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REVIEW ARTICLE

Lung gene therapy—How to capture illumination from the light already present in the tunnel

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Abstract Gene therapy has been considered as the most ideal medical intervention for genetic diseases because it is intended to target the cause of diseases instead of disease symptoms. Availability of techniques for identification of genetic mutations and for *in vitro* manipulation of genes makes it practical and attractive. After the initial hype in 1990s and later disappointments in clinical trials for more than a decade, light has finally come into the tunnel in recent years, especially in the field of eye gene therapy where it has taken big strides. Clinical trials in gene therapy for retinal degenerative diseases such as Leber's congenital amaurosis (LCA) and choroideremia demonstrated clear therapeutic efficacies without apparent side effects. Although these successful examples are still rare and sporadic in the field, they provide the proof of concept for harnessing the power of gene therapy to treat genetic diseases and to modernize our medication. In addition, those success stories illuminate the path for the development of gene therapy treating other genetic diseases. Because of the differences in target organs and cells, distinct barriers to gene delivery exist in gene therapy for each genetic disease. It is not feasible for authors to review the current development in the entire field. Thus, in this article, we will focus on what we can learn from the current success in gene therapy for retinal degenerative diseases to speed up the gene therapy development for lung diseases, such as cystic fibrosis.

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Introduction

In 2008, three research teams independently reported the success in clinical trails of gene therapy treating a rare form of retinal degenerative diseases called Leber's congenital amaurosis (LCA).[1–3] LCA represents a group of inherited blindness with childhood onset.[4] The clinical success has been achieved in treating LCA2, one form of the disease, which is caused by mutations in the retinal pigment epithelium-specific 65-kDa protein gene (*REP65*). *REP65* encodes a protein providing the isomerohydrolase activity for the retinal pigment epithelium to produce 11-*cis*-retinal from all-*trans*-retinyl esters during the visual cycle for regenerating the visual pigment after exposure to light. Without this gene function, 11-*cis*-retinal, the natural ligand and chromophore of the opsins of photoreceptor cells, cannot be regenerated, thus rendering the opsins incapable of capturing light or transducing it into electrical responses for initiating vision. Although this defect in light transduction has an immediate impact on visual function, retinal cell degeneration is delayed in patients, thus making target cells available for gene therapy. The three teams tested the same therapeutic approach in patients by subretinal injection of recombinant adeno-associated virus vector 2 (AAV2) expressing the *RPE65* complementary DNA (cDNA). Patients with treatment showed improvements in visual function without serious adverse events. In 2012, three patients received the same treatment in their other eye and all three demonstrated improvements in visual and retinal function in their second eyes after the treatment, which was administered one-and-a-half to three-and-a-half years after their first eyes were treated.[5] Readministration of the same gene therapy vector caused no harmful immune reactions in patients. In 2014, a gene therapy trial for another retinal degenerative disease, choroideremia, was shown to be successful.[6] Choroideremia is an X-linked recessive disease that is caused by mutations in the *CHM* gene, which encodes the Rab escort protein 1 (REP1). The same gene therapy vector, AAV2, was used in this study. In addition to the eye gene therapy success, progress has been made in other fields as well. For example, as a milestone for using gene therapy as medicine, European Union approved Glybera as the first gene therapy drug for a form of lipoprotein lipase deficiency.[7–9] In this case, AAV1 was used to deliver a naturally occurring functional variant of the LPL gene associated with lower rates of cardiovascular disease and increased efficiency in fat metabolism. These clinical successes provide the proof of concept that the power of gene therapy can be harnessed to benefit human beings.

However, gene therapy developments for other diseases, such as cystic fibrosis (CF) lung disease, are not as successful for eye diseases.[10–13] CF is the most common monogenic fatal disorder in the Caucasian population and it is caused by recessive mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (*CFTR*).[14–16] Although the disease affects multiple organs, including the lung, pancreas, intestine, gall bladder and reproductive organs,[13,17] lung failure due to chronic infection and inflammation is currently responsible for most morbidity and mortality. Therefore, CF gene therapy studies to date have been aimed at treating the pulmonary manifestations. When the cystic fibrosis gene was identified in 1989, it appeared that this disease can be used as an ideal model for the development of gene therapy for lung diseases since airway epithelial cells where the *CFTR* gene is expressed are readily accessible to gene therapy vectors. Yet, all the CF clinical trials conducted so far did not show any evidence of significant therapeutic benefits brought to CF patients.[18–35] Basic research in lung gene therapy developments later identified major barriers to vector delivery and sustained therapeutic gene expression.[10,13,36] Thus, it is useful to look into what is fundamental to the successful gene therapy development for eye diseases to make lung gene therapy fruitful.

In this review article, we will first visit the early developments in CF lung therapy and look into the major challenges encountered in the lung gene therapy field. We will then review the key factors that are critical to the eye gene therapy progress to explain the possible rationale for the clinical success. We will finally discuss strategies that can be translated from the eye gene therapy field to speed up the lung gene therapy development.

Early stages of lung gene therapy developments

Because CF is a monogenic disease and the target cells in lung airway are easily accessible to gene therapy vectors, when the gene was identified, an illusion was created suggesting that lung gene therapy for CF would be available in a few years. The initial excitements inspired many scientists racing in conducting clinical trials. Both viral and non-viral gene therapy vectors were tested. One of the early clinical studies was conducted by Zabner et al in 1993 to examine the safety profile of an adenoviral (Ad) vector with nasal applications.[18] Adenoviruses contain a linear double-stranded DNA and have been widely used as tools for gene delivery because of their ability to infect both dividing and non-dividing cells with a high efficiency, especially epithelial cells. The early generations of Ad vectors were developed by deleting the E1 region within the viral genome to prevent viral proliferation in transduced cells and/or other regions such as E3 or E4 to increase the DNA carrying capacity. There are more than 50 serotypes of adenoviruses identified so far.[37] In this study, a serotype 2 adenoviral vector expressing the human CFTR cDNA was administered to a defined area of nasal epithelium in three patients. Although this initial study showed some functional correction in nasal epithelial cells with no vector-related adverse effects, more extensive studies later demonstrated with similar methods that there was no significant functional correction in nasal epithelia.[19,21] The Ad vectors have also been tested in the lung[24–26] and none of the studies demonstrated functional correction or efficacy in patients.

In addition to the early generations of Ad vectors, recombinant adeno-associated virus (AAV) vectors have also been tested in CF patients.[28,38,39] AAV is a replication-defective parvovirus that depends on a helper virus, either adenovirus or herpes virus, for its propagation during lytic infection.[40] It has a small single-stranded DNA genome (about 4.7 kb). The advantage of AAV as a gene therapy

vector is that it does not elicit strong immune responses. Because of its small DNA carrying capacity, it could not be used to deliver large genes or genes with long DNA regulatory elements. Although there were no major adverse effects even when repeated administration was performed,[38] these studies did not show significant benefits for patients treated with vector delivery.

Non-viral vectors have also been tested clinically in CF gene therapy since these vectors are considered to be safer than viral vectors.[10] Cationic liposomes, which are composed of a cationic lipid and a neutral lipid, are commonly used for gene delivery. There are many types of cationic lipids available[41] which are often mixed with one or two commonly used neutral lipids, dioleoylphosphatidylethanolamine (DOPE) or cholesterol.[10] Many clinical trials have been conducted with cationic liposomes to assess their potential for CF Therapy. The first liposome CF trial was carried out in CF patients through nasal administration[29] and later, several other trials with various liposome formulations were conducted.[30–33] Similar delivery methods were tested in the lung of CF patients and the results were not encouraging.[34,35]

Challenges that slowed the progress in the lung gene therapy

Looking back at the aforementioned clinical studies, it is now understandable; the reason for the lack of clinical progress in the lung gene therapy for CF was that major challenges to lung gene delivery were not fully appreciated at the time. These challenges include 1) lacking efficient vectors for airway gene expression, 2) host immune responses to vectors, 3) lacking efficient and safe methods for vector delivery and 4) difficulty in maintaining long-term therapeutic gene expression in airways where epithelial cells turn over. The following sections will be devoted to explain these challenges as well as progresses being made towards overcoming these challenges.

Efficient vectors for airway gene expression

It is apparent that all gene therapy vectors used in early clinical studies of lung gene therapy were not adequate for achieving the efficiency needed for functional correction in patients. The first generation Ad vectors used in the clinical studies could not confer sustained transgene expression *in vivo*. The issue of lacking sustained transgene expression from adenoviral vectors is often misunderstood because it was interpreted as the lack of vector stability *in vivo*. In fact, Ad vectors are stable *in vivo*; the reason that they cannot confer sustained transgene expression is because they elicit host immune responses (which will be addressed later), which eliminate the transduced cells *in vivo*. Although most Ad vectors have the E1 region deleted from their genomes to prevent viral gene expression and proliferation, leaky expression of viral genes does occur in transduced cells,[42,43] thus providing antigen for the host immune system to attack the transduced cells. Therefore, early generations of Ad vectors are not suitable for gene replacement therapy.

To reduce the host immune responses, helper-dependent adenoviral (HD-Ad) vectors have been developed.[36,44] In these vectors, all the viral coding sequences have been deleted, thus eliminating the leaky viral gene expression and rendering a large DNA carrying capacity to the vectors. These vectors have also been called high capacity or gutless vectors[45] and have been shown to confer long term transgene expression when delivered to mouse livers.[46] Compared to the first generation Ad vectors, HD-Ad vectors elicited reduced levels of inflammation and conferred longer term of transgene expression when delivered to mouse lungs.[47] Our group has demonstrated that the human CFTR gene can be efficiently delivered to mouse lungs using the HD-Ad vector and that the CFTR knockout mice treated with the CFTR expressing vector are protected from acute lung infection with bacteria.[48] We have also showed that HD-Ad vectors can be used to deliver genes to the lungs of rabbits[49] and pigs.[50] We have showed that the human CFTR gene can be efficiently delivered to pig lungs.[50] However, up to date, HD-Ad vectors have not been tested in human lungs.

The reasons for the AAV vector failing to meet the efficiency required for CF lung gene therapy are different from that of Ad vectors. One of the major limitations is the small DNA carrying capacity of AAV vectors; once the *CFTR* cDNA is packed into the vector, there is no room for carrying DNA regulatory elements, such as cell-specific promoter or enhancer, for CFTR gene expression.[51,52] It was reported in 2008 that AAV vectors could deliver as much as 8.9 kb DNA although with a reduced efficiency,[53] but later it was found that a single AAV particle could not deliver the large reporter genes and the cell transduction in this case may be accomplished by two viral particles containing the 5′ and 3′ parts of the reporter genes.[54] In addition, the AAV2 vector is not very efficient for lung gene delivery.[55]

One strategy to expand the DNA carrying capacity is to rely on trans-splicing of mRNAs in target cells. A large gene can be packaged into two AAV vectors to generate two RNA transcripts which are spliced into one functional mRNA.[56,57] Since this strategy required two AAV vectors to carry each part of a gene to transduce the same cell and it is not expected that all RNA transcripts are trans-spliced, the transgene expression efficiency will likely be reduced. Although the DNA carrying capacity of AAV vectors is not easy to change, different serotype AAV vectors with better transduction efficiency for lung airway cells can be selected. For example, AAV5, AAV6, AAV9 and AAV6.2 are better vectors for lung gene delivery[55] than AAV2 that has already been used in clinical trials. In addition, vector tropism can be engineered for lung applications.[58,59]

The lack of success in using nonviral vectors for clinical studies of CF lung gene therapy is understandable because, unlike the viral vectors, there is no specific mechanism for nonviral vectors to send the DNA payload into the nuclei of target cells and perpetuate the existence of the therapeutic gene in the nuclei. For gene delivery with liposomal vectors, the majority of the delivered DNA is degraded by lysosomes before entering nuclei; nuclear entry is another major barrier.[60] Although non-viral vectors might be useful for some other gene delivery applications, in our opinion, it is difficult to use them for lung gene replacement therapy due to the lack of efficiency in gene delivery. Although

nonviral vectors are less toxic, it is unlikely that CF lungs can tolerate weekly or even monthly gene delivery.

Host immune responses

Host immune responses to gene therapy vectors are one of the most important challenges that were previously underestimated in early lung gene therapy development. The lung is a very sensitive immunologic organ[61] capable of producing both strong innate and adaptive immune responses to pathogens and gene therapy vectors. Although the adaptive immune response to viral vectors was recognized early on and investigated extensively,[62–65] the innate immune response was not fully appreciated. This was evident from the first and the only incidence of gene therapy related death in 1999 when a patient who received a high dose of an Ad vector via the hepatic artery succumbed to acute toxicity. Post-mortem confirmed that the patient suffered from systemic inflammation, biochemically detectable disseminated intravascular coagulation, and multiple organ failure within 98 h.[66] Clearly this was the result of patient's innate immune system reacting to the high dose of the Ad vector.

For airway gene delivery, the first innate immune response is mediated by lung macrophages that quickly engulf vector particles, reducing gene delivery to target cells. The macrophages not only take up gene therapy vectors and destroy them, but also initiate the production of proinflammatory cytokines by interacting with lung epithelial cells.[67] Macrophages as well as epithelial cells express Toll-like receptors (TLRs) and RIG-I-like (retinoid-inducible gene-1-like) receptors, which recognize nucleic acid and proteins derived from viral pathogens including viral DNA, single-stranded RNA and double-stranded RNA.[68] Inflammatory cytokines, such as TNF-α, IL-6, MIP-2 and MIP-1α, are dramatically induced in macrophages upon Ad vector delivery to mouse airways within 6 h.[45,69] These cytokines activate airway immune cells and structural cells to produce more proinflammatory cytokines which could lead to airway damage, if the host cannot shut down the cascade. One of the important pathways involved in the induction of inflammatory cytokines is the NF-κB signaling pathway.[70] A variety of inflammatory cytokines, such as IL-6 and IL-8, can be induced by the activation of NF-κB. In humans, a high level of IL-8, a potent neutrophil chemoattractant, can cause neutrophil infiltration that leads to tissue damage.[71,72] Even by using non-viral vectors, bacterial DNA can be recognized by Toll-like receptor 9 which activates the NF-κB pathway.[36,45] It has been recently reported that cytosolic DNA is a danger signal that induces interferons through the production of cyclic guanosine monophosphate–adenosine monophosphate (cyclic GMP–AMP, or cGAMP).[73] Other immune cells, such as neutrophils and natural killer cells, are also recruited in response to viral vector delivery, which can produce inflammatory mediators as well and cause tissue damage. The innate immune response not only leads to acute toxicity, but also enhances the adaptive immune response (will be explained later).

One major strategy to avoid the host immune responses is to avoid using unnecessary high vector dose. Since all gene therapy vectors elicit innate immune responses, the lower the dose the weaker immune responses triggered in the host. In addition, anti-inflammatory drugs may be used transiently to reduce the innate immune responses around the first few days following vector delivery. Finally, noninvasive vector delivery methods should be used to reduce host stress to dampen the host innate immune responses. These points will be further discussed later.

The adaptive immunity relies on T- and B-lymphocytes to produce cellular and humoral responses to infectious agents. In mammals, the adaptive system is developed postnatally. During the development, an extremely diverse repertoire of receptors is generated randomly and each receptor recognizing a unique antigen, is expressed on the surface of one T lymphocyte only. T cells bearing useful receptors are subsequently selected from billions of lymphocytes for clonal expansion by interacting with antigens. Professional-antigen-presenting cells, normally dendritic cells or macrophages present the antigens bound to the MHC II molecules to helper T cells to activate them. Activation of helper T cells by antigen presenting cells also requires a co-stimulatory signal, e.g., CD80 or CD86, on the surface of the antigen-presenting cell to bind to CD28 on the surface of the T cell.[74] The expression of co-stimulatory molecules is regulated by innate immunity,[75] and therefore, the adaptive immunity is regulated by the innate immunity. Helper T cells control other cells in the adaptive immune system, such as activation of cytotoxic T cells to destroy infected cells and B cells to produce antibodies. After elimination of pathogens, some antigen-specific clones of T and B cells remain as "memory" lymphocytes so that the adaptive immune system remembers the antigens and destroy them more quickly upon subsequent exposure.

Early work from several groups[62–64] demonstrated clearly that both cellular and humoral responses are involved in adenoviral vector-mediated gene transfer in mice. Repeated delivery of viral vectors, or primary delivery to individuals with pre-existing immunity, or viral vectors expressing foreign antigens, can cause strong adaptive immune responses. Various strategies can be used to overcome the host adaptive immune responses. First of all, as described above, blocking the innate immune response can reduce the adaptive immune response. Secondly, blocking co-stimulatory pathways can be used to modulate the host adaptive immune responses. Several groups showed that an antibody against CD40 ligand[76,77] or expressing CTLA4Ig, a fusion protein of cytotoxic T lymphocyte-associated protein 4 (CTLA4) and the Fc portion of immunoglobulin G (IgG), by the HD-Ad vector, improved transgene expression in rodents.[78,79]

In addition, "serotype switching"[80] can be used for repeated delivery of recombinant viruses. Gene therapy is initiated with one virus serotype, then switched to a second serotype for a subsequent administration, thereby avoiding attack by neutralizing antibodies specific to the first serotype.[81,82] However, the level and duration of transgene expression following serotype switching may be limited by cross-reactive cytotoxic T lymphocytes that can also target cells infected by the second serotype virus.[82,83] Thus, viral vectors expressing foreign antigens, such as the first generation Ad vectors, cannot be used. Finally, since all viral

gene therapy vectors used in the future should not express any viral coding proteins, in such case transient immune modulation may be sufficient for blocking the adaptive immune response. It was shown that cyclophosphamide alone or in combination with cyclosporine A extended transgene expression mediated by the first generation Ad vector.[62] Our group has demonstrated that transient administration of cyclophosphamide allowed readministration of HD-Ad vectors with efficient expression of transgenes.[84]

Efficient and safe delivery to airway

How to deliver genes safely and effectively into the airway cells is an area that is not extensively studied. In addition to the immune barriers discussed above, there are physical barriers to vector delivery. At the time when the early CF gene therapy clinical studies were conducted, there was not enough information regarding physical barriers to gene delivery, such as the mucosal layer, airway innate immune cells and physical protection by airway epithelial tight junctions. The mucosal layer is the first line of airway defense and it has been shown to inhibit viral transduction.[85–87] For an inflamed lung, such as in CF, excessive secretion of mucus may be even more problematic for gene delivery. In addition, as mentioned above, the airway immune cells, mainly macrophages, can take and destroy a large portion of gene therapy vectors delivered,[69,88] thus preventing them by reaching the target cells. More importantly, viral vectors used in CF lung gene therapy depend on their receptors to gain entry into their target cells which are airway epithelial cells. However, cellular receptors for viral vectors, such as the coxsackie-adenoviral receptor (CAR), are located not on the lumenal side, but on the basolateral side of the airway that is not directly accessible to vectors. Since airway epithelial cells are connected by tight junction proteins, vectors delivered cannot reach the basolateral side of the airway.

There are strategies that can be considered to overcome these barriers. For reducing the inhibition from the mucus layer, mucolytic reagents, such as nacystelyn, may be used.[17,89] In addition, various polycations, such as DEAE-dextran, polylysines, polybrene, protamine, and branched plyethylenimine have been shown to greatly enhance viral vector delivery to mouse lungs.[83] To reduce the loss of gene therapy vector, lung macrophages can be depleted by gadolinium chloride[90] or liposome/dichloromethylene-bisphosphonate.[88,91] However, these approaches may be too toxic to humans even though mice tolerate them well. On the other hand, anti-inflammatory reagents such as rooperol,[92] methyl palmitate[93] and mangiferin,[94] that inhibit the phagocytic activity of macrophages, may be administered by aerosolization to the airway during vector delivery. In addition, dexamethasone has been shown to reduce the phagocytic activity of pulmonary macrophages although it is not effective on peritoneal macrophages.[95]

Although the accessibility of receptors may not be an issue with nonviral vectors, such as liposomes, it is important for gene delivery with viral vectors. Studies with animals show that reagents, such as Ca^{2+} chelator, EGTA, can be used to break the tight-junctions transiently for enhancing viral vector delivery to the lung.[96] It has also been shown that L-α-lysophosphatidylcholine (LPC) can enhance viral vector delivery dramatically to the lung of mice[97] and rabbits[49] although it is not clear whether LPC breaks tight-junctions.

Since gene delivery to lung normally causes some levels of innate immune responses, it is likely that less stress put on the host with less invasive delivery method will elicit weaker innate immune reactions and produce better results of therapeutic gene expression. The delivery method itself may also affect the outcome. For example, an optimized aerosol delivery approach may give a much better vector distribution in the lung than instillation.

Sustained therapeutic gene expression in the lung airway

Sustained therapeutic gene expression is important for lung gene therapy because it is unlikely that gene therapy vectors, no matter viral or nonviral, cannot be frequently administered to the lung due to the host innate and adaptive immune responses to viral vectors and bacterial DNA in nonviral vectors. The challenge has not been paid enough attention because most of the lung gene therapy studies have been focusing on achieving efficient therapeutic gene expression. The sustained therapeutic gene is more difficult to achieve in the lung than other organs, such as the eye because the lung is an immunologically sensitive organ and the airway cells turn over. For the loss of therapeutic gene expression due to the host immune responses, strategies to overcome this problem have to be considered from all aspects of the gene therapy design, such as vector choice, vector dose and delivery methods. These issues are covered in the section of immune barriers and will not be repeated here.

Airway epithelial cells turn over naturally. In mice the average half-life of the ciliated epithelial cells is about 6 months in the trachea and 17 months in the lung.[98] In humans, the lung epithelial cells may have similar life spans, which may be reduced in diseased conditions. Two strategies may be used to cope with the problem. The first strategy is to allow vector re-administration with a long interval, for example once a year, if the adaptive immune responses to vectors can be avoided. This strategy cannot be used if the vector expressed foreign antigens, such as the leaky expression of viral genes seen in the early generation Ad vectors. However, it is expected that all future gene therapy vectors do not express vector encoded viral genes, thus transient immune suppression may be used to control the adaptive immune problem. In fact, it has been demonstrated in mice that HD-Ad vectors can be re-administered through transient immunosuppression.[84] The second strategy is to deliver a vector that allows a therapeutic gene to be integrated safely in the airway progenitor cells. One major concern for integration of therapeutic genes to correct genetic diseases is the risk of random insertion that could cause major side effects including cancer development.[99,100] Now this problem can be solved through engineering site-specific endonucleases to select a safe site for the integration.[101–103] Three types of engineered endonucleases have been studied for their potential

in genetic engineering and therapeutic development. Zinc finger nucleases (ZFNs) are the first type in this group, engineered by combining the nonspecific nuclease domain of the Fok I restriction endonuclease with a zinc finger DNA-binding domain.[104,105] ZFNs have now been used successfully for genome editing and site specific gene insertion[102,106] although engineering ZFNs for a new chromosome site is still time-consuming. Recently, a new class of engineered nucleases called transcription activator-like effector nucleases (TALENs), has emerged.[107–110] Like ZFNs, TALENs use the same Fok I nuclease domains for DNA cleavage. Unlike ZFNs, TALENs can be easily engineered for any new integration site. More recently, a third type of endonucleases was designed based on the CRISPR/Cas9 (CRISPR-associated) system which is involved in genome defense mechanisms in bacteria for destroying foreign DNA.[111–113] This CRISPR/Cas9 system is engineered to contain a single protein, Cas9 and a small RNA.[114,115] Since a relatively short target sequence (only 13 out of 20 is required) is used for determination of the cleavage site,[116] its off-target effects[117–119] have to be extensively characterized before it is used for gene therapy. Since HD-Ad vectors are highly efficient in gene delivery to airway cells with a large DNA carrying capacity, a single vector can carry both the engineered site-specific nuclease genes and a therapeutic gene for clinical applications.

Reasons for the recent clinical success in eye gene therapy

There are several important factors that made the recent success in eye gene therapy possible. First of all, the right gene therapy vector was selected for the disease targeted. For targeting LCA2, AAV2 vector is the right choice because the therapeutic gene *RPE65* is small enough for this type of vectors which are efficient in transducing retinal pigment epithelial cells.[120] The cDNA of *RPE65* is about 3.1 kb[121] which allows AAV type vectors to have 1.6 kb room for DNA elements to control the therapeutic gene expression. For the LCA2 clinical studies, two types of DNA control elements are used. The trial study by Bainbridge et al used the human *RPE65* gene promoter (1.4 kb) and the bovine growth hormone polyadenylation signal to control the *RPE65* expression,[2] whiles in studies[1,3] by Maguire et al and Cideciyan et al, the expression of *RPE65* is under the control of the chicken β-actin promoter together with the cytomegalovirus immediate early enhancer, the rabbit β-globin intro/exon junction and the SV40 polyadenylation signal. In addition to the carrying capacity good enough to meet the delivery of therapeutic gene, AAV2 is efficient in transducing retinal pigment epithelial (RPE) cells.[120]

Secondly, immune barrier to AAV vector delivery is less of a problem for subretinal gene delivery because the anterior chamber of the eye is an immune-privileged site[122] and AAV elicits weaker immune responses compared to other types of viral vectors.[123] Immune privilege of the eye is established through five mechanisms, blood: ocular barriers, absence of lymphatic drainage pathways, soluble immunomodulatory factors in aqueous humor, immunomodulatory ligands on the surface of ocular parenchymal cells and indigenous, tolerance-promoting antigen-presenting cells.[122] Since immune protection against pathogens can injure vital tissues in an innocent bystander manner, immune privilege is regarded as an evolutionary adaptation to enable local protection by immune effectors without disrupting the function of the vital tissues, such as light transduction by retinal cells.

Furthermore, RPE cells are ideal target cells for gene therapy. Essentially, all AAV vectors are efficient in transducing RPE cells although it is not totally clear whether this is due to the phagocytic activity of the RPE or the availability of the viral receptors on these cells.[120] The structural position of the RPE cells allowing them to have more surface area for interacting with the vectors delivered to the subretinal space may be an advantage over other cells, such as photoreceptor cells. In addition to AAV, HD-Ad vectors can also transduce RPE in mice with a high efficiency.[124] For gene therapy, it is important to have the target cells available when the vector is delivered. Although LCA2 is an early onset disease, retinal cells are well preserved in young patients,[125] thus making functional rescue possible. An additional advantage for RPE cells being targets is that there is no cell turnover in the RPE layer so that the therapeutic gene expression can be sustained.

Finally, right animal models have been tested in preclinical studies of LCA2 gene therapy.[125] For gene therapy targeting a particular disease, it is important to test the therapeutic approach in large animal models in addition to rodent models because there are major differences in organ anatomy, composition of cell types and gene function as well as tolerance to foreign substances. For example, mouse lungs do not have submucosal glands as in humans and have different epithelial compositions, such as with more Clara cells and less goblet cells in the large airways.[126–130] When the mouse CFTR gene is knocked out, there are no major lung disorders as in CF patients or other animal models for CF.[131,132] Mice can tolerate endotoxin, LPS, at least 1000 time better than humans[133,134] suggesting that there may be differences in tolerance to other substances such as gene therapy vectors. Before clinical tests in LCA2 patients, several research teams[135–139] demonstrated proof of concept for gene replacement therapy in a canine model that is homozygous for a null mutation in *RPE65* in addition to tests done in mice.[140] These animal studies provided evidence for long term functional correction without apparent adverse effects to support clinical applications.

What can be learned from eye field to improve lung gene therapy

The trajectory of the eye gene therapy research sheds new light into several areas of the lung gene therapy development. First of all, we need to select the right vector for each particular lung disease. If a disease requires long term therapeutic gene expression in airway epithelial cells, such as in CF, a highly efficient vector with a large DNA carrying capacity and capability to confer long term transgene expression, should be considered to avoid frequent readministration. Frequent readministration of gene therapy vectors to lung is unlikely to be tolerated, especially for CF

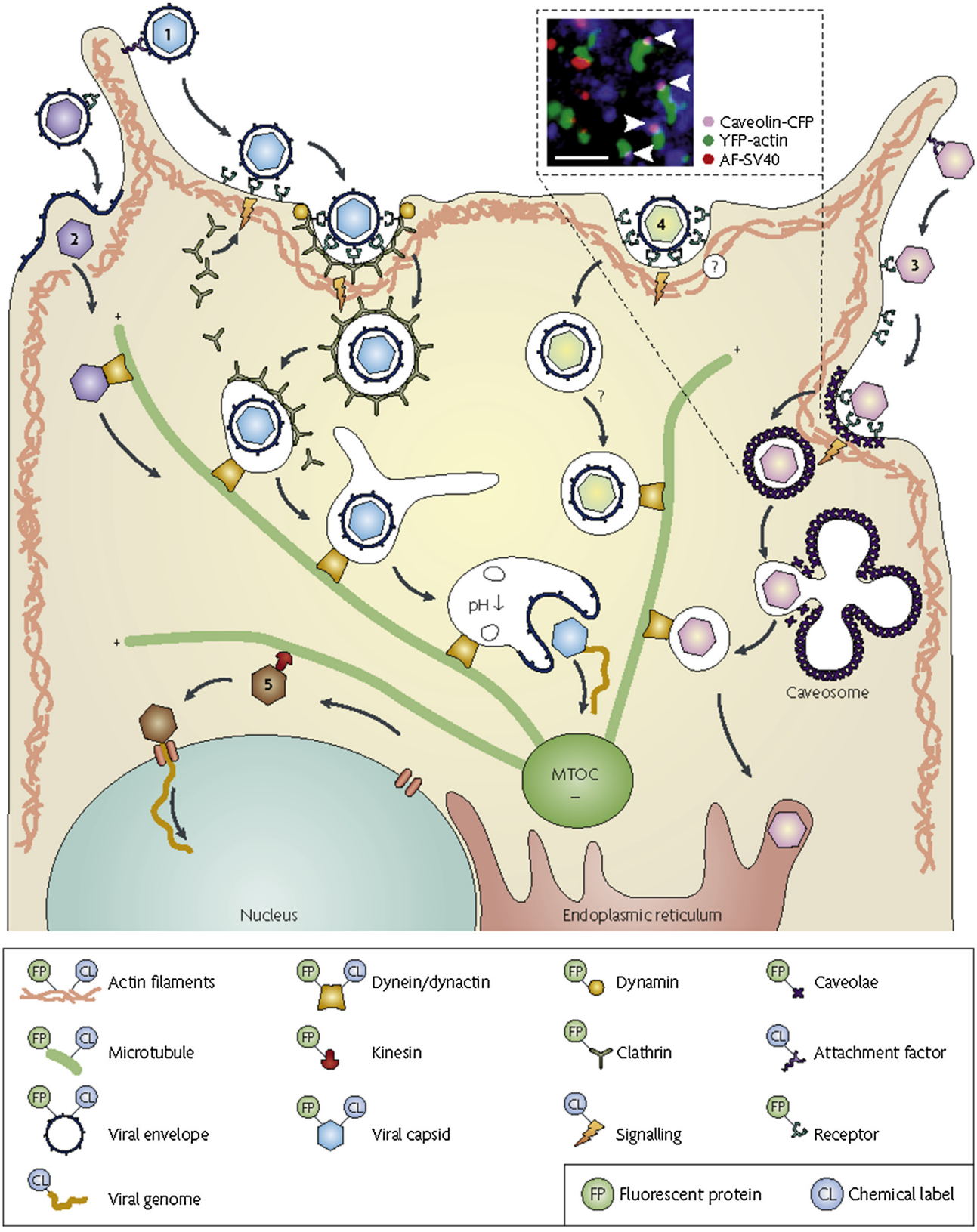

Figure 1 Schematic diagram showing viral entry and transport. This figure was reproduced from the review article by Brandenburg and Zhuang[141] with permission from Nature Publishing Group. The diagram summarizes viral entry and travel in mammalian cells. Viruses attach to the plasma membrane, surf on the cell surface or along the filopodia (**1**—**3**), and bind to specific receptors before entering the cell. Viruses can directly fuse with the plasma membrane (**2**). They also hijack endocytic pathways, including clathrin-dependent (**1**), caveolin-dependent (**3**) or clathrin- and caveolin-independent (**4**) pathways for internalization. After

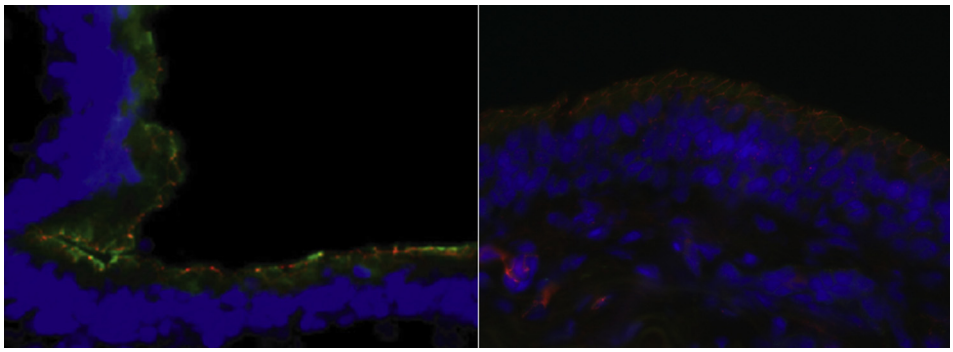

Figure 2 Expression of human CFTR protein in pig airway epithelial cells. An HD-Ad vector containing the human CFTR gene driven by the human cytokeratin 18 gene promoter was aerosolized to pig lungs. One week after delivery, lung tissues were taken and immunostaining was performed on tissue sections to visualize the human CFTR protein. Left panel shows the human CFTR protein located at the apical membrane of pig airway epithelial cells as the green immunofluorescence. The red immunofluorescence was from staining with an antibody against Zou-1(an epithelial tight junction molecule) marking the cells as airway epithelial cells. Right panel, a section of pig lung without vector transduction was immunostained in the same way as a negative control. The blue fluorescence indicates the nuclei stain with DAPI. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

lungs that are under inflammatory conditions. It is likely that a viral vector, instead of nonviral vector, will have a better chance to be successful in clinical applications since current nonviral vectors cannot confer long term therapeutic gene expression. Viruses are professional gene carriers and have evolved molecular mechanisms to enter a cell and inject the DNA into cell nuclei efficiently. As shown in Fig. 1, recent studies using single-virus tracking techniques in live cells revealed many of the molecular mechanisms that different viruses use to gain entry into mammalian cells.[141] All the advanced viral vectors do not have viral genes, but they maintain the ability to send their genomes efficiently into the nuclear compartment of target cells. In addition to the high efficiency in delivering genes to cells, the viral vector DNA is stable in transduced cells as long as host innate immune responses can be minimized. All these features of viral vectors are unmatched by nonviral vectors. Currently, the most hopeful viral vector for lung gene therapy, in our opinion, is the HD-Ad vector because its high efficiency in gene delivery has been demonstrated for transgene expression in mice, rabbits and pigs.[47–50,84]

Second, safe and efficient delivery methods are critical for a clinical success. If the delivery method is too invasive or too stressful, it will likely enhance the host innate immune responses which will reduce the therapeutic efficacy. If the method is not efficient, the therapeutic benefits will be reduced. Over the past decade, our group has been developing safe and efficient methods for vector delivery to airways of large animals. Using the AeroProbe™ catheter and the LABneb™ control system designed by Trudell Medical International (London Ontario, Canada), we have worked out conditions for efficient gene delivery to rabbit airways.[49] In these experiments the HD-Ad vector was formulated with 0.01% or 0.1% LPC in PBS and aerosolized to the rabbit airways through the AeroProbe™ catheter fitted into an endotracheal tube. More recently, we modified the method for efficient gene delivery to pig lungs.[50] Since we use pigs about 30 kg in weight, we insert the AeroProbe™ catheter into a bronchoscope and aerosolize the HD-Ad vectors formulated with 0.01% LPC in PBS into pig lungs. As shown in Fig. 2, extensive human CFTR expression was observed in pig lungs one week post vector delivery. This method should be easily adapted for clinical studies since most instruments, such as the bronchoscope and ventilation machine as well as drugs for anesthesia are the same as used in humans.

Third, since lung is an immune sensitive organ, it is important to avoid the host immune responses. Even by selecting the best vector and most safe and effective delivery method, we will still face the problem of the host immune responses to viral vectors. Strategies to overcome the host immune responses are discussed early in the

internalization and transport through the actin matrix, vesicles that contain virus are transported by dynein or dynactin along microtubules towards the microtubule organizing center (MTOC). This might include trafficking of viruses through endosomes, caveosomes or the endoplasmic reticulum, prior to the release of the virus into the cytoplasm. Capsids can also be transported by dynein or dynactin along microtubules. From the MTOC, capsids can be transported by kinesin towards the replication site of the nucleus (**5**). Some viruses release their genetic material into the cytosol whereas others transport their genomes into the nucleus. The key shows how the different components have been labeled previously. The inset panel shows the caveolin-mediated endocytosis of Simian virus 40 (SV40). The arrowheads indicate SV40-containing caveolae co-localized with actin tails. The dye-labeled SV40 particles are shown in red and the fluorescent protein-labeled caveolin and actin are in purple and green, respectively. Scale bar represents 3 μm. Inset panel reproduced with permission from the article by Pelkmans et al[142] (2002) American Association for the Advancement of Science. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

article. The most practical approaches to minimize the host immune responses are to reduce the vector dose as much as possible and to use transient immunosuppression to reduce the host immune responses.

Another critical consideration for improving lung gene therapy is to examine gene therapy vectors in appropriate preclinical animal models for the targeting disease. For gene therapy targeting CF lung disease, CF mouse models are not adequate enough for assessing the efficacy of gene therapy vectors and delivery methods, because of the major differences in lung biology between mice and humans as mentioned early. Recently, two excellent animal models (pig and ferret) for CF lung disease have been created by scientists at the University of Iowa.[131,132] Unlike CF mice, knocking out the *CFTR* gene in pigs and ferrets results in typical CF lung symptoms, including mucus plugging and spontaneous lung infection. These animal models will be very useful for testing gene therapy vectors and delivery methods for treating CF.

Finally, sustained therapeutic gene expression is required for a clinical success in lung gene therapy. The most important way to maintain therapeutic gene expression is to protect the transduced cells from attack by host immune cells. This point has been discussed already. Although viral vectors are stable in lung cells, lung epithelial cell turnover does occur naturally, which will reduce the therapeutic gene expression. There are two strategies that can be considered to overcome this problem. One strategy is to re-administer viral vectors once a year. This requires transient immunosuppression to avoid host immune responses to vector readministration. In mice, this has been demonstrated.[84] The second strategy is to integrate the therapeutic gene into progenitor cells of airway epithelium. This is a new, hot area of research and it will push gene therapy into a new stage for clinical applications.

Summary

Despite the early unsuccessful clinical trials of CF lung gene therapy, later decades of research has identified major challenges and made progress in overcoming these challenges. Analyzing the factors fundamental to the success in clinical studies of LCA2 gene therapy, we can learn a lot to improve lung gene therapy development. To address these challenges to lung gene therapy, we need to demonstrate that the host immune responses to viral vectors can be controlled effectively and sustained therapeutic gene expression can be maintained. The whole gene therapy field is still in its infancy. Even for eye gene therapy, there are still a lot of challenges, such as, efficient delivery of large genes to photoreceptor cells. With the molecular techniques under constant evolution, more and more successful gene therapy cases will emerge. It is matter of time that gene therapy will be a main approach to treating genetic diseases.

Conflict of interest

No authors have any conflict of interest to declare.

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