Expert Opinion

Targeted gene delivery to the lung

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Gene therapy holds promise for the treatment of a range of inherited pulmonary disorders. However, efficient delivery and expression of the therapeutic transgene at levels sufficient to result in phenotypic correction of the diseased state has proved elusive. This review focuses on the development of gene delivery strategies for the lungs. One of the principal prerequisites for successful gene therapy is the delivery of gene vectors to the target area within a tissue and to target cells within that area. Physical and biological targeting of the gene vectors and its application in various models is discussed. Subsequently, both viral and non-viral vectors are addressed with respect to their transfection efficiency in different lung cells, the longevity of expression and their immunogenicity. Also, the various methods for pulmonary gene delivery are evaluated for their merits and limitations.

Keywords: lungs, magnetofection, non-viral vectors, targeted gene delivery, viral vectors

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1. Introduction

The lung is an appropriate present and future target for gene therapy approaches designed to treat inherited monogenic diseases, eradicate bronchial tumours, transfer pharmacologically active products to the general circulation, express enzymes to catabolise toxins, manage pulmonary hypertension and injury and vaccinate against infection. There have been major advances in the understanding of the molecular pathogenesis of pulmonary diseases in past decades. However, effective translation of this knowledge into viable gene-based therapies and realisation of their clinical potential is yet to be achieved.

In pulmonary gene therapy, the nucleic acid cargo needs to be delivered to cells in the target region of the lung. However, even in cases when these targets are well defined, this is severely limited by the pulmonary architecture, clearance mechanisms, immune activation and the presence of respiratory mucus [1]. Besides these hindering factors, basic issues of efficiency of gene delivery, duration of transgene expression and the toxicity of the gene delivery vectors themselves are currently areas of intense research. Phase I/II clinical trials have already shown that gene transfer to the lung is feasible in principle and, with the development of more efficient gene transfer agents, it is hoped that therapeutically viable gene therapies will soon be available [2].

Although increasing the efficiency of therapeutic gene expression is desirable, the site of expression within the lung is also important. Successful gene therapy for many diseases requires appropriate levels of transgene expression in specific cell types to enhance treatment efficacy and to avoid unwanted side effects caused by expression in inappropriate cell types. An important aspect of these endeavours is strategies that seek to incorporate specific targeting attributes into vector design, thereby optimising gene delivery to the appropriate target cells and ideally also reducing the total dose of vector needed, thus reducing potential vector-related toxicity (**Figure 1**).

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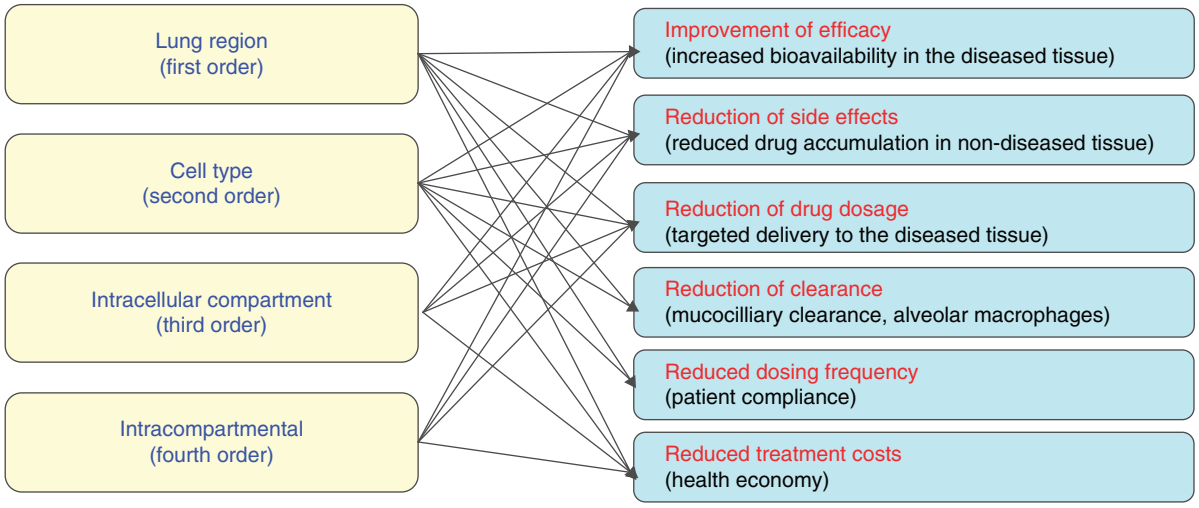

Figure 1. Beneficial aspects of targeted gene delivery. For clinical success, gene vectors need to be delivered in a specific manner to the target region of the organ (first order) and to the specific cell types (second order) in the target region. Once inside the specific cells, the gene vectors need to enter the nucleus for expression (third order, intracellular). Inside the nucleus, the episomal vectors need to be addressed to the nuclear matrix for sustained expression and/or episomal replication. For integrating vectors, the target sites for integration must be accessible and support transgene expression (fourth order, intracompartmental). Each level of targeting addresses different critical aspects of gene delivery and is the subject of current review.

In the following sections different aspects, that is, orders of targeted gene delivery, such as targeting specific lung regions (first order), different cell types (second order), intracellular (third order) and intranuclear (fourth order) [3], are discussed. These are presented diagrammatically in **Figure 2**. Both viral and non-viral vectors have been subjected to numerous modifications aimed at achieving targeted gene delivery. This aspect is also covered in the following sections.

2. Targeting of specific lung regions and gene delivery by physical methods

Although successful gene transfer by non-viral gene vectors to cultured respiratory epithelial cells and to various *in vivo* airway models has been reported [4-10], transfection rates of respiratory epithelial cells remained low in cell culture models [11-13]. Gene transfer to intact airway epithelium was even less efficient [14,15]. Generally, the efficiency of gene transfer systems is limited by three major impediments: i) insufficient enrichment of the applied gene vector at the target tissue; ii) its intracellular trafficking; and iii) nuclear uptake and intranuclear trafficking to active sites of transcription either with or without genomic integration. When gene vectors are topically administered to the airways, physical phenomena such as ciliary beating and mucociliary clearance represent the first extracellular barrier. Mucociliary clearance reduces exposure time of gene vectors to the target cells [16]. Mucus and soluble factors of the airway surface liquid (ASL) covering the airway epithelial cells may interfere with the gene vectors and thus hinder enrichment at the cell surface [14,17-19]. The glycocalix of the cell membrane may impede further the interaction of gene vectors with supposed receptors for endocytosis, as shown for adenovirus-mediated gene transfer [20]. These factors potentially lead to decelerated vector accumulation and consequently lower vector concentration at the target cells. To improve gene transfer efficiency to the airway epithelium, it is necessary to address these limitations.

One major prerequisite for efficient gene transfer *in vivo* is the accumulation of gene vectors at the target tissue. It was postulated that high concentrations of gene transfer particles at the cell surface would lead to enhanced uptake into the cell and thus yield efficient transgene expression. Scherer *et al.* [21] presented magnetofection as a promising new technique to accumulate gene transfer complexes on the target tissue by exploiting magnetic forces. Magnetic targeting of gene delivery is achieved by application of a magnetic field to superparamagnetic iron oxide particles associated with the gene vectors. This method was reviewed in detail by Plank *et al.* [22]. Briefly, cationic polymer polyethylenimine (PEI) coated superparamagnetic nanoparticles (transMAG-PEI) are complexed to plasmid-DNA under the addition of free PEI. The resulting ternary complex is called magnetofectin. The applicability of magnetofection to viral and non-viral vectors has been shown in cell culture experiments in human chronic myeloid leukaemia in blast crisis cells (K562 cells) and in Swiss mice embryo cells (NIH3T3) [21]. In lung cells, Gersting *et al.* [23] applied the principle of magnetofection to transfect both permanent and primary airway epithelial cells. Magnetofection was compared with standard methods used for non-viral gene transfer, such as lipofection or polyfection. Their results

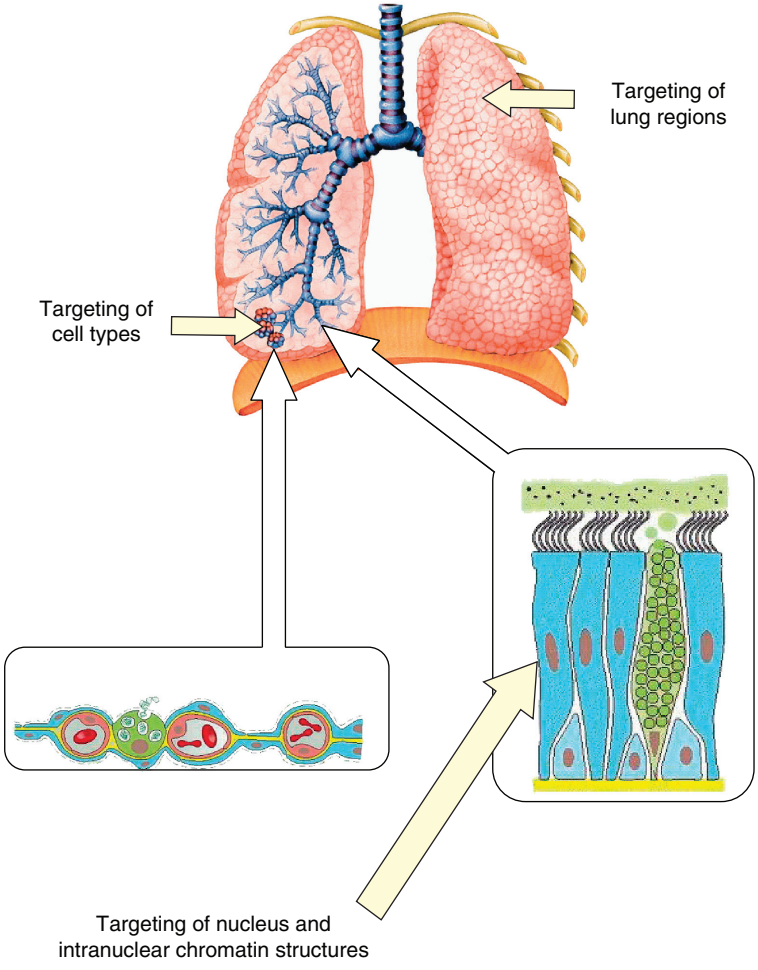

Figure 2. 'Orders' of targeted gene delivery. Different lung regions and cell types are the targets for different diseases. For therapeutic effects, gene vectors need to be delivered to those targets in a specific manner. These aspects of gene delivery using both viral and non-viral vectors are discussed in this review and presented diagrammatically.

indicate that the principle of magnetic drug targeting is an efficient and reliable method to deliver plasmid DNA into airway epithelial cells, although problems associated with colloidal stability of the gene vector solution have to be carefully addressed [24]. Advances in magnetofection and its numerous applications as well as generation of magnetic non-viral gene transfer agents have been reviewed previously [25,26]. More recently, the authors' results in a mouse model have provided evidence that targeted aerosol delivery to the lung can be achieved with aerosol droplets comprising superparamagnetic iron oxide nanoparticles – so-called nanomagnetosols – in combination with a targeted directed magnetic field gradient [27]. Application of other physical methods such as electroporation and sonoporation for gene transfer are reviewed extensively elsewhere [28,29] and are discussed only briefly in the following sections.

Electroporation is a common physical tool to introduce DNA into cells. This technique, also termed electropermeabilisation, exposes the cell membrane to high-intensity electrical pulses that can cause transient and localised destabilisation of the barrier. During this perturbation, the cell membrane becomes highly permeable to exogenous molecules, such as DNA, present in the surrounding medium. The efficiency of gene transfer by electroporation is influenced by several physical (especially pulse duration and electric field strength) and biological (including DNA concentration and conformation, cell size) factors [30]. It follows that the exact conditions for optimal electroporation must be determined for each tissue/cell type. Equally, it has been proposed that different electroporation conditions are required for molecules of different sizes. Short pulses (100 µs) of high electric field strength (> 700 V/cm) were found to be optimal for the delivery of small anticancer drugs, whereas longer pulses (20 – 60 ms) at a lower field strength (100 – 200 V/cm) were preferred for gene transfer [31]. The first evidence that gene transfer could be achieved by electroporation was reported > 25 years

ago by Neumann *et al.*, using mouse lyoma cells [32]. *In vivo* gene transfer began in the early 1990s [33] and since then has been widely applied, with successful gene delivery being reported in many tissues, including muscle [34], skin [35], liver [36] and lungs [37].

Sonoporation enhances cell permeability through the application of ultrasound. Like electroporation, sonoporation is thought to induce the transient formation of small pores in the cell membrane allowing for the direct transfer of genetic material into cells [38,39]. *In vivo* gene delivery mediated by ultrasound has also been reported [39-41]. Optimisation of ultrasound-mediated gene transfer depends on several factors, including transducer frequency, acoustic pressure, pulse duration and exposure duration. Other factors are also important, such as the ultrasound contrast agent concentration and its formulation.

Each of the physical techniques described requires a specific 'hardware', the operation of which is an important factor for its eventual use in practical *in vivo* situations. Further, the technological aspect of each approach also determines the extent to which DNA delivery can be targeted to specific cells or to specific areas of tissue.

Magnetofection requires a formulation that includes magnetic particles (already available commercially) and the application of an appropriate magnetic field. The apparatus needed is simple and inexpensive. Whereas the delivery of anticancer agents has already been performed successfully in humans [42], parallel observations with DNA have not yet been reported. The principal potential advantages of magnetofection are rapid and efficient transfection at low vector doses and remote (i.e., by means of the external magnetic field) targeting *in vivo*. The latter will demand a magnetic field of sufficient strength and focus to localise preferentially the magnetised vectors to their target zone – whether this can be achieved without invasive intervention (i.e., surgical insertion of the field-generating device) requires considerable further study.

Commercial devices are commonly used for electroporation. The method allows specific targeting in that the area to which the gene is transferred is delineated by the positioning of the electrodes. The approach becomes progressively more invasive as accessibility to the target tissue decreases. It must be remembered that electroporation uses high electric fields and therefore the patient must be carefully monitored during the procedure. The electric field distribution is an important factor, as is the choice of an appropriate electrode design, not only to ensure high transfection efficiency, but also because of the volume and location of the target tissue. In a typical treatment, DNA is injected into the tissue and electrodes are placed directly on, or within, the tissue before the electric field is applied. Although this works adequately for solid tumours, for example, the approach is not universally practical for all tissues and organs [43]. It remains to be seen whether current efforts to improve this situation prove successful.

Enhancing the performance of physical methods for gene delivery translates into the need to increase, primarily, the extent of DNA transfer to the cell nucleus while minimising 'collateral' damage to the tissue being treated. Most of the methods described are able to provoke the relatively rapid opening of the plasma membrane barrier and permit plasmid to enter the cell. The challenge, at this point, then becomes how to move the DNA from the cytoplasmic compartment into the nucleus as quickly as possible so as to avoid destruction of the gene by metabolic enzymes. An ideal physical technique, therefore, would propel the plasmid not only through the plasma membrane, but also to the nuclear envelope. Unfortunately, there is only sparse indication that this 'holy grail' is achievable by any single method. Improved gene transfer might presumably be attained by a synergistic combination of techniques – magnetofection already illustrates this possibility, in fact, by associating the use of gene vector technology with a physical force (the magnetic field). Sonoporation also lends itself to a fruitful interaction with an appropriate DNA formulation. It has been used with lipoplexes [44] and polyplexes [45] to some significant benefit. The three techniques described are compared in **Table 1** with respect to their principle, advantages and limitations.

3. Biological targeting of specific lung cells

Delivery of exogenous DNA into cells (both *in vitro* and *in vivo*) can be achieved by various non-viral and viral vector systems. Both classes of vectors have been used in the lungs and continuous improvements are being made to achieve therapeutic levels of gene expression. So far, most gene therapy protocols have used mammalian viral vectors for their efficiency of gene transfer. However, all viral vectors induce an immunological response to some degree and have safety risks (such as insertional mutagenesis and toxicity problems). Furthermore, their cloning capacity is limited and large-scale production may be difficult to achieve. In general, synthetic non-viral vectors are thought to circumvent concerns raised by immunogenicity and safety issues of viral vectors while offering the potential for repeated administration and large-scale production. In the following sections, both groups of vectors are discussed in the context of targeted gene delivery to the lungs.

Among non-viral vectors, polycations and cationic lipids have been used extensively in achieving gene transfer to the lungs [2]. Although both groups of reagents are quite efficient in gene transfer [7-9,46-49], they suffer from weak to non-existing targeting capacity and give rise to significant toxicities [50,51]. Receptor-mediated gene delivery systems have been widely investigated over the last two decades. They have the advantage of being able to recognise specific types of receptor present on surfaces of a variety of differentiated cells and therefore to distinguish between target and non-target tissue. To increase the transfection efficiency and to add target specificity, efforts have been made to combine the unspecific electrostatic polyplex/lipoplex–cell surface interaction with the specific mechanism of receptor-mediated

Table 1. Comparison of physical techniques for gene transfer.

Technique	Principle	Material	Advantages	Limitations
Magnetofection	Magnetic field assisted transport into cells	Magnetic nanoparticles ± transfection reagent, magnetic field	High efficiency Economic Applicable to viral and non-viral vectors	Magnetic gradient field strongly decreases with distance
Electroporation	Electric field induced cell membrane permeabilisation and/or electrophoretic mobility	Electrodes and pulse generator	High efficiency No gene transfer agent needed	Toxicity, invasiveness (electrodes)
Sonoporation	Ultrasound induced cell membrane permeabilisation	Ultrasound probe ± gas microbubbles	Imaging during treatment New microbubble formulations	Efficacy, toxicity to be established

cellular uptake by incorporating cell-binding ligands in the transfection complexes.

3.1 Cellular targeting with non-viral vectors

Polycations examined in gene transfer experiments include natural DNA-binding proteins such as histones, synthetic amino acid polymers such as polylysine, other cationic polymers such as PEI, dendrimers, or carbohydrate-based polymers such as chitosan, and have already been reviewed in detail [46,52,53]. PEI has been reported to promote gene transfection into lungs in various animal models and shows promise, transfecting up to 5% of pulmonary cells after intravenous administration [54]. Administration of the PEI–DNA complexes through the airways resulted in transfection of epithelial cells at the bronchial and/or alveolar levels, as well as, in some instances, macrophages and endothelial cells [55-57]. Many reports focus on the effect of various targeting ligands that are covalently conjugated to a DNA-binding cationic polymer or protein. Numerous targeting ligands, for example small chemical compounds (carbohydrates) or synthetic peptide ligands and proteins (growth factors or antibodies), have been evaluated (**Table 2**).

Successful *in vitro* targeting in cell cultures has been reported, demonstrating up to 1000-fold enhanced gene expression in target cells in comparison with transfection controls and competition experiments with free ligands or ligand-free complexes [58]. In a recent study, lactoferrin as a targeting ligand for receptor-mediated gene delivery to human bronchial epithelial cells was investigated [59]. Molecular conjugation of lactoferrin to branched-PEI (br-PEI) resulted in a significant increase in the transfection efficiency in human bronchial epithelial cells, whereas no effect could be observed on human alveolar epithelial cells. In addition to achieving cell-specific delivery, the cytotoxicity of the transfection complexes was also reduced significantly by conjugating lactoferrin to br-PEI. Targeted polyplexes have also been evaluated in lungs *in vivo* [60,61]. The serpin enzyme complex receptor (sec-R) is expressed on the apical surface of airway epithelial cells, and administration of CFTR plasmid DNA complexed to polylysine linked to the sec-R ligand to cystic fibrosis (CF) mice resulted in correction of chloride channel activity [62] as measured by *in vivo* nasal potential difference measurements and immunohistochemical staining for CFTR. Complexes that lacked the receptor ligands were ineffective, so receptor access was essential. In the same study, mice treated with receptor-targeted lacZ showed β-galactosidase expression in epithelial cells and submucosal glands, but no electrophysiologic correction, so simply accessing the serpin enzyme complex receptor was not sufficient to produce the observed electrophysiologic or immunohistochemical changes. Thus, molecular conjugates targeting the serpin enzyme complex receptor hold promise for gene therapy of cystic fibrosis.

Recently, the authors have successfully demonstrated the use of clenbuterol, a β_2-adrenoceptor (β_2-AR) agonist, to improve the gene transfer efficiency of non-viral gene vectors. Enhancement observed with this ligand was specific for alveolar but not bronchial epithelial cells *in vitro* [61]. Clenbuterol conjugated to fluorescent labelled bovine serum albumin resulted in clenbuterol-specific cellular endosomal uptake into the alveolar epithelial cell line A549, but not the bronchial epithelial cell line BEAS-2B. At optimised concentration and gene vector composition, transfection efficiency with the ligand was found to be 14- and 9-fold higher on human A549 and murine MLE-12 alveolar epithelial cells than for unmodified PEI. No increase of transfection efficiency was observed on human bronchial epithelial BEAS-2B cells. Enhancement of gene delivery was also observed *in vivo* (threefold increase) after aerosol delivery of optimised clenbuterol-modified complexes. In a related study, Weiss *et al.* [58] demonstrated that modification of PEI with mannuronic acid results in significantly higher gene delivery and expression in different lung cell lines. The uptake of the modified conjugates was shown to be mediated by mannuronic acid receptor.

Cationic liposomes form large complexes in which their positively charged side chains interact with DNA and the

Table 2. Ligands used for targeted gene delivery to the lungs with non-viral vectors.

Ligand	Target	Targeted cell type/tissue	Application	Ref.
Anti-PECAM antibody	PECAM	MLECs	*In vitro*, *in vivo*	[142]
EGF	EGF-receptor	Large cell lung carcinoma (H460a, H1299); Squamous lung carcinoma (H322, H226b); Adenosquamous lung carcinoma (H596); Bronchoalveolar cell lung carcinoma (H358a)	*In vitro*	[143]
GE11 (Peptide)	EGF receptor	Human non-small cell lung carcinoma (H1299); H1299 xenograft mouse model	*In vitro*, *in vivo*	[144]
Lactose	Lactose receptor	Primary cultures from human tracheal tissue explants	*In vitro*, *in vivo*	[145,146]
Methyl-α-D-mannuronic acid	Not known	Human bronchial epithelium-derived (16HBE14o-)	*In vitro*	[58]
Lactoferrin	LfRs	Human bronchial epithelium-derived (BEAS-2B)	*In vitro*	[59]
Clenbuterol	β2-adrenoreceptor	Human alveolar epithelium-derived (A549); Murine alveolar epithelium-derived (MLE-12); Mouse lung	*In vitro*, *in vivo*	[61]
Integrin targeting peptide (THALWHT)	α9β1-Integrin	Human bronchial epithelium-derived (16HBE14o-); Human tracheal epithelium-derived (HTEo-)	*In vitro*	[147]
Integrin targeting peptide	α5β1-Integrin	Rat bronchial and alveolar epithel	*In vivo*	[148]
SP5-2 (Peptide)	Not known	Non-small cell lung cancer (CL1-5, H460, A549, PC13, H23, NNM); Human surgical specimens of pulmonary adenocarcinoma	*In vitro*, *in vivo*	[149]

Anti-PECAM: Anti-platelet endothelial cell adhesion molecule; EGF: Epidermal growth factor; LfRs: Lactoferrin receptors; MLECs: Mouse lung endothelial cells.

hydrophobic lipid portion of the liposome enhances fusion with the host cell membrane. Different mechanisms such as endocytosis, fusion with cell membrane and disruption of the cell membrane lipid bilayer have been proposed for internalisation of lipoplexes (complexes of DNA and liposomes). Larger aggregated lipoplexes might also be internalised by phagocytosis [51].

In previous studies, direct intratracheal administration of cationic liposome–DNA complexes led to efficient transfection of the mouse airways [48,63]. Successful transfection of the lungs *in vivo* was observed when the lipoplexes were delivered intravenously [49,64]. In clinical setting, cationic cholesterol derivatives have, however, been hampered by their relatively low transfection efficiency *in vivo* and concerns regarding their pro-inflammatory activity [50,65].

Although lipoplexes often show high levels of transgene expression following direct administration or injection into target tissues, their nonspecific membrane activity usually precludes cell-selective targeting. Moreover, their positively charged surface leads to interactions with plasma proteins and other extracellular proteins, which bind nonspecifically to the lipoplexes and inactivate them [66]. In this regard, protein-resistant lipoplexes have been developed [67]. Also, to facilitate specific uptake into the cells, targeting proteins have been included in liposomes, for example, transferrin [68] and the Sendai virus F protein [69]. Numerous other approaches based on the use of site-directing ligands have been developed for liposome targeting and are reviewed separately [70,71].

In addition to the targeting ligands, choice of the lipid also plays a pivotal role in cell transfection. In a recent study, Tagalakis *et al.* [72] compared several vector systems including GL67, polyethylenimine (PEI) 22 and 25 kDa and two new, synthetic vector formulations, comprising a cationic, receptor-targeting peptide K(16)GACSERSMNFCG (E), and the cationic liposomes (L) DHDTMA/DOPE or DOSEP3/DOPE. The lipid and peptide formulations self-assembled into receptor-targeted nanocomplexes (RTNs) LED-1 and LED-2, respectively, on mixing with plasmid (D). It was shown that LED-1 transfected airway epithelium efficiently, whereas LED-2 and GL67 preferentially transfected alveolar cells.

3.2 Nuclear targeting

A high-affinity association between vector and cell surface receptor is necessary but not sufficient for successful *in vivo* gene therapy. The intracellular trafficking pathway of most surface receptors is degradative in character, with endosomal uptake of the receptor and payload leading to intracytoplasmic fusion with lysosomes, with subsequent enzymatic degradation as well as potential acid denaturation of the vector. For expression, the genetic material must reach the nucleus in a form that enables its transcription. Viruses have evolved

specific mechanisms to destabilise lysosomes [73,74]. However, some of these moieties, such as influenza virus haemagglutinin HA-2 subunit, are highly immunogenic and therefore not appropriate for use in non-viral vectors developed for chronic human diseases. The chemical structure of PEI has been shown to destabilise lysosomes *in vitro* because of its proton sponge effect [75,76]. Whether this mechanism is applicable following *in vivo* gene transfer is less clear in view of likely dilutional effects as well as potential toxicities of the polymer that restrict local concentrations.

Even when the vector escapes the lysosomal compartment or enters the cells through a non-degradative pathway, the journey to the cell nucleus is problematic, as free DNA molecules > 2000 base pairs in length are essentially immobile in the cytoplasm [77]. This observation may explain why microinjection studies have shown that lipid–DNA complexes injected into the cytoplasm do not access the nucleus for gene expression. Carrier-mediated transport to the nucleus is probably required for typical plasmid DNA payloads [78], although small DNA oligonucleotides may be sufficiently diffusive to travel to the nucleus after cell entry [79].

In a study by Chen *et al.* [80], colocalisation of the protein nucleolin with DNA nanoparticles (composed of polylysine – PEG and DNA) was observed. It was shown that only cells expressing nucleolin on the surface took up the DNA nanoparticles and manoeuvres that depleted surface nucleolin also reduced expression of reporter genes from DNA nanoparticles. Moreover, transfection of tagged nucleolin that migrated to the surface increased gene expression, whereas preincubation of the particles with exogenous nucleolin resulted in dose-dependent reduction in gene expression. Thus, nucleolin appears to be an excellent candidate for the receptor for the DNA nanoparticles.

In addition to facilitating cell entry, peptides can also assist in nuclear targeting of plasmid DNA. For example, polylysine linked to nuclear localisation sequence (NLS) from SV40 (simian virus 40) T-antigen resulted in 50% enhancement in transfection efficiency compared with polylysine alone. Interaction of NLS with nuclear import factors importin-α and importin-β was also evident [81]. Similarly, HIV-1 tat protein (GRKKRRQRRRPPQ) increased nuclear delivery of rhodamine-labelled plasmid DNA 30-fold [82,83]. This appeared to be partly mediated by an interaction with cell surface proteoglycans.

In summary, incorporation of targeting ligands to poly- or lipoplexes seems to be a promising tool for non-viral gene delivery in a cell-specific manner and also enhances nuclear transport of the delivered DNA. Lactosylated polylysine enhanced reporter gene expression 100-fold in primary human cystic fibrosis airway epithelial cells when compared with naked DNA, and confocal microscopy demonstrated improved trafficking of rhodamine-labelled plasmid DNA into the nucleus through the nuclear pore when complexed with this vector [84].

4. Viral vectors

Most gene therapy protocols have used viral vectors for their efficiency of gene transfer. However, for *in vivo* gene transfer in human lungs, viral vectors suffered from reduced gene transfer efficiencies compared with other animal species because of discordance of receptors on the apical surface membrane [20,85]. Although several viruses have been developed for their use as vectors, interest has centred on three types: retroviruses (including lentiviruses), adenoviruses and adeno-associated viruses, for gene delivery to lungs (**Table 3**). Each of these is discussed below with respect to their applications for pulmonary gene delivery.

4.1 Retroviral and lentiviral vectors

Moloney murine leukaemia virus (MLV)-based vectors were the first type of recombinant retrovirus used for gene delivery. However, the application of an MLV-based vector system for *in vivo* gene delivery in the lung has been hindered by the inability of the virus to infect non-dividing cells of the airway epithelium. Even in dividing cells, although transgene expression is usually adequate *in vitro* and initially *in vivo*, prolonged expression is difficult to attain. The development of lentiviral and pseudotyped lentiviral vectors, which can infect non-dividing cells, has overcome some of the limitations of the MLV-based vectors. The principal types of lentiviral vector that have been tested in airway delivery models include those engineered from human immunodeficiency virus (HIV) [86], feline immunodeficiency virus [87] and simian immunodeficiency virus (SIV) [88]. However, like adeno- and AAV-2 vectors (discussed later), lentiviruses are hampered by the lack of suitable receptors on the apical surface of airway epithelial cells [47].

Goldman *et al.* [86] showed that a VSV-G pseudotyped HIV vector effectively transduced non-dividing airway epithelial cells *in vitro*. Also, they observed high-level *CFTR* expression in CF bronchial xenografts following transduction with a VSV-G pseudotyped HIV-CFTR vector when the cells were undifferentiated (non-polarised). Transgene expression was stable and corrected the CF defect. Wang *et al.* [87] used a VSV-G pseudotyped FIV lentiviral vector formulated with calcium chelators to allow access to basolateral receptors to deliver transgenes to polarised airway epithelia efficiently. When CF epithelia were treated with an FIV-CFTR vector, correction of the Cl^- channel defect was observed. More detailed, long-term safety studies in animal models will be necessary before the pulmonary applications of these vectors can be investigated in humans.

Pulmonary applications of lentiviral gene transfer *in vivo* have broadened recently. Following successful demonstration of *in vivo* gene transfer to human xenografts [86] and rabbit airways [87], groups have demonstrated efficacy of *in utero* vector delivery. Following intrapulmonary delivery of an HIV-GFP vector into rhesus monkey fetuses, Tarantal *et al.* [89,90] reported gene expression in pulmonary epithelia confirmed

Table 3. Viral vectors for gene delivery to the lungs.

Viral vector	Pseudotype/subtype	Targeted cell type/tissue	Application	Ref.
AAV	Types 1 and 5	Preferentially conducting airway epithelial progenitor cells in the lower airways in mice and long-lived Clara cells and alveolar type II cells	*In vivo*	[150]
AAV	Type 6	Lung parenchyma and epithelia cells in the large airways and MLE-12 (murine adenocarcinoma cells)	*In vivo, in vitro*	[151-153]
AAV	Type 9	Murine alveolar and nasal epithelia (apical and basolateral)	*In vivo*, *in vitro*	[138]
LVV	Filovirus envelope protein	Tracheal epithelium from the apical surface and submucosal glands	*In vivo*	[94,99]
LVV	Baculoviral gp64 glycoprotein	Trachea and upper airways to the bronchioles and mid airways to the distal airways	*In vivo*	[154]
LVV	Vesicular Stomatits Virus glycoprotein	Alveoli and resident macrophages after both adult and neonatal nasal administrations	*In vivo*	[154]
LVV	F and HN gylcoproteins of Sendai Virus	Murine nasal epithelial cells, bronchial epithelium and alveolar epithelial cells Rat airway epithelial cells	*In vivo*	[88,155,156]
LVV	Spike Envelope of Severe Acute Respiratory Syndrome Coronavirus	Airway epithelia, dendritic cells	*In vivo*	[157]
Adenovirus		Cells of the epithelial surface of the small and large airways and alveolar epithelium of rodents	*In vivo*	[106,158]
Sendai Virus		Airway epithelia cells	*In vitro, in vivo*	[159,160]

by direct fluorescence and quantitative polymerase chain reaction. Lim *et al.* [91] had similar success in targeting epithelia in human fetal tracheal xenografts using HIV-LacZ. Transgene expression remained detectable 9 months post-transduction. Postnatal *in vivo* delivery to rodent nasal epithelia and trachea was also achieved following epithelial disruption by sulphur dioxide injury [92] or detergents [15]. Following tight junction disruption, Limberis *et al.* [93] delivered *CFTR* to CF mouse nasal epithelium with a VSV-G pseudotyped HIV vector and observed partial electrophysiological correction for 110 days, after which expression diminished for reasons unknown. One possible explanation is loss of terminally differentiated transduced cells.

Recent success in identifying glycoproteins from other enveloped viruses to pseudotype lentiviruses has circumvented the need for host cell modification and improved postnatal gene transfer. Apical transduction by lentivirus has been achieved both *in vitro* and *in vivo* using several viral envelopes from diverse origins, including filovirus [94,95], baculovirus [96], influenza [97] and parainfluenza [88] viruses. Sinn *et al.* [95] pseudotyped a FIV-based vector with gp64 envelope glycoprotein from *Autographa californica* multicapsid nucleopolyhedrosis virus (GP64-FIV). This pseudotyping conferred new apical entry properties for transduction of polarised primary cultures of human airway epithelia. In another study, Sinn *et al.* [96] also showed that transgene expression after lung application of feline immunodeficiency virus pseudotyped with the baculoviral gp64 envelope applied in a viscoelastic gel formulation was significantly higher than observed with a vesicular stomatitis virus glycoprotein (VSV-G) pseudotyped construct. However, Karlea *et al.* [98] did not see increased apical uptake of gp64/HIV when compared with VSV-G.

Ebola virus envelope glycoprotein has also been used successfully to achieve efficient transduction of the murine lung epithelium and human explants [91,99], although generation of consistently high viral titres has been problematic. Using an HIV vector pseudotyped with deletion mutant of Ebola envelope glycoproteins, Medina *et al.* [94] demonstrated stable and efficient gene transfer in the airway of non-human primates. This particular vector has been shown to transduce efficiently intact airway epithelium from the apical surface in both *in vitro* and *in vivo* model systems.

Recently, cell-specific targeting of a lentiviral vector was achieved by inserting an antibody-binding domain into the envelope glycoprotein [100,101]. Morizono and co-workers specifically targeted an antibody-conjugated lentivirus to transduce a metastatic melanoma target cell population. Such an approach may have applications for targeting airway epithelia.

A limitation for many gene vector systems is the inability to readminister the vector as transgene expression wanes. Mucosal innate and adaptive immune responses against the vector or vector-encoded proteins represent a significant impediment to clinical applications and are well documented for virus vectors such as adenoviral and adeno-associated

viral vectors (reviewed in the following sections). Little knowledge exists regarding the potential for readministration of retro- or lentiviral vectors to the airways. In a recent study, Sinn *et al.* [102] successfully demonstrated the readministration of a GP64 pseudotyped FIV vector to the murine nasal epithelia. With luciferase as a marker gene and quantitative bioluminescent imaging, they showed that consecutive daily dosing achieved a linear increase in gene expression and greatly increased the number of epithelial cells targeted. Reporter gene expression increased additively following each of seven doses of FIV delivered over consecutive weeks (1 dose/week), without the development of systemic or local neutralising antibodies. With erythropoietin as a physiological marker, transduction efficiency achieved following a single dose of FIV expressing mouse erythropoietin was insufficient to increase haematocrit, whereas seven consecutive daily doses significantly increased haematocrit.

A safety risk posed by retro- and lentiviral vectors is their potential to transform cells by integrating near a cellular proto-oncogene and driving inappropriate expression from the long terminal repeats (LTR), or by disrupting a tumour suppressor gene. This event, termed insertional mutagenesis, has led to the development of leukaemia in patients of X-SCID [103] and ever since has been a highly discussed topic in the scientific community. Thus, safety concerns of insertional mutagenesis and immune response of the host need to be addressed before clinical applications of retro- or lentiviral vectors. Encouraging results with respect to lentiviral integration have been obtained by Bartholomae *et al.* [104] in eyes of mice and rats. In contrast to the observations made in different dividing and growth-arrested cells, the authors showed that lentiviral integration site distribution is nearly random in postmitotic tissues. This makes the risk of insertional mutagenesis for lentiviral vectors, under the conditions tested, comparable to non-viral vectors. Successful application of retro- or lentiviral vectors to pulmonary gene transfer will require further advancements in delivery, efficiency, cell specificity and persistent gene expression.

4.2 Adenoviral vectors

Adenovirus-based (Ad) vectors have been the most extensively studied recombinant viral systems for gene transfer to the lung owing to their ability to transduce efficiently a wide variety of proliferating and non-proliferating cells [105,106]. Unlike the genome integrating retroviruses, adenoviruses replicate as episomal elements in the nucleus of the host cell and consequently there is minimal risk of insertional mutagenesis.

The first generation Ad vectors (FGAd) with the E1 region deleted have been the most extensively used vector for pulmonary gene transfer. Although once thought to be an ideal vector for lung gene therapy, more than a decade of research has revealed some serious shortcomings and the enthusiasm for FGAd has diminished. First, pulmonary delivery of FGAd in small animals, large animals and humans is inefficient [107-111]. It was discovered that the cellular receptor for Ad (and other viral vectors) resided on the basolateral surface of the airway epithelial cells and that the tight junctions prevented the vector–receptor interactions required for transduction [112]. A significant finding was that transient disruption of the tight junctions could significantly increase the efficiency of transduction, thus dramatically decreasing the vector dose required to achieve therapeutic levels of transduction. Various strategies have been proposed to improve adenoviral entry into airway epithelia, including calcium phosphate co-precipitates [113], EGTA [114], EDTA [115], polycations [116], polidocanol [117], sodium caprate [118], L-α-lysophosphatidylcholine (LPC) [119] and other agents.

Second, pulmonary delivery of FGAd resulted in dose-dependent inflammation and pneumonia [109,120-123], beginning about 3 – 4 days post-administration and becoming progressively more severe before eventually resolving. This has been attributed to expression of the viral genes present in the vector backbone of FGAds, which is directly cytotoxic and also provokes an adaptive cellular immune response against the transduced cells, consequently resulting in transient transgene expression and long-term, chronic toxicity [124-128]. This dose-dependent toxicity was partially addressed by using second-generation Ad vectors deleted/mutated of other early viral genes, such as E2 or E4, in addition to E1 that diminished further, but did not eliminate pulmonary inflammation and pneumonia, probably as a result of continued leaky expression of the viral late genes [127,129-131]. These studies not only identified some of the major challenges in gene delivery using adenoviral vectors, but also provided sound rationale to improve the viral vectors. Non-clinical studies have explored the potential of pseudotyping the adenovirus vectors to target other more suitable cell surface receptors [132].

Significant improvement in the safety and efficacy of Ad-based vectors came with the development of helper-dependent adenoviral vectors (HDAd), which are deleted of all viral coding sequences [133]. Therefore, in contrast to FGAd, HDAd are able to mediate long-term, high-level transgene expression in the absence of chronic toxicity owing to the absence of viral protein expression in the transduced cells. Application of HDAd vectors in animal models of pulmonary diseases would provide vital information for their applicability in the clinic.

4.3 Adeno-associated viral (AAV) vectors

Adeno-associated viruses (AAV) are members of the Parvovirus family, dependent on a helper virus, usually adenovirus, to proliferate. They are capable of infecting both dividing and non-dividing cells, and in the absence of a helper virus integrate into a specific point of the host genome (19q 13-qter in human genome) at a high frequency [134]. This site-specific integration requires the activity of Rep protein.

When used as a vector, the *rep* and *cap* genes of the virus are replaced by the transgene and its associated regulatory sequences. The total length of the insert cannot greatly exceed 4.7 kb [135], beyond which the titre is significantly reduced. The main interest in AAV vectors has been due to their lack of pathogenicity, ability to transduce non-dividing cells and prolonged transgene expression. Persistent expression of exogenous gene using AAV vectors has been demonstrated in the lungs. Most AAV biology and rAAV vector studies were initiated with serotype 2 vector. The advent of technology for pseudotyping of AAV2-ITR-flanked genomes into capsids of different serotypes, such as type 3, type 5, type 6 and type 9, was conceived as a means to bypass the limitation of receptors on the apical surface of airway epithelium and prevalence of neutralising antibodies to AAV2 in the lung [136-138]. Comparison of AAV serotypes 2, 3, 5 and 6 *in vivo* with respect to their airway transduction potential found AAV6 to be the most efficient [136,137]. In a separate study, Seiler *et al.* [139] reported that compared with vectors made with AAV2 type capsid, those having AAV type 5 or type 6 capsids show high transduction rates in airway epithelia cells, in a range that should be sufficient for treating lung disease. A comparison of AAV5 and AAV6 showed that, whereas both vectors had high transduction rates in well-differentiated human airway epithelial cultures, they showed distinct cell type transduction profiles in mouse lung that were consistent with differences in receptor use [139]. The above studies point to the fact that the numerous AAV serotypes present in the primates differ greatly in their transduction efficiency.

To treat chronic inherited diseases, it is likely to be necessary to maintain transgene expression throughout the life of the treated individual. So far there are no definitive data that measure the turnover of different cell types in the lungs of either humans or mice. However, most of the cells in the lungs are terminally differentiated; therefore, it is expected that transgene expression from the single administration of any gene transfer vector will fall with time unless a cell population with the ability to self-renew is targeted. Until the time that the ability to target well-defined stem cell populations is developed, repeated administration of gene therapy vectors is inevitable. As for other viral vectors, neutralising antibodies to AAV capsids have been observed that hinder the administration of subsequent viral doses of the same serotype [140]. Additionally, pre-existing neutralising antibodies against AAV2, 5 and 6 have been observed in CF patients and normal human populations [141]. About 30% of adults were seropositive for AAV2, 20 – 30% were seropositive for AAV6, and 10 – 20% were seropositive for AAV5. In the same study, CF children were seropositive for AAV types 2, 5, or 6 at rates of only 4 – 15%. These results indicate that AAV type 2, 5 or 6 exposure is low in CF children, and these vectors, especially types 5 and 6, owing to higher transfection efficiences compared with AAV2 [139] and low pre-existing immunity, may be amenable to use as gene therapy vectors in pulmonary gene therapy. Another AAV serotype that is of immense interest for pulmonary gene therapy is AAV type 9. As for AAV5 and 6, type 9-based vectors were reported to be able to transfect airway cells from the apical as well as basolateral side [138]. In *in vivo* experiments in mice, AAV9-based vector was found to mediate 60-fold higher expression compared with type 5 pseudotyped vector. Moreover, the resulting expression was found to be stable for ~ 9 months. With respect to immunogenicity, it has been shown that AAV9 can be readministered if the time between two administrations is extended beyond ~ 28 weeks [138]. These attributes of higher transduction efficiency, low immunogenicity and ability to be readministered make AAV vectors attractive for further development with respect to pulmonary gene delivery.

5. Conclusion

Gene therapy holds great promise for the treatment of many acquired and inherited pulmonary diseases. Although major advances have been made in our understanding of the molecular pathogenesis of pulmonary diseases, the translation of this understanding into effective treatment has remained elusive so far. For effective gene therapy, the gene vectors need to be delivered to the target cells, on one side to minimise the undesired effects resulting from the delivery of gene vectors to non-target cells, and on the other to reduce the total amount of dose required to observe a clinically beneficial effect. Both physical and biological methods are used to attain this targeted delivery. Among physical tools, magnetofection has been shown to achieve targeted gene delivery with non-viral vectors both *in vitro* and *in vivo*. Biological targeting with specific ligands has been applied successfully in various gene delivery systems and shown to work in a cell-specific manner. Different viruses have also been evaluated for gene delivery to the lungs. Targeting of specific cells in the lungs has been achieved by pseudotyping of the viral vectors with capsids from different serotypes. In summary, although targeted gene delivery to the lungs has been demonstrated using various non-viral and viral vectors, the clinical translation of this know-how still needs to be attained.

6. Expert opinion

This review focuses on targeted delivery with emphasis on pulmonary gene therapy. Lungs are attractive organs for gene therapy owing to their easy accessibility through the airways and gene therapy is a promising approach for the treatment of many inherited pulmonary diseases such as cystic fibrosis, α1-antitrypsin and surfactant protein deficiencies, to name a few. For gene therapy to be effective, vectors (both viral and non-viral) need to be delivered to the target tissue and within the tissue, to the target cells. One major prerequisite for efficient gene transfer *in vivo* is the accumulation of gene vectors at the target tissue. Using

physical forces of an applied magnetic field, the proof-of-principle has been established that specific regions of the lungs can be targeted.

Although delivery of exogenous genetic material into the cells has been achieved by numerous viral and non-viral approaches, viruses have led the way by making it into clinics earlier. Their most desirable property has been the high efficiency of gene transfer. Adenoviruses (AdV) were initially the vectors of choice for pulmonary gene delivery owing to their natural tropism for the lungs. However, interest in AdV vectors has diminished with time because of their high immunogenicity, transient expression and, most importantly, the basolateral location of the CAR receptor on the epithelial cells. Another class of viral vectors is those of the AAV group. Several serotypes of AAV, such as 5, 6 and 9, have been shown to mediate efficient transduction of airways and alveolar epithelium and can also be readministered (AAV6 and 9), indicating their low immunogenicity. Lentiviral vectors have also been applied to achieve stable and long-term expression in the lungs. Moreover, by pseudotpying lentiviral vectors with different envelope proteins, cell-specific delivery has been demonstrated in various studies. As major proportion of the cells in the lungs are terminally differentiated, long-term expression resulting from AAV vectors may be more desirable than lentiviral vectors because the former persist in the cells as an episome, thus minimising the risk of insertional mutagenesis. Moreover, for therapy of inherited deficiencies, expression needs to persist for the rest of the life of the patient or the vector should allow for repeated administration. The reduced immunogenicity of AAV vectors and the ability of lentiviral vectors to be successfully readministered make them leading candidates for clinical gene therapy among viral vectors.

Another alternative to repeated vector application is to correct the disease in 'stem cells'. The field of pulmonary stem cells is still developing and there is no definite cell population that can be classified as 'lung stem cells'. Stem cells have been identified in different niches in the respiratory system, such as the submucosal glands, neuroendocrine bodies and bronchial alveolar duct junctions. Even at the defined niches, their proportions are < 1% and at present there are no viable approaches to target them *in vivo* or *ex vivo* followed by reimplantation. Until such techniques are available, repeated administration of the gene vectors would be needed to maintain the therapeutic level of expression. In light of their high immunogenicity, AdV vectors may not be suitable for such diseases. Their immunogenicity may, however, be useful in cancer gene therapy. Shenzhen SiBiono GenTech's Gendicine (first generation adenoviral vector with tumour suppressor p53) for head-and-neck squamous cell carcinoma (HNSCC) has been used in China. With respect to gene therapy in stem cells, both AdV and AAV groups of viral vectors persist in the cells as episomes and so are expected to be lost during cell division. For such requirements, integrating lentiviral vectors are best, but the risk of insertional mutagenesis needs to be evaluated before their wide-scale application.

To overcome the limitations of viral vectors (limited cloning capacity, costly upscale procedures, immunogenicity, insertional mutagenesis), considerable research efforts are underway to develop synthetic non-viral vectors that circumvent the above-mentioned concerns. With non-viral vectors, cell-specific delivery of gene vector complexes to specific lung cell types has been achieved with various ligands. Several 'proof-of-principle' studies have established non-viral gene therapy as a feasible therapeutic approach for genetic diseases. The outcome of currently initialised clinical trials with both viral and non-viral vectors may help to identify further the most promising candidate vector in the near future. However, for this 'future pulmonary medicine' to reach the clinic rapidly, further concerted efforts are needed from the fields of vector design and delivery. Efficient vectors need to be designed keeping in mind the complexity of pulmonary cellular architecture. Delivery strategies, especially with non-viral vectors, need to be optimised further for enhanced efficiency resulting in therapeutic expression. Moreover, joint efforts between basic and applied researches would result in rapid translation of discoveries in research labs to bedside therapies in the clinic.

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