mutation and correction of SMN2 aberrant splicing, by exploiting the non-homologous end-joining (NHEJ) pathway. Plasmids encoding Cas9-GFP under the control of CMV promoter, and selected gRNAs downstream to the Pol-III U6 promoter (Addgene) were transfected in HEK-293T cell line and in immortalized myoblasts derived from either healthy donors or SMA patients. Transfection efficiency was estimated as percentage of GFP-expressing cells (20-50% and 1-10%, respectively) and nuclease activity detected by Surveyor assay and target site sequencing. In particular, in SMA patient-derived myoblasts we detected mutations (indels) at the level of the induced DNA double-strand break at ~30% frequency. Levels of SMN restoration will be investigated by qPCR of the different species of SMN transcripts and by western blotting of SMN protein. The goal of this study is to provide an in vitro proof of principle of effective gene correction in SMA patient-derived cells. In the context of a multisystemic, complicated disease such as SMA, targeted genome editing strategy could represent an additional therapeutic tool.

132. Gene Therapy With Self-Complementary Recombinant Adeno-Associated Virus in Models of Autosomal Dominant Retinitis Pigmentosa Cause by RHO Mutations

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Purpose

Retinitis pigmentosa is the leading hereditary cause of blindness with 30-40% of cases attributable to autosomal dominant retinitis pigmentosa (ADRP). ADRP arises from mutations in at least 24 known genes with 30% arising in the rhodopsin gene (RHO). Given the large heterogeneity of mutations in RHO leading to ADRP, we propose knocking down of endogenous RHO and replacing it with a "hardened" copy, or a RHO with nucleotide changes that preserve the amino acid sequence but decrease the efficiency of knock-down. Here we report the use of a scAAV serotype 8 (Y733F) to express a hardened human rhodopsin (hRHO) under the control of the human opsin proximal promoter (HOPS) and an H1 promoter driven shRNA.

Methods

Four different knock-down methods were tested, ribozyme (Rz) 407 and Rz525, miRNA 301 and shRNA 301 against both the wild-type and hardened RHO target regions. The transfections were done in HEK293 cells (n=6) with (1) the target plasmid psiCheck2 dual luciferase containing RHO target region, (2) plasmid expressing the shRNA, miRNA or ribozyme against the target region and (3) a control miRNA. The reduction in expression of luciferase was measured at 24 and 48 hours post transfection.

We are using adeno-associated virus vectors to deliver these RNA knockdown agents to mouse models of ADRP: Rho I307N and human RHO transgenic T17M and P23H. Mice were injected with AAV-HOP-hRHO and knockdown agents at postnatal day 5. I307N Rho mice were created through the use of N-ethyl-N-nitrosourea (Budzynski et al. JBC 2010). The I307N mouse model exhibits very slow degeneration under ambient light but is reduced in visual response to light by 50% in one week post exposure to 10,000 lux. Intravitreal injections with one of the two constructs, hRHO+shRNA301, or hRHO+shRNA750 in one eye and AAV-HOPS-mCherry or sham injection in the other. The mice were followed using electroretinogram and optical coherence tomography.

Results

The knock-down results show shRNA301 and ribozyme 525 to cause the largest reduction of RHO mRNA. At one month post injection there was statistically significant difference between T17M RHO eyes injected with AAV-hRHO-shRNA750 and the sham injected eyes.

Conclusions

We have generated a series of combination RNA knockdown and replacement AAV vectors that may be useful for the treatment of ADRP. At early time points, our tests of these specific vectors have not been conclusive. The injected mice will be followed for longer intervals and additional mice will be added to the study to determine if the difference in visual function of the experimentally treated eyes versus the control is statistically significant.

133. Use of 2As To Control Protein Subcellular Localization

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A substantial proportion (~39%) of all human proteins are either secreted from the cell, located within the lumen/ membranes of cytoplasmic vesicular structures, or, are plasma membrane proteins. Given that such high proportion of proteins are initially translocated into the endoplasmic reticulum (ER), many therapeutic strategies rely on the ability to co-express multiple proteins – some, or all of which, might be targeted to such sites. This directly applies to in vivo gene therapy strategies, or, when therapeutic proteins may need to be co-expressed with selectable markers (e.g. ex vivo gene therapies).

As was shown before, Picornavirus 2A (foot-and-mouth disease virus 2A; F2A) and '2A-like' sequences are powerful tools that allow multiple proteins to be translated and co-expressed from a single transcript mRNA under the control of only one promoter. When 2A is positioned between sequences encoding two, or more, genes, it mediates a co-translational 'cleavage' at its own C-terminus. A major problem with co-expression of certain proteins targeted to, or transiting through, the ER is that the 'cleavage' activity of short F2As can be greatly inhibited by sequences immediately upstream leading to aberrant sub-cellular localisation of some proteins.

We have also discovered a number of active cellular 2A-like sequences, associated with non-long terminal repeat (non-LTR) retrotransposons, but also with structural and metabolic proteins: ankyrin repeats, sodium dependent neutral amino acid transporters, and NOD-like receptor (NLR) proteins. Examination of the surrounding protein and gene structure revealed that in the majority of cases these 2A sequences occurred as N-terminal features. Interestingly, using SignalP, many of these novel 2As scored highly as N-terminal signal peptides.

Here, we present our latest findings on 2A sequences. A series of test proteins (eGFP and mCherry) were expressed i) followed by 'hybrid' 'self-cleaving' F2A sequences (with different upstream contexts) or ii) downstream of a putative signal 2A. We demonstrate that inhibition of F2A-mediated cleavage in shorter sequences can be overcome by introduction of mutations upstream of 2A changing the context of the sequence between the C-terminus of the upstream protein and 2A sequence. In the case of N-terminal - cellular - (NLR) 2As, 'uncleaved' 2A indeed can act as a signal peptide. If 2A does not 'cleave', it directs a proportion of the newly synthesised reporter protein to the exocytic pathway: if 2A 'cleaves', the protein downstream is localised to the cytoplasm. This type of 2A mediates, therefore, a newly discovered form of dual protein targeting.