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Intra-tracheal delivery of AAV6 vectors results in sustained transduction in murine lungs without genomic integration

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ABSTRACT

Despite the progress made in AAV-based gene therapy targeting different organ systems, lung-targeted gene therapy using AAV vectors has not been effective, mostly due to the poor transduction and un-sustained gene expression in airway epithelium. Furthermore, concerns over possible harmful insertional mutagenesis seen in other cell types, particularly hepatocytes, raised a question about AAV safety. In this study, we evaluate the long-term persistence of this vector in mouse lungs and any possible harmful integration of these vectors into the host genome. AAV6 vectors expressing reporter gene (firefly luciferase) were delivered to the lungs of C57BL/6 mice through intra-tracheal intubation. Despite the large variation among individual animals, most animals had high and sustained luciferase activity with a peak from 2 to 3 weeks post-transduction before a significant decline between 15 and 19 weeks post-transduction. More importantly, even after its decline, most animals maintained detectable luciferase expression for 150 days or more, which was confirmed by post-necropsy qPCR analysis of luciferase gene expression. At the termination point of experiments, an average of one copy of AAV expression cassette per mouse genome was detected. We also found that partial overlaps between the AAV6 expression cassette and the mouse genome were distributed broadly with no apparent systematic preference in any mouse chromosomal map location. In summary, our data suggest that AAV6 mediated long-term gene expression in the lungs with no evidence of genomic integration, and thus, any insertional mutagenesis.

1. Introduction

Lung-directed gene therapy is a potentially promising therapeutic option for genetically determined lung diseases such as Cystic Fibrosis (CF), α1-antitrypsin (α1-AT) deficiency, and for disease modification of non-genetically detriment pulmonary disorders such as chronic obstructive pulmonary disease (COPD), asthma and lung cancer. However, numerous previous attempts at preclinical and clinical gene therapy trials have failed to identify a reliable vector platform for gene transfer to overcome the lung's anatomical and natural defenses (van Haasteren et al., 2018; Guggino and Cebotaru, 2017; Sondhi et al., 2017; Alapati and Morrisey, 2017).

Non-viral vectors, in the form of chemically modified liposomes or encapsulated plasmid DNA or mRNA, can overcome the limitations of viral vectors such as vector size and immunogenicity. However, the relatively low efficiency of gene transfer and the transient therapeutic effect hinders the use of these vectors in clinical trials (Alton et al., 2015; Ramamoorth and Narvekar, 2015; Hill et al., 2016). On the other hand, viral vectors such as adenovirus or lentivirus can be more effective vehicles of gene transfer, but safety concerns related to their high immunogenicity, cytotoxicity and the high potential of harmful insertional mutagenesis in the host genome remain significant drawbacks for their use as well (Wold and Toth, 2013; Alton et al., 2017).

Adeno-associated viruses (AAV) are non-pathogenic parvovirus. Several different serotypes of AAV have been widely explored as a potentially safe and effective clinical-stage vector for gene therapy in a broad spectrum of genetic diseases ranging from Leber's congenital amaurosis to hemophilia B and muscular dystrophy (High and Aubourg, 2011; Mendell et al., 2012; Jacobson et al., 2015). Most recently, AAV-based treatment of rare eye disease and spinal muscular atrophy (SMA) in children became two novel gene therapy treatments ever approved by the US FDA (Smalley, 2017; Keeler and Flotte, 2019). However, similar successes of the AAV-based gene therapy of lung diseases, such as CF, α1-AT or COPD could not be achieved. One reason for this is the inability of previously tested AAV vector serotypes to transfer clinically sufficient levels of the therapeutic gene into the airway epithelial cells

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(AEC) (Kim et al., 2016; Griesenbach et al., 2009; Cooney et al., 2018). Several other barriers need to be overcome for more successful lung gene therapy, such as vector penetration in mucus plugs, high turnover of AEC, and minimization of host immune response. Therefore, identification and development of AAV serotype that has the highest affinity to human AEC (Guggino and Cebotaru, 2017) and the most prolonged duration of gene expression become essential to overcome some of these barriers.

Another concern that remains unresolved in lung targeted AAV-based gene therapies, which holds back any clinical use of AAV vectors, stems from the fact that a large vector dosage is usually needed to target major human organs such as the lungs. A large vector dose can lead to the development of inflammation in the target organ and consequent cell damage due to the exaggerated host immune response against the transduced cells (Gernoux et al., 2017). In addition, the recent discovery of AAV genome integration in human clinical samples of hepatocellular carcinoma (HCC) has stirred discussions regarding the safety of AAV vectors for application in gene replacement therapy (Buning and Schmidt, 2015; Nault et al., 2015). In this study, we demonstrate the possibility of using the AAV6 serotype specifically as an effective and safe vector candidate for lung-directed gene therapy, and provide experimental data showing sufficient and sustained gene transduction in murine lungs without evidence of potentially harmful genomic integration.

2. Results

We have previously evaluated several AAV serotypes (AAV1, AAV2, AAV5, and AAV6) and different capsid-modification (substitution of critical tyrosine, serine, and threonine residues Y705F, Y731F, S663V, and T492V) for their effectiveness in transducing cultured airway epithelial cells (data not shown) (Colon-Cortes et al., 2016). Therefore, only AAV6 was used in this study.

The lungs of five C56Bl6 mice were transduced with AAV6 vector and two other mice were mock treated with PBS. Despite the large variation between animals, all of the five AAV6 transduced animals maintained some level of luciferase expression during the entire duration of the experiment (Fig. 1.a and b), with expression lasting more than 150 days (Fig. 2.a). Luciferase activity was higher soon after infection (peak of 1.3 × 10^5 p/s/cm^2 between weeks 2 and 3). However, the intensity of luciferase activity started to gradually decline, and dropped significantly to approximately one-tenth (1.15 × 10^4 p/s/cm^2) of the maximum expression between weeks 15 and 19 (Fig. 2b).

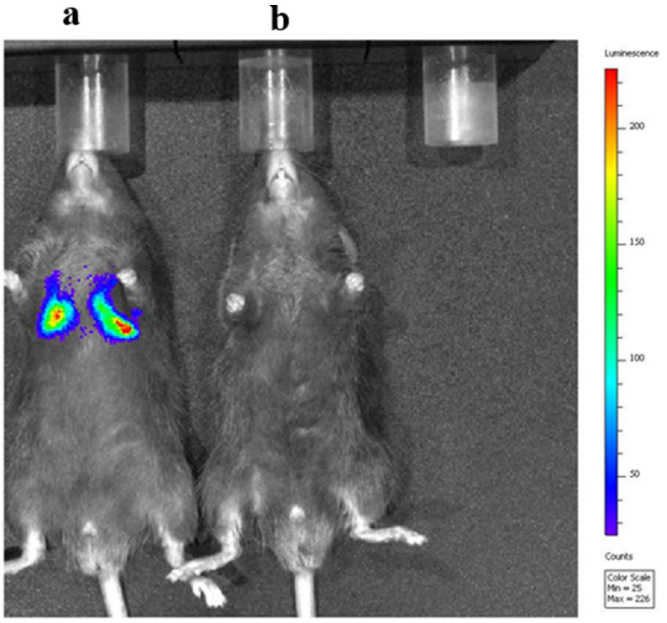

Fig. 1. AAV6 vectors umpire gene expression in mouse lungs. A-B. Representative image of AAV6-fLuc expression in the lungs of transduced C57BL6 mouse compared to non-transduced control.

Despite the drastic but late drop in expression in the transduced animals, luciferase activity remained well above that of the non-transduced control animals (Fig. 2.a). Persistent gene expression in the transduced animals was also confirmed by relative gene expression analysis of the total RNA extracted from lung samples post-necropsy, which was on average 17-fold higher than the mock controls (Fig. 3.a).

Post-necropsy, the mice genomic DNA (gDNA) was extracted, and qPCR were used to identify AAV genome copies present in the mice gDNA using AAV expression cassette specific primers. On average, there were 1,6 × 10 (Ramamoorth and Narvekar, 2015) -4.5 × 10^6 copies of AAV per 100 μg gDNA which corresponds to approximately 0.5–1.5 AAV copies per mouse genome (Fig. 3.b).

The extracted gDNA samples were used to perform analysis of possible genomic integration by creating a pooled and barcoded gDNA library of fragments (150-200 bp) from individual animals. These fragments were hybridized with AAV capture bait of the whole expression cassette (4162 bp), and sequenced on the Illumina MiSeq platform, yielding an average of 1.3 million paired reads per sample. The mean fold increase in AAV-reference target sequence coverage was significantly higher in the AAV transduced lung samples, which ranged between 5000×–23,000×, compared with 26× in the mock control samples.

The Samblaster (VN:0.1.24) was used to identify discordant read-pairs or split reads between AAV-vector and mouse genomic DNA. The fraction of discordant or split reads ranged from 0.75 to 1.2 per 1000 AAV-vector mapped reads (map quality > 3) (Table 1 column 4). However, we found that both discordant reads (Fig. 4.) and split reads (not shown) were randomly localized throughout the mouse genome. There was no difference in the frequency of discordant inter-chromosomal reads between the samples from the AAV transduced and the samples from the mock animals (Table 1 Column 3). Also, the fraction of these AAV-specific reads was extremely small compared to all mapped reads (Table 1 Column 2 and 3).

No apparent “hot spots” (Fig. 4) were identified that suggest any preference of the AAV expression cassette sequences junction in any of the mouse chromosomes. Therefore, the data strongly demonstrates that long-term gene expression in mouse lungs is visible using the AAV6 vectors, and that genomic integration is unlikely, at least within the parameters of our experiments.

3. Discussion

In this study, we specifically addressed the issue of long-term persistence of the AAV6 vectors in the lungs and the possible integration of these vectors into the host genome using the mouse animal model. Our data showed that using the AAV6 serotype as a vector resulted in a strongly visible and long-term reporter gene expression in mouse lungs. It also showed that integration in the host genome was below the detection level of the proposed method.

Several previous studies have advocated for the use of other AAV serotypes as the vector of choice for lung targeting. Studies on ovine perinatal lungs, for example, which has greater anatomical similarity to human lungs, identified AAV9 and AAVrh10 as highly effective vectors to transduce medium and small epithelial cells (McClain et al., 2016). A preclinical study in rhesus macaques has also reported successful lung transduction by only two commonly used AAV1 and AAV5 serotypes using a reporter gene (Guggino et al., 2017). Another study conducted on CF pig airways showed correction of CF phenotype with a modified AAV2 based vector derived from a recombinant library (Steines et al., 2016). AAV6 vectors and their modifications have shown to be effective in lung gene transfer of healthy (Halbert et al., 2010; Kurosaki et al., 2017; Kurosaki et al., 2018; van Lieshout et al., 2018; Strobel et al.,

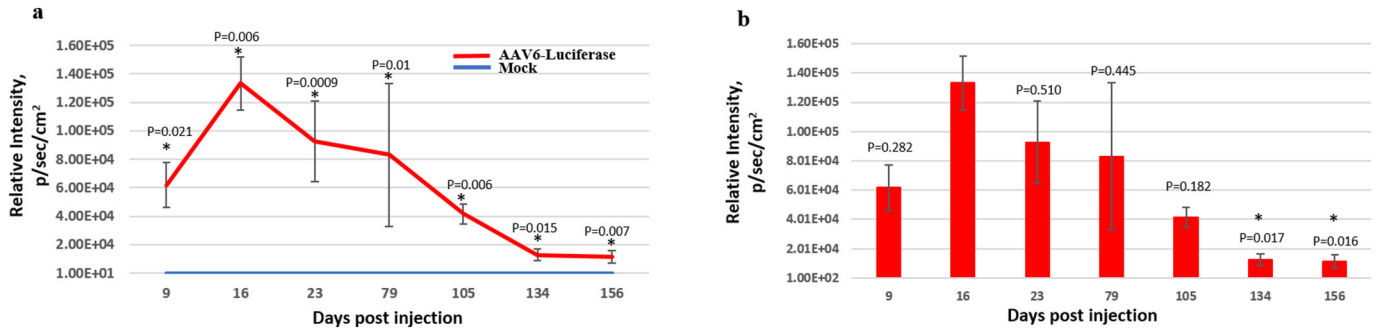

Fig. 2. A. Relative luminescence of signal intensity (mean ± SEM) of AAV6-fLuc over time of 150 days as determined by Living Image 3.2 software (Caliper Life Sciences). The same settings were used for each measurement: FOV 12.5 and exposure time 30 s. The *P* values represent the difference between AAV transduced and mock control animals. **B**. Bar-graph of relative luminescence of signal intensity (mean ± SEM) in transduced mice with P value of difference between peack of expression at day 16 and each other day of measurement.

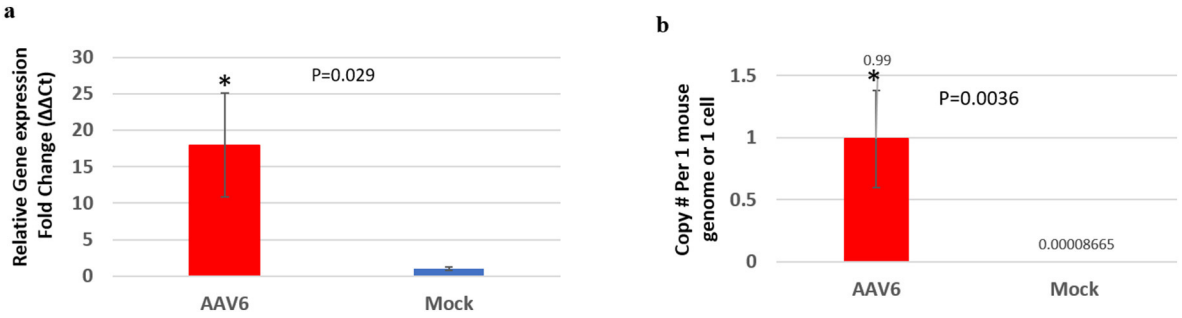

Fig. 3. A. Post-necrotic analysis of relative gene expression (fold change) of AAV6-fLuc on the total RNA extracted from transduced versus non-transduced mouse lungs. **B**. Post-necrotic analysis of AAV copy number/100μg from transduced versus non-transduced mouse lungs gDNA in AAV using qPCR analysis and normalized per single cell.

Table 1
AAV expression cassette discordant mate reads to the mouse genome.

Samples	All mapped reads	Interchromosomal discordant mouse-only per million mouse-only Chr mapped reads	The fraction of discordant reads of all mapped reads x1000
Mock#1	2,144,013	1628	0.75
Mock#2	2,417,009	1901	0.78
AAV6 #1	2,326,206	1850	0.79
AAV6 #2	2,007,712	1909	0.95
AAV6 #3	2,157,526	2707	1.2
AAV6 #4	2,090,392	1940	0.92
AAV6 #5	1,781,210	2182	1.2
Mock sum	2,280,511 ± 1930,37	1765 ± 192	
AAV6 sum	2,072,609 ± 2,00,601 $P = 0.16$	2118 ± 353 $P = 0.08$	

2015), mucus-covered/plugged airways of Scnn1b-Tg mice (Duncan et al., 2018) and dogs (Halbert et al., 2010). More importantly, they have higher efficiency in transducing human AE cell lines and primary cells in the air-liquid interface (ALI) cultures (Kurosaki et al., 2017; Duncan et al., 2018; Song et al., 2009; Limberis et al., 2009). We also showed superior penetration of the AAV6 vectors in freshly isolated human mucus (Duncan et al., 2018) in comparison to other clinically relevant AAV serotypes (Schuster et al., 2014). These findings emphasize the significance of considering the use of AAV6 vectors for clinical application (Duncan et al., 2016).

Naturally, AAV has rare virus properties, which makes it able to integrate to chromosome 19q at a site-specific location termed the AAVS1 (Kotin et al., 1990). This ability of AAV depends mostly on the activity of its replication (Rep) protein (Weitzman et al., 1994) However, the large vector dose requirement for efficient infection, together with the general Rep protein toxicity and the relatively small packaging capacity of AAV vectors makes this proposition challenging and impractical. On the other hand, recombinant AAV vectors were shown to persist in cells, predominantly in an extrachromosomal (episomal) (Nakai et al., 2001) form, but with infrequent random integration in human cells *in vitro* (Miller et al., 2005), and some mouse cell types such as hepatocytes *in vivo* (Nakai et al., 2005; Rosas et al., 2012).

Since AAV associated genotoxicity has been observed previously in mice (Chandler et al., 2017) and since lungs are one of the organs where WT AAV serotypes can be isolated from the genome (Schnepp et al., 2005; Gao et al., 2004), we conducted our study specifically to understand the nature of recombinant AAV-mediated long-term gene expression and its integration in the host genome. Our data indicate that expression of a reporter gene such as luciferase persists in mouse lungs for a significant period (over 150 days) with an average of 1 copy of AAV expressing cassette per mouse gDNA. The relative gene expression in the transduced animals was also confirmed by qPCR analysis of the total RNA extracted from lung samples at necropsy and was approximately 17-fold higher than in control animals.

Analysis of several individual mouse lung samples did not reveal any exclusive chromosomal locations for the specific sequence of the AAV expression cassette used in the experiments. Although the frequency of discordant and split reads for transduced samples was higher compared to the mock control, the overall number of the possible junctions with the mouse genome was extremely low. We concluded that any inference of AAV integration is extremely weak and at best circumstantial. That difference in gene integration in AEC compared to some reported data from mouse and human hepatocytes can be explained, at least in part, by the difference in cellular turn-over between these organs. However, we cannot rule out the possibility of low-frequency AAV integration at levels below the sensitivity of sampling depth. Additionally, the actual sequence of expression cassette used can be a factor, and further experiments need to be conducted with different

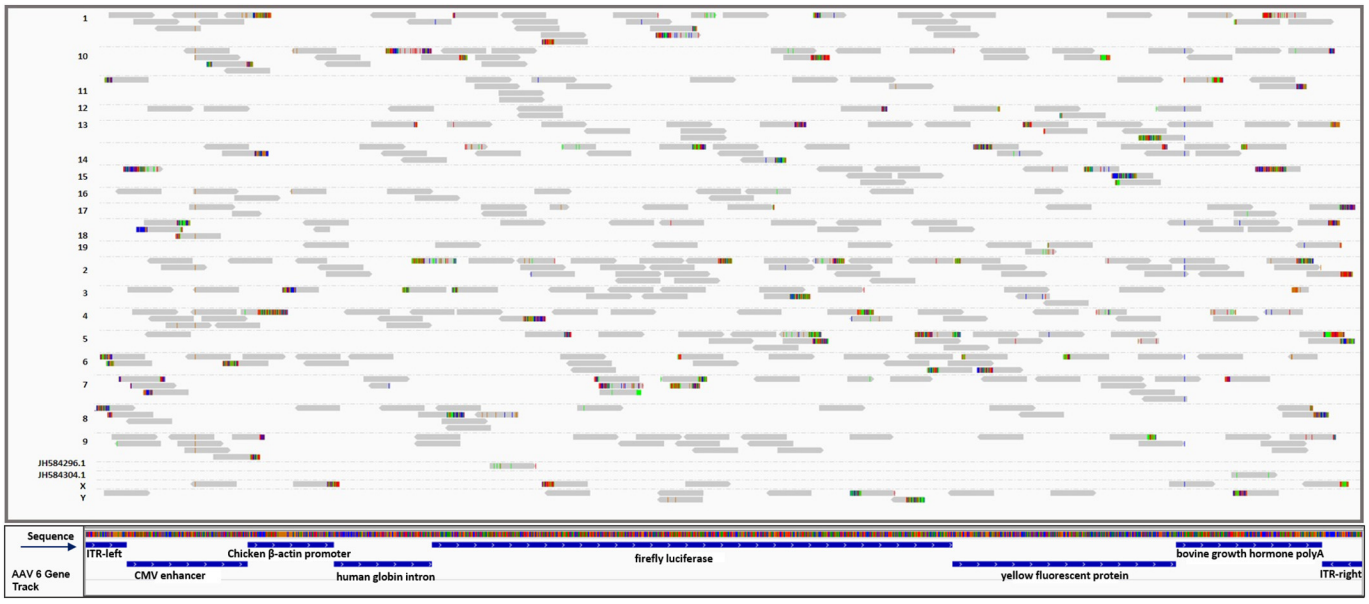

Fig. 4. Visual representation of AAV integration in mouse lungs by depicting AAV expression cassette discordant reads against the mouse genome. Each horizontal gray bar represents one sequencing discordant read (alignment with both gDNA strains) mapped to the part of the AAV6 reference genome having BWA map quality ≥3. Mismatched sequences and soft-clipped bases are shown in color, where nucleotides are color-coded as A-green, T-red, G-orange, C-blue. Discordant reads were mapped to AAV reference according to the pre-defined sequence of the expression cassette fragments: ITRs, promoter and enhancer, intron, firefly luciferase, a yellow fluorescent protein (YFP), and polyA as shown on the bottom. Mapped reads (each of the gray bar) were grouped by the mapped chromosome number of the discordant mate and the arrow points to the direction on the gDNA strain. Chromosome numbers are displayed on the left. The perfect alignment of more than two mapped reads (gray bars) at the exact chromosomal location will be considered as a possible "hot spot" for integration. On the contrary, the figure shows that discordant reads are randomly localized throughout the mouse genome. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

promoters and therapeutic genes.

One possible explanation for the long-lasting gene expression, which requires further evaluation, lies in the heterogeneity of lung cell populations and their permissiveness to infection by different AAV serotypes (Gruntman et al., 2012). Besides the long-lasting expression, the need to target different cell populations in the airway, such as ciliated, club, basal, and goblet cells, for efficient gene expression was also recently underlined (Flotte, 2018). Interestingly, two groups of researchers have recently identified a rare and previously uncharacterized cell type in the airway epithelium called pulmonary ionocytes (Montoro et al., 2018; Plasschaert et al., 2018), which can be a potentially important target for gene therapy. However, further studies need to be performed to analyze interactions of these subsets of the rare cells with AAV6 or any other AAV serotypes.

In summary, our data strongly suggest that AAV6 should be considered as a possible candidate for lung-directed gene therapy. Further investigation using AAV6 vectors in advanced preclinical animal models, including animals with lung anatomy/physiology more similar to humans and in animals affected by human-like lung diseases, such as non-human primates (Guggino et al., 2017) or pigs (Steines et al., 2016), are needed before any clinical use in humans.

4. Materials and methods

4.1. Evaluation of AAV vector activity in mouse lungs

Wild-type capsid of AAV6 vectors (Sequence ID GenBank: AAB95450) were packaged in HEK293 cells by triple-transfection using PEI reagent. The vectors contained a single-strain expression cassette construct of the chicken-b-actin promoter (CB)-driven fusion of firefly luciferase (FLuc) and yellow fluorescent protein (YFP) genes, which allow for an overall evaluation of reporter gene expression in live animals. Vectors were isolated by iodixanol gradient followed by ion-exchange column purified as previously described (Pandya et al., 2013).

We tested the effectiveness and the duration of the AAV6-mediated gene transduction in C57BL/6 mice (Jackson Laboratory, Bar Harbor, ME). The 10-week-old male mice were infected with a single dose of 5×10^{10} vgs per animal through an endotracheal catheter. Briefly, animals were first anesthetized and placed supine over an intubation platform with their incisors secured with an O-ring. An operating otoscope with an intubation speculum was used for tongue retraction and visualization of the glottis. Mice were intubated by sliding a small catheter over a guiding wire threaded into the mouse trachea. A precision syringe with a blunt needle containing 50 ul of AAV-fLuc liquid suspension and 150 ul of air cushion was used to inject and distribute the vector into the lungs (Gruntman et al., 2012; Halbert and Miller, 2004). Other mice were mock-treated with 50 µl PBS and 150 µl air. Post-infection luciferase activity in the live animals was measured and analyzed weekly for the first month and then monthly for a total period of 150 days or more, using a Xenogen IVIS Lumina System (Caliper Life Sciences) as previously described (Sayroo et al., 2015; Aslanidi et al., 2013). All manipulations of the animals were performed according to the principles of the National Research Council's Guide for the Care and Use of Laboratory Animals, and the study was approved by the University of Florida Institutional Animal Care and Use Committee.

4.2. RNA extraction and quantitative PCR

To assure consistency, only the right lungs were harvested from each mouse post necropsy and immediately fresh frozen forRNA extraction. Tissues were homogenized using BeadBug homogenizer (Benchmark, Carlsbad, CA). Total RNA was isolated following on-column gDNA digestion and concentration using Quick-RNA MiniPrep Plus kit (Zymo Research, Irvine, CA). Relative gene expression was analyzed by Real-Time PCR CFX 96 touch (BioRad, Hercules, CA) with SensiFast One-Step master mix (Bioline, Memphis, TN). The following primers were used to isolate target gene: Luciferase-YFP forward 5′-AAGCTGACCCTGAAGTTCATCTGC-3′ and reverse 5′-CTTGTAGTTG

CCGTCGTCCTTGAA-3′. The following primers were used as 2 internal controls: Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) forward 5′-ACCCAGAAGACTGTGGATGG-3′ and reverse 5′-ATGCAGGGATGATGTTCTGG; 18S forward 5′- TCAACACGGGAAACCTCACC-3′ and reverse 5′-CCACCCACGGAATCGAGAAA-3′.

4.3. gDNA extraction and quantitative PCR

The same samples described above were used for Genomic DNA (gDNA) extraction. gDNA was isolated using a DNeasy blood and tissue kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. AAV genome copies present in gDNA were quantified by qPCR using the following primers: CB Forward: 5′-CATCTACGTATTAGTCATCGCTATTACCA-3′; CB Reverse: 5′-CCCATCGCTGCACAAAATAATTA-3′; CB Probe: 6FAM-CCACGTTCTGCTTCACTCTCCCCATC-TAMRA). Results were analyzed using the Sequence Detection Systems (SDS) 2.3 software. AAV vector copy number was normalized per μg of total gDNA and per single mouse genome. The sample that contained greater than or equal to 100 AAV genome copies/μg mouse gDNA was considered positive for vector genome presence (Corti et al., 2017).

4.4. DNA library preparation, MiSeq sequencing, and analysis of integration

Any possible integration of AAV expression cassette was analyzed on the same gDNA samples described above with the conventional gene-enrichment bait approach (Agilent, Santa Clara, CA, USA), followed by MiSeq sequencing and mapping in reference to the mouse gDNA (Nault et al., 2015; Ravi et al., 2018). Briefly, acoustic shearing with a Covaris S220 instrument, hybridized with biotinylated RNA baits at 65 °C for 24 h and captured by streptavidin-coated magnetic beads (Dynabeads MyIne Streptavidin T1, Thermo Fisher, Waltham, MA, USA), fragmented the genomic DNA. Illumina Reagent/kits for DNA library sequencing cluster generation and sequencing were used for DNA sequencing. DNA sequences were then mapped to the mouse reference genome (GRCm38) using BWA (VN:0.7.15-r1140).

4.5. Statistical analysis

All data are shown as mean ± SEM. For statistical analysis unpaired *t*-test for comparison of independent samples (AAV6 treated vs control) with unequal variance was used. The difference was considered significant when P values were < 0.05 (*).

Abbreviation

α1	antitrypsin (α1-AT) deficiency
(AAV)	Adeno-associated virus
(ALI)	Air-liquid interface
(COPD)	Chronic obstructive pulmonary disease
(CB)	Chicken-b-actin promoter
(CF)	Cystic Fibrosis
(AEC)	Epithelial cells
(FLuc)	Firefly luciferase
(FDA)	Food and Drug Administration
(gDNA)	Genomic DNA
(HCC)	Hepatocellular carcinoma
(Rep) protein	Replication
(YFP)	Yellow fluorescent protein

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CRediT authorship contribution statement

Yanerys Colon-Cortes: Conceptualization, Methodology, Investigation, Writing - original draft. **Mutasim Abu Hasan:** Conceptualization, Writing - review & editing, Project administration, Funding acquisition. **George Aslanidi:** Conceptualization, Methodology, Investigation, Writing - original draft, Project administration.

Declaration of competing interest

Authors have no conflict of interest.

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