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Delivery of genome-editing biomacromolecules for treatment of lung genetic disorders

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ABSTRACT

Genome-editing systems based on clustered, regularly interspaced, short palindromic repeat (CRISPR)/associated protein (CRISPR/Cas), are emerging as a revolutionary technology for the treatment of various genetic diseases. To date, the delivery of genome-editing biomacromolecules by viral or non-viral vectors have been proposed as new therapeutic options for lung genetic disorders, such as cystic fibrosis (CF) and α -1 antitrypsin deficiency (AATD), and it has been accepted that these delivery vectors can introduce CRISPR/Cas9 machineries into target cells or tissues *in vitro*, *ex vivo* and *in vivo*. However, the efficient local or systemic delivery of CRISPR/Cas9 elements to the lung, enabled by either viral or by non-viral carriers, still remains elusive. Herein, we first introduce lung genetic disorders and their current treatment options, and then summarize CRISPR/Cas9-based strategies for the therapeutic genome editing of these disorders. We further summarize the pros and cons of different routes of administration for lung genetic disorders. In particular, the potentials of aerosol delivery for therapeutic CRISPR/Cas9 biomacromolecules for lung genome editing are discussed and highlighted. Finally, current challenges and future outlooks in this emerging area are briefly discussed.

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1. Introduction

Lung genetic disorders mainly consist of a variety of respiratory syndromes characterized by airway obstruction in the lungs. The progression of lung genetic disorders eventually entails severe respiratory morbidities that often result in pulmonary disease-associated mortalities, where the most common therapeutic modalities only serve to manage associated symptoms. Among lung genetic disorders, the most common chronic and life-threatening one is cystic fibrosis (CF) caused by single gene defects [1]. Apart from CF, α -1 antitrypsin deficiency (AATD) also represents one of most common genetic disorders, particularly in North America [2]. Other lung genetic-related disorders including lung cancer, asthma, and chronic obstructive pulmonary disease (COPD) are also life-threatening diseases which are yet short of effective treatment options. Over last thirty years, many efforts have been dedicated to the development of gene therapy regimens for these disorders in an attempt to repair the malfunctions at the genetic level; however, the precise correction of these mutated genes is of great technical challenges which are still elusive to date. Recently, clustered, regularly interspaced, short palindromic repeat (CRISPR)/associated protein 9 (CRISPR/Cas9) that is exploited as a therapeutic tool for precise editing of mutated genes opens an avenue for the treatment of lung genetic disorders. Despite its early stage, preliminary studies on the treatment of genetic disorders, such as CF, AATD and lung cancers, through CRISPR/Cas9-based genome editing show encouraging results that shed light on its great therapeutic potentials [3–8]. To deliver CRISPR components into lung, a key consideration is the route of administration which greatly influences the final therapeutic efficacy. Several routes are currently available, such as intravascular administration [9], direct injection [10] and topical administration [11,12], and each has its own pros and cons. In addition, delivery vectors, either viral or non-viral vectors, are essential in transporting therapeutic genome-editing biomacromolecules to the nucleus of target cells.

In this review article, we primarily focus on the critical issues of traditional gene therapy approaches for lung genetic disorders, followed by the recent advances in the development of CRISPR-associated nucleases for the correction of the genetic mutations. Finally, different approaches and delivery routes for gene editing of the lung genetic disorders and the potentials of emerging technologies for the delivery of CRISPR-associated nucleases are also discussed (Fig. 1).

2. Lung genetic disorders and current therapeutic options

2.1. Cystic fibrosis

CF is an autosomal, recessive genetic disorder, which is caused by inherited mutations in the *CFTR* gene [13,14]. CF is one of the most common lung genetic disorders in North America and Europe that mainly causes severe impairment of normal lung functions. It has been generally accepted that the *CFTR* gene encodes a protein that mainly regulates ion and fluid homeostasis across epithelial barriers, and when the gene mutates, chloride and bicarbonate transport in the epithelial tissues of the lung becomes aberrant, leading to the altered hydration of airway surface fluid and reduced mucociliary clearance. Thus, increased susceptibility to lung infections and inflammations are the main symptoms

of CF patients, and the presence of particular bacteria can even cause progressive airway obstruction, leading to severe respiratory morbidity [13]. In addition to lung, *CFTR* gene is also widely expressed in the epithelium of many organs including liver, kidney, and pancreas. Although more than 1990 mutations have been characterized within the *CFTR* gene, the most common mutation worldwide is an in-frame deletion of a phenylalanine residue at amino acid position 508 (dF508, also known as F508del), followed by Gly542X (G542X) and Gly551Asp (G551D) [15,16].

2.1.1. Current available therapeutic options

Several therapeutic options are currently available for the treatment of CF, including inhaled antibiotics, mucolytics (such as Dornase Alfa, a recombinant human deoxyribonuclease), nutritional support and systemic anti-inflammatories [17,18]. These therapeutic regimens rapidly progress from bench to bedside, and progressively extend the median survival. Despite their effectiveness, the main underlying issues in restoring the function of mutant *CFTR* protein at the genetic level remain to be addressed yet. Recent efforts in alleviating *CFTR* dysfunction are emerging as a new therapeutic strategy for the treatment of CF. Ivacaftor, a *CFTR* potentiator, is widely used to treat CF patients with certain *CFTR* mutations by improving the ion channel function of *CFTR* [19,20]. Although ivacaftor prevents lung from progressive deterioration and demonstrates to be effective in improving lung functions, its activity is only limited to several specific classes of mutations (such as G551D mutation) which only accounts for 4–5% cases of CF [21,22]. To broaden the indications, lumacaftor, a drug which enables the proper trafficking of *CFTR* protein to the cell surface, is often used in combination with ivacaftor to improve the lung function in the most prevalent *CFTR* mutation, dF508 mutation [23–25]. Tezacaftor is also explored in combination with ivacaftor to correct the defective *CFTR* protein in the most common mutation of dF508, or the compound heterozygous mutations of dF508/G551D in the phase 1 and 2 studies of recent clinical trials [26,27].

2.1.2. Traditional gene therapy

Despite remarkable advances in developing small molecules for restoring the function of *CFTR* proteins, there is still an urgent need to develop gene therapy approach as a more powerful, mutation-independent therapeutic strategy for the treatment of CF. Though small-molecule drugs contribute to alleviating the symptoms of CF, they do not stand as a radical therapeutic approach. Gene therapy represents a promising, alternative way for CF treatment and targets the genetic disorder at the molecular origin. The mutation-independent feature might open a new avenue for all CF patients that are refractory with the current treatment options. To date, clinical trials for CF gene therapy is mainly accomplished by delivering either DNA or mRNA encoding wild-type *CFTR* protein through both viral and non-viral vectors [11]. In addition, small interfering RNA (siRNA) and single-stranded oligonucleotides (ssODNs) are also emerging for CF therapy [28,29]. Though adenovirus was first reported to deliver *CFTR* gene for clinical trials and extensively studied as the delivery vector for CF therapy, unfortunately, *CFTR* gene transferred into lung by adenovirus has been difficult largely owing to the epithelial barriers of the lower lung [30–34]. Recent clinical trials of CF gene therapy demonstrated that adeno-associated virus

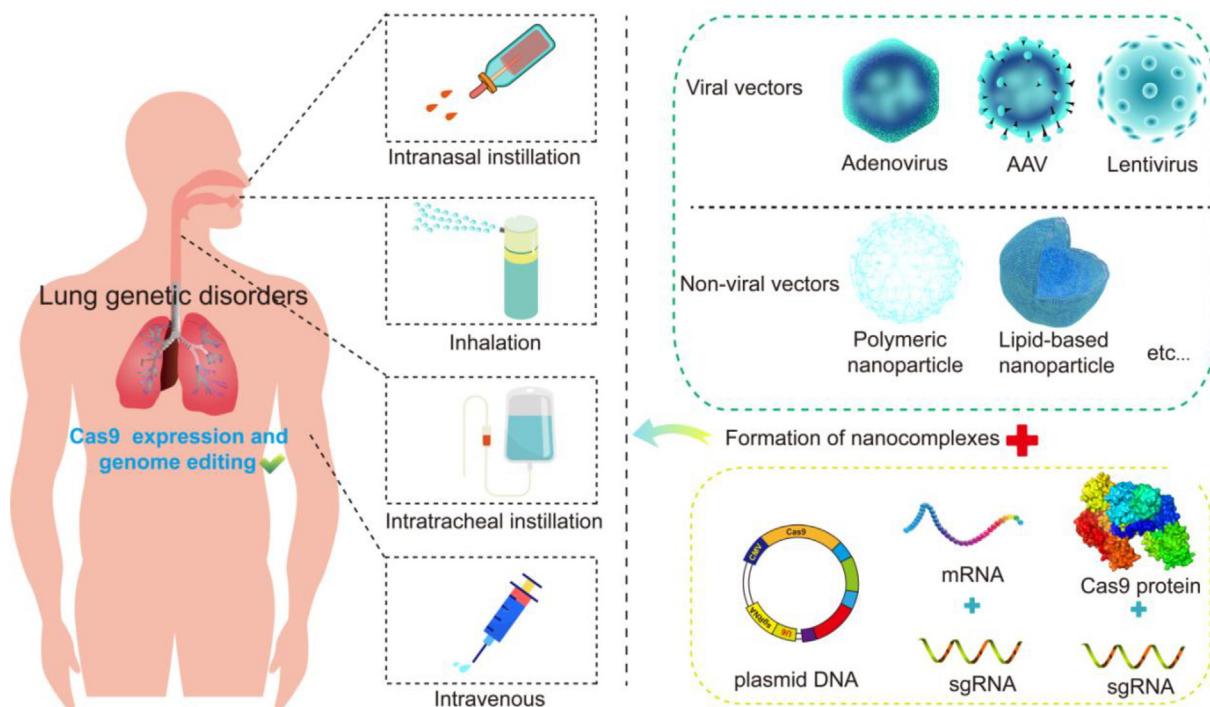


Fig. 1. Pulmonary delivery of therapeutic genome-editing agents mediated by viral or non-viral vectors. There are typically three formats available for Cas9/sgRNA delivery: plasmid DNA encoding Cas9/sgRNA, Cas9-expression mRNA and sgRNA, and Cas9 protein complexed with sgRNA (ribonucleoprotein). The size of Cas9 plasmid is typically greater than 7 kb with the addition of plasmid backbone and other expression assistant elements. Cas9 mRNA and sgRNA are usually produced by *in vitro* transcription using DNA templates, which are driven by T7 promoter for transcription. Cas9 RNP is a complexation comprising a positively charged Cas9 protein and a negatively charged sgRNA. In addition, two types of clinically-relevant gene delivery vectors are currently available to deliver therapeutic agents to the lung, namely viral vectors and non-viral vectors. Intravenous, intratracheal/intranasal instillation and inhalation route can be used to deliver therapeutic genome-editing agents to the lungs of human.

serotype 2 (AAV2) could improve transfection and *CFTR* expression levels in the lung [35–39]. Lentiviral vectors are viruses that are currently considered to be the most active vectors to deliver therapeutic genes for CF treatments. They are linear RNA viruses with the inherent capability of integrating into the host genome of cells that do not undergo divisions, such as muscle or neuron cells, and show the advantages of the sustained expression of wild-type *CFTR* [2,40]. However, the systemic delivery of lentivirus results in relatively poor uptake in airway epithelium due to its common pseudotype with stomatitis virus G, implying its insufficient potential to distribute in the lung for treating CF. In order to improve its uptake in airway apical epithelium, the combination of lentivirus and some adjunct tight junction modulators have been utilized to facilitate transfer into host progenitor cells in the deeper apical epithelium [2]. Collectively, though a number of viral gene therapy trials show the promising clinical benefits for CF therapy, viral vectors still encounter formidable delivery barriers at both extracellular and intracellular levels that have severely impair their effectiveness. In addition, safety concerns such as carcinogenesis, immunogenicity and insertional mutagenesis, also limits their clinical translations [41]. As an alternative, non-viral gene therapy may well circumvent biological barriers and biosafety issues. Safety concerns of insertional mutagenesis, carcinogenesis, and immunogenicity associated with viral delivery offer non-viral approaches as an alternative option for gene therapy [41]. The potential advantages of non-viral vectors, in comparison with their viral counterparts, include decreased immunomodulatory responses and better tolerance to repeated administration [41]. Recent clinical trial reveals that the repeated administration of *CFTR*-encoding DNA (pGM169)/Genzyme lipid 67A (GL67A) gene encapsulated by liposome via a nasal spray device provided a significant benefit in forced expiratory volume in 1 s (FEV₁), as compared with 0.9% saline [42]. However, the cationic lipid-based carrier showed a modest correction of specific *CFTR* mutation *in vivo* with limited evidence of *CFTR* transgene expression. Later, other liposomal formulations

were investigated to access the potential of CF gene therapy [11]. Due to greater stability of cationic polymers over liposomes during nebulization, nebulized delivery of DNA or mRNA facilitated by cationic polymers would be a promising way for non-viral gene therapy of CF [43,44]. As mRNA can be customized to improve certain functions, it is ideal to treat genetic disorders without any confinement of mutation type. Recently, repeated administration of *CFTR* mRNA formulated with hyperbranched poly (beta amino esters) (hpBAEs) through nebulization showed significant improvement in the production of functional proteins, which is more efficient over polyethylenimine (PEI)-mediated nebulized mRNA delivery [44]. Another recent study demonstrated that both self-assembled peptide/poloxamine/DNA ternary complexes or self-assembled peptide/poloxamine/mRNA ternary complexes could generate long-term effect for *CFTR* restoration in the lungs of CF mice via the intratracheal application with low level of associated inflammatory responses [45]. Overall, the peptide-poloxamine carrier is able to deliver multiple genetic payloads (DNA and mRNA) to ensure successful *in vivo* long-term restoration of *CFTR* deficiency with negligible toxicity. In addition, poloxamine-based amphiphilic block copolymers may potentially act as a carrier for genome-editing agents (for example, zinc-finger nucleases and CRISPR/Cas9). Apart from polymers, exosomes are also emerging as a new class of carriers for the delivery of *CFTR* DNA or mRNA, as they have shown their inherent ability to transfer bioactive nucleic acids to their neighbouring and distant parent cells *in vivo*. Exosomes isolated from culture medium of *CFTR*-expressing Calu-3 cells were able to transfer the *CFTR* to human CF cells and restore the genetic defect [46].

2.1.3. Repairing *CFTR* mutations: gene correction with genome-editing nucleases

Beyond traditional gene-therapy approaches, genome editing with CRISPR is an attractive approach for repairing the mutation of CF and AATD (Fig. 2 and 3). CRISPR technology provides a new efficient tool

Cystic Fibrosis

Wild type CFTR

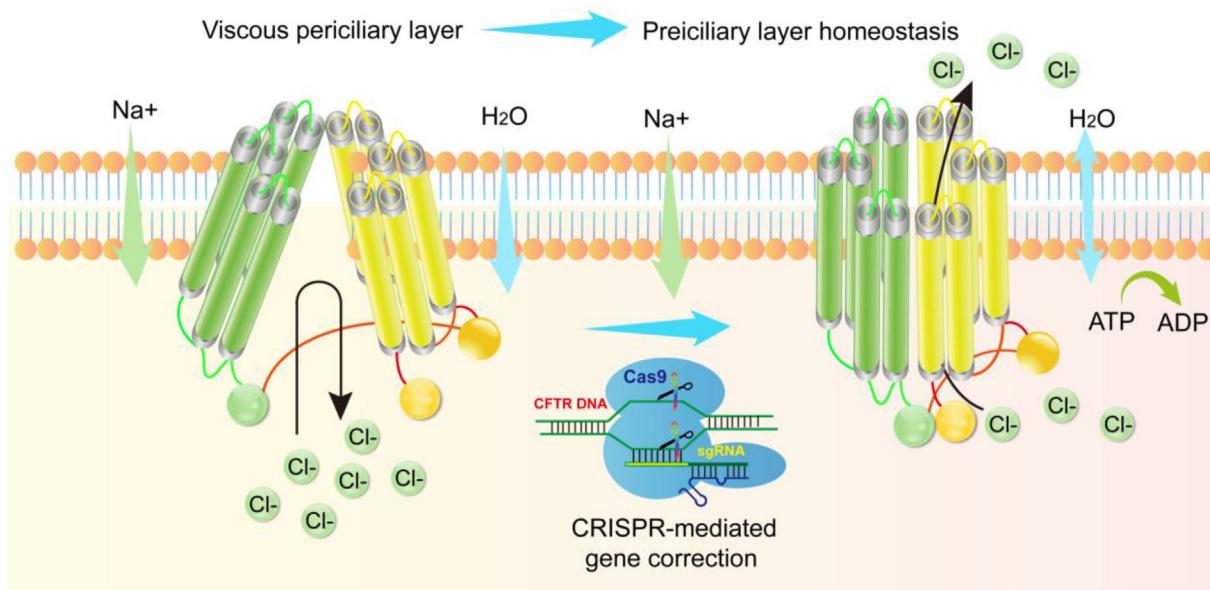


Fig. 2. Schematic illustration of CRISPR/Cas9 technology for the treatment of CF. Hypertonic saline increases airway surface liquid, which is reduced in patients with CF as a consequence of defective chloride and increased sodium absorption. By using CRISPR/Cas9 genome-editing systems to correct the mutant *CFTR* locus in CF patients, the apical calcium-activated chloride channel would be activated.

to directly correct the specific genetic mutations at the genome level. A number of CRISPR-based *ex vivo* and *in vivo* genome-editing therapeutics are currently in clinical trials (Table 1). Whereas traditional gene therapy seeks to transport a new functional gene into the targeted cells to correct the genetic defect, genome editing can precisely delete and insert targeted genes to cure genetic defects with a site-specific manner. The main differences between CRISPR/Cas9, siRNA, DNA-based gene therapy, and small bio/pharmaceutical molecule in the treatment of lung genetic disorders were shown in Table 2. CRISPR/Cas9 has shown its merits to disrupt coding sequence in contributory genes and cryptic splice sites, or to repair specific mutations with high precision. CRISPR/Cas9 can introduce double strand breaks (DSBs) in the chromosome nearby the mutation site, which are then repaired through various DNA repair pathways including non-homologous end joining (NHEJ), homology-directed repair (HDR), homology-mediated end joining (HMEJ) or microhomology-mediated end joining (MMEJ). Thus, *in vivo* genome editing is potentially competent to provide long-term therapeutic benefits. Recent advances in genome-editing technique allow the potential and reasonable design of single-guide RNA (sgRNA) targeting mutant *CFTR* based on mutation types. Note that the design and synthesis of sgRNA is cost-effective and flexible. Even when a patient bears more than one type of mutations, multiple sgRNAs targeting different mutations can be designed simultaneously for multiplex editing. As an innovative therapeutic modality, CRISPR/Cas technology is becoming a potential therapeutic strategy for the therapy of lung genetic disorders. Examples of application of CRISPR/Cas9 systems for lung genetic diseases therapy are summarized in Table 3.

Schwank and co-workers first reported the functional repair of *CFTR* by CRISPR/Cas9 system in primary cultured, small and large intestinal stem cell organoids derived from CF patients with dF508 mutation (Fig. 4) [3]. The forskolin-induced swelling assay was performed to assess the *CFTR* function of the gene-corrected mutant dF508 allele in the organoid system. As expected, the gene-corrected F508 allele becomes fully functional as measured in the primary intestinal organoids *in vitro*. Furthermore, Firth and co-workers recently used CRISPR/Cas9 DNA to target endogenous *CFTR* genomic locus of induced pluripotent stem cell (iPSCs) derived from CF patient with dF508 mutation, and

demonstrated that the recovery of normal *CFTR* expression as well as related function was achieved after the genomic correction of iPSCs (Fig. 5) [4]. With a GFP reporter and puromycin selection, an overall correction rate of 16.7% was demonstrated, and the corrected patient iPSCs could be subsequently differentiated into functional, respiratory airway epithelial cells where the restoration of wild-type *CFTR* expression was accomplished. Although these corrected iPSCs expressed low level of normal *CFTR* protein, upon proper differentiation, they may be differentiated to airway epithelial cells, lung organoids or other types of functional cells that can be used as alternative therapeutic modalities for CF patients. Collectively, though these two proof-of-concept studies have shown the possibilities for the correction of *CFTR* defects in CF patients, the use of viral vectors and reporter enrichment as well as drug selection are still the major concerns that limit the future translation of genome editing-based therapeutics for CF.

Harrison and co-workers used Lipofectamine 3000/Cas9 plasmid DNA complexes for genome editing of three different rare *CFTR* mutations in cystic fibrosis tracheal epithelial (CFTE) cells [47]. These three rare mutations include c.1679+1634A>G (1811+1.6kba>G), c.3140-26A>G (3272-26A>G), and c.3718-2477C>T (3849+10kbC>T), which collectively account for about 3% of individuals with CF worldwide. For these mutations, the delivery strategy showed high efficiency and site-specific excision of the target genomic regions via the NHEJ repair pathway. The overall genome-editing efficiency of NHEJ-mediated targeted excision for these three mutations was ranged from 25–47%. Ruan and co-workers compared different combinations of the transfection methods (electroporation versus lipofectamine) and formats (plasmid DNA versus RNP) to deliver genome-editing elements along with ssODNs to patient-derived iPSCs (Fig. 6) [5]. In contrast with other combinations of the transfection methods, electroporation of Cas9 RNP is highly efficient in delivering CRISPR/Cas9 elements into CF patient-derived iPSCs. Such a method generates a correction rate over 27% by targeting the most commonly found homozygous *CFTR* mutation loci. Thanks to the high genetic correction rate, effective *CFTR* function in adenocarcinoma cell line CFPAC-1, as well as in iPSC-derived proximal lung organoids were recovered after the genetic correction. There are two factors which critically contribute to the high genome-editing

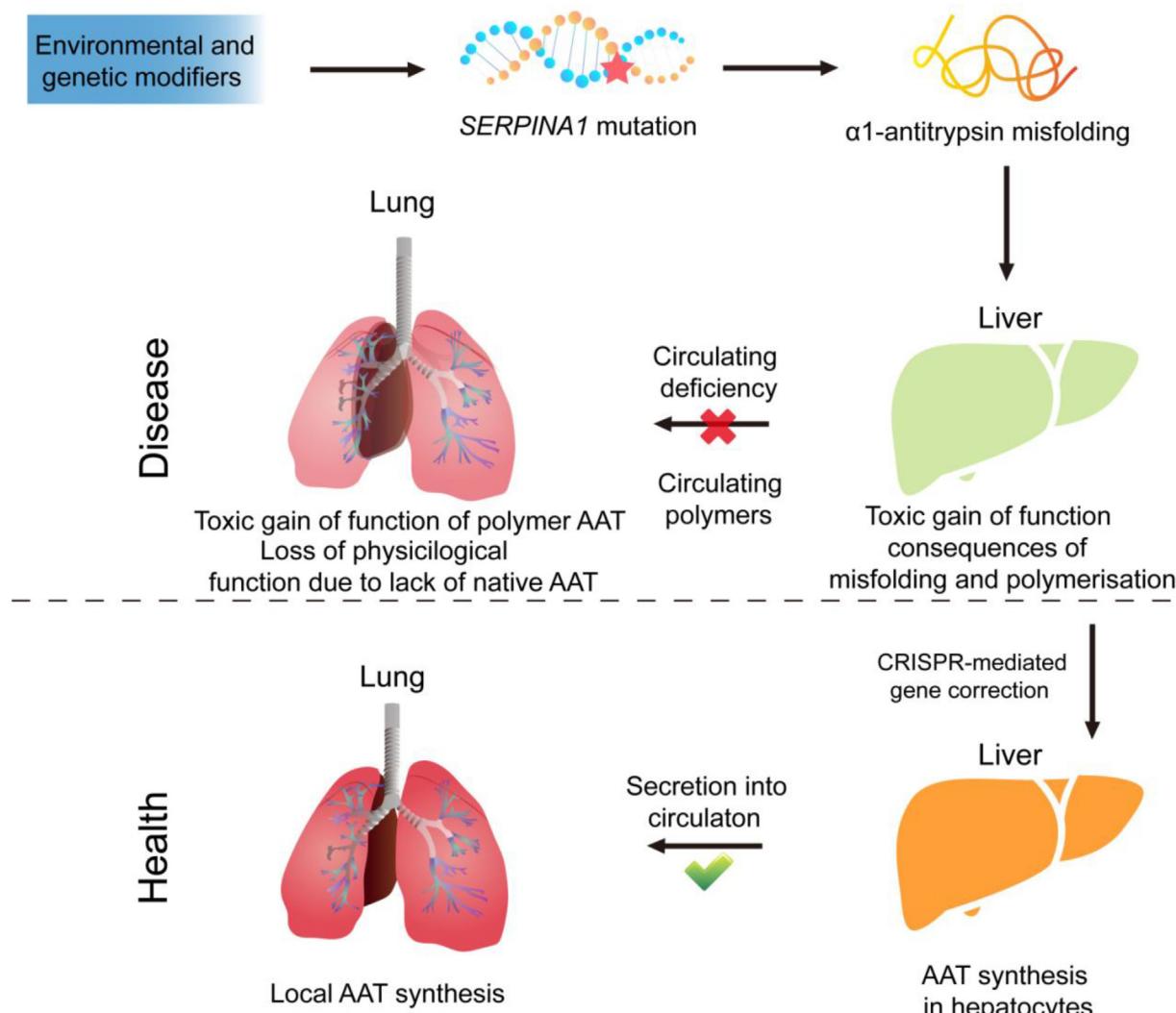


Fig. 3. Schematic illustration of CRISPR/Cas9 technology in the treatment of AATD. Misfolding and polymerisation of AAT lead to AAT accumulation in the liver and lung, which results in oxidative stress and cell death or proliferation. The final outcome is concurrent liver and lung failure. Genome editing through CRISPR/Cas9 would partially enable the restoration of wild-type AAT expression in AATD patients.

efficiency in the electroporation method. One is the smaller size of Cas9 RNP and ssODN (as compared to DNA), which facilitates the delivery of CRISPR elements into cells. The other factor is protospacer adjacent motif (PAM) mutations that are introduced in all the donor ssODNs, thereby significantly improving the overall mutation efficiencies. Furthermore, the authors further proceeded with the electroporation method to create the most prevalent CF-derived *CFTR* mutations in patient-derived iPSCs, including dF508, G542X, G551D mutations, and even the compound heterozygous mutations of dF508/G551D. These *in vitro* isogenic iPSC-based model systems carrying different *CFTR* mutations open an avenue for screening new therapeutic approaches, understanding the mechanisms of disease, and identifying the responders to currently available therapies in the clinic.

In a recent work, Porteus and co-workers used Cas9 RNP and correction template to correct the dF508 mutation in human bronchial epithelial cells (HBECs) and upper-airway basal stem cells (UABCs) obtained from CF patients (Fig. 7) [48]. The genome-editing strategy was mainly divided into two steps. In the first step, electroporation was used to deliver Cas9 complexed with sgRNA modified with 2'-O-methyl 3' phosphorothioate (MS) in the 50 and 30 terminal nucleotides (MS-sgRNA) into airway stem cells, and AAV6 expressing the correction template was added immediately after the electroporation in the second step. In average, 28% allelic correction in UABCs, and 41% allelic correction in HBECs

obtained from homozygous CF patients were observed, respectively. In addition, the authors also observed allelic correction rates of 42% in UABCs from compound heterozygous CF patients. Furthermore, UABCs and HBECs from corrected CF patients were differentiated in air-liquid interface cultures to verify the recovery of normal *CFTR* function. As evident from the increased *CFTRinh*-172-sensitive short-circuit current in edited UABCs and HBECs by Ussing chamber analysis, corrected dF508 homozygous and compound heterozygous samples showed 20%–50% restored *CFTR* function relative to non-CF controls. It must be noted that recovery of 15% *CFTR* function relative to non-CF patients would be therapeutically beneficial for CF patients. Collectively, through genome-editing strategy with proper delivery methods, the authors demonstrated the possibility to rescue of *CFTR* function in differentiated epithelia sheets which derived from gene-corrected UABCs and HBECs.

2.2. AATD

AATD is an inherited liver monogenic disorder caused by over 150 mutations in the human *SERPINA1* gene encoding AAT protein [49]. It is an autosomal, co-dominant genetic disorder that is characterized by low systemic levels of AAT [49,50], often leading to progressive liver and lung diseases. Under normal circumstances, AAT is synthesized predominantly in the liver by hepatocytes, secreted into the serum, and

Table 1
The CRISPR-based therapy in clinical trials.

Disease type	Target gene	Phase	Status	ClinicalTrials.gov identifier
Genetic diseases				
Sickle cell disease, hematological diseases, emoglobinopathies	<i>BCL11A</i>	Phase I/II	Recruiting	NCT03745287
β-thalassemia, thalassemia, genetic diseases, inborn hematologic diseases, hemoglobinopathies	<i>BCL11A</i>	Phase I/II	Recruiting	NCT03655678
Thalassemia	<i>HBB</i>	Early phase I	Not yet recruiting	NCT03728322
Leber congenital amaurosis type 10	<i>CEP290</i>	Phase I/II	Recruiting	NCT03872479
Kabuki syndrome 1	<i>KMT2D</i>	Not applicable	Active, not recruiting	NCT03855631
Infectious diseases				
HPV-related cervical intraepithelial neoplasia	<i>HPV E6/E7</i>	Phase I	Not yet recruiting	NCT03057912
HIV-1-infection	<i>CCR5</i>	Not applicable	Recruiting	NCT03164135
Cancer				
B cell leukemia, B cell lymphoma	<i>TCR and B2M</i>	Phase I/II	Recruiting	NCT03166878
B cell leukemia, B cell lymphoma	Unknown	Phase I/II	Recruiting	NCT03398967
B cell malignancy, non-Hodgkin lymphoma, B cell lymphoma	Unknown	Phase I/II	Recruiting	NCT04035434
Multiple myeloma, melanoma, synovial sarcoma, myxoid/round cell liposarcoma	<i>PD-1 and TCR</i>	Phase I	Recruiting	NCT03399448
Solid tumor, adult	<i>PD-1 and TCR</i>	Phase I	Recruiting	NCT03545815
Solid tumor, adult	<i>PD-1</i>	Phase I	Recruiting	NCT03747965
Metastatic non-small cell lung cancer	<i>PD-1</i>	Phase I	Active, not recruiting	NCT02793856
Gastric carcinoma, nasopharyngeal carcinoma, T cell lymphoma, adult Hodgkin lymphoma, diffuse large B cell lymphoma	<i>PD-1</i>	Phase I/II	Recruiting	NCT03044743
Esophageal cancer	<i>PD-1</i>	Not applicable	Completed	NCT03081715
CD19-positive leukemia, lymphoma	<i>HPK1</i>	Phase I	Recruiting	NCT04037566
T cell malignancies	<i>CD7</i>	Phase I	Not yet recruiting	NCT03690011
Neurofibromatosis type 1 with tumors of the central nervous system	<i>NF1</i>	Not applicable	Completed	NCT03332030

Table 2

The main differences between CRISPR/Cas9, siRNA, DNA-based gene therapy, and small bio/pharmaceutical molecule in the treatment of lung genetic disorders.

Method	Principle	Characteristics	Advantages	Limitations
CRISPR/Cas9	Bacterial acquired immune system; consists of clustered, regularly interspaced, short palindromic repeats and Cas proteins	Composed of a Cas9 protein and a sgRNA molecule; DNA endonuclease; lentivirus and adenovirus transmission; targets endogenous DNA	Low cost; simple manufacture and operation; quick and highly efficient; multiple targeted genomic loci	Relatively high off-target rate
siRNA	A sequence-specific method to silence genes by introducing small double-stranded RNA	The siRNAs are a special type of double-stranded RNAs with a 3' overhang of two nucleotides, and a 5' phosphate group	High efficiency in gene suppression	Rapid clearance <i>in vivo</i> ; poor transmembrane capability
DNA gene therapy	Involving the replacement or addition of genes in place of the defective or disease-causing genes	DNA will undergo transcription and translation using the host cellular mechanisms to produce the required protein	Highly stable; long term persistence; risk of infection is minimum	Disordered insertion of genes into the genome
Small bio/pharmaceutical molecules	Mainly focusing on functional deficit of the normal protein	An important and highly effective component of the bio/pharmaceutical portfolio	High therapeutic efficacy with low dose	High cost of administration

gradually delivered into the lung where it regulates the proteolytic effects of neutrophil elastase to protect alveolar interstitial elastin [51]. The anti-protease activity of AAT plays an important role in neutralizing other pro-inflammatory enzymes [52,53]. The misfolding and accumulation of AAT protein can lead to the airway inflammation, which is a typical symptom in the AATD-related lung diseases [49,52,54]. The aggregation of misfolded AAT protein in the endoplasmic reticulum of hepatocytes often progress into liver diseases and cirrhosis, owing to the gain-of-function toxicity [55]. A large number of AATD patients complicated with emphysema, asthma, COPD and granulomatosis with panniculitis and polyangiitis [49,56]. Furthermore, the gain-of-function phenotype would ultimately lead to endoplasmic reticulum stress, inflammation response, fibrosis, cirrhosis, hepatitis and even hepatocellular carcinoma [57,58]. In contrast, the progressive lung disease is mainly associated with the loss of anti-protease function of ATT [7,53],

which stems from the low or even undetectable circulating levels of AAT that lead to the inadequate proteinase inhibition of alveoli linings and connective tissue in the lung [7,54]. Subsequently, lung elasticity and a variety of pulmonary functions are deteriorated. The classical clinical presentation of AATD-associated lung disease is severe whole-lung panacinar and lower lobe-predominant emphysema [59]. Although AATD shares many similar clinical characteristics with emphysema, the pathology features of AATD fundamentally differ from common emphysema [60]. Patients with AATD can also progress into COPD, which is often triggered by interactions between environmental and genetic factors [61].

2.2.1. Traditional therapeutic approaches

Currently, the intravenous infusion of purified human AAT protein is the gold standard of therapeutic option for the prevention of

Table 3

Examples of application of CRISPR/Cas9 for lung genetic diseases therapy.

Disease type	Target cell or animal models	Target gene	Delivery mode	CRISPR/Cas9 mode	Ref
CF	Intestinal stem cell organoids	CFTR	Lipofectamine 2000	SpCas9 expression DNA and sgRNA expression DNA	[3]
	iPSCs	CFTR	PiggyBac Transposase	SpCas9 expression DNA and sgRNA expression DNA	[4]
	CFTE cells	CFTR	Lipofectamine 3000	(SpCas9 and sgRNA) expression DNA	[47]
	iPSCs and CFPAC-1 cells	CFTR	Electroporation or Lipofectamine 2000	(SpCas9 and sgRNA) expression DNA	[5]
	HBECs and UABCs	CFTR	Electroporation	SpCas9 RNP SpCas9 RNP and correction template	[48]
AATD	Neonatal PiZ mice or adult PiZ mice	SERPINA1	AAV	SpCas9 expression DNA and sgRNA/HDR expression DNA	[6]
	PiZ mice	SERPINA1	Adenovirus	(SpCas9 and sgRNA) expression DNA	[8]
	PiZ mice	SERPINA1	AAV	(SaCas9 and sgRNA) expression DNA SaCas9 expression DNA and sgRNA/HDR expression DNA	[7]
Lung cancer	H1975 cells and A549 cells; xenograft in nude mice	Mutated EGFR	Lipofectamine 2000	SpCas9 expression DNA and sgRNA expression DNA	[98]
	A549 cells; xenograft in nude mice	CD38	DNA-In transfection reagent	SpCas9 expression DNA and sgRNA expression DNA	[86]
	H1650 cells	Mutated EGFR	FuGENE HD transfection reagent	SpCas9 expression DNA and sgRNA expression DNA	[89]
	A549 cells; xenograft in nude mice	Surviving	Fluorinated acid-labile branched hydroxylrich polycation	(SpCas9 and sgRNA) expression DNA	[88]
	A549 cells; xenograft in nude mice	KRAS	Low-molecular-weight protamine	SpCas9 RNP	[90]
	A549 cells; xenograft in nude mice	Plk1	PEI-coated upconversion nanoparticles	SpCas9 RNP	[91]
	A549 cells; xenograft in nude mice	Plk1	Cationic polymer-coated Au nanorod	(SpCas9 and sgRNA) expression DNA	[95]
	NSCLC; xenograft in nude mice	FAK	Retrovirus	SpCas9 expression DNA and sgRNA expression DNA	[92]
	A549 and H1299 cells	NESTIN	Lipofectamine 2000	(SpCas9 and sgRNA) expression DNA	[93]
	H460 cells and H1299 cells	RSF-1	Lipofectamine 2000	(SpCas9 and sgRNA) expression DNA	[96]
	Lewis lung cells; xenograft in B6/C57 mice	CTNNND2	PEI	(SpCas9 and sgRNA) expression DNA	[94]
	HCC827 cells	IGF1R	X-tremeGENE 9 DNA transfection reagent	SpCas9 expression DNA and sgRNA expression DNA	[87]

emphysema patients with AATD [62–64]. The protective action of augmentation therapy is very effective in improving wild-type AAT circulating levels, and lung function can be further improved via weekly or biweekly intravenous infusion of AAT protein [64]. However, several unavoidable downsides are associated with this therapeutic option, such as frequent dosing, inconvenient intravenous infusion and expensive costs [49]. Moreover, owing to the uncertainty of the optimal dose, the immunomodulatory properties of AAT protein may be compromised, leading to the insufficient response during the augmentation therapy [49]. These limitations may be avoided by continuous expression of wild-type AAT by gene therapy. To this end, augmentation therapy with AAV vectors has been implied as an indirect way to improve the gene expression level of wild-type AAT to maintain the steady plasma concentration. As SERPINA1 gene therapy only requires a single systemic administration for AATD treatment, such a way can considerably lower the burden of the expensive, weekly AAT protein infusions in conventional augmentation therapy. Several early studies have also indicated that the systemic delivery of SERPINA1 gene by AAV increased the levels of circulating AAT after intramuscular injection [65–67]. Furthermore, the administration of lentiviral vectors or replication-deficient adenoviral vectors carrying SERPINA1 was also conducted in combination with haematopoietic stem cell therapy [68,69]. These viral gene therapies increased the levels of circulating AAT in tissues outside the liver, which might lead to increased serum levels to protect AATD patients from lung diseases. In addition to above new therapeutic strategies, SERPINA1 gene silencing [70], intracellular degradation of misfolded AAT [71–73], improvement of AAT folding [74–76], and inhibition of AAT polymerization [77–79] have also been explored for the AATD treatment. To silence SERPINA1 gene, RNA interference (RNAi) was exploited to evaluate in preclinical studies. Although liver injury were completely ameliorated following the silence of mutated SERPINA1 genes, the systemic delivery of SERPINA1 siRNA could result

in lung damage, due to the non-specific inhibition of wide-type AAT expression that is critical to maintain the normal lung functions [70]. Thus, transfection with wild-type gene or augmentation therapy is necessary to counteract the severe adverse effects associated with the use of SERPINA1 siRNA. In addition, therapeutic methods that accelerate the intracellular degradation of mutant AAT to enhance autophagy and subsequently reduce the burden of mutation AAT protein in hepatocytes have demonstrated superior anti-inflammation effects for liver disease. These methods reply on the administration of small-molecular drugs like rapamycin and carbamazepine, or the use of iPSCs [71–73]. Moreover, the application of chemical chaperone approaches to improve correct folding of AAT, by using either 4-phenyl butyric acid or suberoylanilide hydroxamic acid, has also shown as a potential supplementary therapeutic strategy for the treatment of liver diseases [74–76]. Lastly, the delivery of peptides have been proven to inhibit AAT polymerization both *in vitro* and *in vivo* [77–79]. Data from *in vivo* animal model experiments have shown that these peptide-based therapeutic approaches can directly modulate the conformational behaviour of AAT. By targeting polymerized AAT themselves, mutant AAT polymerization process can be disrupted to maintain its original confirmation. However, the major caveat associated with these therapeutic peptides is that they have a similar structure to the reactive centre loop of AAT, which can generate complexes with AAT that do not show anti-protease activity.

2.2.2. Recent development of genome editing for AATD

Wiseman and co-workers used adenovirus expressing Cas9 and sgRNA targeting SERPINA1 to ameliorate AATD phenotype in a humanized AATD mouse model (Fig. 8) [8]. One single intravenous dose led to a complete reversal of the PiZ phenotype, which included reduced transaminase and AAT protein levels in the serum. Furthermore, liver histology confirmed the levels of hepatic fibrosis and protein

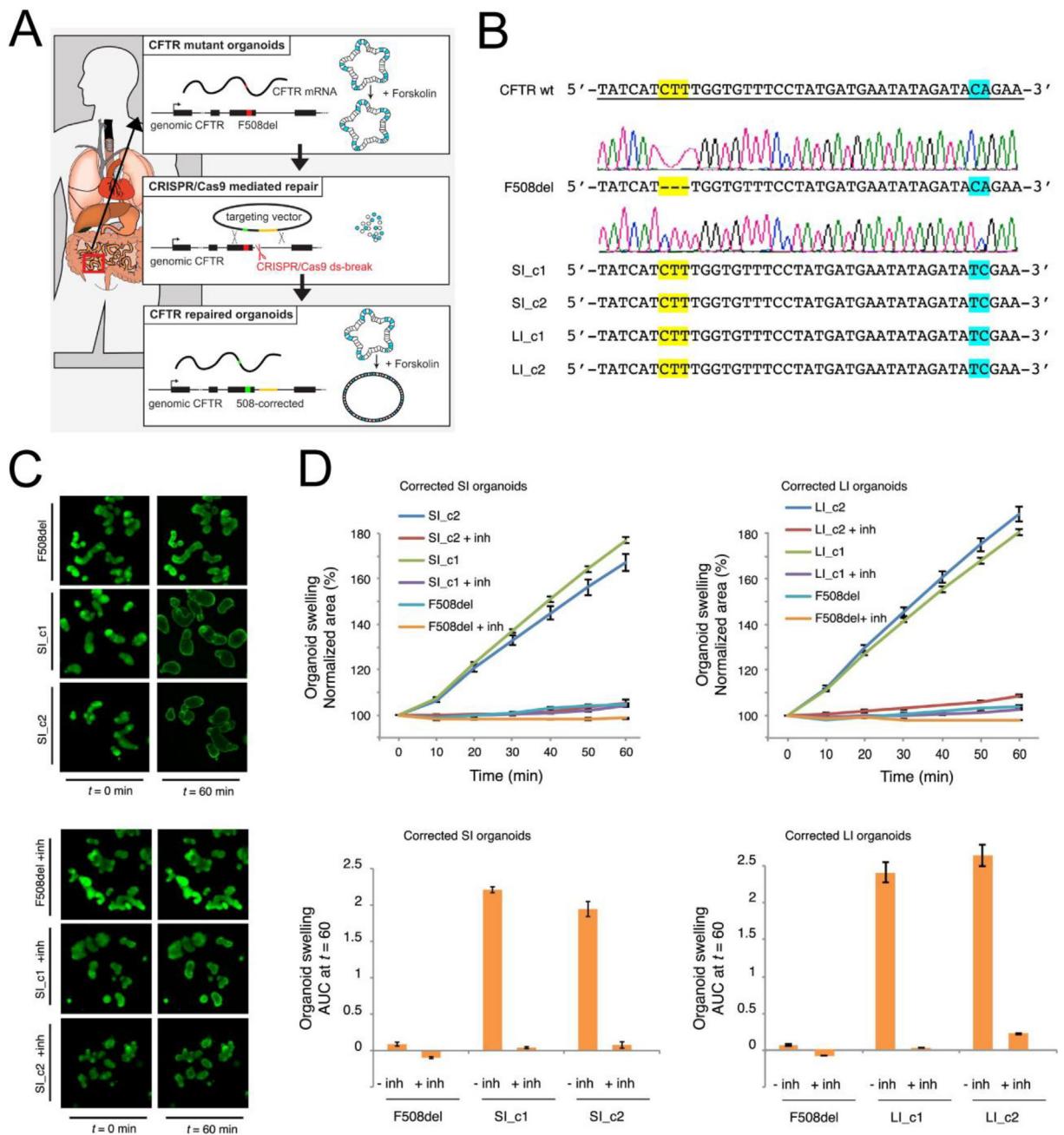


Fig. 4. Functional repair of *CFTR* by CRISPR/Cas9-mediated genome editing in intestinal stem cell organoids derived from CF patients. (A) Schematic illustration of the gene correction by homologous recombination in intestinal stem cells derived from CF patients with a single-gene hereditary defect. (B) Sanger sequencing of the recombinant allele confirmed correction of the F508 del allele. (C) Confocal images of calcine-green-labelled and forskolin-stimulated small intestinal organoids in the absence and in the presence of a chemical *CFTR* inhibitor. (D) Quantification of organoid swelling of corrected small intestinal organoid clones and large intestinal organoid clones. Adapted with permission from [3]. Copyright (2013) Elsevier.

aggregation reduced significantly in gene-edited AATD mice compared to the control. Genomic analysis of liver tissue revealed significant disruption of the *SERPINA1* transgene sequences in gene-edited PiZ mice.

In an attempt to ameliorate AATD phenotype, Xue and co-workers used AAV to deliver Cas9 and sgRNA targeting *SERPINA1*, and demonstrated that CRISPR-mediated AAT correction reduced *SERPINA1* transcription and circulating AAT protein levels, and liver fibrosis in the PiZ mouse via NHEJ (Fig. 9) [6]. Because the PiZ mutation of glutamate 342 to lysine is the most common variant among AATD patients, HDR is therefore the best approach to correct the mutation *in vivo*. To partially ameliorate AAT deficiency in transgenic PiZ mice, viral gene therapy using two separate AAV vectors was explored. Whereas AAV9

vector was used to deliver CRISPR/Cas9, AAV8 vector carried a mutant-allele specific sgRNA targeting AAT and a HDR template. After these two AAVs were co-administered into either neonatal PiZ mice by tail-vein injection or adult PiZ mice by intraperitoneal injection, both common AAT mutation and wild-type AAT in the serum were restored in two models. Furthermore, deep-sequencing analysis confirmed the effectiveness of genome editing and precise HDR in the liver genomic DNA of PiZ mice. This proof-of-principle study establishes a fundamental cue for CRISPR/Cas9-mediated genome editing for the therapy of AAT mutation in the transgenic mouse model.

By using CRISPR/Cas9, Albright and co-workers also developed two therapeutic strategies to decrease AAT protein aggregates in

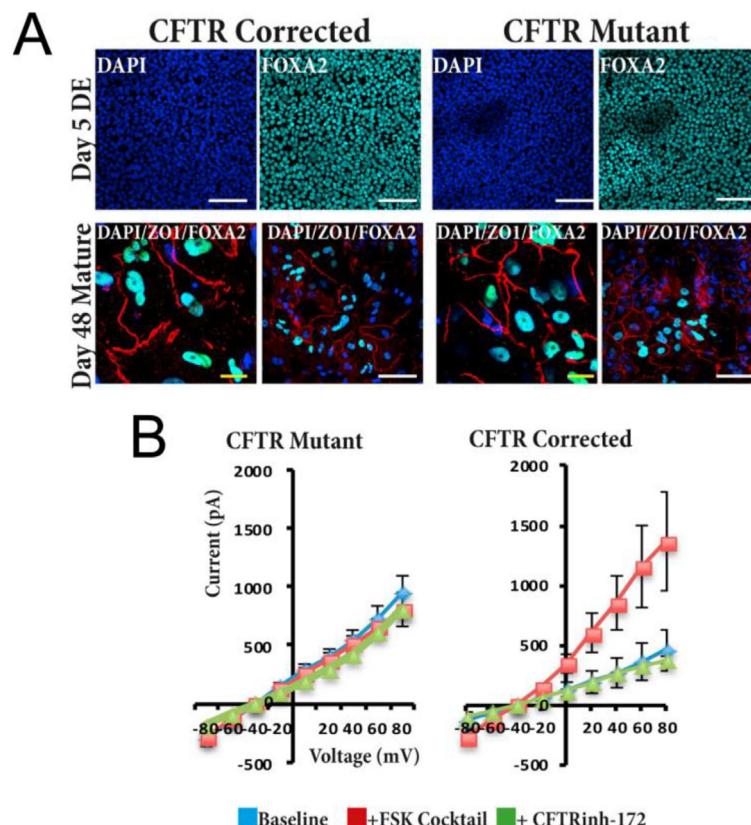


Fig. 5. CRISPR-mediated gene editing in lung epithelial cells generated from patient iPSCs. (A) Representative confocal images show CF-corrected and CF-mutant iPSC differentiation. (B) Averaged CFTR chloride current/voltage plots for baseline (blue), in the presence of forskolin, genistein, and isobutylmethylxanthine (pink) and with the addition of specific *CFTR* inhibitor CFTRinh-172 (green). Adapted with permission from [4]. Copyright (2015) Elsevier. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

hepatocytes and increase systemic wild-type AAT levels in the adult PiZ transgenic mouse model (Fig. 10) [7]. To reduce liver aggregates *in vivo*, SaCas9 and sgRNA components were cloned into one plasmid to obtain the “all-in-one” AAV8-CRISPR vector. Following the systemic treatment of AAV8-CRISPR vector targeting exon 2 of *SERPINA1*, AAT-Z protein expression in hepatocytes was significantly down-regulated in the adult transgenic mice. For HDR repairing, the SaCas9 delivered in tandem with sgRNA/donor template were explored using dual AAV vectors to correct Z mutation *in vivo*. This dual-AAV vector system could introduce targeted genome correction of the Z-mutation in exon 5 of *SERPINA1* locus *in vivo*. These works collectively demonstrate the potential of CRISPR/Cas9 systems for correcting the PiZ mutation in human cells, and offer fundamental mechanisms for the genetic correction of AAT mutation through a humanized mouse model.

2.3. Lung cancer

Lung cancer is becoming one of the most common malignancies with high morbidity and mortality worldwide [80]. The progression of lung cancer involves multiple gene mutations and signalling pathways [81]. The inhibition of specific proteins and the correction of defective genes have been widely explored for the treatment of lung cancers. Most recently, CRISPR/Cas9-based genome-editing strategies has been proposed for the therapy of lung cancers, including editing proto-oncogenes, tumor-suppressor genes, or chemotherapy resistance-related genes (Fig. 11) [82–84].

2.3.1. CRISPR/Cas9-mediated genome-editing of proto-oncogenes

Proto-oncogenes associated with lung cancer include catenin delta 2 (*CTNND2*), remodeling and spacing factor 1 (*RSF1*), *IGF1R*, epidermal

growth factor receptor (*EGFR*), *SRC*, *FAK*, *CD38*, *RAS*, *NESTIN*, *ROS1*, and *FOS*, etc [85–95]. These genes can function as oncogenes to enhance the invasive and metastatic ability of cancer cells, and can also promote lung cancer progression. Oncogene addiction, namely the inhibition of tumor growth following the inactivation of a single proto-oncogene in cancer cells, provides adequate theoretical basis for CRISPR/Cas-based genome-editing technology for cancer therapy. By using CRISPR/Cas9, lung cancer-related proto-oncogenes which are overexpressed, mutated, or overactivated can be knocked out, resulting in the loss of tumorigenic and metastatic functions. In recent years, oncogenes such as *EGFR*, *RSF1*, *NESTIN*, *CTNND2*, *FAK*, and *IGF1R*, have been studied in the context of therapeutic genome editing for lung cancer treatment [82,89,96–98].

2.3.2. CRISPR/Cas9-mediated genome-editing of tumor-suppressor genes

The inactivation of tumor-suppressor genes also plays a key role in the progression of lung cancer. Lung cancer-related tumor-suppressor genes include tumor protein P53 (*TP53*), cyclin dependent kinase inhibitor 2A (*CDKN2A*), *RB*, *APH*, *MCC*, *APC*, and *NM23*, etc [99,100]. The expression proteins of these tumor-suppressor genes can inhibit colony formation and cell proliferation, prevent cell invasion and migration, and exhibit tumor regression during tumorigenesis [100–102]. In recent years, tumor-suppressor genes that have been edited with CRISPR/Cas9 technology include mitofusion 2 (*MFN2*) [103], tumor-suppressor *miR-1304* [104], and Kelch-like ECH associated protein 1 (*KEAP1*) [105]. Among these, one study using CRISPR/Cas9 to knockout *MFN2* in the lung cancer cell line (A549) showed increased cell viability and promotion of cell survival [103]. Another study confirmed that genetic knockout of *miR-1304* in the lung cancer cell line A549 showed promotion of cancer cell survival and growth, colony formation, and metastasis [104].

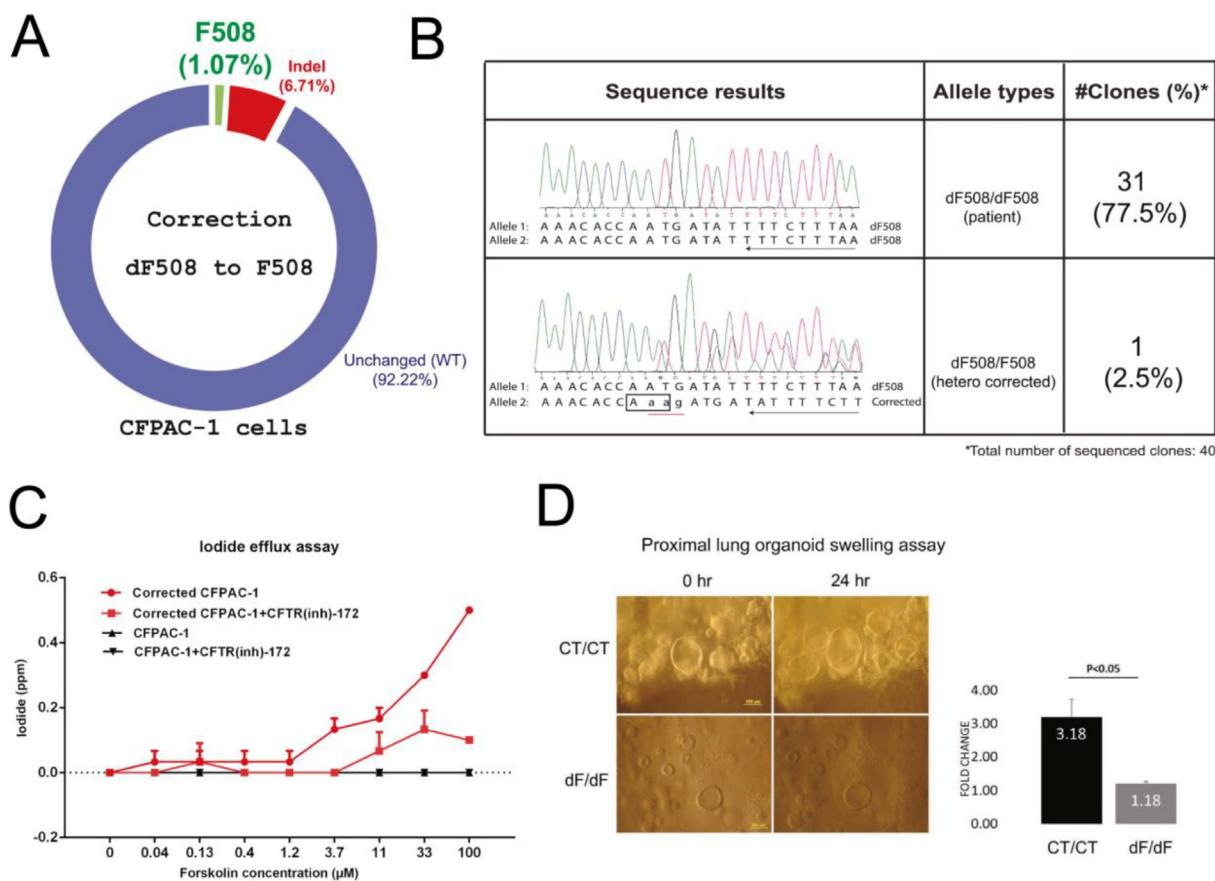


Fig. 6. Restoration of *CFTR* function in CFPAC-1 cells and iPSC-derived proximal lung organoids by CRISPR-mediated gene editing. (A) Correction efficiencies in patient-derived CFPAC-1 cells was determined by deep sequencing. (B) Efficiency in establishing a heterozygously corrected CFPAC-1 cell clone. (C) Iodide efflux assay using uncorrected CFPAC-1 and heterozygously corrected CFPAC-1 cells. (D) Forskolin-stimulated swelling assay using gene-corrected iPSC-derived proximal lung organoids. Adapted with permission from [5]. Copyright (2019) Elsevier.

In addition, the genetic knockout of *KEAP1* gene in a mouse model of lung adenocarcinoma could increase cell viability, and promote tumor survival and growth [105]. These studies reveal that the genetic knockout of tumor-suppressor genes promotes tumor growth, invasion, and metastasis. Thus, the correction of target tumor-suppressor genes which are mutated or poorly expressed in the lung cancer cells by employing CRISPR-based genome-editing technology may inhibit lung tumor growth.

2.3.3. CRISPR/Cas9-mediated genome-editing of chemotherapy resistance-related genes

In clinical practice, multidrug resistance to chemotherapy is the one of the main reasons of the poor therapeutic outcomes in the treatment of malignant lung tumors [106]. By targeting multidrug resistance-related genes, the resistance of tumors to molecular targeted drugs or other inhibitors would become compromised, thereby contributing to the improvement of lung cancer treatments. In recent years, numerous studies have shown that the disruption of drug-resistant genes can improve the sensitivity of lung cancer cells to chemotherapeutics to reverse chemoresistance during cancer treatment. For example, the genetic knockout of *IGF1R* gene in HCC827 cell line (a human NSCLC cell line) could significantly reduce chemotherapy resistance to erlotinib [82]. Likewise, the knockout of nuclear factor 2 (*NRF2*) gene significantly increased the drug sensitivity to cisplatin and carboplatin in A549 cells [107]. The mouse model xenografted with NSCLC cells in which the *AURKB* was knocked out by CRISPR/Cas9 became sensitive to paclitaxel and cisplatin [108].

Collectively, genome-editing of proto-oncogenes, tumor-suppressor genes, and chemotherapy resistance-related genes by CRISPR/Cas9 offers a great potential for lung cancer treatment.

2.4. COPD and asthma

COPD is a common heterogeneous inflammatory disease with high global morbidity and mortality, and it is currently expected to be the third largest cause of chronic illness and related death [109,110]. COPD, which mainly includes chronic obstructive bronchiolitis and emphysema, is characterized by progressive and irreversible airway obstruction, fibrosis, narrowing and remodelling of the small airways and the destruction of alveoli walls [109,111]. Smoking is generally accepted as the major risk factor for COPD development, but the exposure to environmental irritants and genetic predisposition also contribute to the development of COPD [54,109]. Similar to COPD, asthma is also a global health issue characterized by respiratory symptoms that result from a complex interplay between environmental irritants and genetic predisposition [112].

2.4.1. Regaining steroid sensitivity in asthma and COPD

Anti-inflammatory medications, especially inhaled corticosteroids, are the most frequently used anti-inflammatory therapeutic agents for COPD and asthma patients [109,112]. However, most of patients with severe COPD and asthmatics show poor response to corticosteroids. Corticosteroid resistance is now considered as a major therapeutic challenge in the treatment of severe COPD and asthma [113,114]. Thus, the

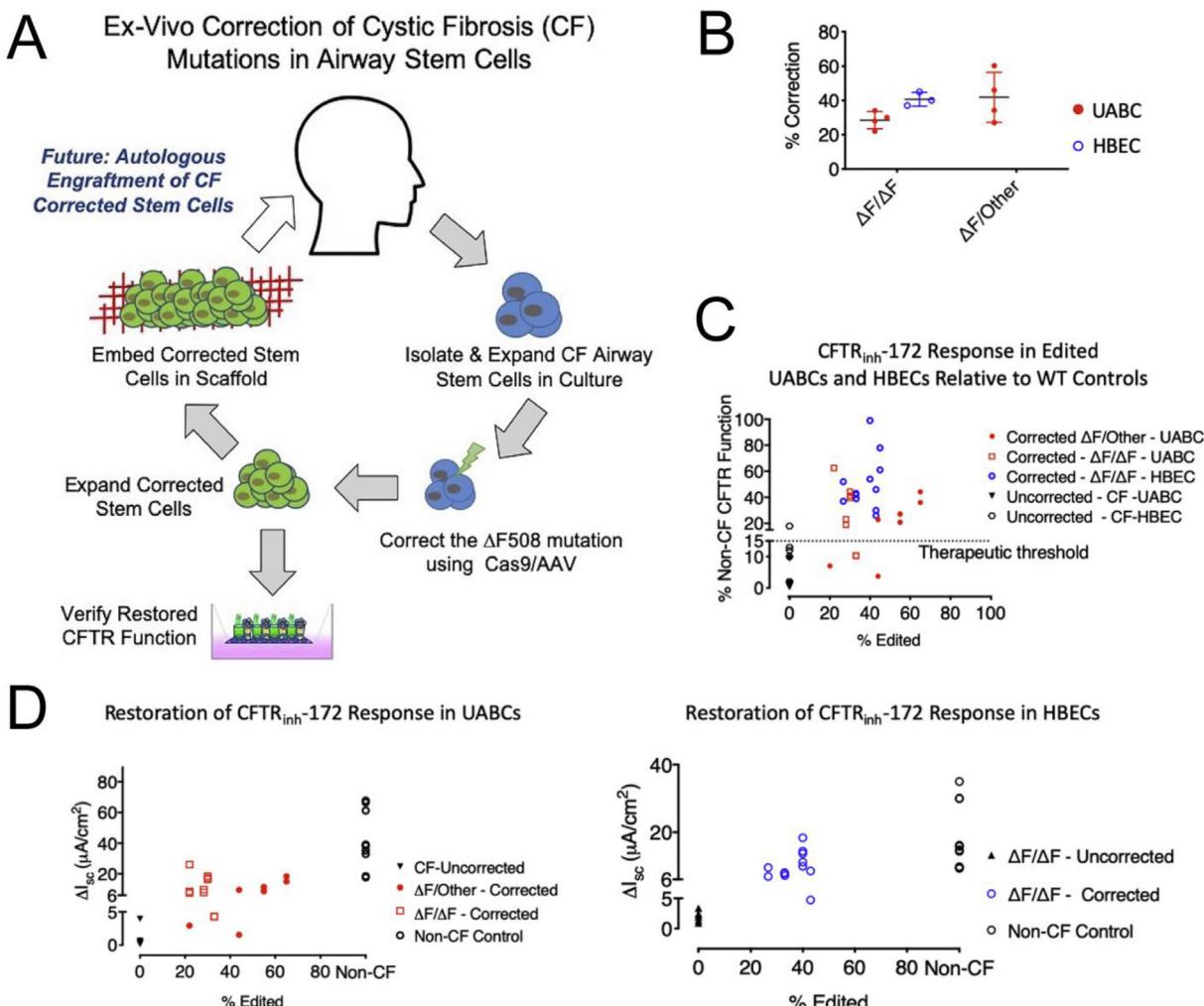


Fig. 7. Restoration of CFTR function in airway stem cells from CF patients by CRISPR-mediated gene editing. (A) Schematic illustration of the ex vivo correction of CF mutations in airway stem cells. (B) Allelic correction in compound heterozygous UABC samples. (C) CFTR_{inh}-172 sensitive short-circuit currents observed in corrected CF samples as a percentage of non-CF controls. (D) Restoration of CFTR_{inh}-172 response in corrected UABCs and HBECs samples. Adapted with permission from [48]. Copyright (2020) Elsevier.

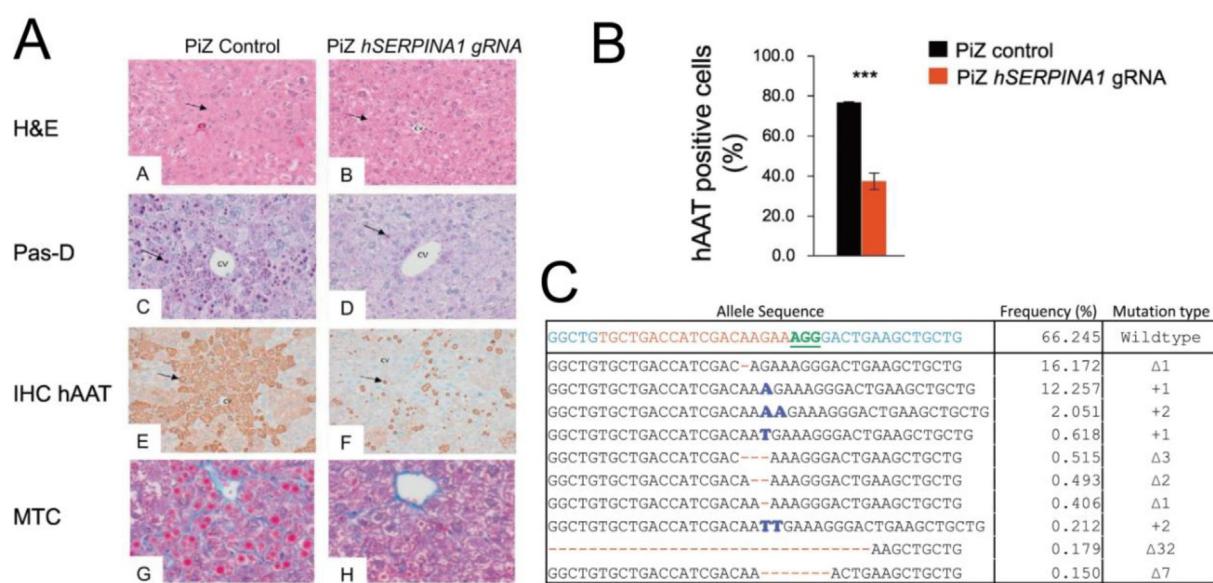


Fig. 8. Therapeutic genome editing with CRISPR/Cas9 in a humanized mouse model ameliorates AATD phenotype. (A) Cas9-sgSERPINA1 mediated reversal of liver globule accumulation, liver fibrosis and AAT protein aggregation in the livers of PiZ mice. (B) Automated quantification of IHC for AAT. (C) Most frequent alleles following therapeutic genome editing at SERPINA1 target site in the liver samples are shown. Adapted with permission from [8]. Copyright (2018) Elsevier.

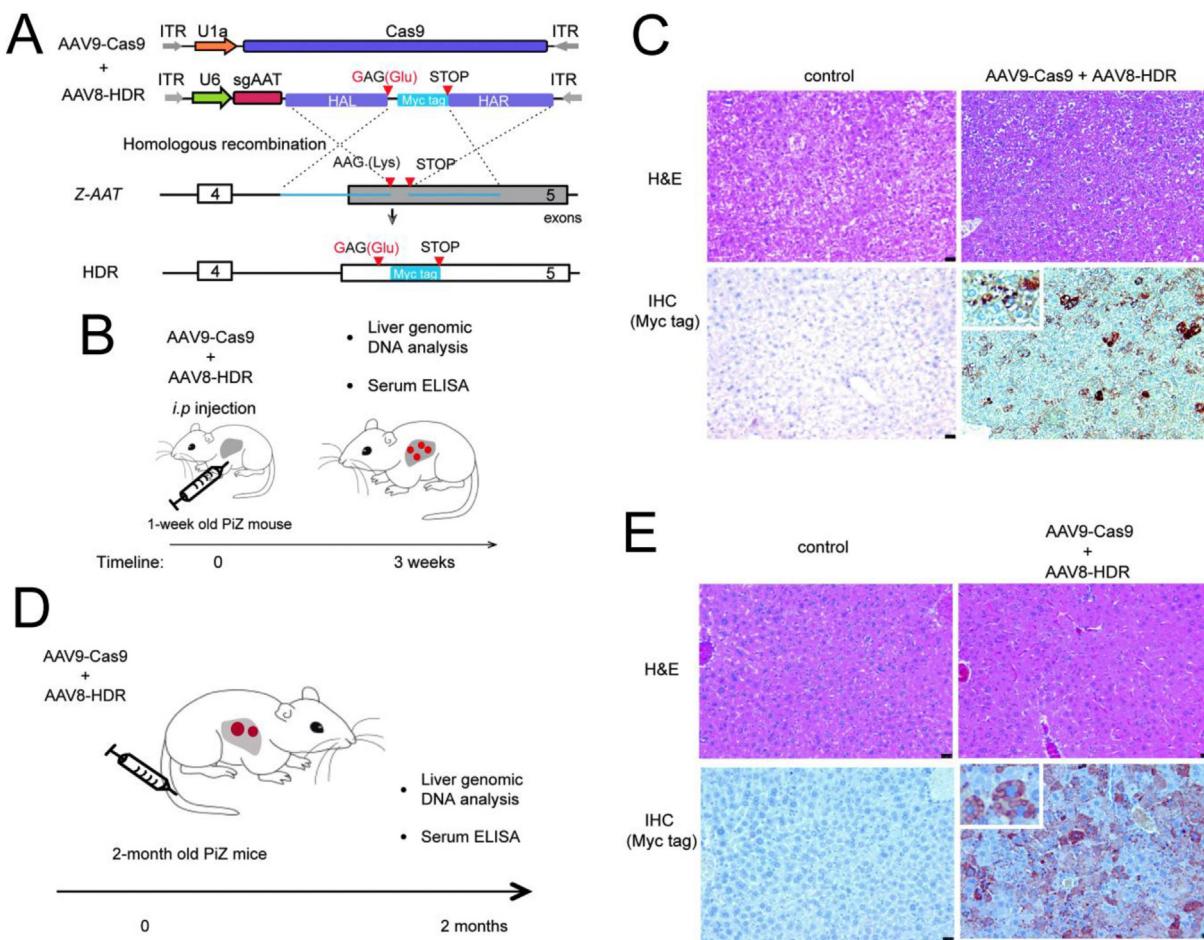


Fig. 9. *In vivo* genome editing partially restores AAT in a murine model of AATD. (A) The design of AAV-HDR template and experiments. (B) *In vivo* delivery of AAV-HDR and AAV9-Cas9 in 1-week-old PiZ mice partially restores AAT in the serum. (C) Immunohistochemistry with Myc-tag antibody. (D) Two-month-old PiZ mice were injected with AAV-HDR and AAV9-Cas9 by tail-vein injection. (E) Immunohistochemistry with Myc-tag antibody. Adapted with permission from [6]. Copyright (2018) Mary Ann Liebert, Inc.

evaluation of effective and innovative therapeutic technologies to regain corticosteroid sensitivity in asthma and COPD is imperative. Recent genetic, biochemical and histological evidence revealed that various elevated pro-inflammatory cytokines, such as thymic stromal lymphopoietin (*TSLP*), *IFN- γ* , *TNF- α* , *IL-17A*, *IL-33* may compromise the anti-inflammatory efficacy of corticosteroid in the treatment of severe COPD and asthma [115]. These pro-inflammatory cytokines together with pathogens, allergens, and other environmental irritants can modulate multiple signaling pathways, such as heightened glucocorticoid receptor (GR) β /GR α ratio, JAK1/2-STAT1/5, PI3Kd/Akt/Mtor, casein kinase 1 (CK1d/e)/cofilin 1, p38MAPK/JNK etc, to impair the anti-inflammatory efficacy of corticosteroids and induce corticosteroid insensitivity [115]. More recently, therapeutic strategies such as specific small-molecule inhibitors, cytokine-specific biologics, and microRNA oligonucleotides have been implicated for the reversal of corticosteroid sensitivity [116–122]. Whereas previous therapeutic strategies are introduced into target cells or tissues by targeting specific abnormal genes at the mRNA and protein level in general, genome editing works by correcting the defective gene at the DNA level. Particularly, genome editing by CRISPR/Cas9 represents a promising approach to reverse corticosteroid resistance. Therefore, the disruption of corticosteroids resistance-related genes which are overexpressed, or overactivated in the airways and alveolar sacs via CRISPR/Cas9 technology may provide better therapeutic outcomes in the long term (Fig. 12). Given its powerful ability to correct defective genes, further systematic and in-depth investigations on the potential of CRISPR/Cas9 genome-

editing technology in treating COPD and asthma will be indispensable to its successful implementation in clinical application, although no study was reported to validate the effectiveness of COPD and asthma treatment so far. The above studies indicate that genome editing of malfunctioning genes related to corticosteroid resistance would be an effective therapeutic option for COPD and asthma, in combination with steroid therapy.

3. Delivery technologies for genome editing

3.1. Viral delivery of genome editing systems

Viral delivery is the most commonly used approach to transfer plasmids encoding genome-editing machinery into cells and/or tissues [123]. Lentivirus, adenovirus and AAV have been extensively applied in delivering plasmids encoding genome-editing machinery into the host genome as well as extrachromosomal expression of the genome-editing machinery both *in vitro*, *ex vivo* and *in vivo* [40]. Although viral vectors still represent the dominant class of delivery systems for *in vitro* and *in vivo* gene therapy, the history of viral vector-mediated gene therapy has also been plagued by serious safety concerns. Viral vectors often induce immunogenicity, insertional mutagenesis and tumorigenic risks [124]. The constitutive expression of CRISPR-associated nucleases by virus-mediated transfection also increases the risk of off-target editing, and the packing capacity of viruses also

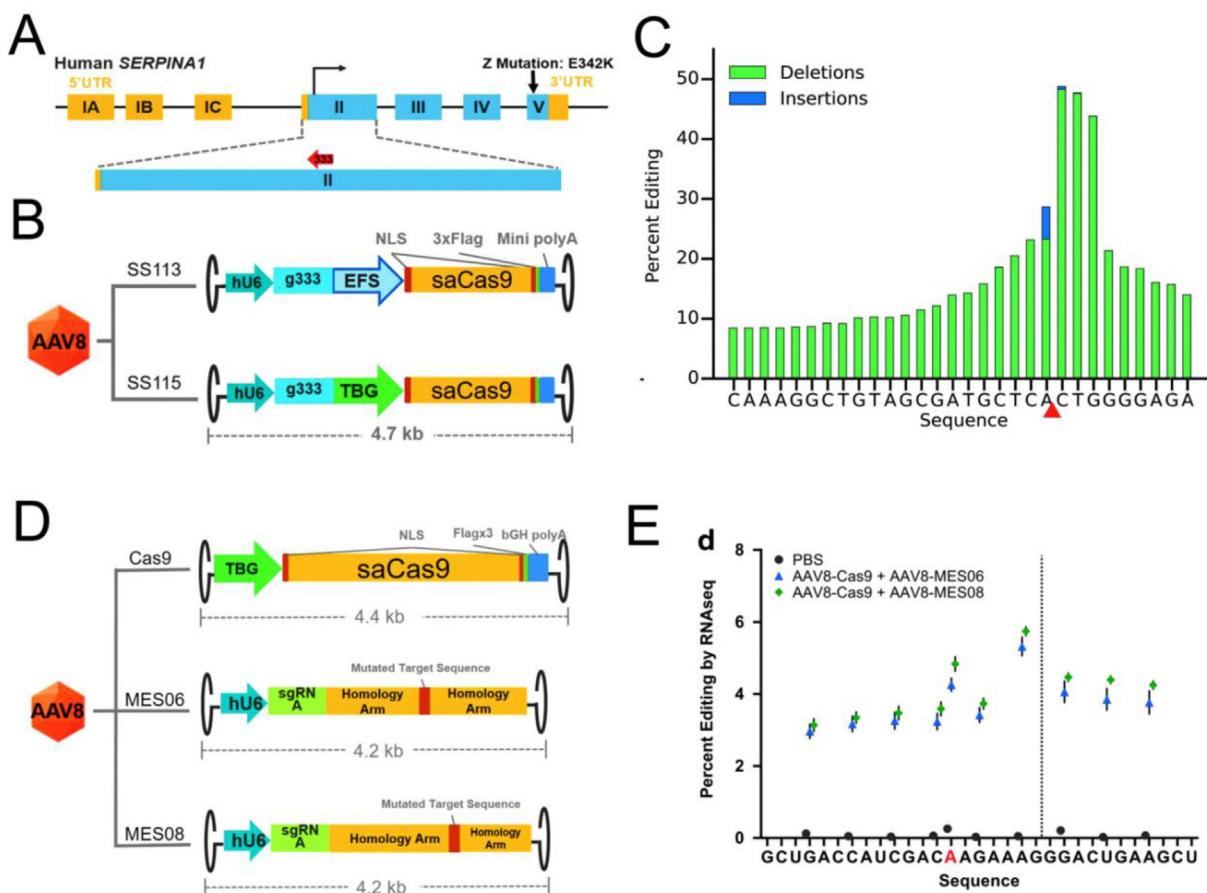


Fig. 10. Amelioration of AATD diseases with genome editing in transgenic mice. (A) Schematic representation of the human SERPINA1 locus in PiZ patients. (B) Configurations of two AAV8-CRISPR vectors tested in adult PiZ transgenic mice. (C) Representative RNAseq analysis near the corresponding Cas9 cut site on SERPINA1 from one PiZ mouse. (D) A dual-vector AAV8-CRISPR system corrects the Z-mutation in adult PiZ transgenic mice. (E) RNAseq analysis showing percentage of nucleotide substitutions near the SaCas9 cut site on SERPINA1 transcripts. Adapted with permission from [7]. Copyright (2018) Mary Ann Liebert, Inc.

hindered their delivery of CRISPR/Cas9 plasmids, the size of which usually exceeds the packing limit of viruses [125].

3.2. Non-viral delivery of genome-editing systems

3.2.1. Nanoparticle-mediated delivery

In comparison with viruses, non-viral vectors show the potential to be safer and less immunogenic owing to their transient expression patterns, allowing for the repeated administration to boost the final therapeutic efficacy. Many types of non-viral vectors have been demonstrated to be effective in delivering genome-editing agents into target tissues, including eyes, ears, livers and muscle tissues, etc. These delivery materials mainly include cationic polymers, lipid nanoparticles, cell-penetrating peptides, synthetic virus-like nanoparticles, DNA nanoclews, gold nanoparticles, cell-derived vesicles, protein-mediated nanocarriers and other inorganic materials [124–126]. Among these materials, polymers and lipids are considered to be the most promising candidates for the cytosolic delivery of CRISPR-associated nucleases, owing to their ease of synthesis and functionalization, biocompatibility and effectiveness in delivering large plasmid [95,127,128]. The detailed, comprehensive review of non-viral vectors for the delivery of CRISPR/Cas9 components could be found in our previous review [124].

3.2.2. Physical methods

Electroporation and other strategies such as microinjection, hydrodynamic injection, and membrane deformation can create transient pores in the cell membrane for effective intracellular delivery of genome

editing agents into the cytoplasm/nucleus [40]. However, local electroporation and other physical methods are limited to a few targeted tissues due to the difficulties for *in vivo* applications. Meanwhile, concerns of cell toxicity and lethal acute side effects associated with physical methods still exist.

4. Delivery routes for genome editing of the lung genetic disorders

4.1. Oral administration

As a non-invasive route, oral administration offers patients rapid onset of action, ease of ingestion, good compliance, painless and safety, as compared with other routes of administration. Although oral delivery of CRISPR/Cas9 elements has not yet been demonstrated to date, the delivery of biomacromolecules like siRNA has previously shown the potential to treat gastrointestinal tract-specific diseases, such as ulcerative colitis and gastrointestinal tract cancer, and to promote systemic therapeutic effects in other organs like lung [129]. For example, Yin and co-workers constructed multifunctional mannose-modified trimethyl chitosan-cysteine nanoparticles for the oral delivery of TNF- α siRNA [130]. These orally delivered nanoparticles reduced TNF- α in the serum and diminished TNF- α mRNA levels in lung macrophages. The above study provides a proof-of-concept example of oral delivery of biomacromolecules as an effective strategy for the treatment of pulmonary inflammation. Nevertheless, for the treatment of lung genetic disorders, the oral route is less favorable for the administration of therapeutic agents. To deliver drugs to lungs, various barriers exist. First,

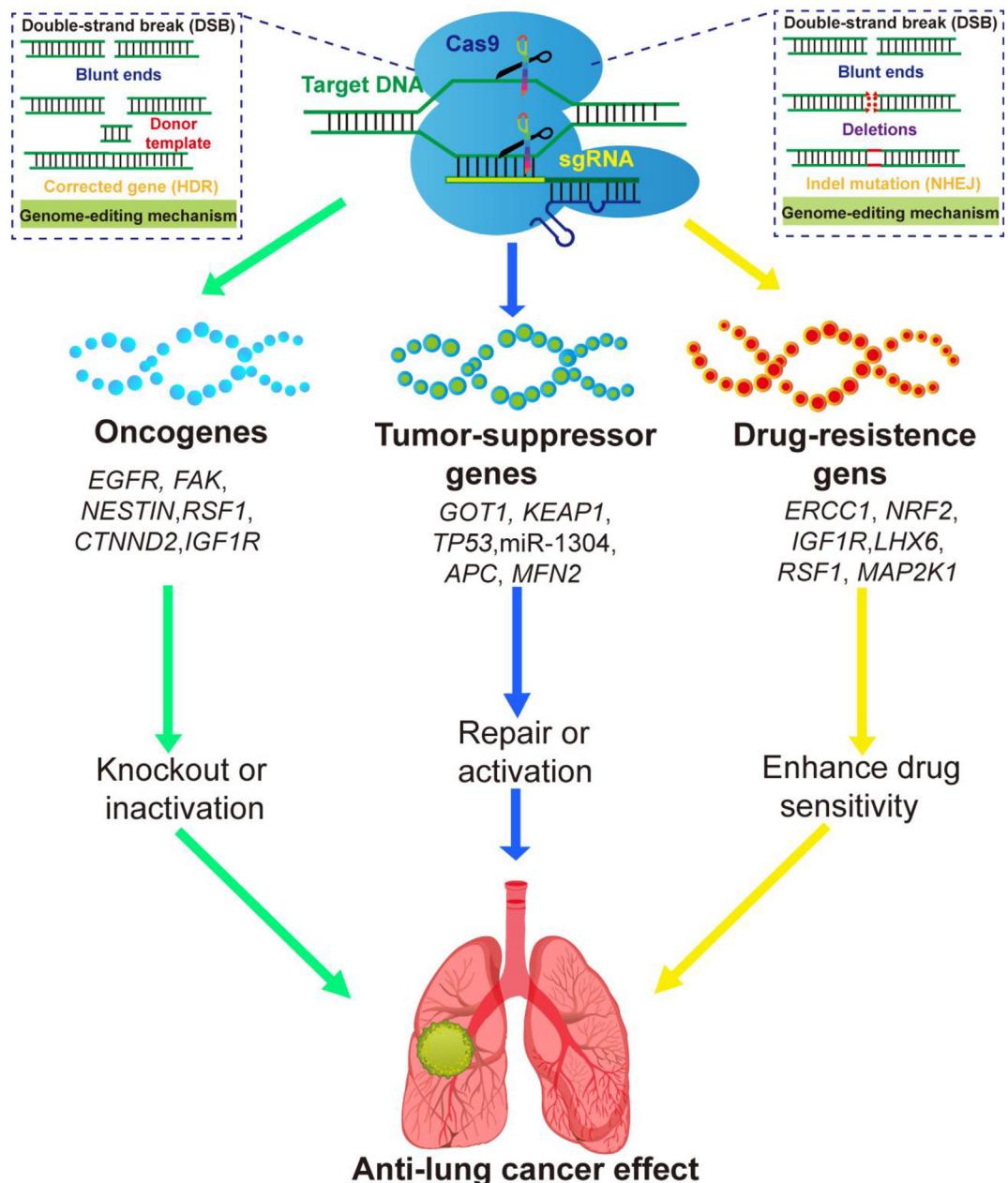


Fig. 11. Applications of CRISPR/Cas9 technology in the treatment of lung cancer. CRISPR/Cas9-mediated gene editing of proto-oncogenes, tumor-suppressor genes, and drug-resistance genes offers great potentials as a treatment modality for lung cancer.

therapeutic agents must enter mesenteric vein blood circulation through the capillaries of the gastrointestinal tract after oral administration. Then, these agents are absorbed by liver through the portal vein. After being processed by the liver, the therapeutic agents enter into the inferior vena cava through the hepatic vein and flow backwards to the right atrium and right ventricle. Finally, those remaining agents appeared in the pulmonary artery from the right ventricle can eventually flow into the capillaries in the lung. As a result, by oral administration, only passing through a series of complicated transport processes can the therapeutic agents finally reach the lung, and such complicated processes would seriously compromise the bioavailability of therapeutic agents. In addition, oral administration of biomacromolecules for lung gene therapy typically reach the lung at extremely low concentrations due to numerous physical and physiological factors such as chemical and enzymatic degradation in the gastrointestinal tract, low

permeability across the gastrointestinal mucosa, first-pass metabolism, and clearance by the liver or the gut mucosa [131]. In order to be therapeutically effective, higher frequency of administrations or doses is essential, which is often associated with severe side effects. To summarize, these numerous factors make the oral route is not preferred for the administration of therapeutic agents.

4.2. Intravenous injection

To date, the systemic administration of therapeutic agents for lung gene therapy is the most favourable administration route in both experimental and preclinical settings [9]. Delivery of biomacromolecules or the combination therapies to simultaneously deliver both biomacromolecules and small molecules via the intravenous route, has been extensively explored for the therapy of lung genetic disorders in

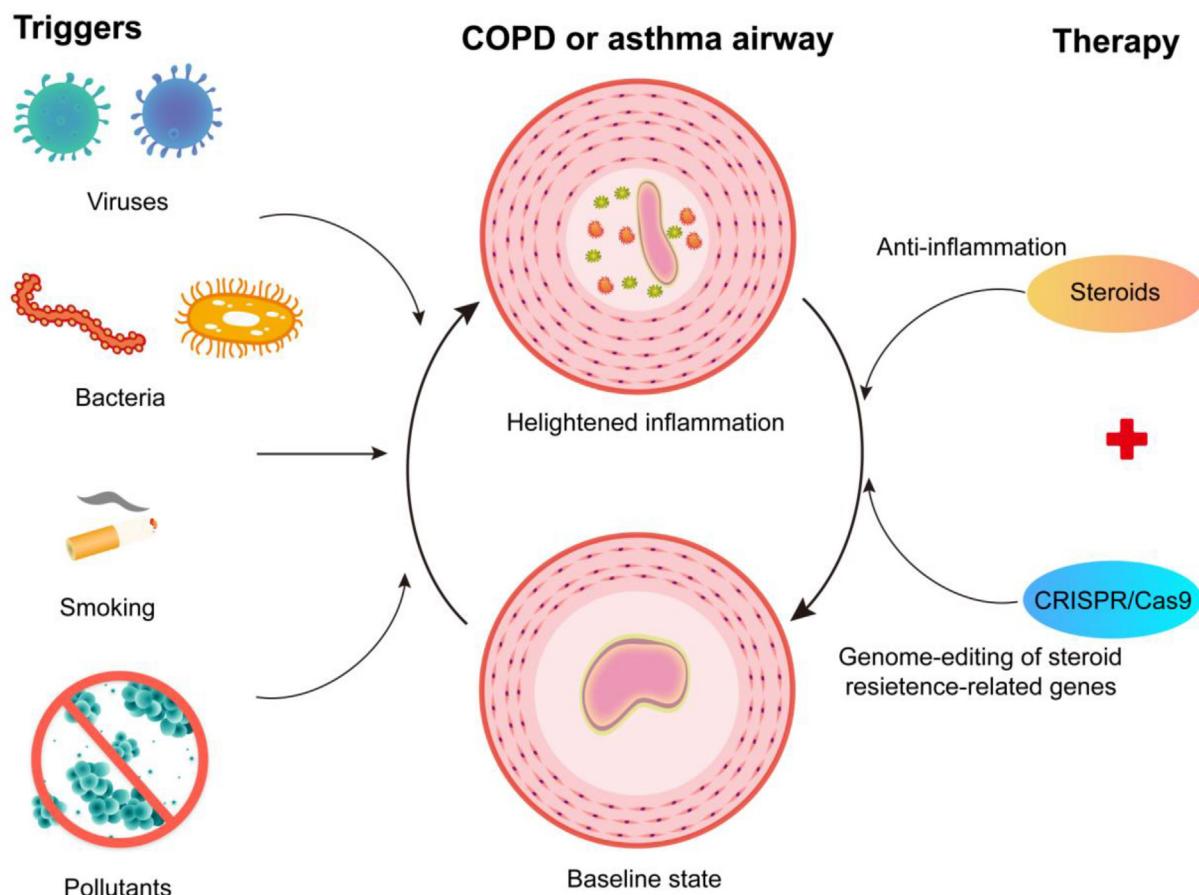


Fig. 12. Applications of CRISPR/Cas9 technology in the treatment of COPD and asthma. Increased inflammation caused by viruses, bacteria, smoking or pollutants results in inflammation of the COPD or asthma airways, which further causes airway narrowing and systemic inflammation. Most of the severe asthmatics and COPD patients show poor response to the anti-inflammatory benefits of corticosteroids. Genome editing of malfunctioning genes related to corticosteroid resistance would be an effective therapeutic option for COPD and asthma, in combination with corticosteroid therapy.

recent years [131]. For example, Zhang and co-workers developed a multifunctional nanoparticles co-delivering Zeste Homologue 2 (EZH2) siRNA and etoposide for the synergistic therapy of NSCLC [132]. *In vivo* distribution studies showed that the pH/redox dual sensitive nanoparticle exhibited a low retention in normal organs, but an obvious distribution in the orthotopic tumor after a single intravenous administration, thereby synergistically suppressing malignant proliferation of NSCLC. One concern with systemic administration is the rapid degradation of these therapeutic biomacromolecules by nucleases and the excretion via the kidneys, and as a result, the intravenous injection of therapeutic biomacromolecules typically reaches the lung at extremely low concentrations. Even when these biomacromolecules are protected by various vectors, the accumulation in the lung is still poor by systemic administration, leading to the unsatisfactory therapeutic outcomes. In order to increase the concentration in the lung, frequent intravenous administrations or high doses are often essential; however, these therapeutic strategies often cause undesirable side effects, such as superficial phlebitis due to drug extravasation injury. More seriously, some patients even suffer from local subcutaneous ulcer and necrosis after frequent intravenous administrations.

4.3. Intratracheal/intranasal instillation

Intratracheal instillation was initially described as an exposure technology to evaluate respiratory tract pharmacological and toxicological properties of test agents, especially some highly toxic, carcinogenic, or radioactive materials. It was later adopted to assess pulmonary delivery of therapeutic agents in animal studies [133]. Intratracheal instillation

exhibits advantages of avoiding the oropharynx deposition, accurate administration of therapeutic agents to the lower respiratory tract, and bypassing upper respiratory tract defenses [134]. Intratracheal instillation also ensures the efficient delivery of biomacromolecules to lung by affording high dose in small volume [135]. Recently, Hattori and co-workers reported that a single intratracheal administration of chitosan/VEGF-siRNA complexes reduced the level of vascular endothelial growth factor (VEGF) in both bronchoalveolar lavage fluid and metastatic lung tumor tissues [136]. Furthermore, the repeated intratracheal administration of chitosan/VEGF-siRNA complexes could significantly suppress the number of visible metastatic foci in lung tissues, as compared with that in the non-target siRNA group. Thus, the intratracheal delivery of biomacromolecules mediated by proper carriers could be a therapeutic strategy for lung cancer. Despite the success of intratracheal route for the delivery of biomacromolecules in animals, it must be noted that intratracheal instillation is not employed as a routine route for human use, due to its non-physiological and extremely uncomfortable characteristics. Therefore, as an artificial way to deliver drugs into the lungs, the clinical practice for intratracheal instillation is very limited. Furthermore, drug deposition by this route tends to be less homogeneous where most drugs are deposited in the basal regions of the lung [137]. Many *in vivo* studies indicate that intratracheal instillation could evoke inflammatory response, especially the serious recruitment of neutrophils to the lung [135,138,139]. Collectively, the intratracheal instillation delivery of biomacromolecules for lung gene therapy is just suitable for proof-of-concept studies.

Intranasal instillation is a local and non-invasive administration route for drug delivery, which is widely used in pulmonary therapy in

animal studies. Several distinguished features of intranasal instillation, such as the easily accessible administration of therapeutic agents, wide absorption area in the nasal cavity, and the high permeability and vascularization of the nasal mucosa, are beneficial for pulmonary drug delivery of therapeutic genome-editing agents [140,141]. By intranasal instillation, Hashida and co-workers developed a cationic liposomes/CpG DNA nanoformulation (CpG DNA lipoplex) to prevent lung metastasis in mice [142]. After intranasal administration, CpG DNA lipoplex mainly distributed in the nose and lung and induced higher IFN- γ production in the lung. In addition, the metastatic nodules were barely detected in the mice treated with CpG DNA lipoplex, whereas metastatic nodules could be obviously observed in the mice treated with naked CpG DNA. These results suggest that intranasal administration of CpG DNA lipoplex particularly influence the colonization of cancer cells in the lung, leading to the considerable inhibition on the incidence of pulmonary metastasis. Edbrooke and co-workers found that intranasal administration of cationic liposomes containing ENac siRNA could induce long-lasting inhibition of ENac expression in human primary bronchial epithelial cells as well as in mice models [143]. Recently, Baldoa and co-workers used liposomes for the nasal delivery of CRISPR-Cas9, aiming to insert a corrective murine α -L-iduronidase (*IDUA*) gene in mucopolysaccharidosis type I (MPS I) mice [144]. The intranasal administration of liposome/CRISPR-Cas9 nanocomplexes in adult MPS I mice significantly resulted in secretion of active *IDUA* enzyme into the plasma and increased *IDUA* enzyme levels in all brain areas. Furthermore, the same group also explored the possibility for nasal delivery of CRISPR/Cas9 by liposomal to correct iduronate-sulfatase (IDS) enzyme deficiency in mucopolysaccharidosis type II (MPS II) mice [145]. Intranasal administration of liposomal CRISPR/Cas9 complexes resulted in sustained serum IDS activity levels in the heart and the lungs. Collectively, these two studies provided proof-of-concept examples for the intranasal delivery of CRISPR/Cas9 elements for the potential treatment of lysosomal disorders. These results indicate the intranasal delivery of biomacromolecules holds great potentials for the treatment of lung diseases. Despite these merits, there are still some drawbacks associated with this delivery strategy. After intranasal instillation, a large amount of administered dose of therapeutics would be trapped in the nasal cavity, leading to the decreased dose of therapeutic agents in the lung [146]. In addition, because mice respire with nose, and the anatomy, microanatomy and physiology of the lungs of mice is different from human, the initial success in pulmonary drug delivery through intranasal instillation in mouse model could not be easily translated to human clinical trials [135].

4.4. Inhalation delivery

As described in the previous studies, inhalation is considered to be the most popular way to deliver therapeutic biomacromolecules into the lungs, and a more friendly physiological way of exposure over intratracheal instillation. Direct delivery of naked therapeutic biomacromolecules into the lungs via inhalation, as well as their complexes with viral and non-viral vectors, allows for the non-invasive, direct access to lungs and avoids the interactions with serum proteins [11,56]. Therapeutic delivery via inhalation also provides other advantages over intravenous administration for lung absorption, such as large alveolar surface area with high vascularization. Inhalable micron-sized liquid droplets generated by nebulizers may carry large amount of genome-editing agents and reach virtually all areas of the lung. Thus, aerosol delivery can ideally generate desirable therapeutic level for lung treatments, and also reduce the dosage of administered biomacromolecular drugs. In addition, the thin air-blood-barrier of the lung is also helpful for absorption, and decreases the rate of drug elimination and metabolism, as oppose to other conventional routes of administration [147]. Last but not least, nebulized delivery of biomacromolecules such as mRNA and DNA well avoids the degradation

Table 4
Advantages and disadvantages of common delivery routes.

Delivery route	Advantages	Disadvantages
Oral administration	Rapid onset of action; ease of ingestion; good compliance; painless and excellent safety	Low bioavailability; higher frequency of administrations
Intravenous injection	Direct access to systemic circulation	Systemic-associated toxicities; low concentrations in the lung
Intratracheal/intranasal instillation	Avoiding the oropharynx deposition; accurate administration of therapeutic agents to the lower respiratory tract; bypassing upper respiratory tract defenses	Non-physiological and extremely uncomfortable characteristics; inflammatory response
Inhalation delivery	Non-invasive; direct access to lungs and avoiding the interactions with serum proteins	Difficulties in developing inhalable formulations

of these nucleic acids by serum nuclease. The advantages and disadvantages of each delivery route were summarized in Table 4. Currently, three main types of inhalation devices are available for the aerosol delivery of drugs or therapeutic nanoparticles to the peripheral airways through inhalation, including metered dose inhalers (MDIs), dry powder inhalers (DPIs) and jet nebulization [9,148]. With appropriate modification and optimization, these various inhalation devices could be tailored for the pulmonary delivery of therapeutic genome-editing agents. Gill and co-workers developed a PEI/CpG-free DNA nanoformulation for aerosol-mediated gene delivery to the lungs in mice [149]. Repeated PEI/CpG-free DNA aerosol formulations to airways showed undetectable toxicity. In addition, high-level, sustained luciferase expression was observed in the lung tissues, indicating the potential for lung gene delivery. Minko and co-workers used liposome to encapsulate doxorubicin (DOX) and ssODNs or siRNA targeting multidrug resistance-associated protein 1 (*MRP1*) for inhalation delivery to the lungs [150]. Noted that the retention of the DOX and ssODNs or siRNA in the lungs were much stronger, as compared with that by intravenous injection. The tumor-bearing mice inhaled with the liposome formulation exhibited significant tumor regression in comparison with the intravenous injection. Recently, Huang and co-workers reported that aerosol inhalation-mediated delivery of a recombinant AAV5 vector harboring a truncated *IL-4* gene can ameliorate the phenotype of asthma in ovalbumin-induced asthma mouse models [151]. Repeated aerosol administration of AAV5 vectors resulted in sustained truncated *IL-4* protein expression in the lung, and further significantly modulated the immune cell infiltration in the lung and also improved the lung function in ovalbumin-induced asthma mice.

To the best of our knowledge, the aerosol delivery of genome-editing biomacromolecules for lung genetic disorders has not been demonstrated yet, which might be associated with difficulties in developing inhalable genome-editing formulations, especially in maintaining the native integrity and physiological activity of biomacromolecules or the physicochemical properties of the nanoparticles during the engineering and delivery processes. Non-viral vectors would be helpful in maintaining the stability and preserving the biological activity of engineered genome-editing agents during the delivery processes. For example, the inhaled vectors should be able to penetrate via mucus, and retain their colloidal stability in the presence of negatively charged pulmonary surfactants (various phospholipids, cholesterols and proteins) that are abundant in the airspace. In addition, a number of physiological barriers that prevent the successful genome editing via inhalation to the lung should be carefully considered, such as airway mucus, mucus gel layer, and periciliary layer (PCL) [152–156]. Moreover, alveolar macrophages residing in the airspace also form an additional barrier that results in low level of gene transfer by inhalation gene therapy [157–160]. In addition to the aforementioned extracellular barriers,

several intracellular barriers, such as acidic vesicles, the molecularly crowded cytoplasm, and the nuclear envelope should be overcome during gene delivery after genome-editing agents are internalized by target cells [161]. Most recently, two main strategies have been proposed to overcome the physiological barriers for inhaled gene therapy, namely modification of gene delivery systems and modulation of various physiological barriers [11]. On one hand, the incorporation of specific ligands to the surface of gene vectors by either covalent or non-covalent conjugation, or through genetic engineering has been widely accepted to reduce multivalent adhesive interactions, improve complex stability, and decrease macrophage uptake, which facilitate the uptake of nanoparticles by target cells or tissues [162–165]. Other approaches have been widely explored to endow viral vectors to be internalized by cells or to accumulate in tissues. Generating libraries of alternative viral capsid types through direct evolution approaches is also helping viral vectors to overcome physiological barriers during the delivery processes [166,167]. In addition, the rational design of capsid pseudotyping, has also been used to efficiently infect cells in the lung to endow apical epithelial tropism [166,167]. On the other hand, a relatively simple strategy to improve the delivery efficacy of inhaled CRISPR-based gene therapy is to modulate physiological barrier properties. Mucus-altering agents, such as recombinant human DNase and N-acetyl cysteine, can enlarge mucus mesh pore size to facilitate the penetration of gene vectors through airway mucus [168,169]. Furthermore, some osmotic agents, such as hypertonic saline and mannitol, can be incorporated into inhaled formulation to increase mucus and/or PCL mesh pore size by hydration as well as to enhance mucociliary clearance [170,171]. In addition, tight-junction disrupting agents (such as sodium caprate, polidocanol, and lysophosphatidylcholine) which transiently disrupt tight junctions in the epithelial layer, help gene vectors to access the basolateral surface [11,172,173]. These well-established strategies are conducive to the rational design of inhalation delivery for genome-editing agents.

5. Summary and future outlook

In the past few years, CRISPR/Cas9-based genome editing has shown enormous potentials in treating a diverse range of genetic diseases. Despite its early stages, encouraging results have established the possibilities of using CRISPR/Cas9-based therapeutic approaches for the treatment of lung genetic disorders, including CF, AATD, and lung cancers. Moreover, the combination therapy for the treatment of other common obstructive lung disorders (asthma and COPD), which can be accomplished by co-delivering corticosteroids and CRISPR/Cas9, opens new avenues to regain chemosensitivity and improved therapeutic effects. Further systematic and in-depth studies are still essential to understand the fundamental issues of CRISPR/Cas9 as a therapeutic genome-editing modality for the treatment of lung genetic diseases before these findings can be designed as specific formulations for pre-clinical studies and clinical trials. Meanwhile, there is growing evidence to indicate that the delivery of genome-editing biomacromolecules can be achieved *in vitro*, *ex vivo* and *in vivo*. Though viral vectors are widely used to deliver CRISPR/Cas9 systems for genome-editing applications in different contexts, their tissue-specific tropism, immunogenicity and tumorigenic risks have apparently limited their applications for humans. Thus, the development of non-viral vectors to deliver CRISPR/Cas9 elements into target cells or tissues is highly essential for future clinical translation. So far, although many types of non-viral delivery systems have been demonstrated to be effective in introducing CRISPR/Cas9 elements into target cells or tissues, they are not ready for the treating lung genetic disorders. To accelerate the future translation, the rational design of non-viral delivery vectors is crucial for safe and efficient *in vivo* delivery of CRISPR/Cas9 elements, and several suggestions are proposed for the delivery of genome-editing agents. First, the construction of delivery vectors with biodegradable and biocompatible materials with low immunogenicity and toxicity contributes to the

safe *in vivo* delivery of CRISPR/Cas9 elements, especially when it comes to the translational investigations. Second, personalized design of vectors is crucial for encapsulating different formats of CRISPR/Cas9 systems, and further protecting their biological activity during their delivery processes. Third, elements of CRISPR/Cas9 should be able to be efficiently released from delivery vectors and transported into intracellular sites to exert their genome-editing effects. Last, the precise control of CRISPR/Cas9-mediated genome-editing activity and spatiotemporal specificity, which can be also manipulated through rational design of delivery vectors, is beneficial to mitigate their unwanted off-target mutations and immunogenic response. For instance, site-specific delivery of CRISPR/Cas9-based genome-editing agents would greatly enhance the therapeutic efficacy at the lesion and reduce genotoxicity caused by non-specific editing in the non-targeted tissues. Apart from installing the tissue- or cell-specific promoters to improve genome-editing specificity, delivery of CRISPR/Cas9 systems in a stimuli-responsive fashion also improve the precision and safety of CRISPR/Cas9-mediated genome editing.

To date, the systemic administration is still the most common route for viral or non-viral delivery of gene therapy agents, although they are known to be rapidly degraded by serum nucleases and excreted via the kidneys upon intravenous injection. These unfavourable pharmacokinetic profiles severely constrain the clinical translation of genome editing towards therapeutic applications. Excitingly, selective lung-targeted delivery by lipid nanoparticles has shown great potentials for the safe and efficient delivery of genome-editing elements very recently [174]. These lipid nanoparticles can be engineered to precisely tune their *in vivo* biodistribution and induce organ-specific genome editing by altering their global apparent pKa values and endowing distinct protein coronas. Thus, careful screening of lipid libraries and their formulation can help identify lipid vectors that exclusively transport CRISPR/Cas9 to the lung to induce effective lung-specific genome editing. Additionally, as an emerging delivery strategy, inhalation provides direct transportation and deposition in the lung where CRISPR/Cas9-based genome editing can work for treating genetic diseases in a relatively non-invasive manner. Towards this goal, careful design of aerosolizable genome-editing delivery systems with either viral or non-viral vectors also contributes to the development of optimal formulation for lung genetic disorders. Though the delivery of genome-editing biomacromolecules by aerosol exhibits numerous advantages, it is of paramount importance to understand the fundamentals of physiological barriers during inhaled delivery, study the mechanism of pulmonary clearance, and select appropriate aerosol devices for the aerosolization of genome-editing agents. Collectively, despite many existing challenges, we are optimistic that the delivery of genome-editing agents will provide a precise therapeutic modality for treating lung genetic diseases in the near future.

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