that VP2/VP3 expression could be improved by using a vaccinia promoter with either stronger overall activity or with higher early activity. Further mimic wtAAV generation using different VV promoters (early, intermediate, and late; strong and weak) may continuously improve rAAV yields in this system.

The AAP protein was reported to function in assembly of the AAV capsid for some AAV serotypes. AAP uses a non-conventional CTG start codon, located between the ACG start codon of VP2 and ATG start codon of VP3. In the current study, the start codon CTG of AAP at 2729 was changed to CCG in the codon-optimized VP1. Thus, there should be no AAP expressed from codon-optimized VP1. Although the open reading frame (ORF) of AAP is still present in VP2 gene, there should be no AAP transcripts when VP2 is expressed in the cytoplasm since there is no cellular promoter for AAP. However, the additional expression of AAP using a vaccinia vector did not lead to a significant increase in rAAV production yield. Nevertheless, the role of AAP in our VV-Ad system remains to be investigated further.

In the current system, the new suspension base cell line, QW158-7, expressed the essential Ela/E1b genes and eliminated the use of wtAd. The complete removal of wtAd from the system greatly simplifies the downstream processing and purification procedures. Unlike the HeLa-S3 cells, QW158-7 cells greatly supported Ad/AAV hybrid replication and provided a sufficient amount of replicated rAAV genomes. VVs were replicated efficiently in QW158-7 cells and expressed abundant Rep and Cap proteins.
Moreover, the additional Reps did not significantly increase rAAV yield, which indicated that the Rep78 and Rep52 expressed by VW22 is enough for rAAV genome rescue and replication in QW158-7 cells. The robust accumulation of replicated rAAV genomes and Cap proteins in QW158-7 cells resulted in efficient rAAV packaging (Table 1). Experiments utilizing a modestly higher cell density (5 x 10^6 cells/mL) conducted in a 6-L stir-tank bioreactor demonstrated a linear increase in vector production. Optimization of rAAV production for high cell density (>1 x 10^7 cells/mL) conducted in a 6-L stir-tank bioreactor demonstrated a linear increase in vector production. Optimization of rAAV production for high cell density (>1 x 10^7 cells/mL) should be able to further increase rAAV yield. Moreover, QW158-7 is stable up to 50 passages. All those properties should make QW158-7 an attractive cell line for large-scale rAAV production.

Taken together, a robust system for packaging high-quality rAAV vectors was presented. This novel system is transfection independent, efficient, stable, safe, and easy to scale up. The ability to produce VP1 superabundant and rcAAV-free rAAV vector makes this system optimal for producing the AAV needed for large animal studies or human clinical trials.

MATERIALS AND METHODS

Cell Lines

All cells (HEK293, HeLa-S3, GM1609, BSC-1, and QW158-7) were grown in DMEM medium supplemented with 10% fetal bovine serum (FBS) (Sigma), and 100 U/mL of penicillin/streptomycin (Invitrogen). All cells were maintained in a humidified, 37°C incubator with 5% CO2. The suspension HeLa-S3 and QW158-7 cells were grown in DMEM media with 10% FBS and 0.1% pluronic F-68 in a 1-L Cell-stir* Spinner Flask (Wheaton Science Products, Millville, NJ).

Generation of Stable Cell Lines

To generate stable cell lines carrying the Ad E1a/E1b genes (QW158-7), HeLa-S3 cells were co-transfected with pCI-CMV-E1a/E1b and pCI-neo (100:1) by Lipofectamine 2000 and selected using 1,000 μg/mL G418. Single-cell-derived cell lines were then evaluated by their abilities to produce rAAV vectors by infecting with Ad/AAV-CMV-EGFP and VW22 in 24-well plates.

Construction and Generation of Recombinant VV Vectors

The plasmid pRB21 (with P7.5 promoter) was digested with Smal (NEB) and used as the backbone to construct all plasmids having different promoters, a DNA sequence containing all promoters was synthesized in the following order: Leo160/Pr4LS5E/pHyb/PrMVA13.5-long/Pr5SE/mH5/pS/ATI (Table S1). The sequence containing genomes in total rAAV genomes. (C) Transduction activity of dsAAV2-CB-EGFP vectors in vivo after intravitreal injection. Confocal images of flat-mounted retinas showing EGFP+ RGCs and Tuj1 immunostaining, and EGFP+ axons in optic nerve (n = 5, *p < 0.05).

Figure 5. Comparison of rAAV Vectors Produced by the VV-Ad System and Triple Plasmid Transfection Methods

(A) Capsid composition of rAAV vectors produced by VV-Ad and triple plasmid transfection (Tri) methods. rAAV vectors (~1 x 10^10 particles) purified after two rounds of CsCl ultracentrifuge were electrophoresed and visualized by silver staining. (B) rcAAV contamination in rAAAV vectors was determined by measuring the percentage of Rep78 containing genomes in total rAAV genomes. (C) Transduction activity of dsAAV2-CB-EGFP vectors in vivo after intravitreal injection. Confocal images of flat-mounted retinas showing EGFP+ RGCs and Tuj1 immunostaining, and EGFP+ axons in optic nerve (n = 5, *p < 0.05).
VP2 expression, and obtained VW22-A (Figure 3A). The VW22 constructs with individual promoters or promoter combinations were generated by deleting additional promoter sequences.

To generate VVs, BSC-1 cells were infected by vRB12 at an MOI of 0.05 for 1 hr followed by Polyjet (SignaGen) transfection of pRB21-derived plasmids. Each kind of VV was subject to three rounds of plaque purification and then propagated in BSC-1 cells. The titer of each VV was determined by counting plaques formed in BSC-1 cells after serial dilution. W5, W8, and VVs carrying Rep78, Rep52, VP1, VP2, and VP3 have been previously.

Ads
Adenoviral vectors used in the current study are based on Ad5. Ad375 was purchased from OD260 Inc. Ad375 is an E1-deleted Ad, with the E3 region intact. All the Ad/AAV hybrids are E1/E3 gene-deleted Ads that contain transgene cassettes flanked on their 5′ and 3′ ends by AAV ITR vector sequences. Ad/dsAAV-CB-EGFP, Ad/dsAAV-CB-Gluc, and Ad/dsAAV-CB-AAT were obtained from a contract company. The Ad/AAV-CMV-EGFP hybrid vector was purchased from Penn Vector Core (Philadelphia, PA). The Ad/AAV-CMV-LacZ was previously described. All Ad vectors used were grown in HEK293 cells and purified by CsCl ultracentrifuge.

Production of rAAV Vectors Using VV-Ad System
To produce rAAV vectors using the VV-Ad system in adherent cells, host cells were infected with Ad/AAV hybrid vectors 16 hr before VV infection. At 36 hr after VV infection, the cells were collected. To produce rAAV vectors in suspension cells, QW158-7 or HeLa-S3 cells were grown in a 1L-Flask (Wheaton Science Products, Millville, NJ). When the cell density reached 1 × 10^6 cells/mL, Ad/AAV hybrids were added (MOI = 50) and incubated for 16 hr. The cells were then collected and re-suspended in 100 mL sera-free DMEM media. Next, VW22 (MOI = 2) was added and incubated at 37°C for 2 hr. After that, the DMEM media was supplemented with 10% FBS and 0.1% Pluronic F-68 at a total volume of 400 mL. rAAV vectors generated by the new Ad-Vv system, as well as by the triple plasmid transfection method, were purified by two rounds of CsCl gradient ultracentrifugation. After extensive buffer exchange against PBS with 5% D-sorbitol, the purified vectors were incubated at 56°C for 1 hr and stored at –80°C.

Titration of rAAV Yield, rAAV, Ad/AAV, and VV Genomes Using qPCR
To titrate the rAAV genomes in the culture system, 2 mL of cell culture infected with VV and Ad/AAV were removed and sonicated to lyse the cells efficiently. After sonication, 10 μL of each sample were added into 90 μL DNase I solution (500 μg/mL, 5 mM CaCl2, 5 mM MgCl2, 50 mM Tris-HCl [pH 8.0]) and incubated at 37°C for 1 hr. DNase I was inactivated by adding 7 μL 0.5 M EDTA (pH 8.0) followed by incubation at 70°C for 20 min. Next, 93 μL of proteinase K solution (40 μg/mL proteinase K, 1 M NaCl, 1% Sarkosyl) were added and incubated at 55°C for at least 2 hr. Samples were then boiled for 20 min to inactivate the proteinase K. After inactivation, samples were serially diluted, and 10 μL were taken to analyze using qPCR. To measure the total rAAV genomes, Ad/AAV genomes, and VV genomes, samples were treated using the same protocol but without DNase I treatment. For titration of purified rAAV, Ad, or VV vectors, 10 μL of each vector were treated with DNase I and proteinase K. Then, the DNA samples were diluted to different ratios and used for analysis via qPCR. The qPCR was conducted using Sybr green reagents in an Eppendorf MasterCycler RealPlex. Samples were denatured at 95°C for 5 min, followed by 40 cycles of amplification (95°C for 10 sec, 60°C for 30 s) and terminated after generating a melting curve. A plasmid containing corresponding targeted sequence was used to make the standard curve.

Analysis of rAAV Vector Genomes Using Southern Blot
For detection of replicated rAAV genomes using a Southern blot, Hirt-extracted DNA was digested by DpnI overnight. For detecting rAAV genomes in the vectors, rAAV vectors (~1 × 10^10) were treated with DNase I and proteinase K, and the DNA was extracted using a GeneJET gel purification kit. All samples were electrophoresed on a 0.8% agarose gel, and a Southern blot was performed using a 32P-labeled EGFP-specific probe.

Silver Staining Analysis of rAAV Capsid Components
The capsid composition of the rAAV vectors was determined by silver staining analysis. Briefly, rAAV vectors (~1 × 10^10 particles) were electrophoresed using a 10% SDS-PAGE gel, and the standard silver staining protocol was followed according to the manufacturer’s procedures (Pierce Silver Stain Kit, Thermo Scientific, Rockford, IL).

Western Blot
Total proteins were extracted with lysis buffer, which consisted of 50 mM Tris (pH 8.0), 150 mM NaCl, 1% Triton X-100, 10 mM DTT, and 1× protein inhibitor (Roche, Indianapolis, IN). Cell lysates were resolved on 10% SDS-PAGE gel and transferred to a nitrocellulose membrane (Bio-Rad, Hercules, California). After blocking the membrane with 5% non-fat dry milk in TBST buffer, which contains 25 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.1% Tween 20, the membrane was incubated with the primary antibody, anti-AAV capsid (B1, American Research Products, Belmont, MA), or anti-AAV Rep (303.9, American Research Products) at a dilution of 1:5000 at 4°C overnight. The membrane was washed and incubated with a horseradish peroxidase (HRP)-conjugated sheep, anti-mouse immunoglobulin G (IgG) antibody (Sigma, St Louis, MI) at a dilution of 1:10,000. The membrane was developed using an enhanced chemiluminescent substrate (Amersham-Pharmacia Biotech, Piscataway, NJ).

TEM of rAAV Particles
For TEM analysis, formvar was coated on a microscope slide and floated onto a water bath. Cleaned 200 mesh grids were placed on this film and collected using parafilm. Five microliters of purified AAV (~1 × 10^13 vg/mL) was placed on the grid and allowed to dry, then 8 μL of 1% phospho-tungstic acid (Electron Microscopy Science, Hatfield, PA) was applied and drawn off. After drying, the
Supplemental Information

Supplemental Information includes eight figures and one table and can be found with this article online at https://doi.org/10.1016/j.omtm.2017.11.002.

Author Contributions

Q.W. helped with study design, performed experiments, analyzed the data, prepared figures, and wrote the manuscript. Z.W., J.Z., H.W., and Z.Z. performed experiments. L.M. and Y.H. performed animal studies. J.F. and L.L. performed TEM experiments. J.F. and Y.D. helped with manuscript writing and editing. W.X. directed the study and wrote the manuscript.

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