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Viral vectors: a look back and ahead on gene transfer technology

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SUMMARY

No matter what their origin, strain and family, viruses have evolved exquisite strategies to reach and penetrate specific target cells where they hijack the cellular machinery to express viral genes and produce progeny particles. The ability to deliver and express genetic information to cells is the basis for exploiting viruses as "Trojan horses" to genetically modify the natural cell target or, upon manipulation of the viral receptor to retarget the virus, to genetically engineer different cell types. This process, known as transduction, is accomplished using viral vectors derived from parental wild type viruses whose viral genes, essential for replication and virulence, have been replaced with the heterologous gene(s) required for cell manipulation. Rearrangement of the viral genome to impede replication or generation of infectious virions but maintaining the ability to deliver nucleic acids has been the object of intense research since the early 1980s. Technological advances and the ever-growing knowledge of molecular virology and virus-host cell relationships have constantly improved the safety profile of viral vectors that are now used in vitro and in vivo to study cellular gene function, correct genetic defects (gene therapy), express therapeutic proteins, vaccinate against infectious agents and tumors, produce experimental animal models, and for other purposes. This review illustrates the strategies used to generate some of the most used viral vectors, and their advantages, limitations and principal applications.

KEY WORDS: Viral vector, Gene transfer vector, Gene therapy, Genetic vaccine, Genetic disease, Gene editing.

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INTRODUCTION

The virus life cycle has two main steps: infection and replication. Infection starts with recognition of the target cell, proceeds with virus entry into the cell (and neutralization of various host defenses) to terminate with the release of the viral genome in a cell location suitable for replication. Replication starts with the expression cascade of viral genes to proceed with the synthesis of viral genome copies. Viral proteins and genomes are then conveyed to a specific "meeting point" where progeny virions are assembled and released from the cell, either by budding or lysis, to start a new infection process in a nearby cell or circulate in the bloodstream until they encounter a susceptible cell. Gene transfer technology relies on, and attempts to exploit, the first step of replication and, at the same time, builds blocks to prevent production of infectious virus. In this context, transduction is defined as a non-replicative or dead-end infection that allows heterologous (i.e. non viral) genetic information to be delivered to a precise cell. To do so, as explained below, the viral genome is radically rearranged to eliminate genes essential for replication and pathogenicity whilst making space for the heterologous genes. Following this makeup, the parental virus becomes a mere carrier of genetic information, hence the name viral vector.

Gene delivery can be used for different purposes. The most common are: functional gene studies

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(Kanvar *et al*., 2011; Kurth *et al*., 2012; Mac Gabhann *et al*., 2010), correction of genetic defects, expression of therapeutic proteins, and immunization against tumors and infectious agents (Edelstein *et al*., 2007; Rollier *et al*., 2011). Compared to most traditional vaccines, which preferentially elicit a humoral response, immunization by means of recombinant viral vectors also triggers a robust cytotoxic T lymphocyte (CTL) response (Chiuppesi *et al*., 2012; Alexander *et al*., 2012) that is particularly efficient in eliminating virus-infected cells, intracellular pathogens, and cancer cells, and extending protection to other strains of the same pathogen by recognizing highly conserved epitopes (Wei *et al*., 2010). Moreover, the understanding that many human diseases have a genetic basis, and the complete sequencing of the entire human genome (Stein, 2004) paved the way for the development of gene therapy strategies.

The idea of using viruses to deliver a package of genetic information is not new and the very first successful demonstration that gene therapy is indeed applicable to treat genetic diseases goes back to 1990 when a four-year-old girl was treated for adenosine deaminase deficiency (ADA), which causes a severe form of severe combined immunodeficiency (SCID). Transduction of her purified T-lymphoid cells with a retroviral vector carrying a functional copy of the ADA enzyme temporarily but successfully restored her immune system efficiency (Blaese *et al*., 1995). Following this success, a burst of trials took place and the race began to produce novel and perfect existing vectors. Ten years after the initial ADA report "Gene Therapy Clinical Trials Worldwide", the internet database of gene therapy clinical trials boasted 578 records in June 2012 (www.wiley.co.uk/genmed/clinical) and in the same period the PubMed database had posted more than 30,000 papers on viral vectors. However, the exponential phase subsided shortly after and, as a consequence of a few dramatic setbacks - an 18-year-old died from an erroneous dose of the therapeutic vector and four out of nine children developed leukemia due to insertional mutagenesis of a retroviral vector (Hacein-Bey-Abina *et al*., 2003) - and other unsatisfactory results, the number of clinical trials stabilized to about 100 a year and has dwindled in the last five years (www.wiley.co.uk/genmed/clinical). It is worth mentioning that most of these trials were performed on experimental animals, few of them were phase III/IV studies (below 4%) and most phase I/II studies did not proceed any further. More than 70% of these studies were carried out with viral vectors - a clear indication that these delivery systems outperform, or are considered to outperform naked DNA plasmids, lipofection, and other non-viral vectors (www.wiley.co.uk/genmed/clinical).

Besides the unexceptional results of most clinical studies, several viral and host factors hamper the progress of gene therapy. Most factors are partly unidentified and possibly distinct in different individuals (Barese and Dunbar, 2011), chief among them are the extra- and intracellular host defenses designed to halt the vector the host invariably sees as an infectious agent (Shayakhmetov *et al*., 2010).

This review describes the principal viral vectors, i.e. the viral delivery systems used in 90% of clinical trials. We will look back at their initial development, highlight subsequent improvements in safety and performances, compare advantages and disadvantages to other viral vectors, and look ahead by describing current and future trends for clinical applications.

Adenoviral vectors

Adenoviral vectors are derived from Adenoviruses (AdV), DNA viruses with a linear double-stranded genome (36 Kilobase pairs, Kbp), a non-enveloped icosahedral capsid with characteristic morphology replicating in the nucleus and producing thousands of progeny virions released by cell lysis. The viral genome encodes about 50 viral proteins, 11 of which are structural and used to physically build the virion. These viruses have been isolated from a large number of species, and in humans they primarily infect the respiratory airways and the gut causing mild and recurrent respiratory and gastroenteric diseases. Human AdVs are organized in more than 40 unevenly prevalent serotypes. Some serotypes are widespread, such that about 80% of healthy people have antibodies against one or more of these serotypes by six years of age, others circulate occasionally (Davison *et al*., 2003).

Because of their low pathogenicity, infectious properties, wide tropism, high level of expression of viral proteins during replication, and the nat-

ural delivery of the viral genome in the nucleus, these viruses have been considered potential candidates for gene therapy since its inception. Unfortunately, such remarkable features are partially obscured by the broad pre-existing immunity in the population that, as described below, prevents the use of vectors derived from the most common serotypes. Furthermore, the high immunogenicity of AdV proteins severely limits the number of vector administrations in the same patient. In fact, the risk of anaphylactic shock upon activation of the complement system, innate immunity, and/or pre-existing immunity increases proportionally with the number of administrations and is highly probable after the third inoculum. To circumvent this problem, AdV are mostly derived from rare serotypes that are also associated with mild infections.

AdV vectors have undergone progressive engineering. In first generation vectors the early gene 1A (E1A), a regulatory gene essential for replication, was deleted. Like most viral vectors derived thus far, deletion of crucial gene(s) abolishes replication competence and, at the same time, makes room for the transgene(s) (Graham and Prevec, 1995). To further reduce the risk of reversion to replication competence, which can occur following recombination with other viral or cellular genome components, E1B and E3 genes that play an important role in modulating AdV-specific immunity were also deleted in subsequent generation vectors (Campos and Barry, 2007). These deletions made room for cloning up to 8 Kbp of foreign DNA (Table 1). It is likely that future generations will bear even more deletions and the capacity of AdV vectors to transport heterologous nucleic acids will further increase (Campos and Barry, 2007).

Like all replication-incompetent vectors and viruses, which lack gene(s) essential for productive replication, viral particles can only be constructed in the presence of a helper virus or DNA constructs that provide the missing functions *in trans*. Vector particles are generated in specific packaging cells, generally adherent cell cultures easy to transfect and manipulate, that are transfected with the vector construct and a helper virus or a DNA construct that provides missing genetic information. For AdV vectors, the missing genes are provided by a single DNA plasmid, called the packaging construct, which contains nearly the whole viral genome except the genomic region necessary for encapsidation of the viral genome into progeny particles (Figure 1). As an advantage over other vectors, the AdV vector can also be produced with ready-to-use packaging cells that stably express the structural and regulatory proteins required for virion assembly.

Notwithstanding the progressive deletion and inactivation of genes causing inflammation or stimulating the host immune response, the AdV vector has the unenviable record of having caused the death of a young patient with ornithine transcarbamylase deficiency. This 18-year-old male succumbed to multiple organ failure four days after a hepatic arterial injection of the therapeutic vector. Autopsy revealed that death was caused by a severe anti-AdV immune response triggered by an excessive inoculation dose of the vector (Raper *et al.*, 2003). This dramatic event put an abrupt stop to these procedures and a general rethinking of the approach. For instance, to cir-

TABLE 1 - *Advantages and disadvantages of adenovirus vectors.*

Advantages	*Disadvantages*
Transduce non-dividing and dividing cells	Highly immunogenic
Carry up to 8 Kbp heterologous DNA	The vector genome does not integrate into the host cell genome
Ensure high levels of transgene expression	Transient expression of the transgene
Well suited as oncolytic vector	High levels of pre-existing immunity
Vector particles produced at high titers (10^{10} pfu/ml*)	

*Number of plaque forming units/ml.

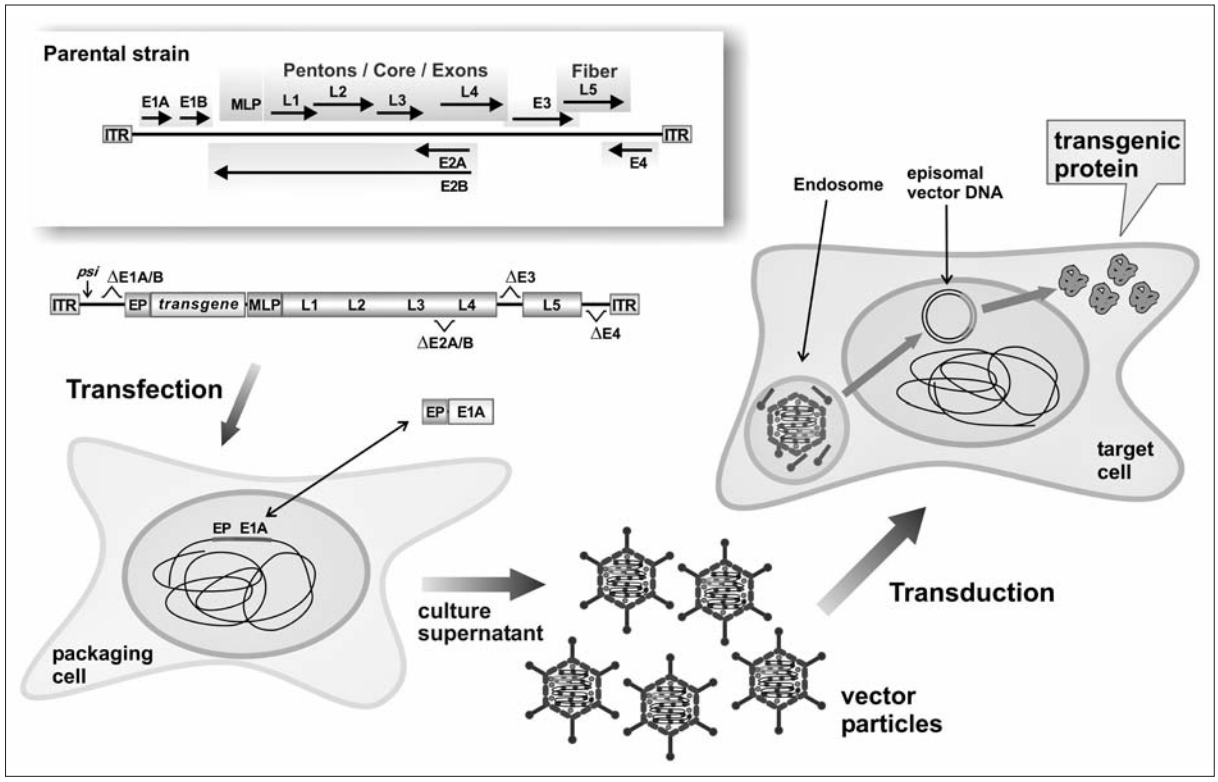

FIGURE 1 - *Production of and transduction with an adenoviral vector. Early (indicated with an E) and other regulatory genes are deleted from the parental strain, usually of genotype 2 or 5, which have lower pre-existing immunity in the population, whereas most structural genes and their relative promoter MLPs (major late promoters) are retained. The transgene and respective eukaryotic promoter (EP) replaced early E1A/E1B genes. Vector particles are produced in packaging cells transfected with the DNA vector and E1A. E1A is provided in trans either by co-transfection of a separate plasmid or stably expressed by the transfected cells. The vector particles released in culture supernatant are collected, purified from contaminant cells and debris and used to transduce the target cells. Here, the vector penetrates the cells by endocytosis and releases the vector DNA in the nucleus where the expression of the transgene takes place. The vector DNA remains as an episome and the transgene expression is transient (modified from Pistello, 2012).*

cumvent the broad pre-existing herd immunity (Table 1) current efforts are focused on:

1. development of AdV derived from parental viruses that rarely circulate in humans;
2. insertion of "gutless" sequences isolating and blocking expression of the viral genes harbored by the vector;
3. elimination of as many viral genes as possible.

In fact, current generation AdV vectors are devoid of most viral genes, contain only the terminal repeats and the encapsidation signal, and are generated with packaging cells that stably express most, if not all, viral genes necessary for virion assembly (Wang *et al.*, 2009). Finally, to avoid over stimulation of the immune system, current clinical trials devised alternate inoculations, whenever possible, of vectors derived from different AdV serotypes.

Initial studies with AdV vectors focused on the respiratory tract, one of the main targets of AdV infection. Subsequently and thanks to precise engineering, AdV proved able to infect a great variety of post-mitotic cells, including cells present in highly differentiated tissues such as skeletal muscle, lung, brain and heart (Howarth *et al.*, 2010). The AdV vector transduces both replicating and quiescent cells. This is an important feature and a plus since the majority of cells in our tissues are non-dividing and mostly refractory to transduction. Conversely, the main disadvantage compared to integrating vectors is that the AdV DNA vector persists as an episome in the trans-

duced cells. Thus, transgene expression is transient and particularly vulnerable to cell silencing mechanisms. Finally, the episome is diluted by duplication of transduced cells. However, lack of integration, as discussed below, is also an advantage as it increases the safety profile. AdV vectors find their primary application in vaccination and clinical trials in which transient rather than stable expression of the transgene is pursued. To achieve durable expression, an active line of research aims to produce chimeric retroviral and AdV vectors that look promising (Kaufmann and Nettelbeck, 2012; Kubo *et al*., 2010), but there is still a long way to go before these chimeric vectors are deployed in clinical settings.

Vaccination aside, the AdV vector excels as an oncolytic vector, i.e. vectors that primarily transduce tumoral cells causing apoptosis, direct cell death or increasing the sensitivity of cancer cells to anti-tumoral drugs. Gutless AdV proved highly safe and effective against glioma, a brain tumor highly resistant to most treatments and with a very poor prognosis (Castro *et al*., 2012; Kaufmann and Nettelbeck, 2012). AdV vectors were also successfully used against other solid tumors. These vectors were engineered *ad hoc* to eliminate the E1B gene that sequesters p53 to block apoptosis. To reduce the risk of adverse effects vectors were derived from AdV rare serotypes and patients were transiently immunodepressed before treatment (Wang *et al*., 2008).

Adeno-associated viral vectors

Adeno-associated viruses (AAV) are small, icosahedral non-enveloped viruses that belong to the genus Dependovirus, family *Parvoviridae*. To replicate, they necessitate co-infection with a helper virus to complete their replication cycle.

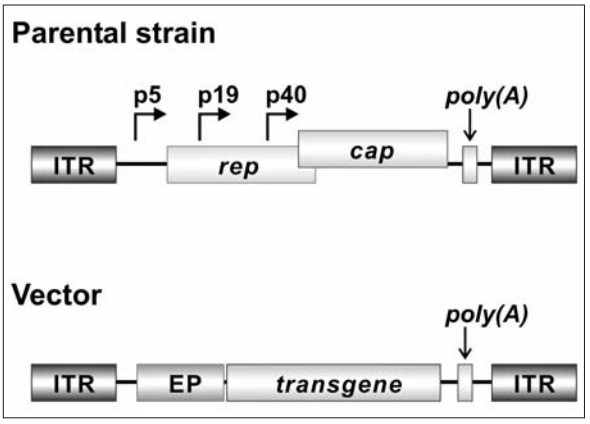

FIGURE 2 - *Genomic organization of a vector derived from an adeno-associated virus (AAV). The parental genome mostly consists of rep and cap encoding replicase and structural proteins. Expression of the AAV proteins is driven by promoters p5, p19 and 40 and all transcripts share the same poly(A). Both poly(A) and inverted terminal repeats (ITR) are maintained in the vector genome. By contrast, rep and cap are replaced by the transgene and the respective eukaryotic promoter (EP) (modified from Pistello, 2012).*

Initially, hence the name, it was thought that AAV depended on AdV. Then it was found that herpes simplex virus (HSV) also helps AAVs to carry out productive infection and replication. Indeed, rather than a specific, missing viral function, AAV complete replication only when the cell is activated by co-infecting AdV or HSV or a genotoxic agent. Under non-permissive conditions, the AAV genome integrates into the q arm of human chromosome 19, where it remains silent until rescued by a helper virus that also induces an AAV lytic cycle. The AAV genome is small (less than 5 Kbp) and contains only two genes: *rep*, replicase, required for viral genome replication; and *cap*, en-

TABLE 2 - *Advantages and disadvantages of adeno-associated virus vectors.*

Advantages	*Disadvantages*
Transduce non-dividing and dividing cells	Carry up to 5 Kbp heterologous DNA
Parental virus apathogenic	High vector titers difficult to achieve
Wide cellular tropism	Need co-infection by helper virus (adenovirus or herpes simplex virus)
Potential site-specific integration	
Low immunogenic	

coding the structural proteins. *Rep* and *cap* genes are flanked by short inverted terminal repeats (ITRs) (Figure 2). AAV infection is highly prevalent but is not known to cause disease and induces mild immune responses. Low immunogenicity, site-specific integration, and the ability to infect dividing and quiescent cells in vitro and in vivo make AAV an attractive candidate for gene delivery (Kay *et al*., 2001; Giacca and Zacchigna, 2012) (Table 2). AAV vectors contain the ITRs that encompass the heterologous DNA (Figure 2). To generate the vector particles, missing *rep* and *cap* are provided *in trans*, often with separate plasmids. Initially, the helper function was provided by a co-infecting AdV then, seeing the difficulty of completely removing the helper virus from vector preparation, AdV was replaced by a plasmid with the missing genes (Allen *et al*., 2000).

The AAV vector also has some disadvantages. The cloning capacity is limited and unsuitable for most therapeutic genes. AAV requires conversion of the single-stranded AAV DNA genome into double-stranded DNA before gene expression can start, thus making AAV vectors "too slow" for some in vivo applications (Coura Rdos and Nardi, 2010), and site-specific integration is lost following Rep deletion (Smith, 2008). To overcome this limitation, hybrid AAV-HSV vectors were produced (Glauser *et al*., 2006) and manufactured for clinical studies (Clément *et al*., 2009). Despite these drawbacks, AAV vectors efficiently transduce a great variety of dividing and non-dividing cells including muscle cells, peripheral and central nervous system cells, hepatocytes, etc., and boast about 100 gene therapy trials mostly treating monogenic diseases (Grieger and Samulski, 2012).

Herpes virus vectors

Herpes virus vectors mainly derive from HSV type-1, a neurotropic large DNA virus (152 Kbp, double-stranded DNA) that comprises more than 80 genes categorized into essential and non-essential genes according to their requirement for viral replication. In its natural life cycle, HSV-1 is spread by contact, infects and replicates in skin or mucous membranes, and is taken up by sensory nerve terminals where it establishes a latent state from which the virus can subsequently reactivate and spread to other individuals (Roizman *et al*., 2007). These features, high infectivity and ability to transduce and persist in dividing and non-dividing cells make the HSV vector a good candidate for gene transfer. The virus contains many non-essential genes involved in subtle interactions with the host cell, decoying the immune system, creating conditions for viral persistence in specific body sites and other functions that, from the vector point of view, are useless or even detrimental, and are therefore removed during vector construction. Removal of non-essential genes makes room for up to 50 Kb heterologous DNA thus making the HSV vector the largest carrier among the viral vectors (Table 3). Another unique feature of HSV vectors is the genetic complexity of the virus genome that has allowed different types of attenuated vectors possessing oncolytic activity to be generated, selectively replicating in and killing cancer cells, or able to invade and persist lifelong in neurons from where the transgenes can be strongly and persistently expressed (Marconi *et al*., 2009).

Currently, three different classes of vectors are derived from HSV-1:

1. replication-competent attenuated vectors;
2. replication-incompetent recombinant vectors;
3. defective helper-dependent vectors known as amplicons.

The first class is obtained by deleting genes not essential for replication but important for pathogenicity. The replication-incompetent vectors have been created by deleting one or more immediate-early genes, which are provided *in trans* by a replication-competent HSV strain, a plasmid construct or, in a few instances, are constitutively expressed by the packaging cell line. The third class, the amplicon, is the safest as it carries minimal viral sequences, has low cell toxicity and immunogenicity. Amplicons are produced by means of a plasmid vector containing the transgene(s) and HSV origin of replication and encapsidation signal. The plasmid was transfected and complemented by packaging cells in a way that has changed over the years and, from older to newer, as follows:

1. infection of packaging cells by a helper virus, usually a replication-defective HSV-1 virus devoid of one or more key viral genes and providing the functions necessary for incorporating the amplicon into a viral particle;
2. development and use of helper virus-free packaging systems. Here a DNA packaging construct provides amplicon missing functions

TABLE 3 - *Advantages and disadvantages of herpes simplex virus vectors.*

Advantages	*Disadvantages*
Wide cellular tropism	Possible residual cytotoxicity
Carry up to 50 Kbp heterologous DNA	The vector genome does not integrate into the host cell genome
Natural tropism for neuronal (HSV-vectors) or B lymphoid cells (EBV vectors)	Transient expression of the transgene
Well suited as oncolytic vector	Risk of recombination with latently herpes simplex virus-infected cells
Vector particles produced at high titers (10^{12} pfu/ml*)	High levels of pre-existing immunity

*Number of plaque forming units/ml.

(Marconi *et al.*, 2010). Compared to the first strategy, the helper virus-free systems reduced the risk of recombination and generation of wild-type particles and eliminated the problem of purifying vector particles from contaminating virions generated by the helper virus;

3. use of separated packaging constructs and/or cells stably expressing some HSV immediate early genes. This strategy is the safest but requires further optimization and refinement as it produces low vector titers, often too low to test the clinical potential of amplicon vectors. A comprehensive and up-to-date description on HSV-1 derived vectors and amplicons is available elsewhere (de Silva and Bowers, 2009; Manservigi *et al.*, 2010).

HSV-1 is endemic and more than 70% people have a specific immune response that, due to the intermittent reactivation of infection, is maintained active and at high levels in most individuals. Thus, the main obstacle in the use of HSV vectors is the pre-existing immunity that efficiently inactivates vector particles and eliminates transduced cells that expose on their surface HSV proteins encoded by the vector or encapsidated in the particle (Table 3). Another safety concern is the presence of latently infected cells that may be transduced and offer a suitable environment for the HSV vector to recombine with the wild-type genome (de Silva and Bowers, 2009; Marconi *et al.*, 2009).

Expression of HSV antigens by transduced cells and residual toxicity have severely limited the range of application of HSV vectors.

Paradoxically, these disadvantages are considered a bonus as therapeutic agents for cancer. If appropriately targeted, HSV vectors provide a direct and indirect mechanism to wipe out cancer cells. Recent studies with replication–conditional vectors, i.e. modified in such a way to replicate only in dividing cells, demonstrated that these vectors are highly toxic to some proliferating tumors (Agarwalla and Aghi, 2012). Other pre-clinical studies in experimental animals showed that to some extent and with some challenges that need to be addressed oncolytic vectors could be injected into the bloodstream to treat solid tumors (reviewed in Friedman *et al.*, 2012; Wong *et al.*, 2012) and visualize cancer metastases (Brader *et al.*, 2012). Because of their inherent ability to target the central nervous system, HSV vectors have been successfully used against gliomas and glioblastomas as well as melanoma, ovarian cancers, and other solid tumors of different histotype (Lentz *et al.*, 2012; Goins *et al.*, 2012; Marconi *et al.*, 2010) Finally, to further increase toxicity, most HSV oncolytic vectors encode HSV thymidine kinase (TK). TK is activated by acyclovir phosphorylation, a nucleoside analogue used to treat HSV infections. The acyclovir triphosphate binds to viral and cellular DNA polymerase and is incorporated in the nascent DNA chain causing its premature arrest. This halts HSV replication, interrupts cellular DNA synthesis and leads to cell apoptosis. A combination of TK-oncolytic vectors and acyclovir therapy has been shown to enhance the toxic activity against some malignances (Pulkkanen and Yia-Herttuala, 2005; Wong *et al.*, 2012).

To circumvent important limitations in gene therapy, HSV vectors have undergone extensive tailoring. Most immediate-early genes, which activate and regulate the gene expression cascade during viral replication and block several host defenses, were removed from the vector to reduce cytotoxic activity and host immune response to viral gene products (Grant *et al*., 2009). As a result, current HSV vectors persist longer in transduced cells, which are spared from immune-mediated destruction. Despite remaining as an episome, the vector genome expresses the transgene(s) for as long as 3 weeks in cell culture and 1 month in vivo (Lentz *et al*., 2012).

A good outcome and safety profile observed in several animal models prompted clinicians to test ad hoc designed HSV vectors against chronic pain (Wolfe *et al*., 2009), rheumatoid arthritis (Burton *et al*., 2001), and several neurological disorders (Goins *et al*., 2012). HSV vectors also proved beneficial and conferred protection against neuron degeneration in a rat model of Parkinson's disease. Thanks to ample cargo capacity, the vector used in these studies expressed the anti-apoptotic peptide Bcl-2, a glial cell-derived neurotrophic factor, a brain-derived neurotrophic factor, and a few enzymes of the dopamine biosynthesis pathway (Sun *et al*., 2005).

A common trait of herpesviruses is their ability to persist in the infected cells. From this standpoint, other herpesviruses naturally infecting other cell types are currently investigated as potential vectors. Epstein-Barr virus (EBV) infects and immortalizes B-lymphocytes. Vectors derived from EBV were used as therapeutic agents for B-cell leukemia/lymphoma cancer, the most common leukemia in the Western world that is largely refractory to conventional therapies. Here the vector was used to transduce the tumoral cell and stimulate the maturation of autologous dendritic cells that, in turn, potentiated immune responses against leukemic cells (Hellebrand *et al*., 2006). In all, these results demonstrate that wide tropism, plasticity and the capacity to transport long stretches of heterologous DNA, make HSV vectors potential tools to treat a wide range of diseases.

Retroviral vectors

Retroviruses are enveloped viruses with a capsid enclosing two copies of a single-stranded, positive sense RNA of 7-11 Kbp. Basically, the retroviral genome has two long terminal repeats (LTRs) at 5' and 3' extremities and encompasses three large open reading frames called *gag*, *pol* and *env*. The LTRs act as promoters and regulate the expression of *gag*, *pol* and *env* that encode the capsid proteins, replication enzymes, and envelope glycoproteins, respectively. Concerning the viral life cycle, once the capsid is inside the host cell, the viral RNA genome is converted to double-stranded DNA by reverse transcriptase, the double-stranded DNA is transported in the nucleus, circularized and eventually integrated into the host cell genome. Integration is more or less random (Wu and Burgess, 2004) and permanent. For this reason and the fact that retroviruses - and retrotransposons, lines, sines, etc. - transduce the infected cells naturally, retroviruses have always been thought of as natural delivery systems and apt tools to permanently modify transduced cells. This idea was strengthened by the observation that except for LTRs and a few viral domains, the remaining viral genome can be deleted to make room for exogenous DNA. In fact, Gag, Pol, and Env are provided *in trans* by one or, usually, two separated plasmids (Figure 3). As mentioned above, the idea of providing viral genes with separate constructs was pursued to minimize the risk of recombination and generation of retroviral-competent particles. For retroviral and lentiviral vectors, however, this technique has also been exploited to retarget the vector, i.e. to modify the spectrum of infectable (or transduceable) cells. This strategy, called pseudotypization, is pursued by swapping the homologous Env with Envs of viruses with diverse, either broaden or restricted, spectra of infection compared to parental retrovirus or lentivirus (Figure 3).

Deletion of *gag*, *pol*, *env*, and other non-relevant sequences enables retroviral vectors, whose parental genome is approximately 9 Kbp in length, to support up to 8 Kbp heterologous DNA. As occurs in true infection, once the vector particle enters the cell its genome is reverse transcribed, transported into the nucleus and integrated into the host genome. Integration thus leads to permanent transduction and stable inheritance of the transgene(s) by the daughter cell. This feature, shared by few other vectors, is an important advantage and, at the same time, a major safety concern (Table 4). Since complemen-

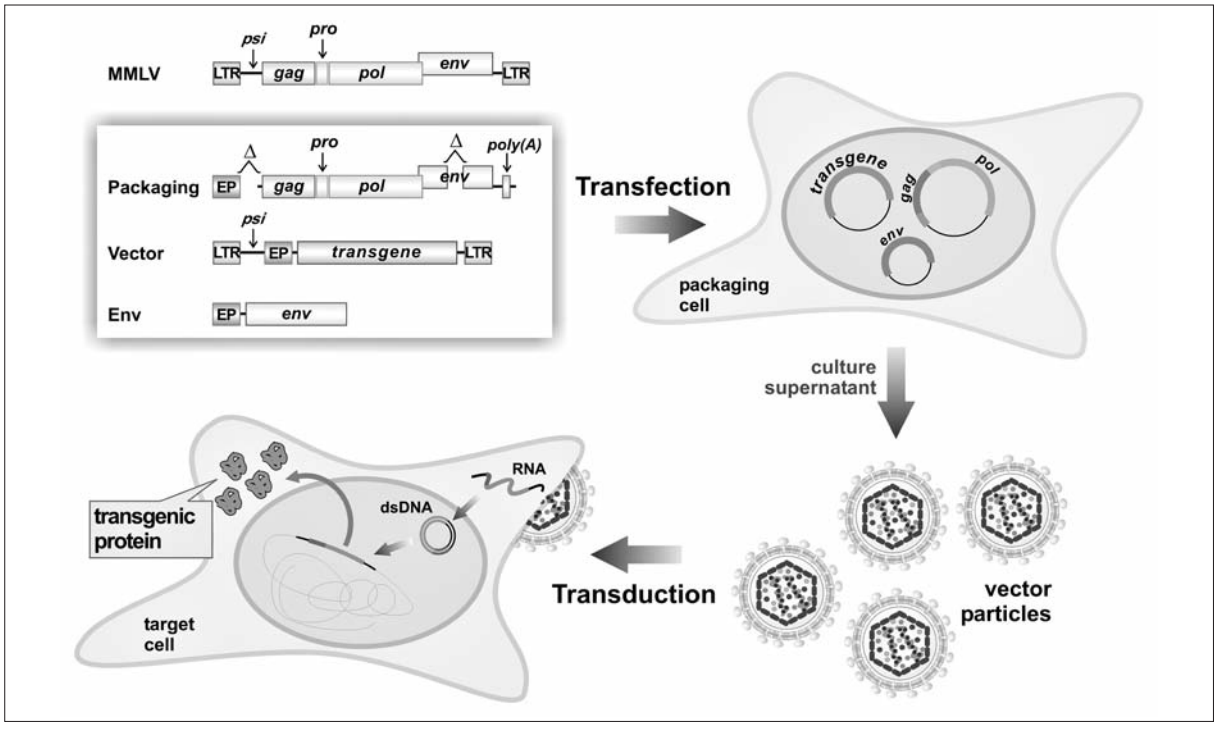

FIGURE 3 - *Production of and transduction with a retroviral vector. Vector and packaging constructs are derived from the parental Moloney murine leukemia virus (MMLV). gag, pol, and env are deleted from the vector; the packaging construct was obtained by total deletion of the encapsidation signal (psi) and partial deletion of env. In first generation vectors, the transgene expression was driven by the long terminal repeats (LTRs). In subsequent generations, the transgene was placed under the control of a separate eukaryotic promoter (EP). In first generation systems, env was provided together with gag and pol by the packaging construct. In subsequent generations, to avoid recombination and creation of replication-competent particles, env was provided in trans. Vector particles are generated by transfecting the vector, packaging and env DNA plasmids in eukaryotic cells (packaging cells). The vector particles released in culture supernatant are collected, purified from contaminant cells and debris and used to transduce the target cells. After fusion and entry of the vector particle into the target cell, the vector RNA is converted into double-strand DNA (dsDNA) and integrated into the host cell genome. The provirus will then stably express the transgenic protein (modified from Pistello, 2012).*

TABLE 4 - *Advantages and disadvantages of retroviral vectors.*

Advantages	*Disadvantages*
The vector genome integrates into host cell genome	Transduce only replicating cells
Carry up to 8 Kbp heterologous DNA	Cellular targeting difficult to achieve
Engineering fairly simple	Unsuitable for non-replicating cells
Wide cellular tropism	Random integration of the retroviral genome
Low immunogenic	High risk of insertional mutagenesis
No (or very low) pre-existing immunity	Low stability
Vector particles produced at high titers (10^6-10^8 pfu/ml*)	

*Number of plaque forming units/ml.

tarity between sequences is not necessary, it was thought that integration of the retroviral DNA genome (or provirus) occurred in random fashion. It was then discovered that retroviruses integrate preferentially, and understandably, in euchromatin, e.g. less condensed and actively transcribed DNA regions, rather than archived, DNA transcriptionally inactive, and highly condensed heterochromatin (Cereseto and Giacca, 2004; Engelman, 2003). Integration in euchromatin may then have different outcomes: the most probable is an invisible effect, i.e. the retroviral genome integrates outside a cellular coding sequence or inside an irrelevant (for neoplastic transformation) gene; death of transduced cells if integration interrupts a vital gene; neoplastic transformation that may result from integration of the provirus within or in close proximity to a proto-oncogene, a cellular gene that regulates cell replication. If integration takes place within the coding sequence, the gene may either produce a truncated protein or a viral-cellular chimeric protein. Conversely, if integration occurs outside the gene but close enough for LTRs, which are strong gene promoters, to deregulate physiological gene expression, the altered gene expression profile may then trigger the molecular cascade that transforms the cell. Since retroviral vectors have few remnants of the parental viral genes, generation of fused proteins is a remote possibility. By contrast, both parental virus and retroviral vector preferentially integrate close to cellular gene promoters (Lewinski *et al*., 2005), thus implying an innate ability to perturb the genomic region flanking the integration site (Cereseto and Giacca, 2004). A good example in this context is a clinical trial carried out a few years ago that employed a retroviral vector derived from Moloney murine leukemia virus (MMLV). The patients were nine children with SCID-X1, a severe combined immunodeficiency disease linked to the X-chromosome and caused by a single mutation in the interleukin-2 receptor subunit gamma (IL2RG) gene. IL2RG encodes for the cytokine receptor common chain, a critical functional component of the receptor for many interleukins, and its disruption manifests in SCID-X1 with a complete lack of T cells and natural killer cells (Leonard, 2001). Patients were treated with their autologous stem cells transduced ex vivo with the MMLV vector encoding a functional IL2RG copy (Cavazzana-Calvo *et al*., 2000). The clinical outcome was a remarkable success, the genetic defect was corrected in eight out of nine patients and the transduced cells were still present after ten years. Seven patients had sustained normal thymopoiesis, a nearly normal T cell repertoire, and improved patient health due to reduced immunodeficiency (Hacein-Bey-Abina *et al*., 2010). Sadly, this clinical trial is mostly known as a demonstration that gene therapy can provoke disasters and that retroviral vectors, despite extensive engineering, maintain their oncogenicity. Four of the nine patients developed acute leukemia and one died. The remaining three patients responded favorably to chemotherapy and fully recovered from leukemia. Restored immunologic functions were not affected by chemotherapy (Hacein-Bey-Abina *et al*., 2003). These results were consistent with two similar trials carried out previously in which of the 20 patients treated five developed leukemia associated with oncogene transactivation by the retrovector's transcriptional control elements (Hacein-Bey-Abina *et al*., 2008; Howe *et al*., 2008). Yet, the clouded outlook on gene therapy, which emerged soon after disclosure of the adverse effects, was progressively replaced by a more optimistic vision thanks to the obvious benefits of these trials. This prompted huge efforts to improve the safety of retroviral vectors and in particular to devise strategies to abate the activity of LTR promoters at proviral level. The two most successful strategies are the entrapment of promoter activity with insulator sequences inserted downstream from the LTRs (D'Apolito *et al*., 2009; Manic *et al*., 2012) and the use of self-inactivating (SIN) vectors. SIN vectors are produced by means of a plasmid construct containing a deletion within the U3 region of 3'LTR. This deletion completely inactivates the 3'LTR. During reverse transcription, which uses both viral RNA copies to synthesize double stranded DNA, the 3'LTRs deletion is "copied" in the 5'LTR. As a result, the integrated provirus has both LTRs partly deleted and is, therefore, completely inactive (Ellis, 2005; Yi *et al*., 2005). This straightforward and quite effective strategy has become common practice in the latest generation retroviral vectors and is also used for lentiviral vectors (see below).

Considerable advances have been achieved since the inception of retroviral vectors that, as men-

tioned, were considered an obvious choice for gene transfer. The added drawbacks of retroviruses are their low immunogenicity and the absence (or very low) pre-existing immune population (Table 4). Yet, retroviral vectors and gene therapy never blossomed. Retroviral vectors never emerged from the shadow of safety concerns over residual oncogenicity and random integration. Further, and unlike their close relatives the lentiviruses, retroviruses are unable to infect (or transduce as vectors) non-dividing cells (Yamashita and Emerman, 2006). Retroviruses lack the pre-integration complex present in lentiviruses and allowing viral double stranded DNA to cross the nucleic membrane, enter the nucleus and integrate into the host cell genome (Figure 3). Integration can occur in replicating cells that during mitosis have their nucleic membrane dissolved and thus the genomic DNA fully accessible (Suzuki and Craigie, 2007). Since most cells in the body are quiescent or divide occasionally, this is an important limitation for retroviral vectors (Urban and Merten, 2011).

Lentiviral vectors

Lentiviruses and retroviruses are closely related. However, lentiviruses can be considered advanced or more complex retroviruses due to the presence in their genome of several regulatory genes beyond canonical common retroviral genes. These regulatory genes have different and highly specialized functions and act in concert to neutralize host cell defenses, blunt immune responses, and regulate viral replication. Some of these proteins are synthesized during viral replication while others are packaged in the infecting particles and intervene in the very early stages of viral replication, for instance, to assemble together with a few cellular proteins to form the pre-integration complex and deliver the reverse transcribed viral genome in the nucleus of non-dividing cells (Bukrinsky and Haffar, 1999; Freed, 2001).

Lentiviral vectors and particularly those derived from human immunodeficiency virus (HIV) are quite effective delivery systems. Upon appropriate modifications, they target a huge variety of cells including quiescent and difficult-to-transduce cells such as hematopoietic precursors, neurons, lymphoid cells, macrophages, and others (Dropuli, 2011; Howart *et al*., 2010; Kay *et al*., 2001; Matrai *et al*., 2010). Even if the engineering and production of a lentiviral vector is generally more complicated than it is for retroviral vectors - some regulatory genes are essential to vector production and function and have either to be provided by the vector, packaging construct, or a separate DNA plasmid - lentiviral vectors are versatile and can deliver up to 9 Kbp heterologous DNA organized in one or more genes. Furthermore, lentiviruses have a low risk of insertional mutagenesis and oncogenicity as they tend to integrate away from cellular promoters (Cereseto and Giacca, 2004; Ciuffi, 2008) and have LTRs with low basal and inducible promoter activity. HIV LTR activity, for instance, increases by more than two orders of magnitude in the presence of HIV viral encoded Tat (Freed, 2001; Tripathy *et al*., 2011).

Other advantages of lentiviruses are the absence of pre-existing immunity in the human population, and the availability of non-human lentiviruses, which do not infect or are apathogenic in hu-

TABLE 5 - *Advantages and disadvantages of lentiviral vectors.*

Advantages	*Disadvantages*
Transduce non-dividing and dividing cells	Possible insertional mutagenesis
The vector genome integrates into host cell genome	Presence of regulatory proteins (tat, rev, and others) in the packaging construct
Carry up to 9 Kbp heterologous DNA	Transient expression of the transgene with integration-defective vector
Prolonged expression of the transgene	
Integration-defective vectors available	

mans but can transduce human cells upon appropriate manipulation (Table 5).

Lentiviral vectors and in particular HIV-derived vectors have undergone extensive changes over the years and three major generations can be distinguished (Figure 4). Vector and packaging constructs were revised to minimize the chances of recombination and generation of replication-competent viruses and reduce the adverse consequences of proviral integration. The first issue was practically solved with the split-component system that produces vector particles by means of

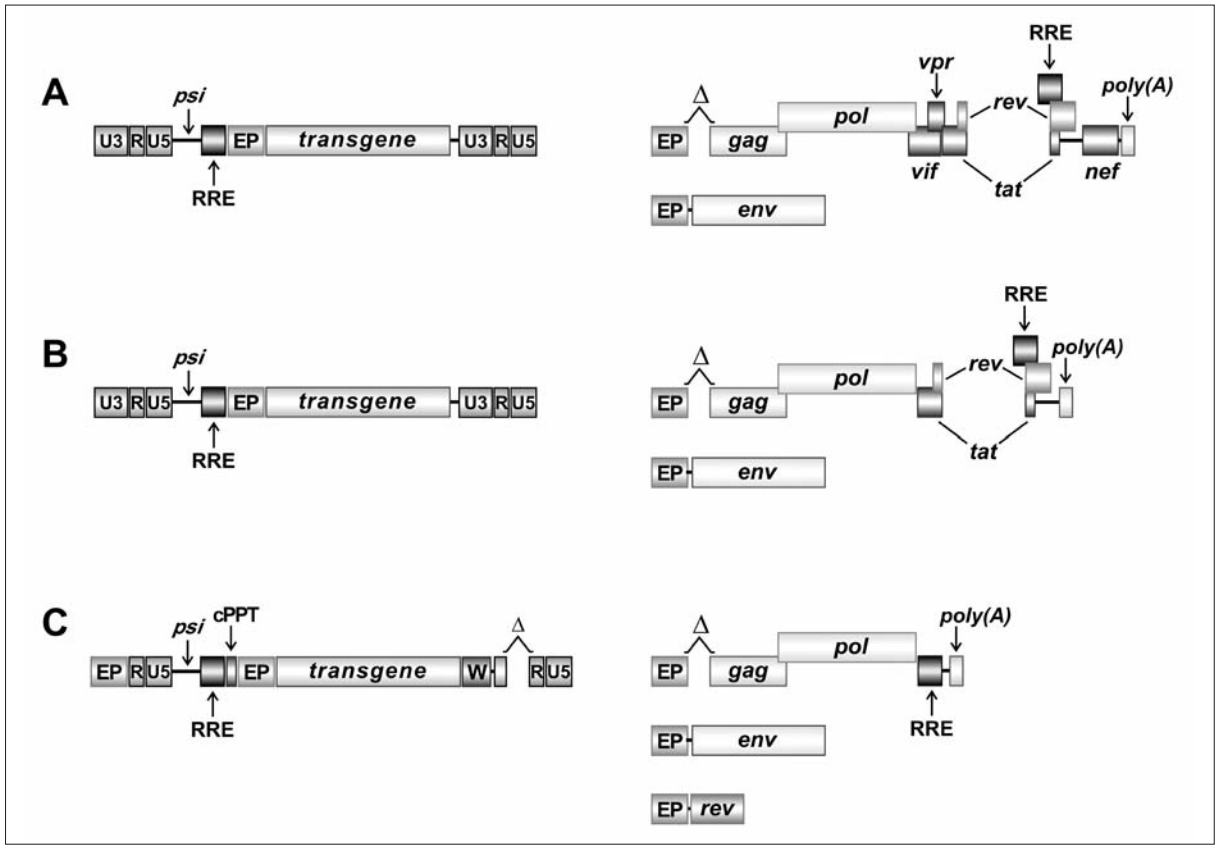

FIGURE 4 - *Vector (shown on the left), packaging and env (right) constructs used to produce first (A), second (B), and third (C) generation lentiviral vectors. To minimize the chances or recombination and insertional mutagenesis, which may lead to the generation of infectious particles and neoplastic transformation, respectively, viral proteins and functions are encoded with separate constructs. (A) First generation system. The vector maintains functional long terminal repeats (LTRs), the ecapsidation signal (psi) and the Rev-responsive element (RRE) necessary for Rev-driven nuclear-cytoplasmic exportat of viral messenger RNAs. The packaging construct retains all viral genes except the encapsidation signal and env. Env is provided in trans. (B) Second generation system. Compared to the previous generation, the vector does not have major changes while all regulatory genes except tat and rev are further deleted from the packaging construct. Tat is necessary for LTR-driven transcription of viral RNA and, as mentioned, Rev is required to export the viral messenger RNAs to the cytoplasm. (C) Third generation system. The vector is a self-inactivating (SIN) type. This is achieved by deleting the U3 region of 5'LTR in the vector DNA construct. U3 deletion abolishes 5'LTR promoter activity that is supplied by a eukaryotic promoter (EP) inserted in place of U3 and drives the expression of the vector RNA in the packaging cells. During reverse transcription in transduced cells, the U3 deletion and, therefore, inactivation of 3'LTR is transferred to 5'LTR. The converted double-strand DNA and integrated provirus thus have both LTRs inactivated. The vector also contains uncoding domains such as the central poly-purine tract (cPPT) and the woodchuck hepatitis post-transcriptional regulatory element (W) that increases the efficiency of vector RNA encapsidation and post-transcriptional processing of transgene RNA, respectively. Env and all regulatory genes including tat and rev are deleted from the packaging construct. Tat is no longer necessary since, as mentioned above, the RNA expression vector is no longer driven by LTR, rev is provided in trans with a separate construct (modified from Pistello, 2012).*

separate constructs providing, as mentioned above, the required structural and regulatory proteins (packaging and *env* constructs) and the vector RNA genome (a third construct). Vector and packaging constructs have undergone extensive modifications since the original description of the split-component system (Dull *et al.*, 1998). First generation packaging contained basically the whole genome except a deletion in *env*. This first generation vector had most viral sequences deleted except the encapsidation signal and Rev recognition element (RRE) necessary to export vector and unspliced packaging mRNAs from the nucleus to the cytoplasm (Freed, 2001). The exporting protein, Rev, is viral codified and provided by the packaging. The second generation was characterized by minimal modifications to the vector and complete removal of *env* and some regulatory genes not essential for vector production. The third generation also brought major changes. The vector was SIN type and bore the polypurine central tract, a short sequence stretch that improves the efficiency of encapsidation. All regulatory genes including *tat*, useless with LTR inactivated vectors, and *rev* that is now provided *in trans* were deleted from the packaging. Both packaging and vector retained the RRE that was displaced from the original location to a position often determined empirically that improved exportation of packaging mRNAs and vector RNA (Figure 4).

Pseudotypization has also improved. Use of homologous or heterologous Env allows transduction of the natural target of infection or retargeting the vector to specific cells, respectively. For retargeting, an apt approach is to pseudotype with the glycoprotein G of the vesicular stomatitis virus (VSV-G). VSV-G interacts with a highly conserved membrane phospholipid for cell entry (Albertini *et al.*, 2012) and therefore confers a broad range tropism to the vector. This strategy has been used to transduce a wide array of human primary cells and continuous cell lines as well as cells of other mammals, zebrafish, drosophila, and other phylogenetically distant organisms. VSV-G pseudotypization is useful in vaccination strategies or approaches where selective targeting is not necessary but is usually impractical for most gene therapy approaches. Many efforts have focused on designing Envs that target specific cells. The choice of Env is determined in part by the target cell or tissue that needs to be transduced. Lentiviral vectors have been pseudotyped with the filovirus envelope to enhance transduction of airway epithelia or endothelial cells, measles and murine leukemia-virus amphotropic envelopes to transduce some T-lymphoid cells, baculovirus and hepatitis C virus envelope to transduce hepatic cells, etc. (Bartosch *et al.*, 2003; Frecha, *et al.*, 2008a; Funke *et al.*, 2008; Kobinger *et al.*, 2001; Mazarakis *et al.*, 2001; Watson *et al.*, 2002; Wong *et al.*, 2004). Pseudotypization has an important constraint. The heterologous Env has to be inserted in the cytoplasmic membrane portion where assembly of the viral capsid is taking place. To circumvent this problem, chimeric Env molecules bearing a retroviral or lentiviral cytoplasmic tail and transmembrane portion showed promising results (Jurgens *et al.*, 2012; Sandrin *et al.*, 2002) and the use of native lentiviral Envs fused with ligands for specific cell histotypes may open novel paths for retargeting (Maurice *et al.*, 2002; Verhoeyen *et al.*, 2003; Yang *et al.*, 2006 Ziegler *et al.*, 2008). Third generation lentiviral vectors are fairly safe and extensively used in various experimental settings. As mentioned, compared to retroviruses, lentiviruses minimally perturb (if any) the expression of genes flanking the provirus, and SIN vectors have disrupted LTRs further reducing the risk of insertional mutagenesis and ruling out the generation of infectious particles by LTR-driven expression of viral mRNA. Last but not least, there is no influence on the promoter that drives transgene expression (Pistello *et al.*, 2007; Sakuma *et al.*, 2012) (Table 5). A detailed description of the modifications and performances of vectors and packaging constructs developed over the years is available elsewhere (Matrai *et al.*, 2010; Sakuma *et al.*, 2012).

Another actively pursued means of achieving specific cell targeting is to work on restricted and specific expression of the transgene. This is done using tissue- or cell-specific promoters, i.e. promoters that are active only in specific cells or cell conditions (Saukkonen and Hemminki, 2004). In all such instances, transduction of specific cells is not mandatory, though certainly preferable. Tissue specific promoters are the object of intense research for all viral vectors and have been tested in vectors derived from AdV, AAV, HSV, etc. (Bos *et al.*, 2009; Dorer *et al.*, 2009; Fujiwara *et al.*,

2011). This approach is extensively used with lentiviral vectors and has showed encouraging results in studying cardiomiopathy and cardiac tissue regeneration, and vascularization (Campan *et al*., 2011; Frecha *et al*., 2008b; James *et al*., 2011). Lentiviral vectors have been developed from several parental viruses. The first, and still the best, vector has been derived from HIV (Naldini *et al*., 1996). From its inception countless studies have been performed to improve safety and performances in vivo. Thanks to these works, HIV vectors are among the most efficient, versatile, and reliable transducing systems in vivo and ex vivo and are used in many fields, including curing HIV infection (Di Nunzio *et al*., 2012; Dropuli , 2011; Kiem *et al*., 2012 Rollier *et al*., 2011; Sakuma *et al*., 2012). However, because of safety concerns over the potential residual pathogenicity of HIV vectors, other lentiviruses have been exploited in this arena. Vectors have been derived from simian immunodeficiency virus, feline immunodeficiency virus (FIV), equine infectious anemia virus and the bovine immunodeficiency virus. The non-human primate lentiviruses were particularly apt since they resemble HIV in some genomic organization and biomolecular features but do not infect humans (Curran *et al*., 2000; Kenyon and Lever, 2011; Pistello *et al*., 2007; Poeschla *et al*., 1998; Valori *et al*., 2008). Vector design and development from animal lentiviruses resemble those of HIV and as such involved deletion of most non-relevant genes, rearranged for packaging signal and other domains, optimized for transduction and expression of heterologous cells, i.e. cells belonging to the non-natural infecting specie(s). The results, especially with FIV, were very encouraging. FIV vectors proved as efficient as HIV vectors in some conditions and were safe in various experimental settings in vivo (Barraza and Poeschla, 2008; Kenyon and Lever, 2011; Pistello *et al*., 2007; Saenz *et al*., 2012).

Thanks to extensive modifications and the use of regulatory elements from HIV and other viruses (Pistello *et al*., 2007; Zufferey *et al*. 1999), FIV vectors proved able to transduce neurons and other cells of the central nervous system, hepatocytes, blood cells endothelial cells, cardiac stem cells, airway epithelial cells and others (Saenz *et al*., 2012). Among other studies, FIV-derived vectors were used to vaccinate against AIDS (Pistello *et al*., 2010) and sexually transmitted herpes simplex virus (Chiuppesi *et al*., 2012). The FIV delivery system was able to induce strong and durable humoral and cell-mediated immune responses, including neutralizing antibodies that correlated to protection in the AIDS study (Pistello *et al*., 2010). A similar FIV vector was also tested in a proof of concept study for hereditary breast cancer treatment. Here the vector delivered a wild type, fully functional copy of breast cancer-associated gene type-1 gene (BRCA-1), an oncosuppressor gene whose impairment has been associated with breast cancer (Trainer *et al*., 2010). Despite the conspicuous size of BRCA-1, the FIV vector stably delivered and transduced the gene that was expressed in such a way to fully restore cellular mutated BRCA-1 in primary cells and continuous cell lines (Vannucci *et al*., 2010). Further, there were no adverse effects even after three years of use in vivo in various animal species. In all, these findings demonstrate that FIV is a versatile and safe vector for in vivo use.

HIV and lentiviral vectors as a whole are employed to treat hematological malignances, various neurodegenerative and genetic diseases (Valori *et al*., 2008; Sakuma *et al*., 2012), and for vaccination (Di Nunzio *et al*., 2012; Rollier *et al*., 2011). Particularly interesting for vaccination purposes are the integration-defective lentiviral vectors bearing inactivated integrase and therefore unable to integrate in the host cell genome. Integration-defective lentiviral vectors express the transgene transiently but long enough to prime and trigger specific immune responses against the delivered transgene (Negri *et al*., 2011; Staunstrup and Mikkelsen, 2011).

Another field of research, actively pursued with lentiviral vectors, is the development of inducible vectors and gene editing in vivo. Inducible vectors are vectors containing promoters whose activity is adjustable and depends on the presence of a drug or specific compound. Inducible promoters switch on or off in the presence or absence of a specific compound administrable in vivo and allow temporal and strictly controlled expression of the transgene. Construction of truly and durably inducible vectors is not easy but there are several important studies in vitro and in vivo demonstrating that quantitative and temporally adjustable expression of the transgene can be achieved (Osten *et al*., 2007). If successful and also applicable to other viral vectors, it is fore-

seeable that inducible promoters will replace all constitutive promoters used hitherto. The second, very exciting novelty in vectorology is gene editing, also known as site-specific recombination. This is an extremely powerful approach: if it fulfills expectations gene editing will revolutionize the gene therapy concept and intervention strategies. This approach relies on zinc finger nucleases (ZFNs) or the more recent transcription activator-like effector nucleases (TALENs). ZFNs and TALENs are artificial restriction enzymes generated by fusing a DNA-binding domain to a DNA-cleavage domain. By acting in concert, these domains recognize a specific sequence and cut in close proximity to the binding domain. The double-strand break is then repaired by the cellular repair machinery that is set in motion after the damage. Repair can be either done with a mere cut and joining that reconnects DNA from either side of the double-strand break. This repair mechanism is imperfect as it may cause insertion, deletion, or chromosomal rearrangement, and hence render a coding sequence non-functional. Alternatively, repair may take place via site-specific recombination by providing a DNA fragment homologous to the damaged DNA. In the presence of this fragment, the repairing enzymes will replace the damaged fragment with the external fragment and reconstitute the DNA integrity. For instance, homologous recombination can permanently repair a genetic defect by replacing the mutated with the wild type DNA sequence or, conversely, stably inactivate a gene by disrupting the coding sequence or, by in situ recombination, with precise introduction of missense mutations (Carroll, 2011). ZFN and TALENs have been used to generate stably modified human embryonic stem cells, induce pluripotent stem cell clones, repair and inactivate genes, create several knockout animals, etc. (Carroll, 2011; Pan *et al*., 2012). ZFNs or TALENs and DNA fragment for in situ-recombination are delivered into cells by viral vectors derived from lentiviruses or adeno-associated viruses (Luo *et al*., 2012; Miller *et al*., 2007; Sakuma *et al*., 2012). One of the most important applications and particularly relevant to microbiology, is the creation of T-lymphoid cells resistant to HIV-1 infection. Based on the seminal observation that subjects bearing a deletion in CCR5, a HIV-1 coreceptor (Wilen *et al*., 2012), are healthy and unusually resistant to HIV infection (Lederman *et al*., 2010), a multidisciplinary team devised a ZFN system that disrupts the CCR5 coding sequence in human hematopoietic stem/progenitor cells (HSPC) (Yao *et al*., 2012). In contrast to wild-type CCR5, which is a transmembrane protein, mutated CCR5 is not transported into the cytoplasmic membrane and eventually degraded. Gene editing was performed ex vivo by collecting blood cell precursors by apheresis from HIV-1 patients. Purified HSPC were transduced with a ZFN system, expanded in vitro and reinfused first into an animal model to test the approach (Holt *et al*., 2010), then into the respective HIV patients (Ledford, 2011). With a mere disruption of 17% total alleles in the transduced population and the production of mono- and bi-allelical disrupted cells, reinfused HSPC differentiated normally and created a population of $CD4^+$ T-lymphocytes that were resistant to HIV-1 infection and expanded at the expense of HIV replication that, in the experimental model, declined by more than one log compared to naïve controls (Holt *et al*., 2010). This result spurred much enthusiasm and opened the way to various approaches, most still in the pre-clinical phase, others in clinical trials, to create genetic resistance to HIV. Several methods are being explored to enhance the genetic barrier or build up host cell defenses (for a comprehensive review see Burnett *et al*., 2012). Of relevance for the purpose of this review is that of the 19 methods published to date, 18 used viral vectors for gene delivery. Of these, two employed an adenoviral vector, one a foamy virus vector belonging to same family but with quite distinct features from retroviruses and lentiviruses (Erlwein and McClure, 2010; Lindemann and, Rethwilm, 2011); the remaining 13 trials used a retroviral or a lentiviral vector (Burnett *et al*., 2012). These numbers are evidence of the extensive use of vectors derived from lentiviruses and, on a smaller scale, retroviruses as gene transfer systems. Safety concerns still curb their use and potential application. It is foreseeable, however, that intense research and preclinical study will enhance their safety and reliability to warrant clinical use.

Poxvirus and other viral vectors

The innate ability of viruses to deliver genetic material into cells makes every virus a potential vector. Thus, besides the major vectors described

TABLE 6 - *Advantages and disadvantages of poxvirus vectors.*

Advantages	*Disadvantages*
Carry up to 30 Kbp heterologous DNA	Potentially cytotoxic
Multiple sites of transgene insertion	Generation of recombinants complicated
Particularly apt as attenuated recombinant vaccine	Transient expression of the transgene
Well suited as oncolytic vectors	Highly immunogenic
Low levels of pre-existing immunity	Heterologous promoters difficult to use

above that cover approximately 80% of vector trials, there is a long list of viruses that served as a platform to develop vectors. Among these, paramixoviruses, alphaviruses, flaviviruses, as well as recombinant and artificial viruses have found a niche or a special application in which they excel (Kaufmann and Nettelbeck, 2012; von Messling and Cattaneo, 2004).

However, special emphasis should be given to poxviruses also known as Vaccinia viruses. Poxviruses are large complex enveloped viruses with a linear double-stranded DNA genome of approximately 190 kbp in length. Other unique features of the poxviruses are the high number of genes (about 250) and the replication cycle that, despite being DNA viruses, takes place entirely in the cytoplasm of infected cells. A conspicuous number of genes are dispensable for replication, whilst important for pathogenicity, and can therefore be deleted (Table 6).

Vaccinia virus can thus accept as many as 25 kbp heterologous DNA, making it useful for expressing large genes. Because of short-lasting but intense expression of the transgene and cytolytic properties, poxvirus vectors are mostly used for production of recombinant protein, oncolytic cancer therapy, cancer immunotherapy, and vaccination (Gómez *et al.*, 2011; Kim and Gulley, 2012). In this regard, the poxvirus vectors have been used to immunize against herpesvirus, hepatitis B, rabies, influenza, HIV, and other viruses (Walsh and Dolin, 2011).

The vaccinia vectors developed to date are highly attenuated, host-restricted, non- or poorly replicating strains. The most used are the Orthopoxviruses modified vaccinia ankara (MVA), NYVAC a derivative of Copenhagen vaccinia strain, and Avipoxviruses ALVAC and TROVAC, derived from canarypox and fowlpox viruses, respectively.

The MVA virus is a highly attenuated strain derived from Vaccinia strain Ankara by long-term propagation in chicken embryo fibroblast cells. This caused the loss of about 10% of the vaccinia genome and its ability to replicate in mammalian cells. MVA has a high safety profile and has been administered to numerous animal species and humans during a smallpox mass vaccination campaign. Recombinant MVA proved suitable as a vaccine vector for its high safety profile and ability to deliver antigens in a highly immunogenic way (Gómez *et al.*, 2011). The canarypox virus vector ALVAC replicates only in avian species and has a high safety profile. This and its inability to replicate in mammalian cells provide important safety barriers for human use. ALVAC has been used to develop several vaccines for animals as well as humans. ALVAC has been actively used as a vaccine vector against HIV-1 (Pantaleo *et al.*, 2010; Vaccari *et al.*, 2010).

CONCLUSIONS

Viral vectors can be applied to different areas and for different goals. They can either be simply used as in vitro tools for biomolecular and gene functional studies, or to accomplish more demanding tasks such as in vivo studies to monitor cell function, drive tissue regeneration and differentiation, cure or prevent infectious diseases, treat genetic disorders, fight cancer, and an ever-growing number of applications.

Due to space constraints this review has superficially examined many aspects, and the trivial conclusion is that a one-fits-all multipurpose viral vec-

tor suitable for all tasks and demands does not exist. Rather, each viral vector has its own advantages, limitations and range of applications. Attentive examination and reflection on these features will help identify the type of vector then select the genome architecture apt for the mission to accomplish. Selection of the best-suited vector is crucial and requires attentive in-depth knowledge of the delivery systems and their performances. The end result is an ongoing search, development of novel and perfection of existing vectors to make gene transfer technology a realistic option to combat tumors, vaccinate against various ailments and correct genetic defects. It is also obvious that new frontiers in medicine mean new opportunities for treatment and prevention, and better healthcare, but also new challenges and hurdles.

Looking back, the path for gene therapy is paved with successes but also serious incidents and victims and, because of these and to quote a famous Nature editorial, gene therapy has long lost its innocence (Hollon, 2000). The scientific community and in particular people using or working on viral vectors are well aware that gene delivery has many unresolved issues, some of which are discussed below. But it is also aware that knowledge and technical advances have considerably improved the field and enhanced the safety and tolerability of viral vectors to levels only dreamed of ten years ago.

The proof-of-evidence came last June when gene therapy had its breaking point. That month, a committee from the European Medicines Agency recommended the approval of a gene therapy drug for the treatment a lipoprotein lipase deficiency, a rare inherited genetic disorder (Gaudet *et al*., 2012). Other treatments had previously been approved in less government rule-restricted countries.

Looking ahead, for full deployment, gene transfer technology needs to:

a. identify the appropriate target, either cell or gene, and strategy to pursue;
b. deliver the genetic information solely into the right cell and in the right amount, i.e. selective and specific targeting and controlled/physiological expression of the therapeutic gene is a must;
c. maintain the gene and its expression in the cell long enough to treat the disease or accomplish the task;
d. restrain the gene from causing short- or long-term adverse effects, e.g. triggering autoimmunity, neoplastic transformation, or other disorders;
e. develop delivery systems with the least possible immunogenicity, and easy to produce and administer, e.g. vectors unable or minimally evoking novel or reactivacting pre-existing immune responses.

In summary, gene transfer supplies the body with healthy genes to compensate for missing, deficient, or defective genes, and protect against and prevent other diseases. Despite cutting-edge technology and the know-how required, this approach has a bright future. This approach could become common practice by the 21st century, able to treat more than 2000 genetic diseases - some of which even before the child is born - and to cure many infectious and neoplastic diseases. This technology is certainly costly, and its routine use is far too expensive in the current economic situation. However, the possibility to permanently cure a genetic disease or stably protect against acquired diseases is alluring and in the long run it may lead to huge economic gains.

Competing interests

The authors declare that they have no competing interests.

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