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# New Vectors for Stable and Safe Gene Modification

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## 1. Introduction

Stable gene modification without affecting normal cellular function is a main goal both for basic and applied science. Current strategies to achieve stable gene modification are either very inefficient (homologous recombination) or very genotoxic (retroviral gene transfer). Retroviral vectors derived from oncoretroviruses (murine leukaemia virus - MLV) were the first to be widely used for stable gene modifications for gene therapy strategies. They are efficient, poorly immunogenic and derived from a non-pathogenic virus. MLV-based vectors were also the first ones to show real therapeutic effect by correcting the immune system of about 50 patients with four different severe immunodeficiencies X-linked Severe Combined Immunodeficiency (SCID-X1), SCID-Adenosine Deaminase Deficiency (SCID-ADA), Wiskott-Aldrich Syndrome (WAS) and Chronic Granulomatous Disease (CGD)(Fischer *et al.*). However, for most applications therapeutic efficacy were incomplete or come together with serious adverse effects such as cell transformation(Neven *et al.*, 2009). Different studies have demonstrated that the enhancer elements present in the old generation retroviral vectors are important in cell transformation(Montini *et al.*, 2009). Therefore, safer integrative vectors are required to replace MLV-based retroviral vectors when stable gene modification is required. New vectors for stable expression must consider three aspects: 1- efficiency, 2- ectopic or unregulated expression of the transgene and 3- genotoxicity (genomic alterations due to vector integrations). Probably the most important feature for a vector to reach clinic is efficiency. Gene Therapy vectors must be able to efficiently transduce their target cells in order to reach therapeutic benefits. High efficiency is especially relevant when the therapeutic protein does not confer any positive advantage to the target cells. It is therefore important to keep in mind that very safe vectors are of no use if the efficiency does not reach a minimum. We can say that, at least for some applications, efficiency is no longer a limitation, and that safety is now the main concern.

The ectopic or unregulated expression of the transgene is other aspect to take into consideration. As conventional therapeutic agents, transgenes have a window where and when to exert their function. In many diseases, the expression of the affected gene is restricted to a particular tissue, to a particular stage of the development and/or in response to environmental conditions. The expression of the transgene in non-target cells as well as the expression of non-physiological transgene levels may cause toxic or deleterious effects.

Therefore, an important safety issue concerning gene therapy is to achieve regulated and/or physiological expression of the transgene (reviewed in Toscano *et al.* 2011).

Genotoxicity can be defined as harmful actions on the integrity of the genetic material. Genotoxic substances are potentially mutagenic or carcinogenic. Integrative vectors are genotoxic due to their ability to integrate into the host DNA since vector integration can result in alteration of gene expression. In the worst scenario, genotoxicity can cause cell transformation and tumor development. Several factors influence vector genotoxicity (Baum *et al.*, 2003) such as integration site preference, presence of strong enhancers in the vector, weak polyadenylation sites or the integration mechanism. To minimize or eliminate genotoxicity problems of integrative vectors we can use safer integration sites (safe harbor), block the effect of the vector on the surrounding chromatin (using insulators) or gene correction strategies (Figure 1).

In this chapter we will discuss the most promising strategies that tackle these issues: 1- improving safety of retroviral-based vectors, 2- novel non-viral systems to achieve efficient transgene integrations, 3- site-specific integrative vector systems and 4- gene correction by enhanced homologous recombination.

## 2. Improving safety of retroviral-based vectors

There are two alternative means of improving safety of retroviral-based gene delivery; 1- by using alternative retroviruses for vector development and 2- by improving the properties of existing retroviral vectors.

### 2.1 Alternative retroviral vectors

One way to improve the safety of stable gene expression is the search for retroviral vectors other than the original Moloney-murine-leukemia-virus (MoMLV)-based vectors. Retroviruses from two different genus from the retroviridae family have been the main focus for the development of new integrative vectors: lentivirus and spumavirus

#### 2.1.1 Lentiviral-based vectors (LV)

Lentiviruses are part of the retrovirus family that includes the human immunodeficiency virus-1 (HIV-1). Viral vectors are useful vehicles for the delivery of genes into target cells and retroviral vectors have become popular because of their ability to integrate into the host cell genome and maintain persistent gene expression. Also, the ability to stably transduce non-dividing cells has made them one of the best platforms for vector development (Ikeda *et al.*, 2003; Cockrell and Kafri, 2007). Lentiviral vectors (LV) offer several advantages over gammaretroviral vectors (GVs) (reviewed in Chang and Sadelain, 2007): 1- Transduction with LV does not require preactivation of non-dividing cells. They are very efficient for stable gene modification of most primary stem cells including neurons, hematopoietic, mesenchymal and embryonic stem cells. 2- LV are more resistant to silencing and different regulatory elements can be incorporated to minimize influence from neighbouring chromatin without substantially affecting vector titre (Ramezani and Hawley, 2010). The modifications include utilization of strong internal enhancer-promoter sequences, addition of scaffold/matrix attachment regions and flanking the transcriptional unit with chromatin domain insulators. 3- The integration “pattern” of LVs is less genotoxic than GV. Contrary to GV, LVs do not have a preferential insertion next to transcriptional start sites and regulatory gene regions. Modlich *et al.* found (Modlich *et al.*, 2009) that the LVs insertion

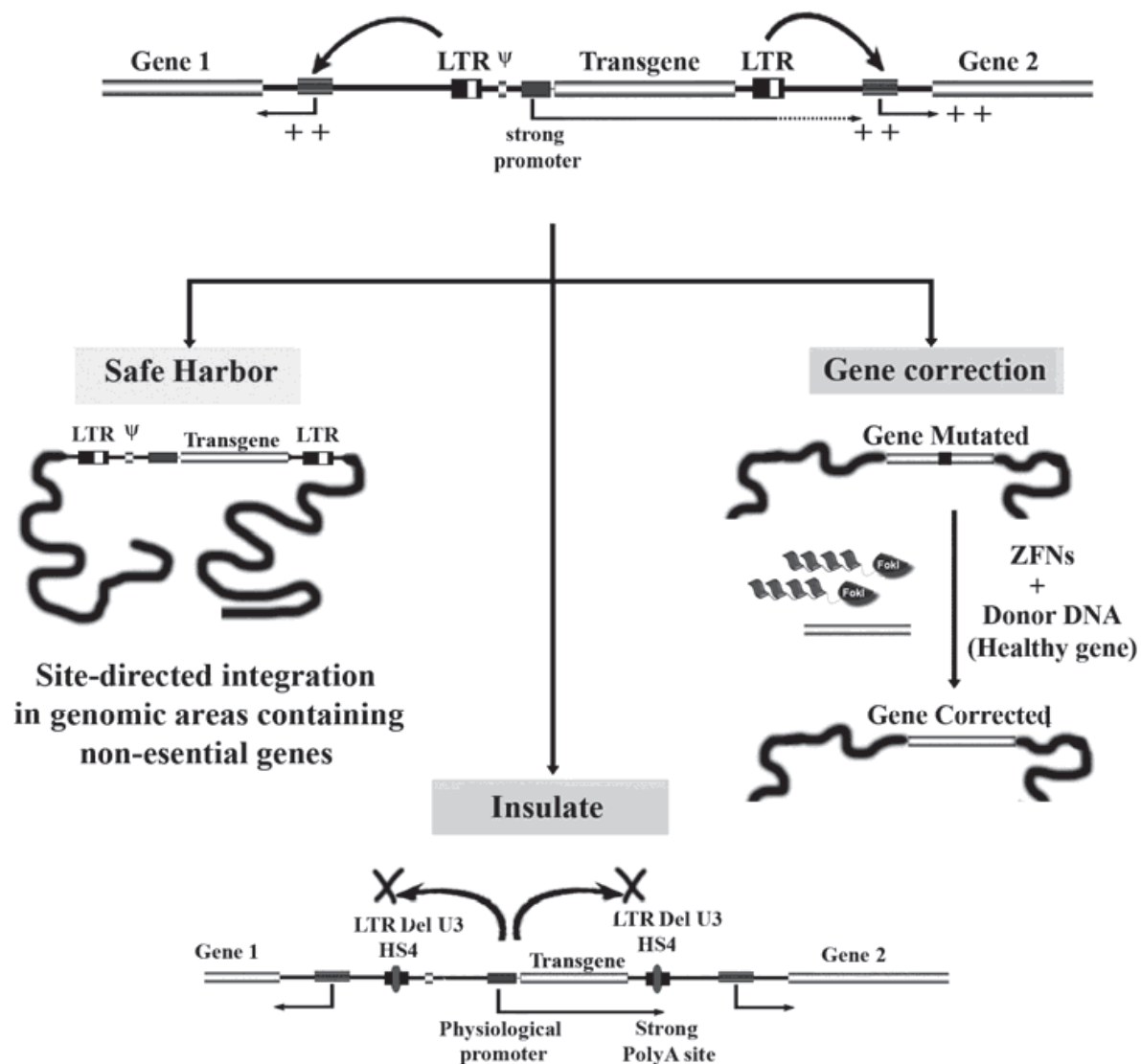


Fig. 1. Main strategies to achieve safer stable genetic modification for gene therapy applications. First generation gammaretroviral vectors (top) contained strong enhancer sequences in their LTR backbones that can influence genes located upstream (Gene 1) or downstream (Gene 2) of the insertion site. In addition, the promoter activity of the LTR (U3 region) or that of a strong internal promoter, can also influence activity of downstream genes by 3' readthrough (dashed lines) during the transcription of the transgene. There are three main strategies to avoid the potential deleterious effects of stable genetic modification: 1- Safe harbor. In this direction scientists search for vector systems that integrate in safer locations than gammaretroviral vectors. 2- Insulate. Another strategy is to modify the integrative vectors by eliminating enhancers from the Long Terminal Repeats (LTRs), by using physiological promoters and by the introduction of insulator boundaries that block the potential residual effects of the internal promoters on the nearby genes. 3- Gene Correction. Recently, a new possibility has come thanks to technologies that dramatically increase the efficiency of homologous recombination for gene editing. It is now feasible to restore the correct version of almost any mutated gene by the use of zinc finger nucleases or meganucleases together with the correct version of the gene.

pattern was approximately threefold less likely than the GVs to trigger transformation of primary hematopoietic cells. 4- The LVs backbone is more flexible to modifications and insertions.

Of all LVs, HIV-1-based are by far the most used for gene therapy strategies. Other lentiviruses commonly used are equine infectious anaemia virus (EIAV), Simian immunodeficiency virus(SIV), Feline immunodeficiency virus(FIV) and Bovine immunodeficiency virus (BIV):

**Human Immunodeficiency Virus-1 (HIV-1) based vectors.** Latest generation LVs are devoid of any HIV coding sequences from the original virus (the most commonly used is HIV-1). Proteins needed for particle formation and enzymatic activities are supplied in trans by separate plasmids encoding *Gag*, *Pol*, *Env*, *Tat* and *Rev* proteins (Figure 2). The transactivator *Tat* (generally used in *trans* to allow vector genome production in producer cells) is also dispensable for the generation of fully efficient LV vector particles. In these vectors, the *Tat*-dependent U3 promoter has been replaced by promoters such as the cytomegalovirus immediate-early enhancer and promoter (CMVp), resulting in *Tat*-independent transcription with no decrease in viral titers(Miyoshi *et al.*, 1998; Delenda, 2004).

HIV-1 based LVs efficiently transduce the most important target cells for gene therapists (neurons, hepatocytes, dendritic cells). They are probably the most efficient vectors for stem cells transduction. They are also safer than other integrative vector systems because they do not have integration preference for transcriptional start sites, although they do integrate semi-randomly in transcriptionally active areas (Montini *et al.*, 2006). In fact several studies have recently demonstrated the lower genotoxicity of LVs compared to oncoretroviral-based vectors(Montini *et al.*, 2006; Gonzalez-Murillo *et al.*, 2008). These characteristics have prompted scientists to propose LVs as one of the most interesting vectors for gene therapy strategies when stable gene expression is required. There are at the moment 29 approved clinical trials using lentiviral vectors ([http:// www.wiley.com /legacy /wileychi /genmed /clinical/](http://www.wiley.com/legacy/wileychi/genmed/clinical/)) for the treatment of primary immunodeficiencies, adrenoleukodystrophy, Parkinson disease, beta-thalassemia, sickle cell disease, Fanconi anemia, AIDS and cancer.

Since HIV-1 is a highly infectious agent for humans, several groups have thought that equivalent vectors derived from lentiviruses that are not pathogens for humans could be of interest. Vectors based on non-human primates (simian) and non-primates (feline, equine and bovine) lentiviruses would theoretically be safer than HIV-1-based vectors, minimizing the risk of generating replication-competent virus:

**Simian immunodeficiency virus (SIV)-based vectors.** LVs derived from the Simian immunodeficiency virus (SIV) have been developed by several groups(Mangeot *et al.*, 2000; Hanawa *et al.*, 2004), with safety issues similar to HIV-1 based vectors. They have been used for efficient transduction of simian and human hematopoietic stem cells (HSCs)(Sandrin *et al.*, 2002). In addition, SIV vectors have been used for correction of CGD in animal models(Naumann *et al.*, 2007). They have also demonstrated to facilitate safe and efficient retinal gene transfer(Murakami *et al.*2010) and for the respiratory epithelium (for cystic fibrosis treatment) when pseudotyped with the envelope protein of the Sendai virus(Mitomo *et al.*2010). Interestingly, integration site studies have demonstrated that while MLV-based vectors integrate predominantly around transcription start sites, SIV integrants strongly favoured transcription units and gene-dense regions of the genome without a preference for transcription start sites(Hematti *et al.*, 2004).



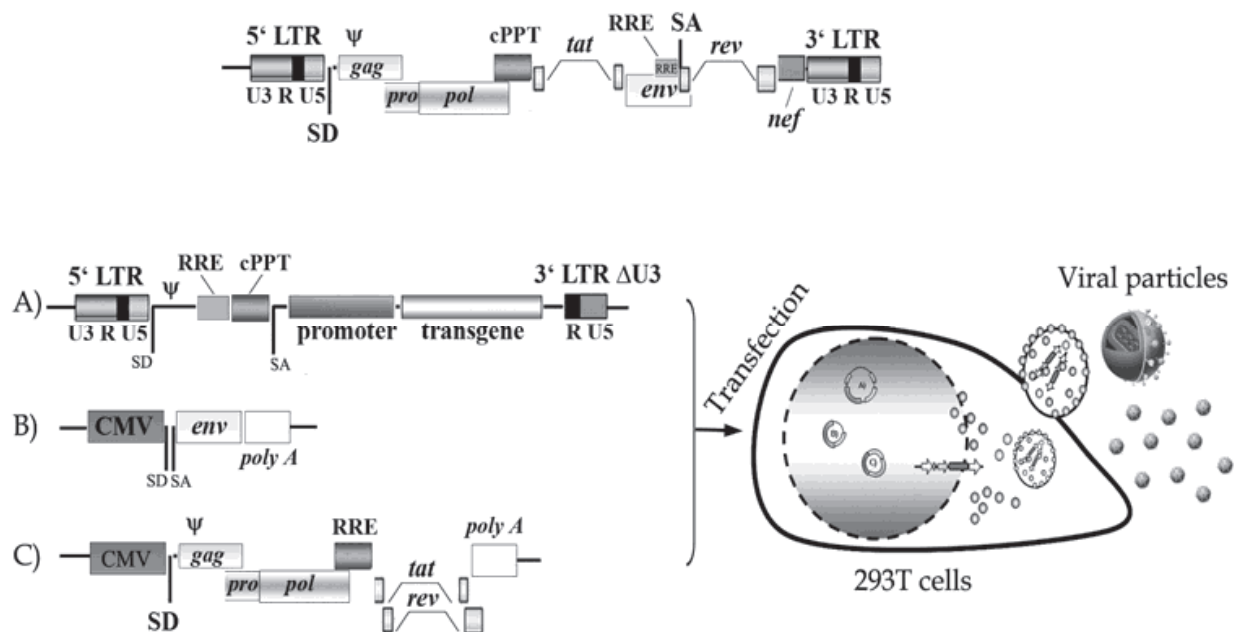


Fig. 2. Schematic representation of HIV-1 provirus DNA and HIV-1 based vector system. In the HIV-1 genome (top), the coding regions are flanked by LTRs (5' and 3') and other *cis*-acting elements such as the Primer Binding Site (PBS), the *Rev* responsive element (RRE) and the packaging signal ( $\Psi$ ). The vector genome (A) does not contain any gene from the original virus and the structural and enzymatic proteins required for particle formation, cell entry, reverse transcription and integration are supplied by other plasmid constructs (B and C) to allow the formation of a functional particle. In the majority of the vector constructs, the removed viral genes are substituted by an expression cassette consisting of an internal promoter driving the expression of a relevant transgene. To produce the viral particles, a set of three or four plasmids coding for the viral genome (A), the *env* gene (B) and the *gag-pol-rev* genes (C) are transfected into a cell line (a packaging cell line) that will be used as a vector factory. After transfection, the vector plasmid will produce the vector genome (RNA) and the other plasmids will drive the expression and synthesis of the *Env*, *Rev* and *Gag-Pol* proteins required for particle formation. *Abbreviations*: LTR, viral long terminal repeat; U3, LTR 3' unique element; U5, LTR 5' unique element; R, repeat element; SA, splice acceptor site; SD, splice donor site;  $\Psi$ , viral RNA packaging signal;  $\Delta$ U3, self-inactivating deletion in U3 region of 3' LTR, cPPT, central polypurine tract; RRE, *Rev* response element; polyA, polyadenylation signal; HEK 293T, human embryonic kidney 293T cells

**Equine infectious anaemia virus (EIAV)-based vectors.** Other groups have focused their attention to equine infectious anaemia virus (EIAV) based vectors (Mitrophanous *et al.*, 1999). Although EIAV-based vectors are about 10-fold less efficient in several human cell lines than HIV-1 based vectors (Ikeda *et al.*, 2002), they have demonstrated good efficiency for treatment of neuromuscular pathologies in animal models (Azzouz *et al.*, 2004; Yip *et al.*, 2006). In addition, they are able to transduce human HSCs (Siapati *et al.*, 2005) and are very efficient *in vivo*. Several groups have demonstrated their potential applicability for gene therapy applications using animal models of neurological disorders, macular dystrophy and choroidal neovascularisation. There is at the moment a Phase I/II study using EIAV vectors for the treatment of Parkinson disease (Prosavin) developed by Oxford BioMedica. The authors observed an average improvement in motor function of 26% (Trial ID: FR-0041).

**Feline immunodeficiency virus(SIV)-based vectors.** Feline immunodeficiency virus has been a good choice to develop LVs(Poeschla *et al.*, 1998) as demonstrated by their success in several fields (reviewed in Barraza and Poeschla, 2008). However FIV-based vectors are very inefficient expressing transgenes in human primary cells due to unknown elements that inhibit expression(Price *et al.*, 2002). This low level of transgene expression has hindered the wide use of FIV-based vectors for gene therapy strategies. In spite of this, several groups have demonstrated the potential of FIV-based vectors in animal models. Grinshpun *et al.* were able to attenuate disease symptoms in a murine model of glycogen storage diseases type I (GSD-Ia) by a double neonatal administration protocol(Grinshpun *et al.* 2010). The authors extended survival, improved body weight, and decreased the accumulation of liver glycogen. Finally, several groups have also demonstrated that FIV integration favoured actively transcribed genes (as all retroviral vectors) but the integration preferences were more similar to those of primate lentiviruses and distinct from those of moloney murine leukemia virus, avian sarcoma leukosis virus, and foamy virus(Kang *et al.*, 2006).

**Bovine immunodeficiency virus based vectors.** Another alternative to HIV-1 based vectors are the LVs based on bovine immunodeficiency virus (BIV)(Berkowitz *et al.*, 2001; Matukonis *et al.*, 2002). BIV-based vectors also transduced a wide panel of gene therapy primary targets including unstimulated human HSCs. However, in spite of the generation of safer packaging cell lines for BIV-based vectors a few groups have followed these LVs as an alternative for gene therapy strategies.

In summary, the use of non-human LVs is a field of intensive research with some promising results in gene therapy. However, based on the published data it is clear that HIV-based vectors are still the vector of choice for most applications since they are more efficient and have at least the same safety profiles than non-HIV-1 based vectors. In fact, there is not a single study demonstrating that non-human LVs are safer than HIV-based LVs.

### 2.1.2 Foamy viral vectors (FVV)

Foamy viruses (FV) share with other retroviruses the principal genetic order of LTR, *gag-pol-env-accessory genes*-LTR, as well as the reverse transcription and integration processes. However they complete the reverse transcription process before budding from the cell membrane. Another interesting property is the stability of the resulting DNA genome that could be the reason for the excellent capability of FV vectors (FVV) to transduce rarely dividing cells, such as HSCs. Other important feature is that the viral envelope mediates access to virtually any cell type, although the viral receptor is still unknown. The viral genome is complex, as it contains not only the *gag*, *pol* and *env* genes, but also genes that encode proteins that are not incorporated into the viral particle (*bel-1 (tas)* and *bet*).

FVV were developed over fifteen years ago(Schmidt and Rethwilm, 1995). The majority of FVV are based on human foamy virus (HFV) which was derived by a rare zoonotic infection of humans by a chimpanzee spumavirus (CFV)(reviewed in Williams, 2008). FVV are highly efficient for HSCs transduction, they have a favourable integration profile, can deliver large DNA fragments and are derived from a non-pathogenic virus. These properties make them a very attractive tool for gene therapy. Novel FVV vectors are devoid of the *env* gene and the internal promoter (Bel1/Tas-independent vectors) and the expression is directed by a heterologous human promoter(Trobridge *et al.*, 2002; Bastone *et al.*, 2007).

For a long time, the low FVV titers have been a major hurdle for FVV clinical applications. However, this problem has been overcome and vector titers above  $10^7$  per ml are routinely produced in different laboratories. Bauer *et al.*(Bauer *et al.*, 2008) described the first

successful use of a FVV to treat a genetic disease. The authors used FVV to treat canine leukocyte adhesion deficiency (CLAD). Four out of five dogs that received infusion of HSCs transduced by a FVV expressing CD18 had complete reversal of the CLAD phenotype for more than 2 years. Integration site analysis showed polyclonality of transduced cells and a decreased risk of integration near oncogenes as compared to GVs. More recently, Park et al. used FFV for conditional expression of short interfering RNAs (siRNAs) in HIV infected cells (Park *et al.*, 2009) and their results showed inhibition of HIV replication by more than 98%.

## 2.2 Improvements of first generation retroviral vectors

In addition to searching for new viruses for vector development, gene therapists are continuously trying to improve safety and efficiency of old (MLV-based) and new (HIV-1 based) retroviral vectors. Below we described the main strategies to achieve better safety profiles:

### 2.2.1 Self-inactivating (SIN) vectors

One of the major drawbacks of first generation retroviral vectors has been the use of the viral LTR U3 region to drive the expression of the transgene. The U3 contains a strong viral promoter and enhancer sequences that have been responsible for the activation of downstream host genes leading to transformation and leukemia-like diseases upon insertion in hematopoietic stem cells (Baum *et al.*, 2003; Modlich *et al.*, 2009). Therefore, one way to improve safety of retroviral vectors is to eliminate the U3 region from the 5' LTR and to use alternative promoters to drive the expression of the transgene. Different authors have included different deletions at the U3 region in the 3' LTR of GVs (Yu *et al.*, 1986; Olson *et al.*, 1994) and LVs (Miyoshi *et al.*, 1998; Zufferey *et al.*, 1998). During the transduction process, reverse transcription transfer this deletion also to the 5' LTR of the proviral DNA. Since the U3 region harbor the promoter and enhancer regions required for RNA transcription, this mutation not only abrogates potential mobilization of the vector but also reduces genotoxicity due to promoter/enhancer interference (Modlich *et al.*, 2006; Modlich *et al.*, 2009).

In oncoretroviruses like MVL or SNV, SIN vector development was compromised since the U3 from the 3' LTR is partially involved in the polyadenylation of the viral RNA. Therefore, most MLV-derived SIN vectors carry a deletion that still maintain significant transcriptional activity in their long terminal repeats (LTRs). Attempts to mutate the TATA box dramatically decreased vector titers, presumably due to weak polyadenylation. However in LVs, the polyadenylation site is located just upstream of the R region of the LTR and therefore tolerate large U3 deletions without functional loss. This has facilitated the use of SIN LVs over SIN GVs counterparts.

### 2.2.2 Insulators

Limitations derived from randomly genome insertion of retroviral vectors have two sides, 1- possible deleterious effects of vector enhancer elements in host cell gene expression (as mentioned above) 2- the effect of chromosomal position in vector expression such as transcriptional silencing. Therefore a main goal of gene therapists has been to isolate integrative vectors from the host chromatin in such a way that 1- enhancers present in the



vector can not influence expression of cellular host genes and 2- that regulatory sequences present in the host chromatin can not influence vector expression (Figure 3).

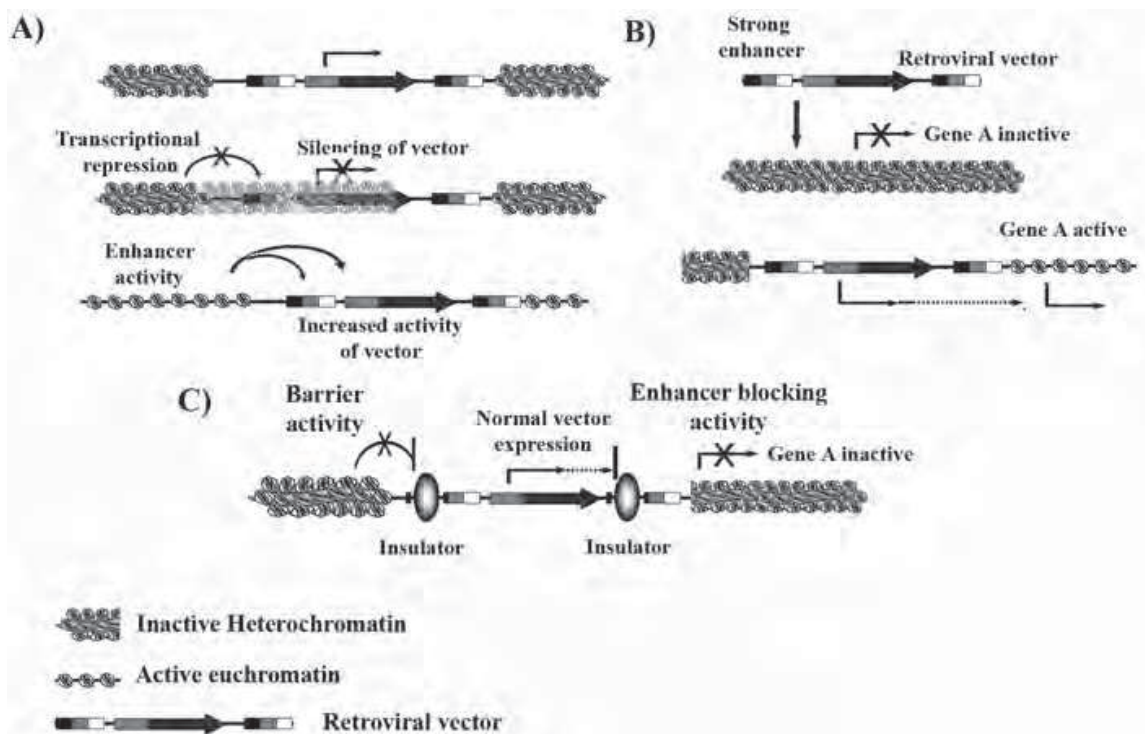


Fig. 3. Insulator functions in vector development. A) Vector expression can be enhanced or silenced if integrated in a transcriptionally active site or near heterochromatin respectively. B) Retroviral vectors can produce the activation or upregulation of poorly active or silent genes. This can lead to cell transformation. C). Retroviral vectors can be modified to reduce both effects by including insulators boundaries such as HS4 in the backbone. These insulators should have two activities; 1- a barrier activity to avoid vector silencing and a 2- enhancer blocking activity to block both the effect of chromatin enhancers on vector expression and the influence of vectors enhancer on nearby chromosomal genes.

Insulators are genetic elements near chromatin domain boundaries that function as barriers against repressive effects of neighbouring heterochromatin or preventing inappropriate activation of a promoter by nearby heterologous enhancers (Bell *et al.*, 2001). The best studied vertebrate element is a 1.2-kilobase (kb) fragment containing the chicken  $\beta$ -globin 5' DNase I hypersensitive site 4 (5'HS4) (Bell *et al.*, 1999). This element can protect against position effects and also provide enhancer blocking function, two important and separable characteristics for an insulator. In addition of HS4, there are several other chromosomal elements that function as insulators in different organisms (Gaszner and Felsenfeld, 2006). Neff *et al.* (Neff *et al.*, 1997) hypothesized that the incorporation of chromatin insulator in gene therapy vectors should lead to improved safety and expression of therapeutic transgenes. However the incorporation of insulator elements may also have undesirable consequences such as reduction in viral titre that need to be solved (Hanawa *et al.*, 2009).

Different insulators have been used to improve safety and expression of various GV and LVs (Emery *et al.*, 2000; Hino *et al.*, 2004; Ramezani *et al.*, 2008). Some authors have combined two different elements such as HS4 and BEAD-1 with good results (Ramezani *et al.*, 2008). The introduction of these elements into the vector backbone has been shown to decrease

genotoxicity and gene silencing (Emery *et al.*, 2000; Hino *et al.*, 2004; Arumugam *et al.*, 2007; Robert-Richard *et al.*, 2007; Ramezani *et al.*, 2008; Li *et al.*, 2009). Nienhuis and colleagues demonstrated that the inclusion of the insulator in the retroviral construct suppressed cellular transformation (Evans-Galea *et al.*, 2007). These important results have pushed gene therapists to incorporate these elements into therapeutic vectors, especially when the strategy involves gene transfer into stem cells.

### 2.2.3 Improved polyadenylation signal

Although long distance enhancer interactions are the most frequent form of retroviral insertional mutagenesis, read-through transcripts (not stopping at the polyadenylation site contained in the vector) could be, theoretically, an important safety problem. In retroviruses, the polyA motif is contained within the R region of the LTRs and is therefore present in both ends of the transcript. To avoid premature termination and polyadenylation in the 5'R region, retroviruses have developed weak polyA sites and additional mechanisms of 5' polyA site suppression. In addition, SIN vectors lack the majority of the U3 region and this increases the probability of read-through. The insufficient termination causes read-through of randomly integrated retroviral sequences into cellular genes and contribute to the up-regulation of cellular proto-oncogenes, potentially triggering malignant transformation (Almarza *et al.*; Schambach *et al.*, 2007). Some groups have shown that the inclusion of upstream polyadenylation enhancer elements improves the efficiency of 3' end mRNA processing in GV and LV SIN vectors (Schambach *et al.*, 2007; Hager *et al.*, 2008). The resulting vectors not only have improved titer and transgene expression in target cells but they also displayed a lower rate of vector mobilization (Koldej and Anson, 2009). Still, whether stronger polyA signals in retroviral vectors will result in an improvement of biosafety must be further analyzed in animal models.

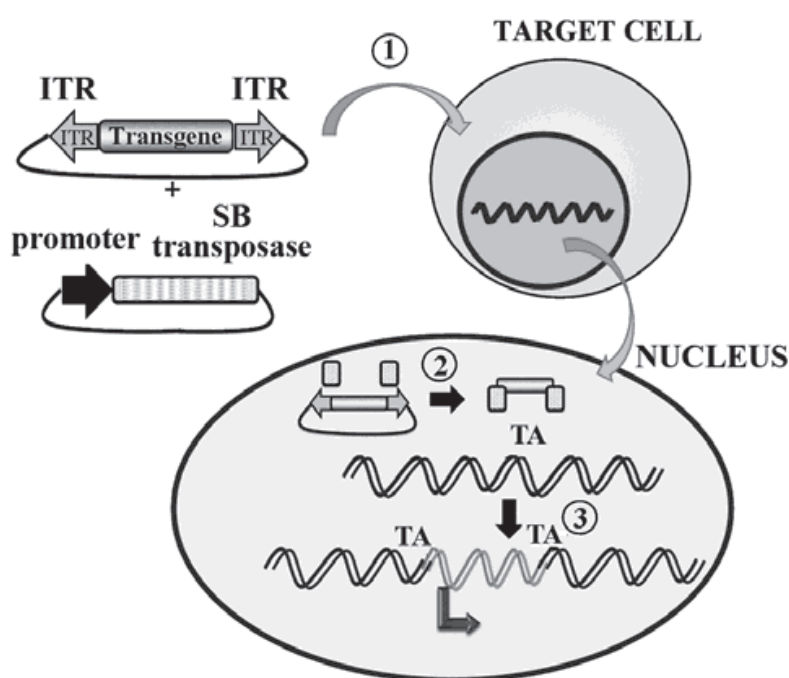
## 3. Novel non-viral systems to achieve efficient transgene integrations

Although most gene therapy approaches use viral-based vectors to obtain stable integration, new non-viral systems may offer some advantages: 1- Viral vector preparations, in particular those for clinical use, are very expensive and time consuming. Non-viral vectors are less expensive and easy to manufacture. 2- Viral vectors need packaging cell lines for their production whereas other non-viral vectors rely on cell free systems with defined components. 3- Non-viral vectors are less immunogenic than viral systems (Yant *et al.*, 2000). 4- Finally, integrative viral vectors have a limited packaging capacity whereas non-viral systems can deliver larger DNA fragments (Zayed *et al.*, 2004; Ding *et al.*, 2005). The use of non-viral vectors has been limited due to the low efficiency, low rate of integration and silencing issues. However there have been important advances in the field in recent years. Two main strategies using non-viral systems, transposons and zinc-finger nucleases, are having a mayor impact in the area:

### 3.1 Transposons

Transposons (in particular the *Sleeping Beauty* (SB)) are becoming serious players as gene delivery tools. Transposons are DNA sequences that can move from one chromosomal location to another utilizing an enzyme named transposase which recognizes two sequences flanking the transposon. Any sequence included between the two flanking regions can also be mobilized and this characteristic makes the transposons attractive as a gene therapy tools

to achieve stable transgene integrations (Figure 4). The mechanism involved behind the mobilization of transposons is described elsewhere (Izsvak *et al.*, 2000). Briefly, the inverted terminal repeats (ITR) flanking the transposon are recognized by the transposase which excises the transposon from the genome (or from a donor plasmid). The transposon is inserted into TA sequences in the genome with no preference for genes or intergenic regions. This characteristic makes the transposon integration semi-random. It seems that they induce low epigenetic changes and no adverse effects have been reported yet (Zhu *et al.* 2010). To avoid further transposition events, the expression of the transposase must be transient in the target cell. To increase the safety level of transposition, three different ways to target SB transposition have been proposed based in the addition of particular DNA binding domains to the SB transposase (reviewed in Izsvak *et al.* 2010).



ITR: Inverted Terminal Repeats; SB: Sleeping Beauty Transposase.

Fig. 4. Transposition mechanism of Sleeping Beauty transposon: 1) Delivery of both the SB transposon (containing the genetic material to be integrated) and the SB transposase (expressed under the control of a constitutive promoter) into the nucleus of the target cell. The transposon is flanked by the ITR. 2) In the nucleus, the SB transposase cleaves the transposon at the ITR sites. 3) The SB transposase integrates the transgene into any accessible TA site in the host genome.

The development of transposases with higher activity (Sleeping Beauty transposase, SB10 or SB100) (Izsvak *et al.*, 2000; Mates *et al.*, 2009) and the new delivery techniques such as lipofection, PEI-complexes, hydrodynamic injection, nucleofection or electroporation have achieved efficient integration of large fragments of DNA into hard to modify cells, as hESCs (Wilber *et al.*, 2007; Mates *et al.*, 2009) or HSCs (Mates *et al.*, 2009). These improvements have allowed the use of SB as a real alternative for therapeutic strategies for diseases such as junctional epidermolysis bullosa, haemophilia A and B, pulmonary hypertension, glioblastoma, inherited tyrosinemia, mucopolysaccharidosis, lung transplantation-associated complications and B-lymphoid malignancies (Izsvak *et al.* 2010). Indeed, three

clinical trials have been approved for the treatment of CD19+ B cell malignancies (Trials ID: US-0922, US-1003 and US-1022) using SB transposons.

#### 4. Site-specific integrative vector systems

Although the systems mentioned above can reduce genotoxicity of first generation integrative MLV-based vectors, they still integrate randomly and have therefore potential genotoxicity consequences. Therefore, several groups have dedicated much effort to achieve site-specific integration to safer genomic areas. This strategy allows the selection of areas that are less rich in genes involved in tumorigenicity and are therefore less likely to cause serious side effects. However as we will see in this section, the low efficacy and/or poor integration specificity of most site-specific integrative systems have been mayor drawbacks of this strategy.

##### 4.1 Adeno-associated viruses (AAV)

Adeno-associated virus (AAV) are nonpathogenic, nonenveloped DNA virus that belong to the parvovirus family. AAV virions contain a single-stranded DNA genome of only 4,680 bases, which is flanked by two inverted terminal repeats (ITRs). AAV can replicate in cell culture only in the presence of a co-infection by a helper virus, either adenovirus (Ad) or herpesvirus (HV). Infection of human cells with wild-type AAV results in site-specific integration into the AAVS1 region of chromosome 19 (Kotin et al., 1990). Recombinant AAV (rAAV) vectors have an excellent safety record with demonstrated efficiency and safety in preclinical studies in thousands of animal studies. AAV have been very successful for human gene therapy strategies in liver, muscle and eye (reviewed in Rolling, 2010; Tang *et al.*, 2010; van der Laan *et al.*, 2011), however, the therapeutic benefits did not correlate with site-specific vector integration. Expression of *Rep78* or *Rep68* is necessary for targeted integration of AAV-derived DNA and, in its absence, AAV genome is kept as episomal DNA or integrates by nonhomologous recombination at random locations. The limited DNA cargo capacity of AAV particles and the need to maintain the *rep* ORF, precludes the use of AAV itself as a site-specific integration vector. It is now well established that the rAAV vector genomes exist in a predominantly episomal form and that transduced dividing cells lose expression with time (Miao *et al.*, 1998). However, the non-specific integration of rAAV vectors allow stable expression in several cell types, including hematopoietic stem cells (Maina *et al.*, 2008) and germ line stem cells (Honaramooz *et al.*, 2008). AAV vectors have been used in preclinic studies of immunodeficiencies like CGD, a heterogeneous group of disorders arising from molecular lesions in the genes encoding a phagocyte-specific enzyme system, the NADPH-oxidase. Vectors based on AAV with a functional copy of the *p47phox* gene have been used to transduce EBV-immortalized B cells derived from patients with CGD, achieving a stable expression of protein and restoration of NADPH-oxidase function in these cells for 3 months without selection (Thrasher *et al.*, 1995). In summary, although rAAV can achieve stable integration in certain cell types, this integration is random as for retroviral vectors and can cause mutations (Nakai *et al.*, 2003) and tumor development (Donsante *et al.*, 2007). Therefore, although AAV vectors can achieve stable gene expression, they are not safer than other integrative systems such as GVs or LVs.

##### 4.2 AAV Hybrid vectors

AAV hybrid viral vectors utilize the cis- (ITRs) and trans- (*Rep68/78*) acting components of AAV viruses to specifically target transgenes into the AAVS1 integration site at



chromosome 19. This technology has opened the possibility of integrating large DNA sequences in a site specific fashion with the aim of minimizing genotoxicity. AAV-Hybrid vectors based on plasmids, baculovirus, Ad and HSV can potentially accommodate genetic material of more than 30.000 base pairs (Smith, 2008). Two factors are limiting in this system, the first one is the delivery efficiency of the transgene flanked by the AAV ITRs and the *Rep68/78* to the target cells and the second is the *Rep68/78* protein toxicity. In fact, *Rep* proteins down-regulate the expression *c-H-ras*, *c-fos*, *c-myc*, and *c-sis* and can inhibit the proliferation of some cell lines. In addition *Rep*-mediated integration at AAVS1 can lead to nonspecific genomic rearrangements at the same locus. The frequency and severity of these undesired effects might be attenuated or suppressed by setting a time limit to the activity of *Rep* proteins in target cells. This has been achieved by transfecting *Rep* proteins or *rep* mRNA directly, by using inducible promoters or by construction of regulated *Rep* chimeras.

#### 4.2.1 Plasmid/AAV

Based on data obtained from AAV integration process, several groups have designed a site-specific integration system based in two plasmids: an integrating plasmid containing AAV ITRs and the second providing the AAV *Rep* proteins. Co-transfection of both plasmids in 293T cells achieved site-specific integration in the AAVS1 integration site. This system has achieved stable expression in several cell lines including hematopoietic cells (Howden *et al.*, 2008). However the low plasmid transfection efficiency in primary cells has limited its use in gene therapy applications. New gene delivery technologies will certainly improve the results obtained with this system.

#### 4.2.2 Baculovirus/AAV

The baculoviruses are a family of large, double stranded DNA (80-180kb), enveloped viruses that infect only invertebrates. The baculovirus *Autographa californica multiple nucleopolyhedrovirus* (AcMNPV)-based vectors have recently been developed as a new tool for gene therapy (reviewed in Hu *et al.* 2010). Latest generation of baculovirus vectors systems are safe, easy to use and amenable to scale up. Baculovirus vectors are able to transduce a wide range of human cell lines, including primary cells such as bone marrow fibroblasts, neural cells, hepatocytes and mesenchymal stem cells. Zeng and co-workers (Zeng *et al.*, 2007) have demonstrated their ability to transduce human embryonic stem cells without altering their characteristics. The hybrid vectors contained three promoters, one driving the expression of the complete baculovirus genome, the second expressing *eGFP* and the third (AAVp5) driving the expression of *Rep78*. However, in spite of their potential, the usefulness of this technology for stable integrations of transgenes into stem cells has not yet been achieved.

#### 4.2.3 Adenovirus/AAV

Adenoviruses (Ad) are non-enveloped non-integrative viruses composed of a nucleocapsid and a double-stranded linear DNA genome. There are 51 immunologically distinct human Ad serotypes, however, the subgroup C serotypes 2 or 5 are the most used for vector development. The wild type Ad genome is approximately 35 kb of which up to 34 kb can be replaced with foreign DNA in the helper dependent (HD) Ad gutless vector. Ad vectors can mediate high level of transduction in a wide variety of both quiescent (post-mitotic cells) and proliferating cells. However, since they are non-integrating, transgene expression in dividing cells is progressively lost. The development of Ad/AAV hybrid vectors (Lieber *et*



*al.*, 1999; Recchia *et al.*, 1999) aim to increase the integration efficiency. Clearly, a vector that combines the advantages of Ad (high titer, high infectivity, and large capacity) with the integration capability of AAV would be advantageous for gene therapy. The Ad/AAV hybrid vectors system requires two Ad vectors; one containing the Ad-ITRs, the Ad packaging signal and the AAV-ITRs flanking the DNA of interest and a second one expressing the AAV *Rep68/78* protein (Figure 5).

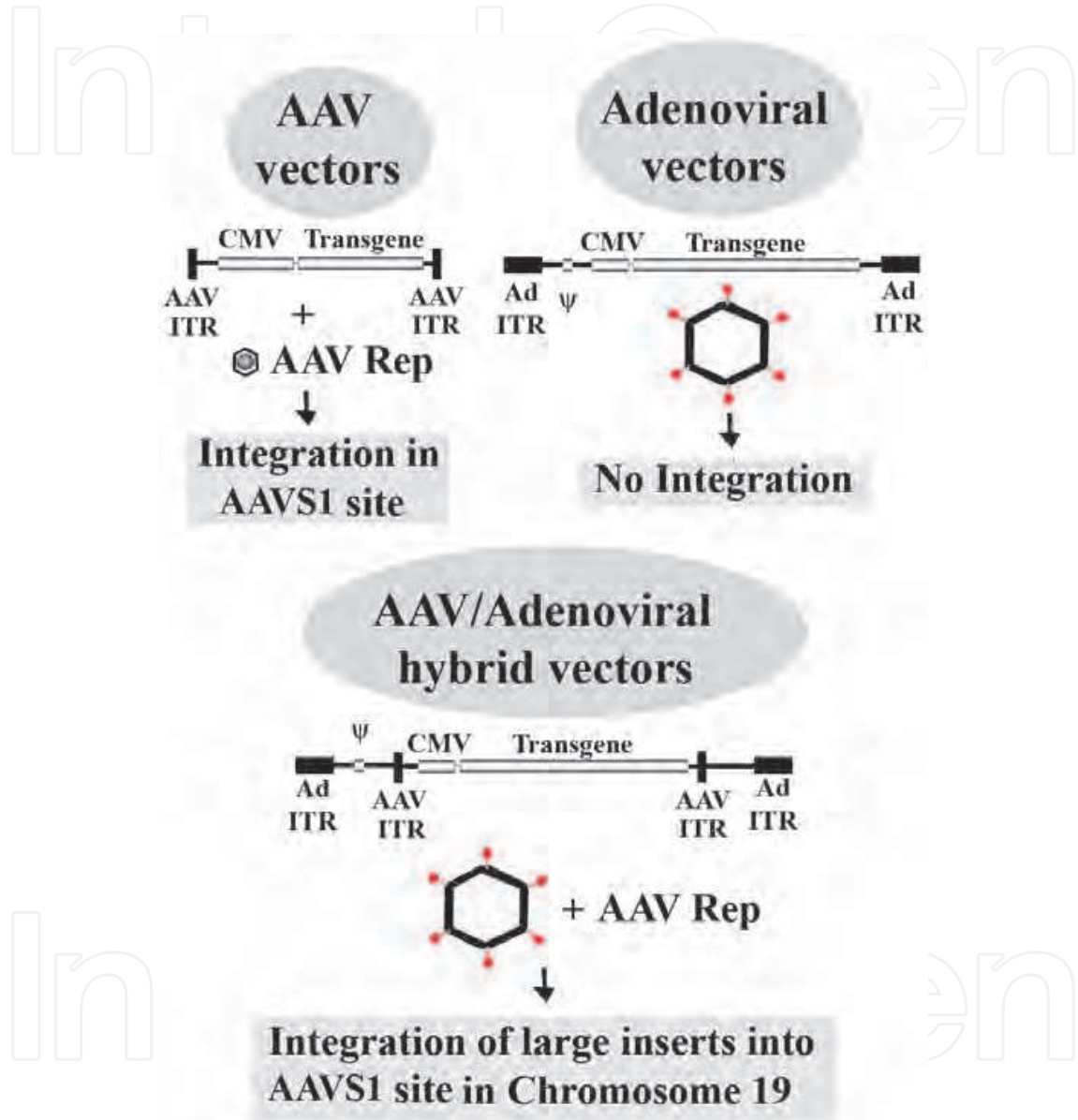


Fig. 5. AAV/Adenoviral hybrid vectors. The AAV/ Ad hybrid vectors combine the large packaging capacity of Ad with the site-specific integration potential of AAV vectors. Either AAV *Rep68* or *Rep78* proteins are sufficient to mediate insertion of AAV-ITR-containing DNA into the AAVS1 on chromosome 19. By including the AAV-ITR into the adenoviral backbone and expressing *Rep68* or *Rep78* we can achieve integration of the adenoviral vector into the AAVS1 site. Only DNA sequences flanked by the AAV-ITR will be integrated into the chromosome 19. The most efficient systems use an additional adenoviral vector to produce the *Rep68/78* proteins required for specific integration.

Although Ad vectors harboring transgenes flanked by AAV-ITRs can be produced easily, the production of Ad expressing *Rep68/78* is more complicated due to the *Rep68/78* inhibition of Ad replication. Recchia and co-workers (Recchia *et al.*, 1999) tackled this problem by expressing *Rep78* gene under the control of promoters with low activity in the packaging cells. Another relevant issue is that transduction of stem cells with first generation Ad vectors is associated with toxicity due to viral gene expression. Therefore helper dependent (HD) Ad vectors depleted of all viral genes should be used for stem cells transduction. In fact most Ad/AAV hybrid systems are based on HD Ad vectors which have demonstrated their ability to stably express several transgenes by specific integration into the AAVS1 integration site, both *in vitro* and *in vivo* achieving expression of therapeutic levels of *Factor VIII* in hemophilia A mice (Gnatenko *et al.*, 2004) and of *dystrophin* in mdx mice (Goncalves *et al.*, 2005).

Although the Ad/AAV hybrid system has achieved specific integration into the AAVA1 site, the efficiency is far from 100%. Recently Wang *et al.* (Wang and Lieber, 2006) demonstrated that Ad/AAV can achieve up to 30% efficiency of stable expression (30% of the cloned cells have stable expression of the transgene). In addition only 30% of these clones stably expressing the transgene were integrated at the AAVA1 site while in the other 70% the stable expression was a consequence of homologous recombination or random insertion. Further knowledge of the mechanism involved in site specific integration into the AAVA1 site and the processes involving homologous recombination will certainly increase the potential of these vectors.

#### 4.2.4 Herpesvirus/AAV

Herpesvirus (HSV) are a large family of enveloped DNA virus that cause diseases in animals, including humans. Their genome is composed of large (over 150kb) double stranded linear DNA encoding 100-200 genes. The DNA is packed by an icosahedral protein structure or capsid that is surrounded by a lipid bilayer membrane (envelope). HSV-1 have several characteristics that make them a promising gene delivery vector such as their capacity to package large amounts of heterologous DNA (over 100kb), their ability to establish persistent, lifelong infections and a broad range of cell tropism including dividing and non-dividing cells (reviewed in Berto *et al.*, 2005)). An important property of HSV-1-based vectors is the ability to deliver a complete genomic locus, in which the native promoter and regulatory regions drive and control expression of the transgene. However first and second generation HSV-1 based vectors require the expression of regulatory viral proteins that hinder their applicability into the clinic. To tackle this problem several authors developed HSV amplicon vectors, bacterial plasmids that contain only the origin of replication and the DNA packaging/cleavage signal from HSV-1. These HSV amplicon vectors are dependent on helper virus function to provide the replication machinery and structural proteins necessary for packaging the amplicon vector DNA into viral particles. Helper packaging function is provided by a replication-defective virus or by packaging methods using a set of 5 overlapping cosmids or bacterial artificial chromosomes (BAC) that encode the entire HSV genome.

The HSV/AAV hybrid vectors combine the high efficiency and large transgene capacity of HSV-1 amplicon vectors with the potential for site-specific chromosomal integration of AAV vectors. The hybrid HSV/AAV vectors contain the origin of DNA replication and the DNA cleavage/packaging signal of HSV-1 and a transgene (ie *lacZ* reporter gene) under the

control of a desired promoter flanked by AAV inverted terminal repeat (ITR) sequences. HSV/AAV hybrid vectors are relatively inefficient in achieving stable expression of the transgene (2-20% depending of the cell line and the vector system), however they are one of the most efficient systems to direct site-specific integration (50-70% of stably-expressing cells). One of the main advantages of HSV/AAV hybrid vectors is their capability to achieve site-specific integration of up to 100kb in a significant proportion of transduced cells. Although HSV/AAV vectors have been successful in animal models of Ataxia-Telangiectasia (Cortes *et al.*, 2003; Cortes *et al.*, 2008) they still have some safety concerns such as the low efficiency in important target cells (HSCs) and the random integrations observed in over 40% of stably expressing cells.

### 4.3 Site-specific integrases

Another alternative used by gene therapists to achieve site-specific integration of therapeutic genes is based on naturally occurring site-specific integrases recognizing specific target in the human genome. One of such enzymes is the *Streptomyces bacteriophage* derived *phiC31* integrase, which mediates site-specific integration of plasmid DNA (pDNA) into mammalian host genomes (Thyagarajan *et al.*, 2001; Olivares *et al.*, 2002). This integrase can insert large DNA elements (over ten kilobases in size) containing an *attB* sequence into genomes containing *attP* sites. Importantly, this integrase recognizes sequences in the mammalian genome termed pseudo-*attP* sites (Sclimenti *et al.*, 2001). These pseudo *attP* sites are also present in the human genome and can mediate efficient *phiC31* -integrase-mediated integration (Figure 6). In addition, directed evolution has rendered new integrases specific for pseudo *attP* sites located at human chromosome 8 (Sclimenti *et al.*, 2001). Using this technology several authors have shown the potential of the *phiC31* integrase system for the treatment of lung diseases (Aneja *et al.*, 2007) and human skin diseases (Ortiz-Urda *et al.*, 2002). Others have shown long-term transgene expression in liver (Olivares *et al.*, 2002) and retina (Chalberg *et al.*, 2005).

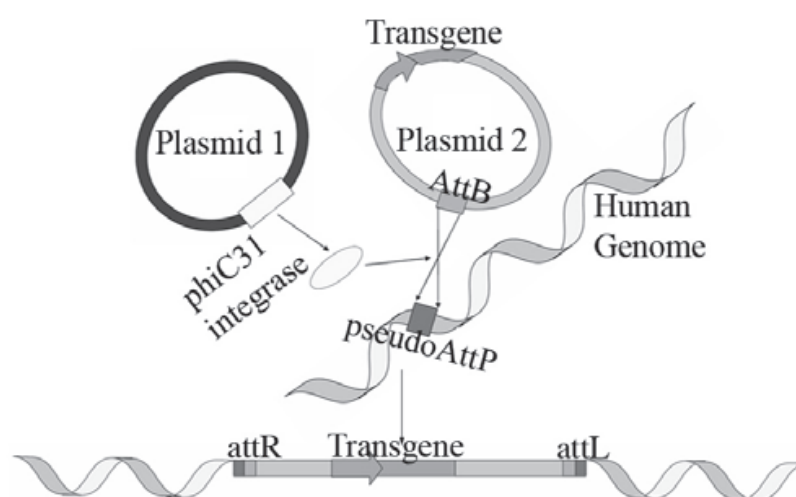


Fig. 6. Site specific integration of *phiC31* integrase in the human genome. *PhiC31* integrase mediates the integration of a plasmid containing an *attB* site and the transgene into *pseudo-attP* sites at the human genome. Once integrated the donor sequence is flanked by *attR* and *attL* sequences.

However safety of *phiC31* integrase-mediated integration has not yet been studied in detail. Several authors have found imprecise integrations into pseudo-attP sites resulting in DNA deletions (Thyagarajan *et al.*, 2001; Ehrhardt *et al.*, 2005) and others have found that the *phiC31* integrase may cause chromosomal instability (Held *et al.*, 2005; Chalberg *et al.*, 2006; Liu *et al.*, 2006). In any case, no serious adverse events have occurred so far in animal models and therefore further studies are required to assess the safety of this vector system.

#### 4.4 Zinc-finger DNA binding domains-transposases chimeras

Other attempts to target DNA integration to specific sites have used transposases from sleeping beauty (SB) (Wilson *et al.*, 2005) or piggyback (Wu *et al.*, 2006) transposons. SB-based vector systems have been successful in several preclinical models (reviewed in Izsvak *et al.*, 2010). However, first generation transposons have two major limitations; the low rate of integration compared to viral-based vectors and the nonspecific integration of the transgene. The incorporation of DNA-binding domains into the transposase can potentially increase efficiency and result in site-specific integration. SB transposase fused to the *Sp1* zinc finger DNA binding domain has achieved specific integration in *sp1* (Wilson *et al.*, 2005). Similarly, Wu *et al.* (Wu *et al.*, 2006) demonstrated targeted integration to *gal4* sites by coupling *Gal4* DNA-binding domain to piggyback transposase. As proof-of-principle, these results are promising while further work is needed to provide more information about non-desired integrations and to target other integration sites. Targeting *sp1* or *gal4* sites is not, in principle, the most appropriate strategy for most gene therapy applications since they could affect gene expression of a wide range of relevant genes.

#### 4.5 Zinc-Finger Nucleases for site directed integration

In addition to the use of ZFN for gene repair (see below in point 5), ZFN can be used to introduce expression cassettes specifically into already identified “safe harbors”, which are particular locations in the genome where the insertion of exogenous DNA is theoretically less genotoxic. This is the case for the adeno-associated virus integration site (AAVS1) locus in human or the ROSA26 locus in mouse. This strategy has already been utilized by some groups (DeKolver *et al.*; Hockemeyer *et al.*, 2009) and tested in cell lines including hESC. This strategy overcomes the need to develop ZFNs for a particular mutation for each particular disease, which nowadays is an expensive, cumbersome and time consuming process.

### 5. Gene correction by enhanced homologous recombination

Gene correction aims to repair a defective gene directly in the cellular genome by gene targeting, a process in which a DNA molecule introduced into a cell replaces the corresponding chromosomal segment by homologous recombination. This is certainly the more logical way to cure primary immunodeficiencies since the “*in situ*” correction of the mutation(s) will restore normal physiological gene function and therefore will warrant sustained and regulated expression of the repaired gene through its endogenous promoter. However, site-specific engineering of the human genome has been limited by the low frequency of homologous recombination (HR), by the requirement of sophisticated targeting vectors and by the use of drug selection. Recently, improvements in DNA-delivery technology and in the development of customized zinc-finger-nucleases (ZFN) (reviewed in Cathomen and Joung, 2008) have awakened new interest in this technology for its use in gene therapy approaches.



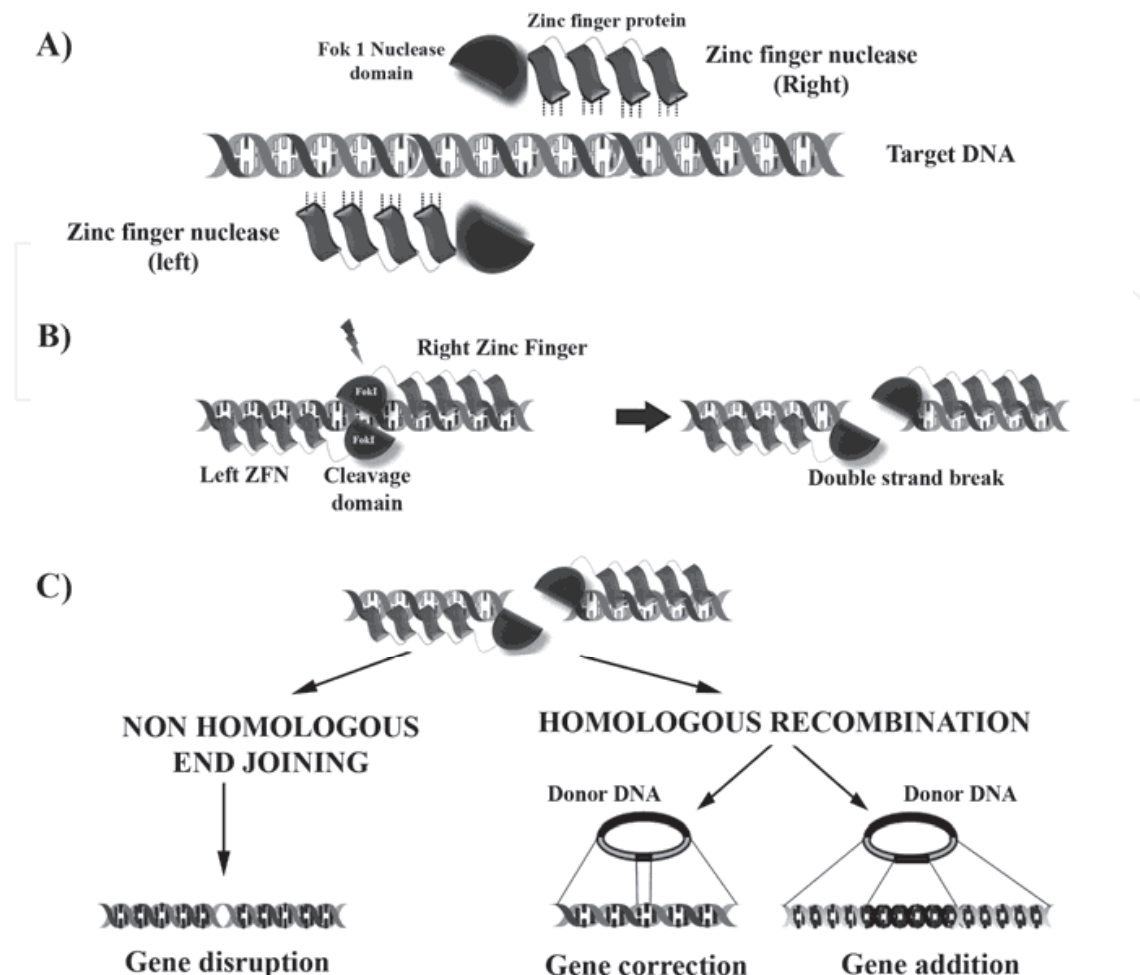


Fig. 7. Gene targeting using zinc finger nucleases. A) Zinc finger nucleases are chimeric proteins containing a zinc finger domain specific for a 9-12 nucleotide sequences and a Fok I nuclease domain that requires dimerization to cut DNA. For targeting specific DNA sequences the system require two ZFNs (right and left) each recognizing a 12 nucleotide sequence separate by a 4-6 nucleotide sequence. B) Once the zinc finger binds to its targets, the nuclease cleaves the DNA creating a double strand break C) The double strand break induces the own cell repair mechanisms to resolve the damage. The two main processes are non-homologous end joining (NHEJ) and homologous recombination directed repair. If a donor DNA (with homologous sequences) is not available the break is repaired by NHEJ yielding small deletions or additions. This process can be harnessed to induce gene disruption. When a homologous DNA sequence is present, the cell can repair the break by homologous recombination. The transfection of donor DNA with homologous regions together with the zinc finger nucleases allows gene editing by including the desired sequences inside the homologous regions. This system can be used to introduce small changes for gene repair or bigger changes to promote gene addition.

*Zinc finger nucleases* can be considered custom-designed molecular scissors. ZFNs combine the non-specific cleavage of an endonuclease (*FokI*) with the specific recognition properties of zinc finger proteins (ZFPs) to cut at the desired chromosomal locus inside the cells (Ashworth *et al.*, 2006) (Figure 7). A single zinc finger domain recognizes three consecutive nucleotides. The ZFN is composed by a ZFP containing three to four zinc finger



domains, which recognize a 9 to 12 nucleotide sequence, and the FokI domain. The DNA cleavage by *FokI* requires dimerization (Miller *et al.*, 2007) and therefore two different ZFPs must properly bind to the DNA side-to-side from the breaking point. Once the target DNA has been cut, the cells' own repair machinery resolves the break by non homologous end joining (NHEJ) or homologous recombination (HR) depending on the absence or presence of a donor DNA (Figure 7). When not present, the DNA break is repaired by NHEJ and when a donor DNA is present HR is the main mechanism.

Using this principle, ZFNs have proven invaluable for stimulating homology-directed gene repair in a variety of cell types. The accuracy and high efficiency of the HR process combined with the ability to design ZFNs that target most DNA sequences make this technology a powerful tool for gene correction. Recent results have shown that ZFNs can be used to create targeting frequencies from 5-50% in the absence of selection in several cell lines (Urnov *et al.*, 2005; Miller *et al.*, 2007) including primary T cells (Perez *et al.*, 2008), hematopoietic stem cells (Lombardo *et al.*, 2007), mesenchymal stem cells (Benabdallah *et al.*) and embryonic stem cells (Hockemeyer *et al.*, 2009).

Recently, ZFNs have shown *in vivo* correction of a humanized mouse model of hemophilia B (Hojun *et al.*, 2010). In this work, *in vivo* administration of AAV expressing the ZFNs achieved cleavage of the target site in up to 45% of the hepatocytes. When co-administered together with donor plasmids containing the correct version of the gene the authors achieved circulating F.IX levels 2-7% of normal which was enough to obtain phenotypic correction of the defect of clot formation.

The ZFNs have also been used in clinic not to correct a mutated gene but to disrupt the expression of a disease-causing-gene. Several clinical trials have been approved to use ZFNs with this aims: Reik *et al* have proposed the use of Zinc finger nucleases targeting the *glucocorticoid receptor* of allogenic transgenic Il13-expressing CTLs with idea to make them resistant to glucocorticoids for the treatment of glioblastoma (ClinicalTrials.gov Identifier: NCT01082926). Other two groups have targeted *CCR5* for the treatment of HIV, used as a co-receptor for HIV entrance (Holt *et al.*; Perez *et al.*, 2008) (ClinicalTrials.gov Identifier: NCT00842634 and NCT01044654).

## 6. Conclusion

Stable gene modification without affecting normal cellular function is a main goal both for basic and applied science. Most gene therapy targets, such as inherited, infectious and degenerative diseases will benefit from a safe and efficient integrative system. Old strategies to achieve stable gene medication are either very inefficient (homologous recombination) or very genotoxic (gammaretroviral gene transfer). Retroviral vectors derived from gammaretroviruses (the most widely used vectors for gene therapy studies) were the only alternative for stable gene modification for years. They were the first vectors to show real therapeutic effect but they also showed a high risk of cellular transformation, producing leukaemia in several patients. As we have seen in this chapter, a variety of different strategies are under development aiming to achieve efficient and stable gene modification with minor or undetected genomic alterations. Although exciting new non-viral technologies have been developed that efficiently integrate the genome into safe harbors in the genome, we are still investigating the real safety benefits of these technologies. By taking together efficiency and safety we can, at this moment, argue in favour of improved viral technologies as the better tools for clinical use. New viral vectors are more efficient and safer

than first generation gammaretroviral vectors used in previous clinical trials. In particular, latest generation of lentiviral vectors have seen a major boost and we are convinced that new exciting clinical results will be coming in the next few years.

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The aim of this book is to cover key aspects of existing problems in the field of development and future perspectives in gene therapy. Contributions consist of basic and translational research, as well as clinical experiences, and they outline functional mechanisms, predictive approaches, patient-related studies and upcoming challenges in this stimulating but also controversial field of gene therapy research. This source will make our doctors become comfortable with the common problems of gene therapy and inspire others to delve a bit more deeply into a topic of interest.

### How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

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