Sequence Integrity of Codon-Optimized RPGR Construct Maintained In Vitro and In Vivo

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INTRODUCTION

AGTC has developed an AAV-based gene therapy product (AAV2tYF-GRK1-hRPGRco) to treat X-linked retinitis pigmentosa (XLRP) caused by mutations in the retinitis pigmentosa GTPase regulator (RPGR) gene. There are multiple alternatively spliced transcripts of the RPGR gene resulting in expression of multiple RPGR protein isoforms. The constitutive transcript, RPGR1-19, is expressed in various tissue types, including the retina[1]. An alternatively spliced transcript containing exons 1 to 15 and a large part of intron 15 (RPGR-ORF15), found abundantly in the retina across different species[2], is predominantly expressed in the retina and localized to the connecting cilia of rod and cone photoreceptors. The retinal ORF15 isoform of RPGR contains a highly repetitive purine-rich region making the natural form unstable and prone to mutations[2,3] during cloning and vector production. We successfully designed and synthesized a codon-optimized human RPGR cDNA (hRPGRco) that was shown to be stable through multiple passages in plasmids, in recombinant herpes simplex virus and during AAV vector production[4].

Here, we further demonstrate the integrity of our hRPGRco drug product through evaluation of transgene and mRNA derived cDNA sequences isolated from treated XLPRA2 dog and Rd9 mouse retinal tissues respectively and evaluation of protein size, post translational glutamylation in rAAV2tYF-GRK1-hRPGRco vector-treated Rd9 mouse retinal tissues. We also examined the functionally crucial protein interaction between RPGR and its binding partner RPGRIP1, in plasmid-transfected HEK293 cells.

METHODS

- To test DNA stability in retinal tissues post subretinal injection, samples from rAAV2tYF-GRK1-RPGRco vector- treated and untreated XLPRA2 dogs were processed for DNA extraction using All-Prep DNA RNA Mini kit (Qiagen, Germantown, MD). AAV vector genome sequence, including the RPGRco cDNA and polyA signal sequence, was PCR amplified in three overlapping PCR reactions. Sanger sequencing of the purified PCR-products was performed at Eurofins Genomics (Huntsville, Alabama).
- For RNA sequencing of hRPGRco transcripts, retinal tissues were collected at 6 weeks post injection in vector-treated or untreated-control Rd9 mice and processed for RNA extraction using the RNeasy mini kit (Qiagen, Germantown, MD). Total tissue RNA templates were reverse transcribed followed by PCR amplification of RPGRco cDNA in three overlapping PCR reactions. Sanger sequencing of the purified PCR-products was subsequently performed at Eurofins Genomics (Huntsville, Alabama).
- RPGR protein expression was evaluated by western blot on whole cell lysates from pTR-RPGRco-transfected HEK293 cells, or on rAAV vector-injected Rd9 mouse retinal tissue lysates. Proteomics analysis was performed by liquid chromatography mass spectrometry (LC-MS/MS) on immunoprecipitated protein products from pTR-RPGRco-transfected HEK293 whole cell lysates.
  In addition, post-translational modification (glutamylation) was evaluated in *in vivo* and *in vitro* samples and protein-protein interaction of RPGR and binding partner RPGR-interacting protein 1 (RPGRIP1), was evaluated *in vitro*.

RESULTS

cDNA Sequencing

- Assembled sequencing reads of codon-optimized RPGR DNA and mRNA extracted from rAAV2tYF-GRK1-RPGRco vector treated HEK 293 cells and Rd9 mice retinal tissues were 100% identical to the reference sequence (Figure 1).

Figure 1: Sequence alignment of cDNA derived from mRNAs extracted from AAV-RPGRco transduced HEK 293 cells or Rd9 mouse retinal tissue to the reference codon optimized human RPGR sequence. Only the GA-rich ORF15 region is shown here.

Protein Analysis

- **Western blot**: Full-length RPGR-ORF15 has 1152 amino acids with a predicted relative molecular mass (Mr) of 127 kDa[5]. However, the C-terminal glutamic acid-rich region results in aberrant migration on SDS-PAGE and an apparent size of approximately 200 kDa[6] for full length RPGR-ORF15 observed in lysates from both plasmid-transfected HEK 293 cells and vector-treated Rd9 mouse retinas (Figure 2A, arrows). We also observed an RPGR-immunoreactive species at approximately 140 kDa (Figure 2A, arrow head).

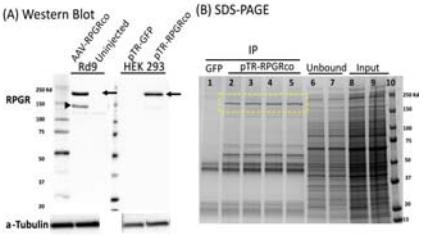

Figure 2: (A) Western blot analysis of lysates from rAAV2tYF-GRK1-RPGRco-treated or uninjected-control retinal tissues (left panel) and lysates from RGPRco-plasmid transfected or green fluorescent protein (GFP) control-transfected (right panel) HEK 293 cells, were probed with an RPGR-specific monoclonal antibody. (B) Coomassie-blue SDS-PAGE of immunoprecipitated RPGRco in plasmid-transfected HEK293 cells.

- **LC-MS/MS**: LC-MS/MS analysis of immunoprecipitated protein from pTR-CB-RPGRco transfected HEK293 cell lysates led to the correct identification of 76.6% of the amino acid residues in the hRPGRco reference sequence (Figure 3). The spatial arrangement of proteolytic sites in the ORF15 region resolved into peptides of undetectable size and thus prevented the identification of the remaining 24.4% of the sequence.

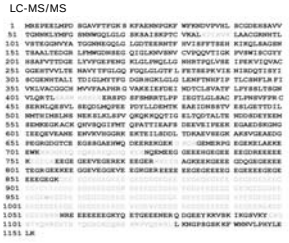

Figure 3: Amino acids in bold were detected by LC-MS/MS; non-bold residues could not be resolved by this method.

Proteomic analysis of the second immunoprecipitation product (~140Kda) by LC-MS/MS led to 68% coverage (data not shown) and conclusively showed that this protein species contains exons 1-14 and the C-terminal end of ORF-15 (LKNGPSGSKKFWNNVLPHYLELK).

RESULTS (CONTINUED)

- **Glutamylation**: Western blot analysis of vector treated retinal tissue lysates using GT-335 antibody (a monoclonal antibody specific for glutamylation, Figure 4), detected the expected 50kDa GT335-reactive tubulin and additional GT335-reactive bands that co-migrated with RPGR-ORF15 immune reactive protein species at ~200kDa and ~140kDa; suggesting that this functionally critical post-translational modification[7] is detected in both full length (~200kDa), or truncated (~140kDa) RPGRco.

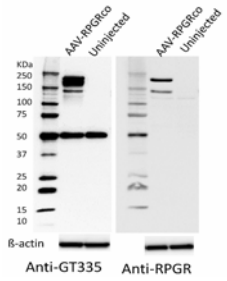

Figure 4: Glutamylation of vector-expressed RPGR protein. Western blots of tissue lysates extracted from rAAV2tYF-GRK1-RPGRco vector treated or control-treated Rd9 mouse retinas, were probed with anti-glutamylation antibody (anti-GT335) (left) or RPGR-specific monoclonal antibody (anti-RPGR) (right). β-Actin control also shown.

- **Protein Interaction**: It has been reported that RPGR mutations impair the interaction between RPGR and RPGRIP1 *in vivo*[8] and that this in turn disrupts normal RPGR function in photoreceptor cells. We assessed the interaction of RPGR-ORF15 and RPGRIP1 in HEK 293 cells transfected with the respective expression constructs, pTR-SmCBA-RPGRco or C-terminus flag-RPGRIP1. Co-immunoprecipitation studies in transfected HEK293 cells demonstrated that full length RPGR interacts with RPGRIP1 as expected (Figure 5). No interaction was detected when either of the binding partners, RPGRco or RPGRIP1, was co-expressed with a GFP construct

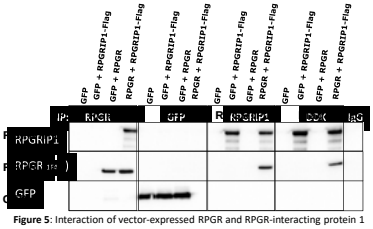

Figure 5: Interaction of vector-expressed RPGR and RPGR-interacting protein 1 (RPGRIP1). Whole cell lysates from HEK293 cells transfected with pTR-GRK1-GFP (GFP), pTR-SmCBA-RPGRco (RPGR) or both pTR-SmCBA-RPGRco (RPGR) and c-terminal flag-pCMV-RPGRIP1 were subjected to immunoprecipitation with anti-RPGR, anti-Flag, anti-RPGRIP1, anti-GFP, or IgG antibodies and subsequently probed with anti-RPGRIP1, anti-RPGR to assess the association of RPGRco and RPGRIP1. Anti-GFP immunoblots assessed non-specific interactions in GFP and RPGR or GFP and RPGRIP1 co-transfections

CONCLUSIONS

Codon-optimized hRPGR cDNA in the rAAV2tYF-GRK1-hRPGRco vector maintained its integrity during transduction and transcription in vitro and in vivo. Vector-expressed RPGR protein was both glutamylated and able to bind to its partner RPGRIP1, each being required for RPGR to fulfill its functional role in retinal cilium.

REFERENCES

1. He S, Parapuram SK, Hurd TW, et al. Retinitis Pigmentosa GTPase Regulator (RPGR) protein isoforms in mammalian retina: insights into X-linked Retinitis Pigmentosa and associated ciliopathies. Vision Res. 2008;48(3):366-376
2. Vervoort R, Lennon A, Bird AC, et al. Mutational hot spot within a new RPGR exon in X-linked retinitis pigmentosa. Nat Genet. 2000;25(4):462-466
3. Wright AF, Shu X. Focus on Molecules: RPGR. Exp Eye Res. 2007;85(1):1-2.
4. Beltran WA, Cideciyan AV, Boye SE, et al. Optimization of Retinal Gene Therapy for X-Linked Retinitis Pigmentosa Due to RPGR Mutations. Mol Ther. 2017;25(8):1866-1880.
5. Megaw RD, Soares DC, Wright AF. RPGR: Its role in photoreceptor physiology, human disease, and future therapies. *Exp Eye Res.* 2015;138:32-41.
6. Pawlyk BS, Bulgakov OV, Sun X, et al. Photoreceptor rescue by an abbreviated human RPGR gene in a murine model of X-linked retinitis pigmentosa. *Gene Ther.* 2016;23(2):196-204.
7. Sun X, Park JH, Gumerson J, et al. Loss of RPGR glutamylation underlies the pathogenic mechanism of retinal dystrophy caused by TTLL5 mutations. Proc Natl Acad Sci U S A. 2016;113(21):E2925-2934
8. Roepman R, Bernoud-Hubac N, Schick DE, et al. The retinitis pigmentosa GTPase regulator (RPGR) interacts with novel transport-like proteins in the outer segments of rod photoreceptors. Hum Mol Genet. 2000;9(14):2095-2105

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