Generation of an Alpha-1 Antitrypsin Knockout Mouse Model Using CRISPR/Cas9 System

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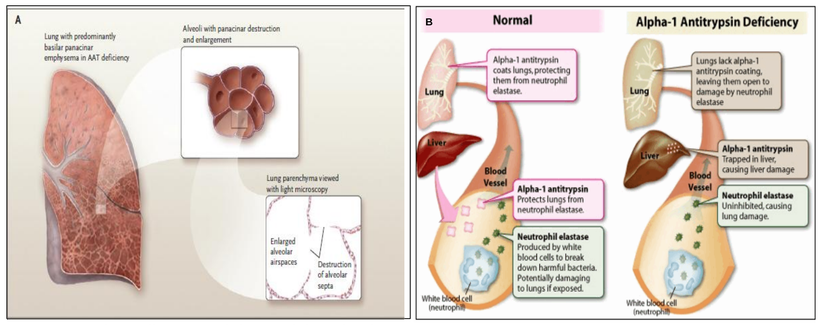
Background

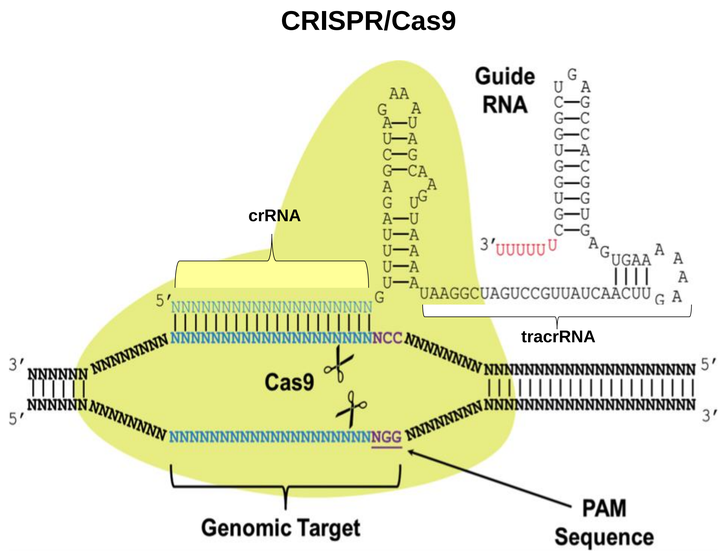
Alpha-1 antitrypsin (AAT) deficiency is a common autosomal co-dominant genetic disorder. This condition affects 1:2500 individuals of European ancestry, leading to the development of lung and liver disease. Within North American and Northern European populations, an estimated 4% of individuals are carriers of deficient genes for AAT. AAT deficiency presents with an emphysema-like phenotype in the lungs of older subjects. AAT deficient subjects also suffer from liver disease of varying severity; however, lung disease is the principle cause of death. Belonging to the serpin family, AAT is a protease inhibitor predominantly synthesized in the liver. Upon secretion into the blood stream, AAT is directed towards the lungs where it inactivates excess neutrophil elastase, thereby preventing damage to the alveoli. Mutations of the SerpinA gene can lead to reduced serum levels of AAT and decreased protein functionality, allowing for unrestricted elastin breakdown, pulmonary inflammation and eventual emphysema. Currently, an animal model simulating the lung condition does not exist, which severely limits the development of innovative therapeutics.



www.sandysandhaus.com/About_Alpha-1.html http://flipper.diff.org/app/items/2843

Figure 1: (A) pathology of tissue with AAT deficiency from an alveolar perspective (B) normal and pathophysiological mechanisms of AAT.

Genomic Editing to Create Knockout



http://2013.igem.org/Team:Paris_Bettencourt/Project/Detect

Figure 2: shows the structure of the CRISPR/Cas system associated with a target sequence of genomic DNA. Diagram includes components of a single guide RNA (sgRNA) including tracr and crRNA, Cas proteins, and a required down stream protospacer adjacent motif (PAM) sequence.

Using the innate adaptive immunity of the CRISPR system, we targeted specific loci within exon 2 of the SerpinA gene to effectively disrupt and silence the gene caused by a DNA repair mechanism known as Non-Homologous End Joining (NHEJ). With 5 copies of the SerpinA gene in the mouse genome, the CRISPR/Cas9 system offered a more effective method to achieve knockout of each individual gene.

Experimental Design

1. Design gRNA's that target coding regions within each SerpinA gene (5 copies per chromosome)
2. Test efficiency of gRNA constructs using a Single Strand Annealing Assay
3. Design primers to screen for AAT mutations (In/Del) using PCR assay
4. *In vitro:* Screen embryonic stem cells co-transfected with gRNA and CRISPR/Cas system
   In vivo: Screen microinjected transgenic zygotes (gRNA: 20ng/uL, Cas9 mRNA:50ng/uL)
5. Perform mouse specific AAT ELISA to validate knockout candidates
6. Sequence bands from PCR products of suspected knockout embryonic stem (ES) cells and transgenic mice

5 Copies of the SerpinA Gene

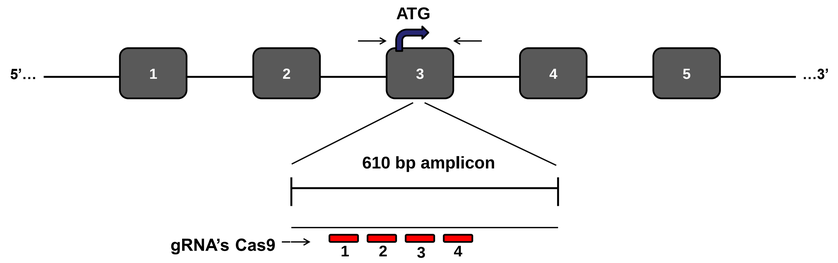


Figure 3: 5 sequential copies of the SerpinA gene exist in the mouse genome, 4 gRNA's were designed to cause In/Dels after NHEJ occurs. The figure shows the 610bp amplicon that results from amplifying wild type genomic DNA. From PCR amplification of samples, various deletion patterns were observed due to differing gRNA/CRISPR/Cas9 activity.

Single Strand Annealing Assay

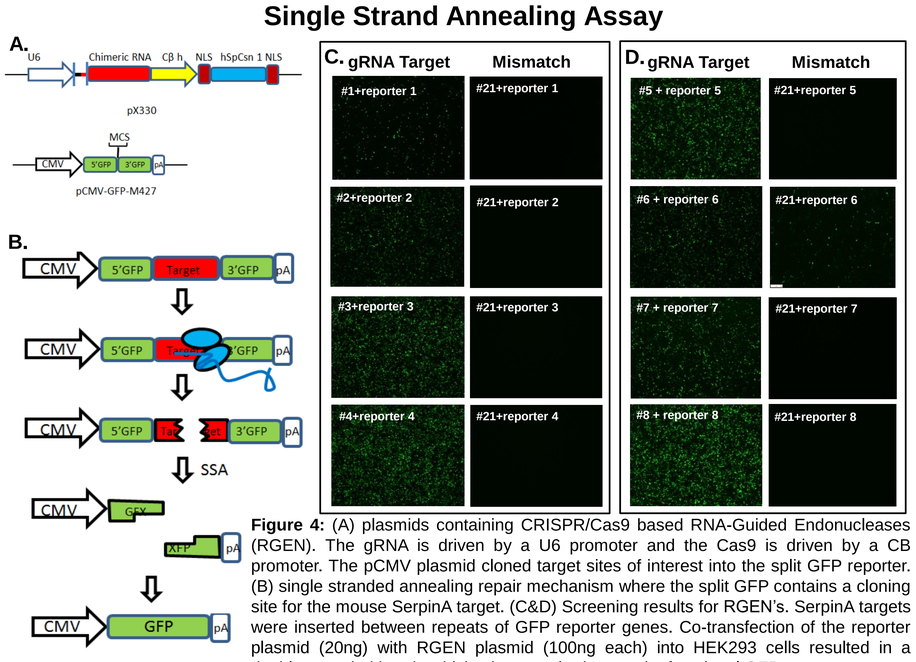
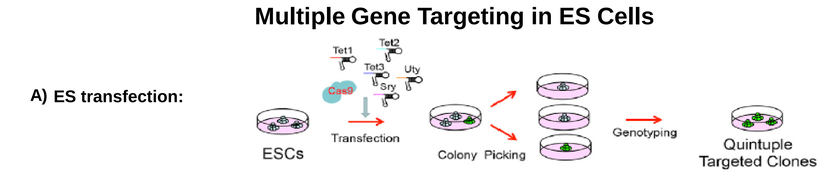


Figure 4: (A) plasmids containing CRISPR/Cas9 based RNA-Guided Endonucleases (RGEN). The gRNA is driven by a U6 promoter and the Cas9 is driven by a CB promoter. The pCMV plasmid cloned target sites of interest into the split GFP reporter. (B) single stranded annealing repair mechanism where the split GFP contains a cloning site for the mouse SerpinA target. (C&D) Screening results for RGEN's. SerpinA targets were inserted between repeats of GFP reporter genes. Co-transfection of the reporter plasmid (20ng) with RGEN plasmid (100ng each) into HEK293 cells resulted in a double-stranded break, which when repaired created a functional GFP gene.

Multiple Gene Targeting in ES Cells



Targeted Mutation (Insertion/Deletion)

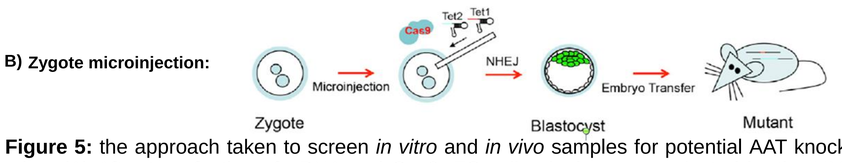


Figure 5: the approach taken to screen *in vitro* and *in vivo* samples for potential AAT knockout mice. (A) After transfection of gRNA and Cas9, ES cell colonies were selected by the UMMS transgenic core and later screened for AAT mutations. (B) gRNA and Cas9 for mouse SerpinA knockout were microinjected into pronucleus and/or cytoplasm of mouse embryos. Surviving injected embryos were implanted into pseudo-pregnant hosts for delivery.

Results

Amplicons Showing Deletion Patterns

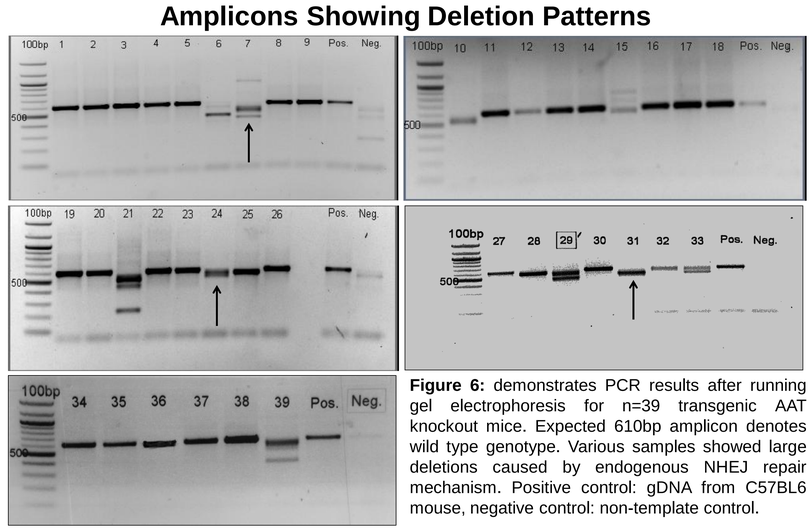
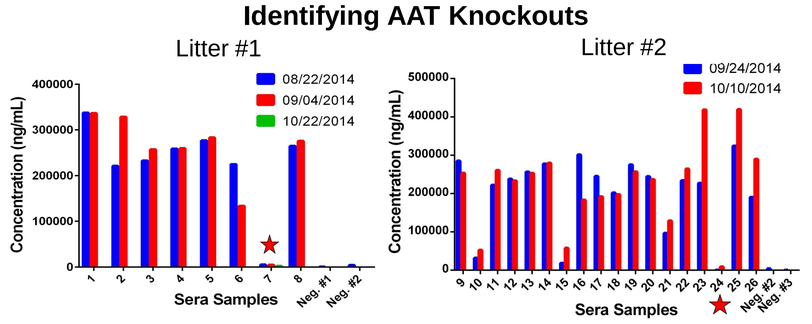


Figure 6: demonstrates PCR results after running gel electrophoresis for n=39 transgenic AAT knockout mice. Expected 610bp amplicon denotes wild type genotype. Various samples showed large deletions caused by endogenous NHEJ repair mechanism. Positive control: gDNA from C57BL6 mouse, negative control: non-template control.

Identifying AAT Knockouts



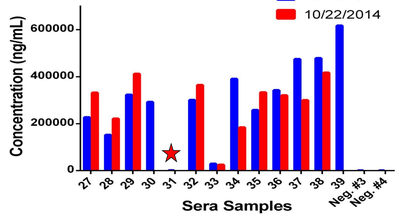
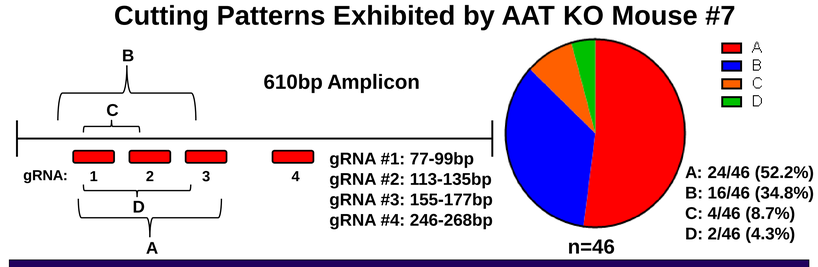


Figure 7: results from AAT mouse specific ELISA kit for transgenic mice of litters #1, 2, 3. Negative control: human serum.

Figure 8: shows various cutting patterns (including statistics) contained within the larger band of transgenic mouse #7 after PCR amplification, extraction, cloning and sequencing.

Cutting Patterns Exhibited by AAT KO Mouse #7



Conclusions

- From screening results, AAT knockout mice were created using CRISPR/Cas9 genome editing technology (mice #: 7, 24, 31).
- Additionally, several other mice exhibit considerable AAT knockdown (mice #: 10, 15, 33).

Future Plans

We will exacerbate the lung condition and characterize this model by performing necessary pathological and histological analysis of affected tissues. With significant clinical relevance, this developmental model presents the opportunity to create novel therapeutics that will help to treat future patients affected by AAT deficiency.

Funding

R24 Grant from the National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) to C.M.

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