HGT

New Directions in Pulmonary Gene Therapy

Amber Vu and Paul B. McCray, Jr.*

Stead Family Department of Pediatrics, Center for Gene Therapy, The University of Iowa, Iowa City, Iowa, USA.

The lung has long been a target for gene therapy, yet efficient delivery and phenotypic disease correction has remained challenging. Although there have been significant advancements in gene therapies of other organs, including the development of several *ex vivo* therapies, *in vivo* therapeutics of the lung have been slower to transition to the clinic. Within the past few years, the field has witnessed an explosion in the development of new gene addition and gene editing strategies for the treatment of monogenic disorders. In this review, we will summarize current developments in gene therapy for cystic fibrosis, alpha-1 antitrypsin deficiency, and surfactant protein deficiencies. We will explore the different gene addition and gene editing strategies under investigation and review the challenges of delivery to the lung.

Keywords: lentivirus, AAV, adenovirus, CRISPR/Cas, gene editing

INTRODUCTION

GENE THERAPY HOLDS promise for many disorders that may currently lack practical or effective treatments. Over the past decade, *ex vivo* gene therapy has advanced, with several treatments approved for clinical use.[1] *In vivo* somatic cell gene therapy for treatment of blindness caused by mutations in the *RPE65* gene is approved by Food and Drug Administration (FDA) and clinical trials for hereditary hemophilias are yielding promising results.[1–3] However, several challenges and setbacks have prevented the advancements for treatment of lung disease. Clinical trials for cystic fibrosis (CF) began in the 1990s using gene addition strategies but did not achieve meaningful improvement in lung function. More than 20 years later, with 27 completed clinical trials, incremental yet substantial improvements in vector design, delivery, and efficacy have occurred.[4]

Continued progress in gene addition approaches has rekindled interest in therapies for lung diseases and other organ systems. In addition, the discovery and development of the clustered regularly interspaced short palindromic repeats (CRISPR)-associated system (Cas) for mammalian gene editing has revolutionized the field, providing new strategies to prevent or treat lung diseases. In this study, we review the advances in both gene addition and CRISPR/Cas-based gene editing approaches to effectively target the lung and treat CF and other genetic pulmonary diseases. We also look at the unique challenges to lung delivery and relevant exciting progress in pulmonary gene therapy.

GENE THERAPY STRATEGIES

Gene Addition

Initial approaches included the addition of a functional gene to affected cells to offset disease phenotypes.[4] This is an excellent approach for recessive monogenic diseases resulting in loss-of-function phenotypes, such as CF, but would be ineffective for dominant negative, or gain-of-function diseases. Gene addition strategies commonly use constitutive promoters to express the gene of interest, but conditional or endogenous promoters are also used. Expression of a gene of interest can be transient or persistent depending on the therapeutic strategy, using genome integrating technologies or episomal maintenance of vector DNA. Examples of gene addition strategies will be discussed in more detail in later sections of this review.

CRISPR/Cas9 Gene Editing

Because of the recent explosion in gene editing technologies, we will briefly review the available tools. CRISPR arrays were first discovered in 1987 in *Escherichia coli* K12 and have since been observed in 90% of archaea and 50% of bacteria.[5,6] CRISPR/Cas systems act as a prokaryotic ''adaptive immune system'' to protect against infecting bacteriophages and other sources of ex-

*Correspondence: Dr. Paul B. McCray, Jr., Stead Family Department of Pediatrics, Center for Gene Therapy, The University of Iowa, Iowa City, IA 52242, USA. E-mail: paul-mccray@uiowa.edu

DOI: 10.1089/hum.2020.166

ogenous DNA.[5,7,8] Although CRISPR/Cas systems are currently classified into six types (I–VI), each with its unique set of Cas nucleases, much of mammalian gene editing has been performed using Cas9 (type II) derived from *Streptococcus pyogenes* (spCas9).[5]

spCas9 is a multifunctional, multidomain protein comprising 1,368 amino acids.[5] During the gene-editing process, Cas9 and guide RNA (gRNA), consisting of a target DNA-specific sequence (crRNA) complementary to the chromosomal sequence and a transactivating CRISPR RNA (tracrRNA) sequence that interacts with the Cas9, are introduced into the cell.[5] The gRNA recognizes and binds to the target DNA near a protospacer adjacent motif (PAM) site. The PAM interaction (PI) domain of Cas9 recognizes and interacts with the PAM site.[5,9] Cas9 cleaves DNA 3 base pairs (bp) upstream of the PAM site by two distinct nuclease domains, an HNH-like endonuclease domain that cleaves the strand complementary to the guide sequence (target strand) and an RuvC-like domain that cleaves the nontarget DNA strand, causing a double-stranded break (DSB) in the genome.[5,9,10] This DSB triggers the cell's DNA repair mechanism, resulting in either (1) error-prone nonhomologous end joining (NHEJ) and resultant insertions or deletions (indels) that often lead to frameshift or nonsense mutations, or (2) homology-dependent repair (HDR) that requires a template in the form of single-stranded DNA (ssDNA) or double-stranded DNA (dsDNA) carrying the desired modification.[5] HDR may also use the other host allele as a template, resulting in a nonediting outcome.[11–13] HDR is substantially less efficient than NHEJ, and it only occurs during the G2 and S phases of the replication cycle, whereas NHEJ can occur throughout the cell cycle.[5,12]

Cas Variants

Although a majority of CRISPR gene editing studies use spCas9, several other Cas variants also show promising results. A second type II Cas protein, termed CRISPR from *Prevotella and Francisella 1* (Cpf1) or Cas12a, was developed for mammalian gene editing.[5,14] Cas12a has four domains: RuvC-I and RuvC-II nuclease domains, a/beta domain, and zinc finger. Unlike Cas9, Cas12a lacks an HNH-nuclease domain, resulting in smaller molecular weight that offers advantages for some delivery approaches.[5] Instead of creating a blunt-end DSB like spCas9, Cas12a causes staggered cuts, providing advantages for knock-in strategies.[5] Cas12a recognizes a T-rich PAM site, rather than the G-rich PAM site recognized by Cas9.[5,15] This difference in PAM recognition can allow access to targeting of additional sites in the genome that were difficult to target with Cas9; however, Cas12a has been noted to have a low indel efficiency.[15] Indel efficiency can be increased by modification of gRNA with addition of an 8-meric uridinylate-rich 3′ overhang.[15] Cas12a also has a lower incidence of off-target edits than Cas9,[16] possibly creating less safety concerns for clinical applications. Cas12a ribonucleoprotein (RNP) delivery to the airways by amphiphilic peptides achieved an average editing efficiency of 12% in the large airways and 10% in the small airways of $ROSA^{mT/mG}$ mice.[17] All cells in these mice contain a tdTomato cassette flanked by *loxP* sequences in the Rosa26 locus, and deletion of tdTomato by dual Cas12a targeting of these *loxP* sequences results in localized GFP fluorescence.[17] This study shows the direct application of Cas12a RNPs in targeting the epithelia of the airways, a prominent area for pulmonary lung disease.

Additional protein engineering has improved the efficiency and specificity of editing with Cas12a and Cas9.[18,19] Using phage-assisted continuous evolution, in which evolution is driven by continuous selection of a desired phenotype linked to production of infectious phages containing the evolving gene,[20] spCas9 variants were developed to recognize non-NGG and non-G PAM sequences.[21,22] Directed evolution or protein engineering of spCas9 can improve target specificity and allow for targeting of sites previously inaccessible, creating better alternatives to native spCas9 gene editing in some instances. At present, evolved Cas9 variants that recognize non-NGG and non-G PAM sequences, xCas9(3.7) and SpCas9-NG, have only been assessed in cultured cells and in plants, so the *in vivo* efficacy and, ultimately, clinical applicability of these Cas variants remains to be determined.[23–26]

A newer Cas variant developed for mammalian gene editing is derived from *Deltaproteobacteria*, termed CasX or Cas12e.[5,27] This is among the smallest Cas proteins, with <1,000 amino acids,[27] offering advantages for adeno-associated virus (AAV) packaging to provide space for other gene editing tools. Like Cas12a, Cas12e also creates a staggered DSB, but utilizes a longer PAM sequence of TTCN.[27] The indel efficiency and off-target incidence is yet to be determined, but its small size and nonpathogenic origin may offer advantages.

Cas variants have also been developed for epigenetic control of targeted genes. Enzymatically deactivated Cas9 (dCas9) that cannot cleave DNA yet retains the ability to bind target sequences is fused to either activator or repressor domains to upregulate or inhibit gene expression, respectively.[5,28–31] Termed CRISPRa when an activator domain is used and CRISPRi when a repressor domain is used, these Cas variants allow for reversible epigenetic control of specific genes. A CRISPRa strategy has been shown to effectively prevent muscle fibrosis and paralysis as well as improve hindlimb paralysis in a muscular dystrophy type 1A (MDC1A) mouse model through the upregulation of the *LamaA1* gene.[32]

Base Editing

Base editing provides a more efficient method to modify a single nucleotide than HDR.[33] Derived from a catalytically inactive spCas9 to a base editor, the Cas9

machinery binds to target DNA and causes displacement of a small segment of ssDNA into an R-loop structure without strand break creation.[33] Base editors then create single nucleotide changes in the R loop. There are two types of base editors: (1) cytidine base editors (CBEs), which can convert a C-G bp to a T-A bp and (2) adenine base editors (ABEs), which convert an A-T bp to a G-C bp. ABEs have a very high product purity ($\sim$99.9%) and low frequency of indels (typically $\leq$0.1%) in cultured cells and mice,[34] compared with the CBE base pair conversion with 60–98% product purity.[33,35] The lower CBE product purity is thought to be associated with uracil *N*-glycosylase (UNG) expression, as $UNG^{-/-}$ cells have increased base editing specificity.[33,35] Base editing has the potential to correct many monogenic disorders, with ABEs offering the potential to correct almost half of all pathogenic human mutations.[36] In CF, 15% of known single base pair mutations can potentially be corrected by CBEs and 46% using ABEs (CFTR2.org). One limitation for base editing is the presence of an applicable PAM site at the target site. ABE variants have recently been developed to use alternative PAM sequences, allowing access to genomic regions that were previously inaccessible.[37] Delivery of messenger RNA (mRNA) encoding CBE and gRNA successfully introduced base edits in mice and pig oocytes.[38,39] Chemically modified mRNA encoding ABE and gRNA can be used to edit otherwise hard-to-transfect cells.[40] Split intein AAV vectors offer another delivery system for base editors. In the short time since the development of base editing, this technique has been used to create novel mouse, rabbit, and sheep animal models, and to correct disease-related phenotypes in various animal models, displaying the broad potential for base editing in gene therapy research.[41–45]

Prime Editing

Prime editing, a newer application of spCas9, allows modification of more than one nucleotide with greater efficiency than Cas9-HDR strategies. In this iteration of CRISPR/Cas, a modified spCas9 (nickase) creates a single-stranded break.[46] Fusion to a reverse transcriptase and use of an engineered guide RNA (prime editing guide RNA [pegRNA]) allows the nicked DNA to act as a primer for reverse transcription of the pegRNA template to DNA, resulting in the desired genetic alteration.[46] This strategy facilitates larger alterations than base editing, but with greater efficiency and less indel creation than conventional CRISPR/Cas9.

Prime editing could significantly expand the scope of gene therapy applications, with the ability to target as much as 89% of disease-causing mutations.[46] To date, this technology has only been tested in cultured cell lines, and its efficacy *in vivo* remains to be determined. Prime editors (PEs) are much larger than Cas9 protein, and effective delivery systems will need to be developed and optimized. In addition, the PE complementary DNA (cDNA) exceeds the packaging capacity of recombinant adeno-associated vectors, a common viral delivery vehicle. Finally, pegRNAs, which may be much longer than conventional gRNAs, could influence delivery efficiency when using a nonviral vector. Alternative or modified methods of PE delivery will likely need to be developed for efficient editing to occur in specific cell types and *in vivo*.

BARRIERS TO DELIVERY: UNIQUE CHALLENGES OF THE LUNG

The complex architecture of the lung allows the epithelial surfaces to be directly targeted for delivery of therapeutic cargoes. Topical administration through inhalation provides direct access to the epithelial cells lining the airways and airspaces. For vector or cell-based therapies, systemic delivery approaches through the vascular system are inefficient at targeting epithelial cells. Systemic approaches may be better suited for delivery to the vascular endothelial cell compartment. Therefore, a majority of pulmonary gene therapy approaches use aerosol administration. Although the lung allows for organ-specific delivery of topical/aerosol therapeutics, lung architecture and immune responses create formidable barriers for efficient delivery.

A mucus layer present in large and small airways comprises a mesh of gel-forming mucins. Mucins are large polymeric macromolecules containing negatively charged glycans.[47] Secreted mucins reside within airway surface liquid (ASL). ASL is derived from the secretions of submucosal glands and surface epithelia and is a rich source of secreted host defense peptides and proteins, antibodies, and the collectins surfactant proteins (SPs), SP-A and SP-D. Viral and nonviral vectors may be immobilized and inactivated in this layer through multivalent interactions with mucins, such as electrostatic and hydrophobic interactions and hydrogen bonding.[48,49] Steric obstruction may also contribute to vector immobilization. Vectors trapped in mucus may also be cleared through mucociliary clearance (MCC). Antibodies can move unimpeded within human mucus and ASL, but Fc regions can form low affinity interactions with the mucus.[49–51] Neutralizing antibodies against viral vectors, especially AAV and AdV, are present in airway secretions of some healthy and diseased individuals.[52–54]

Antibody binding to viral vectors within ASL can cause their aggregation and clearance, reducing transduction. Repeat administration of AdV or AAV vectors results in the development of neutralizing anti-capsid antibodies, potentially reducing efficacy.[55,56] However, readministration of lentiviral vectors (LVs) in mouse models does not elicit development of systemic or local neutralizing antibodies.[57,58]

Recent studies show that immune responses can be elicited by Cas9 variants.[59] *S. pyogenes* and *Staphylococcus aureus* are common human commensals that may also act as pathogens, and it is unsurprising that a subset of

the human population may possess preexisting humoral and/or cell-mediated responses. Studies of AAV delivery of Cas9 in mouse models have shown immune responses can be elicited in response to Cas9 protein expression. Administration of AAV-delivered SaCas9 (AAV-CRISPR) to skeletal muscle leads to infiltration of $CD4^+$ and $CD8^+$ T cells, and the production of anti-Cas9 antibodies.[60] Systemically delivered AAV-CRISPR can also result in a humoral response in mice,[61] the development of SaCas9-specific memory T cells, resulting in reduced efficacy upon repeat dosing,[62] and $CD8^+$ T cell-mediated killing of cells expressing SaCas9 *in vivo*.[63]

Human sera can also contain preexisting antibodies toward Cas9 variants. Immunoglobulin G (IgG) antibodies targeting both SaCas9 and SpCas9 were found in 86% and 73% of donors, respectively, in cord blood from 22 donors.[64] In peripheral blood from 12 healthy donors, 67% of donors had anti-SaCas9 antibodies and 42% had anti-SpCas9 antibodies.[64] In a study with a large sampling size of 200 donors, 19% and 4.5% of donors were identified to potentially positive SaCas9 and SpCas9 antibodies, respectively, in a screening assay.[65] Of these donors, only 5% and 1.5% tested positive for anti-SaCas9 and anti-SpCas9 antibodies upon a more stringent confirmatory assay, suggesting antibodies targeting Cas9 may not be as prevalent in the general population.[65]

Cell-mediated responses toward Cas9 proteins in humans has also been reported. Human peripheral blood mononuclear cells stimulated with SpCas9 or Cas12a lead to activation of T effector and T regulatory cells.[66] Although SpCas9-reactive T regulatory cells could mitigate the proliferation of effector T cells, enriched SpCas9-reactive effector T cells were shown to specifically target and lyse lymphoblastoid B cell lines that express SpCas9,[66] suggesting that the adaptive immune response may counteract therapeutic efforts of CRISPR. Further research into human immune response to SpCas9 and SaCas9 will be required to determine the effectiveness of these proteins in a clinical setting.

Efforts to suppress the host immune response to Cas9 proteins include transient immunosuppression and modification of Cas9. Immunodominant T cell epitopes of SpCas9 for HLA-A*02:01, the most common HLA type in European and North American Caucasians, have been identified, and mutation of MHC-binding anchor residues of these epitopes results in reduced T cell activation.[67] These modifications do not affect SpCas9 function or specificity, suggesting that such an approach could be used to suppress the host immune response to CRISPR-mediated gene therapy.[67] HLA allotype diversity may complicate the creation of a completely immunosilent Cas9 protein and may require allotype-specific modifications for more development of more effective treatments.

Pulmonary surfactant is present in alveolar lining liquid and comprises phospholipids, cholesterol, and SPs such as SP-A, B, C, and D. SP-A and SP-D play roles in host defense, as mice deficient in these proteins are more susceptible to bacterial and viral infections. SP-A and SP-D can decrease the transduction efficiency of nonviral vectors, causing aggregation and clearance.[68–70]

Resident macrophages in the airways and alveoli also play critical roles in host defense and tissue remodeling. Particles in the size range of 250 nm to 3 μm are readily phagocytosed; however, particles smaller than 250 nm are taken up less efficiently.[49,71] As most viral and nonviral gene delivery vectors are below the 250 nm threshold, single particles are not easily targeted by macrophages, but vector aggregation may facilitate phagocytosis. Aggregation and engulfment of 20–110 nm silver nanoparticles was observed in macrophages isolated from bronchoalveolar lavage fluid in rats administered nanoparticles through aerosolization.[72] Opsonization of vectors by SP-A and SP-D may also increase clearance by macrophages.[49]

Vectors must also transverse the physical barriers of the differentiated epithelium. Tight junctions between adjacent cells prevent access to the basolateral surface, a site of many virus receptors.[49] To achieve durable gene editing of the large airways, progenitor basal cells will likely need to be targeted. Basal cells are a population of self-renewable multipotent cells responsible for the regeneration and homeostasis of the conducting airways. Their apical cytoplasm does not reach the lumen, limiting their transduction.[73] In addition, it has been observed that endocytosis from the apical cell surface of differentiated cells occurs at low efficiency,[74] which may prevent transduction by vectors that rely on this cell entry pathway.

Disease-related factors may also create barriers to epithelial cell transduction. In CF, loss of CFTR-dependent anion secretion results in reduced liquid secretion and an acidic ASL pH. This impairs antimicrobial ASL activity and reduces MCC.[75,76] Impaired MCC and reduced host defenses create a permissive environment for bacterial infections that leads to inflammation, airway obstruction, and airway remodeling. CF respiratory secretions contain elevated levels of endogenous DNA, actin filaments, and cellular debris owing to this inflammatory environment, and this may make secretions more viscous.[77] Bacterial biofilms can also form in the CF lung, adding an additional external barrier to epithelial cell access. Elevated oxidative stress can increase disulfide bond cross-links between mucin fibers, leading to smaller pore size of the mucus gel and more elastic mucus.[78,79] Studies have shown that CF mucus can impede both viral and nonviral vector entry to primary human airway epithelial (HAE) cultures.[49,80–84]

METHODS OF DELIVERY: VIRAL AND NONVIRAL APPROACHES

The delivery of cargoes for gene addition and gene editing components may be accomplished using the fol-

lowing formats: (1) delivery of DNA expression cassettes or mRNA encoding the required components, and (2) delivery of protein and DNA or RNA (in the context of CRISPR/Cas system: Cas protein, gRNA, and donor DNA, if required). The vector oftentimes dictates the type of cargo that is used. The delivery vehicle may be viral or nonviral.

Viral Delivery

Viral vectors are commonly used for pulmonary applications. Several viral vector systems are amenable to administration through inhalation and result in robust transduction of a variety of cell types in the lung.

Adeno-associated virus. AAV vectors have been used extensively in airway gene delivery *in vivo* and their safety record for human clinical trials is strong.[73,85–91] AAV transduction provokes little innate or adaptive immune responses upon the first treatment and shows minimal signs of cytotoxicity.[4] The variety of AAV serotypes allows for transduction of different airway epithelial cell types. Clinical trials for AAV delivery for CF used the AAV2 serotype with an excellent safety prolife, but transduction efficiency of the airways was low.[55,92–94] In retrospect, one reason for this limited delivery efficiency could have been receptor availability. The AAV receptor (AAVR), encoded by gene KIAA0319L, is required for infection of AAV serotypes 1, 2, 3b, 5, 6, 8, and 9, and is exclusively localized to the basolateral and perinuclear compartments of HAE cells.[95] AAV2.5T, developed by directed evolution to transduce primary HAE cultures apically, resulted in 100-fold increase of apical transduction compared with the native AAV serotype 5.[96] AAV2.5T uses sialic acid as an attachment factor, and this interaction is required for internalization. Internalization of AAV2.5T is independent of the AAVR, as AAVR antibodies do not affect AAV2.5T transduction.[96,97]

DNA shuffling of capsid subunits of various AAV serotypes can create recombinant AAV (rAAV) vectors with improved targeting and transduction efficiencies. This technique was used to select for AAV capsids that could efficiently transduce hepatocytes in the presence of pooled human antisera, resulting in the identification of an AAV2/AAV8/AAV9 chimera termed AAV-DJ.[98] DNA shuffling was used to identify AAV capsids with high tropism for lung endothelial cells and may be a powerful tool for discovery of novel AAV capsids for other lung compartments, such as airway epithelia or alveolar cells.[99] Display of peptide libraries in the AAV capsid can also be used in combination with capsid shuffling. Random AAV vector peptide display and capsid shuffling libraries have been used to screen capsids for cellular tropism both *in vitro* and *in vivo*.[98,100–103] DNA barcoding can be used in AAV capsid libraries to allow for tracking of capsids in a high-throughput setting.[100,101]

The AAV capsid is an icosahedron consisting of capsid proteins VP1, VP2, and VP3 at a ratio $\sim$1:1:18.[104,105] Surface-exposed protrusions at the threefold axis of symmetry harbor five of the nine ''variable loop'' regions (VRs), which vary in amino acid composition between serotypes.[104,105] Differences between serotypes can primarily be mapped to this surface topology.[104,105] VR composition can affect tissue tropism and some VRs, such as VR-VIII of VP3, can withstand insertion of foreign sequences to alter tropism.[104,105] Selection of cell-type or tissue-specific targeting AAV capsids can be achieved by using peptide or designed ankyrin repeat protein (DARPin) insertion into the AAV capsid at these regions. Deverman *et al.* used a Cre recombination-dependent approach to select for capsids able to breach the blood–brain barrier.[106] Capsid libraries with a 7-mer peptide insertion were selected through administration to an astrocyte-specific Cre-expressing mouse model, resulting in isolation of capsid variants with a 40-fold increase in central nervous system transduction.[106] In a separate study, DARPins specific for Her2, CD4, and EpCAM were inserted into the AAV capsid sequence, resulting in specific targeting of tumors in a mouse model, CD4-positive cells in the spleens of humanized mice, and EpCAM-positive cells in whole human blood, respectively.[107]

The airway mucus may impede efficient transduction of some AAV serotypes. AAV1, AAV2, and AAV5 can adhere to human airway mucus.[80,81] AAV6 transduction, however, is not reduced by endogenous mucus or mucus hypersecretion, whereas AAV1 transduction is reduced in primary HAE cultures or in a mouse model. A single amino acid change in the AAV6 capsid, K531E, increases susceptibility to mucus impairment, indicating glycan-specific interactions of serotypes.[108]

Although AAV vectors can achieve high transduction efficiency in primary human airway epithelia, the packaging capacity is limited to 4.8 kb.[109] This may be a limitation for some gene addition and CRISPR/Cas approaches. The cystic fibrosis transmembrane conductance regulator (*CFTR*) cDNA sequence is $\sim$4.5 kb, which is too large to be packaged within an AAV expression cassette. Creation of a *CFTR* mini-gene through deletion of a portion CFTR regulatory (R) domain (CFTRΔR) and use of a minimal 83 bp synthetic promoter yielded an expression cassette that was effectively packaged into an AAV vector.[110,111] For gene editing, the AAV packaging size is too small to accommodate the full CRISPR/Cas cargo for spCas9. This obstacle can be overcome using a smaller Cas variant such as saCas9 or dual vectors, with one AAV containing the genetic sequence of Cas protein, and a second AAV encoding the gRNA and a marker gene or HDR template. A split Cas system, such as split-intein, can also be utilized, in which the N-terminal region of Cas9 is encoded in one AAV and the C-terminal region of Cas9 is encoded in a second AAV

vector.[60,112,113] This strategy is also effective for delivery of base editors.[112,114,115] Dual AAV vectors were used to correct CFTR function in a CF pig model. One AAV carried an integrating CFTR expression cassette flanked by the piggyBac transposon and another AAV carried the piggyBac transposase, both administered through tracheal aerosolization.[116] Although the transduction efficiency was not determined in this study, delivery was sufficient to correct the anion channel defect.[116] Transduction efficiency of two AAV6 vectors has been shown to occur at similar levels as transduction of a single AAV6 vector in mice lungs, which is approached in 10% of airway epithelial cells.[117] These studies highlight the feasibility of dual AAV vector strategies to effectively target the airways.

Although AAV vectors are very effective at transducing a variety of cell types in the lung, its small carrying capacity creates challenges for delivery of donor DNA for HDR. This application generally requires two vectors. AAV transduction can result in sustained expression of CRISPR components, as packaged DNA can persist episomally for the lifetime of the cell, or with modification, can rarely integrate into the host genome.

Another strategy to overcome genome size limitations is to create a parvovirus chimera in which a rAAV genome is packaged within the capsid of a different parvovirus with a larger packaging capacity. An early demonstration of this technique packaged rAAV genomes into a B19 capsid, resulting in effective targeting of erythroid cells.[118] rAAV genomes have also been packaged into human bocavirus (HBoV) and gorilla bocavirus (GBoV) capsids to increase the packaging capacity by 1 kb.[119–121] These rAAV/HBoV or GBoV chimeras effectively transduced primary human airway epithelia cultures, lung organoids, and ferret lungs.[119–121] Although rAAV/HBOV chimeras using serotype HBoV1 can be effectively neutralized by human IgG, rAAV/HBoV4 and rAAV/GBoV were unaffected by such treatment, suggesting the potential use of these vectors in lung delivery.

A major limitation of parvovirus cross-genera pseudopackaging is production of high titer vector; however, recent progress in understanding HBoV1 viral replication has resulted in better production methods. In the prototypic production of rAAV/HBoV1 vector, transient transfection of HEK293 cells was performed with plasmids containing the (1) rAAV2 genome, (2) necessary adenovirus (Ad) helper genes (*E2*, *E4Orf6*, and *VA RNA*), (3) AAV2 *Rep* genes, and (4) HBoV1 capsids (VP1, VP2, and VP3) and nonstructural (NS) genes (NS1, NS2, NS3, NS4, and NP1).[121] Typical yields were 10- to 20-fold less than that of rAAV2 vector.[121]

It was later discovered that NP1 is the only NS protein required for HBoV1 capsid expression and that it enables transcription of full-length *cap* mRNA that would otherwise be terminated at a polyadenylation (pA) site within the unique region of VP1.[122] Silent mutation of the HBoV1 *cap* cDNA to omit pA sites allowed for the production of rAAV/HBov1 vector in the absence of any NS proteins, further enhancing the production yield.[123]

Another production method of rAAV2/HBoV1 vector uses Sf9 insect cells and baculovirus expression vectors (BEVs).[124] Sf9 cells are infected with two BEVs: one harboring the rAAV2 genome, and the other carrying expression systems for AAV2 Rep78 and Rep52, as well as HBoV1 capsid proteins VP1, VP2, and VP3.[124] Addition of a third BEV that expresses HBoV1 NS protein NP1 enhances vector production yields similar to rAAV2 produced in Sf9 cells, although the proximal pA preventing HBoV1 *cap* expression in the absence of NP1 in mammalian cells do not appear to be effective in insect cells.[124] Overall, production of rAAV2/HBoV1 in Sf9 cells resulted in 10- to 100-fold more vector than rAAV2/HBoV1 produced in HEK293 cells using a transient transfection method of production.[124] Although the biological characteristics, such as transduction ability, of Sf9-produced rAAV are equivalent to HEK293-produced rAAV, rAAV2/HBoV1 produced in Sf9 cells had a five- to sevenfold reduced transduction of cultured HAE cells compared with rAAV2/HBoV1 produced in HEK293 cells.[124–128] This decreased transduction could be because of reduced genome packaging, as rAAV/HBoV1 produced in Sf9 cells had a high level of empty capsids (50–60% full particles) compared with vector produced in HEK293 cells (>95% full particles).[124] Variations in glycosylation of HBoV1 capsid proteins in the two cell lines could also affect transduction. Through further optimization of rAAV2/HBoV1 production methods, use of this vector in animal models and in clinical trials may become more feasible.

Analysis of variant HBoV1 isolates from human samples has identified various hot spots that can affect vector production yields and transduction of primary HAE cultures.[129] One capsid variant (T590S) increases AAV2/HBoV1 vector production without affecting transduction efficiency.[129] This amino acid variation is located in the HI loop of VP3, a hypervariable region that is linked to particle assembly and genome packaging in AAV.[130] Another isolated variant (Y523F) increased transduction of primary HAE cultures.[129] These capsid variants highlight the promising application of HBoV1 capsid modification to further enhance rAAV2/HBoV1 vectors for clinical applications.

Lentivirus. LVs are widely used, and several are FDA approved for use in clinical trials to treat diseases such as SCID, chronic granulomatous disease, adrenoleukodystrophy, and beta-thalassemia. LV therapies for these diseases largely involve *ex vivo* treatment of hematopoietic stem cells that are subsequently returned to the patient. Even with the success of *ex vivo* LV therapies, delivery to the lung remains challenging owing to the barriers previously discussed.

LVs are widely used in gene addition approaches to integrate expression cassettes into the host genome. They can also be used to deliver coding sequences for Cas proteins, gRNAs, and other machinery in a single vector. Multiple gRNAs can be encoded into a single lentivirus vector, each under its own cell type-specific promoter, to edit multiple genes in multiple cell types with one transduction.[131] LVs can be pseudotyped with a variety of viral envelopes to alter tropism. They are commonly pseudotyped with the vesicular stomatitis virus (VSV) envelope, VSV-G, which efficiently transduces the basolateral surface of airway epithelia but has low levels of apical transduction because of receptor availability.[132–135] To enhance VSV-G pseudotyped LV transduction *in vitro* or *in vivo*, transient disruption of tight junctions through calcium chelators,[136] induced injury,[137] or treatment with mild detergents,[138] such as lysophosphatidylcholine (LPC) may be utilized. Multiple screens of different envelope pseudotypes for efficient apical transduction and virus production have been performed, with the envelope from *Autographica californica* multiple nucleopolyhedrovirus (AcMNPV) GP64 being suitable.[139–144] GP64-pseudotyped LVs can efficiently transduce the nasal passages and airways of mice and pigs.[132,141] Pseudotyping an SIV-based LV with modified Sendai virus (SeV) envelope hemagglutinin-neuraminidase (HN) and fusion (F) proteins also achieved robust apical transduction.[145,146] F/HN-SIV transduces the nasal epithelium of mice with 4.5% efficiency and apically transduces fully differentiated primary HAE cultures to generate functional CFTR chloride transmembrane conductance.[145]

Whereas integration and persistent expression is advantageous for some gene addition and CRISPRa or CRISPRi approaches, in the case of gene editing, this may be undesirable. Anti-CRISPR proteins (Acrs), natural bona fide CRISPR-Cas antagonists, have been identified that inhibit Cas activity by preventing DNA binding, cleavage, crRNA loading, or complex formation.[147–149] Acrs can effectively inhibit Cas9 activity when delivered *ex vivo* to hematopoietic stem cells, decreasing Cas-associated cell toxicity, and improving engraftment in a mouse model.[150] Delivery of AcrIIC3 can inhibit Nme2-Cas9 editing in mice, resulting in inhibition of editing in the heart and liver.[151] Integration defective LVs with mutations in integrase may offer uses in this setting but have not been extensively studied to conclude whether integration is 100% deficient or only impaired.[152] Small molecule inhibitors, doxycycline-dependent inactivation, truncated gRNAs, and synthetic oligonucleotides to silence Cas9 activity[153–157] have been developed to limit Cas9 persistence, and such approaches may be needed for editing applications.

Adenovirus. Adenovirus (Ad) vectors are used extensively as the many known serotypes can transduce a variety of cell types. Ad-CFTR vectors of serotypes 2 and 5 transduce primary HAE and were used to successfully transduce nasal turbinate epithelia *in vivo*, although transiently.[4] Serotypes Ad-2 and Ad-5 utilize the coxsackievirus and adenovirus receptor (CAR) as a primary receptor, which is localized to the basolateral surface of human airway epithelia.[158] A chimeric vector Ad-5 carrying a heterologous fiber molecule from serotype 35 (Ad5F35) can transduce the apical surface of human airway epithelia and correct anion conductance in cells carrying the *CFTR* F508del mutation.[159] There are safety concerns regarding clinical use of Ad vectors, as several clinical trials for CF observed inflammatory responses. A nonhuman primate study also reported alveolar inflammation with high-dose administration of an Ad vector.[160,161] High systemic doses of Ad can elicit severe immune responses, as observed in the clinical trial in 1999 for ornithine transcarbamylase (OTC) deficiency, in which administration of 10^{13} viral particles administered through the femoral artery resulted in the death of a patient.[162,163] Owing to these safety concerns, Ad vectors are mostly used in the research setting, or are modified to decrease harmful immune responses. Studies of the innate immune responses to Ad vectors may achieve modifications that improve their utility.[164,165]

Because of a relatively high seroprevalence of Ad-5 in the general population, alternative Ad vectors are being explored. Gorilla adenovirus isolates (GAd) can be converted to replication-deficient vectors that produce high-titer yields. GAd vectors have been used for vaccines against respiratory syncytial virus and Zika virus in preclinical studies to produce high-level antigen-specific antibodies.[166,167] GAd type 9, isolate 46 shows high lung endothelial cell tropism in a mouse model.[168] Like Ad-5, this GAd vector can be modified at the fiber knob to alter tissue targeting.[168] GAd vectors have a low seroprevalence and can undergo capsid engineering, possibly providing an alternative vector for gene delivery.

Helper-dependent adenovirus. Helper-dependent adenovirus (Hd-Ad) is attractive for delivery of large cargoes and CRISPR/Cas strategies that require the use of HDR donors. Hd-Ad has a large packaging capacity compared with AAV, with the ability to encode $\sim$36 kb.[169] This large packaging capacity can accommodate the genetic sequence of the Cas protein, gRNA, and donor DNA within a single vector. In Hd-Ad, all viral coding sequences are deleted to minimize viral protein production and produce a replication incompetent vector.[169] Hd-Ad vectors are less immunogenic than adenovirus vectors. A wide variety of serotypes may be used, but serotype 5 effectively transduces airway basal cells *in vivo* in mouse and pig models, as well as basal cells *in vitro* in primary HAE cultures.[4,170,171] Although it has been reported that Hd-Ad DNA can persist as episomal DNA, one

study reported transient expression of Cas9 in a vector that encoded Cas9 upstream of donor DNA, with complete loss of Cas9 expression after 7 days.[172] Although this phenomenon could be owing to dilution of the Hd-Ad DNA on serial passage, it is thought that the vector DNA is degraded upon donor DNA integration as a result of compromised vector DNA. Hd-Ad vectors may offer an alternative to transiently transduce cells for CRISPR/Cas-mediated gene therapy.

Nonviral Delivery

Nonviral vectors have been less widely used for lung delivery, owing to low transduction efficiency and inflammatory responses. Nonviral vectors continue to be pursued for delivery for the following potential advantages: (1) there is virtually no packaging limit, (2) many nonviral vectors are biodegradable and biocompatible, offering less cytotoxicity and adverse immune responses, (3) scale-up of production is relatively straightforward and economical, and (4) the transient expression of cargo may reduce off-target effects caused by constitutive expression of Cas proteins, host genome integration, and genotoxicity.

There is a resurgence of interest in nonviral delivery for pulmonary applications. The vectors are mainly composed of three configurations: (1) peptide-based, (2) lipid-based, and (3) polymer-based delivery. Other formulations of nonviral delivery systems, such as exosomal delivery, are also being explored. Although further discussion of this topic is outside the scope of this review, we refer the reader to the publications on inhaled nonviral delivery of CRISPR/Cas9 cargo[173] and nonviral strategies for treatment of CF.[174]

GENE THERAPY FOR LUNG DISEASE

Cystic Fibrosis

CF is an autosomal recessive disease resulting from mutations in the *CFTR* gene.[175] *CFTR* encodes an anion channel that conducts chloride and bicarbonate across epithelial cells in the pancreas, liver, intestines, gall bladder, sweat gland, airways, and genital ducts.[176] Mutations disrupting CFTR function alter ASL volume and composition, impair host defenses, and decrease mucociliary transport, leading to bacterial infections, airway remodeling, and reduced lung function.[175,176] CF affects >70,000 individuals worldwide and is the most common life-threatening inherited disease among Caucasians.

To date, >2,000 variants have been identified in the *CFTR* gene, with at least 346 known to be disease causing.[176] Mutations are classified according to their impact on *CFTR* transcription and translation as follows: Class I mutations are nonsense or frameshift mutations that result in truncated or unstable mRNA that preclude the production of any functional protein. Class II mutations, including the most common allele F508del, result in protein misfolding or reduced trafficking to the plasma membrane. Class III mutations are characterized by dysfunctional channel gating. Class IV mutations produce CFTR protein with reduced conductance. Class V mutations are located within introns and cause aberrant mRNA splicing. Finally, class VI mutations produce functional, but relatively unstable CFTR protein at the cell surface. Of importance, a mutation can impair CFTR function in more than one way.

Since 2011, several small molecule CFTR correctors, which facilitate protein folding, and potentiators, which act to keep the CFTR channel open, have been developed as FDA-approved CF therapeutics.[177–180] To date, three correctors (lumacaftor, tezacaftor, and elexacaftor) have been approved for treatment of class II mutations. Tezacaftor and elexacaftor can be used in combination, as each small molecule targets its own specific site, to synergistically enhance protein folding and function.[180] Ivacaftor, at present the only FDA-approved potentiator, targets class III mutations, such as G551D, to increase channel conductance, and has been shown to be effective for some class IV and V mutations as well.[180] A triple therapy of tezacaftor, elexacaftor, and ivacaftor, termed Trikafta is currently approved for individuals 12 years and older.[181] This treatment could benefit $\sim$90% of all individuals with CF within this age range, significantly advancing CF therapeutics.[180,181]

Small molecule therapies are significant advancements but must be taken for the lifetime of the individual. Certain side effects, such as abnormal liver function, may also make these treatment options unsuitable for some patients.[180] Finally, small molecule therapies have little to no benefit to the $\sim$10% of individuals with more rare mutations. Gene therapy strategies have the potential to address these concerns and provide a mutation agnostic cure. Previous studies suggest that achieving 6–50% wild-type CFTR function can restore chloride transport in airway epithelia and could provide significant clinical benefit.[182–187] Several approaches, from gene addition, gene correction, and RNA correction, are currently being studied to restore CFTR function.

Gene addition. The first CF gene therapy studies involved the insertion of an additional copy of the *CFTR* gene, either integrated in the host genome or as an episome. Several viral and nonviral delivery strategies have been applied, all with varying degrees of persistence. Retroviral and LVs were used to deliver and integrate *CFTR* expression cassettes, resulting in restoration of CFTR function in HAE, mouse, pig, and ferret models.[4,188–191] AAV vectors, which persist episomally, are an effective means to deliver and restore CFTR function *in vitro* and *in vivo* in mouse, rabbit, and macaque models.[192–194] However, previous clinical trials utilizing AAV2 vectors did not achieve clinical significance.[4,55,90,92–94,195] Inefficient gene transfer because of

poor airway transduction by the AAV2 serotype, insufficient CFTR expression owing to use of the ITR promoter in the vector construct, and generation of therapy-inactivating host immune responses may have contributed to the disappointing outcomes of these previous clinical trials. Mild stabilization of lung disease was observed in the most recent nonviral delivery phase 2b clinical trial, in which repeated *CFTR* cDNA (pGM169) complexed with liposome complex GL67A was administered through nebulization.[196] Although the improvements in lung function were modest in the pGM169/GL67A-treated group, the investigators concluded that repeated administration of this nonviral therapy is safe and can change clinically relevant parameters of lung function, such as FEV_1.[196]

Liposomes have been extensively studied for inhalable delivery of drugs, vitamins, and nucleic acids. The ''gold standard'' nonviral method of gene delivery to the lung is the cationic liposome GL67A. GL67A complexed with plasmid DNA (pDNA) carrying a *CFTR* expression cassette has shown encouraging results for pulmonary delivery in mice, sheep, and humans. The first clinical trial using GL67A as a vector delivery system resulted in $\sim$25% correction of the CF ion defect, although these results were only observed for 2 weeks. In addition, the treatment caused mild ''flu-like'' symptoms, most likely because of Toll-like receptor-9 (TLR-9) recognition of CpG content in the pDNA. Use of a third-generation, CpG-free plasmid maintained efficient delivery to mice lungs and reduced inflammatory responses to levels nearly indistinguishable to the vehicle control. In a second, phase 2b clinical trial, repeated administration of GL67A/pDNA complexes resulted in stabilization of lung function.

Hd-Ad systems effectively target the human airway epithelium and the airways of several animal models, providing a potential strategy for future clinical trials.[4,170,197,198] Transposase-based methods, such as the *piggyBac* system, which can also be delivered through Ad or AAV vectors, achieved persistent CFTR expression in mice and phenotypic correction in a CF pig model.[4,116,199]

rAAV/HBoV1 chimeras, in which an rAAV genome containing the full-length *CFTR* cDNA coding sequence driven by a CMV-β-actin promoter is packaged into a HBoV1 capsid, effectively transduced primary human polarized airway epithelial cultures from the apical surface and partially corrected CFTR-mediated chloride transport.[121] These chimeras may offer an alternative vector delivery system if there are no blocking antibody barriers. AAV/HBoV1 vectors can also effectively transduce ferret lungs, offering an animal model in which this CFTR-carrying chimeric vector can be tested for phenotypic correction.[120]

CRISPR/Cas9-mediated gene addition was used to insert a *CFTR* expression cassette into safe harbor loci in human and porcine cell lines.[172,200] In these studies, ''all-in-one'' HD-Ad vectors carrying expression cassettes for Cas9, a gRNA targeting either the AAVS1 or GGTA1 safe harbor locus in humans and pigs, respectively, and *CFTR* donor DNA with corresponding homology arms successfully integrated and expressed *CFTR* in a site-specific manner.[171,172,200] Transduced porcine IPEC-J2 cells exhibited an integration efficiency of 10%.[200] Although efficiency was not calculated, transduced IB3–1/CF cells produced measurable CFTR chloride conductance with transient Cas9 expression, most likely owing to instability of vector DNA upon HDR of donor DNA.[201] This CRISPR/Cas gene addition strategy provides high levels of integration in cell lines without persistent expression of the Cas9 protein. Further studies in primary HAE cells and *in vivo* models are required to determine the feasibility of this approach in humans.

Coupling CFTR expression to another gene through the ''GeneRide'' approach is another strategy with therapeutic potential. This approach involves insertion of a promoterless therapeutic gene cDNA in-frame upstream of the stop codon of an endogenous gene, separating the endogenous gene and the therapeutic gene with a 2A ribosomal skipping site.[202] This results in mRNA production containing both endogenous and therapeutic mRNAs but two separate proteins. This technique allowed insertion of the human coagulation factor IX (*hF9*) into the hepatocyte-expressed albumin locus, ameliorating a hemophilia B phenotype in a mouse model.[202] The same albumin locus also used to insert a promoterless human uridine glucuronosyl transferase A1 (*UGT1A1*) cDNA, rescuing a stillborn phenotype in a Crigler–Najjar syndrome type I (CNSI) mouse model, although this did not normalize plasma bilirubin levels.[203] Combining GeneRide with CRISPR/Cas9-mediated gene addition through a two AAV vector design: one carrying cDNA of saCas9 and gRNA targeting the albumin locus and the second vector carrying *UGT1A1* cDNA flanked by albumin homology regions, resulted in a 26-fold increase in targeting efficiency, rescue of the CNSI mouse model stillbirth phenotype, and produced bilirubin levels comparable with wild-type littermates.[204] The GeneRide approach shows potential for clinical applications for a variety of genetic disorders. Future inquiry into potential targeted loci, targeting efficiency, and off-targeting are needed to determine the therapeutic value of this approach for treatment of pulmonary diseases.

Gene editing. Cell penetrating peptides (CPPs), 5–30 amino acids in length that are cationic, amphipathic, or nonpolar can translocate the plasma membrane and facilitate cellular uptake.[205] There are many CPPs that can transport different cargoes to a variety of cell types. Combinations of multiple CPPs may also be used to enhance delivery efficiency.[205] In the context of CRISPR/Cas cargo delivery, CPPs can be linked to RNPs com-

prising Cas protein and gRNAs to mediate cellular uptake.[205] Delivery efficiency can vary widely based on cargo type and the targeted cell type, and extensive optimization of CPPs is required. CPPs also tend to become trapped in endosomal compartments upon internalization.[206,207] Addition of an endosomal leakage domain (ELD) can be used to destabilize the endosomal membrane, allowing escape of Cas RNPs into the cytosol.[208,209] CPP-ELDs carrying Cas9 RNPs or Cas12a RNPs were shown to efficiently transduce primary HAE cultures, as well as the large and small airways of mice.[17] These CPPs offer a promising delivery system for protein-based gene editing approaches for CF treatment. Recent evidence suggests that this Cas protein can effectively target primary human airway epithelia and murine lungs *in vivo*.[17]

Zinc finger nucleases (ZFN) are engineered nucleases that facilitate genome editing by binding a targeted DNA sequence through a DNA-binding domain and introducing a DSB through a DNA-cleaving domain. Bednarski *et al.* uses ZFNs targeting the 5′ end of exon 11 of the *CFTR* gene and introduced a donor construct containing exons 11–27 of *CFTR* to correct the F508del mutation in the CFBE41o− cell line, rescuing expression of *CFTR* mRNA and chloride conductance.[210] Although this approach is yet to be tested *in vivo*, the design of a super-exon could allow correction of all *CFTR* mutation downstream of exon 11.

With CRISPR/Cas technology, correction of disease-causing mutations has also been explored. Studies relying on HDR with donor *CFTR* DNA have corrected the F508del in human induced pluripotent stem cells (hiPSCs), intestinal organoids, and upper airway basal stem cell (UABC) organoids.[171,211–213] Off-target activity as predicted in these studies were relatively low, occurring in 0–4% of alleles sequenced.[211–213] Correction efficiency in these studies, however, has been relatively low or has utilized selection of positive cells or colonies through expression of resistance markers. Gene editing of the CFPAC-1 homozygous F508del human cell line using a viral vector-, drug selection-, and reporter enrichment-free (VDR-free) approach resulted in just 1% of alleles corrected.[214] *In vivo* mouse studies have demonstrated 0.1% HDR-mediated CRISPR-Cas gene editing in the murine lung,[215] well below the threshold of CFTR phenotypic restoration, as well as an indel efficiency >1%.[215]

An alternative strategy is transplantation of gene-corrected cells. Gene-corrected UABCs can be embedded onto pSIS membrane, FDA-approved porcine membranes used for sinonasal repair.[211] This technology could be used to implant gene-corrected basal stem cells in the upper airways, areas that can become reservoirs for antibiotic-resistant bacteria.[211] Although outside the scope of this review, *ex vivo* cell-based gene therapies are another approach under investigation for the treatment of lung diseases. Regardless of the approach, CRISPR/Cas-based HDR gene correction of CFTR mutations will need further progress to overcome the current low correction efficiency and the relatively high indel frequency.

Some mutations result in the creation of alternative splice sites within an intron of *CFTR*, resulting in creation of a pseudoexon or addition of sequence to a current exon.[216] These mutations are amenable to CRISPR/Cas-mediated NHEJ, by excision or disruption of the disease-causing mutation within the intron. Such an approach has been applied to 293T cell lines that contain either the c.1679 + 1634A>G, c.3140–26A>G, or C3718–2477C>T *CFTR* mutations.[217] Mutations c.1679 + 1634A>G and c.3140–26A>G create a splice acceptor site and disrupt 99% and 95% of *CFTR* transcripts, respectively.[218,219] CRISPR/Cas9-mediated NHEJ, using two gRNAs that target intronic sequences flanking the mutation, resulted in upward of 56% excision efficiency and up to 90% splicing rescue.[217] A third mutation, c.3718–2477C>T that creates a splice donor site,[220] was also tested using this approach, achieving 55% excision efficiency and reduction of aberrant splicing to 17%.[193] This degree of editing is 10-fold higher than an HDR-mediated approach using the same cell type and *CFTR* locus.[221] This NHEJ strategy is effective for editing intronic *CFTR* splicing mutations but would not be feasible for mutations affecting canonical splice sites, as this would likely result in deletion of part of the protein coding sequence. NHEJ repair approaches could become a viable therapy for individuals with mutations that currently do not benefit from small molecule therapies.

Base editing has the potential to target many CF-causing mutations, through use of alternative Cas proteins with different PAM sequences, without the need of donor DNA. Of the mutations listed on the CFTR2 database, 11.7% can theoretically be targeted by CBEs and 31.8% by ABEs.[222] In an intestinal organoid model, Geurts *et al.* achieved 8.88% editing efficiency of the rare mutation R553X through adenine base editing using a canonical Cas9 fused to an ABE (cas9-ABE) and editing efficiencies of 1.43% when targeting mutations W1282X and R553X with a noncanonical Cas9 base editor (xCas9-ABE).[222] In this study intestinal organoids were dissociated into single cells and electroporated with plasmids encoding the Cas9-ABE and gRNA. When repeated using organoids derived from nasal brushings target editing of R553X achieved a similar efficiency of 8%,[222] highlighting a promising future for base editing in the treatment of CF lung disease.

Triplex-forming peptide nucleic acids (PNAs) are synthetic nucleotide analogs that can induce DNA repair through sequence-specific triplex formation at targeted genomic sites.[223] Along with short donor DNA, PNAs have been used to edit the human β-globin and CCR5 genes.[224–227] Triplex-forming PNAs and donor DNA that target the F508del CFTR mutation delivered using polymer nanoparticles, achieved a modification frequency

approaching 10% in human CFBE samples, and 5% in the mouse nasal epithelium with very little off-target effects (<0.0001%).[228]

Gene activation. Class V and other CF-causing mutations result in insufficient CFTR expression at the plasma membrane. Increasing production might restore chloride and bicarbonate conductance to near wild-type levels. Targeting a dCas9 activator (dCas9-VPR), in which a dCas9 is fused to the tripartite activator VP64-p65-Rta, with gRNAs that bind upstream of the *CFTR* promoter activated and increased expression of wild-type or F508del CFTR in cultured human nasal epithelial cells.[229] Another epigenetic approach to increase CFTR expression is inhibiting BGas binding to *CFTR*. BGas is a long noncoding RNA (lncRNA) located in the antisense orientation of intron 11 of *CFTR*. BGas functions in concert with several proteins, including HMGA1, HMGB1, and WIBG, to alter chromatin structure and inhibit RNAP-II transcription of *CFTR*.[230] Inhibition of BGas increases CFTR expression and chloride channel activity.[230] Creation of a Gapmer, an antisense oligonucleotide with phosphorthioate bonds at the 5′ and 3′ ends, that targets BGas exon 1 (the exon that binds and suppresses CFTR transcription) increases CFTR expression sixfold, compared with a scrambled gapmer control.[229] Both approaches further enhanced CFTR presentation to the cell surface with the addition of small molecule potentiators and correctors.[229] Whether these documented increases in CFTR expression will provide therapeutic benefit remains to be seen. In addition, treatments using this strategy would not produce a cure but would need to be taken for the life of the individual. Despite these challenges, epigenetic modulation of *CFTR* could prove a useful therapeutic strategy in the future.

RNA editing. Like gene activation, RNA-directed editing can be used to provide a nonpermanent treatment for CF lung disease. Adenosine deaminases that act on RNAs (ADARs) are enzymes that catalyze site-directed mutagenesis of RNA by converting adenine to inosine, which is read as guanosine during translation.[231] This technology has been used to create a modified ADAR, termed λN-DD, in which a lambda phage N protein boxB that binds RNA is fused to the adenosine deaminase region of an ADAR.[231] This yields an ADAR that uses antisense RNA as a guide to bind CFTR mRNA. λN-DD has been shown to correct the W496X *CFTR* mutation *in vitro* with nearly 100% efficiency and within *Xenopus* oocytes to restore functional CFTR currents.[231]

SP Deficiencies

Pulmonary surfactant is a mixture of phospholipids and SPs B and C that function to reduce surface tension at the air–liquid interface in the alveoli.[232] SPs are highly expressed in alveolar type 2 (AT2) cells.[49] Mutations in genes *SFTPB*, *SFTPC*, that encode SP-B and SP-C, and ATP-binding member 3 (ABCA3), which is involved in surfactant metabolism, cause SP deficiencies.[233] SP deficiencies can cause loss-of-function phenotypes (*SFTPB* or *ABCA3* mutations), owing to aberrant surfactant metabolism or SP function, or gain-of-function phenotypes (*SFTPC* mutations), because of cytosolic accumulation of abnormal SP in AT2 cells and disrupted lung development.[233] SP deficiencies often present at birth with severe respiratory failure.[233] Treatment options are mostly supportive or compassionate care, as donor availability for pediatric lung transplants is limited.

All known *SFTPB* mutations are autosomal–recessive, the most common mutation causing a frameshift in codon 121 (pPro133GInsTer95).[233] An initial gene addition study utilized AdV delivery of human *SFTPB* to murine MLE12 cells. These mouse cells retain properties of AT2 cells but have very low SP-B levels and showed increased SP-B expression after Ad gene transfer.[234] Delivery of the same AdV vector to cotton rats resulted in peak human SP-B 48–96 h postadministration, which subsequently diminished to undetectable levels.[234] Administration of a SP-B expression plasmid by electroporation into a SP-B–deficient murine model, with expression controlled by a doxycycline inducible promoter, resulted in an eightfold increase in SP-B expression compared with a control plasmid treatment.[235] SP-B plasmid treatment also resulted in a modest 2.5 to 5-fold increase in survival.[235] Lung organoid models, termed alveolospheres, generated from hiPSCs, contain cells with canonical AT2 function and have been used to study the effectiveness of *SFTPB* gene editing.[236] Lentivirus delivered wild-type (*SFTPB*) to alveolospheres derived from SFTPB(pPro133GInsTer95) hiPSCs resulted in successful transcription and translation of wild-type SP-B, as well as secretion of surfactant bioactive lipids.[237] Electroporation of CRISPR/cas9 in the same model yielded similar results, including increased *SFTPB* mRNA, expression of mature SP-B protein, and generation of lamellar bodies.[236] One study also showed effective *SFTPB* gene editing in a mouse model. Using a mouse with SP-B expression under a doxycycline-inducible promoter, nuclease-encoding chemically modified (nec) mRNA delivery, and donor DNA delivered through an AAV vector were used to insert a CAG promoter upstream of the SP-B start codon through HDR, resulting in prolonged survival of the mice.[201] These studies provide insight into the effectiveness of a gene editing strategy for SP-B deficiency.

The most common *SFTPC* mutation is the I73T missense mutation.[238] Age of onset and disease severity are dependent on the specific *SFTPC* mutation and vary from severe respiratory failure at birth to idiopathic pulmonary fibrosis in adulthood.[233] Knockout approaches may be a sufficient treatment option for this type of SP deficiency, as *SFTPC* null mice develop normal growth and lung

function, whereas *SFTPC*(I73T) mice have an arrest of lung morphogenesis in late sacculation and are not viable.[239] One recent study using CRISPR/Cas9-mediated knockout of *SFTPC*(I73T) in fetal mice shows the potential therapeutic applications of such an approach. An Ad vector encoding spCas9, EGFP, and gRNA was delivered to SFTPC(I73T) fetal mice through intra-amniotic injection, resulting in 64% reduction in AT2 cells that express HA-SFTPC(I73T) and a 22.8% increase in survival.[238] Fetal lung editing through CRISPR/Cas9 has the potential to attenuate gain-of-function SP deficiency.[238]

Alpha-1 Antitrypsin Deficiency

Alpha-1 antitrypsin (AAT) deficiency (AATD) has a carrier frequency of 4% in the United States and is one of the most common genetic disorders in individuals with Northern European heritage.[240] Mutations are named by their protease inhibitor (Pi) phenotype. The most common AAT mutation, E342K, results in the production of the PiZ form of AAT, also termed Z-AAT.[240] Ninety percent of AATD patients are homozygous for PiZ allele. PiZ is prone to polymerization in the rough endoplasmic reticulum, impairing its secretion.[240] PiZ results in two disease phenotypes: (1) a gain-of-function phenotype caused by aggregation of PiZ in hepatocytes that can lead to liver fibrosis, cirrhosis, and hepatocellular carcinoma, and (2) a loss-of-function phenotype with decreased secreted, functional AAT, leading to lung disease.[240] PiZ leads to increased protease-mediated digestion of connective tissues in the lower airway and alveoli that can cause emphysema and chronic obstructive pulmonary disorder.[240] About 5–10% of AATD patients will develop liver disease, and most will develop lung disease in adulthood.[240] Current treatment options are limited for AATD. Patients who develop liver disease may require a liver transplant. Patients generally receive weekly infusions of AAT protein purified from healthy human donors.

AAT is a serine protease inhibitor (Serpin) encoded by the gene *SERPINA1*.[240] AAT is a highly abundant protein, primarily produced by hepatocytes and secreted into the serum, but a small amount is also produced locally by bronchial epithelial cells and monocytes.[240] Baseline human serum AAT levels range from 1,500 to 3,500 μg/mL (20–48 μM).[241] An AAT level of 11 μM in the serum or 1.2 μM in alveolar epithelial lining fluid (ELF) is considered to be protective, based on clinical observation of AATD patients.[242] One important function of AAT is to inactivate neutrophil elastase, an abundant protease released from neutrophil granules in response to various infectious or inflammatory stimuli.[240]

Gene addition strategies for AATD gene therapy involve delivery of the M-AAT coding sequence under the expression of a constitutive promoter. Early gene addition studies used retroviral vectors in murine or canine models of AATD, but these studies did not result in M-AAT expression at the therapeutic level.[75] Preclinical studies utilizing AAV vector serotypes AAV1, AAV2, AAV5, AAV6, AAV8, AAV9, and AAVrh.10 have been performed, with AAV1, AAV2, and AAVrh.10 serotypes moving onto clinical studies.[75,243] Both AAV1 and AAV2 vectors were administered through intramuscular injection and resulted in M-AAT expression below the therapeutic threshold. These trials likely failed because of the method of administration and the resultant targeted cells. Although, theoretically, any cell type can be modified to express M-AAT, as it is ultimately secreted into the serum, the method of administration can greatly affect the efficacy of the therapy. Using a murine model, intrapleural injection of an AAV5 vector resulted in higher human M-AAT levels than intramuscular delivery of the same dose.[75,244] This is most likely because of vector distribution after intrapleural administration, which allows for transduction of both the mesenchymal cells lining the pleura and systemic delivery through vector flow the visceral lymphatics, to systemic circulation, and then to hepatocytes. This would achieve both local M-AAT expression by mesenchymal cells that diffuses into the lung parenchyma and systemic M-AAT expression by hepatocytes that is secreted into the serum. A phase I/II clinical trial exploring intrapleural administration of an AAVrh.10 vector encoding M-AAT has recently concluded.[245] The results of this trial have not yet been published, but murine model data suggest M-AAT expression may be higher than previous trials.

Several *in vivo* studies using a PiZ mouse model have shown therapeutic promise for CRISPR/Cas9-based gene therapy of AATD. One study took two parallel approaches using a dual AAV8 system, with one vector encoding saCas9, a smaller version of Cas9 derived from *S. aureus*, and the other vector encoding either gRNA or gRNA and donor DNA.[246] The first approach used was to solve the gain-of-function phenotype of liver disease by creating indels through NHEJ. This resulted in 98% reduction in circulating PiZ and up to 86% reduction in PiZ aggregates within hepatocytes.[246] The second approach was to correct loss-of-function phenotype of lung disease through HDR. This resulted in 5% correction efficiency. A third study used a similar dual AAV strategy, with one AAV9 vector that has broad cellular tropism encoding Cas9, and a second AAV8 with hepatocellular tropism carrying gRNA and a HDR template.[247] Administration of these vectors resulted in an HDR frequency of 15–20% in newborn and adult PiZ transgenic mice and levels of secreted M-AAT of up to 71 μg/mL.[247] A fourth study used a knock-in approach, targeting the *ROSA26* safe harbor locus of C57B1/CJ mice.[248] A dual Ad5 vector administration with one vector encoding human M-AAT donor DNA and the other vector encoding Cas9 and gRNA, achieved long-term expression of M-AAT lasting over 200 days and stable serum M-AAT level of up to 100 μg/mL.[249]

FUTURE CONSIDERATIONS

Development of improved vectors for airway delivery and advancements in gene editing systems has brought remarkable progress. Major breakthroughs have occurred in CRISPR technology, including the advent of base editing and prime editing. Low indel and off-target frequencies with these approaches suggest that base editing and/or prime editing may be safer and more effective than traditional ZFN or CRISPR/Cas9 gene correction. Further study using *in vivo* models, optimization of correction efficiency, and adaptation to lung delivery will be required to assess therapeutic value.

As progress continues, open questions remain regarding the cell types targeted and the duration of treatment. Many airway epithelial cells that are accessible to vector delivery are terminally differentiated. As cells turn over, readministration may be required to continue to achieve therapeutic value. Investigation of the immune response and the potential for repeated dosing will be critical to future advancements.

Targeting of basal cells and other progenitor cell types could result in a lifelong cure using gene therapy; however, the effect of gene addition and gene editing on these cellular compartments is not fully understood. Determining transcriptomic changes in basal cells and other regional progenitor cell types after gene addition or editing and assessing their ability to differentiate and self-renew will provide important information on targeting these cell types for treatment of lung disease.

ACKNOWLEDGMENTS

The authors thank McCray and Sinn lab members for their critical review of the article.

AUTHOR DISCLOSURE

P.B.M. is on the scientific advisory board and receives support for sponsored research from Spirovant Sciences, Inc. A.V. has no competing financial interests.

FUNDING INFORMATION

This work was supported by the National Institutes of Health (UG3 HL-147366, P01 HL51670, P01 HL091842, P01 HL152960), the Cystic Fibrosis Foundation, The University of Iowa Center for Gene Therapy (DK54759), and the Roy J. Carver Chair in Pulmonary Research (P.B.M.).

REFERENCES

1. Kohn DB. Gene therapy for blood diseases. Curr Opin Biotechnol 2019;60:39–45.
2. Russell S, Bennett J, Wellman JA, et al. Efficacy and safety of voretigene neparvovec (AAV2-hRPE65v2) in patients with RPE65-mediated inherited retinal dystrophy: a randomised, controlled, open-label, phase 3 trial. Lancet 2017; 390:849–860.
3. Morrison C. Fresh from the biotech pipeline-2017. Nat Biotechnol 2018;36:131–136.
4. Cooney AL, McCray PB, Jr., Sinn PL. Cystic fibrosis gene therapy: looking back, looking forward. Genes (Basel) 2018;9:538.
5. Araldi RP, Khalil C, Grignet PH, et al. Medical applications of clustered regularly interspaced short palindromic repeats (CRISPR/Cas) tool: a comprehensive overview. Gene 2020;745: 144636.
6. Ishino Y, Shinagawa H, Makino K, et al. Nucleotide sequence of the iap gene, responsible for alkaline phosphatase isozyme conversion in *Escherichia coli*, and identification of the gene product. J Bacteriol 1987;169:5429–5433.
7. Barrangou R, Fremaux C, Deveau H, et al. CRISPR provides acquired resistance against viruses in prokaryotes. Science 2007;315:1709–1712.
8. Garneau JE, Dupuis ME, Villion M, et al. The CRISPR/Cas bacterial immune system cleaves bacteriophage and plasmid DNA. Nature 2010; 468:67–71.
9. Nishimasu H, Nureki O. Structures and mechanisms of CRISPR RNA-guided effector nucleases. Curr Opin Struct Biol 2017;43:68–78.
10. Nishimasu H, Ran FA, Hsu PD, et al. Crystal structure of Cas9 in complex with guide RNA and target DNA. Cell 2014;156:935–949.
11. Liang F, Han M, Romanienko PJ, et al. Homology-directed repair is a major double-strand break repair pathway in mammalian cells. Proc Natl Acad Sci U S A 1998;95:5172–5177.
12. Heyer WD, Ehmsen KT, Liu J. Regulation of homologous recombination in eukaryotes. Annu Rev Genet 2010;44:113–139.
13. San Filippo J, Sung P, Klein H. Mechanism of eukaryotic homologous recombination. Annu Rev Biochem 2008;77:229–257.
14. Zetsche B, Gootenberg JS, Abudayyeh OO, et al. Cpf1 is a single RNA-guided endonuclease of a class 2 CRISPR-Cas system. Cell 2015;163:759–771.
15. Bin Moon S, Lee JM, Kang JG, et al. Highly efficient genome editing by CRISPR-Cpf1 using CRISPR RNA with a uridinylate-rich 3′-overhang. Nat Commun 2018;9:3651.
16. Kim D, Kim J, Hur JK, et al. Genome-wide analysis reveals specificities of Cpf1 endonucleases in human cells. Nat Biotechnol 2016;34: 863–868.
17. Krishnamurthy S, Wohlford-Lenane C, Kandimalla S, et al. Engineered amphiphilic peptides enable delivery of proteins and CRISPR-associated nucleases to airway epithelia. Nat Commun 2019;10:4906.
18. Kleinstiver BP, Sousa AA, Walton RT, et al. Engineered CRISPR-Cas12a variants with increased activities and improved targeting ranges for gene, epigenetic and base editing. Nat Biotechnol 2019;37:276–282.
19. Jayavaradhan R, Pillis DM, Goodman M, et al. CRISPR-Cas9 fusion to dominant-negative 53BP1 enhances HDR and inhibits NHEJ specifically at Cas9 target sites. Nat Commun 2019;10: 2866.
20. Esvelt KM, Carlson JC, Liu DR. A system for the continuous directed evolution of biomolecules. Nature 2011;472:499–503.
21. Hu JH, Miller SM, Geurts MH, et al. Evolved Cas9 variants with broad PAM compatibility and high DNA specificity. Nature 2018;556:57–63.

22. Miller SM, Wang T, Randolph PB, et al. Continuous evolution of SpCas9 variants compatible with non-G PAMs. Nat Biotechnol 2020;38:471–481.

23. Endo M, Mikami M, Endo A, et al. Genome editing in plants by engineered CRISPR-Cas9 recognizing NG PAM. Nat Plants 2019;5:14–17.

24. Ren B, Liu L, Li S, et al. Cas9-NG greatly expands the targeting scope of the genome-editing toolkit by recognizing NG and other atypical PAMs in rice. Mol Plant 2019;12:1015–1026.

25. Hua K, Tao X, Han P, et al. Genome engineering in rice using Cas9 variants that recognize NG PAM sequences. Mol Plant 2019;12:1003–1014.

26. Huang TP, Zhao KT, Miller SM, et al. Circularly permuted and PAM-modified Cas9 variants broaden the targeting scope of base editors. Nat Biotechnol 2019;37:626–631.

27. Liu JJ, Orlova N, Oakes BL, et al. CasX enzymes comprise a distinct family of RNA-guided genome editors. Nature 2019;566:218–223.

28. Lundh M, Plucinska K, Isidor MS, et al. Bidirectional manipulation of gene expression in adipocytes using CRISPRa and siRNA. Mol Metab 2017;6:1313–1320.

29. Qi LS, Larson MH, Gilbert LA, et al. Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. Cell 2013;152:1173–1183.

30. Lo A, Qi L. Genetic and epigenetic control of gene expression by CRISPR-Cas systems. F1000Res 2017;6:747.

31. Perez Rojo F, Nyman RKM, Johnson AAT, et al. CRISPR-Cas systems: ushering in the new genome editing era. Bioengineered 2018;9:214–221.

32. Kemaladewi DU, Bassi PS, Erwood S, et al. A mutation-independent approach for muscular dystrophy via upregulation of a modifier gene. Nature 2019;572:125–130.

33. Rees HA, Liu DR. Base editing: precision chemistry on the genome and transcriptome of living cells. Nat Rev Genet 2018;19:770–788.

34. Gaudelli NM, Komor AC, Rees HA, et al. Programmable base editing of A*T to G*C in genomic DNA without DNA cleavage. Nature 2017;551:464–471.

35. Komor AC, Kim YB, Packer MS, et al. Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. Nature 2016;533:420–424.

36. Landrum MJ, Lee JM, Benson M, et al. ClinVar: public archive of interpretations of clinically relevant variants. Nucleic Acids Res 2016;44:D862–D868.

37. Richter MF, Zhao KT, Eton E, et al. Phage-assisted evolution of an adenine base editor with improved Cas domain compatibility and activity. Nat Biotechnol 2020;38:883–891.

38. Zuo E, Sun Y, Wei W, et al. Cytosine base editor generates substantial off-target single-nucleotide variants in mouse embryos. Science 2019;364:289–292.

39. Xie J, Ge W, Li N, et al. Efficient base editing for multiple genes and loci in pigs using base editors. Nat Commun 2019;10:2852.

40. Jiang T, Henderson JM, Coote K, et al. Chemical modifications of adenine base editor mRNA and guide RNA expand its application scope. Nat Commun 2020;11:1979.

41. Liu Z, Shan H, Chen S, et al. Highly efficient base editing with expanded targeting scope using SpCas9-NG in rabbits. FASEB J 2020;34:588–596.

42. Zhou S, Cai B, He C, et al. Programmable base editing of the sheep genome revealed no genome-wide off-target mutations. Front Genet 2019;10:215.

43. Lee HK, Willi M, Miller SM, et al. Targeting fidelity of adenine and cytosine base editors in mouse embryos. Nat Commun 2018;9:4804.

44. Villiger L, Grisch-Chan HM, Lindsay H, et al. Treatment of a metabolic liver disease by in vivo genome base editing in adult mice. Nat Med 2018;24:1519–1525.

45. Ryu SM, Koo T, Kim K, et al. Adenine base editing in mouse embryos and an adult mouse model of Duchenne muscular dystrophy. Nat Biotechnol 2018;36:536–539.

46. Anzalone AV, Randolph PB, Davis JR, et al. Search-and-replace genome editing without double-strand breaks or donor DNA. Nature 2019;576:149–157.

47. Cone RA. Barrier properties of mucus. Adv Drug Deliv Rev 2009;61:75–85.

48. Sanders N, Rudolph C, Braeckmans K, et al. Extracellular barriers in respiratory gene therapy. Adv Drug Deliv Rev 2009;61:115–127.

49. Kim N, Duncan GA, Hanes J, et al. Barriers to inhaled gene therapy of obstructive lung diseases: a review. J Control Release 2016;240:465–488.

50. Saltzman WM, Radomsky ML, Whaley KJ, et al. Antibody diffusion in human cervical mucus. Biophys J 1994;66:508–515.

51. Olmsted SS, Padgett JL, Yudin AI, et al. Diffusion of macromolecules and virus-like particles in human cervical mucus. Biophys J 2001;81:1930–1937.

52. Calcedo R, Vandenberghe LH, Gao G, et al. Worldwide epidemiology of neutralizing antibodies to adeno-associated viruses. J Infect Dis 2009;199:381–390.

53. Halbert CL, Miller AD, McNamara S, et al. Prevalence of neutralizing antibodies against adeno-associated virus (AAV) types 2, 5, and 6 in cystic fibrosis and normal populations: implications for gene therapy using AAV vectors. Hum Gene Ther 2006;17:440–447.

54. Chirmule N, Propert K, Magosin S, et al. Immune responses to adenovirus and adeno-associated virus in humans. Gene Ther 1999;6:1574–1583.

55. Moss RB, Rodman D, Spencer LT, et al. Repeated adeno-associated virus serotype 2 aerosol-mediated cystic fibrosis transmembrane regulator gene transfer to the lungs of patients with cystic fibrosis: a multicenter, double-blind, placebo-controlled trial. Chest 2004;125:509–521.

56. Zabner J, Ramsey BW, Meeker DP, et al. Repeat administration of an adenovirus vector encoding cystic fibrosis transmembrane conductance regulator to the nasal epithelium of patients with cystic fibrosis. J Clin Invest 1996;97:1504–1511.

57. Sinn PL, Arias AC, Brogden KA, et al. Lentivirus vector can be readministered to nasal epithelia without blocking immune responses. J Virol 2008;82:10684–10692.

58. Griesenbach U, Inoue M, Meng C, et al. Assessment of F/HN-pseudotyped lentivirus as a clinically relevant vector for lung gene therapy. Am J Respir Crit Care Med 2012;186:846–856.

59. Gough V, Gersbach CA. Immunity to Cas9 as an obstacle to persistent genome editing. Mol Ther 2020;28:1389–1391.

60. Chew WL, Tabebordbar M, Cheng JK, et al. A multifunctional AAV-CRISPR-Cas9 and its host response. Nat Methods 2016;13:868–874.

61. Nelson CE, Wu Y, Gemberling MP, et al. Long-term evaluation of AAV-CRISPR genome editing for Duchenne muscular dystrophy. Nat Med 2019;25:427–432.

62. Moreno AM, Palmer N, Aleman F, et al. Immune-orthogonal orthologues of AAV capsids and of Cas9 circumvent the immune response to the administration of gene therapy. Nat Biomed Eng 2019;3:806–816.

63. Li A, Tanner MR, Lee CM, et al. AAV-CRISPR gene editing is negated by pre-existing immunity to Cas9. Mol Ther 2020;28:1432–1441.

64. Charlesworth CT, Deshpande PS, Dever DP, et al. Identification of preexisting adaptive immunity to Cas9 proteins in humans. Nat Med 2019;25:249–254.

65. Simhadri VL, McGill J, McMahon S, et al. Prevalence of pre-existing antibodies to CRISPR-associated nuclease Cas9 in the USA population. Mol Ther Methods Clin Dev 2018;10:105–112.

66. Wagner DL, Amini L, Wendering DJ, et al. High prevalence of *Streptococcus pyogenes* Cas9-reactive T cells within the adult human population. Nat Med 2019;25:242–248.

67. Ferdosi SR, Ewaisha R, Moghadam F, et al. Multifunctional CRISPR-Cas9 with engineered immunosilenced human T cell epitopes. Nat Commun 2019;10:1842.

68. Ernst N, Ulrichskotter S, Schmalix WA, et al. Interaction of liposomal and polycationic trans-

fection complexes with pulmonary surfactant. J Gene Med 1999;1:331–340.

69. Tsan MF, Tsan GL, White JE. Surfactant inhibits cationic liposome-mediated gene transfer. Hum Gene Ther 1997;8:817–825.

70. Duncan JE, Whitsett JA, Horowitz AD. Pulmonary surfactant inhibits cationic liposome-mediated gene delivery to respiratory epithelial cells in vitro. Hum Gene Ther 1997;8:431–438.

71. Chono S, Tanino T, Seki T, et al. Influence of particle size on drug delivery to rat alveolar macrophages following pulmonary administration of ciprofloxacin incorporated into liposomes. J Drug Target 2006;14:557–566.

72. Anderson DS, Patchin ES, Silva RM, et al. Influence of particle size on persistence and clearance of aerosolized silver nanoparticles in the rat lung. Toxicol Sci 2015;144:366–381.

73. Beck SE, Laube BL, Barberena CI, et al. Deposition and expression of aerosolized rAAV vectors in the lungs of Rhesus macaques. Mol Ther 2002;6:546–554.

74. Pickles RJ, McCarty D, Matsui H, et al. Limited entry of adenovirus vectors into well-differentiated airway epithelium is responsible for inefficient gene transfer. J Virol 1998;72: 6014–6023.

75. Chiuchiolo MJ, Crystal RG. Gene therapy for alpha-1 antitrypsin deficiency lung disease. Ann Am Thorac Soc 2016;13(Suppl 4):S352–S369.

76. Boucher RC. Evidence for airway surface dehydration as the initiating event in CF airway disease. J Intern Med 2007;261:5–16.

77. Duncan GA, Jung J, Hanes J, et al. The mucus barrier to inhaled gene therapy. Mol Ther 2016; 24:2043–2053.

78. Yuan S, Hollinger M, Lachowicz-Scroggins ME, et al. Oxidation increases mucin polymer cross-links to stiffen airway mucus gels. Sci Transl Med 2015;7:276ra227.

79. Suk JS, Lai SK, Boylan NJ, et al. Rapid transport of muco-inert nanoparticles in cystic fibrosis sputum treated with N-acetyl cysteine. Nanomedicine (Lond) 2011;6:365–375.

80. Hida K, Lai SK, Suk JS, et al. Common gene therapy viral vectors do not efficiently penetrate sputum from cystic fibrosis patients. PLoS One 2011;6:e19919.

81. Schuster BS, Kim AJ, Kays JC, et al. Overcoming the cystic fibrosis sputum barrier to leading adeno-associated virus gene therapy vectors. Mol Ther 2014;22:1484–1493.

82. Suk JS, Boylan NJ, Trehan K, et al. N-acetylcysteine enhances cystic fibrosis sputum penetration and airway gene transfer by highly compacted DNA nanoparticles. Mol Ther 2011; 19:1981–1989.

83. Kim AJ, Boylan NJ, Suk JS, et al. Use of single-site-functionalized PEG dendrons to prepare gene vectors that penetrate human mucus barriers. Angew Chem Int Ed Engl 2013;52:3985–3988.

84. Suk JS, Kim AJ, Trehan K, et al. Lung gene therapy with highly compacted DNA nanoparticles that overcome the mucus barrier. J Control Release 2014;178:8–17.

85. Gao GP, Alvira MR, Wang L, et al. Novel adeno-associated viruses from rhesus monkeys as vectors for human gene therapy. Proc Natl Acad Sci U S A 2002;99:11854–11859.

86. Fischer AC, Beck SE, Smith CI, et al. Successful transgene expression with serial doses of aerosolized rAAV2 vectors in rhesus macaques. Mol Ther 2003;8:918–926.

87. Ostedgaard LS, Rokhlina T, Karp PH, et al. A shortened adeno-associated virus expression cassette for CFTR gene transfer to cystic fibrosis airway epithelia. Proc Natl Acad Sci U S A 2005; 102:2952–2957.

88. Flotte TR, Fischer AC, Goetzmann J, et al. Dual reporter comparative indexing of rAAV pseudotyped vectors in chimpanzee airway. Mol Ther 2010;18:594–600.

89. Limberis MP, Vandenberghe LH, Zhang L, et al. Transduction efficiencies of novel AAV vectors in mouse airway epithelium in vivo and human ciliated airway epithelium in vitro. Mol Ther 2009;17:294–301.

90. Flotte TR, Zeitlin PL, Reynolds TC, et al. Phase I trial of intranasal and endobronchial administration of a recombinant adeno-associated virus serotype 2 (rAAV2)-CFTR vector in adult cystic fibrosis patients: a two-part clinical study. Hum Gene Ther 2003;14:1079–1088.

91. Wagner JA, Moran ML, Messner AH, et al. A phase I/II study of tgAAV-CF for the treatment of chronic sinusitis in patients with cystic fibrosis. Hum Gene Ther 1998;9:889–909.

92. Moss RB, Milla C, Colombo J, et al. Repeated aerosolized AAV-CFTR for treatment of cystic fibrosis: a randomized placebo-controlled phase 2B trial. Hum Gene Ther 2007;18:726–732.

93. Aitken ML, Moss RB, Waltz DA, et al. A phase I study of aerosolized administration of tgAAVCF to cystic fibrosis subjects with mild lung disease. Hum Gene Ther 2001;12:1907–1916.

94. Wagner JA, Messner AH, Moran ML, et al. Safety and biological efficacy of an adeno-associated virus vector-cystic fibrosis transmembrane regulator (AAV-CFTR) in the cystic fibrosis maxillary sinus. Laryngoscope 1999;109: 266–274.

95. Pillay S, Meyer NL, Puschnik AS, et al. An essential receptor for adeno-associated virus infection. Nature 2016;530:108–112.

96. Excoffon KJ, Koerber JT, Dickey DD, et al. Directed evolution of adeno-associated virus to an infectious respiratory virus. Proc Natl Acad Sci U S A 2009;106:3865–3870.

97. Hamilton BA, Li X, Pezzulo AA, et al. Polarized AAVR expression determines infectivity by AAV gene therapy vectors. Gene Ther 2019;26:240–249.

98. Grimm D, Lee JS, Wang L, et al. In vitro and in vivo gene therapy vector evolution via multispecies interbreeding and retargeting of adeno-associated viruses. J Virol 2008;82:5887–5911.

99. Korbelin J, Sieber T, Michelfelder S, et al. Pulmonary targeting of adeno-associated viral vectors by next-generation sequencing-guided screening of random capsid displayed peptide libraries. Mol Ther 2016;24:1050–1061.

100. Pekrun K, De Alencastro G, Luo QJ, et al. Using a barcoded AAV capsid library to select for clinically relevant gene therapy vectors. JCI Insight 2019;4:22.

101. de Alencastro G, Pekrun K, Valdmanis P, et al. Tracking adeno-associated virus capsid evolution by high-throughput sequencing. Hum Gene Ther 2020;31:553–564.

102. Lisowski L, Dane AP, Chu K, et al. Selection and evaluation of clinically relevant AAV variants in a xenograft liver model. Nature 2014;506:382–386.

103. Paulk NK, Pekrun K, Zhu E, et al. Bioengineered AAV capsids with combined high human liver transduction in vivo and unique humoral seroreactivity. Mol Ther 2018;26:289–303.

104. Wu Z, Asokan A, Samulski RJ. Adeno-associated virus serotypes: vector toolkit for human gene therapy. Mol Ther 2006;14:316–327.

105. Buning H, Srivastava A. Capsid modifications for targeting and improving the efficacy of AAV vectors. Mol Ther Methods Clin Dev 2019;12: 248–265.

106. Deverman BE, Pravdo PL, Simpson BP, et al. Cre-dependent selection yields AAV variants for widespread gene transfer to the adult brain. Nat Biotechnol 2016;34:204–209.

107. Munch RC, Muth A, Muik A, et al. Off-target-free gene delivery by affinity-purified receptor-targeted viral vectors. Nat Commun 2015;6:6246.

108. Duncan GA, Kim N, Colon-Cortes Y, et al. An adeno-associated viral vector capable of penetrating the mucus barrier to inhaled gene therapy. Mol Ther Methods Clin Dev 2018;9:296–304.

109. Wu Z, Yang H, Colosi P. Effect of genome size on AAV vector packaging. Mol Ther 2010;18:80–86.

110. Yan Z, Sun X, Feng Z, et al. Optimization of recombinant adeno-associated virus-mediated expression for large transgenes, using a synthetic promoter and tandem array enhancers. Hum Gene Ther 2015;26:334–346.

111. Ostedgaard LS, Zabner J, Vermeer DW, et al. CFTR with a partially deleted R domain corrects the cystic fibrosis chloride transport defect in human airway epithelia in vitro and in mouse nasal mucosa in vivo. Proc Natl Acad Sci U S A 2002;99:3093–3098.

112. Truong DJ, Kuhner K, Kuhn R, et al. Development of an intein-mediated split-Cas9 system for gene therapy. Nucleic Acids Res 2015;43:6450–6458.

113. Stevens AJ, Sekar G, Shah NH, et al. A promiscuous split intein with expanded protein engineering applications. Proc Natl Acad Sci U S A 2017;114:8538–8543.

114. Levy JM, Yeh WH, Pendse N, et al. Cytosine and adenine base editing of the brain, liver, retina, heart and skeletal muscle of mice via adeno-associated viruses. Nat Biomed Eng 2020;4:97–110.

115. Yeh WH, Shubina-Oleinik O, Levy JM, et al. In vivo base editing restores sensory transduction and transiently improves auditory function in a mouse model of recessive deafness. Sci Transl Med 2020;12:546.

116. Cooney AL, Thornell IM, Singh BK, et al. A novel AAV-mediated gene delivery system corrects CFTR function in pigs. Am J Respir Cell Mol Biol 2019;61:747–754.

117. Halbert CL, Allen JM, Miller AD. Efficient mouse airway transduction following recombination between AAV vectors carrying parts of a larger gene. Nat Biotechnol 2002;20:697–701.

118. Ponnazhagan S, Weigel KA, Raikwar SP, et al. Recombinant human parvovirus B19 vectors: erythroid cell-specific delivery and expression of transduced genes. J Virol 1998;72:5224–5230.

119. Fakhiri J, Schneider MA, Puschhof J, et al. Novel chimeric gene therapy vectors based on adeno-associated virus and four different mammalian bocaviruses. Mol Ther Methods Clin Dev 2019;12:202–222.

120. Yan Z, Feng Z, Sun X, et al. Human bocavirus type-1 capsid facilitates the transduction of ferret airways by adeno-associated virus genomes. Hum Gene Ther 2017;28:612–625.

121. Yan Z, Keiser NW, Song Y, et al. A novel chimeric adenoassociated virus 2/human bocavirus 1 parvovirus vector efficiently transduces human airway epithelia. Mol Ther 2013;21:2181–2194.

122. Zou W, Cheng F, Shen W, et al. Nonstructural protein NP1 of human bocavirus 1 plays a critical role in the expression of viral capsid proteins. J Virol 2016;90:4658–4669.

123. Yan Z, Zou W, Feng Z, et al. Establishment of a high-yield recombinant adeno-associated virus/human bocavirus vector production system independent of bocavirus nonstructural proteins. Hum Gene Ther 2019;30:556–570.

124. Deng X, Zou W, Yan Z, et al. Establishment of a recombinant AAV2/HBoV1 vector production system in insect cells. Genes (Basel) 2020;11:439.

125. Urabe M, Ding C, Kotin RM. Insect cells as a factory to produce adeno-associated virus type 2 vectors. Hum Gene Ther 2002;13:1935–1943.

126. Virag T, Cecchini S, Kotin RM. Producing recombinant adeno-associated virus in foster cells: overcoming production limitations using a baculovirus-insect cell expression strategy. Hum Gene Ther 2009;20:807–817.

127. Kotin RM. Large-scale recombinant adeno-associated virus production. Hum Mol Genet 2011;20:R2–R6.

128. Urabe M, Nakakura T, Xin KQ, et al. Scalable generation of high-titer recombinant adeno-associated virus type 5 in insect cells. J Virol 2006;80:1874–1885.

129. Fakhiri J, Linse KP, Mietzsch M, et al. Impact of natural or synthetic singletons in the capsid of human bocavirus 1 on particle infectivity and immunoreactivity. J Virol 2020;94.

130. DiPrimio N, Asokan A, Govindasamy L, et al. Surface loop dynamics in adeno-associated virus capsid assembly. J Virol 2008;82:5178–5189.

131. Kabadi AM, Ousterout DG, Hilton IB, et al. Multiplex CRISPR/Cas9-based genome engineering from a single lentiviral vector. Nucleic Acids Res 2014;42:e147.

132. Sinn PL, Cooney AL, Oakland M, et al. Lentiviral vector gene transfer to porcine airways. Mol Ther Nucleic Acids 2012;1:e56.

133. Borok Z, Harboe-Schmidt JE, Brody SL, et al. Vesicular stomatitis virus G-pseudotyped lentivirus vectors mediate efficient apical transduction of polarized quiescent primary alveolar epithelial cells. J Virol 2001;75:11747–11754.

134. Kremer KL, Dunning KR, Parsons DW, et al. Gene delivery to airway epithelial cells in vivo: a direct comparison of apical and basolateral transduction strategies using pseudotyped lentivirus vectors. J Gene Med 2007;9:362–368.

135. Wang G, Davidson BL, Melchert P, et al. Influence of cell polarity on retrovirus-mediated gene transfer to differentiated human airway epithelia. J Virol 1998;72:9818–9826.

136. Wang G, Zabner J, Deering C, et al. Increasing epithelial junction permeability enhances gene transfer to airway epithelia In vivo. Am J Respir Cell Mol Biol 2000;22:129–138.

137. Johnson LG, Olsen JC, Naldini L, et al. Pseudotyped human lentiviral vector-mediated gene transfer to airway epithelia in vivo. Gene Ther 2000;7:568–574.

138. Cmielewski P, Farrow N, Devereux S, et al. Gene therapy for cystic fibrosis: improved delivery techniques and conditioning with lysophosphatidylcholine enhance lentiviral gene transfer in mouse lung airways. Exp Lung Res 2017;43:426–433.

139. Sinn PL, Hwang BY, Li N, et al. Novel GP64 envelope variants for improved delivery to human airway epithelial cells. Gene Ther 2017;24:674–679.

140. Sinn PL, Hickey MA, Staber PD, et al. Lentivirus vectors pseudotyped with filoviral envelope glycoproteins transduce airway epithelia from the apical surface independently of folate receptor alpha. J Virol 2003;77:5902–5910.

141. Sinn PL, Burnight ER, Hickey MA, et al. Persistent gene expression in mouse nasal epithelia following feline immunodeficiency virus-based vector gene transfer. J Virol 2005;79:12818–12827.

142. Sinn PL, Penisten AK, Burnight ER, et al. Gene transfer to respiratory epithelia with lentivirus pseudotyped with Jaagsiekte sheep retrovirus envelope glycoprotein. Hum Gene Ther 2005;16:479–488.

143. Kang Y, Stein CS, Heth JA, et al. In vivo gene transfer using a nonprimate lentiviral vector pseudotyped with Ross River Virus glycoproteins. J Virol 2002;76:9378–9388.

144. Kobinger GP, Weiner DJ, Yu QC, et al. Filovirus-pseudotyped lentiviral vector can efficiently and stably transduce airway epithelia in vivo. Nat Biotechnol 2001;19:225–230.

145. Mitomo K, Griesenbach U, Inoue M, et al. Toward gene therapy for cystic fibrosis using a lentivirus pseudotyped with Sendai virus envelopes. Mol Ther 2010;18:1173–1182.

146. Ferrari S, Griesenbach U, Iida A, et al. Sendai virus-mediated CFTR gene transfer to the airway epithelium. Gene Ther 2007;14:1371–1379.

147. Marino ND, Pinilla-Redondo R, Csorgo B, et al. Anti-CRISPR protein applications: natural brakes for CRISPR-Cas technologies. Nat Methods 2020;17:471–479.

148. Watters KE, Shivram H, Fellmann C, et al. Potent CRISPR-Cas9 inhibitors from Staphylococcus genomes. Proc Natl Acad Sci U S A 2020;117:6531–6539.

149. Eitzinger S, Asif A, Watters KE, et al. Machine learning predicts new anti-CRISPR proteins. Nucleic Acids Res 2020;48:4698–4708.

150. Li C, Psatha N, Gil S, et al. HDAd5/35(++) adenovirus vector expressing anti-CRISPR peptides decreases CRISPR/Cas9 toxicity in human hematopoietic stem cells. Mol Ther Methods Clin Dev 2018;9:390–401.

151. Lee J, Mou H, Ibraheim R, et al. Tissue-restricted genome editing in vivo specified by microRNA-repressible anti-CRISPR proteins. RNA 2019;25:1421–1431.

152. Liu KC, Lin BS, Gao AD, et al. Integrase-deficient lentivirus: opportunities and challenges for human gene therapy. Curr Gene Ther 2014;14:352–364.

153. Maji B, Gangopadhyay SA, Lee M, et al. A high-throughput platform to identify small-molecule inhibitors of CRISPR-Cas9. Cell 2019;177:1067.e19–1079.e19.

154. Li B, Zeng C, Li W, et al. Synthetic oligonucleotides inhibit CRISPR-Cpf1-mediated genome editing. Cell Rep 2018;25:3262.e3–3272.e3.

155. Cui YR, Wang SJ, Chen J, et al. Allosteric inhibition of CRISPR-Cas9 by bacteriophage-derived peptides. Genome Biol 2020;21:51.

156. Rose JC, Popp NA, Richardson CD, et al. Suppression of unwanted CRISPR-Cas9 editing by co-administration of catalytically inactivating truncated guide RNAs. Nat Commun 2020;11: 2697.

157. Kelkar A, Zhu Y, Groth T, et al. Doxycycline-dependent self-inactivation of CRISPR-Cas9 to temporally regulate on- and off-target editing. Mol Ther 2020;28:29–41.

158. Walters RW, Grunst T, Bergelson JM, et al. Basolateral localization of fiber receptors limits adenovirus infection from the apical surface of airway epithelia. J Biol Chem 1999;274:10219–10226.

159. Granio O, Ashbourne Excoffon KJ, Henning P, et al. Adenovirus 5-fiber 35 chimeric vector mediates efficient apical correction of the cystic fibrosis transmembrane conductance regulator defect in cystic fibrosis primary airway epithelia. Hum Gene Ther 2010;21:251–269.

160. Simon RH, Engelhardt JF, Yang Y, et al. Adenovirus-mediated transfer of the CFTR gene to lung of nonhuman primates: toxicity study. Hum Gene Ther 1993;4:771–780.

161. Engelhardt JF, Simon RH, Yang Y, et al. Adenovirus-mediated transfer of the CFTR gene to lung of nonhuman primates: biological efficacy study. Hum Gene Ther 1993;4:759–769.

162. Raper SE, Wilson JM, Yudkoff M, et al. Developing adenoviral-mediated in vivo gene therapy for ornithine transcarbamylase deficiency. J Inherit Metab Dis 1998;21(Suppl 1):119–137.

163. Marshall E. Gene therapy death prompts review of adenovirus vector. Science 1999;286:2244–2245.

164. Atasheva S, Yao J, Shayakhmetov DM. Innate immunity to adenovirus: lessons from mice. FEBS Lett 2019;593:3461–3483.

165. Rollier CS, Spencer AJ, Sogaard KC, et al. Modification of adenovirus vaccine vector-induced immune responses by expression of a signalling molecule. Sci Rep 2020;10:5716.

166. Hassan AO, Dmitriev IP, Kashentseva EA, et al. A gorilla adenovirus-based vaccine against Zika virus induces durable immunity and confers protection in pregnancy. Cell Rep 2019;28: 2634.e4–2646.e4.

167. Johnson TR, Rangel D, Graham BS, et al. Genetic vaccine for respiratory syncytial virus provides protection without disease potentiation. Mol Ther 2014;22:196–205.

168. Lu ZH, Dmitriev IP, Brough DE, et al. A new gorilla adenoviral vector with natural lung tropism avoids liver toxicity and is amenable to capsid engineering and vector retargeting. J Virol 2020;94:e00265–20.

169. Cots D, Bosch A, Chillon M. Helper dependent adenovirus vectors: progress and future prospects. Curr Gene Ther 2013;13:370–381.

170. Cao H, Ouyang H, Grasemann H, et al. Transducing airway basal cells with a helper-dependent adenoviral vector for lung gene therapy. Hum Gene Ther 2018;29:643–652.

171. Palmer DJ, Turner DL, Ng P. A single "all-in-one" helper-dependent adenovirus to deliver donor DNA and CRISPR/Cas9 for efficient homology-directed repair. Mol Ther Methods Clin Dev 2020;17:441–447.

172. Xia E, Duan R, Shi F, et al. Overcoming the undesirable CRISPR-Cas9 expression in gene correction. Mol Ther Nucleic Acids 2018;13:699–709.

173. Chow MYT, Chang RYK, Chan HK. Inhalation delivery technology for genome-editing of respiratory diseases. Adv Drug Deliv Rev 2020.

174. Velino C, Carella F, Adamiano A, et al. Nanomedicine approaches for the pulmonary treatment of cystic fibrosis. Front Bioeng Biotechnol 2019;7:406.

175. Stoltz DA, Meyerholz DK, Welsh MJ. Origins of cystic fibrosis lung disease. N Engl J Med 2015; 372:1574–1575.

176. Yan Z, McCray PB, Jr., Engelhardt JF. Advances in gene therapy for cystic fibrosis lung disease. Hum Mol Genet 2019;28:R88–R94.

177. Clancy JP, Cotton CU, Donaldson SH, et al. CFTR modulator theratyping: current status, gaps and future directions. J Cyst Fibros 2019;18:22–34.

178. Boyle MP, Bell SC, Konstan MW, et al. A CFTR corrector (lumacaftor) and a CFTR potentiator (ivacaftor) for treatment of patients with cystic fibrosis who have a phe508del CFTR mutation: a phase 2 randomised controlled trial. Lancet Respir Med 2014;2:527–538.

179. Wainwright CE, Elborn JS, Ramsey BW. Lumacaftor-ivacaftor in patients with cystic fibrosis homozygous for Phe508del CFTR. N Engl J Med 2015;373:1783–1784.

180. Lopes-Pacheco M. CFTR modulators: the changing face of cystic fibrosis in the era of precision medicine. Front Pharmacol 2019;10:1662.

181. Bear CE. A therapy for most with cystic fibrosis. Cell 2020;180:211.

182. Johnson LG, Olsen JC, Sarkadi B, et al. Efficiency of gene transfer for restoration of normal airway epithelial function in cystic fibrosis. Nat Genet 1992;2:21–25.

183. Goldman MJ, Yang Y, Wilson JM. Gene therapy in a xenograft model of cystic fibrosis lung corrects chloride transport more effectively than the sodium defect. Nat Genet 1995;9:126–131.

184. Farmen SL, Karp PH, Ng P, et al. Gene transfer of CFTR to airway epithelia: low levels of expression are sufficient to correct Cl- transport and overexpression can generate basolateral CFTR. Am J Physiol Lung Cell Mol Physiol 2005;289: L1123–L1130.

185. Zhang L, Button B, Gabriel SE, et al. CFTR delivery to 25% of surface epithelial cells restores normal rates of mucus transport to human cystic fibrosis airway epithelium. PLoS Biol 2009;7: e1000155.

186. Dannhoffer L, Blouquit-Laye S, Regnier A, et al. Functional properties of mixed cystic fibrosis and normal bronchial epithelial cell cultures. Am J Respir Cell Mol Biol 2009;40:717–723.

187. Shah VS, Ernst S, Tang XX, et al. Relationships among CFTR expression, HCO3- secretion, and host defense may inform gene- and cell-based cystic fibrosis therapies. Proc Natl Acad Sci U S A 2016;113:5382–5387.

188. Wang G, Slepushkin V, Zabner J, et al. Feline immunodeficiency virus vectors persistently transduce nondividing airway epithelia and correct the cystic fibrosis defect. J Clin Invest 1999; 104:R55–R62.

189. Cooney AL, Abou Alaiwa MH, Shah VS, et al. Lentiviral-mediated phenotypic correction of cystic fibrosis pigs. JCI Insight 2016;1:e88730.

190. Limberis M, Anson DS, Fuller M, et al. Recovery of airway cystic fibrosis transmembrane conductance regulator function in mice with cystic fibrosis after single-dose lentivirus-mediated gene transfer. Hum Gene Ther 2002;13:1961–1970.

191. Alton EW, Beekman JM, Boyd AC, et al. Preparation for a first-in-man lentivirus trial in patients with cystic fibrosis. Thorax 2017;72:137–147.

192. Afione SA, Conrad CK, Kearns WG, et al. In vivo model of adeno-associated virus vector persistence and rescue. J Virol 1996;70:3235–3241.

193. Halbert CL, Standaert TA, Aitken ML, et al. Transduction by adeno-associated virus vectors in the rabbit airway: efficiency, persistence, and readministration. J Virol 1997;71:5932–5941.

194. Flotte TR, Schwiebert EM, Zeitlin PL, et al. Correlation between DNA transfer and cystic fibrosis airway epithelial cell correction after recombinant adeno-associated virus serotype 2 gene therapy. Hum Gene Ther 2005;16:921–928.

195. Wagner JA, Nepomuceno IB, Messner AH, et al. A phase II, double-blind, randomized, placebo-controlled clinical trial of tgAAVCF using maxillary sinus delivery in patients with cystic fibrosis with antrostomies. Hum Gene Ther 2002;13: 1349–1359.

196. Alton E, Armstrong DK, Ashby D, et al. Repeated nebulisation of non-viral CFTR gene therapy in patients with cystic fibrosis: a randomised, double-blind, placebo-controlled, phase 2b trial. Lancet Respir Med 2015;3:684–691.

197. Parks RJ, Chen L, Anton M, et al. A helper-dependent adenovirus vector system: removal of helper virus by Cre-mediated excision of the viral packaging signal. Proc Natl Acad Sci U S A 1996; 93:13565–13570.

198. Brunetti-Pierri N, Ng P. Progress and prospects: gene therapy for genetic diseases with helper-dependent adenoviral vectors. Gene Ther 2008; 15:553–560.

199. Cooney AL, Singh BK, Loza LM, et al. Widespread airway distribution and short-term phenotypic correction of cystic fibrosis pigs following aerosol delivery of piggyBac/adenovirus. Nucleic Acids Res 2018;46:9591–9600.

200. Zhou ZP, Yang LL, Cao H, et al. In vitro validation of a CRISPR-mediated CFTR correction strategy for preclinical translation in pigs. Hum Gene Ther 2019;30:1101–1116.

201. Mahiny AJ, Dewerth A, Mays LE, et al. In vivo genome editing using nuclease-encoding mRNA corrects SP-B deficiency. Nat Biotechnol 2015; 33:584–586.

202. Barzel A, Paulk NK, Shi Y, et al. Promoterless gene targeting without nucleases ameliorates haemophilia B in mice. Nature 2015;517:360–364.

203. Porro F, Bortolussi G, Barzel A, et al. Promoterless gene targeting without nucleases rescues lethality of a Crigler-Najjar syndrome mouse model. EMBO Mol Med 2017;9:1346–1355.

204. De Caneva A, Porro F, Bortolussi G, et al. Coupling AAV-mediated promoterless gene targeting to SaCas9 nuclease to efficiently correct liver metabolic diseases. JCI Insight 2019;5:e128863.

205. Lino CA, Harper JC, Carney JP, et al. Delivering CRISPR: a review of the challenges and approaches. Drug Deliv 2018;25:1234–1257.

206. Lonn P, Kacsinta AD, Cui XS, et al. Enhancing endosomal escape for intracellular delivery of macromolecular biologic therapeutics. Sci Rep 2016;6:32301.

207. Erazo-Oliveras A, Muthukrishnan N, Baker R, et al. Improving the endosomal escape of cell-penetrating peptides and their cargos: strategies and challenges. Pharmaceuticals (Basel) 2012;5: 1177–1209.

208. Sato H, Feix JB. Peptide-membrane interactions and mechanisms of membrane destruction by amphipathic alpha-helical antimicrobial peptides. Biochim Biophys Acta 2006;1758:1245–1256.

209. Lostale-Seijo I, Louzao I, Juanes M, et al. Peptide/Cas9 nanostructures for ribonucleoprotein cell membrane transport and gene edition. Chem Sci 2017;8:7923–7931.

210. Bednarski C, Tomczak K, Vom Hovel B, et al. Targeted integration of a super-exon into the CFTR locus leads to functional correction of a cystic fibrosis cell line model. PLoS One 2016;11: e0161072.

211. Vaidyanathan S, Salahudeen AA, Sellers ZM, et al. High-efficiency, selection-free gene repair in airway stem cells from cystic fibrosis patients rescues CFTR function in differentiated epithelia. Cell Stem Cell 2020;26:161.e4–171.e4.

212. Firth AL, Menon T, Parker GS, et al. Functional gene correction for cystic fibrosis in lung epithelial cells generated from patient iPSCs. Cell Rep 2015;12:1385–1390.

213. Schwank G, Koo BK, Sasselli V, et al. Functional repair of CFTR by CRISPR/Cas9 in intestinal stem cell organoids of cystic fibrosis patients. Cell Stem Cell 2013;13:653–658.

214. Ruan J, Hirai H, Yang D, et al. Efficient gene editing at major CFTR mutation loci. Mol Ther Nucleic Acids 2019;16:73–81.

215. Platt RJ, Chen S, Zhou Y, et al. CRISPR-Cas9 knockin mice for genome editing and cancer modeling. Cell 2014;159:440–455.

216. Elborn JS. Cystic fibrosis. Lancet 2016;388:2519–2531.

217. Sanz DJ, Hollywood JA, Scallan MF, et al. Cas9/gRNA targeted excision of cystic fibrosis-causing deep-intronic splicing mutations restores normal splicing of CFTR mRNA. PLoS One 2017;12: e0184009.

218. Chillon M, Dork T, Casals T, et al. A novel donor splice site in intron 11 of the CFTR gene, created by mutation 1811 + 1.6kbA—>G, produces a new exon: high frequency in Spanish cystic fibrosis chromosomes and association with severe phenotype. Am J Hum Genet 1995;56:623–629.

219. Beck S, Penque D, Garcia S, et al. Cystic fibrosis patients with the 3272–26A—>G mutation have mild disease, leaky alternative mRNA splicing, and CFTR protein at the cell membrane. Hum Mutat 1999;14:133–144.

220. Highsmith WE, Burch LH, Zhou Z, et al. A novel mutation in the cystic fibrosis gene in patients with pulmonary disease but normal sweat chloride concentrations. N Engl J Med 1994;331: 974–980.

221. Hollywood JA, Lee CM, Scallan MF, et al. Analysis of gene repair tracts from Cas9/gRNA double-stranded breaks in the human CFTR gene. Sci Rep 2016;6:32230.

222. Geurts MH, de Poel E, Amatngalim GD, et al. CRISPR-based adenine editors correct nonsense mutations in a cystic fibrosis organoid biobank. Cell Stem Cell 2020;26:503.e7–510.e7.

223. Ricciardi AS, Quijano E, Putman R, et al. Peptide nucleic acids as a tool for site-specific gene editing. Molecules 2018;23.

224. Schleifman EB, Bindra R, Leif J, et al. Targeted disruption of the CCR5 gene in human hematopoietic stem cells stimulated by peptide nucleic acids. Chem Biol 2011;18:1189–1198.

225. McNeer NA, Schleifman EB, Cuthbert A, et al. Systemic delivery of triplex-forming PNA and donor DNA by nanoparticles mediates site-specific genome editing of human hematopoietic cells in vivo. Gene Ther 2013;20:658–669.

226. Schleifman EB, McNeer NA, Jackson A, et al. Site-specific genome editing in PBMCs with PLGA nanoparticle-delivered PNAs confers HIV-1 resistance in humanized mice. Mol Ther Nucleic Acids 2013;2:e135.

227. Bahal R, Ali McNeer N, Quijano E, et al. In vivo correction of anaemia in beta-thalassemic mice by gammaPNA-mediated gene editing with nanoparticle delivery. Nat Commun 2016;7:13304.

228. McNeer NA, Anandalingam K, Fields RJ, et al. Nanoparticles that deliver triplex-forming peptide nucleic acid molecules correct F508del CFTR in airway epithelium. Nat Commun 2015;6:6952.

229. Villamizar O, Waters SA, Scott T, et al. Targeted activation of cystic fibrosis transmembrane conductance regulator. Mol Ther 2019;27:1737–1748.

230. Saayman SM, Ackley A, Burdach J, et al. Long non-coding RNA BGas regulates the cystic fibrosis transmembrane conductance regulator. Mol Ther 2016;24:1351–1357.

231. Montiel-Gonzalez MF, Vallecillo-Viejo I, Yudowski GA, et al. Correction of mutations within the cystic fibrosis transmembrane conductance regulator by site-directed RNA editing. Proc Natl Acad Sci U S A 2013;110:18285–18290.

232. Nkadi PO, Merritt TA, Pillers DA. An overview of pulmonary surfactant in the neonate: genetics, metabolism, and the role of surfactant in health and disease. Mol Genet Metab 2009;97:95–101.

233. Han S, Mallampalli RK. The role of surfactant in lung disease and host defense against pulmonary infections. Ann Am Thorac Soc 2015;12: 765–774.

234. Yei S, Bachurski CJ, Weaver TE, et al. Adenoviral-mediated gene transfer of human surfactant protein B to respiratory epithelial cells. Am J Respir Cell Mol Biol 1994;11:329–336.

235. Barnett RC, Lin X, Barravecchia M, et al. Featured article: electroporation-mediated gene delivery of surfactant protein B (SP-B) restores expression and improves survival in mouse model of SP-B deficiency. Exp Biol Med (Maywood) 2017;242:1345–1354.

236. Jacob A, Morley M, Hawkins F, et al. Differentiation of human pluripotent stem cells into functional lung alveolar epithelial cells. Cell Stem Cell 2017;21:472.e10–488.e10.

237. Leibel SL, Winquist A, Tseu I, et al. Reversal of surfactant protein B deficiency in patient specific human induced pluripotent stem cell derived lung organoids by gene therapy. Sci Rep 2019;9: 13450.

238. Alapati D, Zacharias WJ, Hartman HA, et al. In utero gene editing for monogenic lung disease. Sci Transl Med 2019;11:eaav8375.

239. Glasser SW, Burhans MS, Korfhagen TR, et al. Altered stability of pulmonary surfactant in SP-C-deficient mice. Proc Natl Acad Sci U S A 2001; 98:6366–6371.

240. Flotte TR. Alpha-1 antitrypsin deficiency as a candidate for gene editing. Hum Gene Ther 2018;29:843–844.

241. Fregonese L, Stolk J. Hereditary alpha-1-antitrypsin deficiency and its clinical consequences. Orphanet J Rare Dis 2008;3:16.

242. Gadek JE, Klein HG, Holland PV, et al. Replacement therapy of alpha 1-antitrypsin deficiency. Reversal of protease-antiprotease imbalance within the alveolar structures of PiZ subjects. J Clin Invest 1981; 68:1158–1165.

243. Stiles KM, Sondhi D, Kaminsky SM, et al. Intrapleural gene therapy for alpha-1 antitrypsin deficiency-related lung disease. Chronic Obstr Pulm Dis 2018;5:244–257.

244. De B, Heguy A, Leopold PL, et al. Intrapleural administration of a serotype 5 adeno-associated virus coding for alpha1-antitrypsin mediates persistent, high lung and serum levels of alpha1-antitrypsin. Mol Ther 2004;10:1003–1010.

245. Chiuchiolo MJ, Kaminsky SM, Sondhi D, et al. Phase I/II study of intrapleural administration of a serotype rh.10 replication-deficient adeno-associated virus gene transfer vector expressing the human alpha1-antitrypsin cDNA to individuals with alpha1-antitrypsin deficiency. Hum Gene Ther Clin Dev 2014;25:112–133.

246. Shen S, Sanchez ME, Blomenkamp K, et al. Amelioration of alpha-1 antitrypsin deficiency diseases with genome editing in transgenic mice. Hum Gene Ther 2018;29:861–873.

247. Song CQ, Wang D, Jiang T, et al. In vivo genome editing partially restores alpha1-antitrypsin in a murine model of AAT deficiency. Hum Gene Ther 2018;29:853–860.

248. Stephens CJ, Kashentseva E, Everett W, et al. Targeted in vivo knock-in of human alpha-1-antitrypsin cDNA using adenoviral delivery of CRISPR/Cas9. Gene Ther 2018;25:139–156.

249. Bjursell M, Porritt MJ, Ericson E, et al. Therapeutic genome editing with CRISPR/Cas9 in a humanized mouse model ameliorates alpha1-antitrypsin deficiency phenotype. EBioMedicine 2018;29:104–111.

Received for publication June 17, 2020;
accepted after revision August 19, 2020.

Published online: August 19, 2020.