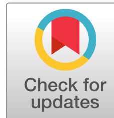

Viral Vectors for Gene Transfer

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Viral vectors are a promising tool for effective delivery of genetic material into cells. They take advantage of the natural ability of a virus to deliver a genetic payload into cells while being genetically modified such that their ability to replicate is crippled or removed. Here, an updated overview of routinely used viral vectors, including adeno-associated viruses (AAV), retroviruses/lentiviruses, and adenoviruses (Ads), is provided, as well as perspectives on their advantages and disadvantages in research and gene therapy. © 2018 by John Wiley & Sons, Inc.

Keywords: adeno-associated viruses • adeno-viruses • gene therapy • lentiviruses • retroviruses • viral vector

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INTRODUCTION

Gene therapy has shown recent progress and there have been numerous advances in the variety of viral vectors available for use (Naldini, 2015). One way viral vectors are categorized is as integrating or non-integrating, based on whether the recombinant vectors (recombinant vectors are those that express a transgene of interest and their ability to replicate has been reduced or removed) integrate their viral genome into the cell genome of the host. Integrating vectors, such as gamma-retroviral vectors, are generally used to transduce actively dividing cells. Lentiviruses, another integrating vector, transduce non-dividing and dividing cells. Non-integrating vectors, such as AAV vectors and Ad vectors, transduce quiescent or slowly dividing cells, as they can be quickly lost from rapidly dividing cells. A vector's tropism for the dividing or non-dividing cell will play a large part into whether there is transduction at all. Other factors when selecting a viral vector are the vector packaging capacity and its tendency to elicit immune responses if applied *in vivo*. The main characteristics of the most commonly used viral vectors are summarized in Table 1.

There are many methods to alter a virus' tropism to enhance specificity for expression in the cell or tissue of interest. These range from altering the capsid, altering the glycoproteins that decorate enveloped viruses, or restricting expression through selected promoters and enhancers. Another clever method to restrict expression to cell types of interest is to employ microRNA target sites in the 3′UTR such that microRNAs naturally expressed in non-desired cells will degrade the expressed transgene (Brown et al., 2007). Target cells, on the other hand, will not express the microRNA, allowing for transgene expression.

AAV

Biology of AAV

AAV is considered a nonpathogenic parvovirus composed of a 4.7-kb single-stranded DNA encapsulated in a non-enveloped, icosahedral capsid (size 20 to 25 nm in diameter). The viral genome is composed of three genes, *Rep*, *Cap*, and *AAP*, flanked by inverted terminal repeats (ITRs) that function as the viral origin of replication and the packaging signal. The *Rep* gene encodes four nonstructural proteins that are required for viral genome replication, transcriptional regulation, and

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Table 1 General Characteristics of Recombinant Viral Vectors

	AAV	Retrovirus	Lentivirus	Adenovirus
Family	Parvoviridae	Retroviridae	Retroviridae	Adenoviridae
Viral genome	ssDNA	ssRNA	ssRNA	dsDNA
Package size	<5 kb	9 kb	9 kb	7.5 kb/30 kb[a]
Cell transduction	Dividing and non-dividing	Dividing	Dividing and non-dividing	Dividing and non-dividing
Integration to genome	No[b]	Yes	Yes	No
BLS category	1	1/2	2	2

[a]Adenovirus vector package size is 7.5 kb in E1, E3 deleted vector backbone, 30 kb in gutted vector.
[b]rAAVs can integrate into sites of DNA damage. The native virus integrates.

packaging. The *Cap* gene encodes three structural proteins (VP 1 to 3), which assemble to form a 60-mer viral capsid. The *aap* gene encodes the assembly-activating protein (AAP) in an alternate reading frame overlapping the *cap* gene. The AAP facilitates nuclear import of the capsid protein and promotes assembly and maturation of the capsid (Samulski & Muzyczka, 2014). However, a recent study examining 12 AAV serotypes, which are variations within a species of virus, found that AAP is not essential in assembling AAV 4, 5, and 11 (Earley et al., 2017).

AAV is a helper-dependent virus. In the presence of a helper virus, such as adenovirus or herpes simplex virus, AAV undergoes viral genome replication and productive infection. Helper viruses either enhance production of AAV proteins to promote AAV replication or provide replication proteins directly. In the absence of a helper virus, the AAV genome can persist in the host as an episome (extragenomic circular DNA), or integrate into the host genome at a specific site on chromosome 19 (19q13.4) (Samulski & Muzyczka, 2014). AAV can infect both dividing and non-dividing cells, but nonintegrated genomes are quickly lost in dividing cells. AAV enters cells by binding to cell surface sugars (such as sialic acid, galactose, or heparin sulfate) and protein receptors (AAV receptor, AAVR; Pillay et al., 2016), and then internalizes via clatherin-dependent or -independent cytosis. Following entry, AAV traffics through the endosomal system, in which AAV undergoes a conformational change in the capsid induced by acidic changes in the endosome. Upon endosomal escape, AAV traffics to the nucleus where subsequent uncoating and gene expression occurs (Pillay & Carette, 2017).

Development of Recombinant AAVs (rAAVs)

rAAVs are generated by inserting a transgene expression cassette between the ITRs in place of the *rep* and *cap* genes. The *rep* and *cap* genes are provided along with helper viral genes *in trans* during vector production. There are 12 serotypes and >100 variants of AAVs, which mediate a broad range of tissue or cellular tropisms. For example, AAV8 has been shown to effectively transduce the liver, whereas AAV1 and AAV5 effectively transduce cells in the central nervous system (Asokan, Schaffer, & Samulski, 2012). AAVs also have broad transduction efficiency in mammalian primary cells and cell lines (Ellis et al., 2013). Different serotypes of rAAV can be produced by merely exchanging the capsid in the helper plasmid, while keeping the same expression cassette within AAV2 ITRs. This is called pseudotyping, and the resulting vector is typically denoted rAAV 2/X, where 2 denotes AAV2 ITRs and the X denotes the serotype capsid. Pseudotyping allows for quick generation of a variety of serotypes to determine which is most efficient for a specific study.

Although different AAV vectors that preferentially transduce many various cell types have been identified, there are cells that are refractory to AAV transduction. AAV receptors, post-attachment steps, and nuclear import all contribute to AAV transduction efficiency (Pillay & Carette, 2017). As a result, different approaches have been used to modify or optimize the viral capsid proteins to meet biomedical needs. The rational design approach utilizes knowledge of AAV to make targeted changes to the capsid to alter transduction efficiency or specificity. For example, tyrosines on the capsid surface have been mutated to

phenylalanines as reducing tyrosine phosphorylation during capsid entry increases transduction efficiency (Zhong et al., 2008). Approaches to broaden the range of tissues that can be targeted by AAV include directed evolution (Nonnenmacher, van Bakel, Hajjar, & Weber, 2015; Yang, Li, & Xiao, 2011) and insertion of peptides into capsid surface loops (Chen, Chang, & Davidson, 2009; Muller et al., 2003). In either case, selective pressure is applied to isolate the variants that reach the tissue or cell of interest.

Advantages and Disadvantages

In most cases, AAV exhibits no pathogenicity or cytotoxicity at doses required for transduction. When delivered *in vivo*, the AAV capsid is less immunogenic than Ad. One advantage of AAVs is that their episomal genomes can persist for many years in non-dividing cells, and their low rate of genomic integration decreases the risk of insertional mutagenesis. AAV vectors have broad host and cell tropism for a number of post-mitotic cell types. While AAV vectors can transduce both dividing and non-dividing cells, a significant shortcoming is the small packaging size (<5 kb). This limits applications for larger genes and more complex multipart regulators. However, this drawback can be addressed by trans-splicing, which involves splitting the expression cassette across two vectors; a functional cassette is reconstituted after recombination in the cell nucleus (Hirsch, Wolf, & Samulski, 2016).

Usage

The AAV vector has been extensively used for gene replacement *in vivo* to test novel therapies in animal models of disease (Katz et al., 2015), to assess gene function (Wang et al., 2018) or to knock down gene expression (Keiser, Monteys, Corbau, Gonzalez-Alegre, & Davidson, 2016). It has also been applied clinically in many scenarios, including various forms of hemophilia (George et al., 2017), and blindness caused by RPE65 mutations (Bennett et al., 2016). AAV is also used as an important research tool, especially in neurobiology to study the anatomy and physiology of the central nervous system (Sizemore, Seeger-Armbruster, Hughes, & Parr-Brownlie, 2016).

Retroviral and Lentiviral Vectors

Biology of retroviruses and lentiviruses

Retroviruses are single-stranded RNA viruses that package two copies of positive-strand RNA surrounded by the capsid and envelope (80 to 120 nm in diameter). The RNA genome of gamma retroviruses encodes the *gag*, *pol*, and *env* genes flanked by long terminal repeats (LTRs). LTRs function as enhancers and promoters. The *gag* gene encodes the structural protein, the *pol* gene encodes the reverse transcriptase and integrase, and the *env* gene encodes the envelope proteins. Lentiviruses, a subcategory of the retrovirus family, are known as complex retroviruses based on the details of the viral genome. In addition to genes found in retroviruses, lentiviruses carry an additional six genes encoding the accessory proteins *tat*, *rev*, *vpr*, *vpu*, *nef*, and *vif*, which encode proteins important for viral replication, binding, infection, and release (Reinhard & Bannert, 2010).

For infection or transduction of recombinant vectors, retrovirus viral envelope glycoproteins attach to host cell receptors. Depending on the type of glycoprotein and the specific retrovirus, the viral envelope fuses with the cellular membrane or endosome membrane of the host with subsequent release of the viral core into the cytoplasm. Double-stranded viral DNA genomes are created by viral reverse transcriptase from the original viral single-stranded RNA. Depending on the retrovirus, viral dsDNA genomes either enter the nucleus of non-dividing cells through the nuclear pore (e.g., lentivirus) or wait until the nuclear membrane disseminates during cell division (e.g., gamma retrovirus). Once in the nucleus, the viral dsDNA integrates into the host genome and remains a permanent part of host cells (Reinhard & Bannert, 2010). Integration sites in the host cell genome are different among retroviruses. Murine Leukemia Virus (MLV; gamma retrovirus) integrates in non-random patterns that actively transcribing favor regulatory elements (promoters, enhancers, evolutionarily conserved noncoding regions) within or around protein-coding genes. Human immunodeficiency virus (HIV; lentivirus) prefers to integrate within the gene but tends not to integrate in regulatory regions (Poletti & Mavilio, 2018).

Viral vector development

Retroviral vectors are mostly derived from MLV. In first generation vectors, transgenes were inserted between LTRs in place of *gag*, *pol*, and *env* genes; the latter genes were provided *in trans* during viral packaging. Deletion of *gag*, *pol*, and *env* and other non-relevant sequences enable retroviral vectors to

support an expression cassette of up to 9 kb. In second generation retroviral vector packaging systems, *env* was separated from *gag* and *pol* to avoid recombination and creation of replication-competent particles. Thus recombinant retrovirus particles are generated by triple transfection with plasmids encoding the transgene, *gal/pol*, or *env*. More recent vector constructs use self-inactivating (SIN) vectors. SIN vectors, in which the enhancer or promoter of the LTR is deleted, decrease the risk of activating a nearby gene (Elsner & Bohne, 2017).

Lentiviral vectors are commonly derived from human immunodeficiency virus (HIV), feline immunodeficiency virus (FIV), and equine infectious anaemia virus (EIAV) (Durand & Cimarelli, 2011). Initially, lentiviral vectors were packaged using three plasmids: a vector plasmid with the transgene between the HIV LTRs, a packaging plasmid containing *gag*, *pol*, and the accessory genes, and a separate envelope plasmid with an *env* gene. To improve safety, the latest generation of lentiviral packaging systems uses four plasmids, one for *rev*, one for *gag* and *pol*, one for the transgene, and one for encoding the envelope. Furthermore, *tat* is eliminated through the addition of a chimeric 5′ LTR fused to a heterologous promoter on the vector plasmid (Elsner & Bohne, 2017). As the glycoprotein envelope determines the retroviral and lentiviral tropism, recombinant vectors are pseudotyped by using envelope glycoproteins derived from other enveloped viruses (Joglekar & Sandoval, 2017). Glycoprotein G of the vesicular stomatitis virus (VSV-G) is commonly used to pseudotype retrovirus and lentivirus, and interacts with a highly conserved membrane phospholipid for cell entry and therefore confers a broad range tropism for the vector. Many other envelopes can also be used, which can alter cellular tropism *in vivo* (Joglekar & Sandoval, 2017; Kobayashi et al., 2017)

Advantages and disadvantages

Retroviral and lentiviral vectors have a packaging size up to 9 kb, which is advantageous over AAVs. Moreover, VSV-G pseudotyped retrovirus and lentivirus vectors have broad tropism. Once the vector genome integrates into the host cell genome, long-term transgene expression can be achieved; however, in some cells, there can be silencing unless insulator elements are included in the transgene expression cassette design (Rivella, Callegari, May, Tan, & Sadelain, 2000). The main disadvantages for retroviral and lentiviral vectors is insertional mutagenesis at the integration site, which is caused by either disrupting or inappropriately activating transcription of a nearby host gene (Poletti & Mavilio, 2018). This is especially true for gamma retroviral vectors where regulatory elements are favored as a site of integration and can cause deleterious consequences (Modlich et al., 2008).

Usage

Retroviral and lentiviral vectors are traditionally used for *ex vivo* gene delivery for hematopoietic disorders (Doering et al., 2018), to treat inherited brain disorders (Rastegar et al., 2009), and to treat metabolic disease (Ungari et al., 2015). Lentiviral vectors have also been utilized to engineer T cells to combat leukemia; T cells transduced *ex vivo* with vectors expressing chimeric antigen receptors (CARs) allows for targeting and killing of cancer cells in patients after reimplantation (Miliotou & Papadopoulou, 2018). Recently, CRISPR-Cas9 has emerged as a promising tool for genomic editing. Lentiviral vectors, which feature a higher packaging capacity than AAVs, are capable of delivering Cas9 and the required guide RNAs needed for genome editing (Kabadi, Ousterout, Hilton, & Gersbach, 2014).

Adenoviral Vectors

Biology of adenovirus

Adenoviruses are non-enveloped and have an icosahedral protein capsid (~90 nm in diameter) encompassing a double-stranded DNA genome. The genomic DNA of Ad ranges from 26 to 45 kb and is flanked by two inverted terminal repeats (ITR) (Flint & Nemerow, 2017).

Adenovirus infection takes place upon binding of the adenovirus fiber protein to the coxsackie and adenovirus receptor (CAR) and the most commonly used serotype (Ad5) employs cell-surface integrin αVβ5 (Arnberg, 2009). Once endocytosed, the viral capsid is partially disassembled and then translocated to the nucleus using the dynein/dynactin motor complex (Scherer & Vallee, 2011). The viral capsid docks to the nuclear pore complex (NPC), and microtubule motor kinesin-1 disrupts both NPC-docked capsids and NPC, thus allowing viral genome nuclear access (Strunze et al., 2011). The Ad viral genome remains episomal in the host cell nucleus.

Development of adenoviral vectors

While >50 serotypes of human adenoviruses exist, most adenoviral vectors have been modified from Ad5. First generation Ad vectors had E1 and E3 genes deleted, allowing for an insert of up to 7.5 kb. While useful for robust transduction in to many tissues, expression of Ad vectors *in vivo* is generally transient due to the expression of non-deleted viral genes and ensuing immune responses to the expressed viral proteins (Brunetti-Pierri et al., 2004). Other serotypes have been employed to expand tropism (Zhang, Fu, & Ehrhardt, 2018).

More useful Ad vectors for sustained expression *in vivo* are known as helper-dependent adenoviral vectors or gutted vectors, in which all viral genes are excised from the vector backbone except for the ITRs and the packaging signals of the wild-type adenovirus. For both Ad vector types, helper virus functions are required to provide the viral proteins necessary for assembly of the viral particles *in trans*. An advantage of gutted vectors is that they result in long-term transgene expression without chronic toxicity and permit a large cloning capacity of 30 kb (Brunetti-Pierri & Ng, 2017).

In addition to helper-dependent first generation Ad or fully gutted Ad, replication competent (or oncolytic) Ads are used. The replication cycle of these viruses lead to an increase of viral copies, a process that results in destruction of the host cell and release of the newly synthesized viral particles into the surrounding area (Rosewell Shaw & Suzuki, 2016).

Advantages and disadvantages

The Ad vector has a large packaging size (up to 30 kb). The Ad vector genome is maintained as an episome in the nucleus and minimizes the integration of the viral genomic DNA into the host's genome, reducing the risk of insertional mutagenesis. Furthermore, Ad5 vectors efficiently transduce a broad range of target cells, including dividing and quiescent cells. The major drawback, however, is the strong innate immune response to the Ad capsid and to genes that are expressed in the first generation vectors. The inflammatory response in target and surrounding tissues results in toxicity to the host and loss of the transduced cells (Brunetti-Pierri et al., 2004). Strategies have been investigated to reduce the host's immune response, including the development of polyethylene glycol (PEG)-shielded Ad vector particles (Leggiero et al., 2013) and immunosuppression (Unzu et al., 2015).

Usage

Ad vectors have been widely employed as vaccine carriers since they are highly efficient vehicles for introducing foreign DNA into target cells and intrinsically induce host immune responses (Fougeroux & Holst, 2017). Furthermore, oncolytic or replication-competent Ad is a promising viral vector for cancer therapy (Rosewell Shaw & Suzuki, 2016).

In summary, viral vectors provide a valuable tool to deliver a genetic payload to desired cell types and tissues, for either transient or persistent expression. Their native host ranges can be augmented by pseudotyping, by engineering, and through alternative expression cassettes that restrict expression to a cell type of interest.

Conflicts of Interest

BLD is a founder of Spark Therapeutics, Inc. and Talee Bio, Inc., and is on the scientific advisory board of Sarepta Therapeutics, Homology Medicines, Prevail Therapeutics and Intellia Therapeutics. The content of this submission was not financially supported or reviewed by any of the aforementioned entities.

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