Exosome-associated SUMOylation mutant AAV demonstrates improved ocular gene transfer efficiency in vivo

Shubham Maurya, Giridhara R. Jayandharan
Department of Biological Sciences and Bioengineering, Indian Institute of Technology, Kanpur, India

Abstract

Exosome associated Adeno-associated virus (AAV) vectors have emerged as a promising tool in gene therapy. Recently, we elucidated the role of SUMOylation post-translational modification in AAV2 capsid and demonstrated that capsid modifications at SUMOylation sites, enhance vector transduction. The present study was designed to study the combinatorial effect of exosome delivery of a SUMOylation site modified AAV2, during ocular gene therapy. In the first set of experiments, we investigated the in vitro gene transfer potential of exosome-associated SUMOylation mutant AAV2 (Exo-K105Q-EGFP) in human retinal pigmental epithelial (ARPE19) cells. Our data showed that, Exo-K105Q vectors had a significantly higher transduction potential in ARPE19 cells when compared to exosomes derived from wildtype AAV2 (Exo-AAV2-EGFP) vector packaging. Subsequently, an intravitreal administration of exosome associated mutant AAV2 vectors in C57BL6/J mice, demonstrated a significant increase reporter gene (EFGP) expression 4 weeks after gene transfer. Further immunostaining, revealed that these exosome-based vectors also had a better permeation across the retinal layers. These data highlight the translational potential of exosome associated SUMOylation mutant AAV for ocular gene therapy.

Keywords
Exosomes; Adeno-associated virus; Gene therapy; SUMOylation

1 Introduction

Ocular gene therapy with viral and non-viral based vectors has been promising for the potential treatment of multiple conditions such as Leber congenital amaurosis (LCA), Retinitis pigmentosa and Stargardt disease(Acland et al., 2001; Ali, 2012; Cashman et al., 2006; Kong et al., 2008; Oliveira et al., 2017). In case of viral vectors, Adeno-associated virus (AAV) has been the vector of choice for intervention in these posterior-segment related disorders(Acland et al., 2001; Cashman et al., 2006; Kong et al., 2008). AAV vectors have
been therapeutic in patients with LCA2 for the delivery of retinal pigment epithelium-65kda protein encoding gene (RPE65) (Albert et al., 2008; Bainbridge et al., 2008; Hauswirth et al., 2008), with its efficacy predominant for the first three years (Bainbridge et al., 2015). Thus, multiple strategies have been proposed to increase the ocular gene transfer potential of AAV2 vectors (Katada et al., 2019; Petrs-Silva et al., 2009).

We(Gabriel et al., 2013) and others(Zhong et al., 2007) have shown the rate-limiting effects of post-translational modifications (PTM) in viral capsid. Modification of AAV2 at its phosphorylation(Zhong et al., 2008), ubiquitination(Yan et al., 2002), SUMOylation(Maurya et al., 2019) and neddylation(Maurya et al., 2019) sites have augmented the efficacy of AAV mediated gene delivery. In another approach, AAV vector associated exosomes harvested from the spent media during packaging, have also been efficacious during intravitreal gene transfer (Gyorgy et al., 2014; Maguire et al., 2012). Exosomes are small extra-cellular vesicles that are secreted from a cell for its intercellular communication(Mathivanan et al., 2010; Raposo and Stoorvogel, 2013; Record et al., 2014) and maintaining tissue homeostasis(Baixauli et al., 2014; Hessvik et al., 2016). These exosomes have emerged as novel carriers for delivering a variety of therapeutics including microRNA, DNA or proteins(Jiang et al., 2019). In an elegant study, exosomes derived from human embryonic stem cells, delivered to the retinal muller cells with Oct4 and Sox2 mRNA, enhanced retinal cell regeneration in an in vitro model(Katsman et al., 2012). Given the significant potential of exosomes and our recent development of SUMOylation site mutant AAV2 vectors for liver and eye-directed gene therapy(Maurya et al., 2019), we wished to further evaluate the therapeutic potential of exosomes / SUMOylation site mutant AAV2 combination for ocular gene transfer in vitro and in vivo.

2 Materials and Methods

2.1 Exosome isolation

Exosome derived AAV2 vectors were harvested after triple transfection of packaging plasmids as described previously(Wassmer et al., 2017). For vector packaging, twenty 150-mm² dishes of AAV-293 cells were cultured with 2% exosome depleted Fetal Bovine Serum (Gibco, Life Technologies, Carlsbad, CA, USA) in Iscove’s Modified Dulbecco’s Medium (IMDM) (Gibco). Upon 70-80% confluency, cells were transfected with plasmids encoding for AAV2 (rep/cap) or AAV2 K105Q, an enhanced green fluorescent protein (EGFP) transgene (p.ds.AAV2 ITR.EGFP) containing plasmid and a AAV-helper (p.helper) plasmid. Cells and supernatant media were collected 68-72 hours post transfection. For exosome isolation, 100 ml media from five 15mm² dishes were centrifuged at 300 g for 10 minutes and 1,500 g for 15 minutes. Next, supernatant media was centrifuged at 20,000 g for 60 minutes to deplete the larger extracellular vesicles. The supernatant was further centrifuged for 1.5 hours at 100,000 g using a Type 90 Ti rotor in an Optima L-90 K ultracentrifuge (Beckman Coulter, Brea, CA, USA). The final exosome pellet was re-suspended in 300 μl of 1xPBS. Physical particle titres of the vectors encapsulated in exosomes were quantified by a quantitative PCR as described elsewhere(Aurnhammer et al., 2012; Maguire et al., 2012).
2.2 Western blotting

To prepare the exosome lysates, the pellets isolated after centrifugation at 100,000 g was dissolved in the radioimmunoprecipitation assay (RIPA) buffer (Thermo Fisher, Waltham, MA, USA). Protein concentration was measured by a Bicinchoninic acid assay (BCA) (Thermo Fisher). About 40 μg of Exo-AAV2 lysate was loaded onto a 10% SDS gel. Proteins were transferred to PVDF membrane (Pall Corporation, New York, USA) and blocked with 5% BSA for 1 hour at room temperature. Membranes were incubated in anti-CD63 antibody (1:1000, Abcam, Cambridge, UK) or anti-AAV (B1) antibody (1:500, Fitzgerald, North Acton, MA, USA) overnight at 4°C. Further, membranes were washed thrice in tris-buffered saline (TBS) with 0.1% Tween 20 and incubated with an anti-mouse HRP conjugated secondary antibody for 2 hours at room temperature. Immuno-reactive bands were detected with a chemiluminescent substrate (SuperSignal™ West Pico PLUS, Thermo Scientific).

2.3 Transmission electron microscopy (TEM)

For characterization of exosome particles, 5 × 10⁹ vector genomes of Exo-AAV2-EGFP was loaded onto a TEM grid and the images were captured in a transmission electron microscope (FEI Tecnai G2 12 Twin TEM 120 kV, Hillsboro, OR, USA).

2.4 In vitro transduction assays

Approximately, 3 × 10⁴ adult retinal pigment epithelium (ARPE19) cells (a kind gift from Dr Krishnakumar/ Dr Sowmya P, Vision Research Foundation, Chennai) were seeded and incubated in a humidified chamber with 5% CO₂ at 37 °C. Cells were either mockinfected or infected with exosome derived AAV vectors (Exo-scAAV2-EGFP or Exo-scAAV2-K105Q-EGFP) at a multiplicity of infection (MOI) of 5 × 10³ vgs. The AAV2-EGFP and AAV2-K105Q-EGFP vector alone infected cells were used as an transduction control. Forty-eight hours after transduction, cells were analysed for GFP expression by flow cytometry (BD Accuri C6 Plus, BD Biosciences, Franklin Lakes, NJ, USA).

2.5 Ocular gene transfer and Fundus imaging

All animal experiments were approved by the Institutional Animal Ethics Committee (IAEC), IIT Kanpur. For intravitreal administration, eyes (n = 8) of C57BL6/J mice were dilated by Phenylephrine and tropicamide (Tropicacyl plus, Sunways India Pvt. Ltd. Mumbai, India). Injections were done after anesthetizing the animals by intraperitoneal injection of ketamine (80 mg/kg) and xylazine (12 mg/kg). Animals were mock-administered (PBS) or with 1 × 10⁸ vgs of scAAV2-K105Q-EGFP, Exo-scAAV2-EGFP and Exo-scAAV2-K105Q-EGFP vectors with a Hamilton syringe fitted with 33 gauge beveled needle. Fluorescence imaging was performed a month later, in a Micron IV imaging system (Phoenix Research Lab, Pleasanton, CA, USA). Image analysis was performed as discussed earlier(Wassmer et al., 2017). Briefly, we used the concentric circle plugin in ImageJ(Schneider et al., 2012), to draw forty circles in each image and the mean intensities were measured at the perimeter to plot the graph.
2.6 Immunohistochemistry

Eyes from mock and vector injected animals were enucleated and fixed in 4% paraformaldehyde. Eyeballs were processed in a sucrose gradient (10%, 20%, 30%) at 4°C. Next day, eyeballs were calibrated and frozen in OCT media (Sigma Aldrich, St Louis, MO, USA). Sections (8 μm) were cut in a cryostat (Leica, Wetzlar, Germany) and washed thrice with PBS and incubated with DAPI (Sigma Aldrich) for 5 minutes. Sections were mounted in FluorSave™ (Sigma-Aldrich). Imaging of the slides was performed in a Zeiss confocal microscope (LSM780NLO, Carl Zeiss, Baden-Württemberg, Germany).

2.7 Immunostaining of retina

For retinal whole mounts, excess tissues were dissected along with the cornea and lens. Eye cups were blocked in 5% normal goat serum in 0.25% Triton X-100 for overnight at 4°C. Eye cups were incubated with an anti-GFP antibody (1:100, Abcam) for 48 hours. Subsequently, eye cups were washed with PBS in 0.25% Triton X-100 and incubated in anti-mouse Alexa Fluor 555 (1:200, Abcam) for 24 hours at 4°C. After adequate washing, 4 radials cuts were made along with the optic disc and retina was dissected and mounted with FluorSave™ (Sigma-Aldrich). Whole mounts were imaged in a Lecia confocal microscope (DMi8, Wetzlar, Germany).

2.8 Statistical analysis

An ANOVA based statistical comparison using Sidak’s multiple comparison test was performed between the test and control groups (GraphPad Prism 7 software, La Jolla, CA, USA). \( p \) values are represented as **\( p<0.01 \), ***\( p<0.001 \), ****\( p<0.0001 \).

3 Results and Discussion

3.1 Exosome fraction of SUMOylation site modified (K105Q) vectors has a significantly higher transduction in retinal cells

As a first step, exosome encapsulated scAAV2-EGFP (Exo-scAAV2-EGFP) vector was characterized for exosome surface marker, CD63 tetraspanin protein (Fig. 1a). The presence of AAV2 capsid proteins in the exosomes was also determined by using an AAV capsid-specific B1 antibody (Fig. 1b). We further performed the morphological characterization of exosomes by TEM analysis. Our analysis revealed that the exosomes isolated were in the size range of 50-150 nm (Fig. 1c).

We then examined the transduction efficiency of Exo-scAAV2-EGFP and Exo-scAAV2-K105Q-EGFP vector in ARPE19 cell lines (\( n = 6 \) replicates). As can be seen in Fig. 2, mock infected ARPE19 cells, did not show any gene expression. Our data showed that the ARPE19 cells infected with the Exo-K105Q mutant vectors had a significantly higher transduction (80.28±2.1% vs. 68.9±2.2% \( p<0.0001 \)) in comparison to Exo-AAV2 vector infected ARPE19 cells (Fig. 2). These data are in agreement with previous studies, where Exo-AAV2 vectors had a 3 to 4.5-fold increase in U87 glioma cells and human 293 T cells(Maguire et al., 2012).
3.2 Intravitreal administration of exosome associated SUMOylation mutant AAV improves retinal gene transfer in vivo

To further investigate the utility of exosome associated AAV vectors for ocular gene transfer, we administered scAAV2-K105Q-EGFP, Exo-scAAV2-K105Q-EGFP and Exo-scAAV2-EGFP vectors at a low dose of $1 \times 10^8$ vgs and PBS (mock group) into the eyes of C57BL6/J mice by intravitreal route. Four weeks post-administration, the eyes ($n = 8$) were examined for transgene expression by fundus imaging. Murine eyes administered with Exo-K105Q vectors had pronounced GFP expression when compared to eyes administered with Exo-AAV2 and scAAV2-K105Q vectors (Fig. 3a). A quantitative analysis of these images by ImageJ software (Schneider et al., 2012; Wassmer et al., 2017), showed a significantly higher increase in transduction of Exo-K105Q vector administered group (Fig. 3b).

To further evaluate the permeation characteristic of exosome associated SUMOylation mutant vectors in the murine retina, we performed cryo-sectioning of eye balls. After tissue fixation, the sections were imaged for GFP positive cells. Our analysis showed that all animals that received the Exo-scAAV2-K105Q-EGFP vectors had a higher proportion of GFP positive retinal cells than other groups. This indicates that the use of Exo-K105Q mutant vectors can promote the permeation of AAV vectors within the retinal cells (Fig. 4). To exclude the impact of any back-ground autofluorescence in this analysis, we further stained the retinal whole mounts for the GFP protein with Alexa Fluor© 555 (red channel, 532 nm), as the secondary antibody (Fig. 5). Eyes administered with Exo-scAAV2-K105Q-EGFP vectors showed a greater number of transduced retinal cells in comparison to eyes administered with Exo-scAAV2-EGFP and scAAV2-K105Q-EGFP vectors.

Our study has confirmed the utility of exosomes for ocular gene transfer, as reported earlier (Wassmer et al., 2017). We have also shown that exosomes containing AAV2 with targeted PTM site mutations, can augment its transduction efficiency (Gabriel et al., 2013; Petrs-Silva et al., 2009; Yan et al., 2002; Zhong et al., 2008; Zhong et al., 2007). The role of SUMOylation in AAV2 transduction of HeLa cells (3 to 4-fold increase in SUMOylation inhibition) was initially postulated by RNA interference based assays (Holscher et al., 2015). Subsequently, we demonstrated the rate-limiting role of SUMOylation during AAV2 infection of host cells, by a targeted gene expression profiling of SUMOylation pathway (SAE1, SAE2, UBC9, SUMO1) (Maurya et al., 2019). Furthermore, a K105Q mutation introduced in the vector capsid, that alters its native SUMOylation recognition site, resulted in phenotypic correction of murine models of hemophilia B and LCA2 (Maurya et al., 2019).

Our findings that exosome associated AAV that are rationally engineered at its capsid has a improved function, opens up several possibilities. In our study, a substantial gene expression was noted in vivo at a relatively lower dose of AAV ($1 \times 10^8$ vgs), which is at least 20 to 500-fold less than previously tested (Meliani et al., 2017; Wassmer et al., 2017) and 3-fold less than our previous study (Maurya et al., 2019). Interestingly, previous reports that investigated exosome-based delivery of AAV5 and AAV8 vectors to deliver human coagulation factor IX (h.FIX) in hemophilia mice has demonstrated this phenomenon with a reduced vector dose required for phenotypic correction (Meliani et al., 2017). A dosage finding experiment [normal dose $5 \times 10^{10}$ vgs vs. lower dose $1 \times 10^9$ vgs of exo-AAV8 and
AAV8] revealed a 5-fold increase in h.FIX in exo-AAV8 administered mice at a dose of $1 \times 10^9$ vgs when compared to AAV8 vector injected animals (Meliani et al., 2017). Taken together, these data highlights that exosomal gene delivery, combined with AAV capsid modifications is likely to be synergistic and generate better AAV/exosomal delivery systems, that are therapeutic at significantly lower doses. Further, it has emerged that exosome associated AAV can bypass circulating neutralizing antibodies (NAb) and are also non-immunogenic in recipient mice (Meliani et al., 2017). A significant protection from pre-existing antibodies was observed in mice preimmunized with intravenous immunoglobulins (anti-AAV8 NAb titer 1:1 to 1:3.16) and treated with exo-AAV8 vectors, whereas in comparison, naked AAV8 vectors were neutralized (Meliani et al., 2017). This observation is crucial, given that 30-70% of humans are seropositive to any of the common ten AAV serotypes (Hüser et al., 2017) and a de novo AAV capsid specific cellular immune response can be pronounced even during local ocular gene transfer (Jacobson et al., 2015). A combination gene delivery based on exosome-AAV thus becomes an attractive avenue to further tailor and engineer such vector associated exosomes, by modification of the putative immunogenic epitopes in vector capsid (Tseng and Agbandje-McKenna, 2014) or by overexpression of decoy or proteins (Stickney et al., 2016) within the exosomal biomembranes, to attenuate such immune recognition mechanisms.

4 Conclusions

Our data has highlighted the therapeutic potential of exosome associated mutant AAV vectors for ocular gene therapy. However further studies, with these vectors carrying a therapeutic transgene such as RPE65 and preferably in a setting of pre-existing antibodies to AAV, will be required to assess its suitability for human applications.

Acknowledgements

This work was supported through a grant support from Wellcome Trust-DBT India Alliance senior fellowship (IA/S/16/1/502355) to GRJ. SM received a study fellowship from Ministry of Human Resource development, Government of India. We thank Dr Ashok Kumar IIT-Kanpur for confocal microscope facility, Dr Sridhar Bammidi, IIT Kanpur for intravitreal injections and Nusrat Khan for the TEM analysis. We also thank Dr Krishnakumar and Dr S. Parameswaran, VRF, Chennai for initial training in intravitreal injections.

Abbreviations

| Abbreviation | Description |
|--------------|-------------|
| AAV2         | Adeno-associated virus type 2 |
| GFP          | Green fluorescence protein |
| vgs          | Vector genomes |
| LCA2         | Leber’s congenital amaurosis type 2 |
| RPE65        | Retinal pigment epithelium 65 |
| Exo          | Exosomes |
| RIPA         | Radioimmunoprecipitation assay |
| BCA          | Bicinchoninic acid assay |
TBS  Tris-buffered saline
HeLa  Human cervical carcinoma cells
ARPE19  Adult retinal pigment epithelium cells
h.FIX  Human Factor IX

References

1. Acland GM, Aguirre GD, Ray J, Zhang Q, Aleman TS, Cideciyan AV, Pearce-Kelling SE, Anand V, Zeng Y, Maguire AM, Jacobson SG, et al. Gene therapy restores vision in a canine model of childhood blindness. Nat Genet. 2001; 28(1):92–95. [PubMed: 11326284]

2. Albert M, Francesca S, Eric A, Edward N, Federico M, Jeannette B, Sandro B, Kathleen A, Francesco T, Enrico M, Richard H, et al. Safety and Efficacy of Gene Transfer for Leber's Congenital Amaurosis. N Engl J Med. 2008; 358(21):2240–2248. [PubMed: 18441370]

3. Ali RR. Ocular gene therapy: introduction to the special issue. Gene Ther. 2012; 19:119. [PubMed: 22318169]

4. Aurnhammer C, Haase M, Muether N, Hausl M, Rauschhuber C, Huber I, Nitschko H, Busch U, Sing A, Ehrhardt A, Baiker A. Universal real-time PCR for the detection and quantification of adeno-associated virus serotype 2-derived inverted terminal repeat sequences. Hum Gene Ther Methods. 2012; 23(1):18–28. [PubMed: 22428977]

5. Bainbridge JW, Mehat MS, Sundaram V, Robbie SJ, Barker SE, Ripamonti C, Georgiadis A, Mowat FM, Beattie SG, Gardner PJ, Feathers KL, et al. Long-term effect of gene therapy on Leber's congenital amaurosis. N Engl J Med. 2015; 372(20):1887–1897. [PubMed: 25938638]

6. Bainbridge JW, Smith AJ, Barker SS, Robbie S, Henderson R, Balagga K, Viswanathan A, Holder GE, Stockman A, Tyler N, Petersen-Jones S, et al. Effect of gene therapy on visual function in Leber's congenital amaurosis. N Engl J Med. 2008; 358(21):2231–2239. [PubMed: 18441371]

7. Baixauli F, López-Otín C, Mittelbrunn M. Exosomes and autophagy: coordinated mechanisms for the maintenance of cellular fitness. Front Immunol. 2014; 5:403. [PubMed: 25191326]

8. Cashman SM, Bowman L, Christoffersson J, Kumar-Singh R. Inhibition of Choroidal Neovascularization by Adenovirus-Mediated Delivery of Short Hairpin RNAs Targeting VEGF as a Potential Therapy for AMD. Invest Ophthalmol Vis Sci. 2006; 47(8):3496–3504. [PubMed: 16877421]

9. Gabriel N, Hareendran S, Sen D, Gadkari RA, Sudha G, Selot R, Hussain M, Dhaikasnaamurthy R, Samuel R, Srinivasan N, Srivastava A, et al. Bioengineering of AAV2 capsid at specific serine, threonine, or lysine residues improves its transduction efficiency in vitro and in vivo. Hum Gene Ther Methods. 2013; 24(2):80–93. [PubMed: 23379478]

10. Gyorgy B, Fitzpatrick Z, Crommentuijn MH, Mu D, Maguire CA. Naturally enveloped AAV vectors for shielding neutralizing antibodies and robust gene delivery in vivo. Biomaterials. 2014; 35(26):7598–7609. [PubMed: 24917028]

11. Hauswirth WW, Aleman TS, Kaushal S, Cideciyan AV, Schwartz SB, Wang L, Conlon TJ, Boye SL, Flotte TR, Byrne BJ, Jacobson SG. Treatment of leber congenital amaurosis due to RPE65 mutations by ocular subretinal injection of adeno-associated virus gene vector: short-term results of a phase I trial. Hum Gene Ther. 2008; 19(10):979–990. [PubMed: 18774912]

12. Hessvik NP, Overbye A, Brech A, Torgersen ML, Jakobsen IS, Sandvig K, Llorente A. PIKfyve inhibition increases exosome release and induces secretory autophagy. Cell Mol Life Sci. 2016; 73(24):4717–4737. [PubMed: 27438886]

13. Holscher C, Sonntag F, Henrich K, Chen Q, Beneke J, Matula P, Rohr K, Kaderali L, Beil N, Erle H, Kleinschmidt JA, et al. The SUMOylation Pathway Restricts Gene Transduction by Adeno-Associated Viruses. PLoS Pathog. 2015; 11(12):e1005281. [PubMed: 26625259]

14. Hüser D, Khalid D, Lutter T, Hammer E-M, Weger S, Heßler M, Kalus U, Tauchmann Y, Hensel-Wiegel K, Lassner D, Heilbronn R. High Prevalence of Infectious Adeno-associated Virus (AAV)
in Human Peripheral Blood Mononuclear Cells Indicative of T Lymphocytes as Sites of AAV Persistence. J Virol. 2017; 91(4):e02137–02116. [PubMed: 27928011]

15. Jacobson SG, Cideciyan AV, Roman AJ, Sumaroka A, Schwartz SB, Heon E, Hauswirth WW. Improvement and decline in vision with gene therapy in childhood blindness. N Engl J Med. 2015; 372(20):1920–1926. [PubMed: 25936984]

16. Jiang L, Gu Y, Du Y, Liu J. Exosomes: Diagnostic Biomarkers and Therapeutic Delivery Vehicles for Cancer. Mol Pharm. 2019; 16(8):3333–3349. [PubMed: 31241965]

17. Katada Y, Kobayashi K, Tsubota K, Kurihara T. Evaluation of AAV-DJ vector for retinal gene therapy. PeerJ. 2019; 7:e6317. [PubMed: 30671314]

18. Katsman D, Stackpole EJ, Domin DR, Farber DB. Embryonic Stem Cell-Derived Microvesicles Induce Gene Expression Changes in Muller Cells of the Retina. PLoS One. 2012; 7(11):e50417. [PubMed: 23226281]

19. Kong J, Kim SR, Binley K, Pata I, Doi K, Mannik J, Zernant-Rajang J, Kan O, Iqball S, Naylor S, Sparrow JR, et al. Correction of the disease phenotype in the mouse model of Stargardt disease by lentiviral gene therapy. Gene Ther. 2008; 15(19):1311–1320. [PubMed: 18463687]

20. Maguire CA, Balaj L, Sivaraman S, Crommentuijn MH, Ericsson M, Mincheva-Nilsson L, Baranov V, Gianni D, Tannous BA, Sena-Esteves M, Breakefield XO, et al. Microvesicle-associated AAV vector as a novel gene delivery system. Mol Ther. 2012; 20(5):960–971. [PubMed: 22314290]

21. Mathivanan S, Ji H, Simpson RJ. Exosomes: extracellular organelles important in intercellular communication. J Proteomics. 2010; 73(10):1907–1920. [PubMed: 20601276]

22. Maurya S, Mary B, Jayandharan GR. Rational Engineering and Preclinical Evaluation of Neddylation and SUMOylation Site Modified Adeno-Associated Virus Vectors in Murine Models of Hemophilia B and Leber Congenital Amaurosis. Hum Gene Ther. 2019; 30(12):1461–1476. [PubMed: 31642343]

23. Meliani A, Boisgerault F, Fitzpatrick Z, Marmier S, Leborgne C, Collaud F, Simon Sola M, Charles S, Ronzitti G, Vignaud A, van Wittenberge L, et al. Enhanced liver gene transfer and evasion of preexisting humoral immunity with exosomeenveloped AAV vectors. Blood Adv. 2017; 1(23):2019–2031. [PubMed: 29296848]

24. Oliveira AV, Rosa da Costa AM, Silva GA. Non-viral strategies for ocular gene delivery. Mater Sci Eng C Mater Biol Appl. 2017; 77:1275–1289. [PubMed: 28532005]

25. Petrs-Silva H, Dinculescu A, Li Q, Min SH, Chiodo V, Pang JJ, Zhong L, Zolotukhin S, Srivastava A, Lewin AS, Hauswirth WW. High-efficiency transduction of the mouse retina by tyrosine-mutant AAV serotype vectors. Mol Ther. 2009; 17(3):463–471. [PubMed: 19065993]

26. Raposo G, Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. J Cell Biol. 2013; 200(4):373–383. [PubMed: 23420871]

27. Schneider CA, Rashand WS, Eliceiri KW. NIH Image to ImageJ: 25 years of image analysis. Nat Methods. 2012; 9(7):671–675. [PubMed: 22930834]

28. Tseng Y-S, Agbandje-McKenna M. Mapping the AAV Capsid Host Antibody Response toward the Development of Second Generation Gene Delivery Vectors. Front Immunol. 2014; 5:9. [PubMed: 24523720]
33. Zhong L, Li B, Jayandharan G, Mah CS, Govindasamy L, Agbandje-McKenna M, Herzog RW, Weigel-Van Aken KA, Hobbs JA, Zolotukhin S, Muzyczka N, et al. Tyrosine-phosphorylation of AAV2 vectors and its consequences on viral intracellular trafficking and transgene expression. Virology. 2008; 381(2):194–202. [PubMed: 18834608]

34. Zhong L, Zhao W, Wu J, Li B, Zolotukhin S, Govindasamy L, Agbandje-McKenna M, Srivastava A. A dual role of EGFR protein tyrosine kinase signaling in ubiquitination of AAV2 capsids and viral second-strand DNA synthesis. Mol Ther. 2007; 15(7):1323–1330. [PubMed: 17440440]
Fig. 1.
Characterization of exosomes. About 40 μg of exosome lysates were immunoblotted for the surface tetraspanin protein, CD63 (a). The presence of AAV capsids in exosomal lysate was verified by Anti-AAV (B1) immunoblotting (b). Transmission electron microscopy demonstrates the size distribution and representative exosomes are indicated by arrows (c).
Fig. 2.

*In vitro* transduction efficiency of exosome associated AAV2 vectors. Transduction potential of Exo-scAAV2-K105Q-EGFP and Exo-scAAV2-EGFP vectors were determined in ARPE19 cells at a multiplicity of infection (MOI) of $5 \times 10^3$ vgs. Mock-treated cells, naked AAV vectors (scAAV2-EGFP and scAAV2-K105-EGFP) were used as controls. The transgene (EGFP) expression was measured by flow cytometry. An ANOVA based Sidak’s multiple comparison test was used for statistical analysis. Error bars represent SD, $n = 6$, **$P < 0.01$, ****$P < 0.0001$. 
Gene transfer potential of exosome associated SUMOylation mutant AAV2 in murine retina. Eyes \((n = 8)\) of C57BL6/J mice were mock-injected or injected with exosome encapsulated vectors (Exo-AAV2-K105Q-EGFP and Exo-AAV2-EGFP) and naked vector (scAAV2-K105Q-EGFP) at the dose of \(1 \times 10^8\) vgs per eye in 1-2 \(\mu l\) volume via intravitreal route. A month later, the eyes were imaged in a Micron IV imaging system. The intensity was set at maximum and gain was set at 18 db, the frame rate was set at 4 fps for imaging of all the groups. Representative set of images has been shown (a). Image analysis was done by using concentric circle plugin in ImageJ software (Schneider et al., 2012) (b). For statistical analysis, ANOVA based Sidak’s multiple comparison test was used. Data are mean + SD. ***\(P < 0.001\), ****\(P < 0.0001\). Representative images from three eyes are shown.
Fig. 4.
Permeation characteristics of exosome associated AAV across the retina. Cryo-sections from eyes, collected after enucleation was stained with DAPI as described in the methods section. Representative images from the mock-administered, scAAV2-K105Q-EGFP, Exo-scAAV2-EGFP, Exo-scAAV2-K105Q-EGFP administered eyes are shown. Images were acquired on a Zeiss confocal microscope (LSM780NLO, Baden-Württemberg, Germany) using 405 nm and 488 nm laser. GCL- Ganglion cell layer; INL- Inner nuclear layer; ONL- Outer nuclear layer; OS- Outer segment; RPE- Retinal pigment epithelium. Exposure settings – Gain [V]: 642; Offset [%]: 3.00%, Magnification 400 ×.
Fig. 5.
Immunostaining of Green fluorescent protein in retinal whole mounts. Eyes, post enucleation, were stained with an anti-GFP antibody (1:100, Abcam) and counterstained with Alexa Fluor® 555 (1:200, Abcam). The retina was dissected and mounted on slides after 4 radial cuts (a). Whole mounts were imaged under a Leica confocal microscope (Wetzlar, Germany) using 532 nm laser. Representative images from mock-administered, scAAV2-K105Q-EGFP, Exo-scAAV2-EGFP, Exo-scAAV2-K105Q-EGFP administered eyes are shown in the above panel (b). Exposure settings – Gain [V]: 809; Offset [%]: 0.00%; Magnification 200×.