CRISPR-CAS9 recombinant ADENO-ASSOCIATED VIRUS MEDIATED CYSTIC FIBROSIS GENE THERAPY

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Abstract

Cystic Fibrosis (CF) is a genetic disease caused by mutations in the cystic fibrosis transmembrane regulator (CFTR) gene. This research focuses on a 3-base pair deletion, F508d, which is a deletion of the amino acid phenylalanine. The F508d is the most common mutation among CF patients, with 90% possessing it. This study utilizes a CRISPR-Cas9 system and recombinant Adeno-Associated Virus (rAAV) to facilitate gene therapy for the F508 deletion. Using 5 different sgRNAs, 5 plasmid constructs were created and their correction efficiencies were tested.

A dual reporter, mRFP-508D-eGFP-CFTR, was created and used to test the homology-directed end repair efficiency of each sgRNA, 2-5, via human kidney cells, HEK293. The cells were then imaged using fluorescence microscopy to detect mRFP and eGFP. Fluorescing mRFP indicates successful transfection, while eGFP indicates a successful correction of the F508d. The cells were imaged with both RFP and GFP overlays to visualize which cells expressed both fluorescent proteins. The images were then analyzed to choose the most efficient sgRNA for this project. sgRNA5 showed the highest correction efficiency. Looking forward, the selected sgRNA, sgRNA5, will be transformed into rAAV-9 in order to be administered in mouse trials. These results show promise for potential rAAV-mediated gene therapy in human cystic fibrosis patients.

Methods

CRISPR-Cas9 System Design

PAM sequences were identified in proximity to F508d. CjCas9 was chosen to fit inside AAV. Crispor was utilized to select sgRNA sequences.

Molecular Cloning of Plasmids Containing CRISPR Construct and sgRNAs 2-5

Blunt ended cloning was utilized. The backbone and insert DNA were then ligated before transformation.

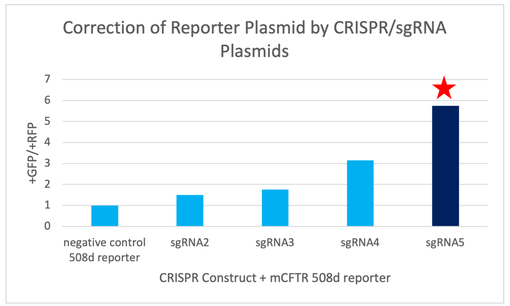
Transformation via Electroporation

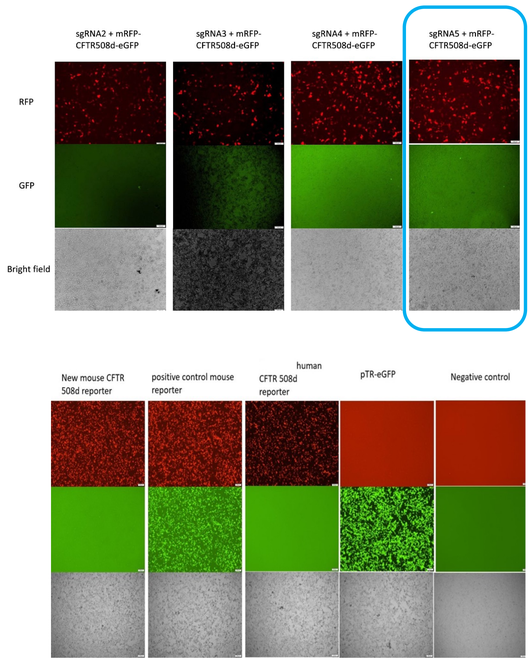
After transformation into E. coli cells, the cells were left to grow overnight. Colonies were excised and plasmids were isolated. A screening enzyme identified the correct clones.

Transfection and Fluorescent Microscopy

Plasmids were transfected into HEK293 cells. Positive and negative controls were created. They were then imaged for RFP and GFP.

Results





Conclusions

sgRNA5 showed the highest level of non-homologous end joining. sgRNA5 was successful in approximately 6 cells. The ultimate goal is to use this system to infiltrate epithelial lung stem cells, which would replicate with the corrected DNA inserted.

Concurrent research was conducted to find the most efficient AAV capsid for lung epithelial cells, which resulted in AAV-9. The next steps will be to package the sgRNA5 construct into AAV-9 and perform in vitro mouse experiments.

As the F508d is the most common mutation among CF patients, this study will work to inform future gene therapy approaches for a large number of people.

References

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CRISPR-Cas9

- CRISPR-Cas9 gene editing was chosen as an alternative to directly introducing CFTR protein into mutated cells. CRISPR-Cas9 would cause a permanent correction of the mutation in impacted cells.
- There are 2 components of CRISPR-Cas9: an sgRNA and a Cas nuclease. The sgRNA is specific to the DNA sequence of interest and requires a downstream PAM sequence. The sgRNA then guides the Cas nuclease to the DNA sequence where it makes a double stranded break.
- This break can then be repaired via non-homologous end joining (NHEJ) or homology-directed repair (HDR). HDR is the goal for this study.
- CjCas9 was chosen due to its compact size, which allows it to fit within AAV.
- 5 sgRNA sequences were chosen to test their HDR correction efficiencies.