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Rescue of Adeno-Associated Virus Production by shRNA Cotransfection

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Adeno-associated virus (AAV) vector technology is rapidly advancing and becoming not only the leading vector platform in the field of gene therapy but also a useful tool for functional genomic studies of novel proteins. As most vectors utilize constitutive promoters, this results in transgene expression during production. Depending on the transgene product, this could induce proapoptotic, cytostatic, or other unknown effects that interfere with producer cell function and, therefore, reduce viral vector yield. This can be a major limitation when trying to characterize poorly described genes. We describe the novel use of shRNA encoding plasmids cotransfected during packaging to limit the expression of the cytotoxic transgene product. This allowed the production of an otherwise unpackageable vector. The approach is simple, versatile, does not require modification of the vector plasmid, and should be easily adaptable to almost any transgene with minimal cost.

Keywords: AAV, shRNA, cytotoxic transgene, GPR78

INTRODUCTION

THE FIELD OF GENE therapy has shown advances in many areas, resulting in effective treatments for many diverse diseases. Recombinant adeno-associated virus (rAAV) vectors are proving to be a reliable gene transfer system for clinical applications, with an increasing body of evidence supporting safety and efficacy. In addition to their therapeutic applications, adeno-associated virus (AAV) vectors are very useful for functional genomic studies due to their relative ease of manipulation and ability to transfer genes into a variety of cell types.

As most vectors utilize constitutive promoters, this results in transgene expression during production, which energetically limits the cellular machinery. Depending on the transgene product, it could induce proapoptotic, cytostatic, or other unknown effects that interfere with producer cell function and, therefore, reduce viral vector yield. This can be a major limitation when trying to characterize poorly described genes.

Incorporation of inducible promoters to regulate expression,[1] riboswitches,[2] transplicing vectors,[3] translational repressor elements,[4] mammalian introns that are silent in insect cells,[5] and microRNA sites that affect mRNA stability[6] have been reported. All of these options would affect the transgene expression both in the producer cell and in the target cell after transduction. Our hypothesis was that the cotransfection of shRNA encoding plasmids during vector production would limit the effect of transgene expression on vector production but not in the target cell. Our findings supported our hypothesis and showed that the cotransfection with shRNA is a simple and versatile method to improve the yield of vectors without altering the transgene cassette.

MATERIALS AND METHODS

Cell culture

HEK293T cells (human embryonic kidney cells) were cultured in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS; HyClone), 2 mM L-glutamine, 100 U of penicillin/mL, and 0.1 mg of streptomycin/mL (Invitrogen). Cells were maintained at 37°C under a 5% CO_2 humidified atmosphere.

Construction of G protein-coupled receptor 78 (GPR78)

Commercial GPR78 plasmid from Origene (RC209203) was digested with *Sal*I and *Sma*I, and the insert (1,265 pb) was cloned into a pAAV2 vector bone[7] that was digested with *Sal*I and *Eco*RV and treated with alkaline phosphatase, calf intestinal (CIP) (4,851 pb). Bands were purified

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from the 0.8% agarose gel by using the MinElute gel extraction kit (QIAGEN), and the ligation reaction was performed for 5 min by using the Quick Ligation kit (NEB), insert:vector bone ratio 10:1. After transformation by using the One shot max efficiency DHαT1 chemically competent cells (Invitrogen), miniprep was performed (QIAGEN) and the correct clones for the pAAV2-GPR78 (6,116 bp) were screened by digestion and DNA sequencing. We selected one clone where the GPR78 insert was in the correct orientation, to be used as our experimental AAV2-GPR78, and another one where the GPR78 was in the inverted (noncoding) orientation, to be used as a control. Maxiprep (QIAGEN) was performed with the selected clones.

shRNA

Five different shRNA clones for the GPR78 gene were purchased from Sigma Aldrich (GPR78 MISSION shRNA Bacterial Glycerol Stock for G protein-coupled receptor 78): TRCN0000008929, TRCN0000008931, TRCN0000008932, TRCN0000008930, and TRCN 0000011663. Individual plasmid preparations were made by using Maxiprep kits (QIAGEN, Carlsbad, CA).

Generation of recombinant virus

Recombinant AAV2 expressing GPR78 protein was produced as previously described.[8] Briefly, 293T cells plated in 150-cm dishes (2×10^6 cells in 20 mL of media per plate) were cotransfected with pAAV2-GPR78 (1 μg/plate), pAAV2 RepCap (6 μg/plate), shRNA (6 μg/plate), and the adenovirus helper plasmid pRS449B[9] (12 μg/plate). Ten plates were prepared per virus preparation. Recombinant vectors (rAAV2-GPR78) were purified by CsCl gradient centrifugation. DNase-resistant genome copy numbers for the vectors were determined by quantitative real-time PCR using TaqMan system (Applied Biosystems) with probes specific to the cytomegalovirus (CMV) promoter contained within the packaged genome. Forward primer-CATCTACGTATTAGTCATCGCTAT TACCAT; reverse-TGGAAATCCCCGTGAGTCA. To check for shRNA contamination, the following sets of primers were used: forward-GTCACCGAGCTGCAAGAA; reverse-CCGATCTCGGCGAACAC. Additional recombinant AAV2 were prepared as controls following the same concentrations of plasmids but with the shRNA being replaced by an empty vector when shRNA is not used.

Preparation of cell lysates and Western blot analysis

For the Rep and Cap protein, 48 h after transfection, the cells were washed twice in ice-cold 1$\times$phosphate-buffered saline; lysed by incubation for 30 min on ice in RIPA buffer supplemented with protease inhibitor, phosphatase inhibitor (Thermo Fisher Scientific); and cleared by centrifugation at 17,000 *g* for 25 min at 4°C. The protein concentrations in collected supernatants were measured by Protein Assay Dye Reagent Concentrate (5000006; Bio-Rad) and bovine serum albumin (BSA) as a protein standard. The supernatants (28 μg per sample) were denaturized with 4$\times$NuPAGE LDS sample buffer (Thermo Fisher Scientific) and 2-mercaptoethanol (Bio-Rad) at 99.8°C for 10 min, resolved by sodium dodecyl sulphate-polycrylamide gel electrophoresis by using 1.5 mm NuPAGE 4–12% bis–tris gel (Thermo Fisher Scientific) and 1$\times$3-(N-Morpholino)propanesulfonic acid sodium dodecyl sulfate (MOPS SDS) running buffer under the running condition with 60 V constant for 30 min followed by 130 V constant for 100 min, and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes (Invitrolon™ PVDF; Thermo Fisher Scientific) by using the Xcell II blot module with 1$\times$NuPAGE transfer buffer supplemented with 20% methanol, using 30 V for 195 min. All membranes were blocked with 6% nonfat milk in 1$\times$Tris-buffered saline (TBS) 0.1% tween 20 (T-TBS) for 1 h at room temperature. Primary antibodies were incubated overnight at 4°C: Rep (dilution 1:50) and Cap (clone: B1; dilution 1:300) antibodies in 5% BSA (Sigma-Aldrich) in T-TBS. After washing thrice in T-TBS, the membranes were reacted with mouse IgG HRP-linked whole antibody (GENA931; Sigma-Aldrich) diluted in 1:4,000, 2% nonfat milk in T-TBS for 1 h at room temperature. Membranes were washed thrice with T-TBS and developed by using Super Signal West Pico Chemiluminescent Substrate or Super Signal West Pico PLUS Chemiluminescent Substrate (Thermo Fisher Scientific) according to the manufacturer's protocol. To confirm loaded protein volume in the samples, the membrane used for detection of Cap protein was incubated with the mixture of β-actin antibody (clone: AC15, A5441; Sigma-Aldrich) diluted in 1:2,000, and Mouse IgG HRP-linked whole antibody (GENA931; Sigma-Aldrich) diluted in 1:10,000, 5% nonfat milk in T-TBS, at 4°C for 15 min. Western blot was analyzed by using ImageJ. Cap and Rep antibodies were kindly provided by Mavis Agbandje-McKenna. For the GPR78 Western blot, protein lysates (20 μg) were denaturated with 4$\times$laemmli sample buffer and 2-mercaptoethanol (Bio-Rad) for 60 min at room temperature. Samples were loaded in a 4–12% 1.0 mm precast Novex gel (Invitrogen), 150 V constant by using MES SDS running buffer 1$\times$, and subsequently transferred to a PVDF membrane by using the Trans-blot turbo transfer pack (Biorad), with the conditions 1.3 A and 25 V for 12 min. The membrane was blocked with 5% nonfat milk in TBS 1$\times$0.1% tween 20 (T-TBS) for 1 h at room temperature. Primary antibody was incubated overnight at 4°C (GPR78 antibody; ab121390; Abcam, dilution 1:300) in 1% nonfat milk in T-TBS. The membrane was washed thrice with T-TBS. Secondary antibody (211-032-171; Jackson ImmunoResearch) was diluted in 1:4,000, 5% nonfat milk in T-TBS for 1 h at room temperature. Membrane was washed five times with T-TBS and developed

by using Supersignal West Pico (Thermo Fisher Scientific). Similar protein loading was confirmed by β-actin-peroxidase antibody (A3854; Sigma-Aldrich).

Cell growth experiment

Overall, 293T cells were transfected with commercial GPR78 plasmid from Origene (RC209203) or with empty plasmid using lipofectamine 3000 (Thermo Fisher Scientific). After 24 h, cells were re-plated with 2×10^5 cells per plate in a 10-cm dish (6 plates/each transfection). To assess live cell growth, the cells from three plates were counted 24 h after re-plating and in the remaining three plates 48 h after re-plating. For the counting process, cells were resuspended on their own media and each plate was rinsed with extra 3 mL of media. Cells were centrifuged at 218 *g* for 5 min at room temperature. The obtained cell pellets were resuspended in 1 mL of media, and the number of the cells for each plate was counted by using the Countess automated cell counter (Thermo Fisher Scientific).

cAMP measurement

HEK293 cells stably transfected with a CREB (cAMP response element-binding protein) reporter plasmid (pCRE-Luc plasmid; Agilent Technologies) were plated in a 24-well plate. When the cells reached around 70% of confluency, they were infected by using 300 μL of media without FBS plus 1 μL of AAV2-GPR78 or AAV2-GFP (titer 1×10^{12} particles/mL). The infections were made in triplicate and incubated for 24 h. After the incubation time, the relative light units of each well were read by using the Luc-Screen® System (Applied Biosystems), on the plate CulturPlate™-96 (Perkin Elmer) inside the machine Victor X2 multilabel reader (Perkin Elmer).

Statistical analysis

Data analysis was performed by using GraphPad Prism (GraphPad Software, San Diego, CA). Data are represented as mean ± standard error mean or mean ± standard deviation. Two-tailed unpaired *t*-test was employed to determine statistical significance between two groups. *p* Values less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

GPR78 expression decreases cell growth and reduces vector yield

G protein-coupled receptor 78 (GPR78) is a poorly characterized 7-transmembrane spanning G(s)-alpha-protein coupled receptor with constitutive cAMP activation activity.[10] One recent paper suggested that GPR78 could be a regulator for lung cancer metastasis[11] and association studies have suggested involvement in bipolar disorders and schizophrenia.[12] Our initial attempts to study this protein by transient transfection of 293T cells showed a dramatic decrease in cell growth rates (Fig. 1), and efforts to produce AAV vectors containing a CMV promoter and encoding GPR78 resulted in yields of less than 1 particle/producer HEK293T cell following a standard AAV triple transfection production protocol. In contrast, using a plasmid with an inverted-GPR78 sequence (noncoding orientation) yielded greater than 10,000 particles/producer cell (Table 1). The inverted-GPR78 plasmid has the GPR78 sequence in the opposite orientation. Due to the sequence being out of frame with the CMV promoter, no GPR78 protein is expressed when the inverted-GPR78 plasmid is used (lane 1; Fig. 2). These findings suggest that GPR78 is toxic in the standard AAV producer cell line.

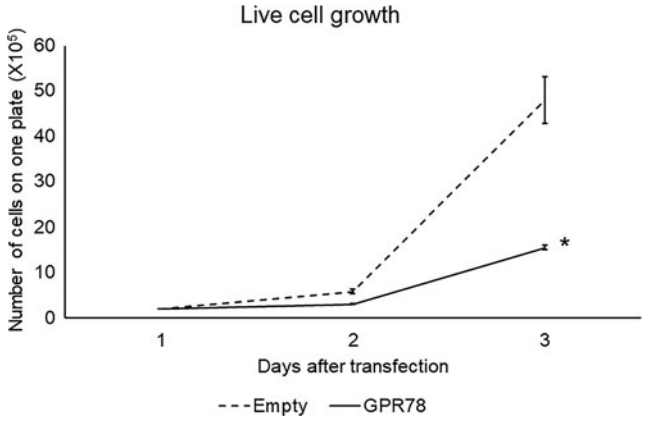

Figure 1. GPR78 expression decreased live cell growth *in vitro*. HEK293T cells transfected with GPR78 expression plasmid present a decreased live cell growth when compared with cells transfected with equal amounts of empty vector. $N=3$, $^*p<0.01$, unpaired Student's *t*-test (mean ± SD).

Cotransfection of GPR78 shRNA plasmid inhibits GPR78 expression

To reduce the toxic effect of GPR78 transgene expression, recombinant vectors were produced by cotransfecting shRNA containing plasmids targeting GPR78 to silence the cytotoxic effect during AAV packaging. Five

Table 1. *Yield of AAV-GPR78 vector after cotransfection with shRNA helper or different vector plasmids*

Plasmid	*Yield (vg/mL)*
AAV2-GPR78	2.0E+02 ± 1.0E+02
AAV2-GPR78 inverted	2.4E+12 ± 7.2E+11
AAV2-GPR78 plus shRNA	4.2E+12 ± 2.8E+12

The yield of vector was quantified from independent preparation of vector ($N=3$) after transfection of $\sim 5 \times 10^6$ cells and purification by isopycnic CsCl gradient fractionation. Virus yield was increased when GPR78 vector was cotransfected with shRNA plasmid (row 3) when compared with GPR78 vector transfection and packaging without shRNA plasmid (row 1). A positive control (row 2) was produced by the packaging of an inverted (noncoding orientation) GPR78 construct. This plasmid has the GPR78 sequence in the opposite orientation, resulting in the sequence being out of frame with the CMV promoter and not allowing the expression of the cytotoxic GPR78.

CMV, cytomegalovirus.

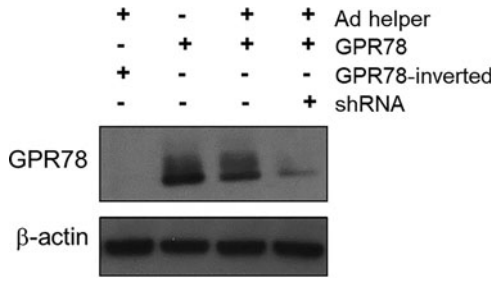

Figure 2. The shRNA (8931) promoted transgene silencing *in vitro* during virus production. HEK293T cells were transfected with the indicated GPR78 plasmid or an unrelated Ad helper plasmid with shRNA (lane 4) or without shRNA (lane 1–3). An inverted (noncoding orientation) GPR78 construct was used as negative control for the GPR78 expression.

different shRNA clones for the GPR78 gene were tested for transgene silencing (Sigma Aldrich; Materials and Methods section). Each shRNA plasmid was tested individually or as a pool for rescue of vector production and two of the five showed improvement in vector yield. The use of the shRNA 8931 led to the most consistent increase in vector production and was further characterized for development. Western blotting shows that, at 24 h post-transfection, 72% of GPR78 expression is inhibited by cotransfection of this shRNA (Fig. 2).

GPR78 silencing increases vector yield

To test the effect of cotransfection of the shRNA plasmid on AAV vector yield during a standard AAV triple transfection on AAV vector production, the shRNA plasmid or an empty carrier plasmid was mixed with the DNA and calcium phosphate precipitate and added to the 293T producer cells and the cell pellet was collected 48 h post-transfection. The packaging of AAV2-GPR78 in the absence of shRNA yielded less than 1 particle/producer cell (Table 1). In contrast, the vector particle yield with cotransfected shRNA 8931 and GPR78 significantly increased to >10,000 particles/producer cell. The vector yield was similar to that of the control vector containing the inverted GPR78 cDNA (Table 1).

To detect whether shRNA plasmid DNA was encapsidated inside AAV particles, a separate set of PCR reactions were prepared with shRNA plasmid specific primers. The level of shRNA plasmid DNA contamination in the final preparation was very low and below the standard limits of detection (100 copies/μL) (Table 1). This would represent $\sim$1 shRNA plasmid copy per 10E+12 particles.

AAV capsid and Rep protein expression increased with GPR78 shRNA cotransfection

AAV vector production requires the coordinated expression of both the AAV nonstructural proteins (Reps) and the three capsid proteins that form the icosahedral shell of the vector particle. To understand whether GPR78 was directly inhibiting AAV protein production during packaging, packaging cell lysates from cotransfection with or without shRNA 8931 were probed by Western blot for Rep or Cap expression. AAV capsid proteins (VP1, VP2, and VP3) are present in the virus prep made with the shRNA, whereas the preparation without the shRNA also shows reduced capsid protein expression (Fig. 3).

A second membrane was probed with an antibody that reacts with Rep proteins (Rep78, Rep 68, Rep 52, and Rep40). As originally reported by Grimm *et al.*, use of an MMTV promoter to drive expression of the P5 Rep proteins (Rep78 and Rep68) significantly reduces their expression compared with the P19 Rep proteins (Rep 52 and Rep 40).[13] Western blotting showed that GPR78 further reduced Rep protein expression with only a faint band for Rep52 in the absence of shRNA 8931. Cotransfection with shRNA 8931 did not completely inhibit the effect of GPR78 and resulted in recovery of some expression of the different Rep and Cap proteins. Although Rep78 was detected on a longer exposure, the most significant recovery was seen in the expression of VP3 and Rep52, supporting their important role in vector production. ShRNA 8931 cotransfection resulted in an 8- and 12-fold increase in VP3 and Rep52, respectively. Although the rationale for this effect on VP3 and Rep52 is not clear, it might be related to the timing of their synthesis in the lifecycle of

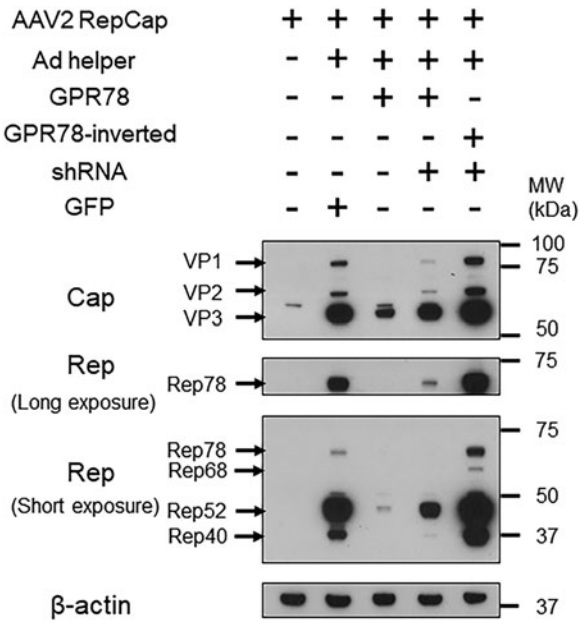

Figure 3. Characteristics of the AAV2 Rep and Cap protein production with shRNA cotransfection. AAV capsid and Rep protein expression were increased after cotransfection of shRNA plasmid with GPR78 vector (lane 4) when compared with GPR78 vector transfection and packaging without shRNA plasmid (lane 3). As a control, Rep and Cap proteins were detected in transfections of the packaging plasmids with a GFP (lane 2) and inverted (noncoding orientation) GPR78 construct (lane 5). A negative control of cell lysate (lane 1) is also included. Western blot detection of β-actin is included as a loading control.

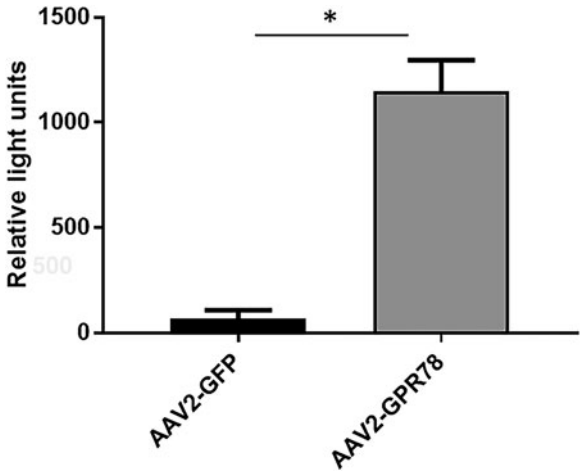

Figure 4. AAV2-GPR78 produced with shRNA cotransfection is active *in vitro*. Luciferase detection assay in HEK293 stably transfected with CREB reporter as a read-out for cAMP induction by GPR78. Cells were plated in 24-well plates and infected with 1 μL of AAV2-GPR78 (1×10^{12} particles/mL). As a control, cells were infected in the same way with AAV2-GFP. $N=3$, $^*p<0.001$, unpaired Student's *t*-test (mean ± SEM). CREB, cAMP response element-binding protein.

AAV, their stability, or requirements for protein synthesis and will require further investigation. Therefore, GPR78 appears to directly affect AAV protein synthesis, which is blocked by the addition of shRNA 8931.

AAV2-GPR78 produced with GPR78 shRNA cotransfection is active *in vitro*

GPR78 is an orphan receptor with a significant level of constitutive activity and its expression triggers intracellular cAMP signaling. To measure the biologic activity of the AAV2-GPR78 transduction, produced with the use of shRNA during packaging, cAMP activity was detected in HEK293 cells stably transfected with CREB luciferase reporter plasmid. The cells transduced with AAV2-GPR78 showed increased luminescence activity when compared with the AAV2-GFP control, supporting functional activity of the packaged vector (Fig. 4). This finding suggests minimal inhibitory effect of any shRNA plasmid used during production.

Final conclusions

Our data suggest that the cotransfection with the shRNA can allow the packaging of toxic or cytostatic proteins. The overexpression of a cytotoxic transgene during the vector production can trigger cellular stress and cell death, negatively impacting vector yield. Several other studies have investigated strategies to improve vector yield. The uniqueness of this study is the use of standard shRNA plasmids that allow for the increase of vector yields without extensive modification of the vector in an inexpensive but efficient manner. Although the exact mechanism is not clear, this work shows that vector transgene expression can have an unexpected impact on AAV protein expression that is necessary for packaging. Experimentation with other low-yield vectors will be required to confirm the generalized nature of this approach. In conclusion, the results of our study demonstrate that the incorporation of shRNA during AAV packaging can be an effective approach to block transgene effects and increase vector yield of toxic or cytostatic proteins.

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AUTHOR DISCLOSURE

No competing financial interests exist.

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