International Journal of Applied Science-Research and Review

ISSN: 2394-9988

Open access

Review Article

Cell Delivery Strategies: Review of the Recent Developments, Challenges and Opportunities

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ABSTRACT

Background: Integrating transgene into the host genome for stable maintenance in dividing cells can have unpredictable effects on gene expression and unintended effects on neighboring genes. Ethical concerns are particularly serious in the case of clinical applications using germ cells of heritable genome editing in humans, i.e., modifications introduced in sperm, eggs, or embryos to "create" genetically engineered children. One major challenge that faces *in vivo* gene editing (GE) technology is the selection of a suitable delivery route to transfer engineered DNA, RNA, or proteins into target cells. This problem originates from the large size of the key components and the low capacity of the delivery vector. In general, cell delivery methods available today can be broadly classified into three categories: chemical, biological and physical.

Methods and findings: The PubMed search was used with each keyword of cell delivery strategies regarding the description of various delivery methodologies and factors that influence transfection efficiency. Due to the exponential growth of papers that appear in the literature in the field and space limitations, the search was limited to data published in the year 2015 up to March 2022. Although the choice of the appropriate cell delivery route may look trivial, it is actually a crucial factor that is often overlooked. Selecting the origin and cell type, as well as proper experimental protocol, is of at most importance to maximize outcomes.

Conclusion: The reader should keep in mind that a side by side comparison of the different methods may be necessary to find the most suitable methodology for a specific goal. No single approach can be applied to all cells and all experiments. The transfer of macromolecules through different cellular routes is much more challenging and technically complex. Hurdles remain to overcome before opening a prospective future in pharmaceutics and gene therapy.

Key Words: CRISPR; Design nucleases; Gene delivery; Gene editing; Gene therapy; Transfection

INTRODUCTION

The critical developments leading to the current state of GE date back to the first gene delivery into mammalian cells, insulin gene cloning, synthesis of human recombinant lysosomal enzymes and human genome sequencing [1]. GE is employed in different gene delivery systems and has evolved to keep pace with technological advances in the field and further refined to distinguish various procedures and cell types. The term 'Transfection" is given to the artificial process of introducing a segment of a foreign nucleic acid into a target cell using different methods [2]. The two main goals of transfection are to generate recombinant proteins with proper folding and post-translational changes needed for its function. This specifically promotes or suppresses gene expression in transfected cells. This process can lead to a change in the properties of the cell, allowing the investigator to follow the function and regulation of genes or their products, for the "creation" of transgenic organisms, and as an efficient approach for gene therapy. Additionally, introducing proteins with readily detectable markers and other alterations into cells enables the study of promoter and enhancer sequences or protein: protein interactions [2]. Furthermore, transfection can be used in a wide range of fields, including

Received:	05-April-2022	**Manuscript No:**	ipias-22-12828
Editor assigned:	07-April-2022	**PreQC No:**	ipias-22-12828 (PQ)
Reviewed:	21-April-2022	**QC No:**	ipias-22-12828
Revised:	26-April-2022	**Manuscript No:**	ipias-22-12828 (R)
Published:	03-May-2022	**DOI:**	10.36648/2394-9988-9.4.60

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Citation Khalil AM (2022) Cell Delivery Strategies: Review of the Recent Developments, Challenges, and Opportunities. Int J Appl Sci Res Rev Vol.9:60

the various forms of bio-production, generation of transgenic animals using embryonic stem cells, model development for diseases, or drug development. One example is the antigens that are being currently developed as vaccines require a delivery vehicle in order to evoke the desired immune activation. Another is the delivery of reprogramming transcription factors that allows the production of induced pluripotent stem cell (iPSC) [3,4]. There are wide differences among the transfection strategies with respect to transfection efficiency, cell toxicity, effects on normal physiology, and level of gene expression. Successful GE must be able to deliver the donor DNA into a large number of target cells in order to have a therapeutic benefit [5]. The present review was carried out with the aim of improving overall understanding of cell delivery technology and to find out how can specific transfection conditions be achieved to maximize chances for successful transfection and consequently perfect editing.

METHODS

An online search using the PubMed database was followed in this study. The snowball method was also used to extract other publications. The keywords used included terms describing various delivery methodologies and the factors that influence transfection efficiency. Thanks to the efforts of a large number of researchers and new ideas for improving equipment and strategies. Due to the exponential growth of papers published in the field and space limitations, only articles between 2015 and March 2022 were retrieved. Titles in non-English language were excluded. After vigorous screening and detailed evaluation, only 136 articles were selected for data extraction.

RESULTS

There are wide differences among the transfection strategies with respect to transfection efficiency, cell toxicity, effects on normal physiology, and level of gene expression. Scientists put transfection methods in two main classes: transient transfection and stable transfection [6].

Types of Transfection

The type of transfection depends on whether the hosted nucleic acid resides in the cell for a restricted period of time (transient transfection) or whether it remains in the cells long term and is passed to the offspring of the transfected cell (stable transfection) (**Table 1**).

Table 1: Basic differences between transient transfection and stable transfection

Aspect of Comparison	Transient Transfection	Stable Transfection
Stability of transfection	Can be lost by environmental factors, nuclease digestion or dilution out during cell division.	Relatively stable
Type of nucleic acid used	Both DNA and RNA	Only DNA
DNA configuration	Highly supercoiled DNA	Linear or circular
Integration of DNA vector	Not integrated, but remains in the nucleus.	Integration is most efficient when linear DNA RNA by itself cannot be stably introduced into host cells.
Number of copies of transfected gene and level of expression	Multiple or large copy number results in high level of protein expression.	Single or small copy number results in lower but level of expression
Expression of the transgene	Short-term Genetic alteration is not permanent	Long-term Genetic alteration is permanent.
Replication of the foreign DNA within the transfected cell	Does not replicate	Replicates
Passage of the foreign DNA to cell progeny	Not passed	Passed stably from generation to generation;
Detection of the transfection	Gene can be easily detected by inserting a reporter gene.	Gene can be easily detected by inserting a selectable marker and selecting through artificial selection on media.
Selective screening for transfectant	Not required	Required
Time needed for cell harvesting	Transfected cells are typically harvested within 24–96 h of transfection.	Requires 2–3 weeks of selection for the isolation of stably transfected colonies.
Suitability for studies using vectors with inducible promoters.	Generally not suitable, and not complex to perform.	Suitable, rare in occurrence, complex, more laborious and challenging

It is important to evaluate the efficiency of the transfection approach, especially in functional investigations that requires high transfection efficacy to assure post-transcriptional control of specific downstream targets [7-9]. Comparison of transfection efficiency can be followed by flow cytometry, fluorescent activated cell sorting, immunofluorescent staining, western blot,or real-time polymerase chain reaction [2,10,11,12].

Certain transfection protocols use cotransfection for particular applications. Cotransfection indicates that a host cell concurrently receives more than one type of nucleic acids, for example, plasmid DNA and small interfering RNA (siRNA) [12,13], multiple plasmid DNAs, siRNA and plasmid DNA, or multiple miRNAs [14,15]. It is a common procedure to produce stable transfection [16].

The plasmid DNA may contain a gene that is easily assayed and acts as a marker. To ensure the occurrence of this, an appropriate marker gene is co-transfected, which gives the cell a selection advantage. The most commonly selectable markers are genes that encode for a fluorescence protein or those that confer resistance to different selection drugs or genes that compensate for an essential gene that is defective in the cell line [17].

Factors Influencing Transfection Efficiency

Successful transfection is affected by numerous factors that can all play a part in the outcome of your transfection experiment [2,18,19,20]. The most important factors include:

Type of target cell: The biological features of the cell type must be carefully considered when planning for transfection experiments because certain cell types are not well suitable for particular trans- fection techniques [2]. Furthermore, different cell types/cell lines respond differently to a given transfection reagent or method [18- 20]. Primary mammalian cells are comparatively less vulnerable to transfection than other cell types due to their limited lifespan and restricted expansion capacity [21,22]. Regular observations suggest that transfection proficiencies are always lower than 40% regardless of the reagents employed and have implicated primarycells as a hard to transfect cell type [2].

Cell health and viability: The viability, density (confluency), and general health of cells before starting a transfection experiment are well-known important sources of variation from one transfection to another [23]. The ideal cell density for transfection varies depending on different cell types, applications, and transfection techniques. To ensure that optimal confluency is achieved at the beginning of transfection, a standard seeding procedure should be maintained from experiment to experiment. Generally, cell survival must be at least 90% before transfection and have had enough time to recover from passaging. As a general rule, using cells from the early passage was recommended to obtain transfection optimal results, especially for transfection which involves primary or stem cells [2,24].

To achieve good transfection outcomes, a routine sub-culturing protocol and passage cultures should be followed once or twice a week at a dilution that enables them to become almost confluent before the next passage [25]. As a whole, using cells that have undergone no more than 30 passages after thawing of a stock culture is recommended. Thawing a fresh vial of frozen cells and establishing low subcultures for transfection experiments enhances the recovery of transfection activity. For optimal reproducibility, aliquots of cells of a low passage number can be stored frozen and thawed as required [26].

Transfection medium: Selection of the most optimal medium for the cell type and transfection method plays a crucial role in transfection protocols. It is very important to use a fresh culture medium, especially if one or more of the gradients are unstable. This is so because a medium that is missing key components and necessary supplements may detrimentally affect cell growth [27]. In general, the serum is important in the culture medium to facilitate transfection with DNA. Most cells remain healthy for several hours in absence of serum in the medium. To retain the health of the cells, lesser amounts of antibiotics should be added when a serum free medium is used than in a serum containing medium.

Type of transfection reagent: The efficiency of chemical transfection depends largely on the type of reagents utilized in the transfection process [2]. The effectiveness of different transfection reagents may differ even in different cell lines originating from the same species. Generally, lipid based or liposomal reagents revealed better transfection performance in most experiments that involved immortalized human and animal cell lines [2]. In comparison, non-liposomal reagents were shown to have higher safety than liposomal based reagents [28]. In both primary and immortalized human cell lines, liposomal reagents were found to cause a higher level of cytotoxicity than non-liposomal reagents [2,28]. Another important factor that plays a role in transfection efficacy and level of cytotoxicity is the ratio of nucleic acids to transfection reagents. When disproportionate and high transfection reagent volume was used, cell toxicity increased resulting in the reduction in the overall outcome of transfection [18].

Type of Delivery: When plasmid DNA is used as a vector for trans- fection, the configuration (linear or supercoiled DNA) and the size of the plasmid affect the efficacy of transfection [29]. Transient transfection is most effective with supercoiled plasmid DNA and generally produces higher efficiency relative to linear DNA, which is more susceptible to digestion by exonucleases [30]. However, asa rule, stable transfection is restricted to DNA vectors since it givesoptimal integration of DNA into the host genome of the host cell [31]. Although the linear DNA produces less DNA uptake by the cells compared to supercoiled DNA, it gives optimal integration of DNA into the genome of the transfected cell [2].

For stable transfection, RNA molecules by themselves cannot be used. When siRNA and miRNA are used they are delivered into host cells as short hairpin transcripts made from a selectable DNA vector. RNA transfection does not mandate genome integration, transcription, and post-transcriptional processing. This may accelerate the synthesis of the wanted protein [32]. However, the expression of a protein is transient following RNA transfection, and RNAs are relatively less stable than DNAs. This makes them more susceptible to breaking down when transported in the cell [33].

Transfection method: It is essential to note that not all of the trans- fection approaches that utilize biological, chemical, or physical methods can be applied to all types of cells. Factors required toreach a high degree of transfection efficacy, minimal cytotoxicity, reduced effects on normal functions physiology, the ideal rate of gene expression etc. depend principally on cell type and the prin- ciples controlling these methods [2,34]. The direct delivery of thegene construct may theoretically sound easy, more efficient, and specific than physical and chemical methods. In practice, it proves to be an inappropriate choice because the successful gene deliv- ery system needs the foreign genetic molecule to remain stable within the host.

A key parameter that influences transfection efficiency is the ionic composition of the electroporation buffer [34]. In addition, the size and concentration of the vector should be carefully chosen when planning an electroporation protocol. In this regard, the large sized plasmid was observed to reduce electroporation transfection [30]. In laser based transfection, the density and duration of laser pulse employed in delivering foreign nucleic acids into the host cells would primarily influence the success of the process. The use of different cell types during laser assisted transfection would also affect transfection efficiencies [2]. On the other hand, when a magnetic assisted transfection approach is used, DNA and RNA have to be coated with metals or other carrier molecules to neutralize them. Also, a magnetic conjugated Adeno associated virus (AAV) vector can be used to introduce the nucleic acid complex into the host cell [35]. Two important factors were described to increase the efficiency of magnetofection; the cell types and the number of pulses [35,36].

Cell Delivery Technologies

t cell types and all experiments. Therefore, the selection of appropriate cell type and optimal nucleic acid size is crucial to guarantee the success of the transfection protocol [2]. Delivery technologies or transfection methods available today can be basically grouped into three classes: chemical, biological, and physical (**Table 2**) (**Figure 1**).

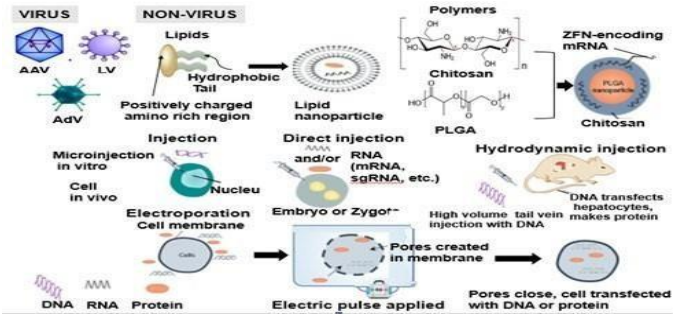

Figure 1: Delivery technologies or transfection methods

Table 2: Summary on comparison between the most common cell delivery strategies for gene editing

Delivery Vehicle	Composition	Most Common Cargo	Capacity	Advantages	Limitations	Ease of Use
Direct microinjection	Needle	DNA plasmid mRNA (Cas9 + sgRNA); RNP	nM levels of Cas9 and sgRNA	Guaranteed delivery into cell of interest	Time-consuming; difficult; generally *in vitro* only	***
Electroporation	Electric current	DNA plasmid; mRNA (Cas9 +sgRNA)	nM levels of Cas9 and sgRNA	Delivery to cell population; well-known technique	Generally *in vitro* only; some cells not amenable	*
Adeno-Associated Virus	Non-enveloped, ssDNA	DNA plasmid	<5 kb nucleic acid	Minimal immunogenicity	Low capacity	***
Adenovirus	Non-enveloped, dsDNA	DNA plasmid	8 kb nucleic acid	High efficiency delivery	Inflammatory response; difficult scaled production	***
Lentivirus	Enveloped, RNA	DNA plasmid	about 10 kb, up to 18 kb nucleic acid	Persistent gene transfer	Prone to gene rearrangement; transgene silencing	***
Lipid nanoparticles/ liposomes/ lipoplexes	Natural or synthetic lipids or polymers	mRNA (Cas9 + sgRNA); RNP	nM levels of Cas9 and sgRNA	Virus-free; simple manipulation; low cost	Endosomal degradation of cargo; specific cell tropism	**
Cell- Penetrating Peptides	Short amino acid sequences	RNP	nM levels of Cas9 and sgRNA	Virus-free; can deliver intact RNP	Variable penetrating efficiency	**
DNA Nanoclew	DNA spheroid	RNP	nM levels of Cas9 and sgRNA	Virus-free	Modifications for template DNA needed	****
Gold Nanoparticles	Cationic arginine-	RNP	nM levels of Cas9 and sgRNA	Inert; membrane-fusion-like delivery	Nonspecific inflammatory response	**

Among many nanocarriers based delivery systems, liposome technology has been particularly useful with many formulations launched into clinical applications [37]. They are well studied and effective gene and/or drug delivery tools, widely utilized in cancer chemotherapy and gene therapy [38-41].

Chemical: Chemical methods utilize carrier molecules to neutralize or transfer a positive charge to the negatively charged nucleic acids. Nanoparticles are defined as solid colloidal particles with a diameter between 10 and 1000 µm. The active ingredient is dissolved, entrapped, or encapsulated within the solid matrix of the particles. In comparison to physical or mechanical transfection methods, chemical transfection involves using specially designed chemicals or compounds to help in introducing the foreign nucleic acid into the host cells [42-44]. Summarizes the advantages and disadvantages of commonly used gene transfer chemical strategies (**Table 3**).

Table 3: Comparison of chemical methods used in gene delivery

Method	Advantages	Disadvantages
Cationic lipid mediated delivery	Fast, simple, and reproducible Purchased commercially with reproducible results High efficacy and expression performance Applicable to a wide range of cell lines with superior efficacy and high-throughput screens Can be used for delivering DNA of all sizes, RNA, siRNA, protein, or oligonucleotides No size limit on the packaged nucleic acid Can be applied to both transient and stable expression systems Unlike other chemical methods, can be used for *in vivo* delivery of nucleic acids to animals and humans.	Optimization may be needed; some cell lines are sensitive to cationic lipids Certain cell lines are not easily transfected with cationic lipids Transfection efficacy may be lowered by the presence of serum which may induce non-specific interactions with serum proteins, immunogenic response, interfere with complex formation, resulting in rapid blood Absence of serum in the medium may enhance cell toxicity Transfection efficiency depends on the cell type and culture conditions, requiring the optimization of transfection conditions. runs seriously short for therapeutics purposes

Calcium phosphate co-precipitation	Well-described, popular, inexpensive and easily mastered Applied to both transient and stable protein production Efficiency is high with many types of cultured cells (cell line-dependent)	Needs careful preparation of reagents; CaPO4 solutions are prone to slight changes in pH, temperature, and buffer salt concentrations Success rate of transfection is comparatively low and requires prior optimization to improve efficiency Reproducibility might be problematic May be cytotoxic to many types of cell cultures, especially of primary cells. Does not work with RPMI medium due to its high phosphate concentration. Not suitable for *in vivo* gene delivery to whole animals
DEAE-dextran	Relatively simple approach Inexpensive and results reproducible High efficacy (cell line-dependent) Ideal for overexpression of recombinant protein in transient transfection Can be applied to *ex vivo* gene transfer therapy	Has a toxic effect on viability in some cell types Useful for restricted transient transfections, but not suitable for generating stable cell lines. Low transfection rate for a range of cell types (typically lower than 10% in primary cells) Need for reduced serum media during the transfection procedure
Delivery by other cationic polymers	Cost-effective, relatively simple and highly reproducible Typically stable in serum and not temperature sensitive	Vary dramatically in their level of transfection efficiency and cell toxicity. Optimization of cationic polymers is needed to improve the gene transfection efficiency. Non-biodegradable (dendrimers)

Cationic lipid transfection: The cationic lipid mediated delivery method represents one of the most popular procedures for introducing foreign genetic material into cells [45,46]. Basically, a cationic lipid consists of a positively charged head group and one or two hydrocarbon chains. The head group controls the interaction between the lipid and the phosphate backbone of the nucleic acid, and mediates DNA condensation. It allows a highly efficient transfection of a wide range of cell types, including adherent, suspension, and insect cells, as well as primary cultures.

In principle, cationic polymers complex with the negatively charged DNA and siRNA aid in the delivery of the cargo into cells through endocytosis. With cationic lipid reagents, the DNA solution is not deliberately encapsulated within the liposomes; rather, the negatively charged DNA binds spontaneously to the positively charged liposomes, forming condensed nucleic acid-cationic lipid reagent complexes via electrostatic interactions between the negatively charged nucleic acid and the positively charged head group of the synthetic lipid reagent.

Calcium phosphate transfection: Calcium phosphate transfection is another common approach that utilizes Ca2+ molecules to induce precipitation of two frequently utilized polymeric vectors for DNA transfer are polyethenimine (PEI) and poly (L-lysine) [47]. Basically, the mechanisms underlying this transfection method involve mixing the negatively charged nucleic acids with the positively charged calcium ions (Ca2+) in a buffered saline/phosphate solution to form a calcium-phosphate-DNA co-precipitate [44]. Branched PEI have elevated charge density, allowing efficient plasmid DNA packing, and pH-buffering ability that helps escape from endosomes. Since branched PEI is cytotoxic, a compromise between the desirable properties of branched PEI and the less toxic linear PEI must be followed for efficient transfection. This route is not suitable for in vivo delivery of nucleic acids to whole animals, and it exhibits relatively poor transfection efficiency in comparison to other chemical transfection techniques such as lipid-mediated transfection.

DEAE-dextran transfection: The cationic diethylaminoethyl (DE- AE)-dextran molecule tightly binds the negatively charged nucleotides. The net positive charge of the resulting nucleic acid-DEAE-dextran complex facilitates the adherence to the cell membrane and entrance into the cytoplasm by endocytosis or osmotic shock induced by Dimethyl Sulfoxide or Glycerol [8]. Biochemical methods of transfection such as calcium phosphate-mediated and DEAE-dextran-mediated transfection have been used for a long time to transfer nucleic acids into cultured mammalian cells [8]. DEAE-dextran-mediated transfection differs from the calcium phosphate co-precipitation method in that it is used for transient expression of cloned genes and not for stable transformation of cells. Another difference is related to the smaller amounts of DNA which are used for transfection with DEAE-dextran than with calcium phosphate co-precipitation. The DEAE-dextran method is suitable for transient transfections with promotor/receptor plasmid or viral vectors in investigating promotor and enhancer functions [8].

Delivery by other cationic polymers: Other cationic polymers used for gene transfer include cationic peptides and their derivatives (e.g., polylysine, polyornithine), linear or branched synthetic poly- mers (e.g., polybrene, polyethyleneimine), polysaccharide based delivery molecules (e.g., cyclodextrin, chitosan), natural polymers (e.g., histone, collagen), and activated and non-activated den- drimers [48]. These polyplexes are formed between a cationic polymer and a nucleic acid-mediated by electrostatic interactions and therefore called polycation/DNA complexes [49]. The complex adheres to the cell membrane through electrostatic interactions and enters the cell by endocytosis. These polymers differ from cationic lipids in that they do not have a hydrophobic moiety and are totally soluble in water. They are, by far, the most widely used non-viral gene delivery vectors. Several variables such as molecular weight (MW), surface charge, charge density, hydrophilicity and the structure of cationic polymers affect gene transfection efficiency of cationic polymers [49].

When compared to DEAE dextran, cationic polymers offer more complex stability, higher reproducibility, and greater transfection efficiencies (Table 3). However, their cytotoxicity and limitation to transient transfection research remain the major concern regarding their applicability. Although higher MW cationic polymers have a very low rate of biodegradation and are more cytotoxic than small polymers, their transfection efficiencies are higher because

of their increased polymer to nucleic acid charge ratio. However, the higher toxicity of larger MW polymers can be reduced by biodegradable cross-linking of low MW polymers into larger polymeric structures [49,50].

Physical

Compares four physical methods usually employed either *in vitro* or *ex vivo* to directly deliver nucleic acids into the cytoplasm or the nucleus of the cell. Other physical delivery technologies use hydrodynamic pressure, magnetic field, or ultrasound to drive naked nucleic acids or nucleic acid-particle complexes into target cells (**Table 4**).

Table 4: Comparison of presently available physical cell delivery methods

Method	Advantages	Disadvantages
Microinjection	Straightforward, simple and rapid Results are reliable with very high efficiency Can be applied in transient and stable transfection of all cell types. Less dependent on cell type and condition Allows single-cell transfection No limit on the size and or number of genes that can be delivered Vector not required Used in clinical applications such as gene therapy	Needs expensive instrumentation, e.g. a microscope precludes the use of in a true *in vivo* setting High precision requires sophisticated personnel technical skills or robotic system Technically demanding and very laborious (one cell at a time) High voltage may result in high level of cell necrosis, apoptosis, and permanent cell damage which are not easily repaired Compared to chemical transfection methods, requires the use of larger quantities of cells Limited to *in vitro* or *ex vivo* applications
Electroporation	Simple principle Reproducible results if optimized Fast transfection of large number of cells if optimized Less dependent on cellular type and condition High efficacy and less constrains on size of the cargo Vector not needed	Requires special instrumentation; Electrical pulse and field strength require optimization More manipulation of cells required High level of cell damage and toxicity Requires large number of cells due to high mortality rate Irreversible membrane damage and cell lysis possible Limited to *in vitro* or *ex vivo* applications
Biolistic particle delivery	Rreliable, fast and highly reproducible Less dependent on cell type and condition Can be used for *in vitro* as well as *in vivo* transfer of nucleic acids in gene therapy Employed to transiently transfect replicating and non-replicating cells Present no limitation to the size and or number of genes that can be delivered Allows both stable and transient transformations Used for transformation of cells with unique growth requirements that are not amenable to other methods of transfection Primarily useful for genetic vaccination and agricultural application Non-toxic, non-allergic and non-mutagenic.	Needs expensive instrumentation Causes mechanical damage to samples Necessitates large number of cells due to the high death rate Preparation of microparticles needed Relatively expensive for research applications In general, efficiency is lower than electroporation or viral- or lipid-mediated delivery Leads to physical cell damage, and needs lage cell numbers because of high mortality.
Laser-mediated transfection	Can be used for delivering DNA, RNA, proteins, ions, micromolecules. Capable of making pores at any location on the cell. Applicable to very small cells Allows single-cell transfection or transfection of large number of cells at the same time Vector not needed High transfection efficacy efficiency Applicable to a wide range of cell lines	Needs expensive laser-microscope system Needs attachment of cells Technically demanding

Microinjection: In spite of all associated complications, this technique has been reported to remain of great value in clinical applications such as gene therapy [25,51,52]. Gene transfer via microinjection is considered the gold standard procedure because it ensures the efficacy of approximately 100% [53]. In microinjection, a micron-scale needle (0.5-5.0 µm diameter) pierces the cell membrane and directly releases a cargo like a nucleic acid into the cytoplasm/nucleus of an individual cell at a time [54,55]. Microinjection into an oocyte or one cell embryo was also able to achieve high efficiency. However, this method is limited to *ex vivo* applications such as introducing genes into oocytes for engineering transgenic animal models or the delivery of artificial chromosomes [52].

Several disadvantages of microinjection were reported [56]. These include the considerable time required to perform sophisticated technical skills that ensure high rates of transgenesis and embryo survival, and it often causes cell death. This makes two different microinjections into a single cell technically impractical. In addition, two microinjections, even if separated by several hours, typically give non-viable cells [55]. Therefore, this method is not appropriate for research that requires the transfection of a large number of host cells.

Electroporation: The most extensively studied physical delivery tool is electroporation (Nucleofection). This technique has been developed to overcome the difficulties encountered in applying microinjection procedures. It is one of the long standing physical methods for delivery of GE components into a population of cells suspended in a buffer. It is a highly effective approach for introducing exogenous charged molecules like DNA, RNA, or protein into a broad spectrum of different cells, including bacteria and mam-

malian cells [57]. Electroporation is widely used because it is less dependent on cell type than other delivery techniques allowing the transfer of cargo into difficult to transfect cells such as primary cells, stem cells, and B cell lines [58]. In electroporation, cells and chosen molecules are suspended in a conductive solution. Then, an adjusted pulsed high voltage electrical current is applied for a few microseconds to a millisecond. This disturbs the phospholipid bilayer of the cell membrane and creates temporary nanometer sized pores making it no more selective structure [59].

The electroporation technique is most commonly used in an in vitro setting, though as with microinjection, *ex vivo* applications are also valid [36]. A drawback of electroporation is that it is typically not suitable for in vivo applications owing to the oftentimes large amounts of voltage needed to be applied across cell membranes. More modern instrumentation has overcome high cell death by distributing the electrical pulse evenly among the cells and maintaining a stable pH throughout the electroporation chamber. The design of the transfection chamber and optimization of pulse and field strength parameters are key benefits that result in increased cell viability and transfection efficiency compared to traditional cuvette based electroporation systems. More recent transfection systems have been designed for electroporation of mammalian cells as well as for other cell types such as insect cell cultures and parasites [60].

Biolistic particle delivery: Briefly, biolistic particle delivery (also known as particle bombardment), involves carrying molecules coated by heavy metal particles (usually gold or tungsten). These particles are accelerated at a high velocity of gas like helium using a ballistic device (i.e., "gene gun"). The loaded particles are introduced to transform target cells in intact animal tissues, animal cells in culture, and animal embryos [45]. Recently, biolistic particle delivery has emerged as an excellent alternative strategy for plant genetic transformation which circumvents the limitations of *Agrobacterium*-mediated genetic transformation [61].

Laser-mediated transfection: Laser-mediated transfection, also known as laserfection, or phototransfection, utilizes a laser pulse to transiently create small holes in the plasma membrane [62]. When the laser perforates the membrane, the osmotic gradient between the medium and the cytosol enhances the passage of nucleic acids or other desired substances from the medium (ions, small molecules, proteins, semiconductor nanocrystals, etc.) into the cell. In spite of its advantages, laser-assisted transfection similar to electroporation poses risks of cell membrane damage and irreversible cell death (Table 4). It should be kept in mind that physical and chemical delivery protocols may result in damage to the cells, be inefficient or be not suitable for the in vivo practices [45].

Biological

Researchers recognize two major vehicles for gene delivery: viral and non-viral (synthetic) vectors (**Table 5**). Biological methods rely on genetically engineered viruses to transfer non-viral genes into cells. Viral vectors are broadly employed to deliver DNA and are typically integrase defective LV, Adenovirus (AdV), and herpes simplex virus.

Table 5: Comparison between viral and non-vital cell delivery strategies

Aspect	Viral delivery	Non-viral delivery
Insert size	Limited to ~10 kb for most viral vectors	Larger (~100 kb)
Therapeutic transfection efficiency	Offer higher levels of transduction efficiency and stable integration of exogenous DNA into the host genome. More efficient for most primary human cells	Generally less efficacious due to anatomical and cellular barriers Demonstrated in animal models, but efficiency remains to be a key obstacle for clinical applications.
Feasibility to manufacture recombinant viruses	Technically expensive, challenging and laborious and time-consuming Transected cell line must have viral receptors	Technically more feasible and cheaper
Level of transient expression	Low	High
Safety concerns	Low safety; high risk for viral infection, cytotoxicity, immunogenicity, insertional mutagenesis and malignant transformation	Much safer, more stable with lower immunogenic response
Potential for repeat administration.	Limited opportunity because of acute inflammatory response, and delayed humeral or cellular immune responses.	Higher potential

The introduction of non-viral vector systems was able to overcome limitations experienced with viral vehicles. Recently, a hybrid of viral and non-viral transfection methods has been used to improve transfection efficiency compared to other transfection approaches using polyplexes alone [63]. The hybrid based transfection has demonstrated safety and the ability to produce stable human cell lines that constantly expressed the proteins of interest [63,64]. However, hybrid techniques seem to be more time and more money consuming due to the necessity for unique glycosylated artificial virus like hybrid vectors [63].

Non-viral delivery

Non-viral vectors are typically based on cationic lipids or polymers. Compared to the popular viral vectors, they have been considered a potential alternative option and are now a successful and fast paced research field in the delivery of donor DNA [64]. Enormous efforts have been made toward the refinement of non-viral approaches, and presently non-viral vectors are receiving significant attention because of their favorable properties. Non-viral delivery cascades include a wide spectrum of transport mechanisms, which

range from nano-sized particles to complex polymers. Non-viral vectors such as polymers, lipids, inorganic particles and combinations of different types. Many factors are considered before the selection of uptake pathways of non-viral gene complexes. These factors include particle size, the density of particle surface charge, particle shape, cell type, and culture conditions [65-69].

Lipid nanoparticles/liposomes: The most developed in vivo non-viral delivery technique is the solid lipid nanoparticles. In principle, when lipids are placed in an aqueous solution, they naturally form nanoparticles, and by simple agitation, cargo can be encapsulated within [52,70]. The FDA has approved this strategy as an appealing option for transferring Cas9 RNPs because of its distinguished efficiency and outstanding clinical history [71]. Lipid nanoparticles have long been employed as delivery tools for a broad range of various molecules to cells and have proved popular for nucleic acid delivery. Nucleic acids are typically unstable extracellularly, and because of their highly anionic nature, they do not easily cross the cell membrane. However, by encapsulating nucleic acids within typically very cationic liposomes, they can be introduced into cells with relative ease. Lipid nanoparticle carriers do not possess any viral components; therefore, the contained load is protected from immunological reactions or enzymatic degradation [57]. They can also, like viral particles (See below; viral delivery section), be used in vitro, *ex vivo*, and in vivo, allowing for extensive testing on different scales of cell types [36]. Furthermore, this procedure is effective in GE and can theoretically be accomplished in a single administration, thereby reducing cytotoxicity and immunogenicity that may result from repeated doses [57]. The nanoparticle size is a key factor that affects all steps in the delivery process. However, it is difficult to define a particle size range for optimal transfection efficiency because the size effect is highly dependent on several variables including vector type, cell membrane permeability, and cellular uptake as well as circulation time in blood, spleen, and liver filtration kidney filtration [64].

Lipoplexes/polyplexes: One class of promising non-viral vectors for nucleic acid delivery into cells is the peptide based cationic lipids [64]. A prominent challenge of using a lipoplex is the low transfection efficiency. To increase polycation and enhance transfection Metallo-liposomes of Ruthenium (Ru) and phospholipid complexes were synthesized [72]. Two peptide lipids containing a tri-ornithine head (LOrn3) and a mono-ornithine head (LOrn1) were generated and the interaction kinetics of the liposome mediated gene delivery was examined [73]. In these studies, the transfection efficiency significantly impacted the length of the Ru-lipid and hydrophobicity and the charge ratios of the peptide lipids, respectively. Further research directions are waiting to advance peptide lipid vectors.

Cell-penetrating peptides: The cell-penetrating peptides (CPPs), also known as protein transduction domains, have been successfully used to deliver a large variety of cargoes, from small particles to proteins, peptides, nucleic acids, and even pharmaceutical nanocarriers to the cell interior in vitro, *ex vivo* and in vivo. They represent a novel approach that has emerged to overcome cell membrane impermeability and deliver a large variety of particles and macromolecules into cells [74]. CPPs are synthetic and natural usually short segments of amino acids (7-30 amino acid residues) that are able to cross cellular membrane [74,75]. These molecules are generally, cationic, and/or amphipathic peptides which make them promising vectors for therapeutic delivery [74]. The mechanism of cellular internalization of these peptides is not clear. But it seems to be dependent on cargo, cell type as well as the experimental conditions (temperature and pH) and believed to involve endocytosis or direct translocation , or both.

The CPPs technique is quite easy, but the challenge of this strategy is the selection of the size, stability, nonspecific versus specific associations, and potency versus toxicity [76]. It needs extensive optimization for each type of cargo and cell. Another drawback of CPPs is the low efficiency of the desired targeted mutation in cells usually around 10%-20%.

It has been used for the treatment of anterior and posterior segment eye diseases [75]. The CPPs administration approach has proven to be non-invasive or minimally invasive, shifting from the capability to obtain intracellular delivery to their ability to cross biological barriers [75]. Recently, CPPs with mitochondrion targeting ability, named mitochondrion targeting peptides, have been engineered for mitochondrion targeted drug delivery in cancer therapy [77].

DNA Nanoclew: A nanoclew, also known as nano-cocoon, is a sphere like structure that measures 150 nm across, made of a single tightly wound strand of DNA. This unique technology has been developed and utilized in in vitro settings by a group of researchers [78]. Once in the cell, the cell's acidic environment cleaves the polymer sheath containing the DNase. Released from its sheath, the DNase quickly slices through the DNA cocoon, spilling its engrafted load; a drug or a nucleic acid [79]. In these settings, nanoclaws demonstrated potent gene knockdown at both mRNA and protein levels, with negligible cytotoxicity to deliver CRISPR/Cas9 components [79].

Overall, nanoclews are relatively easy to manufacture and can serve as a versatile approach that could be adapted for delivering other DNA-binding proteins or functional nucleic acids. However, more testing is guaranteed, particularly on the potential immunogenicity of DNA nanoclews [36].

Gold nanoparticles: Gold nanoparticles (AuNPs) have an advantage over many other nanoparticles in chemicals [80]. Compared to other nanoparticles, AuNPs possess unique advantages of having small sizes (1 nm to 8 µm), different shapes, and a high surface area to volume ratio. They can form stable chemical bonds with S-and N-containing groups. This allows them to link with a wide variety of organic ligands or polymers with a specific function. Consequently, AuNPs have found many applications in bio-nanotechnology, ranging from imaging agents to inert carriers of other constituents [45]. AuNPs as delivery vectors have demonstrated promises in the field of therapeutics due to their high surface loading capacity of oligonucleotides, antibodies, and proteins [80,81]. The easy design and multifunctionality allow a versatile and useful delivery strategy for drugs and genes [80,82,83]. By using a gene gun AuNPs have been extensively used for the delivery of DNA vaccines through the skin epidermis. Contrary to the DNA nanoclew, which depends on a biological molecule to act as a carrier, AuNPs are inert and will not trigger an immune response to the nanoparticle itself [84]. They lack cytotoxicity and are efficiently used in in vitro, *ex vivo*, and in vivo settings [81]. There are several challenges facing AuNPs basic laboratory research that have to be solved before their clinical application. One major technical difficulty in GE of somatic cells for therapeutic purposes is targeting the stem cells of the affected tissue [80].

Viral Delivery

Conventional gene transfer has successfully employed viral vectors to introduce foreign genes into the host genome. Currently, viral cell delivery (also known as transduction) has the highest efficiency amongst gene delivery tools. LV vectors with improved safety characteristics have been found to minimize insertional mutagenesis safety concerns raised in early clinical trials for severe combined immunodeficiency using γ-retroviral vectors [85]. Viral based vectors offer alternative means to transfect cell types that are not amenable to lipid mediated transfection. Many options are available for selecting a viral delivery system that matches specific research needs. A variety of viral vector systems are commonly existing and have been used as the main delivery vectors introducing DNA or mRNA into mammalian cell culture and *in vivo* as well as into insect host cells for protein expression and RNAi studies [20,86] (**Table 6**).

Table 6: Comparison between types of viral cell delivery systems

Aspect	Lentiviruses	Adenovirus	Adeno-associated virus
Type and form of nucleic acid	RNAs	Double stranded DNAs	Single-stranded DNAs
Availability/Potential production	Feasible and shows promising, but costly productivity.	relatively facile	Plentiful
Packaging capacity	Large up to 18 kB.	Up to 35 kB	Small and limited insert <5 kb
Type of transfected cells	Both dividing and non-dividing	Both dividing and non-dividing	Both dividing and non-dividing
Cell transduction efficiency	Highly efficient in most cells	Higher in epithelial than endothelial cells types	High *in vivo* cell infection efficacy
Integration into the host genome	Integrates the transgene into the genome of host cells; RNAs are reverse transcribed into a double-stranded viral DNA before being integrated into the host cell genome	Naturally very low integrators	Does not integrate into the host genome
Expression system of transgene	Offers transient or stable long-term gene expression	Unable to induce prolonged protein expression	Unable to produce sustained gene expression
Amplification, storage and stability	Remains part of the host DNA and is perceived by the cell as normal host cellular DNA.	Easily amplified and stored	Stable at different temperatures and pH values
Risk of insertional mutagenesis and gene disruption	High under certain conditions	Lower but existent risk of triggering mutagenesis	Insertional mutagenesis possible at certain positions
Inflammation/ Immunogenicity and pathogenicity in human	Highly cytogenotoxic	Usually induce strong response	Lower immunogenicity relative to adenoviruses Low toxicity Does not cause any known disease
Safety in *in vivo* somatic cell gene therapy	Low	High laboratory safety	Showed safety profile in phase I clinical trials

An ideal viral infection procedure involves the engineering of the recombinant viral nucleic acid carrying the transgene, amplification of recombinant virus in a packaging host cell line, purification and titration of amplified viral particles, and subsequent infection of the target cells. Although the accomplished transduction efficacies in primary cells and cell lines are quite high (~90-100%), only cells carrying the viral-specific receptor can be infected by the virus. It is also important to note that the packaging cell line used in transduction requires being transfected with a non-viral transfection strategy [86].

DISCUSSION

The lack of an efficient and safe delivery system into cells and tissues remains to slow clinical applications of designer nucleases including Zinc Finger Nuclease (ZNF), Transcription Activator Like Effector Nuclease (TALEN) and Clustered Regularly Interspaced Short Palindromic Repeats/CRISPR-associated (CRISPR/Cas9) [87,88]. Much effort has been devoted to improving and advancing therapeutic medicines and drug delivery methodologies, focusing on specificity and accuracy aspects. Two types of base editors (BEs) have been introduced; cytosine base editors (CBEs)-cytidine deaminase fused with catalytically impaired Cas9-can effectively convert a C-G base pair to a T-A pair. The other one is the adenine base editors (ABEs)-Cas9 nickase (nCas9), which is able A-T to G-C conversions [83]. Another new approach is prime editing (PE) technique or what so-called "search and replace" genome editing method [89]. It mediates targeted insertions, deletions, and all possible base-to-base conversions. It can combine different types of edits with one another.

One disadvantage of the AAV vectors is the limited carrying capacity making them difficult to use for the delivery of TALENs CRISPR [5]. To bypass the packing limit obstacle, many procedures have been explored, including viral and non-viral vectors [90,95]. In general, huge cargoes such as *Streptococcus aureus*(SpCas9), base

editors, and prime editors, containing dead Cas9 (dCas9) along with their accessory proteins exceed the ~4.4 kb loading limit of AAVs [71]. Scientists have introduced the AAV CRISPR/Cas9 delivery method using a version of Cas9 from SpCas9 rather than *S. pyrogenes* (SaCas9). Cargo such as SaCas9 is smaller (about 1 kb shorter) compared to SpCas9 [20]. This version of Cas9 is roughly 70% the size of SpCas9 while retaining the same potent cutting capability. The method makes it easier to package into AAV vectors [96,97]. It was also reported that LV capsids can be used to package SaCas9 mRNA. It was also indicated that the package showed an efficient GE strategy [98]. Further, the SpCas9 proteins were safely delivered with virus like particles (VLPs) [99]. The VLPs-mediated Cas9 mRNA proved to be more successful than sgRNA delivery [100].

The second mechanism to circumvent the packaging issue is by using a dual-vector system to deliver GE constituents separately in viral [11]. The recently developed VLP-delivery method has proved to be efficient and safe in GE. Compared with transferring bacterially expressed genome editing effectors by electroporation or nanoparticles, VLP -mediated mRNA or protein delivery methodology has a series of advantages [100]. The VLPs are easily produced with basic equipment. Additionally, compared with electroporation experiments, VLP-mediated delivery uses much less amount of protein offering it greater specificity [100,101]. Unfortunately, since Cas9 activity is inhibited by nucleosomes, the low dosage advantage of VLP delivery may become a disadvantage when target sequences are associated with heterochromatin because a high dosage is needed for efficient editing. In addition, VLP-mediated RNA transfer is only efficient for Cas9 mRNA but not for sgRNA [102,103]. Therefore, new approaches to deliver Cas9 mRNA and sgRNA in the same particle will no doubt asset the field [100].

The third strategy for evasion of the packaging size limitations associated with viral vector to modify or improve the functional features of viruses is by non-viral vectors by attaching synthetic non-biological components such as polymers and nanoparticles [104]. To date, the search for the use of polymers for the delivery of GE technologies has been minimal. Therefore, there exists an urgent need for a non-viral system to deliver a disruptive platform technology such as CRISPR-Cas9 for clinical translation. Several workers reported the transfer of CRISPR/Cas9 GE components using other nanocomplexes that generally depend on electrostatic interactions. The non-viral methods have been developed to enhance the specificity and safety of viral-mediated GE delivery routes. Non-viral physical delivery tools, such as microinjection, square wave electroporation technique, cationic-lipid transfection, lipid nanoparticles, ligand fusion tags, cell-penetrating peptides, or AuNPs, and lipid nanoparticles [105]. These approaches have been widely used to deliver ZFNs, TALENs, and CRISPR in various different cell lines and animal models. The most significant advantages these methods bring about are enabling the transient nuclease activity, as well as allowing repeated administration of gene therapy reagents, despite being less efficient than viral delivery methods [65,67,71].

There are serious disadvantages to carrying CRISPR/Cas9 components via lipid nanoparticles [36]. First, there are both external and internal obstacles that must be considered. Once the nanoparticle has traveled through the surface of the cell, it is typically internalized within an endosome. The endosome contents can very rapidly be sent by the cell into the lysosomal pathway, resulting in the degradation of all lysosome inclusions. Therefore the cargo has to escape the endosome. Naturally, the cell membrane will prevent the entrance of large sized molecules such as Cas9 and negatively charged ones like gRNA [52]. Also, if the Cas9: sgRNA complex can skip the endosome pathway, it must also translocate to the nucleus, which can also be another potential point of failure. Due to this, it is rare to demonstrate particularly high efficiencies when CRISPR/Cas9 components are delivered via lipid nanoparticles. Besides this, lipid nanoparticles resemble virus particles in that the nature and size of the delivery, as well as the target cell type, which highly influence transfection efficacy and the kind of lipids that are suitable or useful in the system.

A 'Nanoclew' has been developed to deliver CRISPR/Cas9 components. By coating the nanoclew with PEI to induce endosomal escape, the modified version proved about 36% efficiency in transferring CRISPR/Cas9 RNP with the nanoclew (versus 5% with naked Cas9: sgRNA and PEI). This permitted the nanoclew to achieve efficiencies on the order of other high efficiency CRISPR/Cas9 delivery systems, however, still contains no viral material or any exogenous components besides repeating DNA and PEI. More testing is guaranteed, particularly on the potential immunogenicity of DNA nanoclews [36].

Recent research has further highlighted additional major vital limitations in case of regarding viral delivery and repeated gene therapy that should also be considered is to search for other versions of Cas9 that are safe. The conditions influencing delivery efficacy include possibilities of integration of the viral vector into the host genome, the risk of immunogenicity resulting from the virus itself, and/or sustained or high rate of nuclease expression of the Cas9 protein, increasing the potential for off-target effect [71,106,107]. Added to this is the DNA-damage toxicity, for example, ssDNA breaks P53 toxic responses related to GE [71,108]. Therefore, care must always be taken with LVs and AdVs when using them for GE to make the HIV provirus as integration deficient as possible [109]. Currently, it is not possible to totally eliminate the possibilities of integration into the host. In addition, although measures can be taken to make this integration targeted, one cannot ensure that the viral payload goes to the same exact site every time. This can result in an elevation of expression and off target effects, or even potential damage to the cell if the insertion randomly occurs within an important cellular protein [110].

The last available strategy for overcoming the packaging size limitations is to use specially designed transgenic animal models, which can continuously express high levels of Cas9 exclusively in the desired tissue [111]. A pioneered research [112] was carried out in a mouse model of mucopolysaccharidosis type I, a lysosomal storage disease affecting multiple organs, including the heart. The results proved the potential of BE strategy using AAV9-mediated in utero intravascular delivery of an adenine BE (ABE) to successfully correct a nonsense Idua gene mutation. Recently, electroporation has been made viable for cardiomyocytes and, thus for *ex vivo* therapy of cardiovascular diseases. However, *in vivo* electroporation is more challenging because of the complicated choice of parameters and the danger of damaging tissues [57]. The safety of designer nucleases can be enhanced by reducing the expression rate by transferring the nuclease proteins or RNPs using electroporation, CPPs, cationic lipid, and AuNPs [113]. The Cas12a-mediated GE strategy was employed in a canine model of Duchenne muscular dystrophy (DMD) in which the transfer of Cas9: sgRNA

was accomplished with AdV by intramuscular injection [114]. Cas12a-mediated GE was first used in DMD patient-derived iPSCs and also in a murine model of the disease. In both models, the Cas12a-mediated GE strategy rescued the disease phenotype [90]. Lately, scientists succeeded in using the LVLP particles strategy for ABE RNP delivery in human cells [96]. They reported minimal RNA off-target activities making ABE one step closer to possible therapeutic applications.

In addition to muscular and cardioediting, several other animal models, particularly the mouse ones have been designed to treat kidney, liver, and eye inherited diseases, but with varying degrees of success. A growing list of outstanding clinical trials is going on to use CRISPR/Cas9-based gene therapies for several inherited diseases and for cancer. Currently, there are more than 60 ongoing therapeutic attempts using the CRISPR approach are being tested for the treatment of more than 30 different genetic diseases [90], as well as certain types of cancer [115-119].

One interesting observation was observed, that is the presence of less stringent immunologic and physical barriers in the developing embryo in comparison to postnatal stages [120]. This makes in utero genome editing more attractive for the treatment of genetic diseases diagnosed before birth [121]. Furthermore, SpCas9 immune reaction was noticed following postnatal, but not after in utero, AdV delivery of SpCas9-based cytosine BEs (CBEs) [122]. Zygote electroporation needs the setting of appropriate parameters that facilitate uptake of Cas9 but also minimize damage to the embryo. In the beginning, a first pulse series is given to perforate the zona pellucida and the cell membrane of the zygote. After that, a second pulse series is applied to deliver the Cas9 mRNA/crRNA or the Cas9-RNP complex directly into the nuclei of mouse and rat zygotes [123]. A technique for small volume zygote electroporation of Cas12a-crRNA RNPs was developed successfully in 2019 [124]. Researchers demonstrated its use for the induction of large gene deletions. However, certain worries were pointed out regarding the possibility that the creation of large deletions might delete regulatory elements that are not linked to the gene of interest. In this case, effects on other genes might affect the subsequent phenotype. More refinement was made on the zygote electroporation to optimize the transfer of RNP and to ensure zygote survival [125].

In spite of requiring technical experience in dealing with the embryo during zygote electroporation, microinjection skills are dispensable, making this technique accessible to a wide range of researchers. With modification efficiency reaching 100%, microinjection has provided one way to bypass the technical obstacles facing GE in somatic stem cells. Cas9 nucleases in combination with targeting vectors have further facilitated gene targeting in mouse zygotes by pronuclear microinjection [24,126].

In addition, microinjection is not limited by the MW of the cargo, which is a significant limiting factor with viral vector delivery systems [36]. The technique also has the advantage of putting the Cas9 mRNA directly into the cytoplasm, where it can be translated by the cell. This method also allows for the controlled delivery of known quantities of the cargo, making it desirable for improving control over off-target effects [36]. These reports indicate that embryonic stem cell technology might be replaced by direct designing of the zygotic genome for gene targeting. Further, a functional CRISPR RNPs delivery that is produced in vitro by transcription of Cas9 protein and sgRNAs can be microinjected into the zygotes or the gonads (ovaries or testes) of parent animals to generate mutant individuals [127]. GE using embryos has successfully been performed in a variety of animal models [128].

Currently, microinjection gives the 'gold standard' for delivering the CRISPR components into gametes (sperm or unfertilized egg) or cleavage stage of the zygote, the so-called "germline genome editing", or "reproductive CRISPR; rCRISPR" [118,129]. This procedure results in engineered cells that are totipotent, giving rise to all somatic and germ cells. Complement is an important component of the innate immune system that is crucial for defense from microbial infections and for clearance of immune complexes and injured cells.

It must be emphasized that an increasing number of studies have identified striking differences between mouse and human pre-implantation development and pluripotency [130]. A new era of GE for the treatment of human diseases. In the early trials, it was reported that there is a potential for inactivation by the complement system and monocytes in human circulation, restricting gene transfer and promoting vector clearance [131]. This facilitates both efficiency and safety testing and makes them highly versatile delivery vehicles [36]. CRISPR GE was tested in a person for the first time in 2016 [132]. The first-ever approved phase 1 clinical trial using the CRISPR tool aimed at cancer immunotherapy by editing autologous T cells. In this ex-vivo-based therapy, α and β chains are knocked out on endogenous T cell receptors, which leads to an immune reaction, and apoptosis protein 1, which attenuates the immune response. In the last step, the edited genes are returned back into the patients using LV as a vector. The gene which encodes New York esophageal squamous cell carcinoma-11 (NY-ESO-1)-specific T cell receptor has been reported to be highly upregulated in the relapsed tumors [97]. Electroporation was employed to deliver plasmid DNA encoding both Cas9 and mRNA to create colorectal cancer models from Cas9-engineered human intestinal organoids [133], producing an early-onset Alzheimer's disease model in human cells.

Finally, it is worth mentioning that the first successful human germline genome editing has been claimed in what was called CRISPR'd babies [87,88,118]. However, the 'He Jiankui affair' has been questioned. The coming future of humankind's genome editing remains unpredictable. The scientific community must always remember the highlights of Venki Ramakrishnan president of the Royal Society in his speech AAAS annual meeting in Boston, Saturday 18 February 2017: "When considering what we can do with technology, we also need to consider what we should do [134-136].

CONCLUSION

In spite of all the progress that have been made until now, no delivery method has met all needs, including safety, efficiency, easy production, and low cost. Therefore, some mechanistic challenges still exist to fully realize the potential of gene transfer for different applications in GE. One attractive GE delivery system is the CRISPR/Cas9-derived technology is PE, which is composed of reverse transcriptase fused with Cas9n (an RNA-programmable nickase), along with a PE guide RNA (pegRNA) .Currently, the PE strategy, in comparison with CRISPR BE editing, appears to be accompanied by less immunotoxicity, a lower frequency of off-target mutagen-

esis . Clinical trials of the CRISPR technology in humans began a few years ago before being mastered. We have to admit that PE is still in its infancy, and its clinical transformation may not be easily achievable in the near future. However, it promises to be superior to other CRISPR approaches and maybe a major focal point of precision therapy in the coming years . Cell delivery techniques should be further researched to allow better understanding and consequently greater benefits to humankind. If these novel technologies prove to be safe and without any unpredictable complications, then it is reasonable to believe that the future will witness tremendous achievements in the field of GE . We believe that this review will attract lots of attention from the scientific community, including researchers, clinicians, and the drug industry.

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