Simultaneous disruption of five Serpina1 genes in mice using CRISPR/Cas9 to generate the first model of alpha-one antitrypsin deficiency, the leading cause of genetic COPD.

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Background

Alpha-one 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 mutant alleles. AAT deficiency presents with an emphysema-like phenotype in the lungs of older subjects. AAT deficient subjects can also suffer from liver disease of varying severity; however, lung disease is the principle cause of death. AAT is a protease inhibitor predominantly synthesized in the liver that belongs to the serpin family. Upon secretion into the blood stream, AAT enters the lungs where it inactivates excess neutrophil elastase, thereby preventing damage to the alveoli. Mutations of the Serpina1 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 therapeutics. This is due to the higher genomic complexity of mice compared to humans. Indeed due to amplification events, C57BL/6 mice have five genes that are homologous to human SERPINA1.

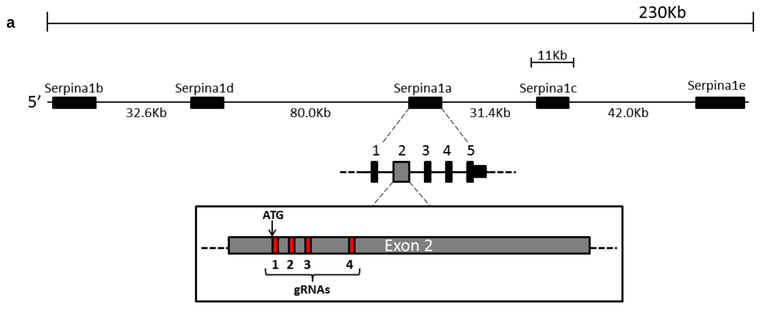
Study design

We generated a quintuple gene knockout using CRISPR/Cas9 and demonstrated absence of hepatic and circulatory AAT protein as well as a reduced capability to inactivate neutrophil elastase.

<u>Hypothesis</u>: Simultaneous disruption of the five Serpina1 genes in mouse via genome editing will recapitulate the respiratory phenotype of alpha-one antitrypsin deficient patients.

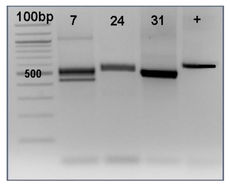
Generation of AAT knockouts

Guide RNAs (gRNAs) were designed within a region conserved in all five genes, in exon 2 (Fig.1a).
Zygotes were microinjected with Cas9 mRNA and a mix of the gRNAs prior to implantation in pseudo-pregnant females, and 39 pups were obtained (Fig.1b).
The genes were amplified by PCR (Fig.1c) and sequenced. Out of 39 candidates, 3 presented deletions in the genes: two males (#7 and # 24) and one female (#31). Deletion patterns include gRNA-gRNA2 (founder #7), gRNA1-gRNA3 (founder #7) and gRNA1-gRNA4 (founder #7). Sequencing data for founder #7 are shown in Fig.1d.



b

Stage	N	%
Injected oocytes	461	
Implanted oocytes	304	66%
Total pups	45	15%
Pups surviving passed newborn	39	87%
Knockouts	3	8%



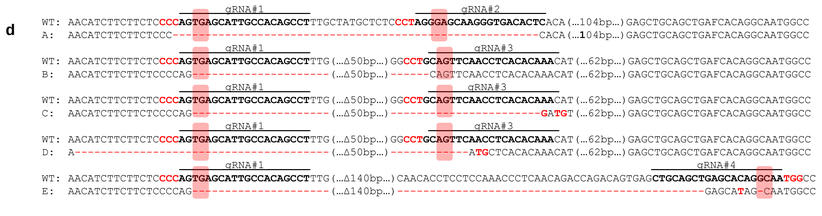


Figure 1. Strategy, generation and screening of quintuple Serpina1 knockouts using CRISPR genome editing.

Characterization of the knockouts

The candidates were further screened by quantification of serum murine AAT levels. Confirming the genotyping data, the three founders present undetectable or background levels of serum AAT (Fig.2).

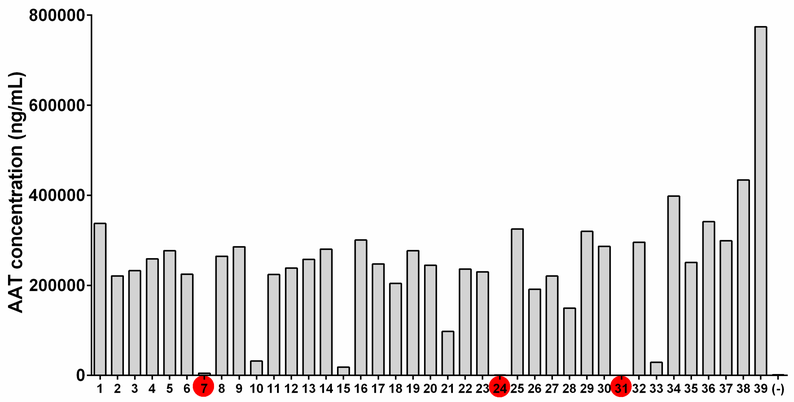


Figure 2. Identification of quintuple knockouts by serum AAT quantification.

AAT is produced and secreted primarily by hepatocytes and normal liver presents high AAT levels (Fig.3a). FFPE liver was sectioned at 5 µm and incubated with the same mouse-specific anti-alpha-one antitrypsin antibody than used previously, and AAT protein was undetectable in liver samples from the knockouts (Fig.3b).

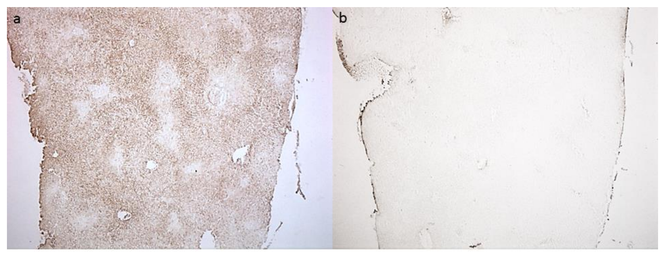


Figure 3. AAT protein is undetectable in livers from AAT knockouts.

Additionally, western blotting was performed and serum samples from the three founders were incubated with the same mouse-specific anti-alpha-one antitrypsin antibody than used previously (in green), and an anti-albumin antibody (in red). Again, AAT protein was undetectable in serum samples from the founders (Fig.4).

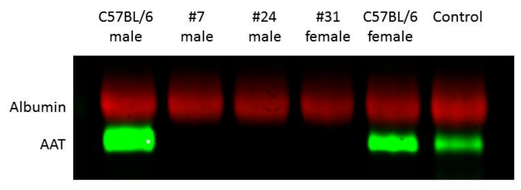


Figure 4. AAT protein is undetectable in serum from AAT knockouts.

One of the endogenous roles of AAT is to inactivate neutrophil elastase. Serum from the founders showed a reduced anti-elastase activity *in vitro* when compared to serum from wild-type C57BL/6 mice (Fig.5).

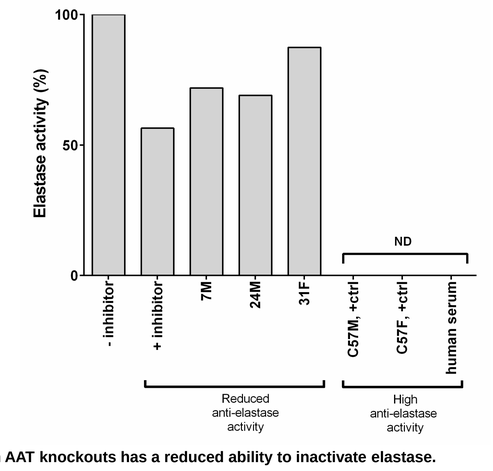
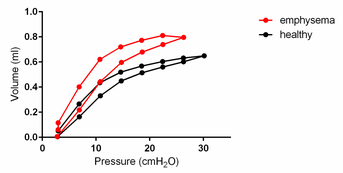


Figure 5. Serum from AAT knockouts has a reduced ability to inactivate elastase.

Respiratory phenotype

Having established the absence of wild-type AAT protein in liver and in serum, as well as a reduced ability to inactivate elastase, we next sought to characterize the respiratory phenotype of the knockouts, which we hypothesized, should recapitulate that of AAT deficient patients.

Clinically, those patients present with emphysema, and are characterized by increased compliance and decreased resistance. As depicted below, the typical pressure-volume (PV) loop will be shifted up and to the left in emphysema.



Wild-type C57BL/6 and AAT knockouts were administered two weekly intratracheal doses of lipopolysaccharide (LPS) to mimic an airway infection and induce neutrophils recruitment to the lungs and neutrophil elastase secretion. Two weeks after the first dose, respiratory physiology was assessed on a Flexivent and lungs were harvested.

As hypothesized, the PV loop from the knockout group (n=12) is shifted up (Fig.6a). This corresponds to a decreased elastance (Fig.6b) and increased quasi-static compliance (Fig.6c), as well as decreased resistance (Fig.6d).
Finally, analysis of the morphometry performed on a subset of the mice (n=4) revealed an increase in mean linear intercept, a measure of volume/surface area (Fig.6e). Total volume and total surface area were also increased (data not shown).

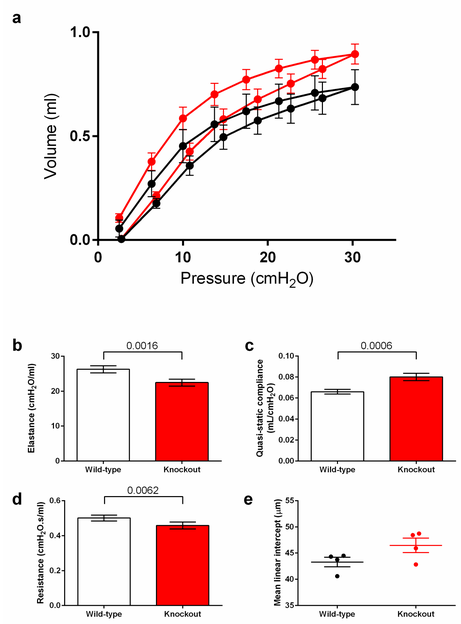


Figure 6. The respiratory phenotype of the AAT knockouts recapitulates that of AAT deficiency patients.

Conclusion

In conclusion, this novel mouse model is the first model of alpha-one antitrypsin deficiency lung disease, and by extension of genetic emphysema. This model may be used to evaluate new therapeutics for alpha-one antitrypsin deficiency.
Future work will include further challenges with porcine pancreatic elastase and cigarette smoke, characterization of the respiratory phenotype as the mice age, as well as genomic characterization.

Acknowledgements

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