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

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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 exo-some-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

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Author contributions

SM performed experiments, GRJ supervised the study, SM and GRJ interpreted the data and wrote the paper.

Declaration of Competing Interest

The authors declare that there is no conflict of interests.

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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-mm^2 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 $15mm^2$ 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).

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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, 5×10^9 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, 3×10^4 adult retinal pigmental 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% CO_2 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 5×10^3vgs. 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 1×10^8 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 anaylsis was performed as discussed earlier(Wassmer et al., 2017). Briefly, we used the concentric circle pluggin 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.

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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 4ºC. 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).

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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 1×10^8vgs 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 (1×10^8 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 5×10^{10} vgs vs. lower dose 1×10^9 vgs of exo-AAV8 and

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AAV8] revealed a 5-fold increase in h.FIX in exo-AAV8 administered mice at a dose of 1×10^9 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 bio-membranes, 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.

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Abbreviations

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 pigmental epithelium cells
h.FIX	Human Factor IX

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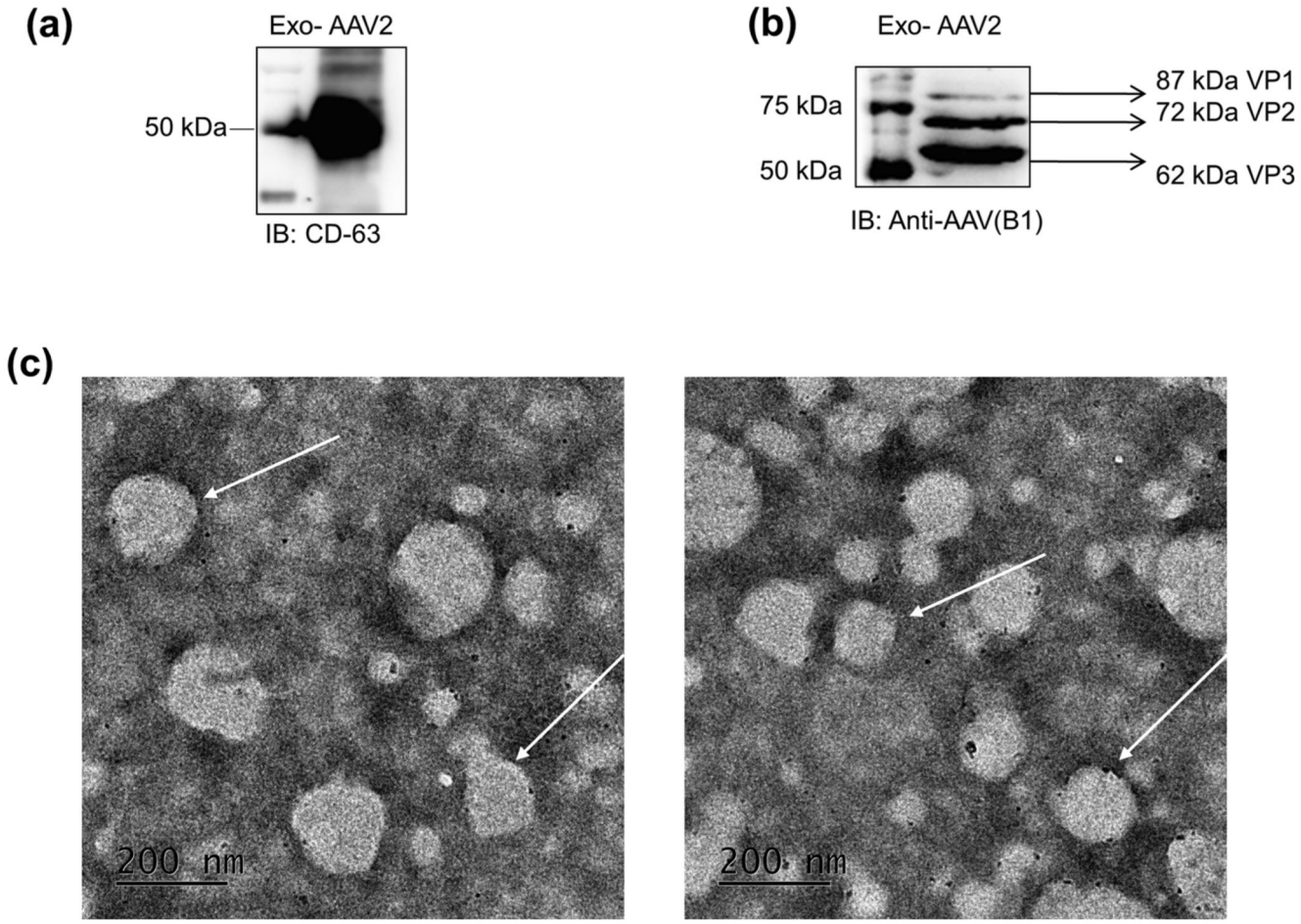

Fig. 1.

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).

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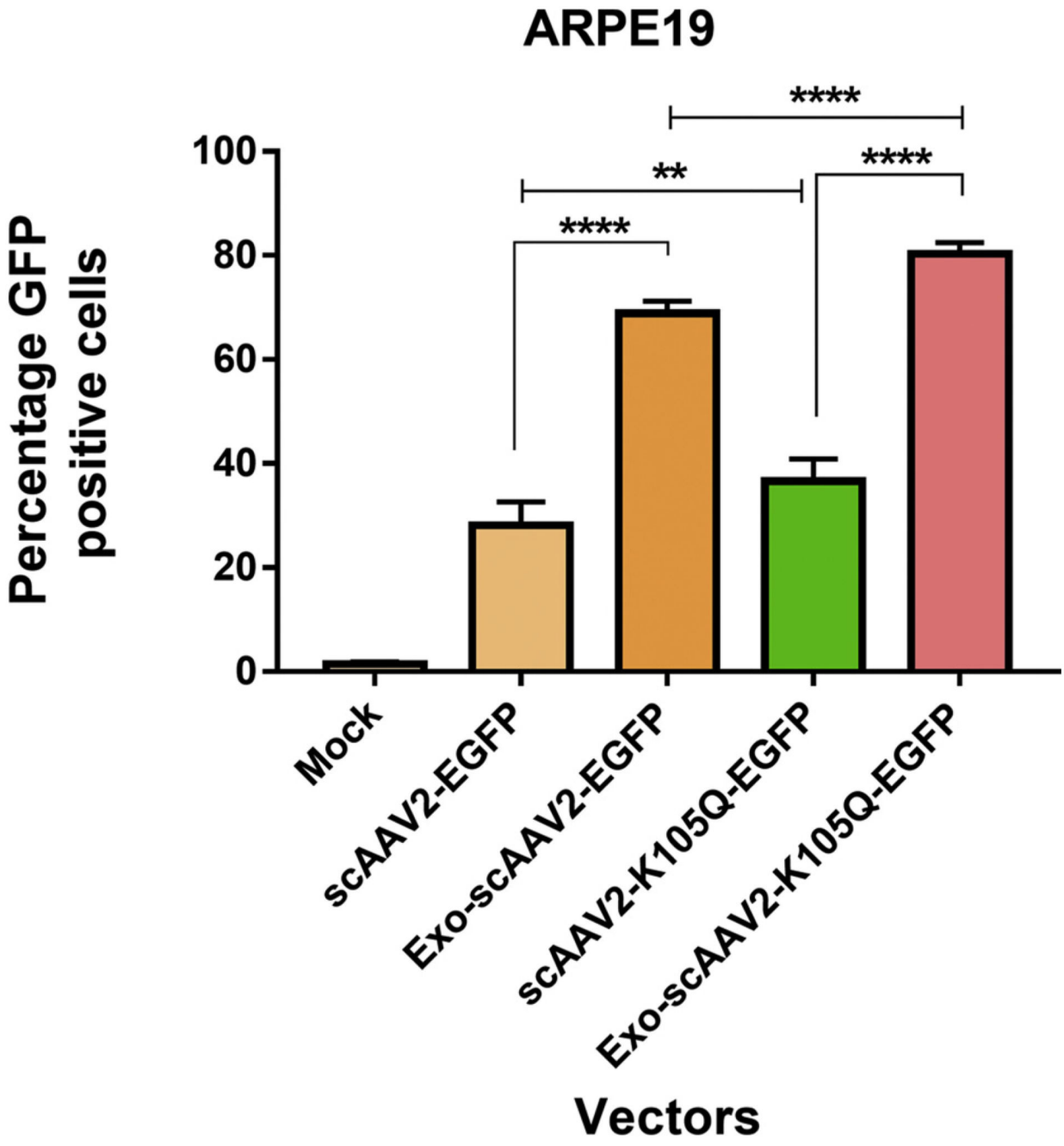

Fig. 2.

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 5×10^3 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.*

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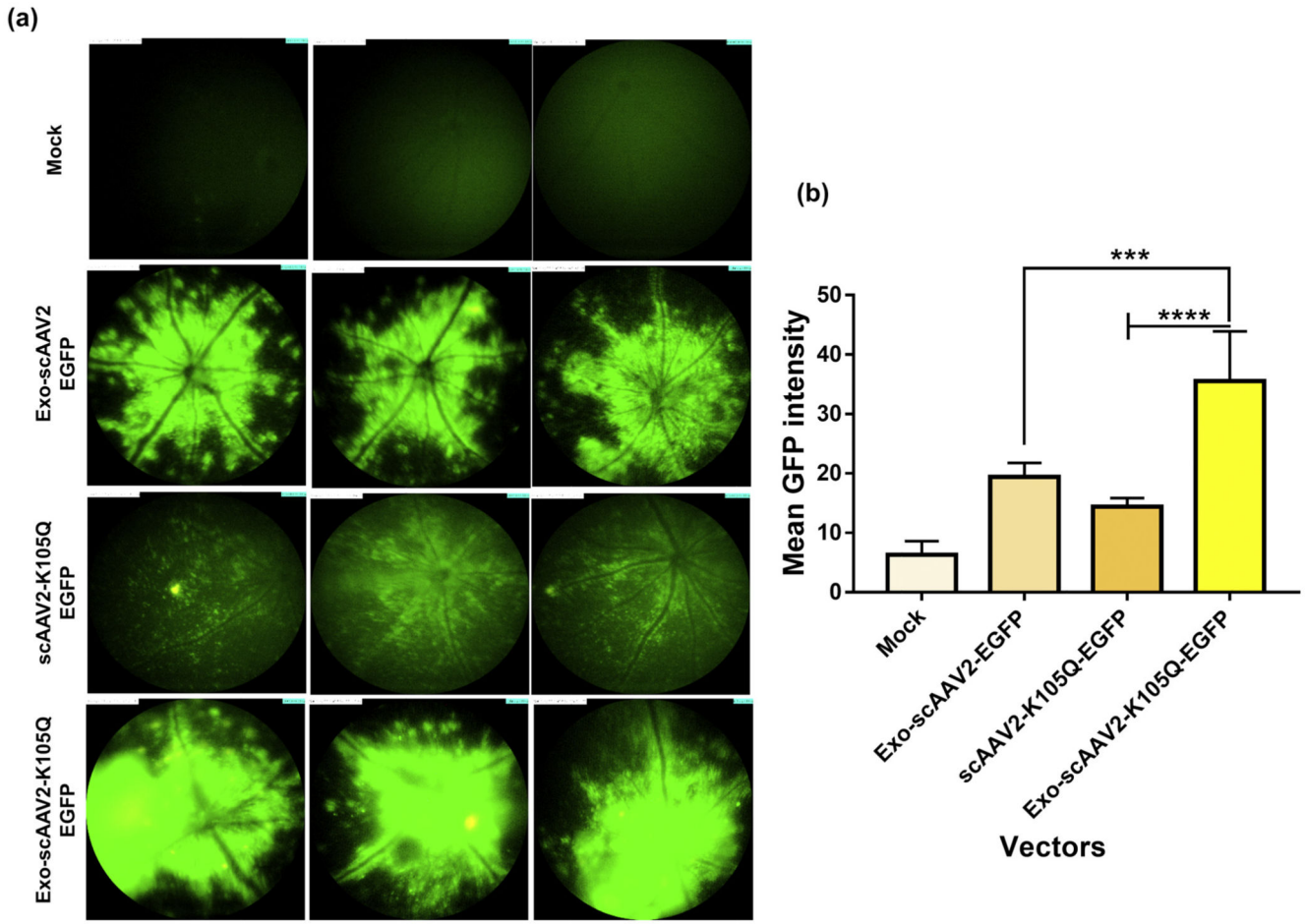

Fig. 3.

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 1×10^8 vgs per eye in 1-2 μl 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.

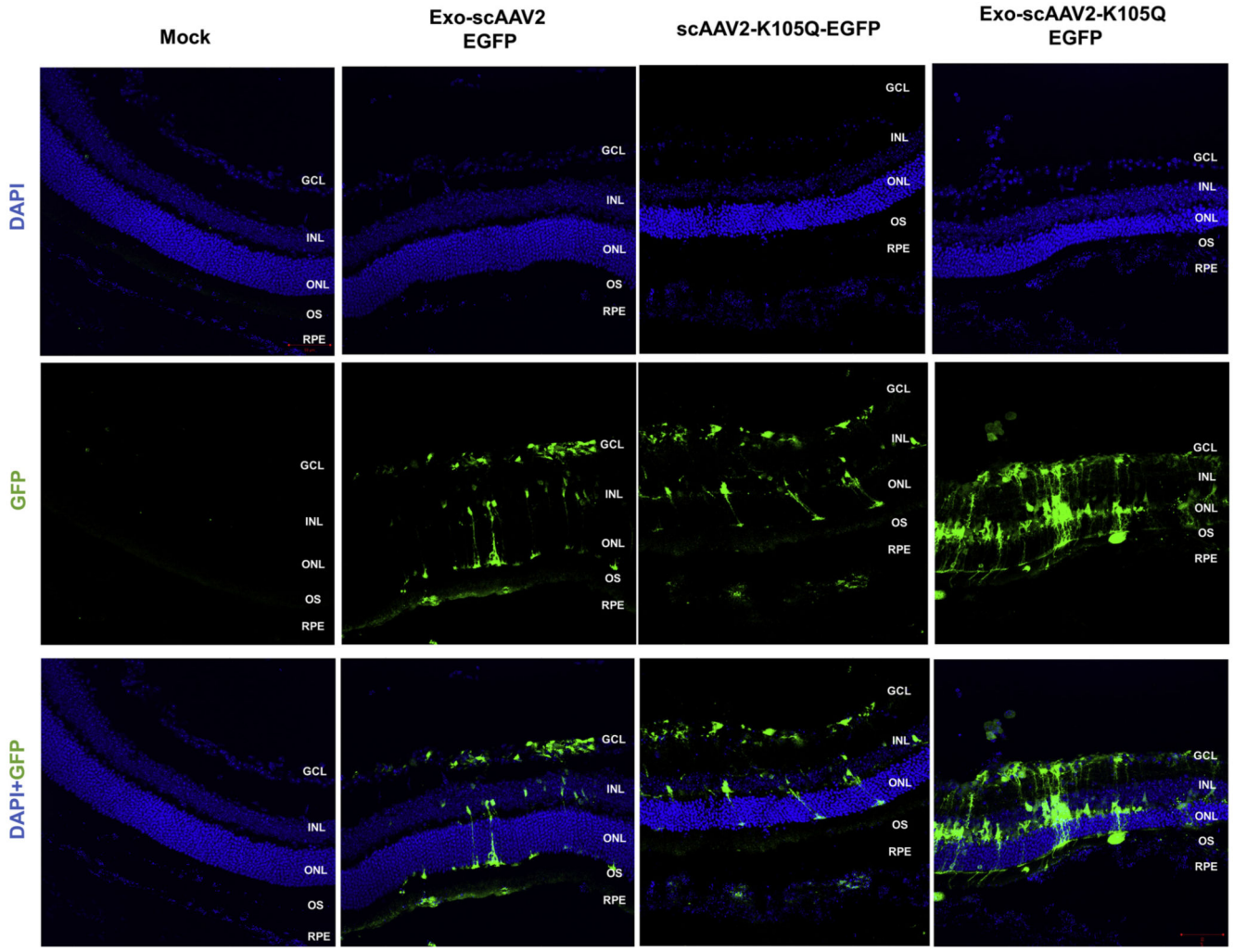

Fig. 4.

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 ×.

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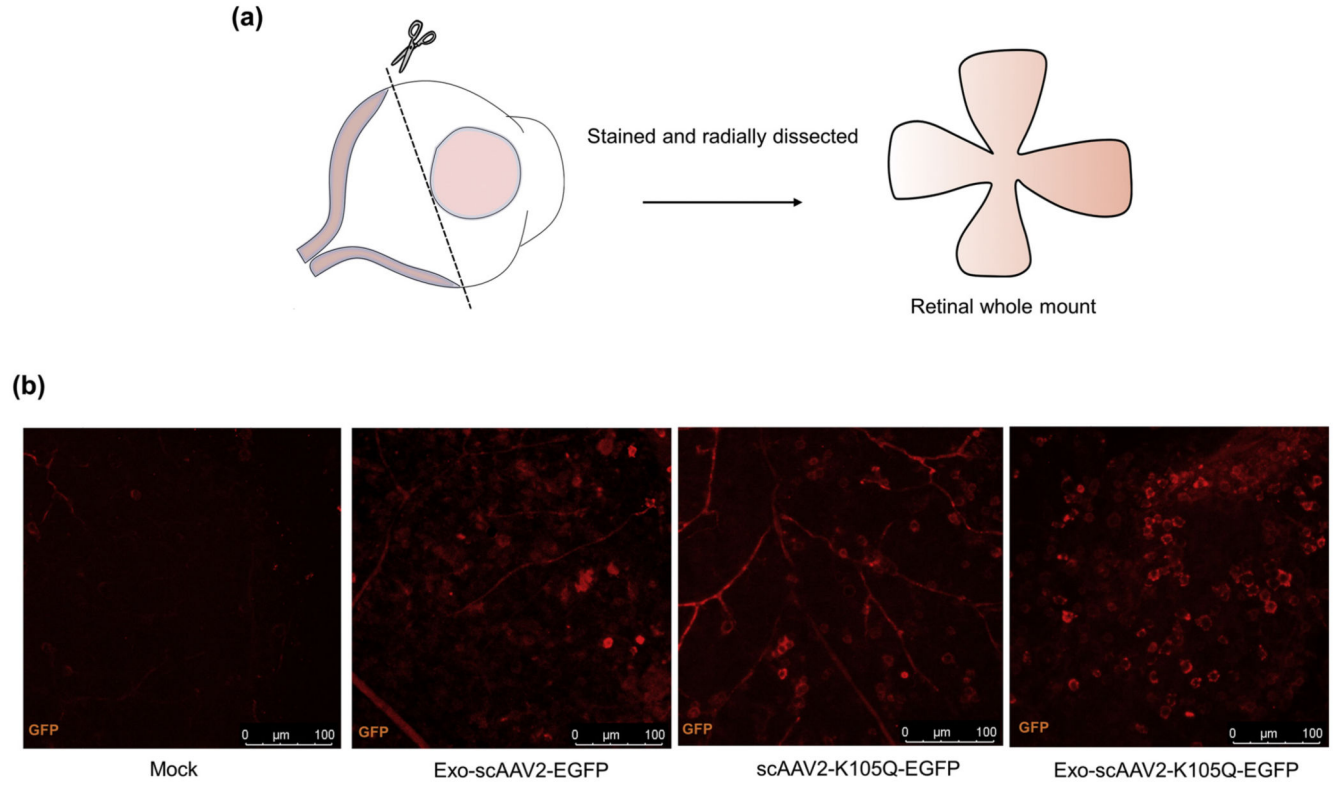

Fig. 5.

Immunostaining of Green fluorescent protein in retinal whole mounts. Eyes, post enucleation, was 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. Representatives 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 ×.

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