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Electroporation-Mediated Gene Delivery

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Abstract

Electroporation has been used extensively to transfer DNA to bacteria, yeast, and mammalian cells in culture for the past 30 years. Over this time, numerous advances have been made, from using fields to facilitate cell fusion, delivery of chemotherapeutic drugs to cells and tissues, and most importantly, gene and drug delivery in living tissues from rodents to man. Electroporation uses electrical fields to transiently destabilize the membrane allowing the entry of normally impermeable macromolecules into the cytoplasm. Surprisingly, at the appropriate field strengths, the application of these fields to tissues results in little, if any, damage or trauma. Indeed, electroporation has even been used successfully in human trials for gene delivery for the treatment of tumors and for vaccine development. Electroporation can lead to between 100 and 1000-fold increases in gene delivery and expression and can also increase both the distribution of cells taking up and expressing the DNA as well as the absolute amount of gene product per cell (likely due to increased delivery of plasmids into each cell). Effective electroporation depends on electric field parameters, electrode design, the tissues and cells being targeted, and the plasmids that are being transferred themselves. Most importantly, there is no single combination of these variables that leads to greatest efficacy in every situation; optimization is required in every new setting. Electroporation-mediated *in vivo* gene delivery has proven highly effective in vaccine production, transgene expression, enzyme replacement, and control of a variety of cancers. Almost any tissue can be targeted with electroporation, including muscle, skin, heart, liver, lung, and vasculature. This chapter will provide an overview of the theory of electroporation for the delivery of DNA both in individual cells and in tissues and its application for *in vivo* gene delivery in a number of animal models.

1. INTRODUCTION

One of the great limitations of nonviral gene delivery has been perceived to be the relative lack of high-level gene transfer *in vivo*. Many nonviral techniques, while much safer in terms of inflammatory and immune responses to the vectors themselves, have generally resulted in much lower levels of gene delivery and expression when compared to their viral counterparts. However, one nonviral approach has proven both safe and highly effective in high-level gene transfer: electroporation. Electroporation uses electric field to facilitate the

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delivery of nucleic acids and other impermeable molecules to cells. Perhaps the most transformative of these molecules, in terms of biology and medicine, is DNA. Neumann and colleagues first demonstrated that moderate electric fields could be used to transfect mammalian cells in culture with plasmids in 1982 and formalized a mathematical and electrical theory for how the process occurred (Neumann, Schaefer-Ridder, Wang, & Hofschneider, 1982). In the past 30 years, numerous advances have been made in the field, from using fields to facilitate cell fusion, transformation in bacteria, delivery of chemotherapeutic drugs into cells and tissues, and most importantly, gene and drug delivery into living tissues from rodents to man (Ausubel et al., 1999; Heller & Heller, 2006; Potter, Weir, & Leder, 1984). Electroporation-mediated *in vivo* gene delivery has proved highly effective in vaccine production, transgene expression, enzyme replacement, and control of a variety of cancers. When a square-wave electric field at the appropriate strength is applied to any number of tissues such as skin, liver, muscle, and a variety of tumors, following direct injection of the plasmid, the levels of gene expression jump between 20- and 1000-fold (Aihara & Miyazaki, 1998; Cemazar et al., 2009; Heller et al., 2000; Lin et al., 2012; Mathiesen, 1999; Mir et al., 1999; Wells, Li, Sen, Jahreis, & Hui, 2000). Most exciting is the fact that a number of Phase I and Phase II clinical trials are either underway or have been completed, clearly demonstrating the efficacy of this approach. This chapter will provide an overview of the theory of electroporation for the delivery of DNA both into individual cells and in tissues and its application for *in vivo* gene delivery in a number of animal models.

2. THEORY OF MEMBRANE ELECTROPORATION

The hydrophobic lipid bilayer of the plasma membrane can be thought of as a simple capacitor that stores a charge and acts as a dielectric between the highly charged conductive environments of the extracellular medium and the cytoplasm. When cells are exposed to an electric field, the membrane can build up a charge in the form of a transmembrane potential (Figure 1(A)). The electric field causes the dipoles of molecules from proteins to carbohydrates to orient themselves with respect to the field. They then distribute within and around the cell such that the side of the cell facing the cathode is “depolarized,” and the other facing the anode is “hyperpolarized” due to the differential accumulation of charge on either side of the plasma membrane (Hibino, Itoh, & Kinosita, 1993). Once this electric field-induced transmembrane potential exceeds the dielectric strength of the membrane (typically around 500 mV), the membrane undergoes a permeation event resulting in the formation of hydrophobic pores that allow water movement and limited ion flow. With increasing time in the field, the destabilized membrane and the hydrophobic pores stabilize to form larger pores that allow the entry and exit of larger impermeable molecules in and out of the cell. Indeed, a study of human embryonic stem cells shows that they required short pulse times of 0.05 ms for loading of propidium iodide and other small molecules, while pulse times of 0.5 ms or more were required for DNA transfection using the same field strengths (Mohr, de Pablo, & Palecek, 2006). Once the field is removed, the pores destabilize and close over time (minute scale), allowing the membrane to reseal.

Computer simulations using recent advances in molecular dynamics approaches to describe membrane behavior under the influence of an applied electric field have suggested that once the membrane reaches the critical point, water molecules begin to gain access across the

membrane and an inverted hydrophilic pore can be formed (Figure 2) (Delemotte & Tarek, 2012; Ho, Casciola, Levine, & Vernier, 2013; Ho, Levine, & Vernier, 2013; Kramar et al., 2012; Polak et al., 2013; Tokman et al., 2013). With continued application in the field, this metastable hydrophilic pore can transition to a larger, more stable pore that can allow the transport of larger charged macromolecules. While considerable disagreements continue in the field as to whether pores actually form (they have not been directly visualized experimentally), there is overwhelming agreement that membrane permeation, resulting in entry of macromolecules, does occur.

The mathematical theory first proposed by Neumann assumes a completely spherical model cell that becomes slightly elongated with the applied electric field, leading to permeation events at the poles (Figure 1) (Neumann et al., 1982). The model proposes that the permeation events occur perpendicular to the field at either apex of the slightly elliptical cells. Experimentation using small fluorescent dyes and either membrane vesicles or isolated cells has shown that this appears to be the case. The entry of small molecules and dyes does not occur uniformly around the cell, but rather at the two opposing sides of the cell within the field (Figure 1(B)) (Golzio, Teissie, & Rols, 2002; Hibino et al., 1993; Tekle, Astumian, & Chock, 1994). As cells move away from the theoretical spheroid shape to more realistic ones with various protrusions and bulges, the distribution of the field and its effects on the transmembrane potential becomes more heterogeneous (Valic et al., 2003). This results in a greater permeabilization at the ends of protrusions with great degrees of curvatures (*i.e.*, at the end of a skeletal myotube or neuron) than along the remainder of the cell body. Additionally, the size of the cell can have a major impact on its ability to be electroporated. As the diameter of the cell increases, the external field needed for effective electroporation decreases. This may also explain why certain cells within tissues can be electroporated using reduced field strengths; if adjacent cells are electrically connected via gap junctions, they may act as a single functionally large cell. Thus, muscle fibers which can be treated as one large “cell” with a number of distinct, but not isolated, myotubes, typically require much lower field strengths for effective electroporation-mediated gene delivery as compared to a variety of other cell and tissue types (Mir et al., 1999).

3. MECHANISM(S) OF ELECTROPORATION-MEDIATED GENE DELIVERY

For electroporation-mediated gene delivery to occur, two things need to be simultaneously present: nucleic acid and the electric field. DNA and other nucleic acids must be present in the tissue or cell suspension before or during the application of electric pulses. In contrast, small molecules and dyes such as propidium iodide or trypan blue can enter cells when added during, or shortly after, application of the electric field and still gain access to cells. When trypan blue was added to adherent cells that had been electroporated at various times, 50% of the cells excluded the dye 6 min after pulsing, however, all cells excluded the dye by 30 min (Gabriel & Teissie, 1995; Rols & Teissie, 1990). This is likely due to the fact that DNA is much larger than any small reporter dye and thus requires larger functional pores for its entry (Figure 1(B)). As the membrane destabilizes and the permeation event occurs, small pores open that then coalesce into larger ones. Increasing the pulse duration or number of pulses can lead to greater functional pore sizes, but the majority of these pores likely close faster than the larger number of small pores, in a simple exponential fashion. Thus, if DNA

is not present at the time of the pulsing, all the pores capable of allowing its transport will be closed by the time of DNA addition.

Experiments with fluorescent dyes have shown that electric field-induced permeation of the membrane occurs at the poles of the cell perpendicular to the applied field. Visualization of the accumulated dye appears rather uniform at these poles, suggesting transport across the membrane at a number of locations within these areas (Escoffre et al., 2009). While DNA also enters the cells at these same poles with respect to the applied electric field, the distribution of DNA at the membrane is not uniform like propidium iodide, but rather is punctate (Escoffre, Rols, & Dean, 2011; Faurie et al., 2010; Golzio et al., 2002). These DNA "spots" aggregate at the pole facing the anode and are between 0.1 and 0.5 μm in size. The fact that DNA accumulates on the anode-facing pole suggests that the electric field causes electrophoretic movement of DNA to this face of the cells (Figure 1(B)). While the number of these DNA spots at the membrane does not increase, they do appear to grow in size as the field is applied, but once the field is removed, the growth halts (Escoffre, Portet, et al., 2011; Golzio et al., 2002). These spots remained visible at the membrane for at least 10 min after removal of the electric field, but by this time the DNA was inaccessible to the extracellular environment. When a DNA-staining dye, TOTO, was added to electroporated cells within several seconds of removal of the field, the DNA at the anode pole was able to bind the dye, suggesting that it is bound to the surface of the cell and not yet internalized. By 30 min after electroporation, the DNA spots had migrated to areas within the cell, and the majority of the fluorescent DNA still appeared to remain in these focused areas as opposed to being diffused throughout the cell. However, if the dye was added 10 min after cessation of the pulses, no staining of DNA by TOTO was detected (Golzio et al., 2002).

The mechanism by which DNA actually enters the cell remains unknown. A number of theoretical studies suggest that DNA gains direct entry into the cytosol, and experiments with planar lipid bilayers and giant unilamellar vesicles have also shown that electroporation can cause direct entry of DNA across the membrane (Hristova, Tsoneva, & Neumann, 1997; Portet et al., 2012, 2009; Riske & Dimova, 2005). However, studies with intact cells are not as clear. The fact that DNA accumulates, concomitant with electroporation, in spots at the membrane that cannot be removed upon reversal of the polarity of the electric field has led to the conclusion that DNA must be trapped within lipid vesicles at the membrane (Golzio et al., 2002). Several groups have interpreted these findings to suggest that DNA is endocytosed or at least encased within electroporation-induced membrane vesicles. In support of this, studies have shown that, although depletion of cholesterol from cells does not alter the number of DNA aggregates/spots that appear at the hyperpolarized membrane upon electroporation, it can decrease transfection efficiency by three- to sixfold in the same cells. Since cholesterol depletion inhibits both clathrin- and caveolae/lipid raft-mediated endocytosis, these results have been interpreted to imply that inhibition of these two modes of endocytosis reduces the internalization of DNA. However, it should be pointed out that no study has directly followed labeled DNA across the membrane in these types of cholesterol depletion studies; therefore, reliance on downstream expression as indicators of distinct pathways of uptake is not definitive. Moreover, if DNA enters the cell via electroporation-stimulated endocytosis, it is unclear whether this population of DNA is actually responsible for productive transfection. In other words, any DNA that enters the cell via electroporation-

induced endocytosis could represent a dead end for transfection, whereas DNA entering the cytoplasm by direct translocation could drive all expression seen. Similar dead-end cell entry pathways have been described and debated for a number of viruses over the years (Sieczkarski & Whittaker, 2005).

Once translocated into the cytosol, DNA must make its way to the nucleus in order to be expressed (Figure 3). Since as early as the 1940s, the cell cytoplasm has been described as resembling a reversible gel–sol system (Chambers, 1940). It is a complex system composed of multiple cytoskeletal elements, including actin microfilaments, microtubules, and intermediate filaments. These elements are organized into a complex, crowded latticework that is constantly remodeling in response to a variety of internal and external stimuli. A number of studies have demonstrated that passive diffusion of either double-stranded DNA fragments greater than 1000–2000 bp or macromolecule-sized solutes in HeLa cells and fibroblasts is essentially nonexistent (Lukacs et al., 2000; Seksek, Biwersi, & Verkman, 1997). Thus, in order to traffic to the nucleus, directed movement must be employed. Indeed, others and we have shown that plasmids utilize the microtubule network and its associated proteins to move to the nucleus (Badding & Dean, 2013; Badding, Lapek, Friedman, & Dean, 2013, Badding, Vaughan, & Dean, 2012; Geiger, Taylor, Glucksberg, & Dean, 2006; Rosazza et al., 2013; Vaughan & Dean, 2006; Vaughan et al., 2008). Within 15 min of electroporation in adherent cells, transfected plasmids have been shown to physically interact with transcription factors, including cAMP response element-binding protein (CREB); this binding is necessary for maximal movement along microtubules (Badding et al., 2012). Since CREB is present only in the nucleus and cytoplasm, its interaction with DNA suggests that at least a fraction of the electroporated DNA has entered the cytosol by this time. Once the DNA has been released into the cytoplasm (either upon escape from any endosomes or after direct entry across the plasma membrane, depending on which mechanism is predominant and productive for transfection), it binds to a number of sequence-specific and nonspecific DNA-binding proteins as well as a number of other proteins to form a large protein–DNA complex (Badding et al., 2013; Miller, Munkonge, Alton, & Dean, 2009; Munkonge et al., 2009). These additional proteins include a number of microtubule accessory proteins and the motors dynein and kinesin, as well as tubulin itself. Real-time particle tracking of quantum dot-labeled plasmids has shown that DNA moves along microtubules with kinetics and dynamics that are in line with those seen for microtubule-based movement of organelles, virus particles, and proteins (Badding & Dean, 2013; Badding et al., 2013, Badding et al., 2012; Rosazza et al., 2013). Further, inhibition of dynein using antibodies or destabilization of microtubules using nocodazole abolishes the ability of DNA to move toward the nucleus (Rosazza et al., 2013; Vaughan & Dean, 2006).

Several recent studies have also implicated a role for actin filaments in the electroporation-mediated cell entry and/or cytoplasmic trafficking of plasmids in addition to that of the microtubule network (Rosazza et al., 2013, Rosazza, Escoffre, Zumbusch, & Rols, 2011). In experiments in which either green fluorescent protein (GFP)-actin or phalloidin-rhodamine-labeled actin was visualized along with DNA immediately or at various times after electroporation, both DNA and actin colocalized to the same spots/aggregates at the permeabilized membrane facing the anode (Rosazza et al., 2011). When latrunculin B was used to destabilize the actin network, the same number of individual DNA aggregates was

still seen at the same membrane locations with respect to the electric field, but only about half as much DNA was present at the spots. In a follow-up study, actin dynamics were perturbed with either latrunculin B or jasplakinolide, and the intracellular movement of electroporated plasmids was followed by single-molecule particle tracking (Rosazza et al., 2013). In this study, either depolymerization or stabilization of the actin network caused slight decreases in the percent of particles showing active transport or the total distance traveled by the particles, respectively. Proteomic studies from our laboratory have found that several actin-based motors (myosin 1B, 1C, and 9) are found in the protein–DNA complexes at early times after electroporation (15 min) along with a number of different microtubule-based motors (Badding et al., 2013), supporting a possible role for actin-based movement of DNA particles, at least at times between entry of DNA into the cytosol and binding to the microtubules.

Following the trafficking of plasmids to the interior of the cell, they must enter the nucleus for productive gene expression to take place. As for microtubule-based movement, it is the proteins that bind to the plasmids that mediate entry into the nucleus in nondividing cells following electroporation or any transfection method. Others and we have shown that when plasmids carry certain DNA sequences termed DNA nuclear targeting sequences (DTS), they form complexes with specific nuclear localization signal-containing proteins that in turn bind to importins for entry of the complex through the nuclear pore complex into the nucleus (Cramer et al., 2012; Dean, 1997; Dean, Dean, Muller, & Smith, 1999; Degiulio, Kaufman, & Dean, 2010; Langle-Rouault et al., 1998; Mesika, Grigoreva, Zohar, & Reich, 2001; Sacramento, Moraes, Denapolis, & Han, 2010; Vacik, Dean, Zimmer, & Dean, 1999). The most common proteins that bind to these sequences are transcription factors which are translated and many times retained in the cytoplasm to regulate their function (Wente & Rout, 2010). If a plasmid containing a DTS is present in the cytoplasm, these cytoplasmic transcription factors can bind to this site before nuclear import to facilitate translocation into the nucleus (Badding et al., 2013, 2012; Miller et al., 2009; Miller & Dean, 2008). Alternatively, any plasmid, regardless of sequence, has the ability to enter into the nucleus during mitosis, once it reaches the area of the nuclear periphery. Either way, these trafficking events through the cytoplasm and into the nucleus are not unique to electroporation, but are seen in all other nonviral methods for gene delivery.

4. PULSE PARAMETERS

Two types of wave forms are used in electroporation of cultured cells: square wave and exponential decay. As for any transfection method, optimization is necessary to determine which works best for desired cell types. Square-wave electroporators deliver pulses to cells with a set voltage for a defined amount of time. These pulses typically last for between 100 μs and 100 ms, and fields are usually between 100 and 1300 v/cm (100–500 V in a 0.4 cm cuvette). Exponential decay electroporators deliver a peak of energy that dissipates exponentially, giving a time constant (τ), a function of the resistance of the sample and the capacitance set on the instrument and which corresponds to the time necessary for the charge to decrease to about 37% of the initial voltage. While both wave forms work well for bacteria, yeast, and cultured mammalian and plant cells in suspension and in *in vivo*

applications in living tissues, square waves have been consistently shown to be safer, induce less damage, and yield better gene transfer and expression.

It has been shown that the duration of the square-wave pulse, the number of pulses, the magnitude of the field, and the frequency with which the pulses are applied, can all influence electroporation-mediated gene delivery (Gehl & Mir, 1999; Gehl et al., 1999; Zaharoff, Henshaw, Mossop, & Yuan, 2008). Studies have shown that several different combinations of pulse lengths and field strengths can be used for electroporation in skeletal muscle and tumors (Lucas, Heller, Coppola, & Heller, 2002; Satkauskas et al., 2002). In several seminal papers using electroporation, high field strengths (1000–2000 V/cm) and multiple short pulses (<100 μs) were used (Heller et al., 1996; Titomirov, Sukharev, & Kistanova, 1991), but lower field strengths (200 V/cm) with multiple longer pulses (10–20 ms) can work in a number of tissues as well (Aihara & Miyazaki, 1998; Mathiesen, 1999; Mir et al., 1999). A more recent report from Mir has suggested that a combination of the two is even more effective in gene transfer (Satkauskas et al., 2002). Regardless of the specifics of low field/long pulses, high field/short pulses, or combinations, empirical optimization by the investigator must be carried out for every tissue and every DNA that will be used. Thus, there is no single "optimized" set of pulse conditions.

Since cell shape, size, and composition can all play a role in how cells sense and respond to the electric field, it is possible to use the field to limit permeabilization and electroporation to specific cell types within a tissue. For example, since larger cells are permeabilized at lower field strengths than smaller cells, it is possible to electroporate specific cells within a tissue if they are sufficiently different in size than other cells within the tissue (Valic et al., 2003). In one study, this fact was exploited to specifically target lipid-laden adipocytes which are much larger (>fivefold) than other cells in adipose tissue for gene transfer. When a series of seven, 20 ms pulses at 500 V/cm were administered to suprascapular white adipose tissue in mice, 99% of cells expressing the delivered transgene were adipocytes, which made up only 16% of the cells within the tissue (Granneman, Li, Lu, & Tilak, 2004).

One major misconception of electroporation, or any gene delivery method for that matter, is that the ultimate goal is to achieve as high a level of gene expression as possible. Indeed, researchers routinely optimize delivery parameters using either luciferase-expressing constructs to measure absolute levels of gene expression or GFP-expressing constructs to measure the fraction of cells expressing their DNA. While maximization of gene transfer and expression is understandable in light of the perception that nonviral gene delivery is "less efficient" than viral gene delivery and that there is a need to reach these viral levels of gene transfer and expression in many cases, or perhaps even all cases, obtaining the maximal amount of a transgene may not be the best approach for therapeutic needs. For example, in studies from our laboratory several years ago, we delivered plasmids expressing subunits of the Na^+/K^+-ATPase to the lungs of mice and rats with the goal of achieving the same levels of transgene expression as seen using adenoviral vectors to transfer the same genes (Factor et al., 1998; Machado-Aranda et al., 2005). Even under "optimized" conditions of electroporation-mediated gene delivery, an adenoviral vector still gave roughly fivefold more expression of the gene product as determined by western blot. However, when we measured the physiological response mediated by the transferred gene product, both adenoviral and

electroporation-mediated transfer gave equivalent activities, which is the ultimate goal. Thus, as the influential twentieth-century modernist architect Ludwig Mies van der Rohe said, often “less is more.”

5. ELECTRODES

Any number of electrode types can be used in applying the electric field—the choice is dependent on the tissue and the goals for expression that are desired. The first types of electrodes for cultured cells were parallel plates inserted into a solution of suspended cells. This was followed up by incorporating the plates into the walls of cuvettes, leading to the electroporation cuvettes that are widely used today. Since animals or parts of them could not be jammed into these cuvettes, other electrode configurations were developed (Figure 4). The first two general designs were based on surface-applied plate electrodes (configured as tweezers or calipers) and penetrating needles for application into the tissue to be electroporated (Gilbert, Jaroszeski, & Heller, 1997). Needle electrodes have come in multiple flavors, from two needles that can be inserted to varying depth to multiple needle arrays containing six or more needles that fire in defined patterns to deliver the field with changing polarity to maximize cell permeabilization, DNA electrophoresis, and gene delivery (Jaroszeski, Gilbert, & Heller, 1997). The needles can be insulated along their shaft with only their tips being able to generate the field. These types of needles are excellent in delivering fields at precise depths within a tissue and act as point charges. The maximal field delivered from these needles is at the tip and falls off as the distance from the tip increases (Sel, Mazeres, Teissie, & Miklavcic, 2003). Such insulated needles can be inserted through the skin into a specific muscle to deliver the electric field only to the muscle and not to all cells and tissues along the shaft. Alternatively, uninsulated needles are very well suited for delivering the field over a wider area (all along the length of the needle at all depths). Since electroporation only occurs where the electric field and DNA overlap, some groups and companies have incorporated needles for DNA delivery into the needle arrays.

Like penetrating needle electrodes, a number of plate electrodes have been developed from simple plates that are placed on the skin to those constrained by tweezers or calipers. The advantage to this type of electrode is that the field applied between the plates is larger and more uniform (Gehl et al., 1999). Again, as in needle arrays, a four-plate electrode has been developed for skin electroporation that is highly effective and can be used to rotate the applied field leading to greater gene delivery (Heller et al., 2007). While these plate electrodes are rather small using electrode surface areas of less than 1 cm^2, larger plate electrodes can also be used safely for electroporation of larger organs or animals. For example, we have used defibrillation paddles and defibrillation electrode pads (10 × 15 cm) to deliver fields across the chests of 40-kg pigs for efficient gene delivery into the lungs (Dean, Barravecchia, Danziger, & Lin, 2011; Emr et al., in press).

Other designs include electrodes based on electroporation cuvettes. Since the vascular wall of vessels, with the exception of very large arteries such as the aorta cannot be injected with DNA, we designed an electrode that resembles a spoon in which the vessel segment to be electroporated can be placed and bathed in a DNA solution (Martin, Young, Benoit, & Dean, 2000; Young & Dean, 2002). Wires on either side of the vessel act as electrodes to apply the

field. Various sizes of this electrode have been used to transfect small mesenteric neurovascular bundles in mice (less than 0.5 mm diameter) and rats (~1 mm diameter), to the rat carotid (3 mm), and even segments of mouse and rat small intestine (up to 0.8 cm diameter).

Another innovative electrode design was developed by Heller for use in the skin (Donate, Coppola, Cruz, & Heller, 2011; Ferraro et al., 2011; Guo et al., 2011; Heller et al., 2010). One negative side effect of electroporation is that the delivery of electric pulses into skin or muscle is accompanied by minor pain that is proportional to the field strength. Thus, if the field strength (and/or pulse length) can be reduced, less pain will be experienced, making the approach more clinically acceptable. The approach taken by the Heller group was to reduce the distance between electrodes so that lower voltages can be applied to the skin, while increasing the number of individual electrode pairs to increase the size of the area to be electroporated. The noninvasive multielectrode array (MEA) has 16 electrodes placed 2 mm apart in a 4 × 4 array (Heller et al., 2010). Thus, to achieve a field of 250 V/cm, only 50 V needs to be applied between electrode pairs as opposed to 200 V for the 8 mm total gap. Pulses are administered in a sequence that utilizes four electrodes at a time forming 2 × 2 mm squares (nine total squares). Pulses are applied in pairs, in two directions, perpendicular to each other (18 pulses) for four rounds of pulsing (72 total pulses). Using this approach, high levels of gene expression can be obtained in the skin that are equal to that seen using plate electrodes with the benefit of using low applied energy for reduced sensation (Heller et al., 2010).

6. TISSUES THAT CAN BE DIRECTLY INJECTED: LIVER AND SKELETAL MUSCLE

Unlike transfection of cultured cells, it has been demonstrated that direct injection of DNA without any carrier or other physical method can result in gene transfer and expression of transgenes in a number of tissues. In 1990, Harold Varmus' group showed that plasmids directly injected into the livers of ground squirrels showed gene expression within 8 weeks in the absence of any carrier (Seeger, Ganem, & Varmus, 1984). Similarly, in the same year, Jon Wolff showed that plasmids can be directly injected into mouse skeletal muscle and drive relatively high-level gene expression within 8 h that lasted for at least 2 months (Wolff et al., 1990). These studies went on to form the basis for all studies in DNA vaccines. Other organs that have shown expression following injection of naked DNA alone include the lung (Pringle et al., 2007), kidney (Barry et al., 1999; Wang, Chao, & Chao, 1995), heart (Li, Welikson, Vikstrom, & Leinwand, 1997; Wang et al., 1995), and tumors (Yang & Huang, 1996), among others. In all cases, the levels of gene transfer are low compared to methods of viral delivery or delivery of the plasmids complexed with lipids, calcium phosphate, or polymers. In a seminal paper, Richard Heller and colleagues showed in 1996 that direct injection of plasmid DNA into the rat liver and immediate application of six square-wave electric pulses (1000 V/cm, 99 μs each, 1 Hz) could increase gene expression by a factor of 10 compared to DNA injection alone (Heller et al., 1996). Field strengths less than this gave no increase in gene expression over DNA alone, and when a field strength of 2000 V/cm was used, necrosis of liver tissue was observed. Expression was detected within 48 h, persisted

for at least 21 days, and was almost entirely localized to hepatocytes. Indeed, between 30% and 35% of hepatocytes in the electroporated liver were found to be positive in transgene expression. Taken together, these experiments demonstrated the utility of this simple procedure for enhanced gene delivery and were the basis for the first "optimized" parameters for electroporation: six to eight short pulses (100 μs) at high fields (1000–1300 V/cm).

In a series of papers published in the late 1990s, multiple groups showed that the application of electric pulses caused up to a 1000-fold increase in the levels of gene expression compared to DNA injection alone in mouse skeletal muscle (Aihara & Miyazaki, 1998; Mathiesen, 1999; Mir et al., 1999). In all cases, DNA was purified and suspended in physiological saline (0.9% NaCl) and injected into mouse tibialis and soleus muscles. In most cases, between 30 and 60 μl of DNA was injected into the muscles that are about twice that in volume. It has been demonstrated that when small volumes are used, the distribution of gene expression is more focused around the site of injection and not dispersed evenly throughout the tissue. While not a major issue in small animals, as muscle electroporation is applied to larger species, this becomes an important variable. Immediately following DNA injection, a series of electric pulses were applied, in all cases using penetrating two-needle electrodes placed into the muscle. These early studies characterized the pulse parameters and outcomes to show that DNA expression was dose dependent with up to 20 ng of luciferase transgene being expressed per muscle by 2 days (Mir et al., 1999). Both the distribution of cells taking up and expressing DNA increases upon electroporation as does the absolute amount of gene product per cell (this is likely due to increased delivery of plasmids into each cell). In a different study, secreted IL-5 was expressed and similar doses of plasmid yielded 20–30 ng of IL-5 per ml of serum (since the blood volume of a mouse is approximately 2 ml, these expression levels are similar) (Aihara & Miyazaki, 1998). Delivery and expression were also dependent on pulse length with high-level expression seen between 10 and 50 ms per pulse, when using fields of 200 V/cm. As in liver, expression was field dependent with a threshold seen around 100 V/cm; below this, expression was similar to that without any applied field. Expression increased with fields between 100 and 200 V/cm and then dropped off at fields above 200 V/cm (Aihara & Miyazaki, 1998; Mir et al., 1999). Pulse number also influenced gene delivery. Even with one pulse (200 V/cm, 20 ms), expression was twofold higher than in non-electroporated muscles. Expression maximized with between six and eight pulses (about 10-fold higher than DNA alone). Thus, with these studies, the second "optimized" set of parameters was established using a series of eight pulses at around 200 V/cm with pulses at durations of around 20 ms.

Based on these studies with two tissues, two types of pulsing parameters were established for *in vivo* electroporation. In the seminal papers using electroporation, high field strengths (1000–1300 V/cm) and multiple short pulses (<100 μs) were used, while later papers shifted to lower field strengths (200 V/cm) with multiple longer pulses (10–20 ms). It should be stressed that neither type is better than the other for all tissues or DNAs to be expressed. Indeed, while early reports from Heller using the liver demonstrated that the short-pulse, high-field combination worked very well, several later reports from different groups showed that longer duration, lower field pulses worked equally as well in liver (Jaichandran et al., 2006; Suzuki, Shin, Fujikura, Matsuzaki, & Takata, 1998) (it should be pointed out that this group used surface plate electrodes, see below). Reports from Mir and colleagues have

suggested that a combination of the two is even more effective for gene transfer (Satkauskas et al., 2002). Since the function of the electric pulses has two components—to permeabilize the membrane and to electrophorese DNA, it was reasoned that a combination of one or more short high-voltage pulses (100 μs, 1000 V/cm) to permeabilize the membrane, followed by long (100 ms) low-voltage pulses for electrophoresis may be beneficial (Andre et al., 2008). While individual researchers have championed these various approaches and conditions, it is imperative to realize that optimization is crucial for any given tissue, electrode, plasmid, and expression goal for success.

7. ELECTROPORATION OF SKELETAL MUSCLE: DNA VACCINES AND USE AS BIOREACTORS

The ease and reproducibility of electroporation-mediated gene transfer to skeletal muscle have made it the most highly used target organ for this approach. Early excitement over the use of muscles to produce antigen for vaccine development following naked DNA delivery alone was amplified even more when electroporation could be incorporated as well. It was found that DNA injection alone into mouse skeletal muscle resulted in highly variable gene expression and subsequent humoral and cellular immune responses (Capone et al., 2006; Widera et al., 2000). These findings were recapitulated in higher animals including pigs and nonhuman primates (Babiuk et al., 2002; Zhao et al., 2006). One great advantage of electroporation is its greater reproducibility of gene delivery and expression, reducing variability between animals (Bettan et al., 2000). In the last 15 years, the use of electroporation-mediated DNA vaccination has exploded and moved to clinical trials and approved for veterinary use. For more detailed examples, several recent reviews are suggested (Gothelf & Gehl, 2012; Liu, 2011).

Following DNA injection and electroporation, plasmids are expressed throughout the muscle, resulting in transgene protein production in myocytes, resident dendritic cells, and monocytes that reside within the tissue. When dendritic cells are transfected directly, the produced transgene can be processed by the host cell machinery and presented with MHC class I or II molecules on the antigen-presenting cell, causing the cells to prime naïve T cells in the draining lymph nodes. When the transgene products form complexes with MHC class I molecules and prime naïve CD8+ T cells, cellular CTL responses are generated. Alternatively, the resident dendritic cells can engulf apoptotic or necrotic transfected myocytes or capture antigen secreted from transfected cells, resulting in the presentation of transgene products with MHC class II molecules, and priming of the immune system.

Although electroporation is touted as being extremely safe at the appropriate fields, some low-level inflammation and tissue redness can often result from the application of pulses. In the case of vaccination, this may provide a benefit (Babiuk et al., 2004). Upon electroporation in muscle or skin, low-level production and release of a number of different cytokines, including MIP-1, IP-10, and MCP-2, has been noted (Peng, Zhao, Xu, & Xu, 2007). These in turn cause the recruitment of neutrophils, monocytes, B cells, CD4+ and CD8+ T cells, and dendritic cells into the tissue (Babiuk et al., 2004; Liu, Kjeken, Mathiesen, & Barouch, 2008). While not enough to induce damage to the tissue, these

chemokines and infiltrating cells may help to set up the perfect environment for enhanced immune priming.

A large number of antigens have been expressed for a variety of bacteria, parasites, and viruses in the development of electroporation-mediated DNA vaccines. Notable targets include *Bacillus anthracis* (anthrax) (Luxembourg et al., 2008), Hepatitis B virus (Luxembourg, Hannaman, Ellefsen, Nakamura, & Bernard, 2006; Zhao & Xu, 2008), Hepatitis C virus (Capone et al., 2006; Weiland et al., 2013; Zucchelli et al., 2000), Herpes virus (Babiuk et al., 2002), HIV (Babiuk et al., 2004; Cristillo et al., 2008; Liu et al., 2008; Widera et al., 2000), Influenza (Chen, Fang, Li, Chang, & Chen, 2005; Kadowaki et al., 2000; Ogunremi et al., 2013; Qiu et al., 2006), *Mycobacterium tuberculosis* (Tollefsen et al., 2002; Zhang et al., 2007), and *Plasmodium falciparum* (malaria) (Ferraro et al., 2013; Kumar et al., 2013; LeBlanc, Vasquez, Hannaman, & Kumar, 2008). One problem hindering DNA vaccine technologies has been that in many studies, success is seen in small animal models, most commonly mice, but there have been issues with translation to larger species, such as pigs or nonhuman primates. However, successes have been found and have led to several clinical trials for electroporation-mediated DNA vaccines, including Phase I trials for HIV (Vasan et al., 2011). In the case of the Phase I trial for HIV, expression of HIV-1 gag, pol, env, nef, and tat elicited an improved HIV-specific cell-mediated immune response, but not a humoral one, compared to DNA vaccination alone.

Another application of electroporation-mediated gene delivery into skeletal muscle has been the use muscles as bioreactors for the systemic delivery of therapeutic proteins. Levels of gene expression following electroporation-mediated delivery into the tibialis muscle can exceed 1 μg of luciferase in the mouse by 2 days and expression can persist for greater than a year, making this an ideal tissue to exploit as a bioreactor (Hojman, Gissel, & Gehl, 2007). The protein that has seen the most activity in this area is erythropoietin (EPO) for anemia and β-thalassemia (Maruyama et al., 2001; Payen, Bettan, Rouyer-Fessard, Beuzard, & Scherman, 2001; Rizzuto et al., 2000; Samakoglu et al., 2001). One elegant study focused on EPO delivery found that optimization of electroporation parameters, plasmid design, and dose could give long-term (>9 months) correction of hemoglobin and hematocrit to levels that could alleviate clinical manifestations of anemia (Hojman et al., 2007). Further, this study used a novel tetracycline-on promoter to drive EPO expression such that in the presence of doxycycline, EPO expression from the plasmid was turned on. Using this plasmid, Gehl and colleagues demonstrated that EPO and the corresponding levels of hemoglobin could be tightly controlled by varying the dose and duration of doxycycline administration. Thus, not only was the muscle being used as a highly efficient bioreactor, but also as one with a sensitive rheostat.

8. ELECTROPORATION OF SKIN: VACCINATION, WOUND HEALING, AND CANCER

The skin of an average adult covers approximately 20,000 cm^2 and as such, provides an ample target for gene delivery. As a consequence, like skeletal muscle, the skin may be a suitable organ for large-scale protein production. Moreover, the skin contains a large number

of dendritic and Langerhans cells for antigen presentation, making it an excellent target for vaccine production. Indeed, Timitrov carried out the first *in vivo* electroporation-mediated gene transfer by delivering the neomycin resistance gene into the skin of newborn mice and subsequently isolating dermal fibroblasts to demonstrate transfer (Titomirov et al., 1991). Since then, a number of different approaches have been developed for the delivery of DNA and application of the electric field to this organ. One of the greatest goals for researchers, clinicians, and pharmaceutical companies is to develop as noninvasive and painless a method for this as possible. However, in almost all cases, the delivery of DNA itself into the skin raises problems. Since the outermost layer of the skin, the stratum corneum, is essentially a layer of flat, keratin-rich dead cells and makes up a formidable impermeable barrier to the cells of the epidermis and dermis underneath, noninvasive delivery of DNA is largely impossible. Thus, for DNA to be delivered to the living cells of the skin, it must be injected directly into the tissue. As for skeletal muscle, the majority of electrodes fall into two categories: penetrating needle electrodes and parallel plate electrodes. In both cases, the electrodes are placed surrounding the site of DNA injection. While needles can be injected into the tissue, the plate electrodes usually pinch an area of skin around the injection site. Both electrode types give good gene transfer (Gilbert et al., 1997; Heller et al., 2007). More recently, the noninvasive MEA developed by Heller has used nonpenetrating needles to apply the field between electrodes that are at a very short distance from one another so that they deliver a low voltage to the tissue, thus reducing pain and increasing control of delivery (Donate et al., 2011; Ferraro et al., 2011; Guo et al., 2011; Heller et al., 2010).

Duration of expression in the skin is less than that seen in skeletal muscle, with expression high within 1–2 days then dropping off between 1 and 2 weeks, depending on the study (Medi, Hoselton, Marepalli, & Singh, 2005; Pavselj & Preat, 2005; Zhang, Nolan, Kreitschitz, & Rabussay, 2002). In order to increase this duration, it has been shown that injections and electroporations of the same plasmid can be done every few days to maintain and prolong expression (Heller, Jaroszeski, Coppola, & Heller, 2008). The distribution of gene expression is also variable, depending on the field and how it is applied as well as how DNA is injected. In several studies using the MEA, gene expression is first detected in cells in the epidermis within 2 days, but by 7 days, these cells were moving into the stratum corneum (Guo et al., 2011). As for other tissues, multiple “optimal” pulse conditions have been found that mediate high-level expression, but these vary based on the electrode type used and the preference of the investigators. Using the MEA, robust expression is seen when fields of 250 V/cm and 150 ms pulses are used (Guo et al., 2011).

Several studies have been undertaken to develop electroporation-based gene therapies to enhance wound healing in the skin. The first used the MEA to deliver plasmids expressing VEGF in an ischemic rat skin flap model (Ferraro, Cruz, Coppola, & Heller, 2009). Electroporation significantly increased VEGF expression in the skin for 5 days and resulted in upregulated endothelial nitric oxide synthase, increased perfusion in the skin flap, and greatly improved wound healing. Another study expressed the LL-37 host defense peptide in full-thickness skin wounds using electroporation and showed that VEGF and IL-6 were upregulated and healing of the wounds improved over controls (Steinstraesser et al., 2014). Taken together, this is a new area that could have great clinical impact.

Since the skin contains an abundant number of professional antigen-presenting cells and is poised as a first line of defense for incoming antigens, its use as a site for vaccine production makes excellent sense. Indeed, a number of studies have expressed various antigens in the skin to take advantage of this high concentration of antigen-presenting cells. As in muscle, expression of antigens following DNA injection and electroporation generates both CD8+ and CD4+ responses (Brave, Nystrom, Roos, & Applequist, 2011; Glasspool-Malone, Somiari, Drabick, & Malone, 2000). An early study in the area by Préat and colleagues optimized expression of and immune response to luciferase in the skin following intradermal injection and electroporation and compared this to expression from muscle (Vandermeulen et al., 2007). While muscle was far superior to skin in terms of expression levels and immune responses generated, IFN-γ secretion by luciferase-stimulated splenocytes suggested that an efficient Th1 response was induced by both delivery routes. This group has also investigated the use of cell-specific promoters for expression in keratinocytes or dendritic cells and found that while the cell-specific promoters were able to drive low-level gene expression, they were unable to mount clear immune responses whereas general promoters that are ubiquitously active and strong (such as the cytomegalovirus (CMV) promoter) generated clear humoral and cellular immune responses, suggesting that generation of the immune response is dependent on the levels of antigen produced (Vandermeulen et al., 2009). As discussed above, the need for “painless” electroporation in the clinic has driven the development of needleless injection systems and nonpenetrating electrodes. The MEA developed by Heller has been used successfully to deliver the hepatitis B surface antigen in guinea pigs, an excellent model for skin vaccination due to its close resemblance to human skin. In these studies, robust expression of hepatitis B surface antigen was detected, as was minor inflammation and significant production of antibodies (Donate et al., 2011). A similar electrode was subsequently developed for use following intradermal DNA injection and has also been shown to elicit cellular and humoral responses against influenza NP antigen in mice, guinea pigs, and macaques (Broderick et al., 2011; Lin et al., 2011).

Electroporation-mediated gene delivery into the skin has also shown extreme promise in production of cytokines, receptor antibodies, and other agents to control cancer. While many of these approaches have been termed “vaccines” for cancer, they are not true vaccines since they are not generating immunity against the causative agent of the cancer (such as against HPV18 for cervical carcinoma), although they do provide protection from subsequent tumor challenge. For example, in one study in hamsters, the HER2 receptor was expressed in skin via electroporation and subsequently, the animals were challenged with HER2 positive carcinogen-induced oral carcinoma cells. Thirty-seven percent of animals that received the HER2 gene developed neoplasia compared to 74% of control animals (Berta et al., 2005).

Perhaps the best developed and most promising candidate for a cancer treatment using intradermal electroporation (or perhaps any type of electroporation) is the delivery of plasmids expressing IL-12 for the prevention, control, and treatment of metastatic melanoma. In 2002, Lucas and Heller demonstrated that expression of IL-12 in mice that had established B16.F10 melanoma tumors cured almost 50% of the mice of the tumor and caused 70% of these mice to be resistant to future challenge with B16.F10 cells (Lucas et al., 2002). Surprisingly, these results were seen only with intratumoral (e.g., intradermal)

injection/electroporation of the plasmids but not following intramuscular injection/electroporation of the plasmids, despite robust expression of IL-12 following both treatment regimens. Subsequent studies show that these mice were essentially cured of the tumors for at least 100 days and that the treatment/protection provided by intradermal/intratumoral injection/electroporation of the IL-12 plasmid also provided treatment of and protection from tumors at distant sites as well (Lucas & Heller, 2003). These studies culminated in a Phase I first-in-man clinical trial, published in 2008 (Daud et al., 2008). Intratumoral injection and electroporation of the IL-12 plasmids caused marked tumor necrosis and lymphocytic infiltrates in a dose-dependent manner. Two out of nineteen patients with distant lesions that were not electroporated showed complete regression of metastases and the disease stabilized or showed partial response in an additional eight patients (Daud et al., 2008). These results suggest that intradermal/intratumoral DNA electroporation is a powerful tool for gene transfer and the treatment of metastatic cancer. A more detailed discussion of these studies and other clinical trials underway is presented elsewhere in this book.

9. ELECTROPORATION OF THE CARDIOVASCULAR SYSTEM

The vasculature presents a challenge to electroporation-mediated gene delivery using standard approaches. With the exception of a few large vessels, such as the ascending aorta, the walls of arteries and veins are too thin to be injected with DNA. Moreover, even if DNA could be delivered to the walls by injection, the architecture of the vessel wall would prevent the even distribution of DNA throughout it. Indeed, with multiple elastic laminas in large vessels and the tubelike structure of the vessel, injected DNA cannot freely diffuse throughout the wall. Consequently, in order to deliver genes to the vascular wall, addition of DNA can be from the outside or the inside of the vessel. For liposome and polymer-mediated delivery, intravascular injection is a simple and highly effective way to target the vascular endothelium, with the majority of gene transfer occurring in regions where the complexes get stuck due to reduced vessel diameter (e.g., the lung microvasculature). Alternatively, by injecting defined vessels that feed only one organ, transfer of DNA to that organ such as the kidney or liver or its proximal vasculature can also be achieved (Kobayashi et al., 2003; Tsujie, Isaka, Nakamura, Imai, & Hori, 2001). However, due to rapid flow rates, intravascular injection of DNA followed by electroporation has not been able to be used as a means to transfect vascular tissue, other than that in the liver or tumors (in this case, the electrodes are placed on the liver following intravascular injection of DNA) (Jaichandran et al., 2006). Various groups have transferred genes to the vasculature from within the lumen (Gunnett & Heistad, 2002; Matsumoto et al., 2001; Miyahara et al., 2006; Young & Dean, 2002). The advantage of this method is that with the appropriate device (e.g., double-balloon catheter or transient ligation; Figure 4), vectors can be delivered to defined regions of the vasculature with relatively simple methods that are clinically routine. The disadvantages are that in order for vectors to be delivered to the vessel lumen using double-balloon catheters, blood flow must be restricted, which may cause ischemia of the downstream vessels and tissues.

Alternatively, vectors can be administered from the adventitial surface of the vessel. The advantage of this approach of targeting defined regions of the vessel is that blood flow is not

restricted, allowing no potential for ischemic injury. However, the major disadvantage is that the region of the vessel to be targeted for gene delivery must be exposed, requiring surgery. Our laboratory developed an electrode system to deliver DNA to the vessel wall from the adventitial surface that resembled a spoon containing two parallel wires as electrodes that flanked a vessel segment that was positioned in the spoon (Figure 4) (Martin et al., 2000; Shirasawa, Rutland, Young, Dean, & Joseph, 2003; Young, Benoit, & Dean, 2003, Young, Zimmer, & Dean, 2008). Not only has this method been used to transfer DNA to vessels, but when catalytic oligodeoxynucleotides (DNAzymes) are added instead of plasmid, significant uptake of the oligonucleotides was detected along with detectable physiological activity (Nunamaker, Zhang, Shirasawa, Benoit, & Dean, 2003).

Most of the studies using this electrode system used the rat mesenteric vasculature as their target tissue. Instead of using an isolated vessel that has been dissected away from all adventitial tissue, we targeted the entire neurovascular bundle containing artery, vein, nerve, adventitial tissue, fat, and other tissue by laying it into the electrode, bathing it in the DNA solution (in 10 mM Tris, pH 8, 1 mM EDTA, and 140 mM NaCl), and applying a series of square-wave electric pulses. We found that 10 ms pulses at 200 V/cm gave very good expression that was sufficient to induce desired transgene responses (Shirasawa et al., 2003; Young et al., 2003, 2008). Using these parameters, an average of 400 pg of luciferase per centimeter of vessel (with a maximum of 2 ng per centimeter of vessel) was obtained 2 days post electroporation using 2 mg/ml DNA. When the field was raised to 400 V/cm, expression levels dropped off dramatically, likely due in part to tissue damage that was seen at this high field strength (Martin et al., 2000). This electrode system has been very successful in gene transfer to mouse and rat mesenteric vessels, the rat carotid, and mouse and rat femoral arteries. Moreover, by increasing the size of the electrode to accommodate larger tissues, we have also been able to successfully transfer genes without damage to segments of the mouse intestine itself.

The other main target organ in the cardiovascular system is the heart. Heller and colleagues first demonstrated that the porcine heart could be effectively electroporated by first injecting DNA into the myocardium and then applying asynchronous pulses to the heart using a penetrating four-needle array (Marshall et al., 2010). Since this study injected the myocardium directly and used electrodes that penetrated the heart, a thoracotomy was required to gain access to the heart. Electroporation using a field of 100 V/cm and 250 ms pulses increased gene delivery and expression of luciferase and VEGF by 25- and 5-fold, respectively, at 48 h compared to DNA injection alone. Although all animals survived following the application of these asynchronous pulses, all of the pigs showed electroporation-induced ventricular fibrillation that had to be treated by defibrillation. When synchronous pulses were applied within the QRS wave, maximal expression of luciferase was seen using a field of 120 V/cm and 20 ms pulses with 15-fold higher expression detected at day 7, compared to DNA alone, and VEGF expression with synchronous pulses was 6-fold higher with electroporation. The need to administer the synchronized pulses within the QRS wave necessitated the shorter pulse lengths, thus perhaps dampening expression levels. Most importantly, using synchronized pulses, no ventricular fibrillation was detected. Subsequent studies testing different electrode configurations found that multiple electrode configurations (with varying depths of tissue penetration) gave

significantly high gene expression compared to DNA alone and resulted in the distribution of gene delivery and expression throughout the myocardium at the sites of injection (Hargrave et al., 2013).

Another study in rats delivered DNA into the occluded coronary artery prior to application of the electric field using two-plate electrodes placed directly on the heart following thoracotomy (Ayuni et al., 2010). The field applied was 200 V/cm with a train of eight, 20 ms pulses. In these experiments, the pulses were applied asynchronously and resulted in no ventricular fibrillation, although a very transient asystole followed the electroporation in several of the animals that corrected itself without intervention.

Two additional studies have used electroporation-mediated gene delivery into the hearts of dogs to study the mechanisms of vagal-induced atrial fibrillation and to develop a therapy. Arora and colleagues demonstrated that atrial-selective attenuation of vagal signaling can be achieved by a specific G-protein C-terminal peptide delivered to the posterior left atrium (Aistrup et al., 2009). To develop a treatment method for this disease based on these findings, plasmids encoding these G-protein peptides were injected at multiple sites in posterior left atrium and a series of eight pulses (150 V/cm pulses at 20 ms each) were applied using two nonpenetrating needles (Aistrup et al., 2011). Not only were the transgenes expressed at the mRNA and protein levels, but they demonstrated physiological effects and were able to abrogate vagal-induced atrial fibrillation. Taken together, these studies show that electroporation has the ability to transfer genes safely to the heart and vasculature for a therapeutic outcome.

10. GENE DELIVERY INTO THE LUNG USING ELECTROPORATION

A number of viral and nonviral methods of gene delivery into the lung have been developed, but many have had limitations. The two most notable limitations are the fact that many delivery approaches, especially viral methods, result in significant inflammation and that all methods result in the delivery of genes only into either the pulmonary epithelium (if delivery is via the airways) or the pulmonary endothelium (if delivery is intravascular). Since many lung diseases are either the result of, or are exacerbated by inflammation (e.g., asthma, acute lung injury, pulmonary fibrosis, etc.), techniques that increase local or systemic inflammation are undesirable. Since electroporation has the ability to drive DNA into different tissue layers, likely through its electrophoretic component, and shows very little inflammatory response in other tissues, it is an excellent choice for gene delivery into the lung.

We developed a simple method of electroporation-mediated gene delivery into the lungs: DNA is suspended in saline and delivered to the lungs by aspiration followed by electroporation using electrodes placed on either side of the chest (Dean, Machado-Aranda, Blair-Parks, Yeldandi, & Young, 2003; Machado-Aranda et al., 2005; Young, Barravecchia, & Dean, 2014). The major benefit of this approach is that it is very fast and simple: animals are lightly anesthetized, held in a position resembling a standing human, and the tongue of the animal is pulled out of the mouth with a pair of forceps. The DNA solution (50–100 μl for mice) is then delivered into the mouth, so 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. 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 are applied to the chest. Flat electrodes, such as disposable, conformable, pediatric pacemaker electrodes, are placed on either side of the chest, usually under the armpits of the animals. A series of square-wave electric pulses are then applied across the chest, which causes the animal to jump slightly, unless paralytics have been administered (Emr et al., in press). 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 post treatment.

We have found that gene transfer and expression are dependent on DNA dose with 10 μg of a CMV promoter-driven, luciferase-expressing plasmid giving about 2 pg of gene product per lung, whereas administration of 40 or 100 μg of plasmid yields 20 pg and 2 ng per lung, respectively, 2 days after electroporation (Dean et al., 2003). The duration of gene expression was dependent on the promoter used in the plasmids: while the CMV immediate-early promoter/enhancer drove robust gene expression for up to 4–5 days in mouse and rat lungs with detectable gene expression appearing as early as 6 h, the human ubiquitin C promoter gave sustained gene expression for up to 6 months (Dean et al., 2003; Machado-Aranda et al., 2005; Pringle et al., 2007). As in other tissues, gene transfer is dependent also on field strength with a high level of expression being seen using trains of eight pulses of between 10 and 20 ms duration each at a field strength of 200 V/cm in mice, rats, and pigs (Dean et al., 2003; Emr et al., in press; Machado-Aranda et al., 2005). Unlike reports from others (Pringle et al., 2007), we have found no gene expression in the lungs without application of electric field (Dean et al., 2003; Machado-Aranda et al., 2005). As seen in the vasculature (Martin et al., 2000), when the field was increased to 400 V/cm, tissue damage, typically hemorrhage or inflammation, was observed in lungs. However, at 200 V/cm, there was no increase in hemorrhage, infiltrating lymphocytes or other cells, pulmonary edema, or alveolar wall thickening compared to control, unelectroporated lungs (Dean et al., 2003). Further, there was no increase in IL-6 or IFN-γ levels in the bronchial alveolar lavage fluid of animals electroporated with or without DNA compared to naïve animals or those receiving DNA only, and only a slight increase in TNF-α levels in electroporated mice compared to DNA-only mice (Dean et al., 2003; Zhou, Norton, Zhang, & Dean, 2007). Since TLR9, the innate immunity sensor in unmethylated CpG motifs on plasmid DNA, resides in the endosomal compartment, if electroporation is driving the bulk of the DNA into the cytosol directly, the DNA would never activate this pathway. Indeed, when plasmids were electroporated into TLR9-expressing or TLR9-knock out cells, IL-8 (a downstream TLR9-activated gene) expression was not increased compared to controls (Zhou et al., 2007). 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.

The only drawback to this approach of pulmonary gene delivery is one of perception. The most common argument against transthoracic electroporation is that the application of an electric field across the chest may likely lead to cardiac arrhythmias, fibrillation, and

dysfunction. In the mouse and rat, application of a 200V/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, the argument goes, if the technique is to be scaled up for human application, the resulting energy would be much higher. We have had no mortality due to electroporation alone (n = 30 mice and 38 rats) and less than 5% mortality due to drugs, surgery, endotracheal tube placement, or fluid delivery (n ≥1000 mice and 400 rats). In 40 kg pigs, the gap between electrodes across the chest is ~15 cm. We found the optimal pulsing parameters, in terms of safety and maximal gene expression, used eight, 150 μs pulses at between 130 and 150 V/cm. Using these conditions, we have seen no deaths (n = 90 treated animals), no cardiac abnormalities, and found that the energy used for electroporation was less than 4 J (0.1 J/kg). This is much less than that used in many pacing modalities in humans and/or pigs and is low enough not to cause any arrhythmias or arrest. Since Advanced Cardiovascular Life Support guidelines use 200–360 J for emergent cardioversion on an adult, and 50–360 J for synchronized cardioversion, this procedure is not unreasonable (Field et al., 2010; Link et al., 2010; Neumar et al., 2010). Further, not only was the total energy 50- to 75-fold less, but the current delivered to the animals was a fraction of that delivered during cardioversion. Thus, the procedure is safe and effective for gene delivery in animals that are roughly the size of humans.

Since electroporation yields high levels of gene expression with little, if any, inflammation or trauma to tissues at the appropriate field strengths, we used this technique to transfer plasmids expressing the rat Na^+/K^+-ATPase β1 subunit to mouse and rat lungs and measured alveolar fluid clearance 2 or 3 days later (Machado-Aranda et al., 2005; Mutlu et al., 2007). This protein mediates movement of Na^+ and K^+ across the alveolar epithelium, resulting in net fluid movement into the interstitium and circulation. Overexpression of this protein could aid in edema removal in lungs with acute lung injury or acute respiratory distress syndrome (ARDS). Transfer of the β1 subunit by electroporation caused an 80% increase in alveolar fluid clearance in isolated rat lungs and a 60% increase in mouse lungs, whereas no significant changes in the rates of fluid clearance were detected in lungs that received DNA without electroporation (DNA only) or an empty plasmid (Machado-Aranda et al., 2005; Mutlu et al., 2007). When applied to a model of acute lung injury using mice exposed to lipopolysaccharide (LPS), we demonstrated that gene transfer of Na^+/K^+-ATPase β1 subunit both protected from subsequent LPS-induced lung injury and reduced injury in a lung that had been injured with LPS prior to any gene delivery (Mutlu et al., 2007). More recently, another group has shown that transfer of the Na^+/K^+-ATPase β1 subunit to mouse lungs can also protect from acute lung injury in a contusion model of lung injury (Machado-Aranda, Suresh, Yu, & Raghavendran, 2012).

In recent studies, we have seen similar improvements in outcome in a model of severe sepsis-induced acute lung injury/ARDS in pigs (Emr et al., in press). In these experiments, we used a “two-hit” model consisting of peritoneal contamination with a fecal-blood clot mixture and intestinal ischemia-reperfusion. Following injury, the animals were maintained in an intensive care unit with round-the-clock medical support for 48 h using the accepted drugs and ventilation strategies that are the clinical standard of care for patients with ARDS. Without intervention, 50% of the animals survived to the 48 h end point. We found that electroporation-mediated gene transfer of the Na^+/K^+-ATPase β1 subunit 4 h after injury

improved lung function, maintained healthy lung architecture, reduced lung inflammation, and improved survival compared to animals electroporated with an empty plasmid (Emr et al., in press). The lung injury that is induced in this model is perhaps the most representative of what is seen in the neonatal, pediatric, and adult intensive care units following sepsis-induced ARDS and provides the most stringent and clinically relevant model in which we can evaluate electroporation for gene transfer in the lungs.

Several other groups have also developed protocols of electroporation-mediated gene delivery in the lungs. Schmid and colleagues routinely perform a thoracotomy to expose the lungs for electroporation. DNA in saline is either delivered intranasally to the lungs prior to thoracotomy and electroporation or to one lobe of the lung using a catheter following thoracotomy (Pringle et al., 2007; Gazdhar et al., 2006, 2007, 2013). In both cases, the electric field is then applied to the left lung using plate electrodes on either side of the exposed lung (not the chest), a chest drain is placed into the hemithorax, and the chest is closed with sutures. As with transthoracic electroporation, gene delivery and expression are 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 1000-fold more expression seen in the electroporated lobes (Pringle et al., 2007; Gazdhar et al., 2006). 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. Another recent study using this approach has shown that delivery of hepatocyte growth factor reduced bleomycin-induced pulmonary fibrosis in mice as assessed by histology, hydroxyproline determination, and designbased stereology compared with controls (Gazdhar et al., 2013). Electroporation has also been used to transfer the gene for keratinocyte growth factor (KGF) to lungs following pulmonary resection to aid in repair (Matsumoto et al., 2009). It is well documented that the lung undergoes compensatory growth after injury or surgical resection in a number of animal models, and that levels of KGF are increased during this proliferation. When a KGF-expressing plasmid was electroporated into one lobe of the lung following removal of the other three lobes (a standard model for pneumonectomy), 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. Taken together, these studies show that invasive and noninvasive methods of pulmonary gene delivery are safe and can have profound benefit for lung diseases in preclinical models.

11. CONCLUSIONS

Of the physical methods of nonviral gene delivery, electroporation is the most developed and has had the greatest impact to date. The benefits of electroporation in gene delivery are evident: reduced inflammation and immune response, ease and relatively low cost of instrumentation, and increasing levels of gene transfer and expression, nearing and perhaps surpassing those of viral vectors. This ease of use, safety profile, and ability to generate high-level gene transfer and expression have evolved from use in isolated cells to applications in living tissues in animals and humans in just the past 30 years. Its use as a platform technology is boundless. This can be seen in the myriad of applications to various tissues, from solid tissues that can be readily injected with DNA and electroporated with

simple needles to fragile internal organs that present different challenges for DNA administration and field application. Regardless of these potential difficulties, the fact that successful and safe gene delivery has been achieved in the laboratory and in human clinical trials points to the potential of this nonviral approach in multiple areas of gene therapy.

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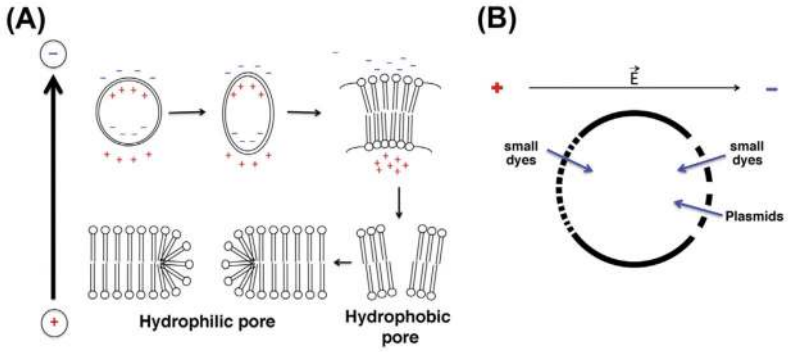

Figure 1. Model for electropermeabilization and electroporation

(A) The presence of an applied electric field induces movement of and redistribution of macromolecular dipoles within and outside the cell, resulting in accumulation of charges across the membrane. Once the transmembrane potential exceeds the dielectric strength of the membrane, transient permeation events occur, generating small hydrophilic pores that stabilize and coalesce into larger pores to allow movement of large molecules such as DNA. (B) The permeabilization of the cell induced by the electric field occurs differentially with respect to pore size: a number of small pores are formed at both poles of the cell, but larger pores capable of allowing DNA entry only form at the anode-facing pole. *Modified from* Somiari et al. (2000) *and* Escoffre, Rols, et al. (2011).

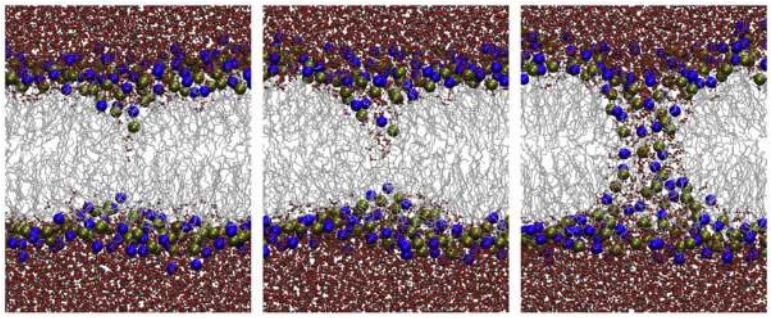

Figure 2. Molecular dynamics solution of the formation of hydrophilic water channels in a membrane in response to an applied electric field

Snapshots of the time evolution of water–lipid–water configurations under an external electric field of 500 MV/m. Panels (left to right) are 5.8, 6.7, and 7.3 ns from the start of the simulation with both water molecules (oxygen—red, hydrogen—gray) and lipid molecules (phosphorus—yellow, nitrogen—blue, lipid tail groups—silver) displayed. *Reprinted with permission from* Tokman et al. (2013). (For interpretation of the references to color in this figure legend see the color plate.)

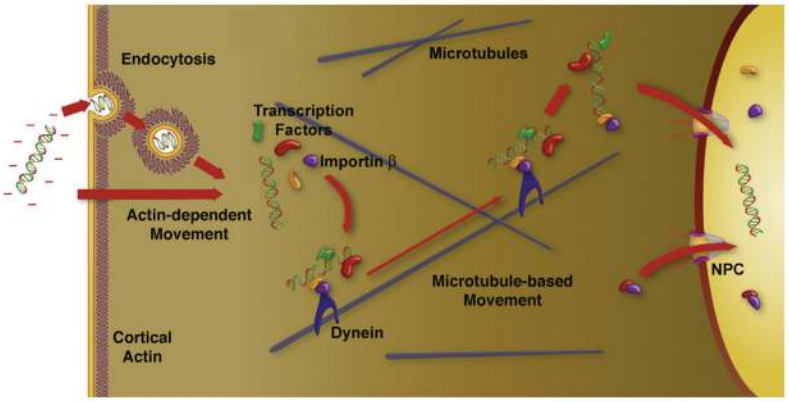

Figure 3. Postelectroporation trafficking of plasmids to the nucleus during gene transfer

Following electropermeabilization of the membrane, plasmids may enter the cell by either endocytosis and/or direct entry into the cytosol (Rosazza et al., 2013; Rosazza et al., 2011). The initial trafficking events near the cell surface appear to involve actin and actin-based movement. Once through the cortical actin layer and free in the cytoplasm, plasmids are rapidly complexed by a number of DNA-binding proteins present in the cytoplasm which in turn bind to other proteins to form large protein–DNA complexes (Badding et al., 2013). Transcription factors bound to DNA interact with importin β and other proteins that in turn link the complex to dynein for movement along microtubules to the nucleus where it falls apart at the nuclear periphery (Badding et al., 2012). Nuclear entry is then mediated by importin β in a sequence- and importin-dependent manner. (For interpretation of the references to color in this figure see the color plate.)

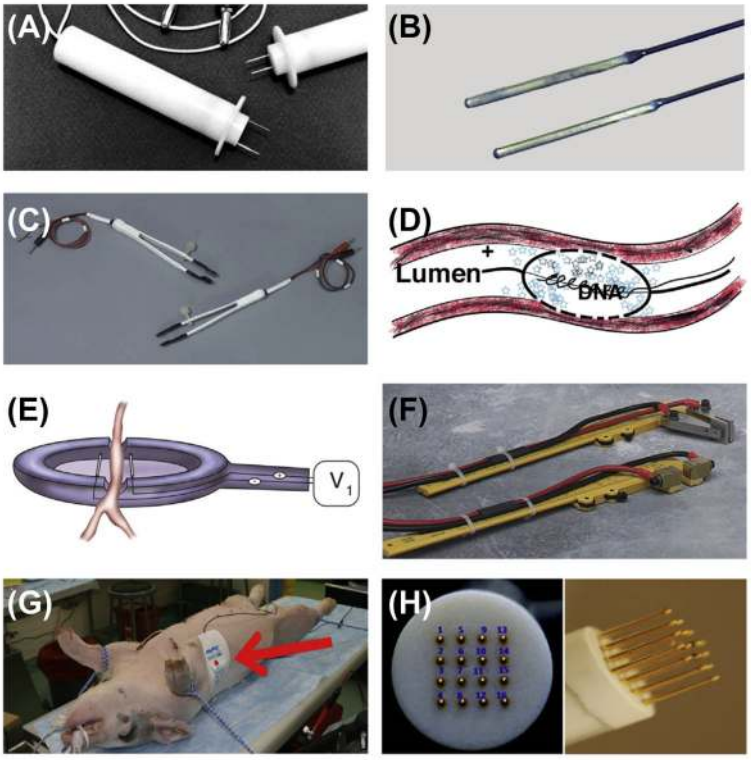

Figure 4. Examples of electrodes for electroporation

(A) Penetrating, two-needle arrays. (B) Nonpenetrating parallel needles (Genetrode electrodes, Genetronics, San Diego, CA, USA). (C) Plate electrodes (Tweezertrodes, BTX, Hollister, MA). (D) Cartoon of a balloon catheter-based electrode for delivery of DNA and electroporation. (E) Spoon electrode for vascular electroporation. (F) Caliper-mounted plate electrodes. (G) Conformable defibrillator pads for electroporation (arrow). (H) Multielectrode array (R. Heller, personal communication). (For interpretation of the references to color in this figure see the color plate.)