Recent Advances in Mammalian Cell Transfection Techniques

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Abstract
Cell transfection is an essential step for gene editing and intracellular delivery of cargoes such as mRNA and proteins. Significant improvements have been made to reduce the cytotoxicity and to improve efficiency associated with transfection over the few decades. Nevertheless, with new, exciting biological questions, the demand for the ideal transfection technique with high throughput, single cell transfection ability and control of cargo dosage has increased. This review focuses on recent innovations in cell transfection techniques, and discusses the pros and cons of each method.
Key words: cell transfection, intracellular delivery, cargo, nanostraws, electroporation

1.0 Introduction

With the discovery of CRISPR/cas9 and improvements in gene editing methodologies, there is renewed interest in using genetic engineering techniques for therapeutic purposes such as editing errant human genome implicated in diseases.[1] One of the key steps for editing the mammalian genome is cell transfection which involves the delivery of components such as DNA or RNA/purified proteins through the cell membrane, and into the cytoplasm and nuclei respectively. This review summarizes articles published in 2017 on the different classes of mammalian cell transfection techniques and the challenges going forward. Readers interested in the timeline of cell transfection techniques up to 2016 can refer to an excellent review by Stewart et al.[2]

2.0 Advances in Cell Transfection Techniques

Mammalian cell transfection techniques can be categorized into a few major classes namely viral, chemical and physical methods.

2.1 Viral Methods for Cell Transfection

Viral transfection commonly makes use of adeno-associated virus (AAV) with lower immunogenicity than most other viruses. Other viral vectors include genetically-modified lentivirus such as retrovirus human immune-deficiency virus (HIV) and herpes simplex viruses (HSV).[3] Some advantages of using viruses include relatively high success rates (~60-80% for cell lines and ~50-60% for primary cells) and stable genetic expressions over several generations of cells or across the lifespan of cells for *in vitro* experiments. Success rates are, however, much lower in complex *in vivo* environments. Viruses have also evolved mechanisms such as tagging their genetic materials with nuclear-localization signals (NLS) and transient disruption of nuclear pore complexes (NPCs) to deliver viral genetic materials across the nuclear envelope.[4] Most notably, they can be further engineered to allow targeted labelling of sub-populations of cells.[3] The ability of viral vectors for specific cell targeting is an advantage of viral method over chemical and physical techniques for cell transfection.

Nevertheless, there is a limit to the type (DNA versus RNA) and size of the genetic materials that can be packaged inside viruses.[3] Most viral vectors will also incorporate viral genome into the host cells which makes them more risky in clinical settings although progress has been made such as in creating non-integrating lentiviruses. Furthermore, high viral load can cause host cells to lyse, and the success rates are also dependent on the health and age of the cells.[5] Some viral vectors such as adenovirus and HSV-1 are known to elicit potent inflammatory responses while others like

lentivirus may induce oncogenesis when given at high viral dosage.[3] Unfortunately, optimal patient-specific viral dosage have not been well-studied which complicates clinical trials using viral delivery mechanisms as potential immune response can stymie the efficacy of CRISPR/cas9-mediated gene therapy.[6] Similar problem also occurs in research application where high cytotoxic viral titer or load are repeatedly administered to increase the number of transfected cells.

To avoid high viral load and its relatively low transduction efficiency, the Gradinaru group recently introduced a strategy for improved gene delivery to *in vivo* nervous system using a cell-type-specific capsid selection method called CREATE (Cre recombinase-based AAV targeted evolution). The team identified AAV capsids AAV-PHP.eB which showed efficient crossing of the blood brain barrier and transduction of neurons and astrocytes throughout the adult mouse brain and spinal cord with low viral load (**Fig. 1A**).[7] Their method involved injection of AAV9 capsids with randomly inserted heptamers into the retro-orbital sinus of adult mice, followed by repeated *in vivo* selection. The team also reported another capsid variant, AAV-PHP.S that displayed tropism towards peripheral neurons including the dorsal root ganglion neurons, cardiac ganglia and enteric nervous system. The group also provided a protocol for generating and purifying these AAV-PHP viruses for biomedical applications.[8]

Recently, Zhu et al. also sought to overcome the cytotoxicity of high viral load by capitalizing on the tropism of Zika virus for neural precursor cells for targeting glioblastoma.[9] The team created an attenuated form of Zika virus i.e. ZIKV-E218A and found that while it had less potency than wild-type Zika virus, it was able to kill different patient-derived glioblastoma stem cells *in vitro*. ZIKV-E218A also had limited replication capacity and induced less toxicity to neighboring healthy, differentiated neural cells. While the utility of engineered Zika virus for *in vivo* work is yet to be evaluated, this study serves as a foundation for further investigation and development of engineered Zika virus for purposes such as targeted neural cell transfection and labelling.

2.2 Chemical Methods for Cell Transfection

The other major class of mammalian cell transfection techniques is using chemicals, with the most common being lipids and calcium phosphate for *in vitro* experiments. Lipid-mediated transfection, also known as lipofection, uses lipids with similar properties to that of cell membrane. The positively charged lipids associate with the negatively charged phosphate groups of genetic materials. The complex then fuses with cell membrane for delivery. It has been found that positively charged lipids produced higher transfection efficiency due to better association with negatively charged cell membrane. The calcium phosphate method involves mixing DNA-calcium chloride mixture into phosphate solution to form precipitate. The precipitate is then taken up by cells via endocytosis. These methods are popular in laboratories as reagents such as lipofectamine are readily available and inexpensive, and calcium phosphate transfection may be performed using chemicals commonly found in the lab inventory. This class of technique is extremely useful for *in vitro* experiments although factors such as presence of serum and high pH can reduce the transfection efficacy. For the past 2 decades, scientists have also created smart polymers with different functional groups and sensitivities towards stimuli such as magnetic fields, temperature and pH for *in vivo* cell transfection purposes.

One challenge of chemical transfection technique has been their inability to target specific cell type. Wang and colleagues attempted to tackle this problem by synthesizing comb-shaped polymers bearing Arg-Glu-Asp-Val (REDV) peptides for selective uptake by endothelial cells.[10] The polymers had low cytotoxicity and improved transfection efficiency compared to polymers without REDV

decorations. This strategy can also be applied to other cell types for selective targeting of sub-populations of cells for gene delivery. Cheng et al. also recently reported a pH-sensitive polymer that efficiently condensed DNA into nanoparticles and endowed high stability to the resulting polyplexes through hydrophobic modification.[11] The polymer also contained an acid-cleavable imine bond that facilitate efficient DNA cargo release in the cytoplasm for enhanced transfection.

2.3 Physical Cell Transfection

Physical forces such as mechanical and electrical forces have also been applied to induce transient opening of cell membrane for transfection. In this class of technique, electroporation is most widely used. As the name suggests, electroporation generates an electrical field across the cell membrane to induce pore opening. Genetic materials can then enter the cells when pores are transiently open. Electroporation has been extremely useful for introducing CRISPR/cas9-associated ribonucleoprotein complexes consisting of guide RNA and purified cas9 protein into sensitive, primary cells such as T-lymphocytes which do not normally uptake foreign DNA easily. They are also popular as it avoids genome integration into transfected cells unlike using viral vectors.

Another popular method in physical transfection technique is magneto-transfection where positively-charged magnetic nanoparticles (MNPs) are associated with negatively-charged genetic materials via ionic interactions. Magnetic fields are then applied to cells cultured onto magnetic plates to induce association of MNP-DNA complexes onto cell membrane and subsequent endocytosis. Physical methods in general offer high throughput in cell transfection but its set-up might be cumbersome or expensive. Equipment that provides uniform physical fields such as magnetic and electrical fields are also necessary for homogenous cell transfection. Nevertheless, newly developed physical tools such as nanostraws[12] can offer single cell transfection, control of cargo dosage and longitudinal delivery of materials to the same exact cell which other transfection techniques cannot.

Micro-tools: Physical cell transfection techniques aim to perturb the cell membrane for delivery of genetic materials, although if effective gene expression is to occur, DNA must also enter the nucleus and integrate into the genome before they are degraded. To address this challenge, Ding et al. integrated physical perturbations of plasma membrane and nuclear envelope with electric fields to enhance nuclear delivery.[13] This named their technique disruption-and-field-enhanced delivery (**Fig. 1B**). The group first utilized a microfluidic channel device to confine and disrupt the plasma membrane through rapid cell deformations. This is followed by exposing the cells to electric fields that induced reversible nuclear envelope rupture and active transport of DNA into cytoplasm and nucleus. Note that the group also made use of hypo-osmolar buffer that rendered the plasma membrane more vulnerable to microfluidic disruption. Their method successfully delivered DNA plasmids to millions of cells per minute in a continuous flow system. The technique can also be applied for the co-delivery of DNA, RNA and proteins for integrated gene editing. However, transfection using the disruption-and-field-enhanced technique might not be suitable if they adversely affect the physiology of sensitive primary cells like stem cells. This become especially important as mechano-sensitive channels and volume-sensitive water in/efflux system can alter stem cell fate.[14]

Nano-tools: While there are various cell transfection techniques for efficient delivery of genetic materials to cells in *vitro*, it is important to note that existing techniques cannot guarantee longitudinal delivery of materials into the same exact cell populations due to poorly understood and/or stochastic delivery mechanisms. Furthermore, no current techniques cannot interface with the same

exact cell populations to both deliver and extract mRNA/proteins.

To directly address this issue, our group has also developed the nanostraw electroporation technology (**Fig. 1C**). This technique facilitates non-destructive, periodic and controlled sampling (>21 days), *and* delivery of multiple materials like mRNA and proteins into the exact same cell populations and single cell[12] with minimal clogging. Their approach offers high spatio-temporal control in dosage, high yield co-delivery of two or more materials, and the flexibility of sequential delivery even on different days.[15] The platform consists of a transwell track-etched polycarbonate polymer membrane with 150 nm diameter alumina nanostraws protruding from the surface. Cells are cultured onto the nanostraw membrane where they adhere, spread and exhibit typical cellular behaviors like on 2D polystyrene surfaces. The nanostraw platform is placed on top of extraction buffer/delivery solution on an indium tin oxide (ITO) electrode. A platinum wire immersed into the cell culture buffer acts as the counter electrode. A small electric field (5-40 V) is applied though the nanostraws to open pores on cell membrane locally (up to 5 mins) for sampling/extraction and delivery of mRNA/proteins.[15] Another advantage of the nanostraw platform is that it can be used to deliver to a wide range of primary cells including stem cells, cardiomyocytes and neurons which are sensitive to other delivery methods; this technique could also sample a panel of 48 different mRNA from the exact same stem cell-cardiomyocytes for >21 days.[12]

One of the common limitation of cell transfection using physical forces is the inability to interface with *in vivo* systems. The Chandan and Langer group overcame this challenge recently by fabricating a nano-channeled device for topical delivery of reprogramming factors to tissues to rescue necrotizing tissues and whole limbs with injury-induced ischemia.[16] They called this the tissue nano-transfection approach. This approach enabled direct cytosolic delivery of reprogramming factors using a high intensity and focused electric field with arrayed nano-channels. The electric fields nano-porated the surrounding tissues while electrophoretically introduced reprogramming factors into the cells. The authors suggested that this technique might enable the use of the patient's own body to produce autologous cells that have been genetically modified. Unfortunately, this study did not investigate the effects of acute and chronic electrophoresis on the animal subjects. The use of electric field might for instance interfere with the bioelectrical signals, both in the central and peripheral nervous systems, that have been recently found to play a role in a plethora of physiological activities such as immunity.[17]

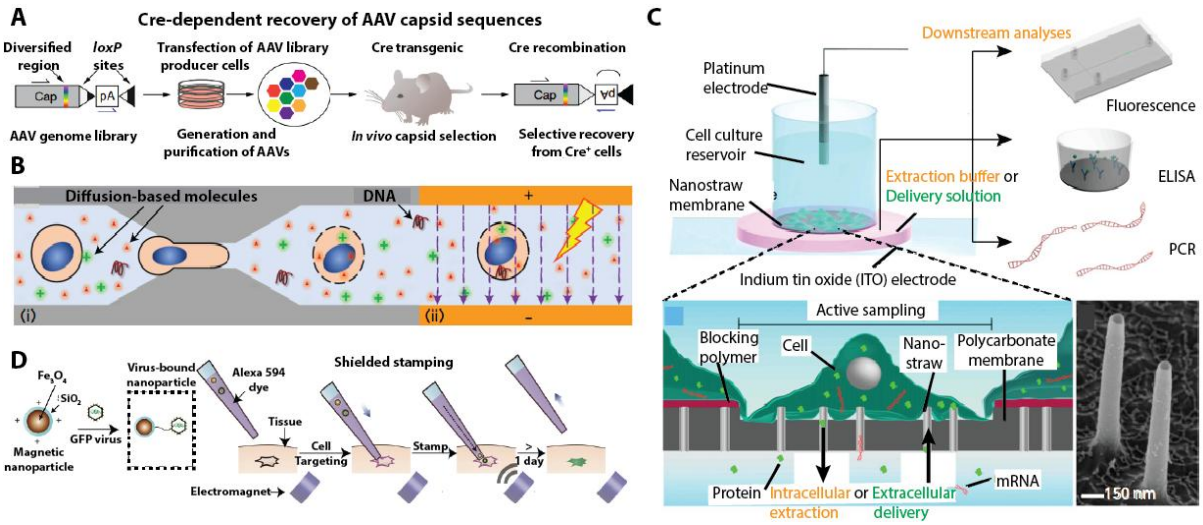

Fig. 1 Recent advances in mammalian cell transfection techniques. (**A**) The CREATE (Cre recombinase-based AAV targeted evolution) technique where AAV with mutated capsids with tropism for different cell types such as neurons are selected *in vivo* (mouse models) over repeated cycles. Permission to reprint was approved by Springer Nature. (**B**) The disruption-and-field-enhanced delivery technique where cells are exposed to hypo-osmolar solution and microfluidic channels to induce reversible cell and nuclear envelope rupture before subsequent exposure to electric fields for active transport of DNA into cytoplasm and nucleus. Permission to reprint was approved by Springer Nature. (**C**) The nanostraws platform where cultured cells on the platform are exposed to local electric fields for controlled electroporation for delivery and extraction of materials such as mRNA and proteins into and outs of the cells. The scanning electron microscopy (SEM) image shows nanostraws of about 1.5 µm in height and 150 nm in diameter. Image reproduced from Proceedings of the National Academy of Sciences. (**D**) The 'viral stamping' technique where viruses which are reversibly bound to MNPs are bought into physical contact with target cell on in tissues (*in vivo*) using magnetic forces for specific single cell transfection. Permission to reprint was approved by Springer Nature.

3.0 Going Forward

Despite advances in each respective class of mammalian cell transfection techniques, it is noteworthy that unique limitations still exist for each of them. For example, the limited loading capacity of viral vectors and the lack of target specificity of chemical polymers. To overcome this, some groups have capitalized on the merits of different techniques and came up with a strategy combining different transfection techniques in their work. The Anderson group made use of systemic delivery of cas9 mRNA by lipid nanoparticles, and delivery of guide RNA/homology-directed repair (HDR) template by AAV to repair the fumarylacetoacetate hydrolase-splicing mutation.[18] The team achieved >6% correction efficiency in hepatocytes which is a drastic improvement compared to their previous 0.4% correction efficiency using hydrodynamic injection of cas9 mRNA and guide RNA/HDR template. Nevertheless, as cas9 mRNA was delivered instead of the typical cas9 protein in ribonucleoprotein complexes, the time needed for mRNA translation and stability of cas9 mRNA might lower the gene editing efficacy. In another example of a combined delivery approach, Schubert et al. made use of MNPs and viruses for targeted single cell transfection. The method named 'viral stamping' technique (**Fig. 1D**) involves viruses being reversibly bound to MNPs and brought into physical contact with target cells on surface (*in vitro*) or tissues (*in vivo*) using magnetic forces for specific single cell transfection.[19]

The choice of transfection depends heavily on the research questions to be answered and the

application of the work – laboratories or clinics. In laboratories, engineered viruses with better specificities will be extremely useful for targeted cell or cellular structure labelling to understand the biology of sub-populations of cells such as neurons. Due to possible immune responses and random gene insertions, the clinical utility of viruses is arguably less compared to chemical and physical cell transfection techniques. Cell type targeting is still a significant challenge for non-viral approaches. The strategy of decorating polymers with cell-specific ligands for cell targeting might help overcome this limitation. Although physical methods such as using light (laser) and magnetic fields are gaining more attention, their use has been largely restricted to *in vitro* applications. There is certainly great value in creating miniaturized devices that can perform *in vivo* cell transfection as the Chandan group achieved. Scientists developing physical methods for cell transfection should also pay careful attention to literature on mechano-biology and bioelectricity in consideration of the compatibility of their approach with cellular physiology, and whether they can exploit inherent physical or electrical properties of their system of interest for enhanced cell transfection efficiency and specificity.

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