Utility of Adenoviral Vectors in Animal Models of Human Disease II: Genetic Disease

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1. Introduction

A disease at the forefront of gene therapy research over the last decade is cystic fibrosis (CF). This hereditary, single gene defect disease although affecting epithelial cells of multiple organs of the body results most often in mortality due to complications associated with the lung. CF lung disease has been considered as a prototypic disease state for "proof-of-concept" gene-therapy strategies. The lack of an alternative long-term treatment for the pulmonary manifestations of this disease, the accessibility of the lung via the airway lumen, and the fact that viruses known to infect the lung were being developed into nonreplicating gene transfer vectors led investigators to believe that administration of gene transfer vectors to the lung could potentially result in an effective treatment of this disease.

Shortly after the cloning of the gene responsible for CF pathophysiology, two groundbreaking observations made gene therapy for CF lung disease appear imminent. First, isolated epithelial cells cultured from the airway epithelium of CF patients could be phenotypically "corrected" by transferring into the cells the cDNA corresponding to the CF gene.[1–5] Second, adenoviral (Ad) vectors engineered to express the CF gene were administered to the airways of experimental animals and transgene expression observed in cells that were considered to require "correction."[6] These initial observations produced a flurry of scientific activity and excitement in both the gene therapy and CF scientific communities and within 3 years of these observations the first clinical trials describing successful Ad-mediated gene transfer to the airway epithelium of CF patients in vivo were reported.[7]

These promising early observations have unfortunately not withstood further investigation. After approximately 20 gene therapy clinical trials for CF lung disease (of which greater than 70% utilized Ad) it has become apparent that gene transfer to airway epithelium in vivo is not a simple procedure. The difficulty lies in the evolution of the respiratory epithelium as an effective barrier to invading pathogens entering the lung (e.g., viruses). The epithelium achieves this "barrier function" by presentation of a host of innate and cell-mediated immune systems, which for gene transfer vectors culminate in reduced uptake and expression of the transgene. In this chapter, I will describe the evidence that led investigators to believe that Ad would be useful in CF

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lung disease, why subsequently this simplistic approach failed and how increasing knowledge of lung biology and viral bioengineering has and will allow novel strategies to be tested. In light of this emphasis on basic research new strategies and models will need to be tested and successful demonstration of efficiency and safety will be required before we once again enter the clinic with Ad for CF lung disease gene therapy.

2. Pathophysiology of CF Lung Disease

CF is a multifaceted disease with major morbidity and mortality resulting from chronic decline of lung function. This disease is the most common fatal inherited disease in Caucasians with 1 in 2500 live births affected.[8] Although CF is most devastating to the lung (accounting for 90% of mortality), resulting in chronic repetitive infections, chronic obstructive pulmonary disease, and respiratory failure, other tissues are also affected including the liver, pancreas, the gastrointestinal tract, and the sweat glands. The abnormal CF gene (250 kb) encodes an mRNA of 6.5 kb which translates into an 180kD protein that has been extensively characterized as a cAMP-activated chloride ion channel, named the CF transmembrane conductance regulator (CFTR).[3,9] In the lung, CFTR is normally expressed in the respiratory epithelium and, although the specific functions of CFTR are complex, is predominately involved in maintenance of ionic homeostasis in this tissue. Over 900 different mutations of CFTR have now been reported resulting in a range of clinical manifestations and differing severity of the disease. However, 70% of these mutations are due to a three base-pair deletion leading to the absence of phenylalanine (F) at position 508 (ΔF508).[10] This particular mutation leads to misfolded CFTR being retained within the endoplasmic reticulum of cells so reducing CFTR function at the plasma membrane.[11] Currently, although the specific localization and functional capacity of ΔF508 CFTR in the different affected organs is a matter of controversial debate,[12] and other mutations can display partial CFTR function, for CF patients, expression of abnormal CFTR in the airway epithelium generally results in reduced chloride ion secretion, hyperabsorption of sodium ions, increased viscosity of airway secretions, impaired mucociliary clearance, chronic bacterial infection, bronchiectasis, and premature death.[8,13] Given that all of these effects are likely primary or secondary to loss of CFTR function, the most efficacious way to treat the broad range of effects would be to replace the defective CFTR gene with a normal copy. Gene therapy for CF lung disease therefore seeks to replace normal CFTR in the airway epithelial cells to hopefully "correct" lung epithelium function.

3. Trials and Tribulations with Ad Vectors for CF Lung Disease

Clinical gene transfer trials with CF patients investigating the safety and efficacy of gene transfer vectors (predominately adenoviral and liposomal vectors) have been

performed in both the US and UK. Details of these trials and the background preclinical studies have been comprehensively reviewed in a recent review.[14] Although preclinical data have been largely promising for lung-directed gene transfer, the trials performed to date have shown, at best, only partial "correction" (<20%) of the CF bioelectrical defect.[7,15–18] This relatively low degree of correction is most likely due to inefficient transfer of the CFTR cDNA to the airway epithelial cells, i.e., a low efficiency of gene transfer, and is most likely not sufficient to be of benefit to CF patients although long-term reversal of disease symptoms were not monitored in this studies.

The gene transfer efficiency required for physiological correction of CF lung disease has been a matter of recent debate. While Johnson and colleagues have shown that "correction" of ~10% of CF cells restores normal chloride secretory function to an epithelium, this degree of "correction" was insufficient to correct the hyperabsorption of sodium.[19] Since "correction" of the sodium defect is likely to be necessary for resolving CF lung disease, then transduction of a higher proportion of epithelial cells will be required.[1,20] Indeed, it has been suggested that greater than 80% of epithelial cells will have to express CFTR to restore the normal sodium transporting capabilities of the epithelium.[20] With regard to efficiency of gene expression on a per cell basis, it appears that CFTR is normally expressed at levels as low as 10 copies per cell and heterozygotes for the CF gene although only expressing 50% of normal CFTR show no disease symptoms. This suggests that the level of expression per cell does not need to be high in order to correct function. On the other hand, overexpression of CFTR has been shown to have deleterious effects on cell function although the effects on polarized airway epithelial cells are not documented.[21]

Issues of safety have arisen due to elicitation of inflammatory responses after Ad instillation in both animal and human experiments.[22–29] These effects have often been due to the large "loads" of vector that has been administered. A current hypothesis is that improvements in gene transfer efficiency may allow smaller quantities of Ad to be administered possibly circumventing much of the inflammatory response.

4. The Airway Epithelium: Cellular Targets for CF Gene Therapy

Airway epithelial cells are present throughout the conducting airways of the lung including the nasal, tracheal, bronchial, and bronchiolar regions. In the upper airway, the surface epithelium lines these structures and is continuous with the tubuloacinar submucosal mucus-secreting glands that invaginate from the airway surface. Airway epithelial cell-type composition is dependent both on the regional location and on the particular species studied and the reader is referred to comprehensive reviews that describe species-specific epithelial cell distribution in more detail.[30,31] The epithelial cell types present in the lung are numerous and include ciliated cells, mucus-secreting cells (goblet), serous cells, Clara cells, and basal cells. The cell types of the alveolar structures of the lung (alveolar Type I and II cells) are not thought to participate in the pathophysiology of CF lung disease. In human airways, the upper airway regions

(nasal, tracheal, bronchial) are composed of a pseudostratified mucociliary epithelium in which ciliated cells predominate with interspersed mucus-secreting goblet cells. The columnar cells overlie intermediary differentiated cells and basal cell layers which interface with the basement membrane. In addition, the human upper airways contain numerous submucosal glands. In the human lower airways, the bronchioles are lined with a simple cuboidal ciliated epithelium containing few mucus-secreting cells, no basal cells, and an absence of submucosal glands. An important morphological difference between the upper airways of human and mice, the most common animal model for investigating airway administration of gene transfer vectors, is that for the mouse upper airway (excluding the nasal cavity epithelium) the columnar cells are roughly an equal distribution of ciliated and Clara cells, compared to the predominance of ciliated cells in the human upper airway.[32] Clara cells are a nonciliated bronchiolar mucus-secreting cell type with distinct properties from ciliated cells. Clara cells although present in human airway are located only in the distal airways and only account for a fraction of the cells present in that region.[32]

The airway basal cells, or at least a subpopulation, are considered to be stem cell precursors for all other airway epithelial cells in the upper airway regions. Basal cells can differentiate into mucus or ciliated cell phenotypes.[33] Whereas mucus cells may also be able to differentiate into ciliated cells, the ciliated cell is considered as a terminally differentiated cell type.

An important observation with regard to experimental models of human airway epithelial cells is that isolation of upper airway epithelial cells for tissue culture purposes results initially in a predominately basal cell-like culture since isolated basal cells proliferate at a greater rate than isolated ciliated and mucus cells. Furthermore, for cells isolated from CF airways, the rate of proliferation of basal cells is even greater than that in normal airway probably reflecting responses to ongoing inflammatory processes.[34] Therefore, morphological differences need to be considered when designing models to study the interactions of gene transfer vectors with airway cells that are presumed to represent the cells in the lung that are exposed to lumenally delivered vectors.

Although CF is a disease of the respiratory epithelium, the exact airway region where CF lung disease initiates is still a matter of debate. It does appear that the first signs of pathology occur in the distal airways with findings of bronchiolitis and mucus plugging in the small airways and although the exact nature of how the CFTR defect initiates the disease is not totally resolved, it does appear that hydration of the periciliary fluid layer in these regions may be a major cause.[35,36] Currently, both the airway surface columnar cells lining the lumen of the small bronchiolar airways and the serous cells of the submucosal glands are candidates for the location for the onset of the disease. The cell type that is believed to be predominately involved in the onset of disease and therefore the specific target for gene transfer is the ciliated cell since these cells exhibit all of the ion- and fluid-transporting functions of CFTR and display abnormal function in patients with CF.[37] However, the submucosal gland serous cell is the highest CFTR-expressing cell type in the lung, suggesting that these cells may also be an important target for gene replacement.[38]

Ultimately it will be important to determine the location of disease initiation since it is likely that for a lumenal gene therapy to be successful, administration of vector

will have to occur early in the life of a CF patient. Later in life, when the airways posses overwhelming mucus plugging and associated bacterial colonization and inflammation, delivery of genes to the target cells will likely become restricted. The current thrust for CF gene therapy strategies is to deliver transgenes to target cell types before such other barriers to treatment are present.

5. Ad Vectors as Gene Transfer Vectors in the Lung

5.1 Animal Models for CF Airway Gene Transfer Studies

The generation of CF mouse models was an important step for understanding the physiology of CF disease. There have now been over 10 different mouse models produced displaying a range of CF-associated genetic mutations.[39] Although most of the models reflect the most common human mutation, either a complete gene knockout or a ΔF508 mutation of the mouse CFTR, other models with less common human mutations (e.g., G551D) have also been reported. The multiorgan pathophysiology associated with the different models has been recently reviewed.[39] Interestingly, although the gastrointestinal phenotype of CF mice is similar to that observed in CF patients, there is no CF-like pathology associated with the CF mouse lung. A comparison of bioelectrical measurements between CF human and CF murine airways has revealed that both species exhibit, relative to normals, hyperabsorption of sodium and an absent or reduced cAMP-induced chloride secretory response. However, it has been deduced that the ion transport defects in the CF mouse airway do not lead to CF-like lung pathology because CF murine airways compensate for the loss of CFTR activity by upregulating an alternative chloride secretory channel that is regulated by changes in intracellular calcium.[40] However, from a practical standpoint, the ability to measure the "bioelectrical defect" in CF mouse airways makes the model useful in terms of monitoring "bioelectrical correction" with gene transfer strategies, but the ability to monitor inhibition or reversal of CF-like pathology induced by transfer of normal CFTR is not possible in these current models. Therefore, the current gold standard for success in CF gene transfer to mouse lung in vivo is correction of the chloride (and sodium) ion transport defects.

Most gene delivery strategies to murine airways have focused on the epithelium of the nasal mucosa and trachea mainly because of accessibility to these regions but also because these regions are similar to those targeted for human CF gene therapy trials. Unfortunately, baseline bioelectric measurements of murine trachea indicate that these tissues do not display sodium hyperabsorption,[41] a key indicator for the human disease, and one that will likely need to be corrected for a treatment to be successful. In contrast, the epithelium of the CF mouse nasal cavity and freshly isolated CF murine nasal mucosa both display sodium hyperabsorption and reduced cAMP-induced chloride secretory activity providing an ideal model for study.[42] A further difficulty with murine airways (excluding nasal epithelium) is the large proportion of Clara cells that are present throughout the upper airway. The distribution of this cell type in the mouse may be misleading when comparing gene transfer efficiency between mouse and human upper airways (see later). The murine nasal mucosa however has few Clara

cells and exists as a pseudostratified mucociliary epithelium with a cell-type distribution similar to human nasal mucosa, again demonstrating the usefulness of this tissue for gene transfer studies.

Therefore, in conclusion, the CF murine models do not display spontaneous or induced pathological signs of human CF lung disease. However, CF murine airways do display bioelectric abnormalities associated with human CF and correction of these parameters by gene transfer can be measured both in vitro and in vivo. Given these considerations, since most clinical trials have focused on studying gene transfer to the nasal mucosa, the CF mouse nose is considered a good model for studying these strategies. In addition, since the epithelial cell-type distribution in human nose is similar to that of the human trachea and bronchus the nasal epithelium would appear a good model for a large proportion of the human airway epithelium.

5.2 Success and Limitations of Ad

5.2.1 Efficiency of Gene Transfer

5.2.1.1 Cell Types

The major cell types that support wild-type Ad infection in the lung are the epithelial cells of the respiratory mucosa lining the airway passages. The tropism of Ad to the respiratory epithelium established this vector as an obvious candidate for delivering transgenes to the lung. Indeed, Ad-mediated gene transfer to airway epithelial cells grown under standard culture conditions in vitro is highly efficient,[43,44] with cellular transduction efficiencies of 90–100% and when the transgene is CFTR, full correction of the spectrum of CF bioelectrical defects is obtained.[1] In contrast, observations from in vivo epithelial cell models derived from cartilaginous (upper airway) regions of the airways of rodents, nonhuman and human primates, show that transgenes are expressed after in vivo dosing in less than 20% of the surface epithelial cells, an efficiency unlikely to benefit to the defective physiology of a CF airway.[43,45] Although the efficiency of gene transfer can be enhanced by prolonging the contact time of Ad with the epithelium for 12–24 h, it is difficult to envision this strategy as being practical in a clinical scenario.[46,47] In the case of intralumenal delivery of Ad to the lower airways of rodents, gene transfer to 10–80% of the airway epithelial cells has been reported with apparently no cell-type-specific selectivity,[48,49] although, in a detailed study of Ad administration to murine airways, only the nonciliated bronchiolar epithelial cells (i.e., Clara cells) were observed to express transgenes.[50] Clara cells are not thought to require correction in the CF lung and this observation casts a shadow on the use of murine airway epithelium as a model for Ad-mediated gene transfer to the human airway epithelium where Clara cells are less common. Therefore, it appears that lumenal facing well-differentiated (WD) airway epithelial cells in vivo, at least in the upper airway regions, are resistant to efficient Ad-mediated gene transfer.

How can we envision that the airway epithelial cells facing the lumen of the airway are not transduced by Ad given the large body of clinical data that show that these cells are targeted in wild-type infections? In a series of studies using human tracheal

epithelium ex vivo and murine trachea in vivo it was discovered that injury to the epithelium by physical abrasion of the columnar cells revealed epithelial cell types that are susceptible to efficient Ad transduction, as depicted in Figure 1.[43,51,52]

This cell-type-specific variable efficiency led to the finding that underlying basal cell-like cells were efficiently transduced by Ad. These cells, as precursors to columnar cells could once transduced, over time proliferate and differentiate into transgene-expressing columnar epithelial cells. Since the epithelial basal cells are probably stimulated to proliferate and differentiate upon injury these susceptible cells were described as "basal cell-like cells" or the poorly differentiated (PD) airway epithelial cells, i.e., injured or regenerating cells, and this cellular phenotype is similar to that displayed by airway epithelial cells grown on plastic that are also highly transducible by Ad.[43,44]

One consideration when comparing wild-type Ad infection to Ad vectors is that the latter rely on delivering many virus particles to a target tissue whereas wild-type Ad needs only access to a small number of cells from which Ad replication and spread can then occur. Therefore, wild-type Ad may be able to take advantage of regions of the airway in which epithelium integrity is compromised or injured. Initiation of wild-type infection in injured regions would then be able to spread as a "basal cellitus" effectively beneath the resistant superficial columnar cells.

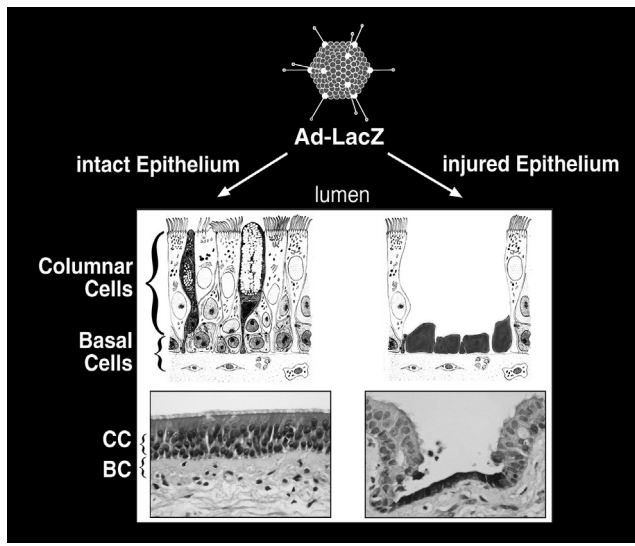

Figure 1 Increased susceptibility of injured epithelium to Ad-mediated gene transfer. Exposure of Ad vectors to intact pseudostratified columnar cells (CC) results in low gene transfer efficiency. Physical abrasion of columnar cells before Ad exposure results in efficient gene transfer to the underlying basal cells (BC). Upper figures show schematic of intact and injured pseudostratified columnar respiratory epithelium and lower figures are intact and abraded human tracheal epithelium exposed to Ad-LacZ ex vivo.
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5.2.1.2 Receptors

The differences in the gene transfer efficiencies for the two cellular phenotypes of airway epithelial cells, the PD and WD columnar cells, suggest that an early step in the virus–cell interaction is deficient for the WD cells. Ad enters epithelial cells by a two-step process: (1) initial attachment of the viral fiber-knob protein to a high affinity receptor, the human coxsackie B and Ad 2 and 5 receptor (hCAR)[53,54] and (2) translocation of the virus into the cell cytoplasm via clathrin-coated pit internalization processes, in part mediated by an interaction of the viral penton base with $\alpha_v\beta_{3/5}$ integrins.[55]

Since quantitative studies of the interactions of Ad with the airway epithelium in vivo are difficult and prone to considerable variation, specialized cell culture models have been generated to aid characterization of the interaction of Ad with both PD and WD cell types. These models have been shown by a number of groups to reproduce[1]: the WD (ciliated) and PD cellular phenotypes and[2] the relative resistance of WD and permissiveness of PD cells to Ad-mediated gene transfer as observed in vivo.[56,57] In addition, although these models were originally generated to ask specific questions regarding gene transfer strategies, they have subsequently become valuable in a whole series of studies were quantitative and qualitative measurement of events in the airway epithelium are difficult to perform in vivo.[35,36,58–63]

Using these models of human airway epithelium, immunofluorescent and functional analyses of the interactions of Ad with human airway epithelial cells have shown that decreased gene transfer efficiency to WD compared to PD cultures is due to limited entry (penetration) of Ad across the apical membrane of WD cultures which reflects a reduced specific Ad attachment due to the absence of hCAR and $\alpha_v\beta_{3/5}$ integrins from the apical surface. Interestingly, columnar cells and basal cell-like cells express all the necessary receptors to efficiently allow Ad entry but for columnar cells these processes are segregated and limited to the basolateral membranes as depicted in Figure 2. In these culture model systems, Ad has been shown to efficiently transduce epithelial cells when applied to the basolateral epithelial surfaces.[56,57,64,65]

It appears that the most significant Ad–cell interaction in determining efficiency is that of the Ad–hCAR interaction. Many cell types usually resistant to Ad infection have been shown to be efficiently transduced after heterologous expression of hCAR, although the status of integrin expression in these cell types is not always clear.[56,66] Earlier observations had suggested that inefficient Ad-mediated gene transfer to a bronchial xenograft model of human *in vivo*-like ciliated airway epithelial cells reflected the absence of $\alpha_v\beta_{3/5}$ integrins from the lumenal membrane of the epithelium.[65] However, $\alpha_v\beta_{3/5}$ integrins may not alone account for decrements in gene transfer efficiency. In support of this hypothesis, Ad mutants lacking penton base RGD sequences (normally required for Ad–$\alpha_v\beta_{3/5}$ integrin interactions) are able to efficiently transduce human epithelial cells although *the rate of* internalization is reduced.[67] In addition, in a β_5 integrin knockout mouse model, airway epithelial cells were equally susceptible to Ad-mediated gene transfer as were wild-type airway cells,[68] again suggesting that $\alpha_v\beta_{3/5}$ integrins may be facilitative rather than necessary for efficient vector entry into the cell.

These observations are important for the design of targeted vectors that attempt to increase gene transfer efficiency to normally unsusceptible cell types.[69,70] Retargeted vectors attached via nonspecific interactions or to noninternalizing receptors

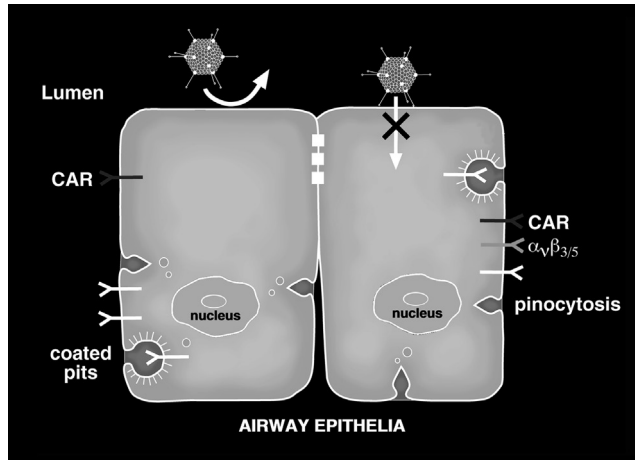

Figure 2 Schematic of polarized epithelial cells displaying resistance of the lumenal surface to adenovirus attachment and entry. The receptors required for Ad entry are located on basolateral membranes and excluded from the apical membrane by the tight junctional complexes.
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will probably depend on nonspecific uptake pathways to enter cells and while this approach is useful for PD cells in vitro, increasing Ad attachment to WD cultures that do not exhibit these pathways is unlikely to improve gene transfer efficiency.[56]

5.2.1.3 The Innate Immune System of the Lung

Despite the progress on the cell biological aspects of vector–cell interactions, surprisingly little attention has been devoted to another fundamental component of innate airway defense that will almost certainly impact on the efficiency of lumenally delivered vectors, the barrier/shielding function of epithelial surfaces by the carbohydrate-rich cell surface glycocalyx. Expression of hCAR, engineered to be expressed at the apical surface of polarized epithelia by incorporation of a glycosylphosphatidylinositol linker (GPI-CAR), identified glycocalyx components as barriers for lumenally applied Ad accessing these receptors as depicted in Figure 3.[71] Electron micrographs demonstrate a "fuzzy coat" on the cell surface,[72,73] termed the glycocalyx, and on epithelial cell apical surfaces it is comprised of several families of carbohydrate-rich molecules, including glycoproteins (most notably the mucins), proteoglycans, and glycolipids. Glycoconjugates are variably modified by sialic acid and sulfate that impart a strong anionic charge to the cell surface. A major component of the airway glycocalyx will likely be the "tethered" mucins and the molecular biologic advances in the mucin field have revealed that the MUC1 and MUC4 are highly expressed in airway epithelium and have transmembrane anchoring (tethering) domains.[74–82] With respect to airway gene transfer, sialoglycoconjugates (including MUC1) comprising the glycocalyx on MDCK cells appear to inhibit Ad gene transfer, presumably due, in part, to

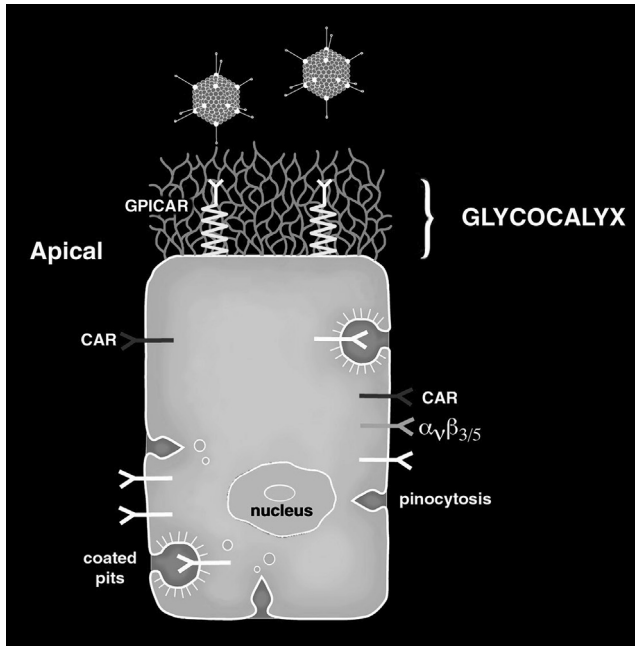

Figure 3 Schematic of polarized epithelial cell expressing reengineered Ad receptors at the apical surface. These studies revealed that the apical surface glycocalyx was an effective barrier to Ad-accessing receptors located on the apical surface.

their negative charge since neuraminidase treatment to selectively remove sialic acid can circumvent the glycocalyx barrier in these cell types.[83,84] Although apical surface mucins expressed on WD cells are also restrictive to Ad, neuraminidase alone is not sufficient to allow Ad permeation through the glycocalyx, and more stringent proteolytic treatments are required.[115,116] Presumably, the mucins, including both tethered and secreted mucins, may also be present in the mucus layer in the airway and may act as false attachment sites for Ad thus effectively reducing the amount of Ad that ultimately reaches the epithelial surface. The reported rheological properties of CF mucus producing a more viscous, more dehydrated, and immobile barrier suggest that this obstacle to gene transfer will be even more pronounced in the CF lung.

Other components of the innate immune system, not studied in specialized cell culture models, may also have barrier effects on gene transfer efficiency. Ordinarily, such barriers occur in the lung as primary defense mechanisms and may be aggravated in the CF lung where airway lumens are inflamed. For example, alveolar macrophages have been reported to sequester up to 70% of Ad genomes within 24 h following tracheal administration to mouse airways.[85] In a mouse nasal model of CF lung bacterial colonization, Pseudomonas infection (PA01 strain) was shown to inhibit Ad gene transfer by 10-fold relative to noninfected control nasal airways.[86]

In conclusion, there appears to be numerous potential barriers to Ad gene transfer in the lung especially in the CF lung that exhibits an overactive inflammatory milieu,

and strategies to circumvent these barriers will likely need to be designed. However, even if all of these barriers are circumvented, the major cause of low-efficiency gene transfer is the lack of entry of Ad into the target cells. Strategies to improve the transduction efficiency will therefore be crucial to proving that the concept of gene transfer into the airway may actually be a feasible one.

In summary, human WD cultures are resistant to Ad-mediated gene transfer because of decreased specific attachment sites and reduced nonspecific entry paths that can internalize a fraction of a large vector load typical of CF gene therapy protocols using Ad. To circumvent the inefficiency of Ad-mediated gene transfer to the respiratory epithelium, either alterations of the host will be required, i.e., ability to access Ad receptors expressed on basolateral cell surfaces or Ad will require retargeting to receptor types that are present in sufficient number on the airway epithelial lumenal surface which allow for efficient uptake of Ad into the cell.

5.2.2 Safety

Initial attempts to improve efficiency of Ad gene transfer to the airway epithelium in vivo have mostly involved delivery of greater doses of Ad to the lung. These doses can represent a relatively large protein load and the subsequent gene expression (even in nonepithelial cells) can produce an unusually high level of transgene in an organ that is designed for monitoring invading pathogen assaults. It is therefore not surprising that inflammatory and immune responses are observed when Ad is delivered to the lung and numerous studies have reported Ad-induced lung inflammation. In general, Ad induces an acute nonspecific mixed cellular inflammatory response and a late-specific, dose-related, lymphocyte-predominant, cell-mediated immune response in all species so far studied.[22,23,25–27,29,87–90] The acute response is nonspecific and likely induced by cytokine production in response to the protein load. It has also been suggested that neurogenic inflammation results after administration of Ad in rat airways, an effect shown to be partially due to vector gene expression but also to the viral proteins of the capsid coat.[91] The later, specific immune response to Ad is mediated by major histocompatibility complex class I-restricted cytotoxic (CD8) T lymphocytes directed against viral gene products and transgene proteins in expressing cells. The subsequent destruction of these cells leads to loss of persistence of transgene expression and so reduces efficiency of gene transfer.[28,92,93] The use of second-generation and high-capacity "gutless" vectors aims to limit the amount of viral gene expression to decrease the effects of this late immune response and these approaches are the topics of other chapters.[94,95]

In addition to cellular immune responses, Ad also elicits humoral immune responses with the production of mucosal and neutralizing antibodies.[25,87,90,96–98] These responses have been shown to be against the viral capsid proteins and are secondary to a helper (CD4+) T lymphocyte response. The production of such an antibody response results in neutralization of subsequent readministration of Ad, resulting in loss of gene transfer assuming the same Ad serotype is used (see later).

Therefore, in addition to the innate immunity of the lung (receptor localization, glycocalyx, macrophages, and mucus) reducing the efficiency of gene transfer, the

cellular and humoral immune systems also respond to Ad delivery into the airway and as a result reduce the efficiency of gene transfer and the persistence of expression in the target epithelial cells.

5.3 Overcoming the Limitations of Ad

5.3.1 Efficiency

The localization of entry pathways for Ad to the basolateral surfaces of airway epithelial cells suggests that a delivery strategy to access these regions would be beneficial to improving gene transfer efficiency. This approach may also allow targeting of the epithelial stem cells (basal cells) resulting in transgene expression in the lung for the lifetime of the individual. This is an important consideration for gene transfer to the airway epithelium since fully differentiated lumenal facing cells (e.g., ciliated cells) have a relatively short lifetime in the order of 40–100 days and targeting these cell types specifically will require regular readministration of vectors.

Access to basal cells/basolateral surfaces may possibly be achieved by intravenous administration of vectors if penetration of the blood vessel wall, the connective tissue, and the basal lamina of the basement membrane were achievable. Unfortunately, studies that have attempted intravenous delivery strategies have not been successful since vectors do not appear to gain access to sufficient lung epithelial cells to make this approach feasible.[99–102] Barrier functions provided by the blood vessel endothelial cells and connective tissue surrounding the airway passages seem impenetrable by Ad. Indeed, the particle permeability of the basal lamina alone is thought to exclude inert particles of greater than 10 nm, which would certainly be restrictive to particles the size of Ad (100 nm). In an in vivo experimental mouse model where Ad was externally administered directly to the tracheal basement membrane, efficient gene transfer to the connective tissue fibroblasts adjacent to the basement membrane was observed without gene transfer to the epithelial cells of the juxtaposed epithelium.[51]

To date, two main strategies to improve intralumenal delivery of Ad vectors have been focused on. One approach is to access the basolateral surfaces of the epithelial cells by disruption of the epithelial "tight" junctions, and the other is to retarget Ad vectors to nonviral receptors that are present on the apical surface of lumenal epithelial cells that allow for entry of Ad into these cell types. Retargeting has so far been achieved by chemically, immunologically, or genetically modifying the Ad capsid coat by incorporating new receptor ligands that can target candidate receptors.

5.3.1.1 Modification of the Host by Opening Tight Junctions

Epithelial cell "tight" junctions (zonula occludens) are collar-like structures composed of a diverse number of proteins that separate the apical and basolateral domains of the lumenal columnar epithelial cells. As well as functioning as a restrictive barrier to mixing of apical and basolateral membrane components, these intercellular junctions limit the transepithelial transport of solutes across the epithelium. A number of disease states have been shown to alter tight junction permeability (e.g., asthma), and reagents to increase the permeability of the junction are available. The key to

successful disruption of tight junctions to allow Ad access to basolateral epithelial cell surfaces will be to use a reagent that open tight junctions sufficiently for Ad to pass through but that is rapidly reversible to limit the passage of other lumenal contents (e.g., bacteria) or serosal fluid into the airway lumen.

A property exploited for this purpose is the calcium ion dependency of the structural integrity of the junction. Walters et al., have successfully shown that treatment of the apical surface of human WD airway cells with the calcium chelator EGTA or hypotonic solutions (e.g., water) allow for improvements in Ad-mediated gene transfer presumably by allowing Ad access to basolateral receptors.[64,103] The slow reversibility of this effect however is problematic, tight junction reformation takes a least a couple of hours, a time period that would be unacceptable in a clinical setting. In vivo studies in mouse airways have confirmed that these treatments improve gene transfer efficiency although parameters of safety were not assessed fully.[62,104]

More specific reagents are available for studying tight junction permeability and the effect on Ad gene transfer. Parsons et al. used a detergent, polidocanol, in murine airways in vivo to enhance Ad-mediated gene transfer, an effect shown to be due to the ability of this reagent to transiently open tight junctions.[86] The short-chain fatty acid, sodium caprate, has also been used to increase Ad-mediated gene transfer to human WD cultures and results in full correction of CF cultures when AdCFTR is subsequently applied to the apical surface. This result is exciting since the effect is rapidly reversible effect and has previously been used clinically for enhancing pharmaceutics absorption across the GI tract, again presumably by an effect on tight junctional permeability.

These studies although fraught with inherent safety issues are beginning to establish that this strategy for delivering transgenes to the lung may be a viable option. The possibility of targeting the basal stem cells by this procedure is reason enough to continue pursuing the usefulness of these strategies.

5.3.1.2 Targeted Ad to Increase Gene Transfer Efficiency

Targeted Ad directed against specific receptors has been used to successfully transduce cell types that are usually refractory to Ad infection. The epidermal growth factor receptor, stem cell factor receptor, fibroblast growth factor receptor, α_V integrins, and T cell receptors (CD3) have all been used as surrogate receptors for Ad entry in a variety of cell types.[105–108] Given the lack of Ad receptors at the apical surface of lumenal airway epithelial cells, a retargeting strategy to receptors known to present on the airway lumen may allow for gene transfer efficiency to be improved. However, a successful targeting strategy to the lung epithelium will require the identification of target molecules that allow for attachment and internalization of AdV across the apical membrane of columnar airway epithelial cells.

The identification of target receptors to which to redirect Ad tropism on the lumen of airway epithelium is difficult because most receptors and entry mechanisms occur on the basolateral surfaces of the cells. Certain members of a specific 7-transmembrane-spanning G-protein-coupled receptor family (i.e., P2Y2 purinoceptors, B2 kinin receptors, and adenosine type 2b receptors) have been identified as putative utile target receptors for redirecting Ad tropism to the surface epithelium of the lung. These

receptors have been shown to be present on the lumenal surface of human airway epithelium and internalized into clathrin-coated pits when activated by their respective agonists.[109] The utilization of clathrin-coated pit internalization pathways for native Ad receptors suggests that the G-protein-coupled receptors may provide an ideal surrogate entry pathway for Ad. The high potency of P2Y2 agonists (e.g., ATP, UTP) combined with the low affinity of these agonists for the receptor suggests that the P2Y2 purinoceptors are abundant in number on the lumenal surface of the human respiratory epithelium.[110] Since pharmacological activation of airway epithelial P2Y2 receptors does not result in untoward effects in human airways, this receptor is an ideal target receptor to redirect Ad tropism. However, since the only available ligands for this receptor are low-affinity, small organic molecules, certain technical difficulties are associated with conjugating these molecules to Ad. Other receptor types suitable for Ad retargeting exist on the airway although specific retargeting data for Ad are lacking. The urokinase plasminogen activator receptor, uPA-R and the SEC-2 receptor have also been proposed as target receptors for Ad and adeno-associated virus (AAV), respectively.[111,112]

5.3.1.2.1 Immunologically Modified Targeted Vectors One immunological approach for targeting gene transfer vectors is using bispecific antibodies linking Ad directly to non-Ad receptor types present on the cell surface.[107,113] For example, chemically conjugated antibodies, one of which is directed against an epitope-tagged Ad coat protein and the other against α_V integrin membrane proteins have been reported to increase gene transfer efficiency by seven- to ninefold compared to that of nonmodified Ad, indicating that increased Ad attachment results in increased gene transfer efficiency.[113] In a similar approach, Ad was retargeted to nonviral receptor types in conjunction with ablation of the natural Ad tropism using an anti-fiber-knob protein antibody conjugated to folate.[114] Folate-conjugated antibody was the ligand of choice since the folate receptor is reported to be upregulated on the surface of malignant cells, thus providing a targeted vector for a variety of cancers. Retargeting Ad to cells expressing folate receptors was shown to be specific and successful with significant increases in gene transfer efficiency.

As "proof-of-concept" studies, an hemagglutin (HA) epitope-tagged P2Y2 receptor expressed at the apical surface of human WD cultures and targeted with bispecific antibodies consisting of antibodies to Ad fiber-knob protein/HA tag has been shown to facilitate Ad entry into these cell types, shown schematically in Figure 4.[115,116] This effect is enhanced by coadministration of exogenous ATP to activate the receptor, an effect that can be reduced by desensitization of the P2Y2 receptors prior to addition of targeted Ad. Importantly, the apical surfaces of the HA-tagged P2Y2-expressing cultures required a brief exposure to specific proteases before targeting was effective suggesting that the apical surface glycocalyx hindered access of the targeted vector to the target receptors.[115] This approach also relied on the expression of an HA-tagged receptor that may be overexpressed relative to the endogenously expressed P2Y2 receptors in the culture system. The number of target receptors and the affinity of the targeting ligand are both likely to be critical parameters for the success of such a targeting strategy.

5.3.1.2.2 Chemically Modified Targeted Vectors Since antibodies to the external domains of P2Y2 receptors are not currently available, a strategy to target Ad to the endogenous P2Y2 receptor was to chemically conjugate small molecule agonists (UTP)

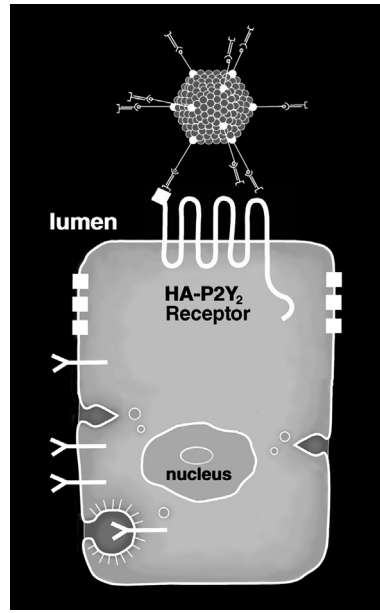

Figure 4 Schematic of targeting strategy used to redirect Ad tropism to P2Y2 receptors on the apical surface of human airway epithelial cells. Bispecific antibodies against the virus and the receptor were used as a targeting link and activation of the receptor results in receptor internalization and entry of Ad with subsequent gene transfer.

to the proteins of the Ad capsid coat. Using chemically reactive biotin derivatives, biotin was coupled to the Ad capsid coat predominately via hexon protein. This strategy is reported to couple 2–300 biotins to a single Ad particle and does not significantly alter the fiber-knob–hCAR interaction. By using commercially available biotin-linked UTP in combination with streptavidin as a "bridge" linking biotin-Ad to biotin-UTP, these molecular conjugates were shown to mediate gene transfer by an interaction specifically with endogenous P2Y2 receptors on the apical surface of WD cultures.[109] Again, the effectiveness of this approach was reduced by the presence of apical surface glycocalyx since gene transfer was only observed in cultures pretreated with agents that degrade this barrier. Regardless, gene transfer efficiency using these conjugates was still inefficient, probably due to the clumsiness of the "streptavidin bridge" and the low affinity of UTP for this receptor. Future experiments using this targeting strategy will require the identification of receptor agonists with higher affinity in addition to improved methods to directly couple the agonist ligands to the Ad capsid coat.

Another method for chemically conjugating receptor ligands to Ad is by the use of polyethylene glycol (PEG) that can be covalently linked directly to the Ad capsid coat. A number of groups have now shown that PEG-conjugated viruses can be used to target Ad.[111,117] For example, Ad conjugated to a 12-amino acid peptide, identified from phage display assays on the apical surfaces of human WD cultures, resulted in a 10-fold increase in gene transfer efficiency to these cell types.[117] Similarly, Ad conjugated via PEG to a peptide that binds to uPA-R has been shown to target Ad to this

receptor type and enhance gene transfer to polarized airway epithelia.[111] An additional bonus of using PEG-conjugated Ad is that these vectors appear to be less immunogenic that non-PEG-conjugated Ad. This effect is due to the masking of antigenic Ad capsid proteins (mainly hexon) from neutralizing antibodies.[118]

5.3.1.2.3 Genetically Modified Targeted Vectors The ideal targeted vector would be one in which the target ligand could be incorporated into the capsid coat with minimal disruption of the physical and biological properties of Ad. For targeting strategies in which a peptide ligand is used, the most desirable method would be to generate an Ad vector genetically modified to express a functional peptide ligand on the viral surface. Such an approach for targeting vectors has been reported, where the Ad viral coat has been genetically modified to express multiple polylysine groups on the C-terminus of the Ad fiber-knob protein.[70] This redirects Ad tropism to heparan sulfate moieties that are present on the surfaces of most mammalian cells. With certain nonepithelial cell types, which lack hCAR, this modified vector has been shown to increase gene transfer efficiency from 10- to 300-fold in comparison to nonmodified Ad. However, the modified vector will likely not be useful for gene transfer to the airway epithelium since heparan sulfate is not expressed at the apical surface of airway epithelial cells.[119] Targeted Ad in which the fiber-knob protein (responsible for Ad attachment to the hCAR) has been modified to express novel ligands that can interact with other receptor types are being developed and the feasibility of this approach has now been reported by a number of groups.[106,120,121] A recent development in this type of approach was reported by Krasnykh et al.,[122] who hypothesized that the HI loop region of the fiber-knob structure can withstand the insertion of heterologous peptide sequences without significantly compromising the tertiary structure of the fiber-knob protein nor the production and infectivity of the modified Ad. These authors incorporated the FLAG octapeptide marker sequence into the HI loop region and were able to produce functional Ad. Importantly, they also showed that the sequence contained within intact virions was accessible to a FLAG-specific antibody suggesting that sequences inserted into this region are capable of interacting with other target substrates such as cell surface receptors.

A significant technical advance in Ad-targeting strategies evolved from studies that deduced the viral sequences in fiber-knob protein that interact with hCAR. Genetic ablation of these sequences from Ad vectors led to the generation of Ad that no longer binds to hCAR and no longer transduces cells that are permissive for normal Ad transduction.[123] The broad cellular tropism of Ad vectors can now be reduced and by the addition of targeting moieties to these Ad vectors specific cell-type targeting is possible. Reduced Ad interactions with nontarget cells will lessen the potential for adverse effects with these vectors. In the lung, however, the significance of natural tropism ablation is unclear since most of the epithelial cells targeted with delivery strategies do not express Ad receptors at the lumenal surface. However, the loss of transduction to other cell types that may interact with Ad delivered to the lung (e.g., macrophages, dendritic cells) may benefit from the hCAR-binding ablation mutant.

Recent developments in immunologically, chemically, and genetically modified targeted Ad suggest that "designer" gene transfer vectors will one day be available. Although Ad vectors, in their present form, may not be ideal for a number of gene

transfer target tissues, notably the lung epithelium, this vector clearly remains at the forefront of gene therapy research since it is still one of the most efficacious gene transfer vectors available and will continue to be useful at least in "proof-of-concept" studies.

5.3.1.2.4 Screening with Other Adenoviral Subtypes Although over 51 different serotypes of wild-type Ad exist, the predominant serotypes used for gene transfer experiments are serotypes 2 and 5. The reason for this is largely historical since these two serotypes have been extensively studied over the last 30 years and understanding of the viral genome has allowed the manipulations necessary to evolve these viruses into gene transfer vectors. With regard to the airway epithelium, other serotypes have been suggested to be efficacious at delivering transgenes to human WD cultures. Serotypes 17 and 12 have been shown to bind/deliver transgenes 10-fold over Ad2 vectors.[124] However as of yet no conclusive results have been presented that suggest that the improvements warrant future investigations with these vectors. One approach to determining if any of the other serotypes may be more efficacious in the lung epithelium could be envisioned using a recently reported system of generating an Ad5 capsid expressing fiber proteins from the other serotypes.[125] This system was used to screen vascular endothelial and smooth muscle cells and the efficiency of gene transfer compared against the efficiency of gene transfer with Ad5. This screening procedure identified Ad5 with Ad16 fibers as being significantly more efficient at gene transfer than Ad5 in these particular cell types. It will be of interest to screen these serotypes on human WD cultures relative to Ad5 to determine whether other Ad serotypes may be of benefit to airway epithelial cell gene transfer. A serotype which may be of particular interest is Ad37, since it has been reported that Ad37 utilizes sialic acid residues that are present on the extracellular surfaces of most cells.[126] An abundance of sialic acid residues on the lumenal surface of airway epithelial cells as components of glycoconjugates may allow for improved gene transfer. Whether attachment of Ad37 to sialic acid residues located on the airway lumen leads to efficient entry and gene transfer awaits further study.

5.3.1.2.5 Other Methods to Increase Gene Transfer Efficiency Nonspecific methods to enhance Ad-mediated gene transfer to airway epithelial cells have been reported.[127,128] Calcium phosphate coprecipitation has been used to precipitate aggregates of Ad and other vectors to increase gene transfer to airway epithelia both in vitro and in vivo. It has been suggested that in in vivo these aggregates increase the rate of nonspecific endocytosis of Ad across the apical membrane of polarized epithelial cells. The possible effects of this technique on cellular and paracellular permeability have not been investigated.

Another method to improve both the delivery and efficiency of Ad to the lung epithelium in vivo is using the inert perfluorochemicals (PFCs). These compounds are liquid in nature but due to high-oxygen saturation capacities can be instilled into the lung for periods of time with maintenance of passive oxygen diffusion. Several studies have now shown that administration of gene transfer vectors (including Ad) with PFC results in increased gene transfer to rodent and nonhuman primate lungs.[129–131] The improvements in gene transfer are predominately localized to the alveolar regions with only modest improvements in the efficiency of gene transfer to the respiratory epithelium. The exact mechanism by which PFCs produce these effects remains to

be determined but may be due to prolonged contact time for the vector on the cells and reduced ingestion of Ad by macrophages and/or due to some nonspecific effect on the paracellular permeability. Nonetheless, this method provides an example of a new strategy to deliver transgenes to the lung without the need for direct instillation or aerosolization, which are both inefficient methods for airway epithelium delivery.

5.3.2 Safety

Strategies that improve gene transfer efficiency, as described above, will allow for lower doses of Ad to be administered to the lung. This achievement alone will be beneficial in reducing the inflammatory responses seen with Ad administration. However, attempts are also been made to reduce the inflammation produced by expression of viral genes that produce the cell-mediated immune responses described above. The identification of specific viral genes that initiate or amplify the immune response has led to the reengineering of Ad vectors to ablate the specific gene expression. For example, vectors deleted of E2a and E4 have been reported to display reduced immune responses and improve persistence of transgene expression.[92,93] The ultimate vector is one that contains no viral genes and the high-capacity "gutless" vectors have been generated and appear to blunt the immune response considerably.[132–134] In contrast, several viral genes have been identified that have evolved to subvert the immune response and the inclusion of these genes into new vectors may be desirable (e.g., E3).[135]

Strategies to circumvent the humoral immune response are also been considered. Since this arm of the immune system results in the inability of readministration of specific Ad serotypes, serotype switching has been proposed as a method to allow repeat administration. Indeed, Ad5 administration but not Ad4 or Ad30 has been reported to prevent the gene transfer obtained with subsequent Ad5 administration to the lung.[97] However, in addition to this being a somewhat limited procedure, it is not yet clear whether these different serotypes are as inefficient for gene transfer to the airway epithelium as Ad5. Transient immunosuppression has also been suggested to reduce the inhibitory effects of neutralizing antibodies. Intratracheal administration of immunosuppressive factors (IL-12, interferon gamma, antibodies to CD40, corticosteroids, and cyclophosphamide) at the time of vector administration have all shown a reduction in generation of neutralizing antibodies.[136–140] The longer-term effects of administering these factors to lung have not been reported. Finally, covalent conjugation of PEG to the Ad capsid coat that permits addition of targeting moieties is also a strategy for the virus to elude neutralizing antibodies by masking capsid coat proteins, especially hexon protein. Although PEGylation of Ad leads to some loss of viral titer and aggregation the ability of this procedure to develop targeted vectors combined with reduction in immune response makes this a promising method for future study.[118]

6. Other Vectors

The focus of this review has been on Ad vectors for use in CF lung disease. However, a number of other vectors have been suggested as candidates for CF lung gene transfer

vectors. AAV, retrovirus, lentivirus, and liposomal vectors have all shown promise in preclinical studies in the lung and some have been tested in clinical trials. The general observation is that all of these vectors, like Ad, do not appear to display the efficiency of gene transfer in WD airway epithelial cells as they do in nonpolarized cells suggesting that these vectors confront similar barriers in the airways as do Ad vectors. Strategies to improve gene transfer efficiency for these other vectors have followed the progression of experiments with Ad, i.e., tight junction modulation, targeting, serotype switching, and immune response reduction, and all have been shown as for Ad to improve efficiency to some degree. Whether efficiency can ever be improved to a point that shows efficacy in the lungs of CF patients remains to be determined. Meanwhile, other viruses (Sendai virus[141] and lentiviruses pseudotyped with filovirus coat proteins[142]) may show promise for gene delivery to the airway and preliminary reports suggest that these viruses or components thereof may one day provide us with a method to deliver transgenes to the lung in an efficient and safe manner.

7. Conclusion

It is clear that the evolution of gene therapy has been aided by many different aspects of basic biological and medical research efforts and the possibility of a gene therapy for CF lung disease will only take time and a continuation of these efforts. These findings will not only be beneficial to the treatment of CF lung disease but also other disease states, which are continually being brought closer to a treatment and perhaps a cure by this new and exciting biomedical technology.

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