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Review

Targeting site-specific chromosome integration✪

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The concept of gene therapy was introduced with great promise and high expectations. However, what appeared simple in theory has not translated into practice. Despite some success in clinical trials, the research community is still facing an old problem: namely, the need for a vector that can deliver a gene to target cells without adverse events while maintaining a long-term therapeutic effect. Some of these challenges are being addressed by the development of hybrid vectors which meld two different viral systems to incorporate efficient gene delivery and large cloning capacity with site-specific integration. The two known systems that integrate genes into specific sites in mammalian genomes are the adeno-associated virus and phage integrases. Recent experiments with hybrid vectors incorporating both of these systems are encouraging. However, extensive research should be directed towards the safety and efficacy of this approach before it will be available for gene therapy.

Keywords: gene therapy, viral vector, hybrid vector, helper-dependent adenovirus, adeno-associated virus, phage C31 integrase

The "holy grail" of gene therapy is the ideal vector. Recent success in clinical trials for patients with severe combined immunodeficiency (SCID) was overshadowed by adverse events (Check, 2003; 2005). The major problem was random integration of the therapeutic gene into patients' chromosomes (Hacein-Bey-Abina *et al.*, 2003). This article will review the current challenges in gene delivery and review the progress made in the design of viral vectors, culminating in the development of hybrid vector systems. Our own experiences with these systems will be discussed.

PROBLEMS WITH VIRAL VECTORS

Conceptually, gene therapy is simple: delivery of corrective genetic materials into target cells reverses the symptoms of disease. However, despite initial optimism, the gene therapy community has come to realize that what is simple in principle does not necessarily translate into practice (Marshall, 1995). In 1990, the first patient with SCID received an injection of cells containing normal adenosine deaminase (ADA). Now, several years after this first clinical application, the field has had to face a slowing of progress and an absence of therapeutic applications (Friedmann, 1996). The original concept for gene therapy was focused on curing genetic diseases (Morgan & Anderson, 1993; Mulligan, 1993). The fundamental problems of gene therapy were appreciated in the beginning including i) lack of safe and effective gene delivery systems, ii) lack of sustained expression, and iii) the host immune response (Marshall, 1995; Verma & Somia, 1997). The latter two limitations are not issues for short-term applications in cancer gene therapy and vaccinations. Despite the lack of clear success, the community had no doubt that it would eventually bear fruit because adverse events had not been common and had been related to the gene delivery strategies, not the gene being transferred (Crystal, 1995).

However, this complacency was challenged in September 1999. A teenaged patient died during

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Abbreviations: Ad, adenovirus; ADA, adenosine deaminase; AAV, adeno-associated virus; EF-1, elongation factor-1; HDAd, helper-dependent adenovirus; eGFP, enhanced green fluorescence protein; HSV, herpes simplex virus; ITR, inverted terminal repeat; LTR, long-terminal repeat; p5IEE, p5 integration efficiency element; REB, Rep binding element; SCID, severe combined immunodeficiency; SCID-XL, X-linked severe combined immunodeficiency; *trs*, terminal resolution site; VSV-G, vesicular stomatitis virus glycoprotein.

an ornithine transcarbamylase gene therapy trial. If he had not participated in the trial, he would have survived with conventional treatments. What ensued were a flurry of reports, congressional hearings, and the halt of several gene therapy trials by the Food and Drug Administration. Important criticisms were raised regarding a conflict of interest of a participating investigator as well as informed consent (Balter, 2000).

Despite this first setback, the gene therapy community was encouraged in the following year with reports of success for the X-linked severe combined immunodeficiency (SCID-Xl) and hemophilia B clinical trials (Cavazzana-Calvo *et al.*, 2000; Kay *et al.*, 2000; Hacein-Bey-Abina *et al.*, 2002) followed by the positive results of the SCID-ADA trials (Aiuti *et al.*, 2002). Unfortunately, over the long-term, these results have not turned out to be as affirmative as first perceived. The success of SCID-Xl was offset by vector-induced leukemia in two patients who received autologous CD34 bone marrow cells transduced by retrovirus carrying γ chain of T-cell receptor (Hacein-Bey-Abina *et al.*, 2003). The leukemia was caused by the retroviral vector genome integration in proximity to the LMO2 proto-oncogene promoter, leading to aberrant transcription and expression of LMO2. The potential for insertional oncogenesis was a recognized risk of integrating viral systems (Li *et al.*, 2002), but its occurrence had not been reported in clinical trials until this point. Therefore, this incident raised concerns about the safety of gene delivery vectors (Williams & Baum, 2003).

Although the adeno-associated virus (AAV) is a promising vector in gene therapy, functioning mostly as an episome, the detection of the AAV genome in germ cells of patients in the hemophilia B trial and minimal but detectable integration into active genes (Nakai *et al.*, 2003) further fueled concerns about risk and ethics in gene therapy clinical trials (Kimmelman, 2005). Recently, a third patient in the SCID-Xl trial was found to have developed cancer (Check, 2005; Couzin & Kaiser, 2005). These incidents have highlighted an old problem: the ideal vector must first be developed before this technology is used in clinical trials and practice.

VIRAL GENE TRANSFER VECTOR: INTEGRAL *VS* NONINTEGRAL

There are encouraging developments in gene therapy using non-viral vectors (Niidome & Huang, 2002). In spite of this, viral vectors remain the agent of choice in clinical trials (for current statistics in gene therapy clinical trials, access http://www.wiley.co.uk/wileychi/genmed/clinical/; also see Kimmelman, 2005; for more resources). There are three major ways to improve the safety in gene therapy: i) to develop vectors with reduced propensity for insertional toxicity and improved safety profiles, ii) to define safe integration sites for integrating vectors, and iii) to reduce the exposure of non-target cells in order to reduce the number of vectors required for treatment (Williams & Baum, 2003).

The three major classes of viral vectors are categorized by genome integration into the host chromosome (retrovirus and lentivirus), persistence in the nucleus as extrachromosomal episomes (adenovirus, AAV, and herpes simplex virus), or a combination of both, the so-called "hybrid vectors" (Thomas *et al.*, 2003). This distinction determines their suitability for a particular gene therapy application. Although research is in progress to identify new viral species for vector development, retrovirus, lentivirus, AAV, adenovirus (Ad), and herpes simplex virus (HSV) are the most popular vectors currently used in gene therapy (Oka & Chan, 2002; 2004; Thomas *et al.*, 2003). Since HSV mainly affects the nervous system, only the first four aforementioned vector systems will be reviewed here (Table 1).

Retroviral vectors

The retroviral vector is a single-stranded RNA virus. Early gene therapy studies employed Moloney murine leukemia virus-based vectors. The current vector in use has been pseudotyped with the vesicular stomatitis virus glycoprotein (VSV-G). This confers a broad host range and stabilization of the vector particles, allowing the vector stock to be concentrated to high titers by ultracentrifugation. The packaging capacity of this vector is approx. 8 kb.

Table 1. Comparison of various viral vectors

Feature	Retroviral vector	Lentiviral vector	Helper-dependent adenoviral vector	Adeno-associated viral vector
Cloning capacity	~7.5 kb	~ 7.5 kb (<18 kb)*	~ 37 kb	~ 4.7 kb (~ 10 kb)**
Integration	Yes (random)	Yes (random)	Essentially non	Minimal (random)
Transduction	Dividing cells	Dividing cells and non-dividing cells	Dividing cells and non-dividing cells	Dividing cells and non-dividing cells
Transduction efficiency	Good	Good	Excellent	Excellent
Immune problems	Few	Few	Moderate	Few

*The titer of the lentiviral vector is dramatically reduced (Kumar *et al.*, 2001). **This cloning capacity can be achieved by an intermolecular joining method.

Transgene expression persists due to integration into the host chromosome. However, the vector genome is integrated randomly, leaving the possibility of oncogenesis. Another disadvantage is that retroviral vectors only work in cells undergoing division because they can only access the nucleus if the nuclear membrane has broken down.

Lentiviral vectors

Lentiviral vectors are derived from human immunodeficiency virus, also a retrovirus, and are pseudotyped with VSV-G. The deletion of the majority of the parental genome in packaging constructs renders the virus self-inactivating since they lack the regulatory elements in the downstream long-terminal repeat (LTR) required for transcription of the packaging signal. Unlike retroviral vectors, most lentiviral vectors can penetrate an intact nuclear membrane and thus transduce non-dividing cells. A vector genome up to 18 kb can be packaged at the expense of a reduction of viral titer (Kumar *et al.*, 2001). Few immunological problems have been reported for both retroviral and lentiviral vectors.

Adeno-associated viral vectors

AAV is a single-stranded DNA virus that can infect both dividing and non-dividing cells. Although it is considered a non-pathogenic virus, there have been reports of a potential role of AAV2 in abortion (Tobiasch *et al.*, 1994) and hepatocellular carcinoma in mice (Donsante *et al.*, 2001). Cell type- or tissue-specific transduction of various AAV serotypes is influenced by the presence of specific cell surface receptors or co-receptors. With a genome size of approx. 4.7 kb, the limited packaging capacity is the major disadvantage of AAV. This problem has been addressed by intermolecular joining of two vectors, each with half of the expression cassette. After concatamerization in the nucleus, a complete expression cassette is created (Duan *et al.*, 2000; Nakai *et al.*, 2000; Sun *et al.*, 2000).

During latency in humans, AAV2 preferentially integrates at a site on chromosome 19q13.3-qtr (Kotin *et al.*, 1990; Samulski *et al.*, 1991), the so-called "AAVS1" site, by targeting a sequence composed of a Rep binding element (RBE) and terminal resolution site (*trs*) identical to the viral terminal repeats (Weitzman *et al.*, 1994; Linden *et al.*, 1996). Similar sites have been identified in CV-1 cells isolated from the African Green Monkey (Amiss *et al.*, 2003) and on mouse chromosome 7 (Dutheil *et al.*, 2004). Upon infection with a helper virus such as Ad or HSV, AAV enters the lytic cycle and undergoes replication and productive infection. Although most recombinant AAV vectors do not contain Rep and will not integrate, the potential for mutation and oncogenesis may exist. In the absence of Rep gene, AAV genome has been shown to integrate randomly into the mouse genome with propensity towards gene regulatory sequences albeit at low frequency (Nakai *et al.*, 2003; 2005).

Adenoviral vector

Early adenoviral vectors are inappropriate for most gene therapy applications due to their potent immunogenicity, toxicity because of leaky viral gene expression, and the transient nature of transgene expression. However, these properties are acceptable for certain applications such as cancer gene therapy or vaccination. Ad is the most efficient vector for delivery of vector genome into the cell nucleus, and it transduces both dividing and non-dividing cells. The helper-dependent Ad (HDAd) vector was developed in an effort to reduce Ad-associated toxicity (for review, see Kochanek, 1999). The HDAd vector lacks all viral coding genes with the exceptions of the inverted terminal repeats (ITR) for replication and the packaging signal. Helper virus, a first generation Ad, provides the necessary viral proteins *in trans* for packaging in culture. An improved safety profile and long-term transgene expression *in vivo* have been reported for HDAd in a number of studies. In addition, HDAd vector has a large cloning capacity up to 37 kb.

Hybrid vector

Hybrid vectors are the newest improvement in gene delivery as they take advantage of the high infectivity of Ad vectors and the gene integration capabilities of other viral systems. These vectors can be categorized based on whether integration of vector genome is random (AAV ITR, *Sleeping Beauty* transposon) or site-specific (AAV-ITR plus Rep). Hybrid HDAd vectors reported include HDAd/retrovirus vector (Soifer *et al.*, 2002), HDAd/lentiviral vector (Kubo & Mitani, 2003), HDAd/retrotransposon (Soifer *et al.*, 2001), and HDAd/Epstein-Barr viral vector (Dorigo *et al.*, 2004). Yant and coworkers developed an HDAd vector that carries a human factor IX transposon flanked by *FRT*, a recognition sequence of *Flp* recombinase. Intravenous administration of this vector with a second vector that expresses the *Flp* and *Sleeping Beauty* recombinases resulted in the generation of transposon circles and the random integration of the human Factor IX gene into mouse liver. This treatment maintained therapeutic levels of Factor IX for more than 6 months despite extensive liver regeneration after multiple partial hepatectomies (Yant *et al.*, 2002).

With the HDAd vector, the transgene flanked by AAV-ITR integrates at random sites on the host chromosome. An advantage of this hybrid is to ex-

pand the packaging limit of the AAV vector while allowing integration (Lieber *et al.*, 1999; Goncalves *et al.*, 2001). Site-specific integration of AAV-ITR flanked transgene has been reported in cell culture using HDAd vector and herpes-virus amplicon vector that express the AAV Rep68/78 proteins (Costantini *et al.*, 1999; Recchia *et al.*, 1999). Recchia and coworkers further improved this vector and reported site-specific integration into mice carrying AAVS1 site (Recchia *et al.*, 2004). Hybrid vectors with the ability to integrate often require additional genes that have no role for the therapeutic outcome. For example, Rep protein is necessary for site specific integration for a HDAd/AAV hybrid vector. However, Rep protein has negative effects on viral replication (Hermonat, 1992; Weitzman *et al.*, 1996) and is toxic to the cell (Schmidt *et al.*, 2000). Recchia and coworkers have addressed this problem by using a tetracycline-regulated Rep expression system so that the protein is only produced in the presence of the antibiotic (Recchia *et al.*, 2004).

ADENO/ADENO-ASSOCIATED VIRUS HYBRID VECTOR

AAV is the only known eukaryotic virus capable of targeted integration. The two elements required for viral genome integration are the ITR and the Rep proteins, either Rep68 or Rep78, which are transcribed from sequences downstream of the p5 promoter (Surosky *et al.*, 1997; Lamartina *et al.*, 1998; Pieroni *et al.*, 1998). The human chromosome 19 AAVS1 site contains an AAV RBE and *trs* identical in sequence to the AAV-ITR required for viral replication. Deletion of *trs* eliminates site-specific recombination (Weitzman *et al.*, 1994; Linden *et al.*, 1996). No large regions of homology are shared between the AAVS1 site and the AAV provirus, suggesting that integration occurs through non-homologous recombination (Kotin *et al.*, 1992). A 138 bp AAV integration efficiency element (p5IEE) in the p5 promoter enhances Rep-mediated integration efficiency independent of the AAV ITR (Philpott *et al.*, 2002a; 2002b; 2004).

AAV genome integration may involve Rep binding to *trs*/RBE on chromosome 19 followed by Rep-mediated unwinding of the RBE and covalent attachment of Rep to the 5′-end of the DNA break point by tyrosine phosphate linkage. This leaves a free 3′-OH substrate to initiate replication. There may be formation of 5′ flaps with covalently attached Rep and subsequent complex formation between actively replicating chromosome sequences and Rep bound to AAV-ITR. Finally, there is resolution of the viral genome into the chromosome (Young & Samulski, 2001; McCarty *et al.*, 2004).

The persistent presence of Rep protein may be toxic to cells. In order to control Rep expression, we have employed a chimeric transactivator, GLp65, which consists of a mutated progesterone receptor ligand binding domain fused to the GAL4 DNA binding domain of the human p65 protein, a component of the NF-κB complex (Burcin *et al.*, 1999). In the presence of the anti-progesterone mifepristone (RU486), this chimeric transcription regulator binds to the 17-mer GAL4 consensus binding sequence and activates transcription of Rep. Unfortunately, a single HDAd vector containing both the GLp65 transactivator and Rep proved to be difficult to produce. This problem was addressed by splitting the components of the system into three vectors: 1) GLp65 under constitutive transcriptional control of the elongation factor-1 (EF-1) promoter, 2) Rep transcription driven by binding of GLp65 in response to RU486, and 3) EF-1 driven reporter/selectable marker eGFPuro, a fusion protein of enhanced green fluorescence protein (eGFP) and puromycin-*N*-acetyltransferase, which is flanked by AAV-ITR. Hep3B, human hepatoma cells, with no rearrangement on chromosome 19, were infected with the three vectors and then incubated with RU486. Two days after infection, puromycin was added to the medium, and the selection was continued for 12 weeks. Puromycin resistant eGFP positive clones were isolated and site-specific transgene integration was characterized by PCR. Six out of seven clones yielded the expected AAVS1/AAV-ITR specific PCR products.

ADENO/PHAGE INTEGRASE HYBRID VECTOR

Phage integrases are enzymes that mediate unidirectional site-specific recombination between a phage attachment site *attP* and a bacterial attachment site *attB* (Groth & Calos, 2004). Some integrases such as ΦC31, and R4 integrases do not require host cofactors, while others act with the help of phage and/or bacterial proteins. ΦC31 integrase is functional in mammalian cells (Groth *et al.*, 2000) and can integrate plasmid DNA containing the *attB* sequence into pseudo-*attP* sites in mammalian genomes (Thyagarajan *et al.*, 2001). ΦC31 integrase-mediated gene transfer has been used in small animal experiments (Ortiz-Urda *et al.*, 2002; 2003; Hollis *et al.*, 2003; Quenneville *et al.*, 2004; Held *et al.*, 2005). Although ΦC31 integrase acts on a circular *attB* containing plasmid, linear DNA may also work, albeit at lower efficiency (Belteki *et al.*, 2003). The integrase system has no apparent upper size limit and readily integrates plasmid DNA, even over 9.7 kb. The use of a plasmid construct for gene delivery has some advantages and disadvantages. The efficiency of plasmid DNA transfer efficiency is low, and plasmid

DNA can be degraded rapidly if not integrated. This might actually be an advantage for plasmids encoding for integrases since it would be eliminated from the cells when recombination is complete.

In order to maximize its utility, we have developed an HDAd-ΦC31 integrase system. We generated two HDAd vectors containing either RU486-regulated integrase and *Flp* expression cassette or β-geo cassette and *attB* flanked by *FRT* sequences. *Flp* circularizes the transgene cassette flanked by *FRT* sequences and provides the substrate for ΦC31 integrase. We first tested whether the expression of the ΦC31 integrase increases integration frequency in a mouse hepatoma cell line, BNL-CL2. We used β-geo as a reporter/selectable gene, which allows G418 selection of the cells carrying the stably integrated β-geo gene. The number of surviving cells incubated with RU486 was 2-fold larger than those without, suggesting increased integration frequency with expression of ΦC31 integrase. We further tested this system in mice. After liver injury by administration of CCl_4, we found clonal expansion of hepatocytes expressing β-geo in mice treated by two HDAd vectors and RU486 but not in mice treated by two vectors alone (Chao *et al*., manuscript in preparation).

Whether transgenes are integrated at safe sites is a major concern for integrating gene transfer systems. Held *et al.* (2005) addressed this problem by serial transplantation of hepatocytes transfected by ΦC31 integrase-mediated fumanylacetate hydrolase (FAH) gene transfer into $Fah^{-/-}$ recipient mice, a model of hereditary tyrosinemia type I. After tertiary transplantation, the authors found normal appearing hepatocytes in recipient mice (Held *et al*., 2005). These results suggest that ΦC31 integrase-mediated gene transfer may be safe and have future utility in gene therapy.

CONCLUSION

Chromosome integration of a therapeutic gene is necessary for transducing actively dividing cells such as bone marrow cells. However, random integration may cause adverse events such as the tragedy that occurred in SCID-Xl clinical trials. AAV and phage integrases are two known site-specific gene integration systems. Although more characterization and optimization are needed, the results from hybrid vectors using these systems are encouraging and warrant future study.

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