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Physical Non-Viral Gene Delivery Methods for Tissue Engineering

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Abstract—The integration of gene therapy into tissue engineering to control differentiation and direct tissue formation is not a new concept; however, successful delivery of nucleic acids into primary cells, progenitor cells, and stem cells has proven exceptionally challenging. Viral vectors are generally highly effective at delivering nucleic acids to a variety of cell populations, both dividing and non-dividing, yet these viral vectors are marred by significant safety concerns. Non-viral vectors are preferred for gene therapy, despite lower transfection efficiencies, and possess many customizable attributes that are desirable for tissue engineering applications. However, there is no single non-viral gene delivery strategy that "fits-all" cell types and tissues. Thus, there is a compelling opportunity to examine different non-viral vectors, especially physical vectors, and compare their relative degrees of success. This review examines the advantages and disadvantages of physical non-viral methods (i.e., microinjection, ballistic gene delivery, electroporation, sonoporation, laser irradiation, magnetofection, and electric field-induced molecular vibration), with particular attention given to electroporation because of its versatility, with further special emphasis on Nucleofection™. In addition, attributes of cellular character that can be used to improve differentiation strategies are examined for tissue engineering applications. Ultimately, electroporation exhibits a high transfection efficiency in many cell types, which is highly desirable for tissue engineering applications, but electroporation and other physical non-viral gene delivery methods are still limited by poor cell viability. Overcoming the challenge of poor cell viability in highly efficient physical non-viral techniques is the key to using gene delivery to enhance tissue engineering applications.

Keywords—Non-viral gene delivery, Microinjection, Ballistic gene delivery, Electroporation, Sonoporation, Laser irradiation, Magnetofection, Electric field-induced molecular vibration, Tissue engineering, Nucleofection.

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INTRODUCTION

Combining tissue engineering and gene therapy for clinical applications is not a new idea; however, figuring out how to successfully integrate them has proven to be a major challenge. Both tissue engineering and gene therapy strategies endeavor to treat degenerative diseases, cancers, trauma, and tissue defects that compromise the functions of organs.[106] However, both groups of strategies seem to utilize opposing methodologies. From a broad perspective, most tissue engineering strategies attempt to manipulate cellular behavior from an "outside-in" approach by varying cellular interactions with biomaterials, growth factors, and mechanical stimuli.[90] Conversely, gene therapy strategies attempt to control cellular behavior through an "inside-out" approach by directly delivering nucleic acids (i.e., DNA, siRNA, shRNA, miRNA, and antisense oligonucleotides) into cells to trigger or stall gene expression.[151,156] Several tissue engineering strategies utilize progenitor cells or stem cells to regenerate damaged tissues by seeding cells into biomaterial scaffolds.[183] The culture conditions, type of biomaterial, and mechanical stimuli can be used to direct progenitor and stem cells toward a specific lineage. Additionally, growth factors have been added to cell culture medium or encapsulated for controlled release from biomaterial scaffolds to promote cell differentiation.[40,95,100,105] However, growth factors can be costly and exhibit short half-lives.[133] Furthermore, once growth factors are deposited into cell culture or into extracellular matrices (ECM), there is no way to control how the growth factors will disperse and interact with cells, meaning that not all cells may interact with the growth factors uniformly or at all. Hence, a strategy where cells could produce, express, and control growth factors needed for differentiation would be beneficial for tissue engineering.

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Gene therapy has been investigated as a potential solution to overcome the challenges associated with using growth factors by delivering DNA to induce gene expression or delivering siRNA, shRNA, miRNA, or antisense oligonucleotides to knockdown gene expression; however, gene therapy has its own set of unique challenges.[33,67,77,81,171] Nucleic acids have proven difficult to deliver to a variety of primary cells, progenitor cells, and stem cells, and the ability to manipulate gene expression in targeted cells has proven challenging as well.[28]

The difficulty behind achieving successful transfection is due in part to the many barriers a delivery vector must overcome to gain access to the cellular membrane, cytoplasmic compartment, and interior of the nucleus before target genes can be expressed (Fig. 1). Nucleic acids must first be stabilized in some form to successfully navigate through the extracellular environment to avoid undergoing degradation from changes in pH, exposure to proteases and nucleases, and opsonization.[1] After navigating through the extracellular environment to the target cell, nucleic acids must properly associate with the cell membrane and cross the plasma membrane *via* penetration, electrostatic interaction, adsorption, or ligand mediated receptor binding.[38,66,88,120,148,150,157,178,188,201] Both Mercer *et al.*[109] and Conner *et al.*[30] have extensively reviewed cell entry methods through various endocytotic pathways. Once the nucleic acids reach the cytoplasmic compartment, nucleic acids must avoid degradation by endocytotic mechanisms and cytoplasmic nucleases.[86] If a nucleic acid enters the cell through an endocytotic mechanism, the complex must successfully escape the endosome before undergoing degradation by a lysosome or before the endosome is recycled back to the cell surface.[4,192,204] Once the nucleic acid has escaped the endosome, it must avoid degradation while trafficking through the highly crowded cell cytoplasm, which slows the diffusion of DNA to less than 1% of its rate in water.[103] RNA complexes and antisense oligonucleotides only need to reach mRNAs located in the cell cytoplasm; however, DNA complexes must cross the nuclear envelope before transcription can occur. We refer the reader to Merdan *et al.*,[110] who have provided a comprehensive review on the ''barriers'' that polymeric gene delivery vectors must overcome.

A variety of methods have been engineered to overcome the barriers to gene delivery, but each of these methods have their own unique advantages and disadvantages. Viral vectors have proven to be the most efficient and effective gene delivery method, and the benefits of viral vectors have been reviewed in depth by Kay *et al.*[82] and Zhang *et al.*[205] However, there are major concerns regarding the safety of viral

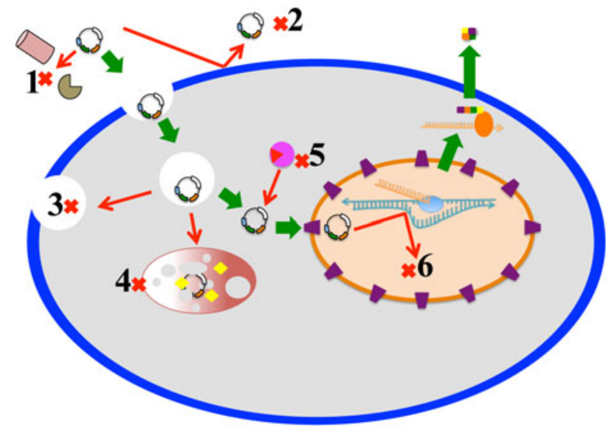

FIGURE 1. Gene delivery barriers. DNA must overcome several barriers during the delivery process to successfully produce desired gene expression. The green arrows are the pathway DNA must follow to induce gene expression, while the red arrows indicate potential barriers that prevent gene delivery. (1) DNA must avoid extracellular nucleases and (2) DNA must associate with the cellular membrane in some form to gain access to the cell *via* penetration, electrostatic interactions, adsorption, or ligand mediated receptor binding. DNA that enters through endocytosis must escape the endosome before the endosome (3) is recycled back to the cell membrane or (4) before the endosome matures into a lysosome, and DNA is degraded. In the cytoplasmic compartment, DNA must traffic toward the nuclear envelope and (5) avoid degradation by intracellular nucleases. Finally, to produce gene expression, (6) DNA must cross the nuclear envelope by transport through a nuclear pore (non-dividing cells) or passively re-locate into the nucleus between the disassembly and reformation of the nuclear envelope during mitosis (dividing cells). Gene expression is produced when enough intact DNA is transcribed in the nucleus into mRNA, and then translated into a protein, composed of amino acids, in the cytoplasm.

vectors such as toxicity, immunogenicity, and oncogenesis from insertional mutagenesis.[24,176] Furthermore, viral vectors possess restricted sequence sizes, and viral vectors can be laborious and costly to engineer. Viral vectors may possess innate tropisms to specific cell types or cell-selective promoters, which may limit their effectiveness in other cell populations.[181]

Non-viral methods are able to circumvent most of the concerns associated with viral gene delivery methods. However, non-viral methods exhibit lower delivery efficacies than viral gene delivery methods. Non-viral gene delivery methods can be broadly separated into chemical and physical approaches. Chemical approaches utilize cationic lipids, cationic polymers, and cell-penetrating peptides that can be engineered to target specific cells locally or systemically.[11–13,87] Chemical vectors avoid some safety concerns associated with disease-causing viral vehicles; however, effective doses of chemical vectors can be toxic, especially to sensitive cell populations because large doses are required to overcome the poor efficiency.[187] Chemical approaches seem to be most effective at

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targeting cancer cells *in vitro* and *in vivo*, and chemical vectors can be customized for specific tissue engineering applications; however, primary cells, progenitor cells, and stem cells have proven more difficult to transfect with chemical vectors. Despite the difficulty in transfecting primary cells, progenitor cells, and stem cells, there has been considerable enthusiasm for the further improvement of chemical vectors for the hope of one day achieving efficacies and efficiencies that could potentially mimic viral vectors.[51,52,61,78,110,112,113,132]

Chemical vectors face many challenges and obstacles because chemical vectors must overcome all of the previously stated barriers. Physical methods, on the other hand, have been shown to be effective at transfecting primary cells, progenitor cells, and stem cells through *in vitro*, *ex vivo*, and *in vivo* approaches.[108] This effectiveness may be in part due to the fact that physical approaches attempt to directly force nucleic acids into the cytoplasmic compartment or nucleus to achieve successful transfection. However, physical delivery methods face different limitations than chemical delivery methods. Depending on the physical delivery method used, the cell may sustain heavy trauma and initiate apoptotic or programmed cell death mechanisms. Thus, physical gene delivery strategies tend to exhibit lower cell viabilities and there is risk that the physical invasion may cause cells to senesce, which could negatively influence cell phenotype. Hence, a major obstacle that limits physical gene delivery in tissue engineering applications is low cell viability.

Over the last decade, significant improvements have been made in areas of microinjection, ballistic gene delivery, electroporation, sonoporation, and laser irradiation, presenting a refreshing opportunity for using non-viral vectors for tissue engineering applications. Nonetheless, different non-viral physical vectors are successful in different cell types. Thus, there is a need to examine which attributes of different non-viral physical vectors enable successful transfection and which physical characteristics of cells enable the ability of the cell to survive the transfection. Comparing the attributes of successful transfection techniques with characteristics of difficult-to-transfect cells that survive transfection methods may provide insight into physical details between the delivery vector and cell that may lead to more efficient gene delivery strategies for tissue engineering applications.

Hence, the goal of this review is to examine the advantages and disadvantages of non-viral physical vectors (i.e., microinjection, ballistic gene delivery, electroporation, sonoporation, laser irradiation, and lesser-known methods such as magnetofection and electric field-induced molecular vibration), with special attention given to electroporation because of its versatility. An additional goal of this review is to identify the physical characteristics of cells that survive and successfully express the target gene for the purpose of determining which physical features between delivery vector and cell type can be used to enhance differentiation strategies for tissue engineering applications.

PHYSICAL GENE DELIVERY STRATEGIES

While much attention has been given to viral and chemical non-viral delivery systems for transporting nucleic acids into cells, physical non-viral gene delivery methodologies have shown promise for transfecting difficult-to-transfect cells. Physical gene delivery methods attempt to deliver nucleic acids directly to the cell, and attempt to avoid complications associated with targeting, endocytotic pathways and immunogenicity.[89] However, physical gene delivery has its own set of advantages and disadvantages, which limits its use for certain applications. Microinjection is a technique that directly delivers DNA to the cell nucleus, whereas ballistic gene delivery uses a projectile to deliver DNA to the cell. Electroporation utilizes electrical potentials to induce the formation of pores in the cell membrane while sonoporation utilizes physical disturbances in the fluid to induce pores in the cell membrane for nucleic acid delivery. Laser irradiation perforates individual cells by focusing a laser beam on a localized area of the cell membrane to enable the entry of nucleic acids. Nonetheless, physical gene delivery has been favorable for tissue engineering applications where *ex vivo* approaches can be utilized. In the following sections, this review highlights some of the advantages and disadvantages of the most common (and uncommon) physical gene delivery methods from a tissue engineering perspective, provide examples of how physical gene delivery has been integrated into tissue engineering, and examine challenges that still need to be addressed to further improve the integration of gene therapy and tissue engineering.

Microinjection

Microinjection is perhaps the most direct nucleic acid delivery method of all of the physical delivery methods. The development of microneedles and the applications for which they can be used have expanded considerably over the past 30 years. Prausnitz *et al.*[144,146] have published excellent reviews regarding the evolution of microneedles for drug delivery applications and developing gene vaccines. Microneedles are no longer confined to the toolbox of cell biologists, but now are widely used by pharmaceutical manufacturers and are gaining popularity among bioengineers.

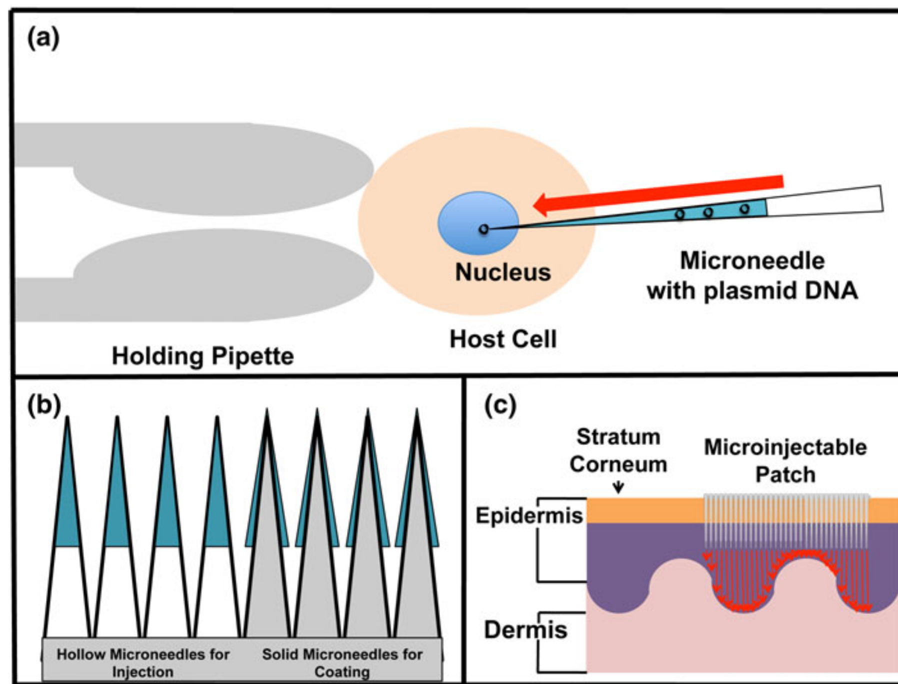

FIGURE 2. Microinjection. Microinjection strategies utilize microneedles to deliver DNA directly to cell nuclei. (a) In traditional microinjection, an individual cell is held in place by the tip of a pipette while a technician uses a microscope to pierce the cell membrane and nuclear envelope with a microneedle to deliver genetic material to the cell nucleus. (b) Microneedles can be fabricated so that the shaft is hollow and able to carry a suspension of genetic material for injection, or microneedles can be fabricated so that the shaft is solid and the tip is dipped in a suspension of genetic material for application to tissues *via* coating or scratching. (c) Microneedles can be arranged in arrays on patches that can be applied directly to the skin. The microneedle patches are capable of penetrating the stratum corneum and delivering drugs or genetic material to the epidermal tissues.

In their earliest form, microneedles were made of glass and used to inject nucleic acids directly into cellular cytoplasm and nuclei as illustrated in Fig. 2a.[193] Microinjection of nucleic acids became a robust method to transfect cells with specific amounts of pure nucleic acids.[92] However, the technique proved to be tedious, and no more than a few hundred cells at best could be transfected using this method. Despite the difficulties associated with microinjection, the technique persisted, and became quite valuable among cellular biologists for studying RNA trafficking,[135] immunocytochemistry,[94] and making transgenic animals.[9,27,32] Specifically, the ability to create transgenic animals became a powerful tool for illuminating functions of uncharacterized genes. Dahlhoff *et al.*[32] successfully created transgenic mice that express *Cre recombinase* in pancreatic B-cells by using pronuclear microinjection in C57BL fertilized oocytes. Today, single microneedles are used for transfecting rat and mouse ova for creating transgenic animals and for facilitating somatic nuclear transfer.

Beyond creating transgenic animals, microneedles are used for transdermal delivery of nucleic acids and drugs.[39] Microneedles can be arranged in arrays, which have proven to be advantageous for transdermal drug delivery as microneedles can penetrate the outer layer of the skin and the stratum corneum, and deliver drugs, nucleic acids, and macromolecules directly to the epidermis by creating microchannels in the stratum corneum.[134] Furthermore, microneedles can be easily fabricated and engineered to accommodate multiple delivery applications. For example, microneedles can be manufactured from silicon, metal, or biodegradable polymers.[131] As such, the size and shape of microneedles can be easily modified for drug delivery applications. Microneedles can be made hollow to be used as an injectable vehicle, or microneedles can be made solid and coated with drug or nucleic acid for direct application to tissue as illustrated in Figs. 2b and 2c.[50,159] Gill *et al.*[50] found that microneedles could be coated with microparticles containing a diameter no bigger than 10 μm, and successfully delivered payloads to the stratum corneum without wiping off on the skin. In addition, Choi *et al.*[26] and Daugimont *et al.*[34] have published exciting investigations on combining electroporation techniques with microinjection techniques for the purpose of creating DNA vaccinations delivered through the skin.

However, despite the benefits of microinjection, there are still limitations to the use of microinjection for tissue engineering. The use of single microneedles is highly inefficient for most tissue engineering applica-

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tions, as typically the transfection of cells is needed on a larger scale than a few hundred cells and on multiple overlapping cell layers. Additionally, rat and mouse ova are large cells that can accommodate microneedles, whereas some smaller cells such as fibroblasts are much more difficult to transfect with a microneedle. The diameter of the pipette tip and timing of injection (within the cell cycle) can play a major role in the ability of the cell membrane to reseal and survive.[182] Thus, a major factor that determines the success of the technique is the technical ability of the individual injecting the cells. Furthermore, when microneedles are used in an array format, care must be taken to ensure that the stiffness of the microneedles are strong enough to endure the shear forces of the tissue so that the needles do not break and tear the tissue layer or fail to distribute drugs or nucleic acids uniformly.

In summary, microneedles are a safe way to deliver nucleic acids to a variety of cell types directly, thus avoiding many of the gene delivery barriers mentioned earlier; however, single cell transfections are inefficient for most tissue engineering applications. Microinjection requires precision and high accuracy for success, which places the majority of the success or failure of the technique on ability of the individual performing the technique. The shape, size, and location of target cells can greatly restrict the ability of the investigator to effectively transfect cells *via* microinjection as well. Furthermore, isolation and immobilization of cells are an additional challenge that requires specialized training for successfully transfecting cells *via* microinjection. Microinjection could be far more attractive for tissue engineering if the process of isolating and injecting the cell of interest could be automated to remove the ''human'' factor from the process.

Ballistic Gene Delivery (Gene Gun)

Interdermal powder injection, biolistics, or ballistic gene delivery are names for a needle-free gene delivery technique originally developed by Sanford *et al.*[154] to transfect plant cells using DNA-coated metal particles. Over the years, the ballistic method was refined and commercialized for use in mammalian cells using both DNA and RNA.[194] Ballistic gene delivery is a needle-free alternative to electroporation and microinjection that allows for DNA or RNA to be precipitated onto gold or tungsten particles, ranging in size from the nanometer to micron scale. The particles are delivered directly to mammalian tissues as a projectile out of a barrel of a pressurized ballistic device, colloquially referred to as a ''gene gun.'' Particles are projected *via* a helium discharge or high-voltage electric spark, and can be propelled directly into the cell cytoplasm or nucleus (Fig. 3). Ballistic gene delivery has gained popularity as a potential delivery method for gene vaccines, as the DNA or RNA can penetrate the stratum corneum of the skin and reach the epidermis.[177] Additionally, investigators have successfully transfected mouse skeletal muscle fibers and liver tissue *in vivo* using ballistic gene delivery.[203] *Zelenin et al.*[203] transfected skeletal muscle fibers in mdx male mice with plasmid DNA expressing human *Dystrophin in vivo*. Approximately 20–30 μg of the *Dystrophin* plasmid were precipitated onto gold/tungsten (1:4) particles ranging in size from 1 to 4 μm in diameter for each discharge. The DNA coated particles were projected onto the tissue from a distance of 10 cm. Immunohistochemistry was used to detect the positive expression of human *Dystrophin* on the skeletal muscle fibers. According to Zelenin *et al.*[203] the number of human *Dystrophin* positive muscle fibers were found to vary from 2.5% on day 17 up to 5% on day 60 post-bombardment. The study published by Zelenin *et al.*[203] was one of the first studies to demonstrate the ability to deliver reporter and therapeutic genes *in vivo* to skeletal fibers in mdx mice *via* ballistic gene delivery. Furthermore, ballistic gene delivery has become not only a method to deliver therapeutic agents, but diagnostic agents as well. Several researchers have used the gene gun to deliver fluorescent dyes to track the functions of neurons.[15,85] Thus, ballistic gene delivery has continued to grow in popularity as an alternative to microinjection for *in vivo* applications.

However, while useful for potential gene vaccine applications, ballistic gene delivery has several limitations. Ballistic gene delivery has a limited tissue depth to which DNA microparticles can be transmitted, thus many studies have investigated gene delivery to the skin. Furthermore, the path of the projectile can cause

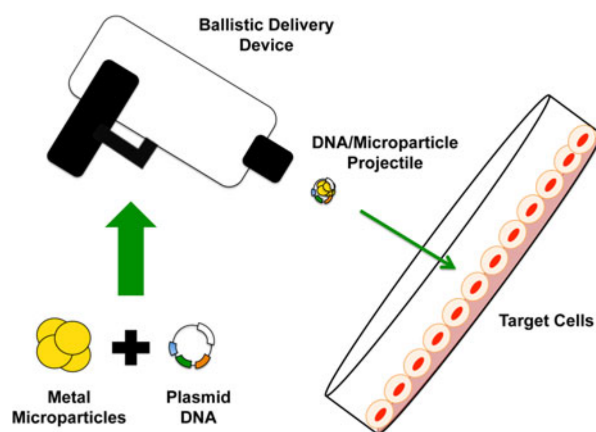

FIGURE 3. Ballistic gene delivery. Plasmid DNA is mixed with gold or tungsten particles ranging in size from nanometers to microns. An electric or plasma discharge is used to propel the DNA/particle complexes into tissues or cell cultures.

inflammation and damage to the target tissue with improper operation of the gene gun, or if the target tissue is bombarded with a high density of microparticles.[169] Moreover, ballistic gene delivery lacks cell specificity, so non-targeted cells may be transfected with the gene of interest if the non-targeted cells are within the dispersal area of the gene gun. In addition, microparticles can accommodate limited quantities of DNA or RNA. Thus, several treatments are needed to transfect a large population of cells if ballistic gene delivery is to be used for tissue engineering applications. Furthermore, there is no reliable way to ensure that multiple treatments would uniformly distribute DNA microparticles, and not produce an inflammatory response in the target tissue. Mitchell *et al.*[116] and Kendall *et al.*[84] explored the effects of temperature, distance, and pressure on the penetration of DNA microparticles on buccal mucosa and porcine skin, respectively, and both groups found that uncontrolled environmental factors can greatly influence the efficacy of using ballistic gene delivery. Kendall *et al.*[83] confirmed that gold particles ranging in size from 0.4 to 2.4 μm were able to achieve a greater impact velocity (420–640 m/s) than glass or polystyrene particles when delivered *via* a contour shock tube (CST). Furthermore, Kendall *et al.*[83] noted that the sizes of gold particles are typically smaller than cells allowing for penetration into the cell cytoplasm when delivered *via* ballistic gene delivery. Thus, the physical parameters need to be tightly regulated to optimize uniform delivery to cell cultures, which may not be possible for *in vivo* applications.

Thus, in summary, ballistic gene delivery can produce transient gene expression by directly delivering DNA to the cell cytoplasm or nucleus; however the delivery of DNA *via* ballistic gene delivery can be quite variable. Ballistic gene delivery is able to transfect primary tissues and difficult-to-transfect cells. However, ballistic gene delivery is limited in tissue engineering applications, as it can only transfect a limited number of cells, and not always in a uniform manner. Despite the drawbacks of ballistic gene delivery, it is an excellent method for developing gene vaccines as the DNA microparticles can readily penetrate the stratum corneum. As more research is conducted on ballistic gene delivery, perhaps a high throughput design will be developed that can consistently maintain precision and accuracy for nucleic acid delivery, or use different particle delivery materials to avoid inflammation and increase nucleic acid payload.

Electroporation

One of the most effective non-viral gene delivery methods, which has been extensively used and studied is electropermeabilization, also known as DNA electrotransfer, and colloquially referred to as "electroporation." Neumann *et al.*[124] introduced electroporation almost 30 years ago by successfully transfecting mouse lyoma cells. Since then, electroporation has evolved rapidly. *In vivo* studies have been well underway since 1996, and since then electroporation technologies have successfully transfected skin, skeletal muscle, liver, tumor tissues *in vivo*.[3,14,36,69,71] Aihara *et al.*[3] injected the tibialis anterior muscles of C57BL mice with 50 μg of plasmid expressing *interleukin-5* (*Il-5*), and electroporated the tissues *in vivo* using a pair of electrode needles inserted to a depth of 5 mm and separated by 5 mm. A square-wave pulse at a frequency of 1 pulse per second lasting no more than 50 ms was administered at 100 V. After 5 days, serum from the mice treated with electroporation contained over 20 ng/mL of *Il-5*, whereas mice only injected with plasmid had serum containing 0.2 ng/mL. Furthermore, Heller *et al.*[69] successfully transfected the livers of male Sprague–Dawley rats *via in vivo* electroporation using the *luciferase* reporter gene. Six 28 gauge acupuncture needles were used as electrodes and inserted into the right median lobe in the pattern of a 1 cm diameter circle so that the electrodes were equidistant from the center of the circle, where 100 μg of DNA was injected. Six pulses lasting a duration 99 μs at a frequency of 1 pulse per second were administered to create a rotating electric field. Approximately 30–40% of the rat liver cells that underwent electroporation expressed maximum luminescence 48 h after transfection. Luminescence was detected up to 21 days after electroporation at 5% of the maximum expression in the electroporated liver cells. Both of the studies conducted by Aihara *et al.*[3] and Heller *et al.*[69] were significant because both studies were some of the first *in vivo* studies that demonstrated electroporation could deliver genes *in vivo* successfully. Thus, it is necessary to look at how electroporation is thought to work to better understand its potential for tissue engineering. Additionally, this review examines a commercial electroporator that has gained much attention to aid in the discussion of how electroporation could be used for tissue engineering and cell differentiation.

In the broadest sense, electroporation is the application of an electrical field to a cell population for a finite amount of time to increase cell permeability to DNA, RNA and small proteins by creating localized transient disturbances in the cell membrane.[7,115,184,185] Electroporation has shown to be highly effective in a wide variety of tissues *in vivo*, and cell cultures *in vitro*. In particular, electroporation has been used to aid chemotherapy for cancer treatment.[70,101,149,164,165,186] In cancer treatments, irreversible electroporation is employed to ablate cancer tissues by inducing perma-

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nent formation of stable, non-resealing pores.[123] In gene therapy, reversible electroporation is employed, which keeps tissues intact because membrane pores are able to reseal.

There are many excellent reviews, in particular those published by Farvard *et al.*,[45] Teissié *et al.*,[174] Cemazar *et al.*,[21] Weaver *et al.*,[185] Mir *et al.*,[114] and Zimmerman *et al.*[208] as well as work published by Golzio *et al.*,[56,57] which collectively explain the physical mechanisms proposed to take place during electroporation. To summarize, when an electric field is applied across a set of cells, hydrophilic pores are thought to form on the sides of the cell facing the electrodes, hence the name "electroporation."[136,147] However, the precise mechanisms by which nucleic acids cross the cell membrane are still under investigation. Electrophoresis has been implicated as a possible process for enabling nucleic acids to diffuse from the extracellular environment into the intracellular environment during the application of the electric field when nucleic acids are tightly associated with the cell membrane.[191] However, other studies suggest internalization of nucleic acids is restricted to nucleic acids bound to the cell membrane.[58] In addition to uptake mechanisms, the subject of how nucleic acids are transported and trafficked through the cell to the nucleus is still widely debated. Wu *et al.*[190] have recently suggested that nucleic acids may be transported *via* an endocytotic mechanism and have provided data showing that GFP gene expression was diminished by over 20% in B16-F10 cells when treated with 80 μM of dynasore, an endocytotic inhibitor. Interestingly, Vaughan *et al.*[179] have provided data supporting trafficking *via* microtubules in TC7 cells and A549 cells. Vaughan *et al.*[179] showed that A549 cells treated immediately after electroporation with 10 μM of taxol, a microtubule network stabilizing agent, produced a 4.5 fold increase in luminescence 2 h after transfection with luciferase DNA. Zaharoff *et al.*[200] and Lukacs *et al.*[103] provided evidence that suggested that DNA does not diffuse through the cell cytoplasm after microinjection and electroporation, but in fact must traffic *via* another mechanism such as endocytosis or *via* some form of convection.

Despite the many discrepancies over how electroporation works, a few general themes have been observed. There are distinct physical and biological considerations that must be tailored for each tissue to achieve maximum transfection efficiency. The size and type of the cell, nucleic acid concentration, and orientation of the cell are important factors to consider when adjusting pulse duration, pulse shape (e.g., square wave vs. exponential decay), and electric field strength, to achieve maximum transfection efficiency. Jordan *et al.*[79] directly addressed how to tailor physical parameters of electroporators to achieve maximum transfection efficiency in a variety of cell lines that are difficult to transfect. For example, morphological characteristics between human umbilical vein endothelial cells (HUVECs) and neuroblastomas differ dramatically.[42,79,107] Thus, the voltage, pulse shape, pulse duration, nucleic acid quantity, and cell density must be experimentally determined for each cell type to achieve maximum transfection efficiency, cell viability, and gene expression.[74,104,162] Mehier-Humbert *et al.*[108] suggested that long pulses (20–60 ms) combined with modest field strengths (100–200 V/cm) produce larger pores in cell membranes that remain open for longer durations. Tailoring electroporation parameters is especially important for improving the stability of gene expression in primary cells such as HUVECs, neurons, and Jurkat cells, which are not as robust to electroporation procedures as skeletal muscle fibers, which are very robust to electroporation.[102,115,121,153,173,198]

Another important component to consider in electroporation is the electrodes that are used to generate the electric field. Normally, electrodes are directly applied to the tissue *in vivo* or a cell culture *in vitro*. A variety of electrodes have been developed for different applications commercially, and several investigators have built custom electrodes for specific applications. The strength, orientation, shape, and homogeneity of the electric field are directly dependent on the geometry and spacing of the individual electrodes.[108] Furthermore, the material used to coat the surface of the electrode that interacts directly with the tissue can affect the transfection efficiency. Stainless steel is commonly chosen to minimize ion stripping during electroporation, which can change the pH of the suspension buffer of cells and increase cell toxicity.[7] A variety of different electrodes have been developed for different applications, which include plate electrodes, needle electrodes, and catheter electrodes.[68] Plate electrodes are commonly used for electroporation of surface tissues and for *in vitro* electroporation of cell suspensions in cuvettes. Needle electrodes are used to electroporate deep tissues *in vivo*, and catheter electrodes have been developed to electroporate blood vessels.[108] Figure 4 illustrates how plate electrodes and needle electrodes can be used to transfect cells *in vitro* and *in vivo*, respectively. Plate electrodes are able to generate more uniform (defined and homogenous) electric fields, but usually require stronger voltages for electroporation. Needle electrodes allow for more flexibility and customizability in setting up electric fields, but at the expense of less homogenous electric fields.[7,68]

Many viral and chemical vectors have limited efficiency in non-dividing cells, but electroporation has successfully transfected both dividing and non-dividing cells.[3,198] For tissue engineering applications, trans-

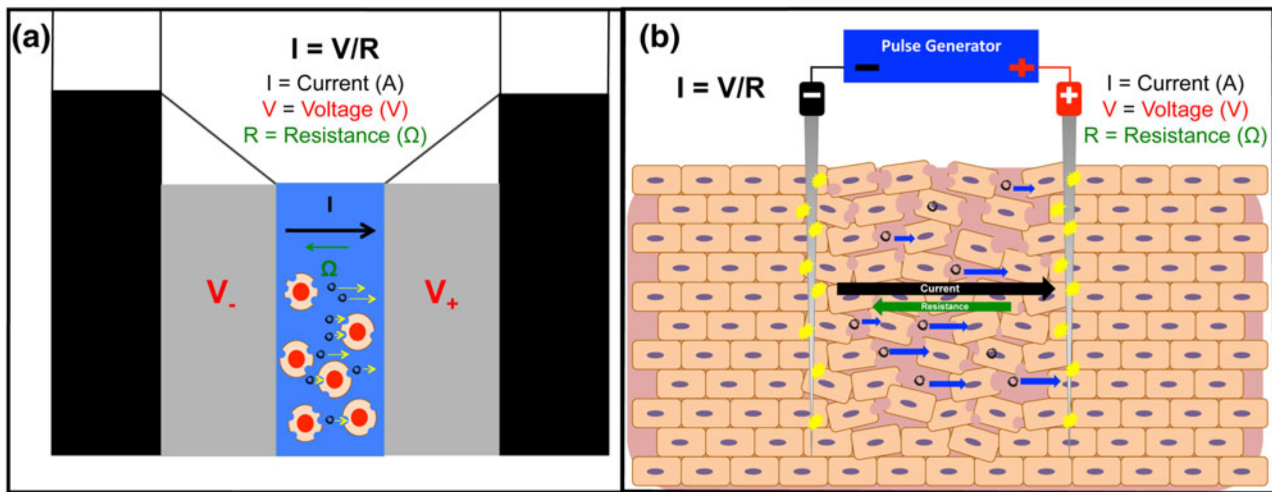

FIGURE 4. Electroporation. Electroporation strategies apply a current across cells or tissues to make cell membranes more permeable to exogenous DNA. (a) Traditional electroporators have a pulse generator and a pair of electrodes that can be applied directly to tissues or cells. A cuvette utilizes plate electrodes to apply a voltage potential across cells in suspension. Since resistance is constant, the current is proportional to the voltage potential. As voltage reaches a critical threshold, hydrophilic pores form in the cell membrane make it permeable to plasmid DNA. The negatively charged DNA is mobile in the electrical field (toward the positive electrode) so DNA transport into permeabilized cells is greater than by diffusion alone. (b) Needle electrodes have been used for *in vivo* applications where needles are inserted directly into primary tissues such as skin or skeletal muscle fibers after DNA has been injected. A current is applied across a very small area of tissue to facilitate the same process as in a cuvette.

fection of non-dividing cells is a highly desirable attribute as several primary cells in cartilage, bone, and neurons have rates of division that are too slow for passive gene delivery.[90,133,153,156] Furthermore, electroporation has been shown to transfect progenitor cells and stem cells, which is another highly desirable attribute for tissue engineering, as many groups focus on utilizing various stem cell sources for differentiation and tissue regeneration.[6,46]

Despite the advantages of electroporation, there are some major limitations restricting its use. First, several physical and biological parameters must be carefully tailored for each tissue to achieve maximum transfection efficiency, which can be tedious. Second, electroporation may be able to efficiently transport nucleic acids into cells, but the benefit usually comes at a cost of low cell viability. Low cell viability is a major disadvantage of electroporation. Low cell viability may be a result of some cells undergoing irreversible electroporation or cells may die because of an increased cytotoxicity occurring from changing pH, resulting from the use of electrodes with poor biocompatibility. Furthermore, when electroporating tissues directly *in vivo*, there is the risk of producing inflammation, as not all cells will survive the procedure, and there may be a deposition of metal ions into the tissues from the electrodes. Another disadvantage of electroporation is that the up-front cost can be expensive, depending on the model of pulse generator and the associated electrodes. Furthermore, if *in vivo* studies are being conducted, a specially trained physician or technician must be present to properly place the electrodes on the subject to prevent injury and ensure proper alignment and generation of the electric field.

In summary, electroporation has great potential in tissue engineering and for gene vaccine applications, as electroporation is able to successfully transfect a variety of cells *in vitro* and *in vivo*, including dividing and non-dividing cells. However, electroporation can be an invasive procedure depending on the target tissue, and electroporation is notorious for producing low cell viabilities. However, unlike other transfection techniques, electroporation can transfect a large number of cells. Furthermore, the electroporative technology is rapidly evolving, and new systems are being developed each year to address the issues noted above. Nucleofection™ by Amaxa is a leading electroporative system that will be further discussed below for tissue engineering applications. The Nucleofection™ system attempts to mitigate the issue of low cell viability while increasing transfection efficiency.

Nucleofection™

Nucleofection™ has had incredible wide-reaching success in tissue engineering and cancer studies, compared against other physical non-viral gene delivery methods, and is therefore highlighted with special emphasis in this review. Nucleofection™ is a patented commercial electroporation system created by Amaxa,

and owned by Lonza. Nucleofection™ is an electroporator that uses a sterile disposable cuvette to facilitate electroporation, Amaxa has developed a variety of cell specific buffers that are proprietary, and are designed to enable maximum transfection while reducing cell death. In addition to the cell specific buffers, the Nucleofector™ comes pre-programmed with an assortment of programs specific to different cell lines that vary voltage, frequency, and pulse duration. However, the voltage, frequency, and pulse duration for each cell type are not revealed to the user, although Amaxa does provide suggested protocols for the user. The Nucleofection™ system has gained great popularity among many researchers as the Nucleofector™ is able to transfect many difficult-to-transfect cells, including several progenitor cells and stem cells. For example, Aslan *et al.*[8] transfected human bone marrow-derived stem cells (hBMSCs) at a density of 5×10^5 with 5 μg of plasmid DNA expressing human bone morphogenetic protein 2 (*hBMP2*) and human bone morphogenetic protein 9 (*hBMP9*) *via* Nucleofection™ and achieved a transfection efficiency of $68 \pm 41\%$. Furthermore, hBMSCs transfected with *hBMP2* produced an 8–16-fold increase in recombinant *BMP2* secretion 24 h after transfection *via* quantification through an enzyme-linked immunosorbent assay (ELISA). Aslan *et al.* confirmed that the Nucleofected™ cells were able to form new bone tissue both *in vitro* and *in vivo* through RT-qPCR, micro computed tomography (μCT), and immunohistochemistry. In an additional example, Bowles *et al.*[17] used a Nucleofector-96-Shuttle™ to transfect naïve dendritic cells at a density of 5×10^5 cells per 100 μL with 0.25 μg of retinoic acid-inducing gene 1 (*RIG-I*) small interfering RNA (siRNA) to knockdown the *RIG-I* viral recognition receptor. Bowles *et al.* determined through RT-qPCR and western blotting that Nucleofection™ enabled a 75% knockdown of the detection of *RIG-I*. In a third experiment, Gonzalez *et al.*[59] successfully generated induced pluripotent stem cells (iPSCs) by Nucleofecting™ mouse embryonic fibroblasts with a polycistronic construct containing octamer-binding transcription factor 4 (*Oct4*), (sex determining region Y)-box 2 (*Sox2*), krueppel-like factor 4 (*Klf4*), and v-myc myelocytomatosis viral oncogene homolog (avian) (*c-Myc*). The identities of the iPSCs were confirmed *via* RT-qPCR, Southern blotting, and western blotting. Furthermore, iPSCs were differentiated *in vitro* toward neuronal lineages, cardiomyocyte lineages, or endoderm lineages. In addition to these studies, several more have summarized the ability of Nucleofection™ to successfully transfect progenitor cells, stem cells, and connective tissues (Table 1), and the Nucleofection™ technique has been successfully used for gene knockdown studies in a variety of cells (Table 2). However, as displayed in the tables, not all cell types tolerate Nucleofection™ well. Some cells types lack desirable cell viabilities. Thus, the cell types lacking high cell viabilities may require more customization of buffer solution, electrical parameters, or a combination of both to increase viability.

Thus, in summary, Nucleofection™ is an effective transfection method for difficult-to-transfect cells, and Nucleofection™ can facilitate high transfection in a variety of cell types, which makes Nucleofection™ an attractive technique for *in vitro* and *ex vivo* tissue engineering applications.

Sonoporation

Similar to electroporation methods, high-intensity ultrasound has demonstrated the ability to induce pore formation in cell membranes, and allow for movement of plasmid DNA into cell cytosol.[48] This method is commonly referred to as ''sonoporation,'' and in contrast to electroporation methods, induces pore formation through physical movement of fluid rather than using an electric field. Ultrasound is used in the clinic for diagnostic imaging, kidney stone treatment, pain relief, and ablation of cancer tissues.[80,127,175] High-intensity focused ultrasound (HIFU) produces localized shear forces in extracellular fluids that facilitate cavitation, or the controlled collapse of air bubbles present in the surrounding fluid, and induce pore formation in cell membranes, increasing the permeability of cells to plasmid DNA and drugs (Fig. 5).[128,143,189] Cavitation can be enhanced with the use of ultrasound contrast agents, such as Optison™, and drugs and nucleic acids can be complexed with contrast agents for systemic delivery.[199] Zhou *et al.*[207] have examined the effects of pore formation in *Xenopus* oocytes, and have found that the resealing of pores is affected by extracellular calcium concentration. Zhou *et al.*[207] observed that pores induced by sonoporation in *Xenopus* oocytes resealed in 6–26 s in the presence of 1.8 mM Ca^{2+}, whereas in contrast pores resealed between 58 and 170 s after sonoporation in the presence of 0.54 mM Ca^{2+}. Sonoporation has gained popularity in clinical settings because it is noninvasive, and already used in the clinic to enhanced transdermal absorption of drugs. Furthermore, Newman *et al.*[125] have reviewed the use of sonoporation on a variety of cell types and tissues, and noted that sonoporation seems to be a less destructive method for delivery of plasmid DNA than electroporation. Currently, sonoporation is primarily used to enhance drug delivery and gene delivery to diseased tissues *in vivo* rather than for tissue engineering applications. Interestingly, Liang *et al.*[98] have noted that sonoporation

TABLE 1. Transfection studies of stem cells, progenitor cells, and connective tissues using Nucleofection™.

Reference	Cell type	Gene(s)	Nucleofector™ program	Cell density	Transfection efficiency	Max gene expression	Cell recovery	Cell viability	Additional notes
Aluigi *et al.*[6]	hBMSC	*GFP, IL-12*	U-23	$4–5 \times 10^5$ cells/100 μL	27.4 ± 2.9%	73.7 ± 2.9%	38.7 ± 2.9%	44.5 ± 3.9%	Mean *Il-12* protein production: 290.5 ± 97.6 pg/mL
Aslan *et al.*[8]	hBMSC	*EGFP, hBMP2, hBMP9*	G-22	5×10^5 cells/100 μL	68.2 ± 4.1%.	N/A	53.6 ± 2.5%	Not reported	2000-fold and 8000-fold increase in hBMP2 and hBMP9 detected, respectively through RT-qPCR 24 h post-transfection.
Baksh *et al.*[10]	HUCPVC	*DsRed*	U-23	5×10^5 cells/100 μL	>50%	>80%	~50%	N/A	
Cesnulevicius *et al.*[22]	SDrMNP	*EGFP, DsRed*	A-033	2.08×10^4 cells/100 μL	47.6 ± 8.6%	47.6 ± 8.6% (*GFP*), 16.7 ± 10.25% (*DsRed*)	40%	N/A	
Cui *et al.*[31]	mPIVDC	*GDF-5*	U-24	1×10^5 cells/100 μL	N/A	N/A	N/A	N/A	*GDF-5* gene delivery *via* Nucleofection™ produced a ~3.5 fold and ~1.5 fold increase in type II collagen and aggrecan expression, respectively.
Duffy *et al.*[41]	hMSC	*GFP, Ephrin-B2*	U-23	5×10^5 cells/100 μL	46.90%	73% (*GFP*), 46.9% (*Ephrin-B2*)	66.3 ± 1.5%	N/A	
Gonzalez *et al.*[59]	mEF	*GFP, Oct4, Sox2, Klf4, c-Myc*	T-020	2×10^6 cells/100 μL	N/A	37% (GFP)	N/A	N/A	GFP was used as a reporter gene in a polycistronic plasmid expressing Oct4, Sox2, Klf4
Motoyama *et al.*[119]	mHep	*EGFP, DsRed, Pdx1, Ngn3*	T-028	7×10^5 cells/100 μL	52.2 ± 3.7%	60.4 ± 16.0 (co-expression Pdx1 & Ngn3)	~90%	~25%	
Sheyn *et al.*[166]	pADSC	*GFP, rhBMP-6*	G-22	$1–2 \times 10^6$ cells/100 μL	62.5%	4.78 ± 0.97 ng/10^6 cells	Not reported	52.9%	
Zhang *et al.*[206]	rSF	*GFP, bFGF, VEGF*	U-30	1×10^6 cells/100 μL	~60%	>250 pg/mL (bFGF), 11 ng/mL (VEGF)	Not reported	95%	

Terms and Abbreviations: Transfection Efficiency, percent of positive expressing cells out of total viable cell population; Max Gene Expression, maximum number of positive cells recorded or maximum quantity of protein detected; Cell Recovery, percent of live cells 24 h after transfection; cell viability, percent of live cells 48 h or more after transfection; hBMSCs, human bone marrow stem cells; hUCPVCs, human umbilical cord perivascular cells; SDrMNPC, Sprague–Dawley rat mesencephalic neuronal progenitor cells; mPIVDC, mouse primary intervertebral disc cells; hMSCs, human mesenchymal stem cells; mEF, mouse embryonic fibroblasts; mHep, mouse hepatocytes; pADSC, rSF, rat skin fibroblasts; *GFP*, green fluorescent protein; *Il-12*, interleukin 12; *EGFP*, enhanced green fluorescent protein; *hBMP2*, human bone morphogenetic protein 2; *hBMP9*, human bone morphogenetic protein 9; *DsRed*, Discoma species Red; *GDF-5*, growth/differentiation factor 5; *Oct4*, octamer-binding transcription factor 4; *Sox2*, sex determining region Y-box 2; *Klf4*, krueppel-like factor 4; *c-Myc*, v-myc myelocytomatosis viral oncogene homolog; *Pdx1*, pancreatic and duodenal homeobox 1; *Ngn3*, neurogenin 3; *rhBMP-6*, recombinant human morphogenetic protein-6; *bFGF*, basic fibroblast growth factor; *VEGF*, vascular endothelial growth factor.

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TABLE 2. RNA studies using Nucleofection™.

Reference	Cell type	Gene(s) targeted by siRNA	RNA dose (format)	Nucleofector™ program	Cell density per transfection	Max transfection efficiency	Max gene knockdown	Cell recovery	Cell viability	Additional notes
Bowles *et al.*[17]	HMDDC	*RIG-I*	0.25 μg siRNA (0.75 mL Matrix Tube)	FF-168	5×10^5 cells/ 20 μL	55%	75%	Not reported	70%	
Bradburne *et al.*[18]	A549	*RelA*	100 nM/250 nM/ 500 nM siRNA (96 well-plate)	DS-150	2.75×10^5 cells/ 20 μL	7.7%	70–95%	17.6%	92.7%	
Merkerova *et al.*[111]	CMLPC	*PCNA, MMP8, MMP9, p38, JNK2, BCR/ABL*	50 nmol	T-20	5×10^6 cells/ 100 μL	36–42%	65% (BCR/ABL), 63%, (PCNA), ~70% (MMP8), ~64% (MMP9), ~70% (p38), ~75% (JNK2)	Not reported	74.7 ± 32.9%	
Mo *et al.*[117]	MDCK	Galectin-3	10 μg	T-23	4×10^6 cells/ 100 μL	~70%	~85%	N/A	N/A	
Moore *et al.*[118]	hES	*GFP*	200, 300, 600 ng	96-CB-150	2×10^5 cells/ 20 μL	74.2 ± 1.4%	71.75%	73.5 ± 3.2%	Not reported	
Schnoor *et al.*[161]	THP-1	*TIP47*	1 μg	Y-001	2.5×10^6 cells/ 100 μL	100%	75%	20–60%	Not reported	
Verreault *et al.*[180]	U251MG	*ILK*	0.0625–2 μg	U-16	Not reported	73%	93%	78%	>90%	

Terms and Abbreviations: Transfection efficiency, percent of positive expressing cells out of total viable cell population; Max gene expression, maximum number of positive cells recorded or maximum quantity of protein detected; Cell recovery, percent of live cells 24 h after transfection; Cell viability, percent of live cells 48 h or more after transfection; hMDDC, human monocyte derived dendritic cells; A549, adenocarcinomic human alveolar basal epithelial cells; CMLPCs, CML primary cells; MDCK, Madin Darby canine kidney cells; hES, human embryonic cells; THP-1, human acute monocytic leukemia cells; U251MG, human glioblastoma cells; *RIG-1*, retinoid-induced gene 1; RelA, v-rel reticuloendotheliosis viral oncogene homolog A (avian); *PCNA*, proliferating cell nuclear antigen; *MMP8*, matrix metallopeptidase 8; *MMP9*, matrix metallopeptidase 9; *p38*, mitogen-activated protein kinase 14; *JNK2*, c-Jun N-terminal kinase; *BCR/ABL*, breakpoint cluster region/Abelson murine leukemia viral oncogene homolog 1; *GFP*, green fluorescent protein; *TIP47*, mannose-6-phosphate receptor binding protein 1; *ILK*, integrin-linked kinase.

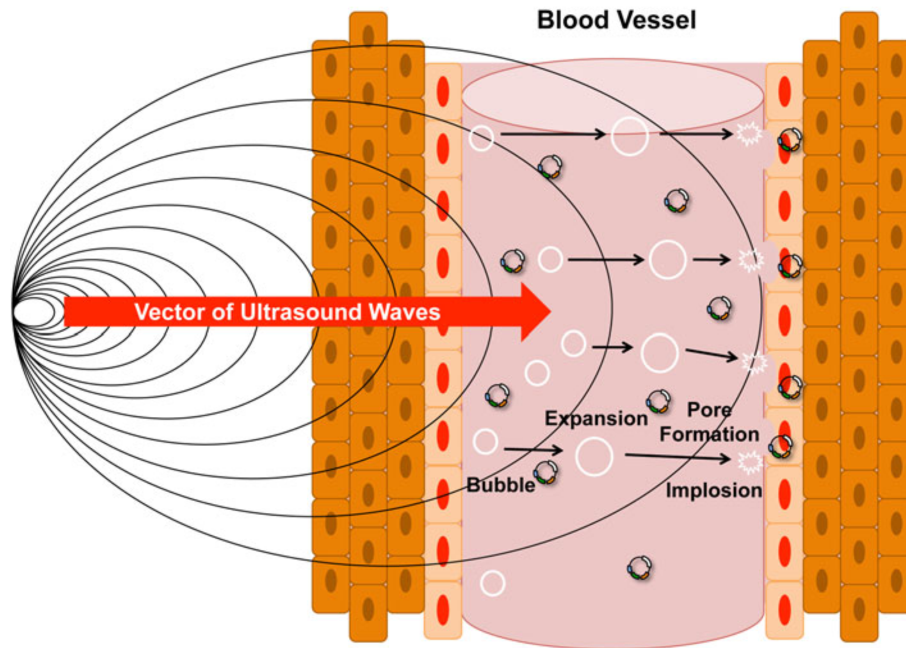

FIGURE 5. Sonoporation. Ultrasonic frequencies are used to induce the cavitation of microbubbles for creating pores in cells contained in culture or tissue. The acoustic waves cause microbubbles to expand and then collapse. When the microbubbles collapse, a microshockwave is emitted that can rupture a cell membrane if the collapsing microbubble is in close proximity to the cell membrane. The ruptured cell membrane forms a pore, which allows cells to be temporarily more permeable to plasmid DNA.

has exhibited enhanced transfection efficiency in tissues such as the heart, blood vessels, lung, kidney, brain, muscles, and the tumors when physical parameters of sonoporation are optimized.

Sonoporation is limited by relatively poor control of the energy localization. While sonoporation can induce cavitation in tissues, there currently is no way to control the uniformity of the cavitation or the entry of DNA. Complexation of DNA and contrast agents have greatly improved targeting; however, each target tissue needs to be carefully evaluated to ensure that cavitation induces pore formation in target cells.[108] Thus, sonoporation cannot be as precisely controlled as in electroporation, where cells are placed between electrodes. Furthermore, sonoporation seems to be more effective *in vivo* for tissues that are in direct contact with blood vessels.

In summary, sonoporation is effective for transfecting cells *in vivo* as it is non-invasive, and already used in the clinical setting. However, sonoporation exhibits lower transfection efficiencies because cavitation cannot be precisely controlled within the tissue. Improving the uniformity of cavitation for membrane pore formation and improving the accuracy of cell contrast could make sonoporation highly effective for tissue engineering.

Laser Irradiation

Laser irradiation is an alternative strategy under investigation for gene delivery applications. Investigators have used neodymium-doped yttrium aluminum garnet (Nd:YAG), holmium-YAG, titanium sapphire, and argon powered lasers to perforate cells to enable the entry of DNA by varying the pulse frequency of the laser.[130,152,172,202] Typically, a laser is focused through an objective onto a localized area of an individual cell in culture and increases the permeability of the cell to exogenous DNA in the culture medium. Interestingly, cells seem to not undergo any lethal injury when perforated by a laser, and they are able to repair the ''holes'' made by perforations in less than a second.[93,130] Furthermore, a laser can be used indirectly to induce stress waves in the medium to perforate cells temporarily to enable the entry of DNA. Yao *et al.*[197] provided a comprehensive review of the different methods to use a laser to facilitate gene delivery in cell culture. Ogura *et al.*[129] demonstrated the precision and efficiency of using laser irradiation by injecting Sprague–Dawley rats with plasmid DNA coding for enhanced green fluorescent protein (EGFP) and luciferase. Ogura *et al.* injected Sprague–Dawley rats with 50 μg of plasmid DNA and irradiated the injection area with six pulses of 1.9 J/cm^2 fluence laser, which produced a luminescent expression of 10^5 relative light units (RLUs) per milligram of protein for up to 5 days. EGFP expression was confined to the exact area of skin irradiated with the laser 24 h after transfection. In another report, Shirahata *et al.*[167] successfully delivered EGFP to HuH-7 and NIH/3T3 cells in culture by using a pulsed 355 nm Nd:YAG laser to

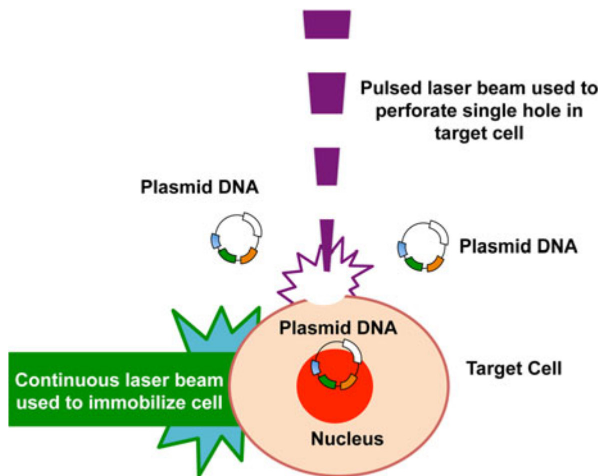

FIGURE 6. Laser Induced Pore Formation. Pulsed lasers have been shown to perforate cell membranes similar to microinjection strategies, but without the use of a needle. Investigators have shown a variety of laser beams of varying wavelengths are capable of making precise "holes" in cell membranes when beam energy, pulse frequency, and exposure duration are manipulated. Investigators can precisely target individual cells in culture or in tissue with aid of a microscope to target specific sites on cells for perforation to allow DNA to enter cells. A second laser with an uninterrupted beam can be used to immobilize individual cells in suspension while a pulsed laser is used to perforate cells.

perforate cells, while a 1015 nm continuous-wave Nd:YAG laser was used to trap individual cells. Figure 6 provides an illustration of a cell undergoing perforation by a pulsed laser while being immobilized by a continuous laser. Shirahata *et al.* achieved a transfection efficiency of 10% in NIH/3T3 cells when DNA was delivered at a concentration of 10 μg/mL.

Laser irradiation has great potential for tissue engineering as it can be used to target precise cells in tissue or in culture. Laser irradiation is less invasive than microinjection or electroporation as no needle is required and individual cells can be targeted. The brief perforation of the cell by a pulsed laser seems not to cause cell death. Furthermore, optical fibers can deliver laser light that can be controlled by computers, and may eventually provide convenient access to tissues inside the body that were previously inaccessible.[197]

However, despite the advantages of laser irradiation, some key limitations exist. While precise and efficient, laser irradiation is still a young technology, and more studies are needed to determine how robust the procedure is on different cell types. Laser irradiation is highly efficient at targeting individual cells, but it is not efficient at targeting large populations of cells efficiently. In addition, optical lasers can be very large and costly. Furthermore, specialized training is required to operate an optical laser properly.

In summary, laser irradiation has great potential for gene therapy and tissue engineering applications as optical lasers can be used to precisely target individual cells in culture or in tissue. However, laser irradiation is still a young technology that needs further investigation, and is costly. Nevertheless, as laser irradiation strategies improve and the technology is further investigated, optical lasers may allow investigators to target tissues for gene therapy and tissue engineering in ways that were previously not possible.

Emerging Techniques in Gene Delivery

In contrast to the techniques described above, there are additional techniques under development that may prove advantageous for specific gene delivery and tissue engineering applications. Here we briefly describe the techniques of electric field-induced molecular vibration gene delivery, a novel technique introduced by Tuan *et al.*,[170] and magnetofection, a technique used as a tool to enhance gene delivery strategies.

Tuan *et al.*[170] developed a unique gene delivery method known as electric field-induced molecular vibrations as an alternative to electroporation to facilitate high transfection efficiencies in mesenchymal progenitor cells and a variety of cell lines. Tuan *et al.*[170] created a unique apparatus where cells and DNA are suspended in a glass dish that undergoes vigorous vibration induced by two electrodes.[170] The electrodes do not directly contact the cells, and no current is applied across the cells; however, the vigorous shaking enables exogenous molecules to penetrate the cell membrane and reach the cytoplasm. According to Tuan *et al.*[170] electric field-induced vibration transfection is economical and efficient because it requires no additional reagents and exhibits high transfection efficiency with low cell mortality. Furthermore, Tuan *et al.*[170] noted that electric field-induced molecular vibration transfection does not interfere with cell proliferation, and provides stable gene expression. Limited literature is available on this technique; however, if these claims and data can be verified then electric field-induced molecular vibration transfection could be a suitable transfection technique for *ex vivo* and *in vitro* tissue engineering applications. Tuan *et al.*[170] report several data values regarding transfection efficiency and cell viability for multiple cell lines using different parameters for the electric field-induced vibration apparatus. Most notably, Tuan *et al.* reported a maximum transfection efficiency of 9.4 $\pm$ 9.3% and a maximum cell viability of 57 $\pm$ 40% for human mesenchymal stem cells (hMSCs) when a frequency of 120 Hz was used in combination with a 12 V setting and a wave ratio of 300 for the parameters of the electric field-induced molecular vibration apparatus.

Magnetofection is a technique that exploits the energy of a magnetic field to enhance the delivery efficiency

of DNA, siRNA, or shRNA *via* viral or non-viral vectors.[35,43,49,160] Paramagnetic particles typically made of iron oxide are coated with viral particles, liposomes, or cationic polymers and combined with nucleic acids.[73,75,160] Several *in vitro* studies have placed a magnetic plate underneath a cell culture vessel once the magnetic particles and gene delivery vector have been combined with the target tissue of cells to preferentially direct or ''pull'' the magnetic particles into cells or tissue explants.[196] Magnetofection does not necessarily improve the transfection efficiency of gene delivery methods; instead, magnetofection increases the speed at which nucleic acids traffic into the cell and nucleus while enabling smaller doses of nucleic acids to be used.[140] Plank *et al.*[139,141] have published insightful reviews on how magnetofection works and the potential benefits of magnetofection on *in vitro* gene delivery applications. In addition, magnetofection has been used to enhance gene delivery to primary cells such as neurons and endothelial cells *in vitro*, and has been applied to enhance gene delivery to the gastrointestinal tract and blood vessels *in vivo*.[19,91,137,155,160,163] Sapet *et al.*[155] were able to achieve a transfection efficiency of approximately 15%, 48 h after transfection in primary neuronal stem cells from P1 mice using a magnetofection reagent, NeuroMag. This finding may be considered impressive because neuronal cells are known to be incredibly difficult to transfect. As new technologies develop, magnetofection could prove quite valuable for enhancing tissue engineering applications.

APPLICATIONS OF GENE DELIVERY TO TISSUE ENGINEERING

The goal behind integrating gene therapy and tissue engineering together is to manipulate the behavior of cells so that cells can be used to produce proteins and associate into tissues that are capable of replacing, restoring, regenerating, or enhancing the function of tissue defects within the human body. The marriage of these two fields is not a new idea. In fact, there are a multitude of examples where gene therapy and tissue engineering have been integrated for enhancing differentiation strategies. Most of the studies have focused on *ex vivo* or *in vitro* approaches for integrating gene therapy into tissue engineering. Multiple groups have used a variety of synthetic polymers (PEI), biodegradable polymers (PLL-PA, PBAE, PLGA), and biological polymers (chitin, fibrin, collagen) to either encapsulate or anchor nucleic acids to the scaffolding material on which cells are seeded for nucleic acid uptake.[16,29,53,54,60,62–64,76,97,99,126,138,158,195] Several of the studies have shown sustained gene delivery for periods up to 2–3 weeks, but the overall transfection efficiency has varied. Polymers provide flexibility in designing scaffolds to accommodate stem cells for differentiation, and the incorporation of nucleic acids to direct differentiation is a natural progression. However, no one polymer has emerged as a reliable vector for primary cells, progenitor cells, and stem cells. Nor should any one polymer be expected to successfully transfer nucleic acids to all cell types. While it seems several investigators within the field of tissue engineering have placed an emphasis on trying to deliver nucleic acids *via* polymers, others have focused on physical methods. Cesnulevicius *et al.*[22] transfected mesencephalic neuronal progenitor cells from Sprague–Dawley rats with fibroblast growth factor 2 (*FGF-2*) linked to EGFP *via* Nucleofection™ and found that the transfected cells tested positive for *nestin*, an important protein for neuron growth. Furthermore, the cells were able to survive transplantation into lesioned rat brains, demonstrating a potential for developing a new primary transplantation method for neuronal tissues. The significance of the work by Cesnulevicius *et al.*[22] is that they achieved a transfection efficiency of 47% in neuronal progenitor cells, which is high for a non-viral method, especially because neuronal tissues are notoriously difficult to transfect. Additionally, Cesnulevicius *et al.*[22] detected *FGF-2* expression up to 11 days after transfection. This study shows promise for using physical non-viral gene delivery vectors, in this case Nucleofection™.

In a different experiment, Duffy *et al.*[41] transfected 5×10^5 hMSCs with 2 μg of plasmid DNA expressing *Ephrin-B2 via* Nucleofection™ and achieved a transfection efficiency of approximately 45%. hMSCs have been known not to transfect easily. Furthermore, in this study, the hMSCs expressed *Ephrin-B2* and took on early endothelial phenotype and are thought to have contributed to the increased detection of VEGF in cell culture, which could potentially promote angiogenesis in ischemic tissues. Like the work published by Duffy *et al.*[41] and Cesnulevicius *et al.*,[22] several investigators are exploring strategies to isolate cells and transform them outside of the body either by chemical or physical methods for therapeutic purposes.[25,37,72] However, while there has been a focus on using *ex vivo* and *in vitro* strategies for integrating gene therapy and tissue engineering, much of the field of gene therapy has focused on using *in vivo* methods to for therapeutic purposes. Thus, perhaps it might be worth reversing the idea of integrating gene therapy into tissue engineering, and instead look at how to integrate tissue engineering strategies into gene therapy. To better understand how to apply tissue engineering strategies to gene therapy, it would be best to focus on the limitations and challenges that restrict gene delivery.

UNDERSTANDING CHALLENGES THAT LIMIT NON-VIRAL GENE DELIVERY

Every single vector must first cross the plasma membrane. However, before a delivery vehicle reaches the cell membrane, there are obstacles to overcome. For example, most cells in a connective tissue are located within the labyrinth of the ECM. Some investigators have suggested that the collagen in the ECM could be hindering the diffusion of large nucleic acids and other macromolecules, preventing them from reaching the target cell surface.[122,142] Thus, one approach to overcome this limitation is to disrupt the ECM. Disrupting the ECM *in vivo* could pose difficulties for the subject; however, if the target tissue is excised, the ECM could be disrupted with trypsin to expose cell membranes. An additional consideration is that electrophoresis may be able to help move nucleic acids through the ECM, but this process, depending on the tissue, may not be able to bring the nucleic acids close enough to the cell membrane for interaction.[71] Thus, the ECM may be an additional barrier to consider when designing delivery vectors.

Beyond the ECM, the cell membrane remains a significant barrier for all delivery vectors. Many chemical vectors attempt to associate the nucleic acid delivery vehicle with the cell membrane through electrostatic interactions, ligand mediated receptor binding, and through adsorption. Several studies suggest that association with the cell membrane is required for entry into the cytoplasmic compartment of the cell.[44,47,55,65,145] However, microinjection and ballistic gene delivery bypass the cell membrane by directly transporting nucleic acids into the cell cytoplasm or the nucleus. Electroporation, sonoporation, and laser irradiation disrupt the cell membrane to facilitate infiltration of nucleic acids. However, microinjection, ballistic gene delivery, electroporation, sonoporation, and laser irradiation display one common weakness. All methods rupture the cell membrane in some fashion, and if the cell is unable to mend the membrane, then the cell dies. Thus, taking a closer look at the function of the cell membrane may provide questions and answers to finding ways to better overcome this important barrier and maximize cell viability.

What is the plasma membrane? Simply, the plasma membrane is a barrier to separate two hydrophilic compartments, namely, the intracellular space and the extracellular space. The plasma membrane is composed of a phospholipid bilayer with proteins permeating both the intracellular and extracellular sides of the plasma membrane.[5,20] Furthermore, the composition of lipids and proteins can vary among cell types, and the plasma membrane is not a rigid structure, meaning that lipids and proteins are not static, but rather moving targets. The composition of the cell membrane can have an influence on the physical and mechanical functions of the cell membrane. For example, cells that are a part of tissues that provide structure and support (e.g., bone) may be more inflexible and rigid, containing fewer unsaturated lipids to maintain a less fluid structure, and hence lower membrane permeability, whereas secretory cells may contain more unsaturated lipids and fewer proteins to maintain a more fluid membrane composition that is more permeable.[5] Furthermore, depending on the target cell and the condition of the tissue (i.e., adherent cells or cells in suspension), access to the plasma membrane may be restricted. Thus, an appropriate questions is "does exposure to cell surface affect localization of nucleic acids on the surface?" Adler *et al.*[2] endeavored to address this very subject by exploring the effect of cell surface topography on transfection efficiency. Adler plated fibroblasts onto micropitted surfaces at varying densities and found a 25% increase in transfection efficiency when using Lipofectamine 2000^{TM} to deliver GFP for cells plated on densely pitted surfaces as opposed to smooth surfaces. This increase could have been attributed to a variety of factors. The cells did spread across the pitted surfaces, but not on the smooth surfaces. So why did transfection efficiency increase? Were delivery vehicles able to associate with the cell membrane because the membrane had an increased surface area? Did the composition of the membrane change because of the cell spreading across the pitted surface? Adler suggested that a consequence of the cell spreading was a loss of integrin mediated cell adhesion, which resulted in the internalization of caveolae, and could have been responsible for a down regulation of particle uptake through competitive mechanisms. The spreading of cells on pitted surfaces did not lead to an increase in cell proliferation. Thus, the rate of cellular mitosis did not increase, which means passive diffusion of DNA into the cell nucleus was not responsible for the increase in gene expression. Adler *et al.*'s study was exciting because it drew attention to the consideration of surface topography, and presented questions about how nucleic acids associate with the cell membrane and how the cell membrane might be altered to accommodate molecules. Perhaps the permeability of a cell membrane can be manipulated mechanically to alter firmness or fluidity for delivery vectors. An exciting and recommended next step would be to see how other non-viral vectors perform when a cell spreads. For example, combining cell spreading with an electroporative technique would be an exciting study to investigate how cell spreading affects the ability of the cell to permeabilize and mend under an electric field. Another question to ask is whether different cell types

produce the same results when cultured on pitted surfaces.

Chalut *et al.*[23] presented an additional insight regarding the influence of changing mesenchymal stem cell (MSC) membrane topography and how deforming the MSC changes the structure of its nucleus. As the nucleus is connected *via* the cytoskeleton to the cell membrane, mechanical forces on the cell membrane act *via* the cytoskeleton on the nucleus. They offered evidence that is consistent with findings in the literature that the nucleus changed shape in response to the deformation of the cell.[23,96,168] If the nucleus can alter its shape in response to mechanical forces exerted on the cytoskeleton, then how does gene expression change? Does the elongation of the nucleus in response to mechanical forces acting on the cell increase transfection efficiency by shortening the distance between the cell membrane and nucleus? These questions need further investigation.

Despite the interest in the membrane topography and deformation of the nucleus as they relate to gene expression, there is an additional parameter to consider. Chemical vectors tend to use an endocytotic route of delivery once the nucleic acids have entered the cell, while physical vectors attempt to deliver nucleic acids directly to the cytoplasm or nucleus. However, chemical methods seem to have higher cell viabilities, but low transfection rates in primary cells, progenitor cells, and stem cells, whereas the opposite is true for physical methods. Thus, the questions arises, ''how do nucleic acids traffic through the cytoplasm?'' Nucleic acids must escape endosomes to avoid degradation by lysosome enzymes, but then how do the nucleic acids reach the interior of the nucleus? Dividing cells provide an opportunity on a regular interval as the nuclear envelope deconstructs during mitosis, and reforms at the conclusion of mitosis. In non-dividing cells, the nucleic acids must enter through a nuclear pore. Thus, how do the nucleic acids reach the nuclear pore? Zaharoff *et al.*[200] suggested the nucleic acids do not diffuse through the cytoplasm, but move by some other mechanism such as convection. Lukacs *et al.*[103] presented work that was consistent with Zaharoff *et al.*[200] in that DNA did not seem to diffuse through the cytoplasm. Vaughan *et al.*[179] provided evidence that suggested that nucleic acids may traffic *via* microtubules. Unfortunately, there is still very little that is known about how nucleic acids traffic through the cytoplasm. Elucidating how nucleic acids traffic through the cytoplasm will be crucial to improving future vectors.

What is really needed now are studies that focus on applying tissue engineering approaches to gene therapy, and elucidating the mechanism by which cells can be manipulated to better take up DNA, siRNA, shRNA, miRNA, and antisense oligonucleotides to affect gene expression. Adler *et al.*[2] and Chalut *et al.*[23] presented interesting data on manipulating the physical parameters and behaviors of cells using chemical delivery methods. The next step forward should be to apply the same cell manipulations to a physical delivery method such as ballistic gene delivery or electroporation to determine whether the physical manipulations of the cell can enhance physical gene delivery methods for tissue engineering applications. Increasing the quantitative assessment of studies that combine both gene delivery and tissue engineering are key to enhancing both the fields. Furthermore, approaching gene therapy from a tissue engineering perspective might be a fresh way to reveal more details about how cells take up and express exogenous nucleic acids.

FUTURE DIRECTIONS AND OPPORTUNITIES

Improving physical non-viral gene delivery methods for tissue engineering applications requires an examination of the fundamental mechanisms utilized by each physical non-viral gene delivery method as well as the reason different cell types are more or less responsive to each gene delivery method. Elucidating the basic mechanisms by which physical non-viral gene delivery methods work and understanding why different cell types are responsive to different gene delivery methods will allow investigators to exploit the positive attributes of gene delivery methods and different cell types to enhance tissue engineering applications.

Microinjection is perhaps the most efficient and direct method for delivering nucleic acids to cells; however, the major weaknesses associated with microinjection are the restricted access to tissues, and the inability to transfect large numbers of cells. Likewise, ballistic gene delivery lacks access to tissues and is restricted by the quantity of nucleic acids that can be delivered. However, both of these methods have significant potential if the weaknesses previously stated can be overcome, as both methods can directly control the amount of nucleic acids directly delivered to individual cells.

In addition, electroporation, sonoporation, and laser irradiation seek to transiently disrupt the cell membrane to increase permeability of nucleic acids to the cells. Sonoporation is attractive because it is already used in a clinical setting, but the tissues that are being targeted need to be extensively evaluated to produce maximum efficiency. Electroporation suffers the same weakness as sonoporation, yet electroporation has more flexibility for targeting cells, as the electric field can be controlled *via* a pulse generator and electrodes can be designed specifically for individual

applications. However, electroporation still suffers from low cell viability. Laser irradiation can precisely target individual cells; however, laser irradiation is not efficient for targeting thousands of cells in different layers of tissues. In contrast, several non-viral chemical vectors exhibit high cell viabilities, but limited transfection efficiencies. Thus, it is necessary to look at which physical features enable the high cell viability of most non-viral chemical transfection vectors and which physical features enable moderate to high transfection in non-viral physical vectors. Perhaps it is best to consider these questions from the point of view of the cell and the environment of the cell to gain a better understanding of what affects cell viability and limits transfection.

If the cell can be physically manipulated, how else can non-viral vectors be improved? Is it possible to make cells more susceptible to electroporation or sonoporation by adjusting the osmolarity of the extracellular fluid? If the extracellular fluid is made to be hypotonic to the intracellular fluid of the cell to induce swelling of the cell, will the swelling produce similar responses in the cell membrane as cell spreading? These questions are important to consider when designing new vectors. Furthermore, can delivery vectors be combined to achieve higher transfection efficiencies? Is there a way to design a combinational polymer scaffold to where one polymer acts as an electrode and another polymer acts as an anchor for nucleic acids and an attachment platform for cells seeded into the scaffold to permit increased transfection efficiency?

Increasing the abilities of non-viral vectors to manipulate gene expression and mitigate cell death depend on finding ways to improve the uptake of nucleic acids into cells and minimizing the trauma to the cell membrane from points of entry. The physical methods described in this review are capable of overcoming the limitation of the cell membrane entry by directly acting on the cell membrane and forcing nucleic acids into the cytoplasm or even the nucleus. However, the method of membrane disruption can directly influence the cell's ability to mend the membrane. The diameter of the ''holes'' created in the cell membrane and the duration for which these ''holes'' remain open seem to directly correlate with the cell's ability to survive. As suggested by Mehier-Humbert *et al.*,[108] larger pores that remain open for increased durations increase the uptake of nucleic acids; however, larger pores permit the exchange of additional agents that normally cannot cross the cell membrane, increasing the risk that homeostatic concentration gradients will be disrupted leading to cell death. Thus, a balance needs to be struck between facilitating the entry of nucleic acids without compromising the homeostatic concentrations of ions such as Na^+ and K^+ inside and outside of the cell.

As investigations continue into how cell membranes and gene expression can be manipulated from a chemical and mechanical perspective, new mechanisms of how the cell membrane reseals and how nucleic acids are trafficked within the cytoplasm in different cell types are bound to be proposed in the literature. Elucidating these fundamental mechanisms will contribute to developing new delivery strategies that enhance the delivery of nucleic acids with minimal risks to compromising the cell membrane. Perhaps even combinational approaches may yield beneficial consequences for gene transfer into target tissues. Tissue engineering currently focuses on manipulating cellular behavior externally by applying mechanical stimuli and different biomaterials to simulate native environments to aid in the differentiation of progenitor cells and stem cells. Perhaps considering the external environment as part of the nucleic acid delivery system is the key to changing the behavior of the cell to better accommodate nucleic acid delivery and improve differentiation of cells into target tissues for regeneration and tissue engineering applications.

The improvements in physical gene delivery methods over the past three decades have been impressive and have greatly enabled increased gene expression in difficult-to-transfect cells; however, the fundamental challenges still remain. Non-viral physical methods still focus on deforming the cell membrane in some manner to increase transfection rates at the expense of cell viability. However, investigators are working to elucidate mechanisms of how nucleic acids can cross the cell membrane and traffic through the cytoplasm to the nucleus. These endeavors are expected to lead to the development of new vectors that can increase the gene expression in cells without compromising significant numbers of cells *in vitro* or *in vivo*. Furthermore, exploration of how cellular behavior can be manipulated externally to achieve a desired behavior is of interest in tissue engineering, which may be key in developing new strategies to better facilitate cell differentiation. Thus, it would seem that applying a tissue engineering approach to gene therapy rather than a gene therapy approach to tissue engineering may be a potential solution for providing a fruitful integration of these two fields together to expand approaches for cell differentiation and tissue formation in tissue engineering and regenerative medicine applications.

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