Minireview

Pulmonary gene delivery—Realities and possibilities

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Impact statement

This work provides an update on basic, pre-clinical, and clinical gene delivery to the lung, highlighting improvements and remaining challenges to effective *in vivo* gene therapy. This provides important information on questions and problems in the field that should be addressed to further translation of work from the laboratory to the clinic. We further highlight new/undeveloped techniques of interest to the field. The information contained within has not been presented from this viewpoint or format previously, to our knowledge.

Abstract

Delivery of genetic material to tissues *in vivo* is an important technique used in research settings and is the foundation upon which clinical gene therapy is built. The lung is a prime target for gene delivery due to a host of genetic, acquired, and infectious diseases that manifest themselves there, resulting in many pathologies. However, the *in vivo* delivery of genetic material to the lung remains a practical problem clinically and is considered the major obstacle needed to be overcome for gene therapy. Currently there are four main strategies for *in vivo* gene delivery to the lung: viral vectors, liposomes, nanoparticles, and electroporation. Viral delivery uses several different genetically modified viruses that enter the cell and express desired genes that have been inserted to the viral genome. Liposomes use combinations of charged and neutral lipids that can encapsulate genetic cargo and enter cells through endogenous mechanisms, thereby delivering their cargoes. Nanoparticles are defined by their size (typically less than 100 nm) and are made up of many different classes of building blocks, including biological and synthetic polymers, cell penetrant and other peptides, and dendrimers, that also enter cells through endogenous mechanisms. Electroporation uses mild to moderate electrical pulses to create pores in the cell membrane through which delivered genetic material can enter a cell. An emerging fifth category, exosomes and extracellular vesicles, may have advantages of both viral and non-viral approaches. These extracellular vesicles bud from cellular membranes containing receptors and ligands that may aid cell targeting and which can be loaded with genetic material for efficient transfer. Each of these vectors can be used for different gene delivery applications based on mechanisms of action, side-effects, and other factors, and their use in the lung and possible clinical considerations is the primary focus of this review.

Keywords: Lung, gene delivery, viral vectors, liposomes, nanoparticles, electroporation, exosomes

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The current state of gene therapy

Clinical gene delivery is a recent development, with only 17 FDA-approved therapies available as of August 2019. Most of these FDA-approved therapies involve the reprogramming of autologous cells externally before transplant of the reprogrammed cells into a patient. The majority of these approaches are for immunotherapy for various cancers, and many employ CAR-T cells. Indeed, only two current FDA-approved drugs are for gene therapy directly, Luxturna and Zolgensma, using viruses to transfer genes to treat congenital blindness and spinal muscular atrophy, respectively. However, none of the approved gene therapies currently target the lung, although over 180 Phase I, II, and III clinical trials have been completed and another 250 are currently progressing (ClinicalTrials.gov, 2019). Transplantation strategies come with many potential problems due to pre-existing immunity and failed engraftment. Some of these therapies may also face ethical challenges due to questions concerning the sources of the transplanted cells used.[1–4] Additionally, prices for patients are upwards of hundreds of thousands to millions of dollars for these early cell-based gene therapies.[5–8] Given these drawbacks, several different methods are being developed in order to deliver genetic therapies *in vivo* without having to transplant cells. These methods aim to decrease cost for the patient and reduce the potential of adverse effects.

The main challenges of *in vivo* gene delivery include off-target effects of the vector itself or the genetic material and

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delivery efficiency. Early approaches for gene therapy were largely focused on gene overexpression or methods to repair a mutant gene using viral or non-viral delivery strategies. Inefficiency of gene transfer, immunological responses, and non-specificity of cell targeting are just a few of the problems associated with viral approaches for gene delivery. By contrast, many methods of non-viral delivery have been less robust, yielding lower levels of transfection *in vivo*. When successful, it has even been shown that overexpressing plasmids sometimes can outcompete host gene expression in a competitive manner, making it difficult to modulate appropriate levels of transgene expression. Early gene correction technology focused on zinc-finger nucleases and TALENS, though these are now being replaced by more efficient CRISPR-Cas technologies. As gene therapy has evolved, it has been realized that more than simply overexpression or gene replacement can be done. One of these approaches have been to knockdown aberrant or other gene expression utilizing RNA interference (RNAi) technologies. Many different nucleic acids can be used for RNAi, including siRNA, shRNA, bifunctional shRNA, lncRNA, and miRNA. The different nucleic acid species used can have large differences in off-target effects and the duration and degree of target gene knockdown; both siRNA and shRNA are highly specific for a given target gene, while lncRNA and miRNA are more promiscuous due to less stringent homology needed for binding to their targets and thus can target multiple genes in entire pathways. While many RNAi approaches have used direct transfer of modified RNAs, both shRNA and miRNA can be expressed in target cells via transferred plasmids for longer term expression as well.[9–11] Functional delivery of all of these genetic materials *in vivo* has remained problematic due to issues with vector off-target effects, delivery efficiency, and perceived and real safety issues.

Disease targets in the lung

The lung is a prime target for gene delivery due to its importance in maintaining homeostasis. Abnormal lung function is a common cause of death and is strongly correlated with the onset and severity of other pathologies. Indeed, the moto of the American Lung Association is "when you can't breathe, nothing else matters". The lung is the primary organ affected in many acute and chronic pathologies including cystic fibrosis, acute respiratory distress syndrome (ARDS), familial emphysema, pulmonary fibrosis, cancer, and numerous bacterial and viral infections. For example, cystic fibrosis (CF) is caused by a familial defect in CFTR, a gene responsible for chloride ion transport and necessary for normal function of the lung, gut, and pancreas. Abnormal secretion of mucus in the lung as a result of altered CFTR localization and activity decreases mucociliary clearance in the lung, decreases oxygenation, allows persistent pathogenic bacterial colonization, and eventually leads to death.[12,13] Several recent drugs (e.g. Trikafta from Vertex Pharmaceuticals) have been developed that tremendously improve treatment for some genetic variants of CFTR by improving protein folding and channel activity, but many mutations are not effectively treated in this manner, especially nonsense mutations of the gene in which no full length CFTR is produced. Treatment for such mutations, which account for approximately 10% of all cystic fibrosis patients, thus necessitates some form of genetic therapy.[12,13,16] Another example is ARDS, which has a high mortality rate and no therapeutic approach besides basic symptomatic and supportive care strategies. ARDS is caused by many different insults, most commonly sepsis, lung trauma, pneumonia, acid or toxic gas inhalation, and viral infections, most recently highlighted by the COVID-19 pandemic, all of which result in the breakdown of the alveolar-capillary barrier, increased systemic inflammation, reduced gas exchange, hypoxemia, and ultimately multi-organ failure and is usually concurrent with other trauma or pathology making clinical care and treatment more difficult.[14,15] Again, as with cystic fibrosis, since many traditional drugs have failed to show any activity against ARDS, a genetic approach seems plausible. Familial emphysema results from a deficiency of alpha-1 antitrypsin, causing widespread damage to and simplification of alveoli (causing greatly reduced gas exchange and lung function) requiring plasma or serum perfusions weekly-monthly.[17–19] Like cystic fibrosis, this single gene mutation (or deletion) has long been a prime target for gene therapy approaches. Chronic obstructive pulmonary disease (COPD) is a largely smoking-associated disorder that also has aberrant alpha-1 antitrypsin activity, simplified alveolar structure, greatly reduced lung function, and similarly has no treatment currently.[20–22] Interstitial lung disease, more commonly known as pulmonary fibrosis, is a broad category of disease where lung tissue undergoes a fibrotic transformation in response to injury or disease caused by perturbations of cellular and genetic signaling. As for ARDS and COPD, no single gene is responsible for the disease and as such, treatment will require a complex genetic approach in order to generate a therapeutic response.[14,15,21,22] The lung is also a common site of cancer metastasis and has a relatively high possibility of primary tumors as well.[23,24] Finally, due to the lung's interaction with the environment, viral, bacterial, and fungal infections are also common and often associated with devastating consequences. Significant examples include tuberculosis, invasive pulmonary aspergillosis, and bacterial and viral pneumonia. These latter two can and often do lead to ARDS, as the world is currently experiencing with the novel COVID-19 coronavirus pandemic. In all cases and for all of these diseases, genetic therapies are of great importance due to lack of effective and widely available pharmacological therapeutics and the limitations of clinical care.

Barriers to gene delivery in the lung

While the lung is well suited to many forms of gene and drug delivery primarily due to its accessibility (via nose or mouth) and extremely large epithelial surface area, a number of physical and biological barriers are present (Figure 1). Any lung-targeted therapy must first pass a number of innate barriers, including mucus, pulmonary surfactant, ciliary beating and clearance, airway branching,

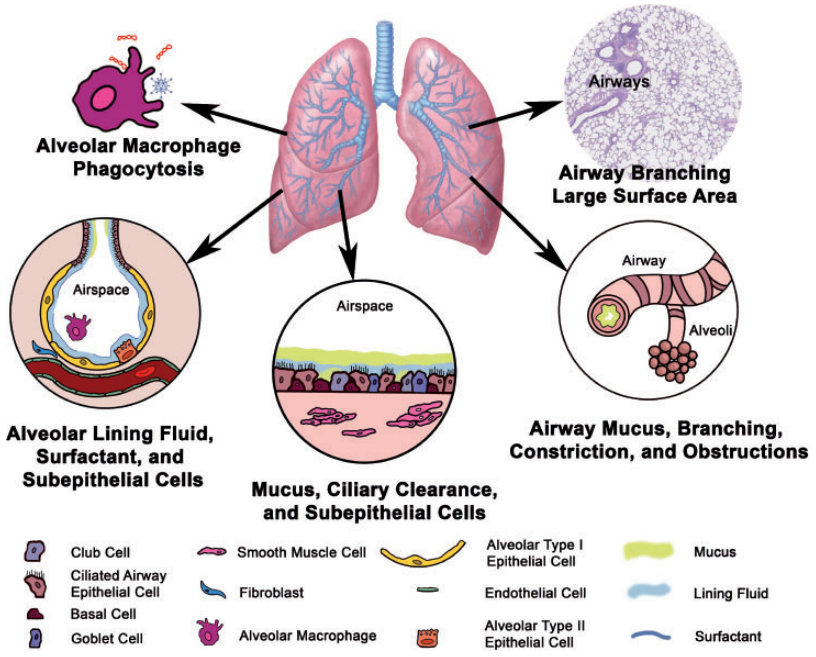

Figure 1. Barriers to gene delivery in the lung. A number of physical, chemical, and physiological barriers for gene and drug delivery in the lung are shown.

innate immune responses, and local inflammation. Even in healthy individuals, the mucus and surfactant present in the lung has small nanosized pores which can impair or even prevent large molecules from passing through the lining fluid layer to the target cells below. This is even more of an issue in individuals with certain pathologies such as cystic fibrosis or asthma where mucus hypersecretion is a hallmark of the diseases. In the healthy lung where lining fluid is not abnormally viscous, the cilia constantly beat to clear the airways of unwanted particles, bacteria, and viruses. While this is beneficial for protecting the lung, the clearance can also cause reduced gene delivery by the same mechanisms.[25,26] Airway branching also makes it difficult for drugs that cannot be aerosolized to target more than limited portions of the lung. Although bolus delivery of drugs and nanoparticles has been shown to mediate relatively even distribution to the deep lung in animal models,[27] this is dependent on bolus volume and rate of administration, both of which are often not amenable to humans. Further, phagocytosis of delivered particles and by resident lung macrophages and their activation to induce widespread pulmonary and systemic inflammation can make drug design more challenging. This coupled with robust inflammatory responses caused by the induction and release of pro-inflammatory cytokines and chemokines by the pulmonary epithelium and endothelium can cause significant, and even deadly, inflammation, greatly limiting any effects of gene transfer. Lastly, if cells other than the epithelium are to be targets for gene delivery following airway delivery, the epithelium itself becomes a major barrier. Indeed, most gene delivery agents transfect or transduce only those cells in which they come in contact with, leaving the sub-epithelial cells largely untouched by the delivery agent unless damage to the epithelial lining occurs. Thus, when most viruses or non-viral vectors are used, no gene transfer to the endothelium, fibroblasts, smooth muscle, or other subepithelial cell is obtained. Bypassing these various barriers and targeting specific cells is therefore a large goal of gene delivery technology.

Viral vectors

Viral vectors are one of the oldest non-chemical methods of gene delivery. First developed in the 1970s, viral vectors have largely remained the forerunners in gene therapy development. Initially, the viral vectors used for gene delivery were integrating retroviruses. While no longer commonly used for direct *in vivo* delivery, they are used extensively for *ex vivo* transduction of T-cells for cell therapies. Currently, adenovirus, adeno-associated virus (AAV), and lentiviruses are the most common vectors for viral gene delivery. *In vivo* use of viral vectors has had difficulty with immune responses, and even FDA-approved viral gene therapies require package insert warnings of possible cytokine release storm, a potentially lethal overactive immune response, as a side effect as well as other types of possible damage. Newer designs of viral gene delivery vectors have tried to limit their immunogenicity and replication capabilities to reduce these possible side effects.

The greatest advantage of all viral vectors is their inherent infectivity, or their ability to enter target cells, deliver cargo contained within the viral capsid, and lead to highly efficient gene delivery and expression (Figure 2, Tables 1 and 2). Due to the physiochemical properties of many capsid proteins and the small size of viral particles, viruses can often overcome several of the physical barriers of the lung, including mucus, surfactant, ciliary clearance, and airway branching. Viruses, such as retroviruses and lentiviruses, have innate mechanisms to integrate into the genome, allowing for long-term gene expression of the integrated transgene(s). The benefit or detriment of integration

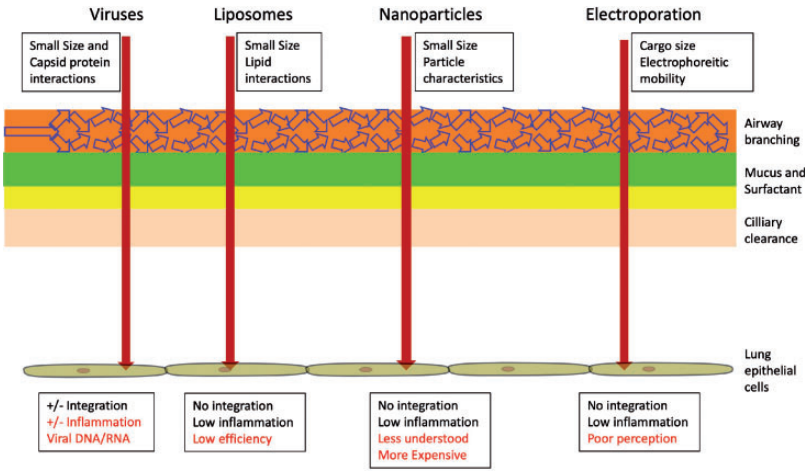

Figure 2. Relationship between gene therapy vectors and delivery barriers in the lung. Properties and abilities to overcome various delivery barriers in the lung are shown for the major classes of viral and non-viral methods of gene delivery (liposomes, nanoparticles, electroporation).

is context-dependent on whether sustained long-term or transient short-term expression is desired. Integration drives long-term expression, while a lack of integration for viruses most often provides short-term expression. In the treatment of inherited diseases, like cystic fibrosis or familial emphysema, integration for long-term expression may be preferred since long-term gene expression/replacement is needed to treat the disease. In contrast, an acute pathology, such as ARDS, is better treated in a transient manner, since the disease itself is also transient. This avoids potential side effects from integration and long-term upregulation or downregulation of pathways. A variety of endogenous viral capabilities allow vector choice to be made based on the type of pathology being treated and its specific needs. Due to these characteristics, viral vectors are already in use for several FDA-approved therapies for both short and long-term gene expression. The three most common viral vectors currently used, adenovirus, AAV, and lentiviruses, are each briefly discussed below.

Adenovirus

Adenovirus is a non-enveloped, dsDNA virus with a capsid of approximately 80–100nm in size. Modified adenoviral genomes used for gene transfer are ~30kb and can deliver ~8kb of recombinant DNA.[28] Wild-type adenoviruses cause a transient infection of the pulmonary tract in immunocompetent hosts. The transiency of these infections is due to high immunogenicity of the virus itself, both against capsid proteins and virus-encoded regulatory and replication proteins. Modifications to the adenoviral genome, removing most or all replication capability and immunogenic portions of the capsid proteins, have become standard when using helper-dependent and late generation adenoviral vectors *in vivo*. However, even with these modifications, most adenovirus-driven expression still lasts only one to two weeks *in vivo* due to a lack of integration and eventual immune clearance.[29–31] While adenovirus appears to be a vector of choice for pulmonary gene therapy in the laboratory and has gene transfer efficiencies of almost 95% *in vitro*, the values are usually much less *in vivo* and require upwards of 10^9 to 10^{10} plaque forming units delivered intratracheally in the rat or mouse. Consequently, cell damage and inflammation are frequently observed, even with late generation helper-dependent viruses. Additionally, the major receptor for adenovirus sits in the basolateral membrane of the airway epithelium, requiring barrier disruption in order for viral transduction to occur.[21,22] This makes its widespread clinical use for most diseases in the lung doubtful. Additionally, pre-existing immunity to many adenoviral serotypes limits their use for gene transfer in many patients and remains one of the greatest obstacles to its widespread clinical use.[29–31] However, despite these issues, numerous studies have been published using adenoviral vectors for gene delivery to the lungs of animals due to the high levels of transgene expression obtained and have allowed researchers to test a variety of different genes in various disease models, including ARDS, cystic fibrosis, alpha-1 anti-trypsin deficiency, non-small cell lung carcinoma, and surfactant protein deficiencies.[32,33] Further, this immunogenicity and transient expression have been fortuitous for using adenoviruses for *in vivo* tumor ablation and for vaccine development, where immunogenicity is advantageous.[34] Some adenoviral serotypes can even specifically target cancer cells due to the increased glycosylation or polysialic acid on the tumor cells and a preference for binding to them by the vector.[35,36] This type of cancer targeting can be used for direct lysis or modification of the cancer cells to improve other means of therapeutic targeting. Priming of the immune response by the vector itself can be desired for effective vaccination as it helps generate a memory response, reducing or eliminating the number of booster shots needed. In terms of human use, phase I trials of adenoviral vectors expressing TNF-alpha or interferon beta that target cancer cells in the lung for mesothelioma have been performed and showed some benefit, reducing tumor burden and increasing overall survival length in epithelial types of mesothelioma.[37] Adenovirus is also used in FDA-approved therapies, where it is used *ex vivo* to deliver gene modification systems (such as CRISPR and sgRNA) to autologous cells to modify them for transplant, further

Table 1. Vectors for gene delivery and delivery barriers in the lung.

	Viral			MNon-viral			
Delivery barriers	**Adenovirus**	**AAV**	**Lentivirus**	**Liposomes**	**Dendrimer nanoparticles**	**Cell penetrating peptide nanoparticles**	**Electroporation**
Size	80–100 nm	20 nm	80–100 nm	~30–1000 nm	Up to 100 nm	~5 nm	Size of DNA or RNA cargo
Dispersion	Yes	Yes	Yes	Yes	Size- and delivery-dependent	Delivery-dependent	Delivery-dependent
Removed by mucociliary clearance	Low	Low	Low	Some	Low	Low	No
Pre-existing immunity	Yes; serotype-dependent	Yes; serotype-dependent	No	No	Formulation-dependent		No
Penetration of mucus /surfactant	serotype-dependent	serotype-dependent	Yes	Low	Good	Good	Good
Transfection of sub-epithelial cells	No	No	No	No	?	?	Yes

Table 2. Common gene delivery vectors used in the lung.

	Viral			Nonviral			
	Adenovirus	**AAV**	**Lentivirus**	**Liposomes**	**Dendrimer nanoparticles**	**Cell penetrating peptide nanoparticles**	**Electroporation**
Genome size	26–48 Kb	4.7 Kb	9–10 Kb	N/A			
Size of transgene	8 Kb max	4.5 Kb max	15 Kb max	No max; size proportional to 1/efficiency	Formula-dependent	Unknown	No max
RNAi (siRNA, miRNA, shRNA)	Yes[a]	Yes	Yes	Yes	Yes	siRNA and mi-RNA	Yes
Integration[b]	Very low	Very low	Yes	No	No	No	No
Long-term expression	Uncommon	Yes	Yes	Dependent on promoter used			
Exogenous material	Yes	Yes	Yes	No	No	No	No
Multiple deliveries	If serotype changes	If serotype changes	Unknown	yes			

[a]Adenovirus endogenously interferes with RNAi machinery (M. Anderson 2005, Virology).
[b]Without additional factors such as transposases, for laboratory strains.

expanding usage potential.[38,39] However, despite its many advantages for high level gene transfer and expression *in vivo*, its use for vaccination, and studies in cancer settings using suicide gene therapy in both lab animals and humans, it has met relatively limited clinical success.[33,40]

Adeno-associated virus

Adeno-associated virus (AAV)-based vectors are much less inflammatory, common alternatives to adenoviral vectors. AAVs have greatly reduced pathogenicity compared to adenovirus, causing only a mild inflammatory response. This coupled with their ability to provide long-term gene expression without integration has led to their position in the gene therapy arsenal. AAV has a capsid that is only 20 nm in size, but can package and deliver ~4.5 kb of recombinant DNA, almost the same size as the full AAV genome.[41,42] Unlike adenovirus, AAV has a linear, single-stranded DNA genome. First discovered in the 1960s, wild-type AAV primarily integrates at a specific locus in chromosome 19. However, recombinant lab strains have reduced integration to 0.1% and any integration occurs at random sites throughout the genome. AAV remains in a lysogenic cycle forming an episomal, plasmid-like structure in the cytoplasm if the cell is not infected with a helper virus (such as adenovirus). Depending on the helper virus or helper viral proteins used, AAV can remain lysogenic, integrate into the genome, or enter an infectious lytic cycle. Reduced, but

not non-existent, immunity and concern for integration has made AAV a favorable research tool. However, these same characteristics make it more difficult to use in cases where integration would be preferred for stable long-term expression. AAV helper viruses are also prevalent in the environment, making their use clinically less ideal than they would be otherwise.[30,43] These environmental helper viruses could lead to unintentional gene integration or activate a lysogenic cycle and immune clearance of delivered AAV. Further, various levels of antibody-mediated immunity to most AAV serotypes are present throughout the population, and coupled with antibody responses generated following administration of recombinant AAV vectors, have limited repeat administration of the vectors to achieve long-lasting gene expression.[33]

While AAV serotypes have been used extensively in laboratory and pre-clinical studies in the lungs of multiple animal models to effectively delivery transgenes to a number of organs including the liver, eye, the CNS, and skeletal muscle, these vectors have been less successful in the lung.[33,44] Nonetheless, AAV has been used with favorable results *in vivo* to treat CF in a pig CF model.[43,45,46] An unexpected advantage of at least some serotypes of AAV, notably AAV6, is that this virus appears to be able to penetrate mucus with increased diffusion rates and distribution both in cultured cells and in mice with airway mucus obstruction, a property that could be very advantageous for CF therapies.[47] Outside of the lung, two gene therapies using AAV as a vector have been FDA-approved, Luxturna and Zolgensma, to treat retinal dystrophy and spinal muscular atrophy, respectively. Luxturna uses a recombinant AAV2 to correct a mutation in *RPE65* and Zolgensma uses an AAV9 capsid to deliver a normal SMN1 gene to motor neurons. Both are one dose only and carry hefty price tags in addition to caveats of childhood treatment and low disease severity.[48–52]

Lentivirus

Lentiviruses are positive-sense strand RNA viruses that form an 80–100 nm enveloped virion and have a delivery capacity of at least 15 kb. These viruses, like other retroviruses, contain reverse transcriptase to convert the RNA genome to a double-stranded DNA intermediate and integrase for its integration into the host genome.[53] Because of its ability to integrate into the host genome, it allows for long-term gene expression and passage to any daughter cells. Perhaps the major advantage of lentiviruses over other retroviruses is their ability to successfully infect and transduce non-dividing cells. This allows them to transduce quiescent cells, including several critical targets, including T-cells and terminally differentiated somatic cells. Taken together, these abilities have made lentiviruses among the most-favored viral vectors for gene therapy. However, a major drawback to lentivirus use is also tied to its integration: lentivirus is able to integrate into germline cells making undesired longitudinal transfer to progeny a possibility. Further, the association of the lentiviral genus with HIV (and other immunodeficiency viruses) is not psychologically ideal.[30,54] Perceived association with immunodeficiency viruses may affect the ease of clinical deployment due to patient concerns. The association with HIV is also not completely without merit; many lentiviral vectors used in research are in fact derived from HIV after removal of replication and virulence factors. As a result, research using lentivirus still requires protocols and personal protective equipment at biosafety level 2 to prevent the risk of infection to personnel.[55,56] Affecting germline cells makes possible off-target and safety issues a matter for future generations as well. Further, since lentiviruses preferentially integrate in active gene sites, they have higher potential to affect normal cellular functions including oncogenic concerns.[30,54] The exact factors through which lentivirus integration sites are determined are being studied and altered to make this process conducive to inactive gene integration, which could reduce the concerns associated with active site integration.

Of the viral vectors, lentivirus and similar retrovirus seems to be closest to widespread *in vivo* clinical deployment. The innate integration capability of lentivirus and other retrovirus has been key in developing chimeric antigen receptor T cells used in FDA-approved cell anti-cancer therapies (Kymriah and Yescarta).[50–52] Further, lentiviral vectors are in phase 1/2 trials to treat many gene defects via *ex vivo* stem cell transplant. Stem cells harvested have a gene correction or defect-correcting alteration made and then are transplanted into an affected patient after ablation of the patient's resident cells. Specific examples of this transplant approach include Duchenne muscular dystrophy,[53] leukodystrophies,[57,58] and beta-thalassemia.[59] Lentiviral vectors have also been used effectively for gene transfer directly in the lung, primarily in small animal models. Simian immunodeficiency virus (SIV)-based vectors have been used to overexpress factor VIII and alpha-1 antitrypsin in the mouse lung following intratracheal administration, essentially turning the lungs into a bioreactor for protein overexpression.[60] Other lentiviral vectors have been used to overexpress IL-10 in donor lungs either prior to (*ex vivo*) or after orthotopic lung transplant (*in vivo*), limiting inflammation and allograft rejection.[61] Other studies have shown success in transferring genes to decrease disease severity in mouse ALI/ARDS and asthma models, as well as in CF.[62–64] Based on these and others preliminary successes, clinical trial of lentiviral gene delivery to treat CF is also in progress.[63,65] Despite these many early clinical trials and seeming benefit, the worry of oncogenic gene integration remains. HIV and laboratory lentiviruses have shown a causative effect of oncogenesis in mouse and human. This concern can be reduced by modifications removing replication and integration activities but this also removes a major lentiviral benefit.[66–68] Lentivirus-transduced cells are tested for these issues prior to transplant but such testing is not amenable for a direct treatment approach in which the virus is administered directly to the patient. Future modifications to lentivirus may reduce or eliminate these concerns. With successes starting to be shown, lentivirus has entered the clinic for indirect transplant approaches and direct treatment approaches are likely in the near future.

Non-viral vectors

Liposomes

Liposome-mediated transfection, or lipofection, was first developed in the late 1980s and has flourished *in vitro* with relatively low cytotoxicity compared to a host of other transfection methods. However, usage *in vivo* has been limited due to interference from serum and lower efficiencies than other types of vectors. Newer liposome formulations have been able to increase efficiency and reduce serum interference allowing better *in vivo* efficiencies. The greatest advantage, however, of liposomes and other non-viral methods of gene delivery is that they are much less inflammatory and immunogenic than their viral counterparts. Lipofection uses cationic lipid complexes to encapsulate negatively charged cargo yielding a net positively charged complex that can then further interact with the cell's membrane. Specialized formulations and combinations of lipids may be specific for complexing DNA, RNA, or even nucleoprotein complexes, and can even be specific for the size of the cargo being delivered. These lipopolyplexes are often preferred over other methods due to their relatively low cost, speed, and ease of use. Additionally, at least *in vitro*, liposome-mediated transfection often has reduced cytotoxicity compared to viruses or electroporation, albeit in a formula-dependent manner, though it most often does not share the same efficiency as these other methods for cargo delivery.[69–71] Understanding of the precise mechanisms of lipofection is incomplete, although most lipid complexes are thought to be endocytosed prior to fusion with the endosomal membrane and the resulting cargo release into the cytoplasm.[72] Once endocytosed and released into the cytosol, liposomes are dispersed in cells by Brownian motion, avoiding lysosomal or endosomal degradation, thereby allowing them to deliver RNA as well as DNA. However, if they are not unpackaged to release their DNA or RNA as lipid-free species, they do not efficiently form protein-DNA or -RNA complexes for association with microtubule-based motors and fail to efficiently traffic to the nucleus, thereby making nuclear delivery of cargo inefficient. Nucleic acid delivery is limited by the size of the formed liposomes although to a much lesser degree than viral vectors. As a result lipofection can deliver supercoiled plasmid DNA and small RNAs for RNAi, but may have difficulty with large mRNAs.[72] The efficiency of lipofection varies by cell type, serum, and antibiotic presence, limiting *in vivo* capabilities to more topical tissues without further understanding of the necessary formulations. Current lipofection technology is also unable to transfect all cell types, though with better understanding of uptake mechanism and cell membrane characteristics of specific cell types, this may change. Finally, since liposomes carry just the DNA and no associated proteins for recombination, integration of delivered genes occurs extremely infrequently, especially for supercoiled plasmids.

Perhaps the greatest advantage of liposomal transfection *in vivo* is that fact that most liposomes are much less inflammatory than any viral vectors used. Liposomes have low immunogenicity since there are no protein epitopes to target. Reduced inflammation allows liposomes to be used under already inflammatory conditions to deliver genes with less potential for adverse side-effects that immunogenic (viral) vectors may have. Currently, delivery of anti-inflammatory agents by this approach has focused on delivering traditional pharmaceuticals[69–71,73–75] but delivery of nucleic acids, such as for RNAi, is also possible depending on formulation. Low immunogenicity also allows multiple doses to be delivered without alteration of the vector or cargo.[76–78] Multiple doses allow for multiple delivery attempts and for a dose-number increase in efficiency that is not possible for viral vectors. Proof of concept multi-dose delivery approaches using liposomes have been performed that successfully tested this hypothesis in both mice and humans.[77,79,80]

Perhaps the greatest successes for liposome-mediated transfection in the lung have been for delivery of CFTR, giving partial correction of the CFTR defect in mice, sheep, and other models. In most cases, various cationic liposomes or combinations of cationic and other lipid derivatives have been used. While the first use of aerosolized liposomes in the lung delivered a reporter gene in 1992,[81] less than one year later CFTR was delivered to the lungs of mice using the same approach using either lipofectin or DC-cholesterol/DOPE,[82,83] achieving correction of ion channel defects in at least some animals. Moreover, several clinical trials for CF have been carried out using liposomes and plasmids. The first use of liposomal gene delivery to treat cystic fibrosis occurred in a 1999 double-blind phase 1 trial. The trial showed some short-term benefit but patients in both treatment and placebo (liposome without vector) groups did experience some pulmonary side-effects that spontaneously resolved.[76] A more recent phase 2 b trial in 2015 by the same group used a CpG-free plasmid design and a CMV instead of GM-CSF promoter resulting in reduced side-effects and longer term expression.[80] Unfortunately for both trials, the results while favorable were minimal due to delivery efficacy.[65,76,80] Future work with liposomes *in vivo* will likely remain limited without further modifications to formulations and plasmid design.

Nanoparticles

Nanoparticles (NPs) are defined as particles on the nanometer scale and, for the purpose of this article, are defined as being 100 nm or less. We further exclude lipid-based formulations (e.g. liposomes), which may otherwise fall under this classification, and which are increasingly being referred to as nanoparticles. NPs have been studied for decades, but only recently have practical applications for their use become feasible. NPs show great promise but have not been as well characterized in comparison to other vectors in terms of vector-host interactions. There are hundreds of thousands of natural or synthetic NPs possible, along with derivatives of each. These derivatives can each have their own chemical/biological properties, making characterization difficult and individualized for given particles. Some common types of NPs include dendrimers, cell penetrant peptides (CPPs), copolymers

(e.g. PEGylated poly-l-lysine), and nucleic acid aptamers, among a host of others (for a brief review on clinical NPs, see Bobo *et al.*[84]) Dendrimer NPs function similarly to liposomes, forming a spherical structure around the desired cargo before being endocytosed and fusing with the plasma membrane of a cell for delivery. Dendrimer NPs tend to be made of negatively charged amino acids and are often PEGylated in order to aid the formation of the structure.[26,85,86] Cell penetrant peptides (CPPs), smaller modified or synthetic peptides, are positively charged, able to associate with negatively charged cargo, such as nucleic acids, and are endocytosed through poorly characterized mechanisms. These CPPs are derivatives of bacterial and viral peptides such as TAT, penetratin, transportan, or oligoarginines, all of which penetrate cellular membranes and can be modified for purposes of gene delivery.[70,87] Copolymers like PEGylated poly-l-lysine enter cells by micropinocytosis avoiding endosomes and lysosomes.[88] Nucleic acid aptamers can have various properties but are similarly endocytosed for delivery to the cell cytoplasm. A major benefit of some NP designs is a lack of immunogenicity, opening options for multiple repeat doses for treatment of chronic pathology or during inflammatory conditions less conducive to other approaches. Most NPs pass through mucus and surfactant due to their size and general dispersion properties but must be instilled or aspirated as larger droplets in order to reach the deep lung.[26,27]

Early usage of CPPs for delivery was often somewhat troublesome, as delivered cargo was sequestered in endosomes or had altered function due to complexation with the peptide. Further, synthesis of these peptides was expensive and difficult to do accurately. As a result, other vectors for delivery have been preferentially studied and used. However, commercially available Pepfect and Viromer, as well as the recent development of fmoc solid-phase peptide synthesis has greatly improved the financial feasibility of creation and use of peptide-based nanoparticles.[89,90] Trying to overcome the alterations to cargo caused by complexation with the various peptides has resulted in several modifications of design, such as inclusion of redox sensitivity and cyclization of the peptdies.[70,91] Some peptides, such as a redox sensitive cyclic amphipathic peptide, have improved efficiency of RNAi delivery to the lung compared to both Pepfect and lipofection.[70] However, CPPs and these cyclic peptide derivatives have not been used to deliver larger plasmid DNAs, a primary approach to gene delivery. While other larger polymer-based NPs such as dendrimers, nucleic acid aptamers, and copolymers, can deliver plasmids with varying efficiencies depending on formulation and target cells, most NPs seem to show greater abilities for smaller RNAi delivery applications. Some NPs including CPPs can also deliver small proteins, opening a potential use over viral vectors for CRISPR-related technologies. One concern of NPs is a buildup of the particles in cells or in filtering organs, such as the kidney or liver, and work still needs to be done to characterize biodistribution of nanoparticles used for gene delivery.[26,70,85,89,92] Despite these possible concerns, a number of NPs have been used for *in vivo* gene delivery to the lung with some early success, including for delivery of plasmids up to 20 kb[93] and siRNA[70] in mice. Studies using PEGylated poly-l-lysine copolymers that form <20 nm particles, copernicus therapeutics has also shown safety and short-term efficacy of delivered plasmids in mice and in CF patients.[94–96] Unfortunately, long-term expression of delivered plasmids was not obtained though this could potentially be overcome by altering plasmid design or by using other cargo. With a better understanding of uptake, delivery, and biodistribution characteristics, NPs will likely enter the clinic for anti-inflammatory or multi-dose uses.

Electroporation

Electroporation (EP) was first used *in vivo* in the late 1980s and is currently the only physical method that is practical or feasible for use in the lung.[97] *In vivo* EP for the purpose of gene delivery, or gene electro transfer (GET) has been used sparingly, but electrochemotherapy (ECT; electroporation at similar fields to gene delivery but for chemotherapeutic drug delivery) and irreversible electroporation (IRE; electroporation at much higher field strengths than for gene transfer) have been used for multiple cancer treatment approaches.[98,99] In research settings, EP is a staple laboratory technique for *in vitro* gene delivery, especially in hard-to-transfect cell lines. EP creates transient pores in the plasma membrane due to charge distribution across the membrane and electrochemical rearrangement of membrane phospholipids into transient non-selective pores. Pores from electroporation vary in number and size (~1–400 nm) depending on cell membrane characteristics, voltage, duration, and type of electric pulse.[100,101] Nucleic acids and other material in the extracellular solution can then enter the cell via these pores. Following removal of the electric field, the pores close through endogenous membrane repair mechanisms. One of the prime benefits of using a physical method of gene delivery is that DNA, RNA, or other cargo can be delivered with no carrier (e.g. complexed lipid or viral proteins) to alter cellular distribution or cargo activity. Exogenous DNA from viruses or bacteria does not enter a cell unless purposefully included as cargo themselves. This feature has become of great use for ribonucleoprotein CRISPR complexes to deliver functional CRISPR-Cas while bypassing the need for exogenous components.[102–104]

EP is highly adaptable; the voltage, pulse, and other characteristics can all be altered to fit the cell type being transfected, as well as the amount and sizes of the pores. Because EP physically affects the phospholipids that are components of all cells, it can be used on all cell types with appropriate empirical changes to the voltage, pulse duration, and other variables. Unfortunately, *in vitro* EP often has an unfavorable effect on cell viability, with upwards of 30–40% cytotoxicity. However, *in vivo* cellular and tissue recovery from GET are much more robust with no significant harm under appropriate settings.[15,105,106] Usage of EP in IRE and ECT has shown that EP is safe to use *in vivo* in humans even for highly vascularized tissue such as liver, pancreas, and lung.[107–110] Clinical use of GET has generally been applied to the skin, as simple electrical pads and an adjustable power supply are all that is

required. This approach has been highly successful for overexpression of immune regulators to treat melanoma or for DNA-based vaccine delivery.[111–114] For delivery to internal organs, such as the lung, we have achieved success using simple defibrillation pads on the chest in mice, rats, and pigs.[15,105,106,115] Once purified, plasmid DNA is administered to the lungs by aspiration, intratracheal injection, nebulization, or aerosolization, electric pulses are delivered across the chest. This approach has been used successfully for gene transfer of reporter genes in mice,[116] rats,[117] pigs,[118] and sheep,[119] as well as a number of therapeutic genes to treat ALI/ARDS,[15,117,120–125] pulmonary fibrosis,[126–128] and surfactant protein B deficiency.[129] Following electroporation, plasmid DNA is transferred to and expresses in all cell types throughout the lung, including surface airway and alveolar epithelial cells as well as cells underneath the epithelium, including interstitial fibroblasts, airway and vascular smooth muscle cells, and endothelial cells. By manipulating the promoter or other sequence elements on the plasmid, this means that targeted gene transfer to specific cell types can be achieved.[128,130,131]

The current clinical focus for EP is mainly on IRE, a method for tissue ablation using targeted high energy EP, and ECT, using EP to target cancerous tissue with normally cell-impermeant chemotherapeutic drugs.[107,108,132,133] IRE applies >2000 V/cm fields with multiple pulses up to 30 ms each, while ECT and GET typically use 250–500 V/cm for 1 ms or less to instead deliver chemotherapeutic drugs and genes, respectively.[107–110,113,132–135] At all of these field strengths, EP has proven safe in animal models and humans. In human-sized 50 kg pigs, delivery of EP pulses across the chest for lung gene delivery uses less than 4 J of energy for gene transfer. By comparison, an automated external defibrillator (AED) uses 360 J in a single 1 ms pulse to defibrillate a patient and is deemed safe. Perception of GET as being related to electroshock therapy or electrocution, and the confusion with IRE and its use as an ablative therapy, has hindered its translation to the clinic. However, as safety has been clearly demonstrated and with more research on the necessary delivery characteristics, EP for gene delivery to the lung will likely translate to the clinic in the future as well.

Future uses

Viruses

Viral vectors, such as lentivirus, are still some of the best vectors for long-term expression of transgenes with genome integration. Additionally, co-infection of cells and tissues with constructs containing transposons can allow for integration of transgenes carried by other viruses, including adenovirus, thus overcoming the limitation of this virus for only short duration expression. Such transposons can also be used in conjunction with non-viral gene delivery approaches. An example of a transposon, *piggybac*, functions to integrate delivered genes into the host genome based on the transposase recognition of short repeat sequences.[8] Transposon-based integration allows for long-term maintained expression, but there are multiple integration sites due to the short recognition sequence of the transposase, and possible off-target effects have not fully been elucidated. Transposons are not limited to combined use with viral vectors. Indeed, delivery of plasmid-based transposons to the lung has also been achieved using polymers, nanoparticles, and electroporation to achieve integration and long-term expression.[136,137] Modified viral vectors for *in vivo* gene delivery also are being further developed for vaccine creation with the benefit that they induce mild local and systemic inflammation which can aid in generating an immune response. Viruses are used in many of the FDA-approved cellular/gene therapies where cells are removed, modified, and reinjected, but this type of *ex vivo* to *in vivo* usage is not suited for scenarios of acute disease where time may be of the essence or with other difficulties of transplantation. Viral targeting is often not unique to specific cells or tissues as receptors utilized for viral uptake are often widespread. Viral vectors are also limited by their genome size and the amount of DNA/RNA that they can package in their capsids, thus reducing their capacity to deliver large genes or multiple genes. However, even with these limitations, viral vectors are an established means of gene transfer that will likely have a long future of clinical use.

Lipofection

Despite being the major "go-to" technology for cellular transfection in the laboratory, *in vivo* lipofection will likely remain a secondary strategy unless its efficiency in the living animal can be increased. One long perceived limitation of non-viral methods has been a lack of long-term expression. This is not the case, since non-viral based plasmids with the appropriate promoter choice are suitable for long-term expression, but without any side effects caused by viral vectors. These non-integrated plasmids delivered by a host of non-viral methods have shown long-term expression in a variety of tissues in mice, with reports of expression six months to one year after delivery to mice.[128,138–140] In conjunction with other technology such as electroporation or nanoparticles, lipofection may have greater potential than its use alone. Further improvements in lipid chemistry and structure may also increase their use *in vivo*. A major advantage is that liposomes have essentially no size constraints for cargo, allowing delivery of large genes and also have a relatively low inflammatory profile.

Nanoparticles

NPs have only recently become practical to use, and recent data has attempted to explain how NPs work. Mechanisms of entry can be different between cell types and NPs making "full" understanding of all particle types an ongoing effort. Functional understanding of how each specific NP is internalized will lead to better structural formulations for improved targeting and uptake. There are many currently untested NP formulations that will allow us to further understand how NPs "capture" cargo, are endocytosed, and allow the cargo to remain functional. NPs are highly versatile, can have low immunogenicity, low cytotoxicity, lack exogenous DNA or RNA (unlike exosomes),

and may have higher efficiency compared to viral vectors, lipofection, or EP methods in certain circumstances. NPs are able to deliver large genes and plasmids depending on formulation. As yet, NPs have not been shown to induce long-term expression of delivered plasmids and are therefore a short-term delivery approach without additional factors or appropriate promoter choice. Repeated dosing approaches may increase their versatility but may also generate side-effects, and biodistribution and clearance will be important to this use. NPs as drug and gene delivery mechanisms are likely to progress relatively slowly as they remain more expensive than more classical approaches but may gain usage as production becomes cheaper and mechanistic understanding is improved. Clinically, since they are seen as very similar to classical pharmacologic drugs, their acceptance as "safe", along with supporting safety data, may greatly aid their acceptance.

Electroporation

Electroporation is likely to be developed more quickly for both tumor ablation (IRE) and chemotherapy (ECT), improving parameters for efficiency and targeting. EP has already entered the clinic for these anti-tumor uses and will likely remain prior to the development of more personalized therapies. IRE and ECT showcase EP as safe, therefore *in vivo* EP for gene delivery may see a rise in research and clinical usage. Electroporation for gene delivery will also certainly be further developed both as a stand-alone delivery approach for classic overexpression of plasmid DNA and when complexed with other technology, such as CRISPR. EP can deliver large genes and multiple genes or components in a single delivery event. EP is targeted primarily through the placement of electrodes and parameters used. Future possibilities of EP would see further development of research technologies that improve targeting of gene delivery to specific organs or cell types. EP could also potentially be used in combination with nanoparticles or liposomes for nuclear or organelle specific delivery. Use of EP with integration factors or long-term expressing plasmids (CpG reduced) is also in progress.[113,141] Due to the lack of any exogenous components (i.e. lipid, polymers, other carriers) other than the nucleic acid that are needed for delivery, electroporation is very likely to attain FDA-approval for gene therapy. Indeed, there are currently over 95 clinical trials for electroporation-mediated DNA gene transfer underway, including a Phase II trial for a SARS-CoV2 vaccine against Covid-19, attesting to its clinical appeal.

Exosomes

Exosomes were first discovered in 1983 as microvesicles less than 150 nm that are released from cells. They are endogenous cell-membrane derived vesicles that have been shown to contain RNAs, proteins, and other molecules involved in cell–cell signaling.[23,142] Because exosomes are composed of normal cellular membranes, they enter cells by innate clathrin-dependent and macropinocytic mechanisms after ligand-receptor or membrane interactions.[143] Further, since they are seen as "self", they largely avoid initiating an inflammatory response. Some tissue-specific targeting of exosomes dependent on integrins expressed by the exosomes has been shown but its mechanisms are not completely understood.[23] The main limitation for their use comes from challenges in their isolation and purification at scales needed for commercial or human use. Separating exosomes containing the correct factors from other exosomes and cell debris requires density gradients or ultracentrifugation, and/or other filtration techniques, which can vary in terms of what specific exosomes are isolated. Further, keeping exosomes stable long-term has proven difficult, as they are prone to aggregate and degrade.[144–149] Additionally, methods for loading cargo into exosomes currently rely on nanoparticles or electroporation-based delivery to the exosome.[144] A potential drawback is that cellular components internal to cellularly-produced exosomes may also be delivered along with any loaded cargo.[142,150–152] Exosomes have been used in mice for delivery to brain[144]and lymph nodes[151] without noticeable toxicity as long as the cell source was syngeneic. Safety studies will be the first major hurdle for exosome delivery to humans since unapproved exosome containing products (from the cells in which the exosomes are produced) have been warned against by the FDA.[153] However, recent methods for exosome production from red blood cells may greatly reduce some of these issues.[154]

Exosomes are farthest from clinic but likely to increase in usage due to favorable characteristics of a cellular origin and endogenous uptake. The development of technology allowing greater specificity in the separation of specific exosome subsets from other subsets and other vesicles will be important to understanding and clinical usage. Further development of a solution/method that is better at maintaining exosome stability will also be important to any widespread clinical usage and pharmaceutical feasibility. Small particle flow cytometers are now being deployed in many research cores and will help further understand exosome characteristics in conjunction with proteomics and genomics. Understanding of exosome characteristics will allow better tissue specificity, and possible generation of synthetic exosomes. No cargo size limit is known for exosomes, but it will likely be difficult to load large plasmids without compromising exosome membrane integrity. Exosomes can deliver multiple plasmids in multiple doses but as a single dose would be limited by "loading" of exosomes with plasmids and the uptake of target cells. Exosome delivered RNAi or plasmid expression have not been studied long-term, with 48 h or less timepoints being common.[144,151,155] The duration of delivered nucleic acid(s) persistence in tissues will be important to clinical usage. Improvement of storage media and conditions may help prolong exosome stability and increase pharmacologic capabilities.

Conclusions

Despite drawbacks of the reviewed methods of gene delivery to lung, they are currently the most prominently used in

research and clinical trials. Several viruses and electroporation gene delivery uses are already approved or being tested in clinical trials. Liposomes will be limited in use and used primarily for non-integrative treatment approaches. Nanoparticles will likely overtake liposome use by recapitulating necessary characteristics along with other modification. Nanoparticles will likely be developed for other uses, finding niches where lipofection falls short as they enter clinical trials. Exosome use is novel and while favorable due to endogenous characteristics is dependent on further development of associated technology. Better comprehension of necessary characteristics has the potential to develop exosomes into targeted treatments. Understanding these gene delivery techniques from both a basic science and clinical perspective is necessary to ensure proper patient care and further development of the technologies.

AUTHORS' CONTRIBUTIONS

All authors participated in the interpretation of the studies and review of the manuscript. UKB wrote the majority of the manuscript and DAD revised the manuscript, providing additional sources, and guidance.

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