Chapter 15
Gene Electrotransfer to Lung

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Abstract Electroporation has proven to be a highly effective technique for the in vivo delivery of genes to a number of solid tissues. In most of the reported methods, DNA is injected into the target tissue and electrodes are placed directly on or in the tissue for application of the electric field. While this works well for solid tissues, the lung requires a different approach. We and others have developed safe, simple, and highly effective methods for DNA delivery to the lungs using electroporation. These methods have been used in both small and large animal models to transfer both reporter genes and therapeutic ones and have resulted in effective treatments for several pulmonary diseases in these models. The current chapter will discuss these methods and their applications.

Keywords Electroporation • Nonviral gene therapy • Plasmid • Lungs

Introduction

The lung is an attractive target for gene therapy. Multiple genetic, acquired, and infectious diseases are manifested in the lung and it is quite amenable to different delivery strategies [1]. Because of this, multiple techniques for gene delivery to the lung have been developed, including the use of adenoviruses, adeno-associated viruses, lipoplex, and polyethyleneimine [1, 2]. Further, vector administration has been achieved either by tracheal delivery to target the pulmonary epithelium or by vascular delivery to target the pulmonary endothelium. However, all of these approaches have limitations, including inefficiency of gene transfer, immunological responses, inflammation, nonspecificity of cell targeting, and low levels of gene expression. Based on the successful application of electroporation to multiple tissues in vivo, we and others have developed electroporation methods to transfer genes to the lung, both ex vivo and in vivo.

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Ex Vivo Gene Transfer

Several groups have demonstrated effective gene delivery to excised rodent lungs using electric fields [3, 4]. These types of experiments using excised organs usually serve as exploratory studies to determine whether the technique can be applied to a given organ. However, they also may have clinical utility for organ transplantation. In one report, DNA was added to the lungs after removal from the animals, and in the other, DNA was administered intranasally prior to lung removal and subsequent electroporation. In the first case, immediately following removal of mouse or rat lungs *en bloc*, a solution of luciferase-expressing plasmid DNA or 10 mM Tris, pH 8, 1 mM EDTA, and 140 mM NaCl was administered into the bronchi of the lungs. For mice, 200 μL were delivered, and for rats, 500 μL were used. Flat, 0.7 cm diameter electrodes were placed on either side of each lobe and an electric field of 200 V/cm was applied in a series of 8 pulses of 10 ms duration, each using a BTX Instruments ECM830 electroporator. The lungs were maintained in growth medium for 24 h in a humidified CO_2 incubator and luciferase expression was then measured. Experiments using intranasal delivery were carried out in a similar manner, but 100 μL of plasmid in water were delivered to the lungs through the nose immediately prior to lung removal and electroporation. As in the first case, flat plate electrodes were placed on either side of the lungs for field delivery. Several field strengths were evaluated: 20, 200, and 800 V/cm; all using 8 pulses at 2, 20, or 200 ms each. In both studies, application of a 200 V/cm electric field gave approximately 100-fold more gene expression at 24 h compared to DNA alone. Further, the levels of expression were substantial: over 5 ng of gene product per gram wet weight of lung were expressed in the mouse following delivery of 20 μg of plasmid. Levels of expression in the rat lung were less efficient, requiring 0.5 mg of DNA to produce 1 ng of gene product per gram wet weight. One possible explanation for this difference is that the volume of plasmid delivered to the mouse lung was much higher relative to the lung itself than in the rat, and thus greater plasmid distribution could be achieved and result in transfer to a greater number of cells.

Transthoracic Electroporation

Based on the success of the ex vivo gene transfer, we tested whether genes could be transferred to the lung in living animals. While placement of electrodes directly on the surface of the lungs generated a high level of gene transfer and expression, we reasoned that this was too invasive an approach to be used routinely in animals. Thus, plasmids were suspended in saline or 10 mM Tris, pH 8 containing 1 mM EDTA, and 140 mM NaCl and delivered via the airways to the lungs of mice and rats. As such, several delivery methods have been used successfully by our lab and others. Also, injection of DNA into the trachea through cartilage rings following cutdown on the neck is a very effective way to achieve good distribution of the DNA. However, this requires an incision which can lead to infection and poor wound closure. Intubation of mice or rats using a small gauge catheter can be relatively easy to accomplish with practice and this method has been used successfully to deliver DNA into the airways. A benefit of this approach is that no incision is made, thus allowing faster recovery of the animals. Finally, aspiration has been used recently and results in a very good distribution of DNA within the lungs. The major benefit of this approach is that it is very fast and simple: animals are lightly anesthetized with isoflurane or halothane, held in a position resembling a standing human, and the tongue of the animal is pulled out of the mouth with pair of forceps. The DNA solution (50–100 μL for mice) is then delivered into the mouth using a pipettor such that the tip pushes down on the tongue, immobilizing it such that the animals aspirate the solution into the lungs. If a finger is placed over the nares, faster and more robust aspiration is

achieved. After the solution has been aspirated, the animals are returned to a supine position and allowed to recover. This method has been used extensively for allergen administration such as ovalbumin-sensitization in mouse models of asthma. The major benefit is that it is also very fast, results in good distribution of DNA into all lobes of the lung, and allows the use of inhaled anesthetics creating faster recovery and no depression of the respiratory drive.

Once DNA has been delivered to the lungs, the animals are allowed to regain a normal breathing pattern (usually 15–30 s) and a series of electric pulses is applied to the chest. Flat electrodes are placed on either side of the chest, usually under the armpits of the animals (Fig. 15.1b). We have found that disposable, conformable, and pediatric pacemaker electrodes (Medtronics) work the best. A series of square wave electric pulses is then applied across the chest, which causes the animal to jump slightly. Following electroporation, the animals are placed on their side and allowed to recover. The animals recover and survive with no apparent trauma until the experiments are terminated at the desired times, typically between 1 and 21 days posttreatment.

We have had no mortality due to electroporation alone ($n = 30$ animals). However, we have seen up to 15% mortality due to drugs, surgery, endotracheal tube placement, or fluid delivery ($n \geq 400$ mice and/or rats). The greatest mortality appears to be when the interval between fluid delivery and electroporation is decreased to less than 15 s and seems to be related more to fluid delivery than application of the electric field. To decrease this, we have lengthened the interval

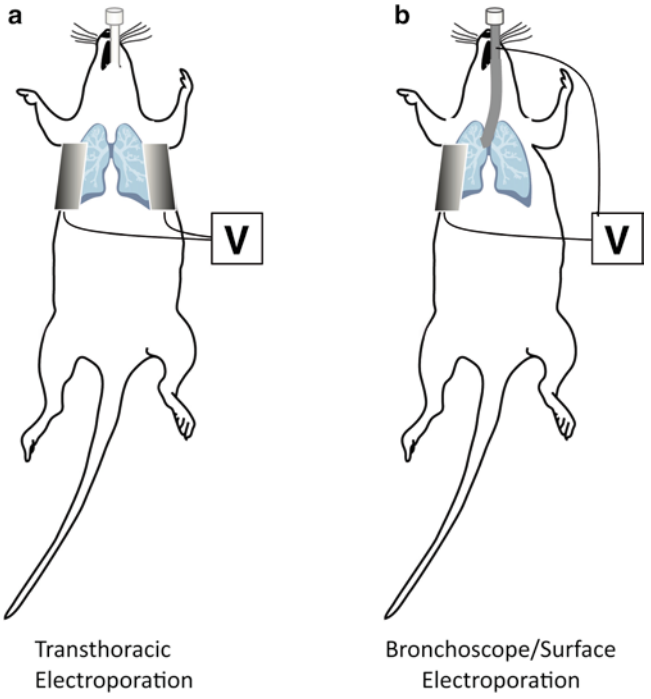

Fig. 15.1 Cartoon of electrode placement for pulmonary electroporation. For transthoracic electroporation (**a**), the DNA solution is delivered to the airways via an endotracheal tube or by aspiration. Electrodes are placed on either side of the chest, under the armpits, and immediately following DNA administration, the electric pulses are given. For bronchoscope-mediated delivery and electroporation (**b**), a bronchoscope is inserted into the airways, positioned in the desired lobe and location, and the DNA solution is delivered through the central channel. The port for the channel on the body of the bronchoscope is attached to the electroporator and a second surface electrode is placed on one side of the chest (same side as delivered DNA) for pulse delivery

between DNA delivery and application of the pulses to 30 s to allow the animals to distribute the DNA evenly within the lungs and regain a normal breathing pattern. We have also found that brief ventilation of the mice (less than 2 min) using room air immediately following electroporation reduced this mortality to about 5%. This is one benefit from using an endotracheal tube for delivery of DNA: it can be used for ventilation. Other labs have noted similar increases in mortality. Pringle and colleagues found up to 75% mortality using this approach [4]. Although anecdotal, in reports from other labs that have used these methods, we find that there is a steep learning curve with this technique in mice for the first 10–20 animals used and it is not uncommon for up to 75% of the animals to die. This is likely due to user training and getting used to the delivery techniques. However, after these initial attempts, mortality decreases substantially to the levels we have reported [3, 5].

Gene expression following transthoracic electroporation is dose-dependent. In the mouse, administration of 10 μg of a CMV promoter-driven, luciferase-expressing plasmid gives about 2 pg of gene product per lung, whereas administration of 40 or 100 μg yields 20 pg and 2 ng per lung, respectively, 2 days after electroporation [3]. Gene transfer and expression is also dependent on the field strength. These levels are very high compared to those achieved by other nonviral methods, including Polyethylenimine or DNA-liposome complexes [4, 6, 7]. In all of our experiments, we have used trains of 8 pulses between 10 and 20 ms duration each, based on early studies in skeletal muscle showing that this approach works well [8–10]. We have found very little, if any, gene expression in the lung when no electric field is applied following DNA delivery and seen optimal expression at 200 V/cm [3, 5]; very little expression was seen at 50 or 100 V/cm, and although expression was detected at 400 V/cm, the level was lower than that at 200 V/cm. Further, at 400 V/cm, tissue damage is observed in lungs and other tissues [3, 11, 12]. Other studies have seen detectable expression in the absence of electroporation, but have shown that the electric field can increase gene expression about 50-fold over DNA alone [4]. Similar damage is seen at higher field strengths [4]. There is also a direct relationship between pulse length and expression, with maximal expression being seen at 10–20 ms compared to 0.01 and 0.5 ms [5].

The duration of gene expression seen following electroporation-mediated delivery depends entirely on the cells being transfected and the promoter being used to drive transgene expression. Although the CMV promoter drives high-level, long-term expression in skeletal muscle [13], in the lung, it is silenced within 5 days [3, 5]. By contrast, the Ubiquitin C (UbC) promoter can be used in the lung following delivery by liposomes or electroporation and gives high-level gene expression for up to 6 months [4, 14, 15]. We have also found that cell-specific promoters can also be used to direct gene expression to specific cell types with success [16].

Perhaps the greatest advantage to pulmonary electroporation is that gene delivery and expression is seen throughout multiple cell layers in the lung (Fig. 15.2). While most gene expression is seen in the airway and alveolar epithelial cells, a significant amount of expression is seen in endothelial cells, vascular smooth muscle, and airway smooth muscle cells, all of which lie beneath the epithelium [3–5]. This makes electroporation the only method that can be used to deliver genes beyond the epithelium without damage to the epithelium itself. Further, the most exciting application of this is the ability to transfer genes to airway smooth muscle cells, a primary target for any gene therapy approach treating bronchoconstriction associated with asthma. However, this ability to transfect all cell types and layers in the lung should not imply that all cells in the lung receive transgenes. Rather, gene delivery and expression is controlled by two major factors: distribution of the delivered DNA and distribution of the applied field. We have found that if DNA is delivered only to one lobe of the lung and the field is applied across the entire lung, gene expression is detected only in the lung receiving the DNA. This results in nonuniform distribution of gene expression – some areas have a high percentage of transfected cells, while other areas show sparse expression. Further research is required to optimize these parameters to obtain uniform high-level delivery and expression.

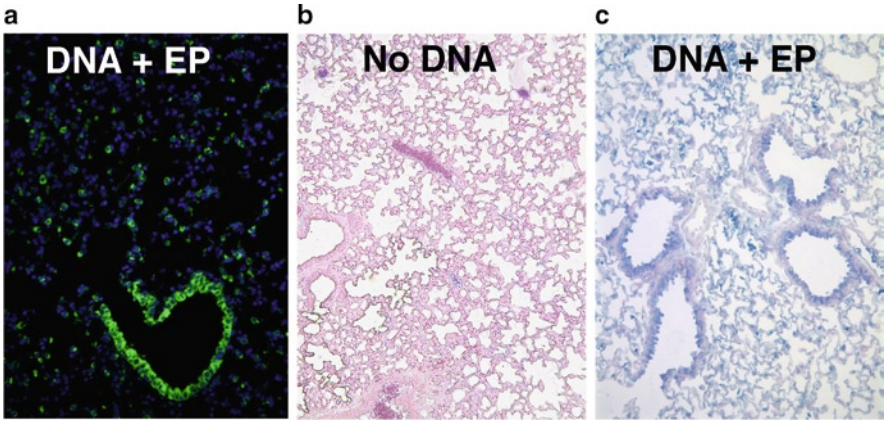

Fig. 15.2 Gene expression in the lung following electroporation. Plasmids expressing GFP (**a**) or LacZ (**b**, **c**) were delivered to the lungs through an endotracheal tube. Animals received a series of eight 10 ms long pulses of 200 V/cm each (**a, c**) or no electric pulses (**b**). Gene expression was visualized 2 days later directly using either fluorescence microscopy (*green*; **a**) or immunohistochemistry for LacZ (*blue*; **b, c**). Sections were counterstained with DAPI to visualize nuclei (*blue*; **a**) or with eosin (*pink*; **b, c**)

Invasive Thoracotomy-Mediated Electroporation

Several groups have also developed methods to transfer genes to the lungs using electroporation and more invasive approaches to apply the electric field. For instance, Schmid and colleagues routinely perform a thoracotomy to expose the lungs for electroporation. In some experiments, DNA in water or saline is delivered intranasally to the lungs of anesthetized mice and the mice are intubated and ventilated prior to thoracotomy and electroporation [4]. In other experiments, DNA is delivered to the left lung using a catheter following thoracotomy [15, 17]. In both cases, the electric field is then applied to the left lung using plate electrodes on either side of the exposed lung, a chest drain is placed into the hemithorax, and the chest is closed with sutures. The chest drain is removed when the animals resume spontaneous breathing, and the animals are extubated. One advantage to this approach is that no mortality was reported in three different studies, suggesting that it may be safer than the transthoracic method. However, the relative invasiveness of the approach may limit clinical acceptance.

As with transthoracic electroporation, gene delivery and expression is both DNA dose-dependent and field strength-dependent. When DNA is delivered to the entire lung and only the left lung is electroporated, there is between 100- and 1,000-fold more expression seen in the electroporated lobes [4, 15]. Although a field strength of 800 V/cm using 2 ms pulses gave the highest levels of gene expression, there was significantly more lung injury at this strength than at a field of 200 V/cm using 20 ms pulses. It should be pointed out, however, that in one study, even at 200 V/cm, there appeared to be a fair amount of lung injury, determined histologically [4]. By contrast, two other studies showed very little tissue damage or evidence of inflammation at these lower field strengths [15, 17]. It is possible that the tissue damage observed could have been close to the surface of the lungs and be the result of the electrodes being placed directly on the lungs themselves. Indeed, we and others have found that electroporation to skin can result in transient reddening and tissue damage reminiscent of a minor sun burn (at low field strengths) to more severe burns (at very high field strengths).

Gene expression following electroporation is detected in many cell types in the lung, including alveolar type I and type II epithelial cells, airway epithelial cells, endothelial cells, and vascular smooth muscle cells [15]. Further, as with every other delivery technique, the duration of gene

expression was also dependent on the promoter used. Two studies used the UbC promoter to drive gene expression and detected significant levels of gene expression for up to 40 days [4, 15]. By contrast, expression using the CMV promoter peaked by one to two days and decreased immediately thereafter, giving almost undetectable levels by day 5. These are exactly the results we have seen in our studies. Unfortunately, gene expression in studies using thoracotomy-mediated electroporation was reported only as relative light units per mg protein, and consequently, cannot be directly compared to levels of gene expression reported in other studies, due to the intrinsic nature of the "relative light unit." However, in comparing our in vivo transthoracic delivery and ex vivo experiments (where electrodes were placed directly on the lungs), we found that expression in the in vivo lungs was much less than that achieved in our ex vivo lungs [3]. It is likely that when electrodes are placed directly on the lung, the lung "sees" all of the applied field. In contrast, in the case of in vivo delivery, the field must travel through multiple layers of different tissues, including skin, fat, muscle, bone, cartilage, and interstitial fluid, all of which have different resistive properties, prior to reaching the lungs. As such, it is not clear what field strength the lungs themselves actually see, but it is very possible that it is less than 200 V/cm. Thus, it is possible that higher levels of gene expression can be achieved using this more invasive approach, but further studies directly addressing this are needed.

Bronchoscope-Mediated Electroporation

Although several groups have had success using electroporation to transfer genes to the lungs of animals following thoracotomy and open-chest surgery [4, 15, 17], this relatively severe method would be unattractive to many physicians and patients. Over the past 5 years, we have developed less and less invasive methods for pulmonary electroporation. In our initial experiments, we delivered DNA following exposure of the trachea by incision, but required suturing of the animals [3]. We next simply intubated the animals and delivered DNA via an endotracheal tube which required no incision, and mice and rats recovered quicker and easier [5]. More recently, we have found that plasmid can be delivered efficiently to the lungs of mice and rats by placing the solution on the back of the tongue while the tongue is lightly pulled and held to the side. Along these same lines, although we know that application of the electric field to either side of the chest does not result in arrhythmia or fibrillation of the heart, does not cause any burning, and causes no inflammation, trauma, or injury to the animals, many people may perceive this as a harsh and dangerous method. If this promising approach is to be used in humans, we must have a method for electric field application that is less "scary" to people so that it will be accepted and used.

Pringle and colleagues were the first to develop a bronchoscope-based electrode that was able to deliver DNA (through its access channel) and an electrical field [4]. They did this by attaching two insulated wires to the outside of the bronchoscope and connecting them to the pulse generator. Sheep were anesthetized and ventilated in a negative pressure ventilation box and luciferase-expressing plasmid (30 mg) was delivered via the channel on the bronchoscope. The two wires, separated by a 0.5 cm gap, were placed on either side of an airway segment bifurcation, and the field was applied. Only fivefold more gene expression compared to DNA only was detected when a series of 800 V/cm pulses of 2 ms duration was applied to the segments, but almost 100-fold more expression was seen when a field of 200 V/cm (20 ms) was applied. This was approximately 50-fold greater than the level of expression seen following delivery of the same amount of identical plasmid using 25 kDa polyethyleneimine.

We have taken a different approach and used a bronchoscope-based electrode as a "point charge" inside the lung and then placed a plate electrode on the surface of the chest on the same side of the lung (Fig. 15.1b). In this way, the heart is isolated from the applied field. We should stress that even

though the use of two surface plates as electrodes on either side of the chest may be seen as potentially dangerous, all of our data to date demonstrate that this is a safe procedure at the field strengths and pulse lengths used.

To carry out these experiments, we used pigs as a large animal model. The internal channel of most bronchoscopes, including ours, is stainless steel and the body is coated with an insulated polymer. The pigs (35–45 kg) were anesthetized, intubated, and ventilated. The bronchoscope was inserted through the endotracheal tube and directed to the right lower lobe (pigs have three lobes on the left side and four on the right, the largest of which is the right lower lobe). The total lung capacity of pigs of this size is approximately 1,200 mL, and we delivered 50 mg of plasmid in 50 mLof saline (a volume that would routinely be used for obtaining bronchoalveolar lavage fluid (BALF) in a human). Within 10 s of DNA delivery, the channel is connected to the pulse generator and a defibrillation electrode (10 × 15 cm) is placed on the chest under the armpits outside the right lung. Although application of the electric field caused the animals to jerk, no changes in cardiac rhythm or output were detected at fields lower than 250 V/cm using pulses of 10 ms duration. We did have several animals enter ventricular fibrillation when the field was raised to 400 V/cm using 10 ms pulses, but could go to higher field strengths with much shorter pulse lengths (0.1 ms) with no trauma. Gene expression was very high two days postelectroporation with most of the expression seen radiating from the site of DNA delivery and internal electrode placement (since DNA is delivered around the electrode, this makes perfect sense), and no gene expression was detected in any other lobe of the lung. The levels of gene expression are relatively high and similar to those seen in the mouse lung on a per gram wet weight comparison. While electroporation of 100 μg of plasmid gave 10 ng luciferase per gram wet weight of lung in the mouse [3], electroporation of 50 mg of plasmid using the bronchoscope and an external electrode gave approximately 2 ng luciferase per gram wet weight of lung in the pig. Since the sizes of the lungs are so different, this corresponds to 2 ng of total gene expression per mouse and almost 400 ng of total expression in the pig.

Safety

As in all in vivo applications of electroporation, at the appropriate field strengths, pulmonary electroporation is safe and well tolerated. However, at much higher fields, the technique can cause significant morbidity and mortality. We have found that high levels of gene transfer can be obtained with minimal, if any, tissue damage by application of eight 10 ms pulses at 200 V/cm across the chest of the animal. In the mouse and rat, this results in no damage to the skin and no discernable damage to the lung upon histology between 1 h and 2 days after electroporation [3, 5, 15, 18, 19]. However, when the field is applied directly to the lungs using a thoracotomy approach, much higher levels of tissue damage are seen directly under the areas of the electrodes, perhaps due to the intense electric field at this point [4]. Indeed, we have noted that a transient skin burn develops immediately after pulsing in the pig when surface electrodes are placed on the chest. This only develops when greater than 1,000 V are applied to the chest for durations of greater than 0.1 ms (since the chest is about 15 cm across, this would correspond to a field of roughly 70 V/cm). However, the “burn” dissipates and disappears by 30 min, and upon histology at 2 days, faint signs of tissue damage are noted that resemble a mild sunburn.

A number of criteria have been used to evaluate the degree, or lack thereof, of potential injury in the lung following transthoracic electroporation. Histological analysis at either 1 or 24 h postelectroporation failed to notice any differences in hemorrhage, infiltrating lymphocytes or other cells, pulmonary edema, or alveolar wall thickening between control, unelectroporated lungs, or those receiving a field (200 V/cm) [3]. There was also no increase in IL-6 or IFN-γ levels in the BALF of animals electroporated with or without DNA compared to control animals or those receiving DNA only and only a slight increase in TNF-α levels in electroporated mice compared to DNA only mice

[3, 19]. This is somewhat surprising given the wealth of literature demonstrating that bacterial DNA with its unmethylated CpG motifs interacts with Toll-like receptor (TLR) 9 to initiate the innate immune response [20, 21]. Since TLR9 is located within the endosomal/lysosomal compartment, the electroporation-mediated delivery of DNA directly into the cytoplasm across the plasma membrane seems to bypass the activation of TLR9 and the innate immune response [19]. Indeed, when plasmids were electroporated into TLR9-expressing or TLR9-null cells, IL-8 (a downstream TLR9-activated gene) expression was not increased compared to controls. By contrast, when cells were transfected with the same plasmid using liposomal complexes, a significant induction of IL-8 was measured in TLR9-expressing cells, but not in the TLR9-null cells.

Perhaps the most common argument against transthoracic electroporation is that the application of an electric field across the chest will likely lead to cardiac arrhythmias and dysfunction. In the mouse and rat, application of a 200 V/cm field with 10 ms pulses delivers roughly 0.1 J of energy. This is very low and insufficient to alter cardiac electrical function. However, if the technique is to be scaled up for human application, the resulting energy would be much higher. We measured this in the pig and found that while the energy delivered is indeed much higher, it is still well within safe levels. Since the chest of a 40 kg pig is roughly 15 cm across, 3,000 V must be delivered to generate a 200 V/cm field. Because the pulse generator that we were using could not deliver pulses longer than 150 μs at this voltage, we delivered eight 150 μs pulses at 3,000 V and measured a resistance of 15 Å and an energy of just over 4 J. This is well below the 200–360 J routinely used for defibrillation. Moreover, at these fields, no changes in EKG were noted, and out of eight initial pigs exposed to this field strength, only one died; at lower field strengths (100–150 V/cm), we have had no deaths but still good gene transfer. Thus, the approach appears well tolerated and scalable.

Therapeutic Applications

While this approach for gene transfer to the lung is still in its infancy, a number of groups have used these approaches for therapeutic or biological means and have seen very positive outcomes. The first nonreporter gene to be transferred to the lung using electroporation was the β subunit of the Na^+, K^+-ATPase [5]. While minimizing pulmonary edema is known to improve outcomes in acute lung injury and the acute respiratory distress syndrome, current methods using pharmacologic interventions are suboptimal in both efficacy and side effects. Gene therapy may offer a novel and useful treatment option. It had been demonstrated that transfer of the β1 or α2 subunit of the Na^+, K^+-ATPase using recombinant adenoviruses can result in increased alveolar fluid clearance [22, 23]. A drawback to using adenovirus is that the effects on alveolar fluid clearance could only be experimentally measured 7 days after gene delivery because of the induction of inflammatory responses by the viral vector. As such, the timing of this response is not early enough to elicit meaningful effects on the injured lung. When the gene for the β2 subunit was transferred to the lungs of healthy rats, a fivefold increase in the levels of the β2 protein was detected in the lungs [5]. Moreover, alveolar fluid clearance increased twofold over that seen in control rats or those receiving β2 plasmid alone, electroporation alone, or an empty plasmid that was electroporated. This twofold increase in alveolar fluid clearance was identical to that seen using recombinant adenoviruses, the gold standard for gene transfer [22]. More importantly, the increased fluid clearance seen using electroporation was evident just 2 days after gene transfer and was not associated with any procedure-induced inflammation or injury. Therefore, these results suggest that this is a much better approach for treating the injured lung.

While transfer of genes that increase pulmonary edema clearance to the healthy lung is intriguing, it does not address whether this is a viable approach to treat the injured lung. Thus, a follow-up study demonstrated that electroporation-mediated transfer of either the α1 subunit, the β1 subunit,

or a combination of the two subunits for the Na^+, K^+-ATPase provided protection from subsequent lipopolysaccharide (LPS)-induced lung injury [18]. Addition of LPS to the lungs of mice results in a significant inflammatory response and formation of pulmonary edema. Transfer of subunits of the Na^+, K^+-ATPase decreased the severity of LPS-induced lung injury as measured by a number of criteria, including wet-to-dry ratios, BALF cellularity and protein content, and lung resistance. Although this is a promising result, this is clinically irrelevant: no one would treat a patient before he/she develops acute lung injury since it is impossible to predict who will develop the disease (which is usually the result of some form of trauma or sepsis). A more meaningful experiment would be to ask whether electroporation of these genes could have any effect on the previously injured lung. Thus, we determined whether the same approach could be used to reverse previously induced lung injury by electroporating the Na^+, K^+-ATPase subunit genes into LPS-injured lungs [18]. As such, lungs were injured by administration of LPS (4 mg/kg) and, 24 h later, plasmids expressing the $\alpha 1$ or the $\beta 1$ subunit of the Na^+, K^+-ATPase were electroporated into the lungs either individually or in combination. As predicted, transfer of these genes increased alveolar fluid clearance in the LPS-injured lungs, reduced pulmonary edema, and reduced the cellularity and protein content of BALF. More intriguing was the fact that by histological analysis, less inflammation was noted in the lungs of LPS-injured mice that later received the combination of both subunits of the Na^+, K^+-ATPase. Perhaps most exciting about these results was that this was the first time that gene therapy was used to treat a previously injured lung. Taken together, these results suggest that electroporation of these genes may be a viable treatment for the acutely injured lung.

Schmid and colleagues have used electroporation to deliver genes to reduce the severity of bleomycin-induced pulmonary fibrosis [17]. Pulmonary fibrosis is characterized by excessive collagen deposition in the lung, impaired gas exchange, hypoxia, and high morbidity and mortality. A number of studies have suggested that damage to the alveolar epithelium and aberrant repair contribute to the development of this disease [24]. Thus, this group asked whether delivery of the gene for hepatocyte growth factor (HGF), a potent mitogenic factor for pulmonary epithelial cells, could modulate development of pulmonary fibrosis. One reason that gene delivery is attractive for this is that the half-life of the HGF protein is very low, preventing use of a protein therapeutic [25]. Alveolar epithelial cell injury, inflammation, and fibrosis were induced by intratracheal instillation of bleomycin. Seven days later, lungs were exposed by thoracotomy, and plasmids expressing HGF or empty control plasmids were transferred to the lungs of mice using electroporation with electrodes placed directly on the lungs. After, the chest was closed and the mice were allowed to recover. Transfer of HGF plasmids prevented bleomycin-induced loss of body weight in the mice and reduced fibrosis measured by collagen content and histology. Further, transfer of the HGF plasmids caused increased proliferation of alveolar epithelial cells and a decrease in the apoptosis of these cells. Finally, TGF-$\beta 1$ levels, which are tightly linked to development of pulmonary fibrosis, were reduced in HGF plasmid electroporated lungs, suggesting that TGF- $\beta 1$ may be involved in the HGF-induced reduction of bleomycin-induced lung fibrosis.

More recently, another group has used electroporation to transfer the gene for keratinocyte growth factor (KGF) to lungs following pulmonary resection to aid in repair [26]. It is well documented that the lung undergoes compensatory growth after lung injury or surgical resection in a number of animal models, and that levels of KGF are increased during this proliferation. However, the exact mechanisms governing this regrowth are unknown. It has been shown that KGF is a mesenchymal cell-derived factor that is an epithelial cell mitogen, and addition of KGF protein can enhance alveolar proliferation and compensatory lung growth after pneumonectomy in adult rats [27]. Three of the four right lobes of the rat lung were removed and plasmid expressing either FLAG-tagged KGF or the empty FLAG vector was injected into the remaining fourth lobe and electroporated by placing electrodes directly on the surface of the lung. Increased proliferation of cells and increased PCNA staining (a marker of proliferation) were detected in the lungs of animals receiving the KGF vector, but not in animals electroporated with the empty vector. Although levels

of FLAG-tagged KGF were not measured in the lungs of these animals, these increases in measures that are controlled by KGF suggest that KGF levels were indeed increased.

A final example of electroporation-mediated transfer, expression, and use of a nonreporter gene is using a physiological gene as a novel reporter. Mizgerd and colleagues were interested in elucidating the mechanisms of neutrophil recruitment in the lung following *Streptococcus pneumoniae* infection [28]. As with infection with many microbes, TNF-α and IL-1 are rapidly induced upon infection. When knockout mice for either of these two cytokines were infected with *S. pneumoniae*, neutrophil recruitment was inhibited as was pulmonary expression of KC and MIP-2. Since all of these cytokines are controlled by NF-κB activation, the authors wanted to evaluate NF-κB activity directly in mouse lungs in response to *S. pneumoniae* infection. To do this, wild-type and TNF receptor/IL-1 receptor knockout mouse lungs were electroporated with NF-κB transcription reporter plasmids [28]. These plasmids contain 4 tandem NF-κB-binding sites upstream of a minimal promoter driving luciferase expression so that increased NF-κB levels induce increased luciferase expression. In essence, they created a conditional transgenic NF-κB reporter mouse, not by breeding that would take months, but rather instillation of plasmid and electroporation which takes minutes and can be done in any strain background. Using this approach, they were able to demonstrate that both TNF and IL-1 receptor pathways are needed for *S. pneumoniae* activation of NF-κB.

Conclusion

These studies demonstrate that electroporation can be used effectively to deliver DNA to the lungs of living animals, both small and large. The techniques are rapid, easy to perform, and reproducible. They mediate the highly efficient delivery of DNA (and most likely oligonucleotides) to the lung and result in levels of gene expression that approach and even surpass those achieved with the best viral and nonviral means. In contrast to all other techniques developed to date, electroporation promotes gene transfer to multiple cell layers and cell types within the lung without the need to damage the pulmonary epithelium itself. Moreover, the technique is safe at the appropriate field strengths. Thus, based on the ease, efficiency, and nontraumatic nature of these electroporation methods for pulmonary gene transfer, its use may be of great experimental and clinical potential.

References

1. West J, Rodman DM. Gene therapy for pulmonary diseases. Chest. 2001;119(2):613–17.
2. Weiss D. Delivery of gene transfer vectors to lung: obstacles and the role of adjunct techniques for airway administration. Mol Ther. 2002;6(2):148–52.
3. Dean DA et al. Electroporation as a method for high-level non-viral gene transfer to the lung. Gene Ther. 2003;10(18):1608–15.
4. Pringle IA et al. Electroporation enhances reporter gene expression following delivery of naked plasmid DNA to the lung. J Gene Med. 2007;9(5):369–80.
5. Machado-Aranda D et al. Gene transfer of the Na+, K+-ATPase b1 subunit using electroporation increases lung liquid clearance in rats. Am J Respir Crit Care Med. 2005;171:204–11.
6. Gautam A et al. Enhanced gene expression in mouse lung after PEI–DNA aerosol delivery. Mol Ther. 2000;2:63–70.
7. Li S, Huang L. *In vivo* gene transfer via intravenous administration of cationic lipid- protamine-DNA (LPD) complexes. Gene Ther. 1997;4(9):891–900.
8. Aihara H, Miyazaki J. Gene transfer into muscle by electroporation *in vivo*. Nat Biotechnol. 1998;16(9):867–70.
9. Mathiesen I. Electropermeabilization of skeletal muscle enhances gene transfer *in vivo*. Gene Ther. 1999;6:508–14.

10. Mir LM et al. High-efficiency gene transfer into skeletal muscle mediated by electric pulses. Proc Natl Acad Sci USA. 1999;96:4262–7.
11. Blair-Parks K, Weston BC, Dean DA. Gene delivery to the cornea by plasmid injection and electroporation. J Gene Med. 2002;4:92–100.
12. Martin JB et al. Gene transfer to intact mesenteric arteries by electroporation. J Vasc Res. 2000;37(5):372–80.
13. Wolff JA et al. Long-term persistence of plasmid DNA and foreign gene expression in mouse muscle. Hum Mol Genet. 1992;1:363–9.
14. Gill DR et al. Increased persistence of lung gene expression using plasmids containing the ubiquitin C or elongation factor 1alpha promoter. Gene Ther. 2001;8(20):1539–46.
15. Gazdhar A et al. *In vivo* electroporation and ubiquitin promoter – a protocol for sustained gene expression in the lung. J Gene Med. 2006;8(7):910–18.
16. Degiulio JV, Kaufman CD, Dean DA. The SP-C promoter facilitates alveolar type II epithelial cell-specific plasmid nuclear import and gene expression. Gene Ther. 2010;17(4):541–9.
17. Gazdhar A et al. Gene transfer of hepatocyte growth factor by electroporation reduces bleomycin-induced lung fibrosis. Am J Physiol Lung Cell Mol Physiol. 2007;292(2):L529–36.
18. Mutlu GM et al. Electroporation-mediated gene transfer of the Na+, K+-ATPase rescues endotoxin-induced lung injury. Am J Respir Crit Care Med. 2007;176(6):582–90.
19. Zhou R et al. Electroporation-mediated transfer of plasmids to the lung results in reduced TLR9 signaling and inflammation. Gene Ther. 2007;14(9):775–80.
20. Kaisho T, Akira S. Toll-like receptor function and signaling. J Allergy Clin Immunol. 2006;117(5):979–87; quiz 988.
21. Hemmi H et al. A Toll-like receptor recognizes bacterial DNA. Nature. 2000;408(6813):740–5.
22. Factor P et al. Augmentation of lung liquid clearance via adenovirus-mediated transfer of a Na, K-ATPase beta1 subunit gene. J Clin Invest. 1998;102(7):1421–30.
23. Adir Y et al. Na, K-ATPase gene transfer increases liquid clearance during ventilation-induced lung injury. Am J Respir Crit Care Med. 2003;168(12):1445–8.
24. Selman M, King TE, Pardo A. Idiopathic pulmonary fibrosis: prevailing and evolving hypotheses about its pathogenesis and implications for therapy. Ann Intern Med. 2001;134(2):136–51.
25. Dohi M et al. Hepatocyte growth factor attenuates collagen accumulation in a murine model of pulmonary fibrosis. Am J Respir Crit Care Med. 2000;162(6):2302–7.
26. Matsumoto K et al. Keratinocyte growth factor accelerates compensatory growth in the remaining lung after trilobectomy in rats. J Thorac Cardiovasc Surg. 2009;137(6):1499–507.
27. Kaza AK et al. Keratinocyte growth factor enhances post-pneumonectomy lung growth by alveolar proliferation. Circulation. 2002;106(12 Suppl 1):I120–4.
28. Jones MR et al. Lung NF-kappaB activation and neutrophil recruitment require IL-1 and TNF receptor signaling during pneumococcal pneumonia. J Immunol. 2005;175(11):7530–5.