

Evolutionary Timeline of Genetic Delivery and Gene Therapy



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Abstract: There are more than 3,500 genes that are being linked to hereditary diseases or correlated with an elevated risk of certain illnesses. As an alternative to conventional treatments with small molecule drugs, gene therapy has arisen as an effective treatment with the potential to not just alleviate disease conditions but also cure them completely. In order for these treatment regimens to work, genes or editing tools intended to correct diseased genetic material must be efficiently delivered to target sites. There have been many techniques developed to achieve such a goal. In this article, we systematically review a variety of gene delivery and therapy methods that include physical methods, chemical and biochemical methods, viral methods, and genome editing. We discuss their historical discovery, mechanisms, advantages, limitations, safety, and perspectives.

Keywords: Nonviral, CPPs, viral, ZFNs, TALENs, CRISPR, gene therapy, genetic delivery, genome editing.

1. INTRODUCTION

Hereditary diseases and disease predisposition have been associated with particular genes. Over 3,000 genes have been linked to hereditary illnesses and around 500 have been identified that increase risk for certain diseases [1]. For example, cystic fibrosis is a recessive illness occurring in individuals carrying two copies of a mutant *CFTR* (cystic fibrosis transmembrane conductance regulator) gene [2]. Patients carrying two recessive β -globin genes with a single base substitution present with sickle cell anemia [3] and patients with one copy of a dominant mutation in the huntingtin (*HTT*) gene develop Huntington's disease [4]. Historically, these diseases are not curable and symptoms can only be managed. Some treatments, such as proteins and small molecules, are difficult to apply due to bioavailability, stability, specific targeting, and other pharmacokinetics issues [1].

The idea of gene therapy first arose in the 1970s [5]. Initial applications focused on gene replacement therapy to treat inherited disorders by supplying target cells with a copy of a normal gene. In the last fifty years, these delivered treatments have advanced to include protein-coding complementary DNA (cDNA) sequences and non-coding small nucleic acids that regulate a broad spectrum of cellular behaviors and functions. Both of which are becoming mainstream therapies and hold tremendous potential in revolutionizing medicine. In addition to replacing a target gene, protein-coding cDNAs are being widely used to manipulate neurotrophic factors in neurodegenerative diseases such as Parkinson's and Alzheimer's diseases, modulate regulatory proteins that involve cell survival and apoptosis of cancer,

produce angiogenic factors in cardiac ischemia, and immune-modulate human immunodeficiency virus (HIV) and other immune diseases [6]. In the past decade, non-coding small nucleic acids represented a new shift of paradigm in gene therapy. Non-coding nucleic acids include oligonucleotides, catalytic RNAs or DNAs, antisense RNAs, and aptamers. These non-coding nucleic acids have been used to completely silence or partially regulate functions of certain genes to mitigate disease severity or progression in cancer, neurodegenerative diseases, and cardiovascular diseases, among others [7-9].

Missing genes may be added into cells using various techniques in order to combat illnesses [10-13]. Genome-editing technologies may be able to edit or replace defective genes and eliminate genetic diseases all together [1]. Genome editing is the key to advancing the treatment of inheritable diseases and human medicine. In theory, these techniques sound simple. In reality, the development of genome editing is sophisticated and has experienced numerous setbacks. One of the most notable setbacks was the death of Jesse Gelsinger in 1999, the first death associated with gene therapy [14]. Gelsinger took part in an experimental gene therapy trial to treat a rare metabolic disorder known as ornithine transcarbamylase deficiency. His body overreacted to the viral vector used and he died after multiple of his organ systems failed. His death shocked the research community and no gene therapy clinical trials relating to ornithine transcarbamylase deficiency were proposed until 2016 [15]. This clinical case is a manifestation that efficacy and safety are equally important in developing gene therapies.

In vitro and *in vivo* DNA deliveries are key to numerous aspects of life science research, which include but are not limited to the discovery of fundamental principles in biology (e.g., gene structure, regulation, and function), understanding the nature of human diseases (e.g., genetic defect and

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correction), and biomedical applications (e.g., gene therapy, drug delivery, and DNA vaccination). One of the major obstacles in DNA delivery is the mammalian cell membrane due to its non-polar and hydrophobic nature. Over the course of evolution, cells have survived by making their membranes selective. On the one hand, selectivity allows for the efficient passage of nutrients, minerals, and other essential materials into cells for growth and removal of cellular wastes [16]. On the other hand, selectivity helps fend off harmful materials from entering into cells. Polyanions, such as nucleic acids, are poorly internalized but can be delivered into cells using various carriers and methods. Before the benefits of DNA therapy can be relished, inefficiency in delivery must be addressed.

Delivery of nucleic acids into mammalian cells can be generally divided into two main strategies: viral and nonviral delivery. Nonviral delivery tools can be based on physical methods or various classes of chemicals, such as cationic lipids, polymers, peptides, or carbohydrate analogs [17]. Physical nonviral methods discussed in this review encompass microinjection, bioballistics, hydrodynamic force, ultrasonic nebulization, and electroporation. Chemical and biochemical nonviral methods include calcium phosphate coprecipitation, dendrimers, and membrane infiltration mediated by artificial lipids and peptides. Importantly, small delivery peptides, termed cell-penetrating peptides (CPPs), have gained a great interest among these nonviral tools. Viral delivery of genetic material takes advantage of the natural life cycle of viruses, using viruses with modified or synthetic genomes to inject therapeutic genes into cells during infection. Depending on the virus used, delivered genes may exist as a plasmid inside cells or be integrated into the host genome. Viral vectors discussed here include adenovirus, adeno-associated virus, gamma-retrovirus, and lentivirus.

Each nucleic acid delivery method has its benefits. Viral methods are efficient but may cause adverse immune responses. Nonviral gene transfer is likely to be nonimmunogenic but often suffers from lower transfection rates and toxic carriers. Nonviral methods of delivery also are often limited by transient transfection [5], with the expression of delivered genetic material often only lasting for a short period due to low integration rates [18]. This is due to the low chance that plasmid DNA can enter the nucleus, with the only time period to enter being when the nuclear membrane is destabilized (e.g., during replication) [19]. Integrating viral vectors have higher rates of transient transfection than nonviral vectors; however, it should be noted that integration at random locations in the genome can result in mutagenesis and oncogenesis [1]. Nevertheless, the importance of viral vectors as a DNA delivery tool cannot be overlooked, as they have been a pivotal partner in revolutionary genetics, cell biology, molecular biology, and medical discoveries.

Genome editing, also known as gene editing, is defined as a group of technologies used to change an organism's genome, according to the US National Institutes of Health (NIH) [20]. These technologies allow genetic material to be artificially added, removed, or altered at particular locations

in the genome. Many strategies are currently available for programmable and targeted genome editing, including zinc finger nucleases (ZFNs) and transcription activator-like effector nucleases (TALENs). The clustered regularly interspaced short palindromic repeats (CRISPR)-CRISPR-associated protein (Cas) system is a next-generation genome editing technology, which is originally based on a system used by bacteria and archaea to combat recurrent viral infections.

The number of clinical trials involving gene therapy has steadily increased over the years, with approximately 2,600 trials up until 2017 [21]. The majority of clinical trials use viral vectors, including adenovirus (20.5%), retrovirus (17.9%), adeno-associated virus (7.6%), lentivirus (7.3%), vaccinia virus (6.6%), poxvirus (4%), and herpes simplex virus (3.5%). Naked DNA (16.6%), often in combination with electroporation, and lipofection (4.4%) are the most common nonviral methods used in clinical trials [21]. It should be kept in mind that certain types of gene delivery methods will become more or less popular as techniques are refined, efficiency is fine-tuned, or new technologies are developed.

Here, historically important discoveries for gene transfection and gene editing methods are discussed. The advantages and disadvantages of the methods are also presented. A timeline of significant milestones in gene delivery and gene therapy is provided in Fig. (1).

2. PHYSICAL METHODS

Physical methods of gene delivery act by directly penetrating or compromising the cell membrane in order for nucleic acids to pass into cells. They are some of the earliest methods of gene delivery, though cell viability and transfection efficiency for many of the techniques are undesirable. Physical methods are diagrammed in Fig. (2).

2.1. Microinjection

Microinjection, or the direct injection of genetic material into cells [22], was one of the first methods used to transform and transfect cells. It is a simple method, but delicate and difficult to carry out [23]. It does not rely on a carrier for DNA, making it not immunogenic or toxic [24]. Nucleic acids are injected into a single cell using a needle. DNA can then be localized to the nucleus after injection into the cytoplasm or directly injected into the nucleus to transfect a cell. Microinjection is perfect for cloning and single-cell manipulation. The first case of microinjection dates back to 1911, when Marshall Barber used the technique to clone bacteria [25]. The first animal clones were created by transferring the whole nucleus of one embryonic frog cell to the enucleated oocyte of another in 1952 [26]. However, the older the transplanted nuclei, the less likely a normal tadpole was to develop. In 1974, microinjection was used to create the first transgenic animal by injecting viral DNA into a mouse blastocyst [27]. In the 1990s, regional transfection of tissue by DNA injection was demonstrated [18]. However, it was not until 1997 that adult mammalian cells were able to be used to create viable clones, resulting in the famous Dolly the sheep

[26, 28]. Using small amounts of DNA, the technique is cost-effective [29]. While efficiency is high when DNA is injected directly into the nucleus, transfection can be low if DNA is degraded by cytoplasmic nucleases [23]. A mouse cell line showed expression in 50 to 100% of cells given genes by direct nuclear injection [30]. The process can be slow and tedious when many cells are to be transformed [22]. On the other hand, the method is not dependent on cell type, so it can be used with cells that are difficult to transform or transfect [24]. The benefits of this technique that have gained attention are the precise control of the amount of genetic material passed to cells and the surety that most cells treated will receive genetic material [31], with the nucleus receiving around 90% of fluid directly injected [30]. More recent advances in microinjection techniques have led to the development of devices used to inject multiple cells at once [32].

2.2. Bioballistics

One forceful method of genetic transduction is bioballistics. In this technique, a “gun” is used to accelerate metal particles covered in genetic material through cells [18]. Any particles that travel through the cells have a chance to leave behind the nucleic acids that were on the surface of the metal. Tungsten, silver, and gold particles are most commonly used as the carrier and are accelerated using pressurized in-

ert gases or electric charges [18, 33]. The size and speed of the particles play a major role in gene transfer efficiency [33]. Klein *et al.* first applied this method using plant cells in 1987 [18, 34]. The group used tungsten particles coated in nucleic acid and accelerated them using a gunpowder blast [34]. A downside to bioballistics is that if a cell is hit with a large number of particles, viability is reduced [34]. Low accuracy, tissue damage, and low efficiency [35], in part due to high DNA degradation [36], are also issues to be considered. Only around 3 to 15% of cells targeted with bioballistic gene transfer show high expression [35]. The cost of materials is also high [23], which can be a detriment to researchers if a large number of transformations must be performed. However, the method is relatively easy to carry out and the stability of the carrier is not a concern [35]. The size and properties of the metal particles also enable them to deliver multiple or large DNA molecules that other methods may not [35]. A large concern from a toxicological perspective is that the ultimate fate of the particles is often not known [37]. Metal particles may oxidize in biological systems and result in unintended toxicity if they are not removed. Bioballistics can be applied to both organisms and cells [23]. Advances in this method have resulted in the development of a hand-held gun, the Helios Gene Gun, which allowed *in situ* transformation more easily [36]. Xia *et al.* used the Helios Gene Gun to transfect the skin and livers of mice with bioluminescent reporter genes [38].

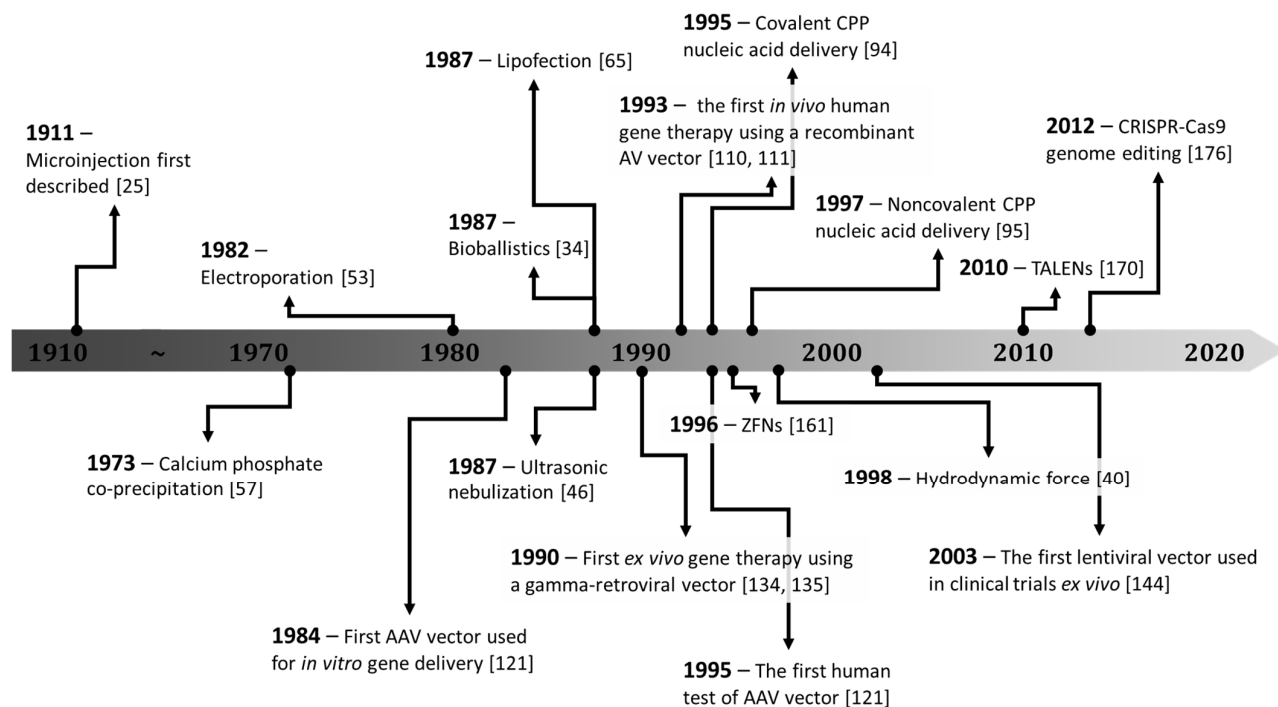


Fig. (1). Significant milestones in gene delivery and gene therapy. Adeno-associated virus (AAV), adenovirus (AV), cell-penetrating peptide (CPP), zinc finger nuclease (ZFN), transcription activator-like effector nuclease (TALEN), clustered regularly interspaced short palindromic repeats (CRISPR), CRISPR-associated protein 9 (Cas9). (A higher resolution / colour version of this figure is available in the electronic copy of the article).

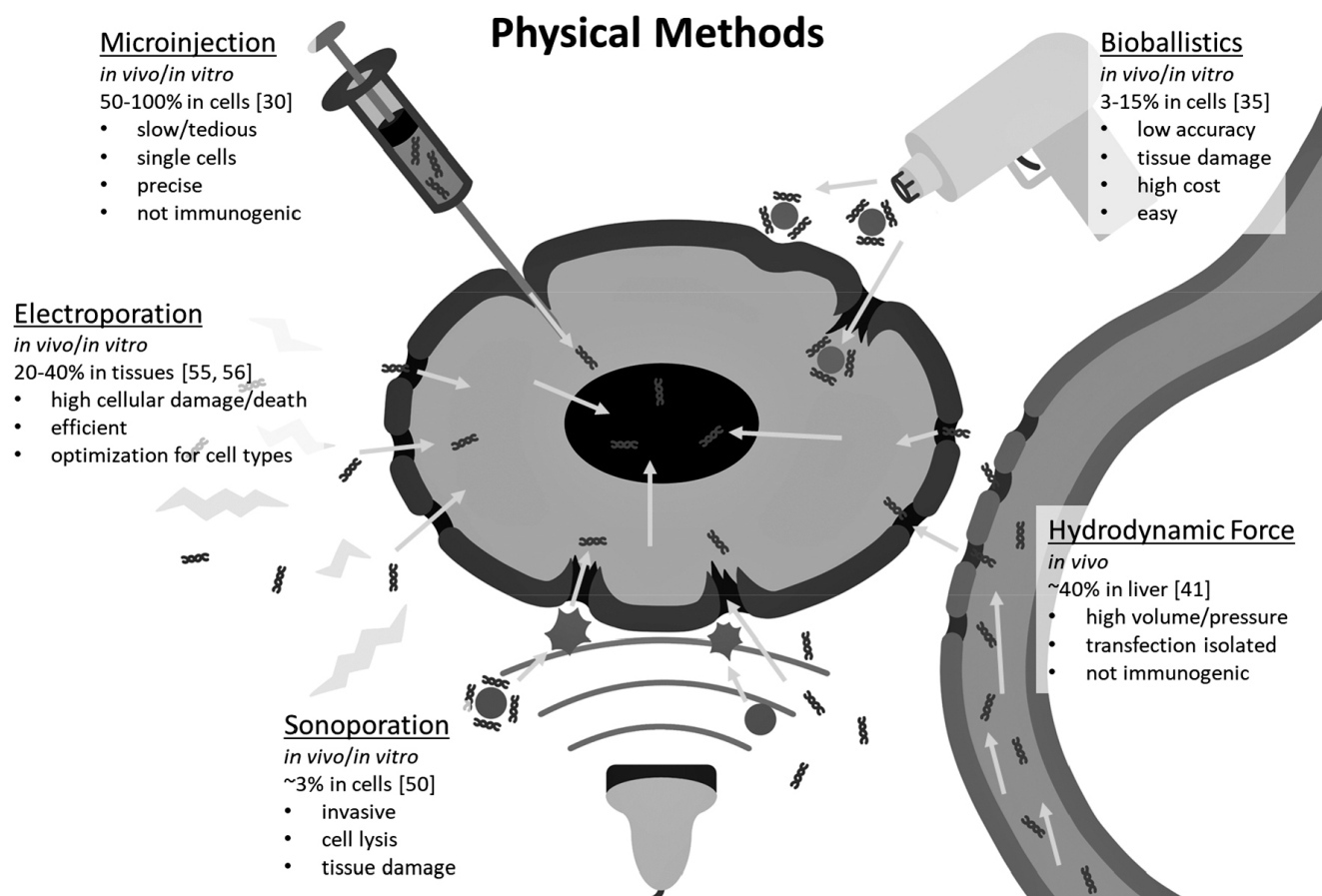


Fig. (2). Physical methods of gene delivery. The cell membrane is compromised by different means in order to facilitate nucleic acid internalization. Microinjection, bioballistics, hydrodynamic force, ultrasonic nebulization (a.k.a. sonoporation), and electroporation are common physical delivery techniques. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

2.3. Hydrodynamic Force

Hydrodynamic force uses pressure in the circulatory system to increase the permeability of cells [18]. The first successful application of hydrodynamic force for gene delivery is attributed to Budker *et al.* [39, 40]. This method was designed for use *in vivo*, originally in rodents, but has been used in various animals, including rabbits, dogs, fish, pigs, monkeys, and humans [39]. Immunogenicity and toxicity of carriers are not a concern as naked DNA is used in the solution [41]. A solution of about 8 to 12% of the subject's body weight is injected quickly, in 5 to 7 seconds, to achieve the systemic pressure needed to facilitate nucleic acid uptake [39, 41]. Transfection rate is highly dependent on injection volume, injection time, and DNA concentration [41]. However, large injection volumes have been deemed unsafe for people [18] as too much fluid in the circulatory system can lead to cardiac arrest [39]. Along with this, large volumes of DNA may be difficult to cultivate for each individual injection. Due to the need for a pressurized system, this method is not applicable to individual cells [39]. Transfection often takes place in specific organs, especially the liver [41, 42].

Lui *et al.* showed that 40% of the hepatocytes of mice injected with plasmid DNA *via* the tail vein expressed a reporter gene, while the lung, kidney, spleen, and heart all had expression levels less than 1,000-fold of the liver expression [41]. This makes the technique particularly useful for gene expression in specific organs but not useful if the expression is desired elsewhere. This method has been used to develop a mouse model of hepatitis B infection, which normally is unable to infect rodents, by forcing viral genomes into mice hepatocytes [39, 42, 43]. One technique used to decrease injection volumes in animals larger than rodents is to use a balloon catheter to isolate a particular organ, so pressure can be increased locally [44]. When balloon catheters were used to isolate the liver of pigs, injection volumes could be reduced from 10% to 1.25% of the individual's body weight [44]. This also allows organs and tissues with lower affinity for transfection in whole body injections to be targeted.

2.4. Ultrasonic Nebulization

Fecheimer *et al.* were the first to demonstrate cell transfection using ultrasonic nebulization, also known as sonopo-

ration [45, 46]. This method relies on the formation of pores in cell membranes by ultrasonic waves [33]. The intensity and frequency of the ultrasonic waves used, as well as tissue type, determine the biological effects [18]. Typically, ultrasound in the 2 to 20 MHz range, with maximum intensities limited to 720 mW/cm^2 by the US Food and Drug Administration (FDA) [47], is used for medical imaging [48]. However, only 1 to 3 MHz, with higher intensities ranging from 0.5 to 2.5 W/cm^2 , are used for gene delivery [18]. Initial studies demonstrated sonoporation using 20 kHz [48]. Ultrasound can result in heating and cavitation within tissues, depending on the intensity of the waves [48]. Cavitation can concentrate ultrasonic energy and occurs when ultrasound waves produce and interact with microbubbles in solution, resulting in wave deflection, resonance frequency vibration of bubbles, and bubble imploding [45, 48]. Cavitation of microbubbles causes mechanical damage in cells and tissues, which can be observed as pore formation or cell lysis [48]. The utilization of ultrasound in gene delivery was dramatically improved by combining ultrasound wave application with injected microbubbles in the mid-1990s [18, 45]. The addition of microbubbles “lowers the threshold for cavitation,” [45], as bubbles do not need to be generated. Injected microbubbles are gas-filled vesicles with a shell that can be composed of various substances, including phospholipids, proteins, and polymers [18], and can vary between 1 to $10 \mu\text{m}$ in diameter [45]. Clinically, microbubbles are routinely used as contrast agents for ultrasonic imaging [45]. During gene transfection, DNA can be injected as a solution or integrated into microbubbles [33]. Ultrasound is applied to a target location, limiting where cavitation occurs. Sonoporation has potential for *in vivo* applications as it is non-invasive [45]. Ultrasound and site-injected phospholipid microbubbles were able to successfully transfer a luciferase reporter gene into mouse muscle tissue [49]. However, despite the potential applications, ultrasound has not been applied for clinical gene therapy due to low efficiency and high cell death [45]. Primary rat cells treated with ultrasound only showed a 2.4% transient transfection rate and a 0.3% stable transfection rate, with only 50% of cells surviving the process [50].

2.5. Electroporation

It has been known since the 1960s that electricity alters membrane permeability [18]. Electric fields can result in the formation of membrane pores, cell fusion, and cell movement [51]. When electric pulses are applied to cells, the pores formed allow nucleic acids to move across the plasma membrane [51]. The electric field causes the cell membrane to polarize and breakdown temporarily [33]. Timing and intensity of the electric pulses, as well as buffers, affect the delivery of nucleic acids [51]. Electroporation parameters must be optimized for individual cell types because if the intensity used is too high, cell membranes may not be able to close [51, 52]. Applying electric pulses to cells can result in death, in part due to membranes not being able to be sealed [22]. Different cell types are more effectively transfected with spe-

cific pulse length and strength [33]. It can be time-consuming to determine the best buffers and electric intensity to use for various cell types [52]. However, the technique is useful for cells that are hard to transfect, such as T cells, hMSC, and HUVECs, and is efficient when low cell viability is not a concern [51]. Electricity was first applied for genetic transfection in 1982 in mouse lymphoma cells by Neumann *et al.* [53], who coined the term for electroporation [51]. In 1991, the first *in vivo* transfection using electroporation was demonstrated in mouse skin cells [18, 54]. In this process, a target location in the body is injected with DNA. Electrodes are then used to apply pulses of electricity to induce cell membrane permeability [18]. The efficiency is dependent on the distribution of the electric field, which may be altered by electrode type and placement [51]. This method has been demonstrated locally in the testis and eyes of mice and the forebrain of zebrafish [51]. When DNA was directly injected into rat livers and treated with electric pulses, 30 to 40% of hepatocytes expressed a reporter gene [55]. Additionally, Heller *et al.* reported that 20 to 30% of skin cells expressed a luciferase gene after transfection using electroporation [56]. However, the ability to access different organs noninvasively with electrodes *in vivo* limits its applications [18].

3. CHEMICAL AND BIOCHEMICAL METHODS

Chemical and biochemical methods of gene transfection involve complexing nucleic acids with organic or inorganic compounds to facilitate cellular uptake. Complexes interact with the cell surface and cell membrane molecules to induce endocytosis or internalization. Chemical methods are diagrammed in Fig. (3).

3.1. Calcium Phosphate Co-precipitation

Calcium phosphate co-precipitation was first described by Frank Graham and Alex van der Eb in 1973 [22, 57]. This method utilizes the phosphates lining the backbone of nucleic acids. A stable ionic complex is formed between the backbone phosphates and divalent metal cations, originally Ca^{2+} but Mg^{2+} , Ba^{2+} , and Mn^{2+} have also been used in similar co-precipitations [58]. The ion-DNA phosphate complex can be endocytosed by cells after coming in contact with the cell surface [22]. To form and precipitate complexes, phosphate buffered saline is added to a mixture of DNA and calcium chloride in solution [59]. Precipitates used for gene delivery usually range in size from 10 to 100 nm [59]. The method is relatively cheap, easy to carry out [24], and benefits from the biodegradability of calcium phosphate [59]. It can be used with many cell types and is often used when transfecting large numbers of cells [24]. However, cytotoxicity is a concern as intracellular calcium levels are generally low [22, 59]. Increased intracellular calcium levels could be a particular issue for muscle cell transfection, as calcium ions regulate muscle contraction [60]. Ca^{2+} membrane pumps may be able to mitigate calcium toxicity by pushing excess Ca^{2+} outside of cells [61].

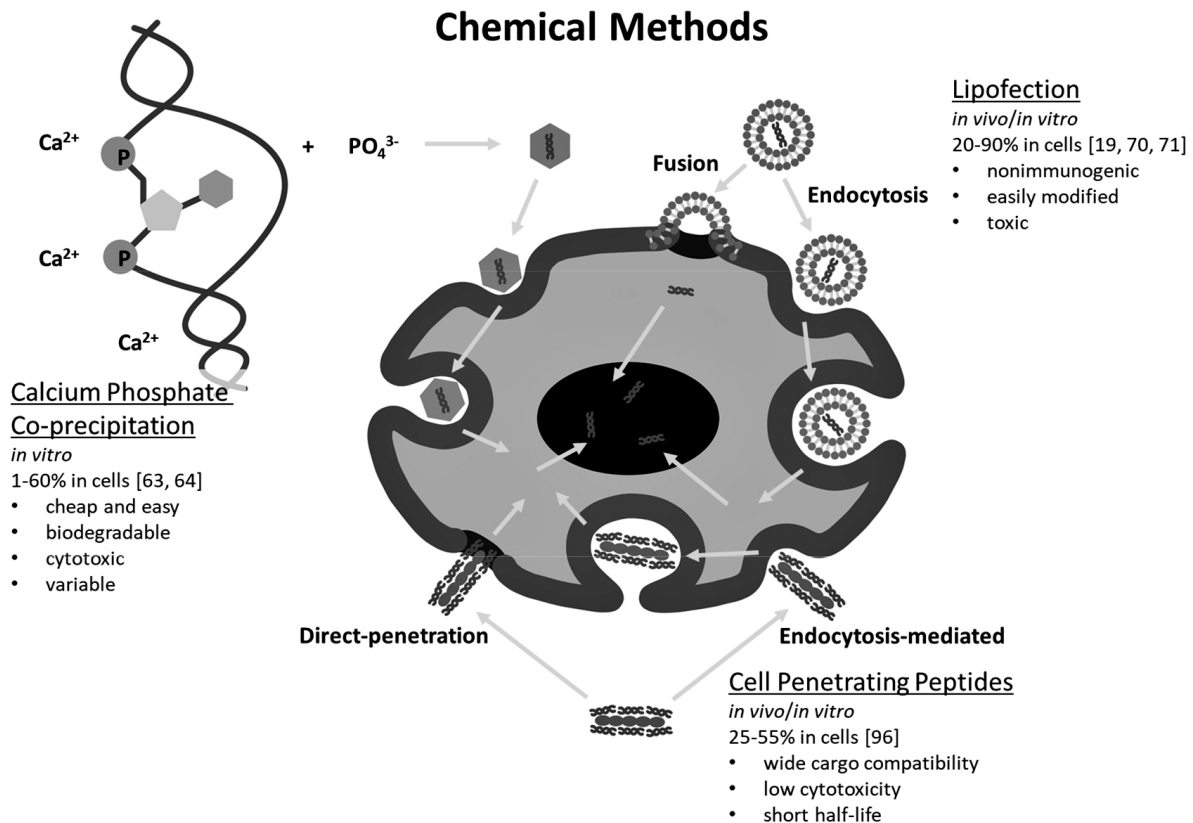


Fig. (3). Chemical methods of gene delivery. Chemical methods complex with nucleic acids and mediate uptake by interacting with the cell membrane. Calcium phosphate co-precipitation, as well as peptide and lipid carriers, are commonly used chemical transfection methods. (*A higher resolution / colour version of this figure is available in the electronic copy of the article.*)

Results from calcium phosphate co-precipitation can be highly variable due to differences in complex size [22]. Transfection efficiency is controlled by the cell type and the precipitation conditions, including calcium chloride and DNA concentration, pH, temperature, and crystal growth time [59]. Smaller precipitates show higher transfection rates than larger particles [62]. As such, precipitates are not able to transfect cells if they are stored for long periods due to nanocrystals growing into microcrystals [33, 59]. Jordan *et al.* demonstrated transfection efficiencies of approximately 60% in CHO cells and approximately 40% in HEK-293 cells using small particles, but efficiencies dropped to 3 to 5% when large particles were used [63]. The variation in transfection efficiency between cell types is also apparent, as transfection efficiency in neuronal cells is generally low, ranging from 1 to 5% [64]. Efficiency variation and low reproducibility may be due to rapid nucleation and growth of the particles [61]. This method is difficult to apply *in vivo* [22, 58], in part due to “poor colloidal stability and uncontrolled growth” [61]. However, calcium phosphate precipitates modified with polymer and lipid coatings have shown promise for *in vivo* delivery [61]. Roy *et al.* modified calcium phosphate precipitates with a polymer and a targeting ligand to increase gene delivery to liver cells in mice by 400 to 500% compared to unmodified particles [58].

3.2. Dendrimers

Dendrimers are cationic polymers composed of highly branched structures [22]. They are three-dimensional, monodispersed, synthetically-created macromolecules that generally range in size from 1 to 20 nm [65, 66]. The central molecule of a dendrimer is referred to as the core and each repeating branched junction is called a generation [65, 66]. The number of generations in a dendrimer determines its size [66]. Chemical reactions are used to grow each generation, allowing the dendrimer size to be well controlled [67, 68]. They are created by either divergent or convergent synthesis strategies [67, 69]. Small molecules can bind in between the branches of dendrimers, allowing them to be used for controlled drug release and delivery [67, 68]. Dendrimers were first synthesized in 1978 by Fritz Vögtle’s group and were further developed by other researchers in the mid-1980s [69].

A high number of surface functional groups makes dendrimers easy to functionalize [67, 68] and associate with therapeutic genes [65]. Complexing DNA to dendrimers can protect the genetic material from degradation [68]. DNA molecules are associated with dendrimers primarily by electrostatic interactions with anionic groups on the dendrimer surface [68]. Polyamidoamine (PAMAM) dendrimers are one of the most studied dendrimers [68] and were first used

for the delivery of genes in 1993 by Haensler and Szoka [69, 70]. PAMAM gene delivery is dependent on the dendrimer hydrophobicity [71], generation, and surface charge [72]. These dendrimers have also been used to deliver antisense oligonucleotides and siRNA [65].

Cationic dendrimers interact with negatively charged membrane molecules [73] and are internalized by endocytosis [65]. After endocytosis, dendrimers may act as “proton sponges” inside endosomes [68]. Certain polymers, such as dendrimers, are able to buffer the pH of endosomes and slow acidification as they transition into lysosomes [18, 74]. The high influx of protons during this process causes the vesicles to internalize chloride ions to balance the charge difference, which then results in increased osmotic pressure and lysosomal bursting as water influxes to the high solute concentration. This allows carried molecules to escape into the cytoplasm.

Shakhbazou *et al.* showed that PAMAM dendrimers of different generations and function groups had transfection efficiencies ranging from 0 to 78% using a fluorescent reporter gene in HEK 293T cells [71]. PAMAM dendrimers have shown effectivity in a variety of mammalian cell types, including fibroblasts and melanoma [72]. PAMAM dendrimers have also been utilized *in vivo*. One group demonstrated significantly higher and localized lung transgene expression in mice after tail vein injection with PAMAM compared to naked DNA [75]. Although dendrimers have been investigated as gene delivery vehicles for cancer therapy and cardiovascular diseases [66, 68], their toxicity has been a concern in their application [73]. Cytotoxicity is highly dependent on the dendrimer generation, core, and surface characteristics; however, surface functionalization can be utilized to decrease dendrimer toxicity [73, 76].

3.3. Lipofection

Gene transfection using lipids, also called lipofection, is one of the most used nonviral methods for genetic transfer [18]. Lipofection was developed and first demonstrated by Felgner *et al.* in 1987 [52, 77]. A variety of lipids are used in lipofection but most are cationic and amphiphilic, generally being composed of a hydrophilic amine head, linker, and hydrophobic hydrocarbon tail [78]. These lipids form micelles and liposomes when placed in aqueous solutions, with hydrophobic tails associating together and hydrophilic heads interacting with the environment [78]. Negatively charged DNA can be electrostatically complexed with the positively charged amine head of the lipids; the complexes are often referred to as lipoplexes [78]. DNA is protected from nucleases in the lipoplex as the nucleic acid becomes surrounded by lipids [18]. Additionally, positively charged heads are able to interact with the negatively charged cell membrane molecules, such as proteoglycans and glycoproteins, mediating internalization [18]. Lipoplexes can be endocytosed or can merge into the cell membrane, depending on the properties of the lipids and cells used [79]. Since lipids are basic components of cell membranes, lipofection is nonimmunogenic [78].

The lipids are easily modified to improve efficiency [78]. “Helper” lipids, such as dioleoylphosphatidylethanolamine (DOPE) or cholesterol, can be included in the lipoplex mixture to improve gene delivery [78]. Lipid composition and the ratios of helper lipids can be optimized to improve efficiency for specific cell lines [19]. Functional groups, such as polyethylene glycol (PEG), may also be added [78]. Transfection efficiency is influenced greatly by the size of the lipoplex [80]. Larger lipoplexes are more efficient at delivering DNA *in vitro* [81]. However, larger lipoplexes are cleared from the blood faster than smaller ones *in vivo* [80]. The type of lipids used, lipid and DNA concentration, and transfection media also affect delivery efficiency [81]. One of the most commonly used and commercially available lipids for transfection is lipofectamine [19, 82, 83]. Using Lipofectamine® 2000, Dalby *et al.* showed transfection in 20 to 30% of primary rat neurons [82]. However, lipofectamine and other lipoplexes can be toxic. For example, lipofectamine and several lipoplexes containing helper lipids reduced the viability of A549 and H1299 cells by around 20% [83]. Kulkarni *et al.* treated primary chicken embryonic cells with lipofectamine and saw only 33% cell survival, with around a 50% transfection rate [19]. The group was able to increase survival to 85% and transfection to 90% in the same cells by modifying the ratios and composition of the lipids used.

3.4. Cell-Penetrating Peptides (CPPs)

3.4.1. Introduction to CPPs

CPPs, also known as protein transduction domains (PTDs), Trojan peptides, or membrane transduction peptides, are short peptides generally containing 5 to 30 amino acids [17, 84-87]. They are characterized by their remarkable ability to translocate through plasma membranes and enter cells, tissues, and even organisms [88, 89]. CPPs possess the ability to traverse biological membranes efficiently in a process termed protein transduction [90]. Importantly, CPPs are capable of transporting numerous cargo molecules, such as DNA, RNA, oligonucleotides, liposomes, proteins, and nanoparticles, both *in vitro* and *in vivo* [85, 88, 89].

CPPs have very different origins and are ambiguous in many ways [17]. CPPs may originate from naturally occurring peptides in living organisms, chimeric peptides, naturally modified proteins, and synthetic peptides [85]. Many early CPPs were identified from naturally occurring protein sequences that were found to possess membrane-translocating properties. The first protein discovered to translocate into the nucleus was the HIV-1 transcription-transactivating (Tat) protein, demonstrated by Frankel and Pabo in 1988, with the minimal sequence isolated in 1997 [91]. Penetratin, a 16-residue CPP isolated from the *Drosophila antennapedia* (ANTP) homeodomain, was discovered in 1994 [91]. Protein/peptide engineering has developed a combination of domains with different properties to generate chimeric CPPs. Subsequently, growing knowledge based on the identified properties of CPPs has led to the development of novel CPPs with completely designed sequences. Based on their

physicochemical properties, CPPs may be classified as cationic peptides with positively charged surfaces, hydrophobic peptides with a high hydrophobic amino acid content, or amphipathic peptides with both hydrophobic and hydrophilic fragments [85]. As summarized in Table 1, independent of CPP origin or classification, several algorithms have recently been built to allow for the prediction of amino acid sequences that potentially have translocating properties [92-103].

Intracellular delivery of various cargos is mediated by binding to CPPs either covalently or noncovalently [17, 84, 88]. CPPs can be directly attached to their cargo molecules through covalent linkage, termed covalent protein transduction (CPT) [88]. However, CPT involves relatively expensive and labor-intensive synthesis, and may not be suitable for the delivery of nucleic acids and nanoparticles. Noncovalent protein transduction (NPT) utilizes noncovalent association, such as electrostatic interactions and hydrophobic effects, between CPPs and cargo molecules. The major advantages of NPT over CPT are simplicity of preparation, cargo versatility, and low working concentrations, which possibly contribute to reduced toxicity. However, NPT may suffer from premature dissociation of cargos from CPPs and off-target effects within cells due to the relatively weak interacting forces between CPPs and cargos.

3.4.2. Mechanisms of CPP Action

Due to their inherent ability to cross plasma membranes, CPPs have been employed extensively to facilitate the transport of cargo molecules into cells. However, the detailed cellular uptake mechanism of CPPs is not well understood. Generally, it is accepted now that CPPs and CPP-cargo complexes are predominantly internalized into cells by two main pathways: endocytosis and direct translocation [17, 88, 89, 104]. However, the exact cellular uptake mechanism of CPPs and CPP-cargo complexes is determined by numerous factors, such as the amino acid sequences of CPPs (hydrophobicity and net charge), extracellular concentration of CPPs, cargo properties, cell type, and the assay temperature

[104]. No matter what mechanism, the electrostatic interactions between the positively charged residues of CPPs and negatively charged glycosaminoglycans, especially heparan sulfate proteoglycans, of the membrane are the first crucial step for cellular uptake of CPPs and CPP-cargo complexes [104].

Endocytosis is a natural and energy-dependent process, occurring in almost all cells by direct interaction with the plasma membrane or by electrostatic interactions with cell surface proteoglycans [88, 104]. Endocytosis can be classified as phagocytosis (cell eating) or pinocytosis (cell drinking) [88]. Different types of pinocytosis include macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis, and clathrin- and caveolae-independent endocytosis [105]. The internalization mechanism for CPP-cargo complexes may involve a combination of specific pathways [88]. Moreover, several endocytic pathways can be used in parallel, or alternative pathways can compensate for the inhibition of specific pathways [17].

Direct translocation, also known as direct membrane penetration, is an energy-independent process where CPPs and CPP-cargo complexes directly penetrate through cellular membranes [88]. This direct translocation model involves a passive membrane diffusive or destabilization process that does not require binding to proteinaceous cell surface receptors. Direct physical interaction between the cationic residues of CPPs and the anionic phospholipids of the plasma membrane leads to direct membrane penetration [17]. Direct translocation usually completes in a short timescale, as little as 5 minutes. Energy-independent internalization of CPP-cargo complexes can be observed by incubating cells at low temperatures (4°C), since low-temperature treatment seizes all energy-dependent movement across the cell membrane. CPP-cargo complexes that directly penetrate the membrane have similar uptake at 4 or 37°C [17]. So far, the mechanism of direct translocation can be explained by three main models: the inverted cell model, the pore formation model, and the carpet model [104].

Table 1. Predictors of cell-penetrating peptides.

Name	Website	References
CPPsite 2.0	http://crdd.osdd.net/raghava/cppsites/	[92]
CPPpred	http://bioware.ucd.ie/~compass/biowareweb/Server_pages/cpppred.php	[93]
KELM-CPPpred	http://sairam.people.iitgn.ac.in/KELM-CPPpred.html	[94]
CellPPD	http://crdd.osdd.net/raghava/cellppd/submission.php	[95]
C2Pred	http://lin-group.cn/server/C2Pred	[96]
CPPred-RF	http://server.malab.cn/CPred-RF/	[97]
SkipCPP-Pred	http://server.malab.cn/SkipCPP-Pred/Index.html	[98]
MLCPP	http://www.thegleelab.org/MLCPPExample.html	[99]
CPPred-FL	http://server.malab.cn/CPred-FL/	[100]
G-DipC	N/A	[101]
StackCPPred	https://github.com/Excelsior511/StackCPPred	[102]
TargetCPP	N/A	[103]

N/A: not available.

3.4.3. Applications of CPPs in Clinical and Gene Therapies

Today, there is an urgent need to develop new therapeutic agents. Many conventional treatments are outdated and less desirable due to drug resistance, low selectivity, and poor solubility [86]. Therapeutic peptides are a promising and novel approach to treat many diseases, including cancers and genetic disorders. Therapeutic peptides have several advantages over proteins or antibodies, as they are easy to synthesize, have high target specificity as well as selectivity, and have low toxicity. Nevertheless, therapeutic peptides do have significant drawbacks related to their stability and short half-life [86].

In 1995, one of the earliest examples of a CPP used in gene therapy was demonstrated. The ANTP CPP was covalently linked to an antisense DNA of amyloid precursor protein, which gave the antisense treatment direct access to the cell cytosol and nucleus [90, 106]. Internalized antisense oligonucleotides mediated by ANTP decreased amyloid precursor protein expression, resulting in the inhibition of neurite outgrowth [106]. Later, in 1997, the first non-covalent delivery of oligonucleotides was demonstrated using MPG, a 27-residue chimeric CPP [91, 107]. Kardani *et al.* demonstrated reporter gene expression in 25 to 55% of mammalian cells transfected with CPPs [108]. Accordingly, numerous CPPs have been reported to assemble antisense oligonucleotides, small interfering RNA (siRNA), or plasmid DNA into CPP--cargo nanoparticles, possessing positive charges that allow them to interact with cellular membranes and to internalize into cells [85, 109, 110].

In vivo delivery of nucleic acids is a challenge that has to be solved before therapeutic gene applications can be translated into the clinics [111]. Preferably, systemic administration should be made applicable as this enables the delivered gene particles to reach all corners of the body. Despite intensive research over the last 20 years, only a few gene therapeutic vectors have been approved for use in clinics [112]. Many clinical studies have revealed safety issues along with inefficiency problems. In 2012, the first statistically significant clinical trial opened the door for clinical delivery of macromolecular therapeutics [113]. Subsequently, there were over 25 clinical trials performed predominantly using CPPs in 2015 [114], and many pre-clinical and clinical trials with CPP-derived therapeutics were conducted in 2017 [115].

In addition to positive or negative regulation of gene products, CPPs may be used for the delivery of genome editing tools [85, 110, 116, 117]. In fact, an innovative application of CPP-mediated delivery of the CRISPR-Cas9 system in genome editing was reported in 2014 [118]. Both covalent conjugation of Cas9-CPP by a thioether bond and noncovalent CPP/single-guide RNA (sgRNA) complexing led to efficient gene disruptions in several human cell types with low off-target incision effects. This study demonstrated CP-

P-enabled direct delivery of both recombinant Cas9 protein and sgRNA into cultured mammalian cells [110, 117]. However, the genome editing frequency of this Cas9-CPP and CPP/sgRNA treatment tended to be low (less than 15% after three rounds of treatment) [119].

Collectively, CPPs have long been regarded as promising therapeutic delivery vehicles, not only because of their high internalization ability but also their potential for modification [104]. As promising carriers, CPPs generally have several advantages, such as low cytotoxicity, ease of preparation, and a wide variety of cargo type compatibility [86, 104, 120]. However, there are still shortcomings for drug delivery *in vivo*, such as cell-free specificity, short duration of action, and lack of oral bioavailability [86, 104, 120].

4. VIRAL METHODS

The goal of any virus is to infect and exploit host cells by injecting nucleic acids into their cytoplasm and taking over the host's cellular machinery. The life-cycle of a virus makes it a particularly promising carrier for gene therapeutics. However, for safety reasons, viral and retroviral vectors have to be modified to reduce their immunogenicity and cytotoxicity. Modification usually involves deletion or truncation of viral genes necessary for viral replication. Currently, viruses used as vectors for nucleic acid delivery have recombinant, replication-defective genomes that have had therapeutic genes added into them. Often, helper vectors or viruses are used to package the replication-defective viruses that carry therapeutic genes, which reduces the chance for viruses to gain competency. However, viral vectors may still be affected by mutational pressure, resulting in unpredictability [121]. In the below sections, applications, advantages, and disadvantages of viral and retroviral vectors as carrier platforms are discussed. Viral delivery methods are diagrammed in Fig. (4).

4.1. Adenovirus (AV) Vectors

Adenoviruses (AVs) were first isolated from human adenoid tissue in 1953 [122]. These viruses were the first viruses used for gene therapy in the 1990s and later they began to be tested in clinical trials [122]. In 1993, the first *in vivo* human gene therapy was performed in a cystic fibrosis patient using a recombinant AV vector [123, 124]. However, sufficient precautions were not taken with this early vector and later trials led to the death of Jesse Gelsinger in 1999 [14].

Adenoviruses are non-enveloped viruses with icosahedral capsids ranging from 60 to 90 nm in size. They contain a linear, double-stranded DNA genome of 30 to 40 kb and enter host cells by clathrin-mediated endocytosis [125]. The genome of adenoviruses includes various transcriptional units, which can be categorized based on when they are transcribed [6]. The units are early genes (E1A, E1B, E2, E3, and E4), delayed early (IX and Iva2), major late (ML), and late genes used in post-translational processing (L1, L2, L3, L4, and L5) [6]. The E1 region and a portion of E3 region in

the AV genome were eliminated in the first-generation of recombinant AVs in order to accommodate a transgene. In addition to the potential of gaining replication competency in packaging cell lines, recombinant AVs tend to elicit a powerful immune response and cytotoxicity. To alleviate these undesirable outcomes, vectors were further modified in a second generation where the E2 and E4 regions, which are responsible for immune reactions, were deleted [126, 127]. This also allowed the transgene packaging capacity of the vectors to increase from 5 kb to 14 kb. Further manipulations to produce a third generation of AV vectors nearly depleted the entire AV genome while allowing it to accommodate up to 37 kb target genes for delivery [128]. Genome components crucial for viral DNA replication and packaging were retained [6]. The onset of expression can occur as early as 16 to 24 hours after infection.

Adenoviral DNA cannot integrate into the genome and is unable to replicate during cell division, which imposes limitations for broader gene therapy; on the other hand, this eliminates the possibility of chromosomal rearrangements

that could lead to potential tumor formation [129]. One group showed gene transduction efficiencies of 55 to 93% after using 4 different adenoviral vectors to infect CD34+ cord blood cells *in vitro* [130]. Their highly efficient transduction of most tissues, high levels of protein expression, and transient gene expression make them attractive in gene transfer and therapy [131]. Including trials up to 2017, 20.5% of gene therapy trials used adenoviral vectors [21].

4.2. Adeno-associated Virus (AAV) Vectors

Adeno-associated virus (AAV) was discovered in the mid-1960s when scientists were studying adenovirus and later identified AAVs in human tissues [132, 133]. A journey of 20 years to understand AAV biology eventually yielded the first AAV vector used for *in vitro* gene delivery in 1984. The first human test of AAV vectors was carried out to treat a patient with cystic fibrosis in 1995 [134]. In 2012, the European Medicine Agency approved the first AAV vector-based gene therapy drug to treat lipoprotein lipase deficiency.

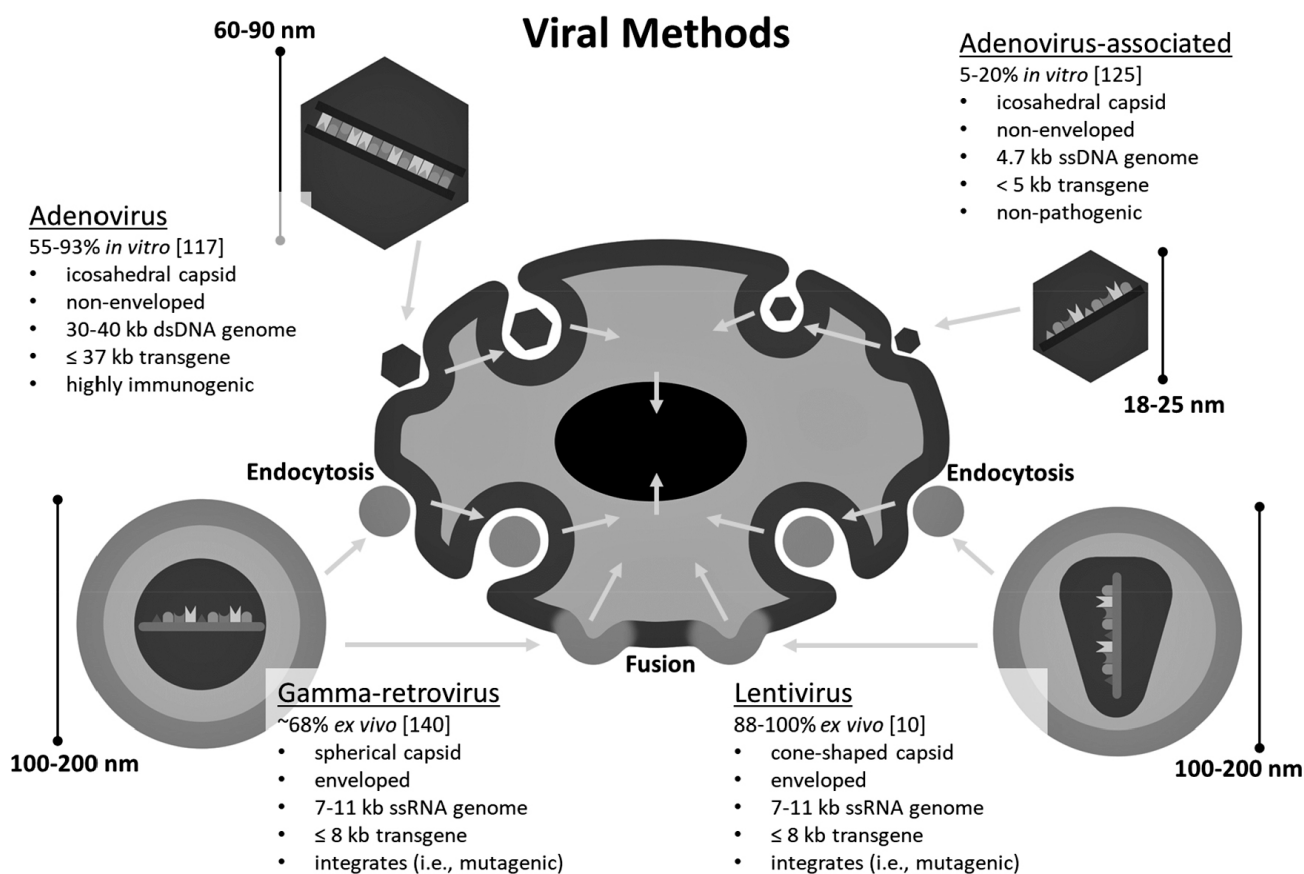


Fig. (4). Viral methods of gene delivery. Viral vectors vary in particle size, genome size and type, transgene capacity, capsid geometry, and whether they have an envelope or not. Vectors enter either by endocytosis or through membrane fusion. Integration of delivered nucleic acids into the host genome is dependent on the virus type. (A higher resolution / colour version of this figure is available in the electronic copy of the article).

AAVs are non-enveloped, single-stranded DNA viruses with 4.7 kb genomes that are encapsulated in icosahedral capsids 18 to 25 nm in diameter. Wild-type AAVs require a helper virus to complete their life cycle. The non-pathogenic nature and the ability to package various transgenes make AAVs an ideal vehicle for gene therapy [134, 135]. AAVs are internalized by endocytosis mediated by clathrin [6]. The genome of the most commonly used serotype, AAV2, contains genes that encode regulatory Rep proteins and structural Cap proteins. The former carry out genome excision from the host chromosome, replication, packaging, and integration whereas the latter produce capsid proteins. Inverted terminal repeats flanking the genome possess regulatory cis-acting sequences needed for the virus to complete its life cycle and for integration into the host genome. In making AAV vectors, Rep and Cap were removed from AAV DNA to make room for therapeutic genes. Required proteins are provided to the virus by helper vectors during vector production [136]. An example of vector production in HEK293 cells would involve the functions of Rep and Cap being supplied in a separate helper vector, with an additional helper vector containing the E4, E2a, and VA regions required for replication [136].

The primary disadvantages of AAV vectors are that delivery is limited to smaller sized genes of interest (less than 5.0 kb of DNA), a slower onset of expression (2 to 7 days for *in vitro* and 3 to 21 days for *in vivo*), and relatively low levels of protein expression that leads to a necessity of re-administration of AAV vectors. Another significant drawback of AAVs is that they often trigger immune responses. For instance, neutralizing antibodies lead to rapid clearance of AAVs from the circulatory system by opsonizing viral particles and thus facilitate uptake by phagocytic cells. To overcome this issue, certain strategies have been employed that include re-engineering AAV vectors, use of capsid decoys, changing the route of administration, plasmapheresis, disruption of B cell activation and reduction of the number of activated B cells, and targeting T cell activation. Each method has disease-dependent pros and cons [137].

Currently, more than nine different serotypes of AAV vectors have been used in clinical trials. The infectivity of AAVs in different cell types can be increased by utilizing different serotypes. Liver, eye, heart, muscle, brain, and bone have been targeted for therapy. Most of these trials are in phase I and/or II, while only a few have entered phase III. Around 7.9% of gene therapy clinical trials used AAV vectors up until 2017 [21]. The transduction of AAV vectors is rate-limited by the necessity for AAV single-stranded DNA to be converted to double-stranded DNA before transcription [134]. It was demonstrated that an AAV alone was only able to transduce approximately 5 to 20% of HEK293T or HepG2 cells [138]. However, this has been improved by using methods, such as self-complementary vectors, to overcome the rate-limiting step [139].

4.3. Retroviral Vectors

Retroviruses are enveloped viruses with two identical copies of single-stranded RNA that range in length from 7 to

10 kb [140-143]. The entire viral particle is about 100 to 200 nm. Retroviruses are classified as simple retroviruses or complex retroviruses according to their genomic organization and biological features. Gamma-retroviruses and lentiviruses are representative simple retroviruses and complex retroviruses, respectively. All retroviruses enter cells either by receptor-mediated endocytosis or membrane fusion [144, 145]. The unique features of retroviruses lie in their capability to reverse transcribe their RNA into cDNA *via* reverse transcriptase activity and to integrate the cDNA into the host genome *via* integrase activity. The integration allows for hijacking the host's replication machinery for viral reproduction for further infection. In the below sections, two major types of retroviral vectors for gene therapy are discussed.

4.3.1. Gamma-retroviral Vectors

Gamma-retrovirus possesses a simple genome structure. The *gag* gene encodes structural proteins and proteins pertaining to the budding process. The *pol* gene codes for a protease, a reverse transcriptase, and an integrase. The protease cleaves the *gag* polyprotein. The reverse transcriptase is needed for the generation of viral cDNA from viral RNA and the integrase aids integration of viral cDNA into the host cell genome. All regulatory elements involved in viral RNA processing lie in the long terminal repeat (LTR) regions of the viral genome.

The most commonly used retroviral vector for gene therapy was derived from murine leukemia virus (MLV), which is a simple gamma-retrovirus [145] with a nearly spherical capsid [146]. The first *ex vivo* gene therapy trial was approved in 1990 by the FDA. This trial treated the white blood cells of two young adenosine deaminase deficiency patients with a modified MLV vector carrying a normal gene [147, 148]. The efficiency of the therapy was debated [147]. Throughout decades, safety considerations have directed the evolution and development of the MLV vector system. The goal of manipulating vector arrangement was to remove its replication competence. The most advanced third generation gamma-retroviral vectors utilize a split-genome approach comprised of three plasmids. The main retroviral vector contains the LTR, a primer binding site for reverse transcription, and the packaging signal, as well as the transgene which can be up to 8 kb long. The structural and enzymatic retroviral genes are located in two helper plasmids. The *gag*, *pol*, and *env* genes in the helper plasmids do not contain retroviral elements pertaining to packaging, thereby reducing the probability of recombination [145]. To further improve safety, self-inactivating (SIN) vectors were developed by making the 3'-LTR nonfunctional. Even with so many modifications, once genes are delivered to cells, insertional mutagenesis, enhancer interaction, or premature termination may occur, which can lead to severe side effects.

Gamma-retroviral vectors have been used to transfer genes to hematopoietic stem cells to correct blood-related genetic disorders and skin diseases, such as Wiskott-Aldrich syndrome, SCID-X1, SCID-ADA, epidermolysis bullosa, and melanoma [11, 149-156]. In an *ex vivo* trial using gam-

ma-retroviruses to treat SCID-X1, Gaspar *et al.* demonstrated an approximate 68% transfection efficiency in CD34+ bone-marrow stem cells [153]. Currently, approximately 17.9% of gene therapy clinical trials use retroviral vectors [21]. However, treating patients with integrating viruses is risky as integration has been associated with mutagenesis and oncogenesis when normal genes are disrupted or oncogenes are activated. For instance, in one trial of nine SCID-X1 patients, four patients treated with gamma-retroviral vectors developed T cell leukemia after gene therapy [149, 152].

4.3.2. Lentiviral (LV) Vectors

Lentivirus (LV) vectors are derived from HIV-1. They were first used in clinical trials in 2003 to deliver an anti-sense sequence for the HIV-1 envelope gene to CD4+ cells *ex vivo* [157]. In addition to *gag*, *pol*, and *env* found in gamma-retroviruses, the HIV-1 virus contains two regulatory genes, *tat* and *rev*, that are essential for replication. Tat and rev regulate transactivation of gene expression and nuclear export of mRNAs. Importantly, HIV-1 contains four accessory genes that encode critical virulence factors for virus transmission enhancement. The lentiviral capsid is cone-shaped, rather than spherical [146]. In order for HIV-1 to be used safely in gene therapy, significant modifications to its genome were carried out [158].

The first generation of lentiviral vectors consisted of a major portion of the HIV genome, including retention of the *gag* and *pol* genes. The envelope protein (Env) was supplied by another virus called VSV-G, which broadened the range of mammalian cells that could be transduced. The second generation took out accessory virulence genes, such as *vif*, *vpr*, *vpu*, and *nef*, without compromising transduction efficiency. The third generation of lentiviral vector systems underwent significant genome rearrangement and contained four plasmids: a vector plasmid, two packaging plasmids (containing *gag*, *pol*, and *rev* separately), and one envelope plasmid (containing *env* from VSV-G). The split-genome approach was intended to maximize the segregation of *cis*- and *trans*-acting functions and to minimize the possibility of homologous recombination events in order to reduce the generation of replication-competent lentiviruses. The removal of all accessory genes in the vector plasmid achieved a higher safety level. The gene *tat* was replaced by a modified LTR with a constitutively active promoter sequence [159]. The LTR sequence was further modified to become self-inactivating (*i.e.*, SIN 5' and 3' LTR) [144]. To enhance viral titers and transgene expression, central polypurine tract/central termination sequence (cPPT/CTS), an enhanced promoter, and a woodchuck hepatitis virus posttranscriptional regulatory element were included [136]. Similar to gamma-retroviral vectors, lentiviral vectors can deliver transgenes up to 8 kb in length [79].

The most distinctive advantage of LVs over other retroviral vectors is their ability to penetrate the nuclear envelope of non-dividing cells. This extraordinary characteristics allow lentiviral vectors to be used in neurons and other non-di-

viding cells in adult organisms [160]. In addition, lentiviral gene therapies have been used in vascular transplantation, chronic granulomatous diseases, prostate cancer, hemophilia A, rheumatoid arthritis, and diabetes mellitus [161-164]. In the past decade, the clinical use of LVs has gained significant attention for gene transfer into CD34+ hematopoietic stem cells to treat a variety of genetic disorders, such as β -thalassemia, X-linked adrenoleukodystrophy, metachromatic leukodystrophy, and Wiskott-Aldrich syndrome [10, 12, 13, 165, 166]. *Ex vivo* transduction of hematopoietic stem cells with a lentiviral vector to treat Wiskott-Aldrich syndrome resulted in 88 to 100% gene transfer efficiencies in CD34+ bone-marrow stem cells [10]. Approximately 7.3% of gene therapy clinical trials utilized lentiviral vectors up until 2017 [21].

5. GENOME EDITING

Zinc finger nucleases (ZFNs), transcription activator-like effector nucleases (TALENs), and CRISPR systems recognize and cut DNA sequences and have been utilized for genome editing [1]. ZFNs, TALENs, and CRISPR systems function through a DNA-targeting domain and a non-specific nuclease domain, resulting in a double-strand break (DSB) in the DNA at the target site. The DNA-targeting domain is unique to each system, but the cleavage and cellular repair of a break are similar.

After a double-stranded DNA break occurs in a eukaryotic cell, the conserved homology-directed repair (HDR) or non-homologous end joining (NHEJ) pathways are used to repair the gap [167]. NHEJ repairs the DNA break by quickly ligating the strands; however, it is not accurate and frequently results in short insertions and/or deletions that generate loss-of-function mutations [167, 168]. This may be desirable if gene inactivation is the goal. NHEJ-mediated repair is dominant in cells in the G₁ and G₂ phases of the cell cycle [169]. In HDR, a strand of template DNA similar to the breakage site is used to repair the gene [167, 168]. Homologous DNA with corrected bases can be delivered with gene editing tools and used as a template to fix the gene or add in a new gene [167]. However, HDR is restricted to cells in the S and G₂ phases of the cell cycle [169].

ZFNs and TALENs rely on proteins to specifically interact with DNA sequences and CRISPR systems use an RNA guide sequence [1, 170]. The use of RNA for targeting makes CRISPR-Cas9 systems easier to manipulate than ZFNs and TALENs, as proteins must be engineered to bind new DNA sequences [170]. The rate of mutations induced by these systems varies, with ZFNs having low success rates ranging from 0 to 19% [171], TALENs ranging from about 2 to 56% [172], and CRISPR ranging from approximately 2 to 80% [173]. CRISPR systems have emerged as the front-runner for genome editing due to higher success rates and simpler target alteration. However, in order to function, editing tools need to be delivered into cells [1]. Some of the above methods may be used, but the safety and efficiency of these delivery systems still need to be considered. Genome editing techniques are diagrammed in Fig. (5).

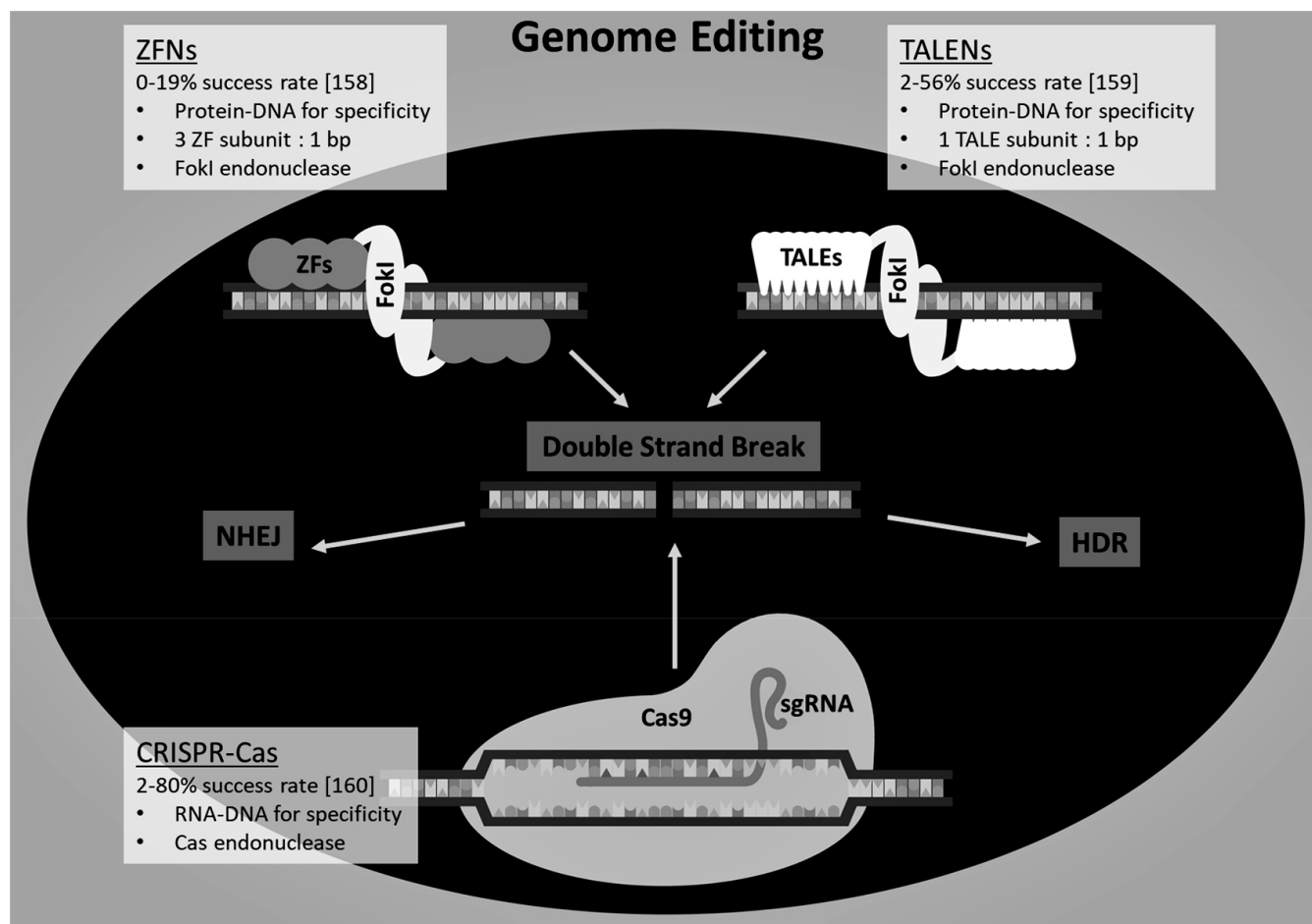


Fig. (5). Genome editing techniques. All methods function by causing double-strand breaks in DNA at targeted sequences. Cells repair the damage through non-homologous end joining (NHEJ) or homology-directed repair (HDR). ZFNs and TALENs share the same endonuclease (FokI) to induce breaks but target sequences using either zinc finger (ZF) or transcription activator-like effector (TALE) protein subunits. CRISPR uses a Cas endonuclease and targets DNA with a single-guide RNA (sgRNA). (A higher resolution / colour version of this figure is available in the electronic copy of the article).

5.1. Zinc Finger Nucleases (ZFNs)

Zinc finger nucleases (ZFNs) are site-specific endonucleases that induce double-strand breaks in DNA [167]. They can be designed to target specific DNA sequences. ZFNs are zinc finger proteins, a type of eukaryotic transcription factor, linked to a FokI restriction enzyme nuclease domain, which is of bacterial origin [167, 174]. The zinc finger proteins provide specificity and the restriction enzyme enables the DNA to be cut [167]. Both segments can be modified individually for optimization and then joined. The combination of modular DNA sequence-specific proteins and the non-specific FokI nuclease domain was first developed in 1996 [170, 174], with initial applications demonstrated in mammalian cells and *Drosophila* [170]. ZFNs have been used to target gene sequences in CHO cells, human cells, *Drosophila*, zebrafish, tobacco plants, and nematodes [168].

Zinc finger proteins are very diverse in nature and very few residues are conserved between them [175]. They often contain cysteine and histidine amino acids that provide essential interactions with a zinc ion, which stabilizes the folding of the protein [175, 176]. Zinc fingers containing two cysteines and two histidines (Cys_2-His_2) play roles in protein interactions with DNA, RNA, and other proteins [175] and many are transcription factors [176]. Zinc fingers used in ZFNs are of this type [167]. Cys_2-His_2 zinc fingers often fold into a two-stranded antiparallel β -sheet and an α -helix, which bind around the zinc ion [175]. Generally, the Cys residues are located at one end of one β -sheet and the His residues are in the α -helix C-terminal area, with variable spacing. Loss of function results from the alteration of either the Cys or His residues, indicating their essential role. The fingers in a protein are linked with a spacer sequence that influences “the spacing of the fingers along the DNA site” and is important for DNA binding affinity [175]. When binding

DNA, the protein wraps around the helix, with each α -helix of individual fingers interacting with the major groove. The residues along the surface of the finger's α -helix seem to be responsible for base-specific interactions [175, 176]. Finger proteins also interact with the phosphate backbone of the same DNA strand base interactions are made with, further securing the protein [175].

The FokI domain is only functional as a dimer, formed by two separate ZFNs binding DNA and arranging their nuclease monomers into proper orientation [167]. Each finger binds specifically to about 3 DNA base pairs, with early studies incorporating 3 fingers and more recent ones using up to 6 per monomer, allowing 18 to 36 base pairs to be recognized with the dimer [167]. Libraries of zinc fingers have been assembled that specifically bind combinations of three base pair sequences, allowing zinc fingers to be selected and bound sequentially in order to target the desired DNA sequence [177]. Using different combinations of fingers in the set of dimers allows for longer sequences to be targeted [167]. However, designing ZFNs with sequence-specificity can be difficult because combining natural and synthetic fingers modularly can result in unforeseen interactions between other fingers and DNA bases [167]. Engineering new synthetic fingers can be complex [1].

Off-targeting is a major concern with ZFNs. Fingers may bind to and cut similar sequences in the genome, other than the one intended [167]. If two different ZFNs are paired together to target a sequence, off-targeting may also occur because homodimers can form. However, this particular issue has been resolved by developing ZFNs that only function as heterodimers [167, 178]. ZFNs can also be toxic, likely due to off-targeting, with only 20 to 60% of 293T cells surviving transfection with ZFN plasmids [179]. ZFNs may not efficiently break a sequence if chromatin structure is too dense due to the necessity of the protein fingers wrapping around the DNA helix [167]. ZFNs have not been used extensively since their development because of the complexity in designing fingers to target new sequences and the difficulty in validating binding [170].

5.2. Transcription Activator-like Effector Nucleases (TALENs)

Similar to ZFNs, transcription activator-like effector nucleases (TALENs) cause double-strand breaks in DNA using a FokI nuclease domain joined with a DNA-targeting domain [180, 181]. TALENs were created in 2010 [182, 183], originally with FokI because the targeting domain was inserted into the same plasmid used to create ZFNs [184].

The DNA targeting domain of TALENs, transcription activator-like effectors (TALEs), was discovered in *Xanthomonas*, a genus of bacterial plant pathogens [184]. TALEs mimic eukaryotic transcription factors and are secreted by the bacteria to weaken plant cells, making them more vulnerable to attack after their gene transcription is altered. TALEs can be engineered for DNA binding specificity [181]. Since their

discovery, TALENs have been applied in a variety of cells and organisms, including yeast, *Drosophila*, human cell lines, zebrafish, frogs, rice, and roundworms [181].

The potential of TALEs for DNA targeting is greater than zinc fingers. Individual TALE subunits can be modularly combined in order to produce a sequence-specific binding protein [177]. TALE subunits are composed of a highly conserved amino acid sequence ranging from 33 to 35 residues in length and were discovered because of their repeating sequence [181]. TALE repeats in the natural protein are flanked by amino- and carboxy-domains, which are also incorporated into TALENs. Each subunit in a TALE array binds one nucleotide. Hypervariable residues at positions 12 and 13 are responsible for base-specificity [184]. Multiple of these diresidues can bind the same base with varying efficiency. The most common diresidues used in research to bind the DNA bases A, T, G, and C are the amino acid pairs NI, NG, NN, and HD, respectively; however, NN has also been shown to bind A, so NH and NK have been implemented to decrease off-targeting [184]. The conserved amino acids in each monomer fold into α -helices on either side of the hypervariable residues, forming a v-shape [181]. These v-shaped subunits fit into the DNA major-groove so that their diresidues make contact with the bases and together wrap around the DNA to form a superhelix [181].

Due to the use of FokI, TALENs also function in pairs [184]. Targeting domains are designed to bind to opposite DNA strands with 12 to 25 base pair spacing between the binding points. TALENs are often created to target 18 or more base pair sequences, though specificity may decrease with longer chains [180]. TALENs have lower toxicity and higher specificity than ZFNs [178]. Production and validation of TALENs are also easier [170]. However, off-targeting may still be a concern and they are much larger than ZFNs, making them more difficult to deliver to cells as many delivery methods have limits with respect to cargo size [178]. Despite this, off-targeting seems to be less when TALENs are used to target the same genes as ZFNs [180]. Each pair of TALENs still must be engineered when a new DNA sequence is to be targeted [182], which may have contributed to their limited application [170]. Regular use of TALENs may also have been hampered by the discovery of CRISPR-Cas9 shortly after they were developed.

5.3. CRISPR-Cas Systems

5.3.1. Development History

The CRISPR-Cas system was derived from an adaptive immune system of bacterial defense against foreign invaders, such as viruses, phages, and certain plasmids. In 1987, clusters of short palindromic DNA repeats separated by hypervariable spacer sequences were discovered in *Escherichia coli* [185]. It was revealed that the bacteria captured DNA segments from invading viruses or foreign DNA,

and used them to create DNA repeats, termed clustered regularly interspaced short palindromic repeats (CRISPR), in 2002 [186]. These CRISPR allow the bacteria to retain a history of previous viral infections or transformed DNA in the bacterial genome. When the viruses or closely related ones attack again, the bacteria produce RNA segments transcribed from these CRISPR to target the invasive genetic material, similar to current uses of siRNA. CRISPR-associated (Cas) genes are usually located adjacent to each CRISPR locus and code for a variety of polymerases, nucleases (both DNA and RNA), helicases, and RNA-binding proteins. The bacteria use Cas9 or a similar enzyme to cut foreign DNA apart, which disables the viruses or destroys other harmful DNA.

In 2012, two research teams first developed the CRISPR-Cas9 system for genome editing based on the bacterial defense system [187-190]. As summarized in Table 2, the original developers have launched their own biotechnology companies. They shared the 2016 Tang Prize in Biopharmaceutical Science for their work in “the development of CRISPR/Cas9 as a breakthrough genome editing platform that promises to revolutionize biomedical research and disease treatment” [191]. They were also winners of the Canada Gairdner International Award in 2016. Ultimately, Emmanuelle Charpentier and Jennifer Doudna won the 2020 Nobel Prize in Chemistry “for the development of a method for genome editing”.

This genome editing approach has inarguably revolutionized the field of molecular biology and medical research, and has had a profound and rapid impact on the development of more effective strategies to conquer human genetic diseases and cancers. In terms of experimental practice, the CRISPR-Cas system is characterized by its simplicity to use, high success rate, and easiness in design, construction, as well as delivery [169]. Additionally, targeted mutations in multiple genes (also known as multiplex genome engineering) are possible with the CRISPR-Cas system. Thus, these features make the innovative CRISPR-Cas system an extremely valuable tool for the evaluation of investigational gene therapies.

5.3.2. Mechanism of CRISPR-Cas Action

The CRISPR/Cas system is a ribonucleoprotein complex and comprises two key components: a chimeric single-guide RNA (sgRNA) and a DNA endonuclease Cas protein for genome editing [117, 192]. Together, an appropriate sgRNA carries a Cas nuclease to target a specific genomic sequence. The detailed mechanism of CRISPR-Cas system comprises three phases, which are the adaptation of spacer sequences, expression and mutation, and interference [193]. As of 2018, CRISPR-Cas systems are classified into three main types of Cas variants (and a dozen subtypes) that have been developed and differ in their mechanisms of action [188, 192].

The first Cas system is the wild-type Cas9 protein from the type II CRISPR system of *Streptococcus pyogenes*, and is commonly used as a genome editing tool [189]. This Cas9 protein cleaves DNA at specific sites, resulting in the creation of DSBs. DSBs are then subjected to the error-prone repair by NHEJ or HDR [169]. The efficiency of CRISPR-induced HDR may be very low *in vivo* due to HDR being limited to S and G₂ phase cells. The second Cas9 variant is Cas9D10A (a.k.a., nickase) [194], a mutant form of the Cas9 protein which cleaves only one DNA strand or modifies only one specific nucleotide [188, 192]. This activates the high-fidelity HDR pathway and downregulates NHEJ-mediated repair. The third Cas9 version is dCas9 (dead Cas9, nuclease-deficient Cas9, or CRISPR interference; *i.e.*, CRISPRi) [195], in which certain mutations have been introduced to inactivate the protein’s cleavage activity but retain the DNA-binding activity. This dCas9 is a DNA complexing protein that can specifically interfere with transcription and modulate gene expression. It shall be remarked that the number and diversity of CRISPR-Cas systems are continuously expanding. Recently, a new evolutionary classification has been introduced to include 2 classes, 6 types, and 33 subtypes [196]. The class 1 systems include multiple Cas proteins that form a CRISPR RNA-binding complex. By contrast, class 2 systems have a single, multidomain CRISPR RNA-binding protein, such as Cas9 in type II systems.

Several methods have also been developed related to CRISPR-Cas systems. Prime editing is a genome editing technique that produced higher precision and a wider selection of applications, potentially enabling researchers to correct up to 89% of known genetic variants [197]. This versatile and precise genome editing method uses a catalytically impaired Cas9 fused to an engineered reverse transcriptase and a prime editing guide RNA (pegRNA) that both specify the target site and encode the desired edit. Anti-CRISPRs (Acrs) are small proteins that have been identified to inhibit the RNA-guided DNA targeting activity of CRISPR-Cas proteins [198]. Three inhibitors, AcrIIA13, AcrIIA14, and AcrIIA15, were found to block CRISPR-Cas-mediated genome editing in human embryonic kidney cells. These inhibitors share a conserved N-terminal sequence that is required in DNA cleavage inhibition. Notably, a new base editor was developed by fusing dCas9 and cytidine deaminase that enabled the direct, irreversible conversion of one target DNA base into another in a programmable manner, without DSBs [199]. Instead of possible disruption of the entire genome, this base editor creates point mutations at a targeted genomic locus with an efficiency of up to 75% using a CRISPR framework. This tool is like using an eraser and pencil to fix just a single letter. Another new CasRx ribonuclease effector was recently identified to exhibit favorable efficiency and specificity relative to RNA interference across diverse endogenous transcripts [200]. CasRx can be flexibly packaged into AAVs to manipulate alternative splicing, reducing pathological tau isoforms in a neuronal model of frontotemporal dementia.

Table 2. Three original developers of the CRISPR-Cas9 system.

Principal Investigator	Jennifer A. Doudna	Emmanuelle Charpentier	Feng Zhang
Institute	University of California, Berkeley, USA	Umea University, Sweden	Massachusetts Institute of Technology, USA
Discovery	The use of CRISPR-Cas9 system to edit DNA.	The use of CRISPR-Cas9 system to edit DNA.	The application of CRISPR-Cas9 in mammalian cells.
Company founder	Caribou Biosciences, Inc. and Editas Medicine, Inc.	CRISPR Therapeutics Co.	Editas Medicine, Inc.
References	[189]	[189]	[190]

5.3.3. Applications of CRISPR-Cas in Gene Therapy

In the post-CRISPR-Cas era, we have observed incredible increases in laboratory research from academic institutes and clinical applications from pharmaceutical companies. It was estimated that the genome editing market would be worth more than US\$5 billion by 2021 [201]. Although the driving force of this rapid development was certainly CRISPR-Cas, both ZFNs and TALENs platforms reached the clinical stage before CRISPR-Cas. Many teams and companies have been working to translate CRISPR-Cas technologies into safe and effective human therapeutics [1, 201-205]. For instance, the Genome Project-write (GP-write) is an open, international research project, which focuses on whole genome engineering of human cell lines and other organisms of agricultural and public health significance [206]. In any case, genome editing must be accurate, efficient, and deliverable to the desired cells or tissues for safe and effective clinical use *ex vivo* and *in vivo* [207]. Currently, there are 10 clinical CRISPR studies under either recruiting or not yet recruiting status, according to ClinicalTrials.gov [208]. In 2020, there were 19 ongoing clinical trials using CRISPR-based gene editing [209]. However, none of the drugs proposed in these clinical trials have been officially approved as new drugs by the FDA [203].

Since the discovery of the CRISPR-Cas system in 2012, the progress toward human trials has been slow [210]. For example, FDA halted the proposed trial of CTX001 to use the CRISPR-Cas system for a single genetic change in patients with sickle cell disease. The status of the European trial using the same treatment in patients with beta-thalassemia was unaffected by this FDA decision, and was still planned to be initiated later in 2018. In 2015, it was reported that the first CRISPR-Cas human clinical trial took place in China [203, 211]. There were 86 patients with various cancers that participated in these clinical CRISPR-Cas trials from 2015 to 2017. At least 15 patients died during the trials, although the directors of clinical research claimed they died from their own cancers. One of the clinical trial directors from Hangzhou Cancer Hospital revealed that the cure rate of an *ex vivo* clinical CRISPR-Cas trial after 11 months was approximately 40% from 21 patients with esophageal cancer [212]. However, no official or complete results from Chinese CRISPR-Cas human clinical trials have been reported to date. In November 2018, a Chinese biophysics researcher, Jiankui He, sent shockwaves across the world by claiming that he had utilized CRISPR technology to edit the genomes of two twin babies, Lulu and Nana

[213]. His team attempted to introduce a mutation into their *CCR5* genes, a gene encoding the T cell receptor that HIV viruses bind to, in order to prevent HIV infection. However, the ethically controversial announcement led to his dismissal from the Southern University of Science and Technology in Shenzhen, China. He was also fined three million yuan and imprisoned for three years by the Chinese People's Court in 2019.

The ongoing global pandemic of coronavirus disease 2019 (COVID-19), caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), is one of the most devastating viral outbreaks in the past 100 years [214]. A rapid (less than 40 minute), easy-to-implement, and accurate CRISPR-Cas12-based diagnostic assay was developed for detecting SARS-CoV-2 from RNA extracts of respiratory swabs [215]. This DNA endonuclease-targeted CRISPR trans reporter (DETECTR) assay provides a visual and faster alternative to general assays. Additionally, a CRISPR-Cas13-based antiviral strategy was recently developed to target conserved sequences of coronaviruses and influenza A virus [216]. This strategy was named prophylactic antiviral CRISPR in human cells (PAC-MAN) and effectively degrades RNA from SARS-CoV-2 and influenza A virus in human lung epithelial cells. Bioinformatic analysis showed that a group of only six CRISPR RNAs can target more than 90% of all coronaviruses.

5.3.4. Concerns of CRISPR-Cas in Gene Therapy

Serious safety concerns about the use of CRISPR-Cas have been raised after the development of therapeutic genome editing applications [217]. CRISPR-Cas technology has enabled efficient genome editing and modifications in several model organisms, and has successfully been applied in biomedicine and biomedical engineering [218]. Much attention has also been focused on the development of potential CRISPR-Cas therapies to cure complex heritable diseases in humans [219]. However, major challenges related to the effectiveness, specificity, and safety of the CRISPR-Cas system remain. Notably, safety guidelines for preclinical trial studies and ethical issues concerning genome editing and genomic analysis at the population level remain unsettled.

CONCLUSION

Gene therapy has evolved and improved over the past five decades. Methods that were initially non-specific and

non-targeting have become specific and precise with the ability to target genes, which need to be corrected, in order to eliminate the disease. Correction can be an addition or a simple edit to a small stretch of a gene. Genetic delivery has been achieved at the cellular level and organismal level. CRISPR systems have gathered the main attention of gene therapy research as the system is viewed as an accurate and efficient technique with the potential to challenge all diseases. Gene delivery and genome editing will continue to develop in the twenty-first century, with advances in delivery efficiency and targeting specificity, though progress may be gradual due to necessary safety precautions. We have learned from past mistakes, where safety was not fully considered, and have faced morbidity and mortality among patients. Certain methods are likely to become more effective and safer for disease management, but other methods will still remain competitive due to ease of preparation and use, low cost, simplicity, and will still contribute to fundamental scientific endeavors in cell and molecular biology. As these techniques continue to develop, more applications will arise, treatments will be further tailored to individuals, and diseases with more complex genotypes will be able to be treated. As genome therapy becomes safer and highly efficient, we will be faced with more cases of modified genes in humans and the questioning line between disease treatment and playing God with our genetic code.

LIST OF ABBREVIATIONS

AAV	= Adeno-associated Virus
ANTP	= Antennapedia
AV	= Adenovirus
Cas	= CRISPR-associated Protein
cDNA	= Complementary DNA
CPPs	= Cell-penetrating Peptides
CPT	= Covalent Protein Transduction
CRISPR	= Clustered Regularly Interspaced Short Palindromic Repeats
DSB	= Double Strand Break
FDA	= Food and Drug Administration
HDR	= Homology-directed Repair
HIV	= Human Immunodeficiency Virus
LTR	= Long Terminal Repeat
LV	= Lentivirus
MLV	= Murine Leukemia Virus
NHEJ	= Non-homologous End Joining
NPT	= Noncovalent Protein Transduction
SARS-CoV-2	= Severe Acute Respiratory Syndrome Coronavirus 2

sgRNA	= Single-guide RNA
SIN	= Self-Inactivating
siRNA	= Small Interfering RNA
TALE	= Transcription Activator-like Effector
TALENs	= Transcription Activator-like Effector Nucleases
ZFNs	= Zinc Finger Nucleases

AUTHORS' CONTRIBUTIONS

Writing of original draft, N.J.H., H-J.L., Y-W.H.; writing, review and editing, N.J.H., H-J.L., Y-W.H.; writing, technical editing and formatting, N.J.H.; figures design and production, N.J.H. All authors have read and agreed to the published version of this manuscript.

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REFERENCES

- [1] Yin H, Kauffman KJ, Anderson DG. Delivery technologies for genome editing. *Nat Rev Drug Discov* 2017; 16(6): 387-99. <http://dx.doi.org/10.1038/nrd.2016.280> PMID: 28337020
- [2] Burney TJ, Davies JC. Gene therapy for the treatment of cystic fibrosis. *Appl Clin Genet* 2012; 5: 29-36. PMID: 23776378
- [3] Hoban MD, Orkin SH, Bauer DE. Genetic treatment of a molecular disorder: gene therapy approaches to sickle cell disease. *Blood* 2016; 127(7): 839-48. <http://dx.doi.org/10.1182/blood-2015-09-618587> PMID: 26758916
- [4] Dickey AS, La Spada AR. Therapy development in Huntington disease: From current strategies to emerging opportunities. *Am J Med Genet A* 2018; 176(4): 842-61. <http://dx.doi.org/10.1002/ajmg.a.38494> PMID: 29218782
- [5] van Gaal EV, Hennink WE, Crommelin DJ, Mastrobattista E. Plasmid engineering for controlled and sustained gene expression for nonviral gene therapy. *Pharm Res* 2006; 23(6): 1053-74. <http://dx.doi.org/10.1007/s11095-006-0164-2> PMID: 16715361
- [6] Giacca M, Zacchigna S. Virus-mediated gene delivery for human gene therapy. *J Control Release* 2012; 161(2): 377-88. <http://dx.doi.org/10.1016/j.jconrel.2012.04.008> PMID: 22516095
- [7] Laham-Karam N, Pinto GP, Poso A, Kokkonen P. Transcription and translation inhibitors in cancer treatment. *Front Chem* 2020; 8: 276. <http://dx.doi.org/10.3389/fchem.2020.00276> PMID: 32373584
- [8] Bennett CF, Krainer AR, Cleveland DW. Antisense oligonucleotide therapies for neurodegenerative diseases. *Annu Rev Neurosci* 2019; 42: 385-406. <http://dx.doi.org/10.1146/annurev-neuro-070918-050501> PMID: 31283897

- [9] Laina A, Gatsiou A, Georgiopoulos G, Stamatelopoulos K, Stellos K. RNA therapeutics in cardiovascular precision medicine. *Front Physiol* 2018; 9: 953. <http://dx.doi.org/10.3389/fphys.2018.00953> PMID: 30090066
- [10] Aiuti A, Biasco L, Scaramuzza S, et al. Lentiviral hematopoietic stem cell gene therapy in patients with Wiskott-Aldrich syndrome. *Science* 2013; 341(6148): 1233151. <http://dx.doi.org/10.1126/science.1233151> PMID: 23845947
- [11] Aiuti A, Cassani B, Andolfi G, et al. Multilineage hematopoietic reconstitution without clonal selection in ADA-SCID patients treated with stem cell gene therapy. *J Clin Invest* 2007; 117(8): 2233-40. <http://dx.doi.org/10.1172/JCI131666> PMID: 17671653
- [12] Cartier N, Hacein-Bey-Abina S, Bartholomae CC, et al. Hematopoietic stem cell gene therapy with a lentiviral vector in X-linked adrenoleukodystrophy. *Science* 2009; 326(5954): 818-23. <http://dx.doi.org/10.1126/science.1171242> PMID: 19892975
- [13] Cavazzana-Calvo M, Payen E, Negre O, et al. Transfusion independence and HMGA2 activation after gene therapy of human β -thalassaemia. *Nature* 2010; 467(7313): 318-22. <http://dx.doi.org/10.1038/nature09328> PMID: 20844535
- [14] Stolberg SG. The biotech death of Jesse Gelsinger. *The Best of the Best American Science Writing*. New York, NY: HarperCollins Publishers 2010; pp. 30-44.
- [15] Gene-therapy trials must proceed with caution. *Nature* 2016; 534(7609): 590. <http://dx.doi.org/10.1038/534590a> PMID: 27357758
- [16] Lodish H, Berk A, Matsudaira P, et al. Transport of ions and small molecules across cell membranes. *Molecular cell biology*. (5th ed.). New York, NY: W. H. Freeman and Company 2003; pp. 245-300.
- [17] Lehto T, Ezzat K, Wood MJA, El Andaloussi S. Peptides for nucleic acid delivery. *Adv Drug Deliv Rev* 2016; 106(A): 172-82. <http://dx.doi.org/10.1016/j.addr.2016.06.008>
- [18] Al-Dosari MS, Gao X. Nonviral gene delivery: principle, limitations, and recent progress. *AAPS J* 2009; 11(4): 671-81. <http://dx.doi.org/10.1208/s12248-009-9143-y> PMID: 19834816
- [19] Kulkarni JA, Myhre JL, Chen S, et al. Design of lipid nanoparticles for *in vitro* and *in vivo* delivery of plasmid DNA. *Nanomedicine (Lond)* 2017; 13(4): 1377-87. <http://dx.doi.org/10.1016/j.nano.2016.12.014> PMID: 28038954
- [20] Genetics Home Reference. What are genome editing and CRISPR-Cas9? 2020. Available from: <https://ghr.nlm.nih.gov/primer/genomeresearch/genomeediting>
- [21] Ginn SL, Amaya AK, Alexander IE, Edelstein M, Abedi MR. Gene therapy clinical trials worldwide to 2017: An update. *J Gene Med* 2018; 20(5): e3015. <http://dx.doi.org/10.1002/jgm.3015> PMID: 29575374
- [22] Luo D, Saltzman WM. Synthetic DNA delivery systems. *Nat Biotechnol* 2000; 18(1): 33-7. <http://dx.doi.org/10.1038/71889> PMID: 10625387
- [23] Jinturkar KA, Rathi MN, Misra A. Gene delivery using physical methods. *Challenges in delivery of therapeutic genomics and proteomics*. (1st ed.). Elsevier Inc. 2011; pp. 83-126. <http://dx.doi.org/10.1016/B978-0-12-384964-9.00003-7>
- [24] Kaestner L, Scholz A, Lipp P. Conceptual and technical aspects of transfection and gene delivery. *Bioorg Med Chem Lett* 2015; 25(6): 1171-6. <http://dx.doi.org/10.1016/j.bmcl.2015.01.018> PMID: 25677659
- [25] Korzh V, Strähle U. Marshall Barber and the century of microinjection: from cloning of bacteria to cloning of everything. *Differentiation* 2002; 70(6): 221-6. <http://dx.doi.org/10.1046/j.1432-0436.2002.700601.x> PMID: 12190984
- [26] Di Berardino MA, McKinnell RG, Wolf DP. The golden anniversary of cloning: a celebratory essay. *Differentiation* 2003; 71(7): 398-401. <http://dx.doi.org/10.1046/j.1432-0436.2003.7107002.x> PMID: 12969332
- [27] Tonelli FMP, Lacerda SMSN, Tonelli FCP, Costa GMJ, de França LR, Resende RR. Progress and biotechnological prospects in fish transgenesis. *Biotechnol Adv* 2017; 35(6): 832-44. <http://dx.doi.org/10.1016/j.biotechadv.2017.06.002> PMID: 28602961
- [28] Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KH. Viable offspring derived from fetal and adult mammalian cells. *Nature* 1997; 385(6619): 810-3. <http://dx.doi.org/10.1038/385810a0> PMID: 9039911
- [29] Chow YT, Chen S, Wang R, et al. Single cell transfection through precise microinjection with quantitatively controlled injection volumes. *Sci Rep* 2016; 6: 24127. <http://dx.doi.org/10.1038/srep24127> PMID: 27067121
- [30] Capecchi MR. High efficiency transformation by direct microinjection of DNA into cultured mammalian cells. *Cell* 1980; 22(2 Pt 2): 479-88. [http://dx.doi.org/10.1016/0092-8674\(80\)90358-X](http://dx.doi.org/10.1016/0092-8674(80)90358-X) PMID: 6256082
- [31] Dean DA. Microinjection. In: Maloy S, Hughes K, Eds. *Brenner's encyclopedia of genetics*. (2nd ed.). Cambridge, MA: Academic Press 2013; pp. 409-10. <http://dx.doi.org/10.1016/B978-0-12-374984-0.00945-1>
- [32] Donnelly RF, Raj Singh TR, Woolfson AD. Microneedle-based drug delivery systems: microfabrication, drug delivery, and safety. *Drug Deliv* 2010; 17(4): 187-207. <http://dx.doi.org/10.3109/10717541003667798> PMID: 20297904
- [33] Ramamoorth M, Narvekar A. Non viral vectors in gene therapy-an overview. *J Clin Diagn Res* 2015; 9(1): GE01-6. <http://dx.doi.org/10.7860/JCDR/2015/10443.5394> PMID: 25738007
- [34] Klein TM, Wolf ED, Wu R, Sanford JC. High-velocity microprojectiles for delivering nucleic acids into living cells. *Nature* 1987; 327: 70-3. <http://dx.doi.org/10.1038/327070a0>
- [35] Davidson JM, Krieg T, Eming SA. Particle-mediated gene therapy of wounds. *Wound Repair Regen* 2000; 8(6): 452-9. <http://dx.doi.org/10.1046/j.1524-475x.2000.00452.x> PMID: 11208172
- [36] Finer JJ, Finer KR, Ponappa T. Particle bombardment mediated transformation. *Curr Top Microbiol Immunol* 1999; 240: 59-80. http://dx.doi.org/10.1007/978-3-642-60234-4_3 PMID: 10394715
- [37] Wagner DE, Bhaduri SB. Progress and outlook of inorganic nanoparticles for delivery of nucleic acid sequences related to orthopedic pathologies: a review. *Tissue Eng Part B Rev* 2012; 18(1): 1-14. <http://dx.doi.org/10.1089/ten.teb.2011.0081> PMID: 21707439
- [38] Xia J, Martinez A, Daniell H, Ebert SN. Evaluation of biolistic gene transfer methods *in vivo* using non-invasive bioluminescent imaging techniques. *BMC Biotechnol* 2011; 11: 62. <http://dx.doi.org/10.1186/1472-6750-11-62> PMID: 21635760
- [39] Suda T, Liu D. Hydrodynamic gene delivery: its principles and applications. *Mol Ther* 2007; 15(12): 2063-9. <http://dx.doi.org/10.1038/sj.mt.6300314> PMID: 17912237
- [40] Budker V, Zhang G, Danko I, Williams P, Wolff J. The efficient expression of intravascularly delivered DNA in rat muscle. *Gene Ther* 1998; 5(2): 272-6. <http://dx.doi.org/10.1038/sj.gt.3300572> PMID: 9578848
- [41] Liu F, Song Y, Liu D. Hydrodynamics-based transfection in animals by systemic administration of plasmid DNA. *Gene Ther* 1999; 6(7): 1258-66. <http://dx.doi.org/10.1038/sj.gt.3300947> PMID: 10455434
- [42] Bonamassa B, Hai L, Liu D. Hydrodynamic gene delivery and its applications in pharmaceutical research. *Pharm Res* 2011; 28(4): 694-701. <http://dx.doi.org/10.1007/s11095-010-0338-9> PMID: 21191634
- [43] Yang PL, Althage A, Chung J, Chisari FV. Hydrodynamic injection of viral DNA: a mouse model of acute hepatitis B virus infection. *Proc Natl Acad Sci USA* 2002; 99(21): 13825-30. <http://dx.doi.org/10.1073/pnas.202398599> PMID: 12374864
- [44] Kamimura K, Yokoo T, Abe H, et al. Image-guided hydrodynamic gene delivery: current status and future directions. *Pharmaceutics* 2015; 7(3): 213-23. <http://dx.doi.org/10.3390/pharmaceutics7030213> PMID: 26308044
- [45] Tomizawa M, Shinozaki F, Motoyoshi Y, Sugiyama T, Yamamoto S, Sueishi M. Sonoporation: Gene transfer using ultrasound. *World J Methodol* 2013; 3(4): 39-44. <http://dx.doi.org/10.5662/wjm.v3.i4.39> PMID: 25237622

- [46] Fechheimer M, Boylan JF, Parker S, Sissen JE, Patel GL, Zimmer SG. Transfection of mammalian cells with plasmid DNA by scrape loading and sonication loading. *Proc Natl Acad Sci USA* 1987; 84(23): 8463-7.
<http://dx.doi.org/10.1073/pnas.84.23.8463> PMID: 2446324
- [47] Nelson TR, Fowlkes JB, Abramowicz JS, Church CC. Ultrasound biosafety considerations for the practicing sonographer and sonologist. *J Ultrasound Med* 2009; 28(2): 139-50.
<http://dx.doi.org/10.7863/jum.2009.28.2.139> PMID: 19168764
- [48] Miller DL, Pislaru SV, Greenleaf JE. Sonoporation: mechanical DNA delivery by ultrasonic cavitation. *Somat Cell Mol Genet* 2002; 27(1-6): 115-34.
<http://dx.doi.org/10.1023/A:1022983907223> PMID: 12774945
- [49] Shapiro G, Wong AW, Bez M, *et al.* Multiparameter evaluation of *in vivo* gene delivery using ultrasound-guided, microbubble-enhanced sonoporation. *J Control Release* 2016; 223: 157-64.
<http://dx.doi.org/10.1016/j.jconrel.2015.12.001> PMID: 26682505
- [50] Kim HJ, Greenleaf JF, Kinnick RR, Bronk JT, Bolander ME. Ultrasound-mediated transfection of mammalian cells. *Hum Gene Ther* 1996; 7(11): 1339-46.
<http://dx.doi.org/10.1089/hum.1996.7.11-1339> PMID: 8818721
- [51] Luft C, Ketteler R. Electroporation knows no boundaries: the use of electrostimulation for siRNA delivery in cells and tissues. *J Biomed Screen* 2015; 20(8): 932-42.
<http://dx.doi.org/10.1177/1087057115579638> PMID: 25851034
- [52] Dean DA, Gasiorowski JZ. Nonviral gene delivery. *Cold Spring Harb Protoc* 2011; 2011(3): top101.
<http://dx.doi.org/10.1101/pdb.top101> PMID: 21363958
- [53] Neumann E, Schaefer-Ridder M, Wang Y, Hofschneider PH. Gene transfer into mouse lymphoma cells by electroporation in high electric fields. *EMBO J* 1982; 1(7): 841-5.
<http://dx.doi.org/10.1002/j.1460-2075.1982.tb01257.x> PMID: 6329708
- [54] Titomirov AV, Sukharev S, Kistanova E. *In vivo* electroporation and stable transformation of skin cells of newborn mice by plasmid DNA. *Biochim Biophys Acta* 1991; 1088(1): 131-4.
[http://dx.doi.org/10.1016/0167-4781\(91\)90162-F](http://dx.doi.org/10.1016/0167-4781(91)90162-F) PMID: 1703441
- [55] Heller R, Jaroszeski M, Atkin A, *et al.* *In vivo* gene electroinjection and expression in rat liver. *FEBS Lett* 1996; 389(3): 225-8.
[http://dx.doi.org/10.1016/0014-5793\(96\)00590-X](http://dx.doi.org/10.1016/0014-5793(96)00590-X) PMID: 8766704
- [56] Heller LC, Jaroszeski MJ, Coppola D, McCray AN, Hickey J, Heller R. Optimization of cutaneous electrically mediated plasmid DNA delivery using novel electrode. *Gene Ther* 2007; 14(3): 275-80.
<http://dx.doi.org/10.1038/sj.gt.3302867> PMID: 16988718
- [57] Graham FL, van der Eb AJ. A new technique for the assay of infectivity of human adenovirus 5 DNA. *Virology* 1973; 52(2): 456-67.
[http://dx.doi.org/10.1016/0042-6822\(73\)90341-3](http://dx.doi.org/10.1016/0042-6822(73)90341-3) PMID: 4705382
- [58] Roy I, Mitra S, Maitra A, Mozumdar S. Calcium phosphate nanoparticles as novel non-viral vectors for targeted gene delivery. *Int J Pharm* 2003; 250(1): 25-33.
[http://dx.doi.org/10.1016/S0378-5173\(02\)00452-0](http://dx.doi.org/10.1016/S0378-5173(02)00452-0) PMID: 12480270
- [59] Sokolova V, Epple M. Inorganic nanoparticles as carriers of nucleic acids into cells. *Angew Chem Int Ed Engl* 2008; 47(8): 1382-95.
<http://dx.doi.org/10.1002/anie.200703039> PMID: 18098258
- [60] Kuo IY, Ehrlich BE. Signaling in muscle contraction. *Cold Spring Harb Perspect Biol* 2015; 7(2): a006023.
<http://dx.doi.org/10.1101/cshperspect.a006023> PMID: 25646377
- [61] Xie Y, Chen Y, Sun M, Ping Q. A mini review of biodegradable calcium phosphate nanoparticles for gene delivery. *Curr Pharm Biotechnol* 2013; 14(10): 918-25.
<http://dx.doi.org/10.2174/1389201014666131226145441> PMID: 24372244
- [62] Chowdhury EH, Sasagawa T, Nagaoka M, Kundu AK, Akaike T. Transfecting mammalian cells by DNA/calcium phosphate precipitates: effect of temperature and pH on precipitation. *Anal Biochem* 2003; 314(2): 316-8.
[http://dx.doi.org/10.1016/S0003-2697\(02\)00648-6](http://dx.doi.org/10.1016/S0003-2697(02)00648-6) PMID: 12654319
- [63] Jordan M, Schallhorn A, Wurm FM. Transfecting mammalian cells: optimization of critical parameters affecting calcium-phosphate precipitate formation. *Nucleic Acids Res* 1996; 24(4): 596-601.
<http://dx.doi.org/10.1093/nar/24.4.596> PMID: 8604299
- [64] Jiang M, Deng L, Chen G. High Ca²⁺-phosphate transfection efficiency enables single neuron gene analysis. *Gene Ther* 2004; 11(17): 1303-11.
<http://dx.doi.org/10.1038/sj.gt.3302305> PMID: 15229630
- [65] Manjila SB, Baby JN, Bijin EN, Constantine I, Pramod K, Val-salakumari J. Novel gene delivery systems. *Int J Pharm Investig* 2013; 3(1): 1-7.
<http://dx.doi.org/10.4103/2230-973X.108958> PMID: 23799200
- [66] Sandoval-Yañez C, Castro Rodríguez C. Dendrimers: amazing platforms for bioactive molecule delivery systems. *Materials (Basel)* 2020; 13(3): E570.
<http://dx.doi.org/10.3390/ma13030570> PMID: 31991703
- [67] Abbasi E, Aval SF, Akbarzadeh A, *et al.* Dendrimers: synthesis, applications, and properties. *Nanoscale Res Lett* 2014; 9(1): 247.
<http://dx.doi.org/10.1186/1556-276X-9-247> PMID: 24994950
- [68] Abedi-Gaballu F, Dehghan G, Ghaffari M, *et al.* PAMAM dendrimers as efficient drug and gene delivery nanosystems for cancer therapy. *Appl Mater Today* 2018; 12: 177-90.
<http://dx.doi.org/10.1016/j.apmt.2018.05.002> PMID: 30511014
- [69] Duřeć C, Uchegbu IF, Schätzlein AG. Dendrimers in gene delivery. *Adv Drug Deliv Rev* 2005; 57(15): 2177-202.
<http://dx.doi.org/10.1016/j.addr.2005.09.017> PMID: 16310284
- [70] Haensler J, Szoka FC Jr. Polyamidoamine cascade polymers mediate efficient transfection of cells in culture. *Bioconjug Chem* 1993; 4(5): 372-9.
<http://dx.doi.org/10.1021/bc00023a012> PMID: 8274523
- [71] Shakhbazov A, Isayenka I, Kartel N, *et al.* Transfection efficiencies of PAMAM dendrimers correlate inversely with their hydrophobicity. *Int J Pharm* 2010; 383(1-2): 228-35.
<http://dx.doi.org/10.1016/j.ijpharm.2009.09.020> PMID: 19770028
- [72] Kukowska-Latallo JF, Bielinska AU, Johnson J, Spindler R, Tomalia DA, Baker JR Jr. Efficient transfer of genetic material into mammalian cells using Starburst polyamidoamine dendrimers. *Proc Natl Acad Sci USA* 1996; 93(10): 4897-902.
<http://dx.doi.org/10.1073/pnas.93.10.4897> PMID: 8643500
- [73] Madaan K, Kumar S, Poonia N, Lather V, Pandita D. Dendrimers in drug delivery and targeting: Drug-dendrimer interactions and toxicity issues. *J Pharm Bioallied Sci* 2014; 6(3): 139-50.
<http://dx.doi.org/10.4103/0975-7406.130965> PMID: 25035633
- [74] Pei D, Buyanova M. Overcoming Endosomal Entrapment in Drug Delivery. *Bioconjug Chem* 2019; 30(2): 273-83.
<http://dx.doi.org/10.1021/acs.bioconjchem.8b00778> PMID: 30525488
- [75] Kukowska-Latallo JF, Raczka E, Quintana A, Chen C, Rymaszewski M, Baker JR Jr. Intravascular and endobronchial DNA delivery to murine lung tissue using a novel, nonviral vector. *Hum Gene Ther* 2000; 11(10): 1385-95.
<http://dx.doi.org/10.1089/10430340050057468> PMID: 10910136
- [76] Duncan R, Izzo L. Dendrimer biocompatibility and toxicity. *Adv Drug Deliv Rev* 2005; 57(15): 2215-37.
<http://dx.doi.org/10.1016/j.addr.2005.09.019> PMID: 16297497
- [77] Felgner PL, Gadek TR, Holm M, *et al.* Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc Natl Acad Sci USA* 1987; 84(21): 7413-7.
<http://dx.doi.org/10.1073/pnas.84.21.7413> PMID: 2823261
- [78] Wasungu L, Hoekstra D. Cationic lipids, lipoplexes and intracellular delivery of genes. *J Control Release* 2006; 116(2): 255-64.
<http://dx.doi.org/10.1016/j.jconrel.2006.06.024> PMID: 16914222
- [79] Carter M, Shieh J. Gene delivery strategies. In: Farra N, Ed. *Guide to research techniques in neuroscience*. (2nd ed.). Cambridge, MA: Academic Press 2015; pp. 239-52.
<http://dx.doi.org/10.1016/B978-0-12-800511-8.00011-3>
- [80] Zhao Y, Huang L. Lipid nanoparticles for gene delivery. *Adv Genet* 2014; 88: 13-36.
<http://dx.doi.org/10.1016/B978-0-12-800148-6.00002-X> PMID: 25409602
- [81] Ross PC, Hui SW. Lipoplex size is a major determinant of *in vitro* lipofection efficiency. *Gene Ther* 1999; 6(4): 651-9.
<http://dx.doi.org/10.1038/sj.gt.3300863> PMID: 10476225

- [82] Dalby B, Cates S, Harris A, *et al.* Advanced transfection with Lipofectamine 2000 reagent: primary neurons, siRNA, and high-throughput applications. *Methods* 2004; 33(2): 95-103. <http://dx.doi.org/10.1016/j.ymeth.2003.11.023> PMID: 15121163
- [83] Khatri N, Baradia D, Vhora I, Rathi M, Misra A. Development and characterization of siRNA lipoplexes: Effect of different lipids, *in vitro* evaluation in cancerous cell lines and *in vivo* toxicity study. *AAPS PharmSciTech* 2014; 15(6): 1630-43. <http://dx.doi.org/10.1208/s12249-014-0193-9> PMID: 25145330
- [84] McClorey G, Banerjee S. Cell-penetrating peptides to enhance delivery of oligonucleotide-based therapeutics. *Biomedicines* 2018; 6(2): E51. <http://dx.doi.org/10.3390/biomedicines6020051> PMID: 29734750
- [85] Gagat M, Zielińska W, Grzanka A. Cell-penetrating peptides and their utility in genome function modifications (Review). *Int J Mol Med* 2017; 40(6): 1615-23. <http://dx.doi.org/10.3892/ijmm.2017.3172> PMID: 29039455
- [86] Marqus S, Pirogova E, Piva TJ. Evaluation of the use of therapeutic peptides for cancer treatment. *J Biomed Sci* 2017; 24(1): 21. <http://dx.doi.org/10.1186/s12929-017-0328-x> PMID: 28320393
- [87] Liu BR, Huang YW, Aronstam RS, Lee HJ. Identification of a short cell-penetrating peptide from bovine lactoferricin for intracellular delivery of DNA in human A549 Cells. *PLoS One* 2016; 11(3): e0150439. <http://dx.doi.org/10.1371/journal.pone.0150439> PMID: 26942714
- [88] Chang M, Huang YW, Aronstam RS, Lee HJ. Cellular delivery of noncovalently-associated macromolecules by cell-penetrating peptides. *Curr Pharm Biotechnol* 2014; 15(3): 267-75. <http://dx.doi.org/10.2174/1389201015666140617095415> PMID: 24938892
- [89] Huang YW, Lee HJ, Tolliver LM, Aronstam RS. Delivery of nucleic acids and nanomaterials by cell-penetrating peptides: opportunities and challenges. *BioMed Res Int* 2015; 2015: 834079. <http://dx.doi.org/10.1155/2015/834079> PMID: 25883975
- [90] Schwarze SR, Dowdy SF. *In vivo* protein transduction: intracellular delivery of biologically active proteins, compounds and DNA. *Trends Pharmacol Sci* 2000; 21(2): 45-8. [http://dx.doi.org/10.1016/S0165-6147\(99\)01429-7](http://dx.doi.org/10.1016/S0165-6147(99)01429-7) PMID: 10664605
- [91] Heitz F, Morris MC, Divita G. Twenty years of cell-penetrating peptides: from molecular mechanisms to therapeutics. *Br J Pharmacol* 2009; 157(2): 195-206. <http://dx.doi.org/10.1111/j.1476-5381.2009.00057.x> PMID: 19309362
- [92] Agrawal P, Bhalla S, Usmani SS, *et al.* CPPsite 2.0: a repository of experimentally validated cell-penetrating peptides. *Nucleic Acids Res* 2016; 44(D1): D1098-103. <http://dx.doi.org/10.1093/nar/gkv1266> PMID: 26586798
- [93] Holton TA, Pollastri G, Shields DC, Mooney C. CPPpred: prediction of cell penetrating peptides. *Bioinformatics* 2013; 29(23): 3094-6. <http://dx.doi.org/10.1093/bioinformatics/btt518> PMID: 24064418
- [94] Pandey P, Patel V, George NV, Mallajosyula SS. KELM-CPPpred: kernel extreme learning machine based prediction model for cell-penetrating peptides. *J Proteome Res* 2018; 17(9): 3214-22. <http://dx.doi.org/10.1021/acs.jproteome.8b00322> PMID: 30032609
- [95] Gautam A, Chaudhary K, Kumar R, *et al.* Open source drug discovery consortium. *In silico* approaches for designing highly effective cell penetrating peptides. *J Transl Med* 2013; 11: 74. <http://dx.doi.org/10.1186/1479-5876-11-74> PMID: 23517638
- [96] Tang H, Su ZD, Wei HH, Chen W, Lin H. Prediction of cell-penetrating peptides with feature selection techniques. *Biochem Biophys Res Commun* 2016; 477(1): 150-4. <http://dx.doi.org/10.1016/j.bbrc.2016.06.035> PMID: 27291150
- [97] Wei L, Xing P, Su R, Shi G, Ma ZS, Zou Q. CPPred-RF: a sequence-based predictor for identifying cell-penetrating peptides and their uptake efficiency. *J Proteome Res* 2017; 16(5): 2044-53. <http://dx.doi.org/10.1021/acs.jproteome.7b00019> PMID: 28436664
- [98] Wei L, Tang J, Zou Q. SkipCPP-Pred: an improved and promising sequence-based predictor for predicting cell-penetrating peptides. *BMC Genomics* 2017; 18 (Suppl. 7): 742. <http://dx.doi.org/10.1186/s12864-017-4128-1> PMID: 29513192
- [99] Manavalan B, Subramaniam S, Shin TH, Kim MO, Lee G. Machine-learning-based prediction of cell-penetrating peptides and their uptake efficiency with improved accuracy. *J Proteome Res* 2018; 17(8): 2715-26. <http://dx.doi.org/10.1021/acs.jproteome.8b00148> PMID: 29893128
- [100] Qiang X, Zhou C, Ye X, Du PF, Su R, Wei L. CPPred-FL: a sequence-based predictor for large-scale identification of cell-penetrating peptides by feature representation learning. *Brief Bioinform* 2018. <http://dx.doi.org/10.1093/bib/bby091> PMID: 30239616
- [101] Wang S, Cao Z, Li M, Yue Y. G-DipC: an improved feature representation method for short sequences to predict the type of cargo in cell-penetrating peptides. *IEEE/ACM Trans Comput Biol Bioinform* 2020; 17(3): 739-47.
- [102] Fu X, Cai L, Zeng X, Zou Q. StackCPPred: a stacking and pairwise energy content-based prediction of cell-penetrating peptides and their uptake efficiency. *Bioinformatics* 2020; 36(10): 3028-34. <http://dx.doi.org/10.1093/bioinformatics/btaa131> PMID: 32105326
- [103] Arif M, Ahmad S, Ali F, Fang G, Li M, Yu DJ. TargetCPP: accurate prediction of cell-penetrating peptides from optimized multi-scale features using gradient boost decision tree. *J Comput Aided Mol Des* 2020; 34(8): 841-56. <http://dx.doi.org/10.1007/s10822-020-00307-z> PMID: 32180124
- [104] Zhu P, Jin L. Cell penetrating peptides: a promising tool for the cellular uptake of macromolecular drugs. *Curr Protein Pept Sci* 2018; 19(2): 211-20. PMID: 28699510
- [105] Conner SD, Schmid SL. Regulated portals of entry into the cell. *Nature* 2003; 422(6927): 37-44. <http://dx.doi.org/10.1038/nature01451> PMID: 12621426
- [106] Allinquant B, Hantraye P, Mailloux P, Moya K, Bouillot C, Prochiantz A. Downregulation of amyloid precursor protein inhibits neurite outgrowth *in vitro*. *J Cell Biol* 1995; 128(5): 919-27. <http://dx.doi.org/10.1083/jcb.128.5.919> PMID: 7876315
- [107] Morris MC, Vidal P, Chaloin L, Heitz F, Divita G. A new peptide vector for efficient delivery of oligonucleotides into mammalian cells. *Nucleic Acids Res* 1997; 25(14): 2730-6. <http://dx.doi.org/10.1093/nar/25.14.2730> PMID: 9207018
- [108] Kardani K, Hashemi A, Bolhassani A. Comparison of HIV-1 Vif and Vpu accessory proteins for delivery of polyepitope constructs harboring Nef, Gp160 and P24 using various cell penetrating peptides. *PLoS One* 2019; 14(10): e0223844. <http://dx.doi.org/10.1371/journal.pone.0223844> PMID: 31671105
- [109] Borrelli A, Tornesello AL, Tornesello ML, Buonaguro FM. Cell penetrating peptides as molecular carriers for anti-cancer agents. *Molecules* 2018; 23(2): E295. <http://dx.doi.org/10.3390/molecules23020295> PMID: 29385037
- [110] Rádis-Baptista G, Campelo IS, Morlighem JRL, Melo LM, Freitas VJF. Cell-penetrating peptides (CPPs): From delivery of nucleic acids and antigens to transduction of engineered nucleases for application in transgenesis. *J Biotechnol* 2017; 252: 15-26. <http://dx.doi.org/10.1016/j.jbiotec.2017.05.002> PMID: 28479163
- [111] Park K. *in vivo* DNA delivery with NickFect peptide vectors. *J Control Release* 2016; 241: 242. <http://dx.doi.org/10.1016/j.jconrel.2016.10.005> PMID: 27751254
- [112] Bender E. Gene therapy: Industrial strength. *Nature* 2016; 537(7619): S57-9. <http://dx.doi.org/10.1038/537S57a> PMID: 27602741
- [113] Glogau R, Blitzer A, Brandt F, Kane M, Monheit GD, Waugh JM. Results of a randomized, double-blind, placebo-controlled study to evaluate the efficacy and safety of a botulinum toxin type A topical gel for the treatment of moderate-to-severe lateral canthal lines. *J Drugs Dermatol* 2012; 11(1): 38-45. PMID: 22206075
- [114] Lönn P, Dowdy SF. Cationic PTD/CPP-mediated macromolecular delivery: charging into the cell. *Expert Opin Drug Deliv* 2015; 12(10): 1627-36. <http://dx.doi.org/10.1517/17425247.2015.1046431> PMID: 25994800

- [115] Guidotti G, Brambilla L, Rossi D. Cell-penetrating peptides: from basic research to clinics. *Trends Pharmacol Sci* 2017; 38(4): 406-24.
<http://dx.doi.org/10.1016/j.tips.2017.01.003> PMID: 28209404
- [116] Lostalé-Seijo I, Louzao I, Juanes M, Montenegro J. Peptide/Cas9 nanostructures for ribonucleoprotein cell membrane transport and gene edition. *Chem Sci (Camb)* 2017; 8(12): 7923-31.
<http://dx.doi.org/10.1039/C7SC03918B> PMID: 29619166
- [117] Huang YW, Lee HJ. Cell-penetrating peptides for medical therapeutics and targeted gene delivery. In: Koutsopoulos S, Ed. *Peptide applications in biomedicine, biotechnology and bioengineering*. Oxford, UK: Woodhead Publishing 2018; pp. 359-70.
<http://dx.doi.org/10.1016/B978-0-08-100736-5.00013-2>
- [118] Ramakrishna S, Kwaku Dad AB, Beloor J, Gopalappa R, Lee SK, Kim H. Gene disruption by cell-penetrating peptide-mediated delivery of Cas9 protein and guide RNA. *Genome Res* 2014; 24(6): 1020-7.
<http://dx.doi.org/10.1101/gr.171264.113> PMID: 24696462
- [119] Oude Blenke E, Evers MJ, Mastrobattista E, van der Oost J. CRISPR-Cas9 gene editing: delivery aspects and therapeutic potential. *J Control Release* 2016; 244(B): 139-48.
- [120] He Y, Li F, Huang Y. Smart cell-penetrating peptide-based techniques for intracellular delivery of therapeutic macromolecules. *Adv Protein Chem Struct Biol* 2018; 112: 183-220.
<http://dx.doi.org/10.1016/bs.apcsb.2018.01.004> PMID: 29680237
- [121] Khrustalev VV, Khrustaleva TA, Stojarov AN, Sharma N, Bhaskar B, Giri R. The history of mutational pressure changes during the evolution of adeno-associated viruses: A message to gene therapy and DNA-vaccine vectors designers. *Infect Genet Evol* 2020; 77: 104100.
<http://dx.doi.org/10.1016/j.meegid.2019.104100> PMID: 31678645
- [122] Goswami R, Subramanian G, Silayeva L, *et al.* Gene therapy leaves a vicious cycle. *Front Oncol* 2019; 9: 297.
<http://dx.doi.org/10.3389/fonc.2019.00297> PMID: 31069169
- [123] Crystal RG. Adenovirus: the first effective *in vivo* gene delivery vector. *Hum Gene Ther* 2014; 25(1): 3-11.
<http://dx.doi.org/10.1089/hum.2013.2527> PMID: 24444179
- [124] Zabner J, Couture LA, Gregory RJ, Graham SM, Smith AE, Welsh MJ. Adenovirus-mediated gene transfer transiently corrects the chloride transport defect in nasal epithelia of patients with cystic fibrosis. *Cell* 1993; 75(2): 207-16.
[http://dx.doi.org/10.1016/0092-8674\(93\)80063-K](http://dx.doi.org/10.1016/0092-8674(93)80063-K) PMID: 7691415
- [125] Gonçalves MA, de Vries AA. Adenovirus: from foe to friend. *Rev Med Virol* 2006; 16(3): 167-86.
<http://dx.doi.org/10.1002/rmv.494> PMID: 16710837
- [126] Chirmule N, Propert K, Magosin S, Qian Y, Qian R, Wilson J. Immune responses to adenovirus and adeno-associated virus in humans. *Gene Ther* 1999; 6(9): 1574-83.
<http://dx.doi.org/10.1038/sj.gt.3300994> PMID: 10490767
- [127] Liu Q, Muruve DA. Molecular basis of the inflammatory response to adenovirus vectors. *Gene Ther* 2003; 10(11): 935-40.
<http://dx.doi.org/10.1038/sj.gt.3302036> PMID: 12756413
- [128] Alba R, Bosch A, Chillón M. Gutless adenovirus: last-generation adenovirus for gene therapy. *Gene Ther* 2005; 12 (Suppl. 1): S18-27.
<http://dx.doi.org/10.1038/sj.gt.3302612> PMID: 16231052
- [129] Yoon SO, Lois C, Alvarez M, Alvarez-Buylla A, Falck-Pedersen E, Chao MV. Adenovirus-mediated gene delivery into neuronal precursors of the adult mouse brain. *Proc Natl Acad Sci USA* 1996; 93(21): 11974-9.
<http://dx.doi.org/10.1073/pnas.93.21.11974> PMID: 8876247
- [130] Lv Y, Xiao FJ, Wang Y, *et al.* Efficient gene transfer into T lymphocytes by fiber-modified human adenovirus 5. *BMC Biotechnol* 2019; 19(1): 23.
<http://dx.doi.org/10.1186/s12896-019-0514-x> PMID: 31014302
- [131] Ramos-Kuri M, Rapti K, Mehel H, *et al.* Dominant negative Ras attenuates pathological ventricular remodeling in pressure overload cardiac hypertrophy. *Biochim Biophys Acta* 2015; 1853 (11 Pt A): 2870-84.
<http://dx.doi.org/10.1016/j.bbamcr.2015.08.006> PMID: 26260012
- [132] Atchison RW, Casto BC, Hammon WM. Adenovirus-associated defective virus particles. *Science* 1965; 149(3685): 754-6.
<http://dx.doi.org/10.1126/science.149.3685.754> PMID: 14325163
- [133] Blacklow NR, Hoggan MD, Rowe WP. Isolation of adenovirus-associated viruses from man. *Proc Natl Acad Sci USA* 1967; 58(4): 1410-5.
<http://dx.doi.org/10.1073/pnas.58.4.1410> PMID: 4295829
- [134] Wang D, Tai PWL, Gao G. Adeno-associated virus vector as a platform for gene therapy delivery. *Nat Rev Drug Discov* 2019; 18(5): 358-78.
<http://dx.doi.org/10.1038/s41573-019-0012-9> PMID: 30710128
- [135] Berns KI, Linden RM. The cryptic life style of adeno-associated virus. *BioEssays* 1995; 17(3): 237-45.
<http://dx.doi.org/10.1002/bies.950170310> PMID: 7748178
- [136] Merten OW, Gaillet B. Viral vectors for gene therapy and gene modification approaches. *Biochem Eng J* 2016; 108: 98-115.
<http://dx.doi.org/10.1016/j.bej.2015.09.005>
- [137] Tse LV, Moller-Tank S, Asokan A. Strategies to circumvent humoral immunity to adeno-associated viral vectors. *Expert Opin Biol Ther* 2015; 15(6): 845-55.
<http://dx.doi.org/10.1517/14712598.2015.1035645> PMID: 25985812
- [138] Liu Y, Kim YJ, Ji M, *et al.* Enhancing gene delivery of adeno-associated viruses by cell-permeable peptides. *Mol Ther Methods Clin Dev* 2014; 1: 12.
<http://dx.doi.org/10.1038/mtm.2013.12> PMID: 26015948
- [139] Wu Z, Asokan A, Samulski RJ. Adeno-associated virus serotypes: vector toolkit for human gene therapy. *Mol Ther* 2006; 14(3): 316-27.
<http://dx.doi.org/10.1016/j.ythme.2006.05.009> PMID: 16824801
- [140] Briggs JA, Simon MN, Gross I, *et al.* The stoichiometry of Gag protein in HIV-1. *Nat Struct Mol Biol* 2004; 11(7): 672-5.
<http://dx.doi.org/10.1038/nsmb785> PMID: 15208690
- [141] Fuller SD, Wilk T, Gowen BE, Kräusslich HG, Vogt VM. Cryo-electron microscopy reveals ordered domains in the immature HIV-1 particle. *Curr Biol* 1997; 7(10): 729-38.
[http://dx.doi.org/10.1016/S0960-9822\(06\)00331-9](http://dx.doi.org/10.1016/S0960-9822(06)00331-9) PMID: 9368755
- [142] Kingston RL, Olson NH, Vogt VM. The organization of mature Rous sarcoma virus as studied by cryoelectron microscopy. *J Struct Biol* 2001; 136(1): 67-80.
<http://dx.doi.org/10.1006/jsbi.2001.4423> PMID: 11858708
- [143] Yeager M, Wilson-Kubalek EM, Weiner SG, Brown PO, Rein A. Supramolecular organization of immature and mature murine leukemia virus revealed by electron cryo-microscopy: implications for retroviral assembly mechanisms. *Proc Natl Acad Sci USA* 1998; 95(13): 7299-304.
<http://dx.doi.org/10.1073/pnas.95.13.7299> PMID: 9636143
- [144] Milone MC, O'Doherty U. Clinical use of lentiviral vectors. *Leukemia* 2018; 32(7): 1529-41.
<http://dx.doi.org/10.1038/s41375-018-0106-0> PMID: 29654266
- [145] Maetzig T, Galla M, Baum C, Schambach A. Gammaretroviral vectors: biology, technology and application. *Viruses* 2011; 3(6): 677-713.
<http://dx.doi.org/10.3390/v3060677> PMID: 21994751
- [146] Zhang W, Cao S, Martin JL, Mueller JD, Mansky LM. Morphology and ultrastructure of retrovirus particles. *AIMS Biophys* 2015; 2(3): 343-69.
<http://dx.doi.org/10.3934/biophys.2015.3.343> PMID: 26448965
- [147] Wirth T, Parker N, Ylä-Herttuala S. History of gene therapy. *Gene* 2013; 525(2): 162-9.
<http://dx.doi.org/10.1016/j.gene.2013.03.137> PMID: 23618815
- [148] Vargas JE, Chicaybam L, Stein RT, Tanuri A, Delgado-Cañedo A, Bonamino MH. Retroviral vectors and transposons for stable gene therapy: advances, current challenges and perspectives. *J Transl Med* 2016; 14(1): 288.
<http://dx.doi.org/10.1186/s12967-016-1047-x> PMID: 27729044
- [149] Howe SJ, Mansour MR, Schwarzwaelder K, *et al.* Insertional mutagenesis combined with acquired somatic mutations causes leukemogenesis following gene therapy of SCID-X1 patients. *J Clin Invest* 2008; 118(9): 3143-50.
<http://dx.doi.org/10.1172/JCI35798> PMID: 18688286
- [150] Hacein-Bey-Abina S, Le Deist F, Carlier F, *et al.* Sustained correction of X-linked severe combined immunodeficiency by *ex vivo* gene therapy. *N Engl J Med* 2002; 346(16): 1185-93.
<http://dx.doi.org/10.1056/NEJMoa012616> PMID: 11961146

- [151] Gaspar HB, Bjorkegren E, Parsley K, *et al.* Successful reconstitution of immunity in ADA-SCID by stem cell gene therapy following cessation of PEG-ADA and use of mild preconditioning. *Mol Ther* 2006; 14(4): 505-13. <http://dx.doi.org/10.1016/j.yymthe.2006.06.007> PMID: 16905365
- [152] Hacein-Bey-Abina S, Garrigue A, Wang GP, *et al.* Insertional oncogenesis in 4 patients after retrovirus-mediated gene therapy of SCID-X1. *J Clin Invest* 2008; 118(9): 3132-42. <http://dx.doi.org/10.1172/JCI35700> PMID: 18688285
- [153] Gaspar HB, Parsley KL, Howe S, *et al.* Gene therapy of X-linked severe combined immunodeficiency by use of a pseudotyped gammaretroviral vector. *Lancet* 2004; 364(9452): 2181-7. [http://dx.doi.org/10.1016/S0140-6736\(04\)17590-9](http://dx.doi.org/10.1016/S0140-6736(04)17590-9) PMID: 15610804
- [154] Mavilio F, Pellegrini G, Ferrari S, *et al.* Correction of junctional epidermolysis bullosa by transplantation of genetically modified epidermal stem cells. *Nat Med* 2006; 12(12): 1397-402. <http://dx.doi.org/10.1038/nm1504> PMID: 17115047
- [155] Dudley ME, Wunderlich JR, Robbins PF, *et al.* Cancer regression and autoimmunity in patients after clonal repopulation with antitumor lymphocytes. *Science* 2002; 298(5594): 850-4. <http://dx.doi.org/10.1126/science.1076514> PMID: 12242449
- [156] Morgan RA, Dudley ME, Wunderlich JR, *et al.* Cancer regression in patients after transfer of genetically engineered lymphocytes. *Science* 2006; 314(5796): 126-9. <http://dx.doi.org/10.1126/science.1129003> PMID: 16946036
- [157] McGarrity GJ, Hoyah G, Winemiller A, *et al.* Patient monitoring and follow-up in lentiviral clinical trials. *J Gene Med* 2013; 15(2): 78-82. <http://dx.doi.org/10.1002/jgm.2691> PMID: 23322669
- [158] Dull T, Zufferey R, Kelly M, *et al.* A third-generation lentivirus vector with a conditional packaging system. *J Virol* 1998; 72(11): 8463-71. <http://dx.doi.org/10.1128/JVI.72.11.8463-8471.1998> PMID: 9765382
- [159] Escors D, Breckpot K. Lentiviral vectors in gene therapy: their current status and future potential. *Arch Immunol Ther Exp (Warsz)* 2010; 58(2): 107-19. <http://dx.doi.org/10.1007/s00005-010-0063-4> PMID: 20143172
- [160] Buchschacher GL Jr, Wong-Staal F. Development of lentiviral vectors for gene therapy for human diseases. *Blood* 2000; 95(8): 2499-504. <http://dx.doi.org/10.1182/blood.V95.8.2499> PMID: 10753827
- [161] Dishart KL, Denby L, George SJ, *et al.* Third-generation lentivirus vectors efficiently transduce and phenotypically modify vascular cells: implications for gene therapy. *J Mol Cell Cardiol* 2003; 35(7): 739-48. [http://dx.doi.org/10.1016/S0022-2828\(03\)00136-6](http://dx.doi.org/10.1016/S0022-2828(03)00136-6) PMID: 12818564
- [162] Barde I, Laurenti E, Verp S, *et al.* Lineage- and stage-restricted lentiviral vectors for the gene therapy of chronic granulomatous disease. *Gene Ther* 2011; 18(11): 1087-97. <http://dx.doi.org/10.1038/gt.2011.65> PMID: 21544095
- [163] Zhang KX, Moussavi M, Kim C, *et al.* Lentiviruses with trasuzumab bound to their envelopes can target and kill prostate cancer cells. *Cancer Gene Ther* 2009; 16(11): 820-31. <http://dx.doi.org/10.1038/cgt.2009.28> PMID: 19373278
- [164] Shi Q, Wilcox DA, Fahs SA, *et al.* Lentivirus-mediated platelet-derived factor VIII gene therapy in murine haemophilia A. *J Thromb Haemost* 2007; 5(2): 352-61. <http://dx.doi.org/10.1111/j.1538-7836.2007.02346.x> PMID: 17269937
- [165] Biffi A, Montini E, Lorioli L, *et al.* Lentiviral hematopoietic stem cell gene therapy benefits metachromatic leukodystrophy. *Science* 2013; 341(6148): 1233158. <http://dx.doi.org/10.1126/science.1233158> PMID: 23845948
- [166] Sessa M, Lorioli L, Fumagalli F, *et al.* Lentiviral haemopoietic stem-cell gene therapy in early-onset metachromatic leukodystrophy: an ad-hoc analysis of a non-randomised, open-label, phase 1/2 trial. *Lancet* 2016; 388(10043): 476-87. [http://dx.doi.org/10.1016/S0140-6736\(16\)30374-9](http://dx.doi.org/10.1016/S0140-6736(16)30374-9) PMID: 27289174
- [167] Urnov FD, Rebar EJ, Holmes MC, Zhang HS, Gregory PD. Genome editing with engineered zinc finger nucleases. *Nat Rev Genet* 2010; 11(9): 636-46. <http://dx.doi.org/10.1038/nrg2842> PMID: 20717154
- [168] Carroll D. Progress and prospects: zinc-finger nucleases as gene therapy agents. *Gene Ther* 2008; 15(22): 1463-8. <http://dx.doi.org/10.1038/gt.2008.145> PMID: 18784746
- [169] Lamprecht Tratar U, Horvat S, Cemazar M. Transgenic mouse models in cancer research. *Front Oncol* 2018; 8: 268. <http://dx.doi.org/10.3389/fonc.2018.00268> PMID: 30079312
- [170] Doudna JA, Charpentier E. Genome editing. The new frontier of genome engineering with CRISPR-Cas9. *Science* 2014; 346(6213): 1258096. <http://dx.doi.org/10.1126/science.1258096> PMID: 25430774
- [171] Sander JD, Dahlborg EJ, Goodwin MJ, *et al.* Selection-free zinc-finger-nuclease engineering by context-dependent assembly (CODA). *Nat Methods* 2011; 8(1): 67-9. <http://dx.doi.org/10.1038/nmeth.1542> PMID: 21151135
- [172] Reyon D, Tsai SQ, Khayter C, Foden JA, Sander JD, Joung JK. FLASH assembly of TALENs for high-throughput genome editing. *Nat Biotechnol* 2012; 30(5): 460-5. <http://dx.doi.org/10.1038/nbt.2170> PMID: 22484455
- [173] Kim H, Kim JS. A guide to genome engineering with programmable nucleases. *Nat Rev Genet* 2014; 15(5): 321-34. <http://dx.doi.org/10.1038/nrg3686> PMID: 24690881
- [174] Kim YG, Cha J, Chandrasegaran S. Hybrid restriction enzymes: zinc finger fusions to Fok I cleavage domain. *Proc Natl Acad Sci USA* 1996; 93(3): 1156-60. <http://dx.doi.org/10.1073/pnas.93.3.1156> PMID: 8577732
- [175] Wolfe SA, Nekludova L, Pabo CO. DNA recognition by Cys2His2 zinc finger proteins. *Annu Rev Biophys Biomol Struct* 2000; 29: 183-212. <http://dx.doi.org/10.1146/annurev.biophys.29.1.183> PMID: 10940247
- [176] Laity JH, Lee BM, Wright PE. Zinc finger proteins: new insights into structural and functional diversity. *Curr Opin Struct Biol* 2001; 11(1): 39-46. [http://dx.doi.org/10.1016/S0959-440X\(00\)00167-6](http://dx.doi.org/10.1016/S0959-440X(00)00167-6) PMID: 11179890
- [177] Gaj T, Gersbach CA, Barbas CF III. ZFN, TALEN, and CRISPR/Cas-based methods for genome engineering. *Trends Biotechnol* 2013; 31(7): 397-405. <http://dx.doi.org/10.1016/j.tibtech.2013.04.004> PMID: 23664777
- [178] Lino CA, Harper JC, Carney JP, Timlin JA. Delivering CRISPR: a review of the challenges and approaches. *Drug Deliv* 2018; 25(1): 1234-57. <http://dx.doi.org/10.1080/10717544.2018.1474964> PMID: 29801422
- [179] Cornu TI, Thibodeau-Beganny S, Guhl E, *et al.* DNA-binding specificity is a major determinant of the activity and toxicity of zinc-finger nucleases. *Mol Ther* 2008; 16(2): 352-8. <http://dx.doi.org/10.1038/sj.mt.6300357>
- [180] Gupta RM, Musunuru K. Expanding the genetic editing tool kit: ZFNs, TALENs, and CRISPR-Cas9. *J Clin Invest* 2014; 124(10): 4154-61. <http://dx.doi.org/10.1172/JCI72992> PMID: 25271723
- [181] Joung JK, Sander JD. TALENs: a widely applicable technology for targeted genome editing. *Nat Rev Mol Cell Biol* 2013; 14(1): 49-55. <http://dx.doi.org/10.1038/nrm3486> PMID: 23169466
- [182] Dunbar CE, High KA, Joung JK, Kohn DB, Ozawa K, Sadelain M. Gene therapy comes of age. *Science* 2018; 359(6372): eaan4672. <http://dx.doi.org/10.1126/science.aan4672> PMID: 29326244
- [183] Christian M, Cermak T, Doyle EL, *et al.* Targeting DNA double-strand breaks with TAL effector nucleases. *Genetics* 2010; 186(2): 757-61. <http://dx.doi.org/10.1534/genetics.110.120717> PMID: 20660643
- [184] Nemudryi AA, Valetdinova KR, Medvedev SP, Zakian SM. TALEN and CRISPR/Cas genome editing systems: tools of discovery. *Acta Naturae* 2014; 6(3): 19-40. <http://dx.doi.org/10.32607/20758251-2014-6-3-19-40> PMID: 25349712
- [185] Ishino Y, Shinagawa H, Makino K, Amemura M, Nakata A. Nu-

- cleotide sequence of the *iap* gene, responsible for alkaline phosphatase isozyme conversion in *Escherichia coli*, and identification of the gene product. *J Bacteriol* 1987; 169(12): 5429-33. <http://dx.doi.org/10.1128/JB.169.12.5429-5433.1987> PMID: 3316184
- [186] Jansen R, Embden JD, Gaastra W, Schouls LM. Identification of genes that are associated with DNA repeats in prokaryotes. *Mol Microbiol* 2002; 43(6): 1565-75. <http://dx.doi.org/10.1046/j.1365-2958.2002.02839.x> PMID: 11952905
- [187] Perez Rojo F, Nyman RKM, Johnson AAT, *et al.* CRISPR-Cas systems: ushering in the new genome editing era. *Bioengineered* 2018; 9(1): 214-21. <http://dx.doi.org/10.1080/21655979.2018.1470720> PMID: 29968520
- [188] Razzouk S. CRISPR-Cas9: A cornerstone for the evolution of precision medicine. *Ann Hum Genet* 2018; 82(6): 331-57. <http://dx.doi.org/10.1111/ahg.12271> PMID: 30014471
- [189] Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* 2012; 337(6096): 816-21. <http://dx.doi.org/10.1126/science.1225829> PMID: 22745249
- [190] Cong L, Ran FA, Cox D, *et al.* Multiplex genome engineering using CRISPR/Cas systems. *Science* 2013; 339(6121): 819-23. <http://dx.doi.org/10.1126/science.1231143> PMID: 23287718
- [191] Tang Prize Foundation. Biopharmaceutical Science. 2016. Available from: <https://www.tang-prize.org/en/owner.php?cat=11&y=3>
- [192] Yang H, Jaeger M, Walker A, Wei D, Leiker K, Weitaio T. Break breast cancer addiction by CRISPR/Cas9 genome editing. *J Cancer* 2018; 9(2): 219-31. <http://dx.doi.org/10.7150/jca.22554> PMID: 29344267
- [193] Barman A, Deb B, Chakraborty S. A glance at genome editing with CRISPR-Cas9 technology. *Curr Genet* 2020; 66(3): 447-62. <http://dx.doi.org/10.1007/s00294-019-01040-3> PMID: 31691023
- [194] Li K, Wang G, Andersen T, Zhou P, Pu WT. Optimization of genome engineering approaches with the CRISPR/Cas9 system. *PLoS One* 2014; 9(8): e105779. <http://dx.doi.org/10.1371/journal.pone.0105779> PMID: 25166277
- [195] Qi LS, Larson MH, Gilbert LA, *et al.* Repurposing CRISPR as an RNA-guided platform for sequence-specific control of gene expression. *Cell* 2013; 152(5): 1173-83. <http://dx.doi.org/10.1016/j.cell.2013.02.022> PMID: 23452860
- [196] Makarova KS, Wolf YI, Irazo J, *et al.* Evolutionary classification of CRISPR-Cas systems: a burst of class 2 and derived variants. *Nat Rev Microbiol* 2020; 18(2): 67-83. <http://dx.doi.org/10.1038/s41579-019-0299-x> PMID: 31857715
- [197] Anzalone AV, Randolph PB, Davis JR, *et al.* Search-and-replace genome editing without double-strand breaks or donor DNA. *Nature* 2019; 576(7785): 149-57. <http://dx.doi.org/10.1038/s41586-019-1711-4> PMID: 31634902
- [198] Watters KE, Shivram H, Fellmann C, Lew RJ, McMahon B, Doudna JA. Potent CRISPR-Cas9 inhibitors from *Staphylococcus* genomes. *Proc Natl Acad Sci USA* 2020; 117(12): 6531-9. <http://dx.doi.org/10.1073/pnas.1917668117> PMID: 32156733
- [199] Komor AC, Kim YB, Packer MS, Zuris JA, Liu DR. Programmable editing of a target base in genomic DNA without double-stranded DNA cleavage. *Nature* 2016; 533(7603): 420-4. <http://dx.doi.org/10.1038/nature17946> PMID: 27096365
- [200] Konermann S, Lotfy P, Brindeau NJ, *et al.* Transcriptome engineering with RNA-targeting type VI-D CRISPR effectors. *Cell* 2018; 173(3): 666-76.
- [201] Cornu TI, Mussolino C, Cathomen T. Refining strategies to trans-late genome editing to the clinic. *Nat Med* 2017; 23(4): 415-23. <http://dx.doi.org/10.1038/nm.4313> PMID: 28388605
- [202] Carroll D. Genome editing: past, present, and future. *Yale J Biol Med* 2017; 90(4): 653-9. PMID: 29259529
- [203] Cyranoski D. Chinese scientists to pioneer first human CRISPR trial. *Nature* 2016; 535(7613): 476-7. <http://dx.doi.org/10.1038/nature.2016.20302> PMID: 27466105
- [204] Hudacek V, Györfy B. Genome engineering using the CRISPR-Cas9 system and applications in cancer research. *Magy Onkol* 2018; 62(2): 119-27. PMID: 30027940
- [205] Montaña A, Forero-Castro M, Hernández-Rivas JM, García-Tuñón I, Benito R. Targeted genome editing in acute lymphoblastic leukemia: a review. *BMC Biotechnol* 2018; 18(1): 45. <http://dx.doi.org/10.1186/s12896-018-0455-9> PMID: 30016959
- [206] GP-write. 2019. Available from: <https://engineeringbiology-center.org/>
- [207] Doudna JA. The promise and challenge of therapeutic genome editing. *Nature* 2020; 578(7794): 229-36. <http://dx.doi.org/10.1038/s41586-020-1978-5> PMID: 32051598
- [208] Clinical Trials. Available from: <https://clinicaltrials.gov/>
- [209] Wang D, Zhang F, Gao G. CRISPR-based therapeutic genome editing: strategies and *in vivo* delivery by AAV vectors. *Cell* 2020; 181(1): 136-50. <http://dx.doi.org/10.1016/j.cell.2020.03.023> PMID: 32243786
- [210] Haridy R. FDA hits pause on one of the first US human clinical trials to use CRISPR. 2018. Available from: <https://newatlas.com/us-crispr-human-trial-hold-fda/54862/>
- [211] Censorship hinders development? Should we be more cautious about human trials of gene editing, or should we give it a go? 2018. Available from: <https://theinitium.com/roundtable/20180124-roundtable-global-Crispr-Cas9/>
- [212] China uses gene editing to treat chronic human diseases. 2018. Available from: <http://big5.sputniknews.cn/china/201802051024637731/>
- [213] Normile D. Shock greets claim of CRISPR-edited babies. *Science* 2018; 362(6418): 978-9. <http://dx.doi.org/10.1126/science.362.6418.978> PMID: 30498103
- [214] Abduljalil JM. Laboratory diagnosis of SARS-CoV-2: available approaches and limitations. *New Microbes New Infect* 2020; 36: 100713. <http://dx.doi.org/10.1016/j.nmni.2020.100713> PMID: 32607246
- [215] Broughton JP, Deng X, Yu G, *et al.* CRISPR-Cas12-based detection of SARS-CoV-2. *Nat Biotechnol* 2020; 38(7): 870-4. <http://dx.doi.org/10.1038/s41587-020-0513-4> PMID: 32300245
- [216] Abbott TR, Dhamdhare G, Liu Y, *et al.* Development of CRISPR as an antiviral strategy to combat SARS-CoV-2 and influenza. *Cell* 2020; 181(4): 865-76.
- [217] The Lancet. Genome editing: proceed with caution. *Lancet* 2018; 392(10144): 253. [http://dx.doi.org/10.1016/S0140-6736\(18\)31653-2](http://dx.doi.org/10.1016/S0140-6736(18)31653-2) PMID: 30064637
- [218] Roy B, Zhao J, Yang C, *et al.* CRISPR/Cascade 9-mediated genome editing-challenges and opportunities. *Front Genet* 2018; 9: 240. <http://dx.doi.org/10.3389/fgene.2018.00240> PMID: 30026755
- [219] Fellmann C, Gowen BG, Lin PC, Doudna JA, Corn JE. Cornerstones of CRISPR-Cas in drug discovery and therapy. *Nat Rev Drug Discov* 2017; 16(2): 89-100. <http://dx.doi.org/10.1038/nrd.2016.238> PMID: 28008168