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STATE-OF-THE-ART HUMAN GENE THERAPY: PART I. GENE DELIVERY TECHNOLOGIES

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Abstract

Safe and effective gene delivery is a prerequisite for successful gene therapy. In the early age of human gene therapy, setbacks due to problematic gene delivery vehicles plagued the exciting therapeutic outcome. However, gene delivery technologies rapidly evolved ever since. With the advancement of gene delivery techniques, gene therapy clinical trials surged during the past decade. As the first gene therapy product has obtained regulatory approval and reached clinic, human gene therapy finally realized the promise that genes can be medicines. The diverse gene delivery techniques available nowadays have laid the foundation for gene therapy applications in treating a wide range of human diseases. Some of the most urgent unmet medical needs, such as cancer and pandemic infectious diseases, have been tackled by gene therapy strategies with promising results. Furthermore, combining gene transfer with other breakthroughs in biomedical research and novel biotechnologies opened new avenues for gene therapy. Such innovative therapeutic strategies are unthinkable until now, and expected to be revolutionary. In Part I of this Review, we introduced recent development of non-viral and viral gene delivery technology platforms. As cell-based gene therapy blossomed, we also summarized the diverse types of cells and vectors employed in *ex vivo* gene transfer. Finally, challenges in current gene delivery technologies for human use were discussed.

1. Introduction

Genes have long been considered as medicines (Wirth *et al.*, 2013). Since the emergence of recombinant DNA technology that allows for the creation of a functional gene-expressing unit, how to effectively and safely deliver this therapeutic agent to human body has been a major challenge to gene therapists. Current gene delivery vehicles, namely vectors, are

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DISCLOSURE

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categorized into two classes: DNA (non-viral) vectors and viral vectors **(Figure 1)**. Circular plasmid DNA can enter cells in its naked form, or being covered with chemicals to enhance stability and delivery efficiency. Viral vectors take advantage of the infectious nature and gene-shuttling capability of certain viruses, but are deliberately engineered to minimize harm by removing as many viral genes as possible. Both types of vectors can directly deliver genes into human body. Alternatively, gene transfer may be applied to isolated human cells, which can be then reinfused into patients to function. In Part I of this Review, we briefly summarize the recent advances in gene delivery technologies in the context of gene therapy. This Review should serve as an updated introduction of gene therapy to a wide audience with diverse background. We apologize to our colleagues for not being able to highlight much of their original work as we wish due to length limit.

2. Gene delivery vectors: the toolkit for gene therapy

2.1 DNA (non-viral) vectors

A therapeutic gene expression cassette is typically composed of a promoter that drives gene transcription, the transgene of interest, and a termination signal to end gene transcription **(Figure 1)**. Such an expression cassette can be embedded in a plasmid (circularized, double-stranded DNA molecule) as delivery vehicle. Plasmid DNA (pDNA) can be directly injected *in vivo* by a variety of injection techniques, among which hydrodynamic injection achieves the highest gene transfer efficiency in major organs by quickly injecting a large volume of pDNA solution and temporarily inducing pores in cell membrane (Wang *et al.*, 2013). To help negatively charged pDNA molecules penetrate the hydrophobic cell membranes, chemicals including cationic lipids and cationic polymers have been used to condense pDNA into lipoplexes and polyplexes, respectively. These nanoparticles shield pDNA from nuclease degradation in extracellular space and facilitate entry into target cells (Jin *et al.*, 2014).

Following cellular uptake, pDNA travels with cytoplasmic vesicles known as endosomes, where cellular surveillance mechanisms that clear foreign DNA pose significant barriers to achieving efficacious transgene expression. For example, the toll-like receptor 9 in endosomes senses unmethylated CpG dinucleotides in pDNA, and mounts a destructive innate immune response (Barton *et al.*, 2006). A major advancement in DNA vector design is minicircle DNA (mcDNA), which differs from pDNA in the lack of bacteria-derived, CpG-rich backbone sequences (Mayrhofer *et al.*, 2009). When administered *in vivo*, mcDNA mediates safer, higher and more sustainable transgene expression than conventional pDNA (Gill *et al.*, 2009). Novel methods for large-scale production of mcDNA will boost further evaluation on its therapeutic efficacy (Kay *et al.*, 2010). Another DNA vector design is to embed pDNA backbone within transgene expression cassette, which was recently shown to result in robust transgene expression both *in vitro* and *in vivo* (Lu et al., 2013). A fraction of DNA vector can eventually find its path to the nucleus, where it exists as non-integrating, episomal DNA and leads to transgene expression. However, as cells go through cell division, the non-replicable episomal DNA is gradually diluted and lost, resulting in only transient transgene expression. In the gene therapy cases where prolonged transgene expression is desired, a scaffold matrix attachment region (S/MAR) can be included in

vector design to replicate and retain episomal DNA in daughter cells. S/MAR is a eukaryotic DNA sequence that attaches to the nuclear scaffold/matrix, and by doing so is capable of driving the replication of episomal DNA along with duplication of host genomic DNA during cell division (Argyros *et al.*, 2011).

DNA vectors possess several valuable advantages over viral vectors, such as easy scale-up production, amenable to carrying large genes, and lack of any viral component, hence low immunotoxicity. Although significant efforts have been made to improve cellular uptake, and to battle against cellular mechanisms for robust and sustainable transgene expression, DNA vectors nonetheless remain much less effective in therapeutic gene delivery than viral vectors.

2.2 Viral vectors

Viruses that infect mammals are naturally evolved gene delivery vehicles for gene therapy. The surface proteins on viral particles can interact with their corresponding receptors on target cells, which triggers the cellular uptake process known as endocytosis. Once inside a target cell, viruses eventually deliver their genetic information in the form of DNA into the nucleus for viral gene expression. Many mammalian viruses have been explored as gene delivery vectors (Giacca & Zacchigna, 2012; Kay, 2011; Vannucci *et al.*, 2013). Counterintuitively, vectors based on some pathogenic viruses that cause infectious diseases, such as human immunodeficiency virus (HIV), are among the most widely used in gene therapy. Replacing most of the viral genes with a therapeutic gene cassette, and meanwhile retaining signal sequences that are essential for *in vitro* replication and packaging in producer cell lines formulate the common theme of viral vector genome engineering **(Figure 2)**. Viral vector production commonly employs a *trans*-packaging system in cell culture, requiring the co-existence of one to four components **(Figure 3)**. Vectors based on gammaretrovirus, lentivirus, adenovirus (AdV), adeno-associated virus (AAV) and herpes simplex virus (HSV) are among the most widely used viral vectors in current gene therapy studies, and are more advanced in clinical translation than other types of viral vectors (Herzog *et al.*, 2010). Here we review some of their unique capabilities and gene therapy applications.

Gammaretrovirus and lentivirus are both retrovirus, which is characterized by a RNA genome, and utilizing virus-derived reverse transcriptase and integrase to insert their proviral complementary DNA into the host genome. Gammaretrovirus can only transduce replicating cells, whereas lentivirus can also transduce non-replicating cells, which makes lentiviral vector more favorable in many gene therapy settings (Sakuma *et al.*, 2012). Vector development based on these two viruses has greatly benefited from engineering their envelope glycoproteins that are amenable to modification for specific tissue and/or cell tropisms. For example, replacing the envelope glycoprotein with the G glycoprotein from vesicular stomatitis virus significantly increases vector stability (hence easier purification procedure and higher titers), and expands tropism to a wide range of cell types (Vannucci *et al.*, 2013). For targeted gene delivery to a specific cell type, retroviral vectors can be pseudotyped with a viral glycoprotein that binds to a specific membrane receptor of that cell type. Furthermore, a viral glycoprotein can be fused with a ligand protein or antibody that

recognizes cell type-specific surface molecules, providing a versatile way of cell type-specific gene delivery (Vannucci *et al.*, 2013). Integration into host genome, the distinctive feature of retroviral vectors, is a double-edged sword when it comes to gene therapy. Genomic integration ensures the stability of transgene and persistent transgene expression in daughter cells following genome replication and cell division, but its randomness results in the risk of insertional mutagenesis by potentially disrupting tumor suppressor genes or activating oncogenes (Hacein-Bey-Abina *et al.*, 2010). Therefore, improving the safety profile of retroviral vectors represents another active and ongoing line of research. Currently, most retroviral vectors are based on a self-inactivating (SIN) vector design (Zufferey *et al.*, 1998). The long terminal repeat (LTR) of conventional retroviral vector harbors enhancer/promoter sequences that, following integration into host genome, are capable of activating the expression of neighboring genes including oncogenes. In SIN vector design, these enhancer/promoter sequences in LTR are removed, thus greatly reducing the likelihood of activating oncogenes. Other approaches aiming at reducing the risk of insertional mutagenesis include the development of integration-deficient lentiviral vectors by mutating the integrase (Yanez-Munoz *et al.*, 2006), and site-directed integration using the zinc finger nucleases (Lombardo *et al.*, 2007; Provasi *et al.*, 2012).

In contrast to retrovirus, AdV contains a DNA genome that episomally resides in host nucleus, which prevents insertional mutagenesis. Several features of AdV make it a useful vector particularly ideal for two applications: oncolysis (infecting and killing tumor cells) and vaccination. AdV is able to transduce a broad range of quiescent and proliferating human cells including tumor cells. Deleting certain viral genes can make AdV replication-competent in only tumor cells, thus lysing tumor cells but leaving normal cells unaffected (Choi & Yun, 2013). However, it should be noted that the tumor destruction effect exerted by engineered AdV vector is most likely the combined outcome of its oncolytic capacity and strong immunogenicity (Russell *et al.*, 2012). The human immune response associated with systemic AdV vector administration can be as strong as lethal (Lehrman, 1999). When injected into tumor mass, AdV vector triggers antitumor immunity in the tumor microenvironment, which contributes to tumor killing (Russell *et al.*, 2012). Furthermore, using AdV vectors to express immunostimulatory factors in tumor cells, such as granulocyte macrophage-colony stimulating factor (GM-CSF), can enhance the antitumor immunity and therapeutic efficacy (Cerullo *et al.*, 2010). Another major application of AdV vector is to serve as genetic vaccines against pandemic infectious diseases caused by other viruses, such as HIV and influenza (Lasaro & Ertl, 2009). AdV vector expressing an antigen can mount an initial immune response to the vector, which boosts the later antigen-specific, protective immune response against viral infection (Barnes *et al.*, 2012). Currently, the most commonly used AdV vectors are derived from AdV serotype 5 (AdV5) that naturally infects a large human population. Therefore, pre-existing antibodies that neutralize AdV5 vectors are prevalent and can impede vaccination efficacy (Lasaro & Ertl, 2009). Developing AdV vectors derived from other primate species can potentially bypass pre-existing immune neutralization, thus holding the promise for more efficacious vaccine strategies for humans (Ertl, 2012).

AAV is a group of small, simple, helper-dependent, nonpathogenic and single-stranded DNA viruses. Recombinant AAV (rAAV) vector carrying inverted terminal repeats as the only viral component entered the gene therapy arena much later than retroviral and AdV vectors, but have quickly gained popularity (Mingozzi & High, 2011). For rAAV vectors, it is largely the capsid that determines the tropism and transduction profile in different cell types. Therefore, great efforts have been focused on developing AAV capsids that have unique characteristics. Tropism of several natural AAV capsids has been well characterized in mouse and larger animal models (Gao *et al.*, 2005). Surprisingly, vectors derived from some AAV serotypes such as AAV9 can cross the blood-brain barrier and transduce cells of the central nervous system (CNS) following a single intravenous injection (Duque *et al.*, 2009; Foust *et al.*, 2009; Yang *et al.*, 2014; Zhang *et al.*, 2011). These discoveries hold great promise in treating CNS disorders that affect a large area of brain and/or spinal cord (Nagabhushan Kalburgi *et al.*, 2013). In addition to relying on natural diversity, AAV capsids can be decorated by peptides or “shuffled” to generate novel capsids that suit specific needs (Li *et al.*, 2008). For example, a chimeric AAV capsid “shuffled” from five parental natural AAV capsids was recently found to efficiently transduce human liver cells in a humanized mouse model (Lisowski *et al.*, 2014). Similar to AdV vector, rAAV vector can transduce both dividing and non-dividing cells, and its recombinant viral genome stays in host nucleus predominantly as episome. Interestingly, single or multiple copies of rAAV vector genome can circularize in a head-to-tail or head-to-head configuration in host nucleus, thus enhancing stability of the episomal rAAV DNA genome and mediating long-term transgene expression in non-dividing cells such as muscle fibers (Wang *et al.*, 2014). This feature has recently prompted researchers to utilize rAAV vector to produce secretory antibody, which may potentially provide protection against infectious diseases throughout lifetime. This preventive strategy has shown encouraging results when recently tested in animal models against HIV infection and influenza (Balazs *et al.*, 2013; Balazs *et al.*, 2012; Johnson *et al.*, 2009). It is exciting to examine whether this innovative approach will live up to expectation in clinical trials (Balazs & West, 2013). A major drawback of rAAV vector is that the transgene cassette is limited to ~4.5 kilobase (kb), which precludes delivering large therapeutic genes (Wang *et al.*, 2014). Nevertheless, rAAV vector finds its broad applications in treating numerous diseases. In 2012, the first human gene therapy product Glybera, a rAAV vector expressing lipoprotein lipase (LPL) that treats LPL deficiency, obtained official approval in Europe, representing a milestone in rAAV gene therapy (Flotte, 2013).

HSV is a naturally neurotropic virus. After initial infection in skin or mucous membranes, HSV is taken up by sensory nerve terminals, travels along nerves to neuronal cell bodies, and delivers its DNA genome into nuclei for replication (Manservigi *et al.*, 2010). Therefore, HSV vectors are well suited to gene therapy of neurological disorders. In particular, as the mechanisms of chronic pain unraveled during the past decade, using HSV vectors to target sensory neurons and to express pain reliever genes has shown promising results in pre-clinical testing (Goss *et al.*, 2014). Aside from their natural neurotropism, HSV vectors are also valuable for oncolytic virotherapy. Similar to AdV vectors, HSV vectors can also transduce numerous tumor cell lines, and attenuated HSV vectors with certain viral genes deleted can replicate only in tumor cells resulting in oncolysis

(Manservigi *et al.*, 2010). Most recently, transgene expression and the oncolytic activity of HSV vectors have been combined to synergistically eradicate cancer. For example, talimogene laherparepvec (T-VEC) is an oncolytic HSV vector expressing the immunostimulating factor GM-CSF (Miest & Cattaneo, 2014). The efficacy of T-VEC in treating melanoma has been demonstrated in Phase II and Phase III clinical trials (Goins *et al.*, 2014).

3. *Ex vivo* gene transfer and cell-based gene therapy

Some diseases primarily affect specific cell types, such as the red blood cells in beta-thalassemia. In cell-based gene therapy, affected human cells are isolated, cultured and genetically modified *ex vivo* through non-viral or viral vector-mediated gene transfer. The modified cells are usually enriched and then reinfused into patients to realize therapeutic effects (Naldini, 2011). In this way, human body is not directly exposed to gene delivery vectors, which may improve safety and target cell specificity. In addition, the cells that undergo desired modification can be selected and expanded before or after reinfusion to enhance efficacy. Importantly, the autologous cells isolated from a patient but genetically modified are used to treat him/herself, thus preventing graft-versus-host disease as commonly seen in hematopoietic stem cell transplantation between two individuals.

3.1 The choice of cells

Cell-based gene therapy often utilizes stem or progenitor cells with transgene integrated into host genome, so that their gene-modified progeny can provide long-term therapeutic effects. Hematopoietic stem cells (HSCs) are blood cells that give rise to a variety of cell types including red blood cells and major immune cells. HSCs are easy to isolate and manipulate *ex vivo*, providing an ideal cell population to take up therapeutic genes (Bigger & Wynn, 2014). Several HSC-based gene therapy strategies have advanced into clinical evaluation at different stages. For example, adrenoleukodystrophy (ALD) is a neurological disorder caused by the deficiency of a gene that enables oligodendrocytes and microglia to maintain myelin sheath in the brain. A cell-based gene therapy strategy involves inserting a normal copy of the gene into patient-derived HSCs, and reinfusing the HSCs back to the patient. As a result, the gene-corrected HSC progeny migrate to the brain and further differentiate into microglia and support myelin maintenance. The treatment successfully halted progressive demyelination in two ALD patients (Cartier *et al.*, 2009). A Phase II/III clinical trial has been initiated to further evaluate efficacy and safety. Several other promising clinical studies involve a similar *ex vivo* gene transfer strategy in *ex vivo* HSCs, namely replacing with a functional version of the defective gene, such as the clinical trials to treat adenosine deaminase-severe combined immunodeficiency and beta-thalassemia (Aiuti *et al.*, 2009; Payen *et al.*, 2012).

T cells are a heterogeneous cell population derived from HSCs, and play important roles in immunity. T cell receptors (TCRs) recognize specific molecules on the surface of target cells, and mediate target cell-specific killing. The target cell-specific killing capability of T cells has recently been harnessed to destroy tumor cells. This is achieved by equipping T cells with receptors that redirect T cells to target tumor-specific surface molecules (Song, 2013) **(Figure 4)**. Such receptors include TCRs cloned from natural tumor-targeting T cells,

and chimeric antigen receptors (CARs) that are engineered by fusing an antibody fragment targeting tumor antigen with intracellular signaling domains triggering T cell activation (Kerkar, 2013). Several early phase clinical trials have demonstrated the efficacy of CARs recognizing CD19, a B cell surface molecule widely expressed in B cell malignancies. Adoptive transfer of genetically modified T cells expressing anti-CD19 CAR led to partial remission of lymphoma in a patient with advanced-stage lymphoma (Kochenderfer *et al.*, 2010). Currently, a handful of clinical trials are underway to treat B cell malignancies with anti-CD19 CAR T cells (Kochenderfer & Rosenberg, 2013). In addition, *ex vivo* modified regulatory T cells, a subtype of T cells that can dampen immune response, have been explored to modulate autoimmune diseases (Jethwa *et al.*, 2014).

Induced pluripotent stem cells (iPSCs) are generated by introducing a set of gene transcription factors into *ex vivo* somatic cells such as skin fibroblasts, which converts the somatic cells to pluripotent stem cells (Takahashi & Yamanaka, 2013). Combining the iPSC and *ex vivo* gene transfer technologies to develop cell-based gene therapy is an appealing strategy, since iPSCs have the potential to differentiate into almost all cell types, such as muscle, blood and neuronal cells (Ellis *et al.*, 2010). Inserting a functional beta-globin transgene into a “safe harbor” in the genomes of beta-thalassemia patient iPSCs, and then differentiating the transduced iPSCs to erythroid lineage yielded therapeutic levels of beta-globin expression (Papapetrou *et al.*, 2011). As the targeted genomic editing technology is rapidly evolving, many studies have shown that iPSCs derived from patients with various diseases are amenable to non-viral or viral vector-mediated genomic editing. Importantly, patient iPSCs or their differentiated progeny with a corrected genome demonstrated normal or improved cellular phenotype compared to the uncorrected, diseased counterpart (Lisa Li *et al.*, 2014). In July 2013, the world’s first clinical trial involving iPSCs was officially approved in Japan to treat age-related macular degeneration (Clinical trial ID: JPRN-UMIN000011929). In the future, iPSC combined with *ex vivo* gene transfer is expected to greatly broaden the clinical application of cell-based gene therapy.

3.2 The choice of vectors

In cell-based gene therapy, *ex vivo* gene transfer aims to permanently modify the host genome, so that the beneficial modification is not lost along with cell expansion before or after reinfusion into human body. To this end, integrating viral vectors such as gammaretroviral and lentiviral vectors are commonly used. Due to its improved safety profile and ability to transduce both dividing and non-dividing cells, lentiviral vector is generally preferred to gammaretroviral vector. In addition, DNA transposons have emerged as a powerful tool for *ex vivo* gene transfer (Hackett *et al.*, 2011). Expression of transposase mediates a cut-and-paste mechanism that efficiently inserts a designer transposon harboring a transgene cassette into host genome (Skipper *et al.*, 2013). The DNA transposon/transposase system can be delivered *in vivo* or *ex vivo* in the simple form of plasmid DNA. Recently, the *Sleeping Beauty* transposon/transposase system was used in clinical trials to express anti-CD19 CAR in T cells for the treatment of B-cell lymphoma (Singh *et al.*, 2014). Instead of inserting extra DNA material into host genome, another approach to permanently correcting a diseased genome is through targeted genomic editing. Designer DNA endonucleases such as the CRISPR/Cas system (Mali *et al.*, 2013) can be engineered

to cut genomic DNA in a sequence-specific manner, allowing for disruption or repair of that region (Lisa Li *et al.*, 2014). The genes orchestrating this process need only to be transiently expressed in cultured cells, whereas the mark in the genome is left permanent. In this case, non-integrating vector that does not suffer the risk of insertional mutagenesis is well suited, such as adenoviral vector (Li *et al.*, 2013).

4. The challenges ahead

4.1 Safety concerns

Since the finding that some patients treated with gammaretroviral vector-mediated gene transfer developed leukemia due to insertional mutagenesis (Hacein-Bey-Abina *et al.*, 2010), the risk of genotoxicity remains a major safety concern particularly for the cell-based gene therapy despite vigorous efforts to improve. This risk is associated with not only integrating gene transfer platforms such as retroviral vector or transposon/transposase-based systems (Hackett *et al.*, 2013), but also “non-integrating” gene transfer vectors such as AAV vector that may integrate at a much lower rate (Kaeppel *et al.*, 2013). Developing safer gene delivery methods should be coupled with thorough characterization and evaluation of genotoxicity. As *ex vivo* gene transfer and cell-based gene therapy enters a personalized medicine era, rapidly evaluating genome modification profile by genome-wide sequencing and screening for safe cell clones will be required. Another safety issue is immunotoxicity that mainly concerns *in vivo* gene transfer using viral vectors. The lethal immunotoxicity of AdV vector greatly limits its clinical use (Lehrman, 1999). Although the low immunogenicity of AAV vector has not caused acute life-threatening side effects, in several clinical trials it certainly triggered immunotoxicity that could eliminate transduced cells and hamper therapeutic outcome (Masat *et al.*, 2013; Mingozzi & High, 2013). Further understanding of the immune responses against both viral and therapeutic antigens following *in vivo* gene delivery, and deliberately modulating host immune responses will be crucial for developing clinical gene therapy particularly to treat systemic diseases.

4.2 Transgene expression

In many gene therapy applications, targeted gene delivery and controllable transgene expression is preferred to achieve maximal therapeutic efficacy, and meanwhile to prevent side effects associated with off-target and non-physiological transgene expression. Current approaches include engineering viral vector surface proteins to modify tropism, and designing expression cassette that is regulated by host cellular machinery or pharmacologically inducible (Wang *et al.*, 2014). Deciphering the interactions between viral vector and host cell receptors from a structural biology perspective is expected to pinpoint the key factors determining vector tropism and immunogenicity, and to facilitate the development of novel vectors. The abnormal cellular behavior of diseased cells is usually studied as the target of treatment. However, the unique environment of the diseased cells, albeit aberrant, can be explored as a natural way to confine transgene expression therein (Jazwa *et al.*, 2013). Engineering such regulation into vector design will require deeper knowledge of specific diseases and their impact on gene expression.

4.3 From bench to bedside

Human gene therapy development follows the same route as other drug development, *i.e.* from pre-clinical testing in animal models to clinical trials. However, several studies revealed that gene delivery in animal models does not always predict the outcomes in humans. The discrepancies range from adverse immune response to vector tissue tropism (Lisowski *et al.*, 2014; Pien *et al.*, 2009). Therefore, creating reliable animal models to perform pre-clinical evaluation will be valuable for clinical testing. Although rodent models are widely used in preclinical gene therapy research, larger animal models have proved to represent more truthful testing platforms for clinical translation. In particular, nonhuman primate models will continue to play a pivotal role in studying toxicology and biodistribution of gene delivery vectors. Production of clinical grade vectors poses another challenge to broad clinical applications of gene therapy. Certain applications require a large quantity of vectors for efficient gene delivery, such as using AAV vector to target all muscle tissues affected in muscular dystrophy. Developing scalable approaches to manufacturing high quality vectors will significantly expedite clinical translation (Kotin, 2011; Segura *et al.*, 2011; Segura *et al.*, 2013).

5. Conclusion

Gene delivery technologies have been powering human gene therapy applications. The major setbacks in the early age of human gene therapy only stimulated surging demand for vector development. The recent advances in gene delivery techniques proved to be much improved in both safety and efficacy. Viral vectors are more commonly used than non-viral delivery due to their superior gene transfer efficiency. Viral vectors inherit many intrinsic features from their parental viruses. In many cases these features are double-edged. Harnessing the unique capabilities of each type of viral vector, and meanwhile diminishing the downside in specific gene therapy applications represent the mainstream of viral vector development. Merging seamlessly with other innovative technologies, gene delivery techniques will continue to drive human gene therapy. In Part II of this Review, we will discuss various gene therapy strategies to meet diverse therapeutic needs.

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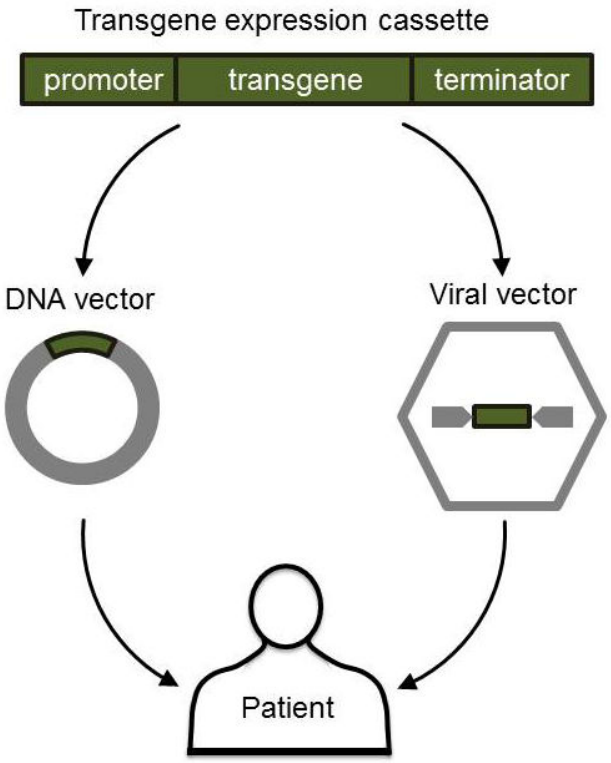

Figure 1.
Gene delivery vectors. A therapeutic transgene expression cassette can be carried by a DNA vector (left) or a viral vector (right) for delivery.

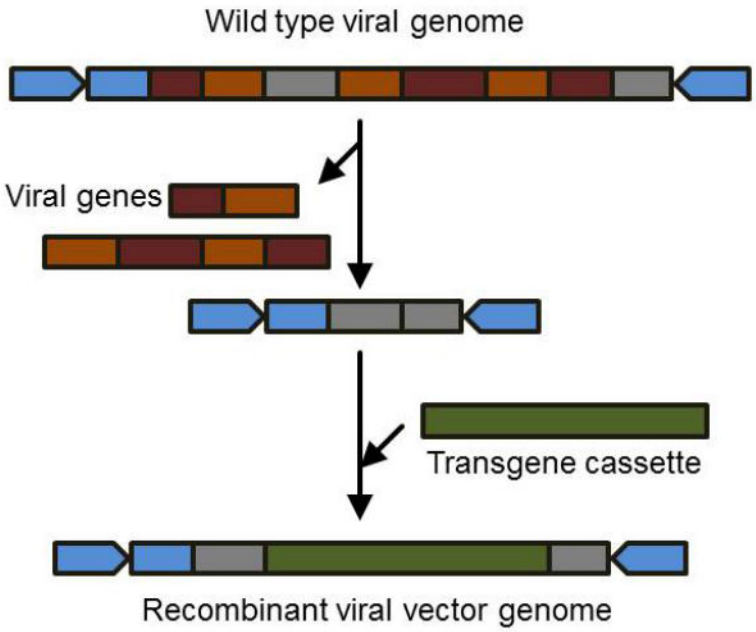

Figure 2.
Viral vector engineering. A wild type viral genome contains viral genes (red, orange and gray) and sequences that serve as packaging signal (blue). Removing most of the viral genes creates space for a transgene expression cassette (green) and minimizes virulence.

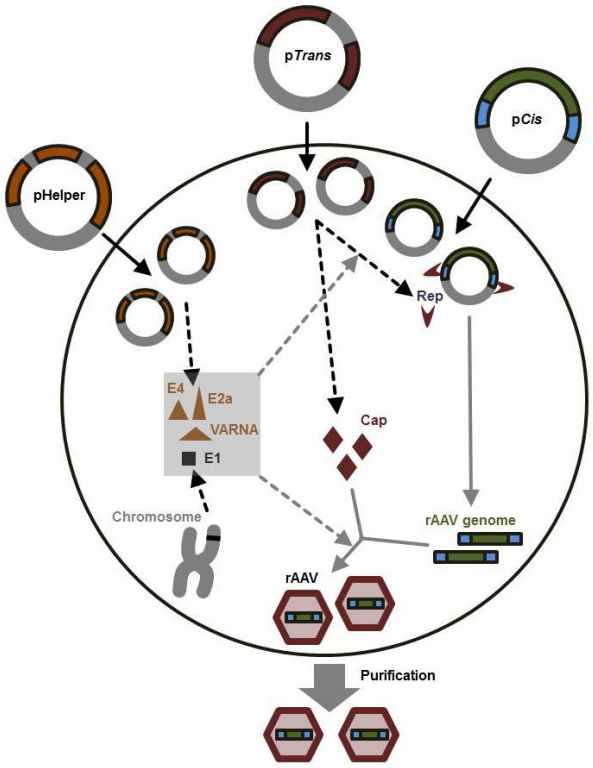

Figure 3.

An example of viral vector production process. Production of recombinant adeno-associated virus (rAAV) is shown. Four components are essential: (1) a *cis* plasmid carrying a recombinant viral genome that contains a therapeutic gene cassette (green) and packaging signals (blue), (2) a *trans* plasmid carrying genes encoding viral capsid proteins (Cap) and replication proteins (Rep), (3) a helper plasmid carrying some genes from adenovirus, and (4) a stable producer cell line expressing the adenoviral protein E1. Co-transfection of the three plasmids into the producer cell line leads to the expression of the carried genes (orange and purple). The adenoviral genes carried on the producer cell chromosome and pHelper facilitate Rep expression. Rep recognizes the packaging signal sequences (blue), and excises the rAAV genome from p*Cis*. With the help of some adenoviral proteins, the released rAAV genome and Cap are assembled into rAAV particles, which are further purified. Black solid arrows: co-transfection. Black dashed arrows: gene expression. Gray solid arrows: assembly line of rAAV particles. Gray dashed arrows: roles of the adenoviral proteins (Gao & Sena-Esteves, 2012). For production of other viral vectors, refer to Vannucci *et al.*, 2013.

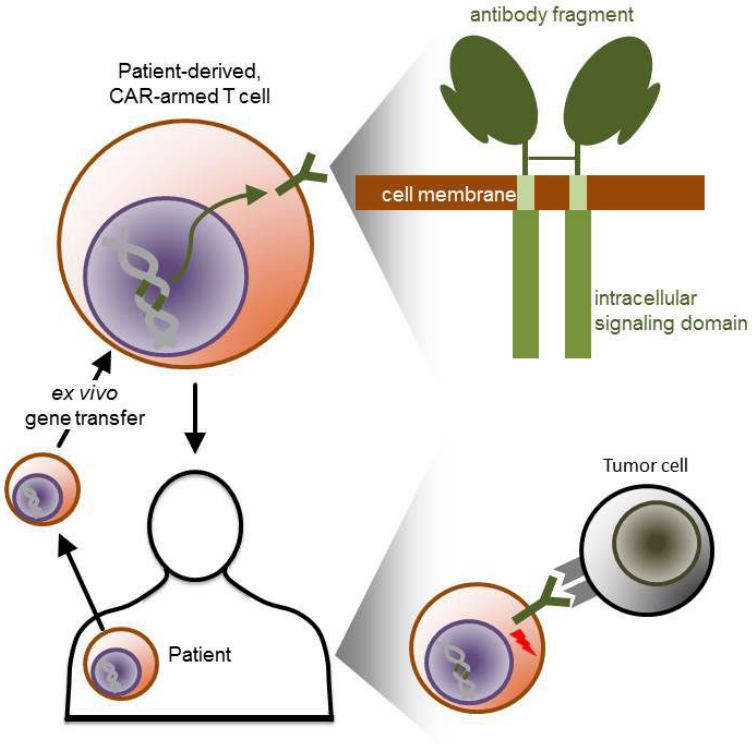

Figure 4.

CAR-armed T cell in cancer gene therapy. Isolated T cell population is genetically modified by *ex vivo* gene transfer to express CAR on cell surface. After the CAR-armed T cell is reinfused into human body, the CAR recognizes specific molecule on the tumor cell surface. The interaction triggers a cascade of cell signaling event (red bolt), and eventually allows the T cell to kill the tumor cell.