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REVIEW

Lentiviral Vectors in Gene Therapy: Their Current Status and Future Potential

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Abstract The concept of gene therapy originated in the mid twentieth century and was perceived as a revolutionary technology with the promise to cure almost any disease of which the molecular basis was understood. Since then, several gene vectors have been developed and the feasibility of gene therapy has been shown in many animal models of human disease. However, clinical efficacy could not be demonstrated until the beginning of the new century in a small-scale clinical trial curing an otherwise fatal immunodeficiency disorder in children. This first success, achieved after retroviral therapy, was later overshadowed by the occurrence of vector-related leukemia in a significant number of the treated children, demonstrating that the future success of gene therapy depends on our understanding of vector biology. This has led to the development of later-generation vectors with improved efficiency, specificity, and safety. Amongst these are HIV-1 lentivirus-based vectors (lentivectors), which are being increasingly used in basic and applied research. Human gene therapy clinical trials are currently underway using lentivectors in a wide range of human diseases. The intention of this review is to describe the main scientific steps leading to the engineering of HIV-1 lentiviral vectors and place them in the context of current human gene therapy.

Keywords Gene therapy · Lentivirus-based vector

Abbreviations

DC	Dendritic cell
ds	Double-stranded
env	Envelope-encoding gene
gag	Gene encoding structural proteins
pol	Gene encoding viral enzymes
HIV	Human immunodeficiency virus
LTR	Long terminal repeat
MMP	Metalloproteinase
MLV	Murine leukemia virus
NILV	Non-integrating lentivector
PPT	Polypurine tract
PSA	Prostate-specific antigen
SCID	Severe combined immunodeficiency
ss	Single-stranded
TCR	T-cell receptor
VSV G	Vesicular stomatitis virus glycoprotein

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A Brief History of Gene Therapy

Gene therapy can be broadly defined as the treatment of a disease or medical disorder by the introduction of therapeutic genes into the appropriate cellular targets. These therapeutic genes can correct deleterious consequences of specific gene mutations or re-program cell functions to overcome a disease. For successful gene therapy, the exogenous therapeutic gene has to be specifically, efficiently, and stably incorporated into the target cell.

The concept of gene therapy is not at all novel or recent and it has been accompanied by controversy since the moment it was proposed. Therefore it is appropriate to approach this subject with a brief historical perspective. As is the case with many scientific revolutions, everything started with a simple question: How are characteristics passed from generation to generation? It cannot be questioned that the work carried out by Gregor Mendel during the 1850s to early 1860s and the later work of Ronald Fisher during the early twentieth century represent landmarks in genetics (Weiling 1991). Their work confirmed that organisms contain "encoded information" in the form of some kind of discrete biological material, termed "gene" in 1909 by the Danish botanist Wilhelm Johannsen (Falk 1984). During the early twentieth century it became clear that many human medical conditions, such as hemophilia, were transmitted from parents to offspring. There was also evidence that some diseases, such as diabetes, retinoblastoma, and colon cancer, presented some underlying genetic contributions. Correction of these defects posed a medical challenge because it was not possible to manipulate genes directly (Keeler 1947). Several breakthroughs in genetics, biochemistry, and molecular biology from the 1940s to 1980s dramatically changed that view (1976). In a relatively short period of time, DNA was identified as the genetic material (Avery et al. 1979), its structure was solved (Watson and Crick 1953a; Watson and Crick 1953b), the genetic code was broken (Ochoa 1963), and gene cloning was becoming routine (1981; Cline 1985; Hamer et al. 1979; Mantei et al. 1979; Mulligan et al. 1979; Nagata et al. 1980).

By the late 1970s, the molecular bases of many genetic disorders were well understood and gene therapy provided the "ideal and clean" solution (Friedmann 1976): restore the gene, cure the disease. Gene therapy was no longer considered an alternative treatment for genetic human conditions, but a potential solution (Friedmann 1976; Friedmann and Roblin 1972). In fact, the first human gene therapy trial was carried out in the early 1970s. Three hyperargininemic patients were intravenously inoculated with Shope papillomavirus so that the virus-encoded arginase could correct the disease. Actually, intravenous administration in rabbits was asymptomatic and therapeutic. However, no reduction in blood arginine levels was observed in the treated patients and the authors attributed the failure to the virus's instability (Friedmann and Roblin 1972; Terheggen et al. 1975). In any case, the rapid scientific development suddenly placed human gene therapy from an unrealistic scenario to a near-future possibility, raising concerns within the scientific and nonscientific community. Some of these issues remain, such as human cloning and "designer babies" (Neville 1976), having been raised again by the more recent advances in somatic cloning, stem cell biology, and regenerative medicine.

By the mid 1980s, gene transfer to mammalian cells was routinely performed. In fact, retrovirus-based gene transfer presented major advantages due to the stable integration of their genome in the host-cell chromosomes. However, gene therapy was still controversial, largely due to the poorly understood field of gene regulation, concerns about the possible influences of exogenous DNA in the host cell, and other major ethical issues (1981; Williamson 1982). Some of these ethical issues materialized after the controversial unauthorized human gene therapy trial carried out in 1980 in two β-thalassemia patients (Cline 1985; Mercola and Cline 1980).

The proof-of-principle of γ-retrovirus gene transfer into hematopoietic stem cells was demonstrated in the early 1990s (Brenner et al. 1993; Deisseroth et al. 1994; Dunbar et al. 1995; Rill et al. 1994) and the first approved clinical trial to correct severe combined immunodeficiency (SCID) was carried out by Anderson and colleagues in 1991 (Anderson et al. 1990; Blaese et al. 1993; Levine and Friedmann 1991). Peripheral blood $CD34^+$ cells from patients were transduced with a γ-retrovirus driving the expression of adenosine deaminase. Although the direct benefits of this gene therapy trial are still under deliberation, this can be considered the first "successful" human gene therapy trial, at least regarding safety issues. A major breakthrough in gene therapy came in 2000, when X-SCID was corrected in 11 children by the introduction of the common interleukin receptor γ-chain in bone marrow using a retrovirus vector based on mouse leukemia virus (MLV) (Cavazzana-Calvo et al. 2000). A similar approach was later published by Adrian Thrasher's group in London (Gaspar et al. 2004).

Regrettably, in both clinical trials, several cases of leukemia directly associated to the gene therapy itself were later reported, highlighting retrovirus-induced insertional mutagenesis as a major complication. Interestingly, this problem had already been taken into consideration from a theoretical point of view in the early 1980s (Cline 1985; Hacein-Bey-Abina et al. 2003; Howe et al. 2008). Following a similar approach to the X-SCID clinical trials, the correction of X-linked chronic granulomatosis was reported in 2006, again using γ-retrovirus vectors encoding gp91 phox in bone marrow (Gottlieb 2006; Moreno-Carranza et al. 2009). Clinical efficacy was observed, together with clonal amplification of corrected cells, probably as a result of insertional activation of MDS1-EVI1, PRDM16 and SETBP1 (Ryser et al. 2007).

In 2006, the first successful gene therapy trial for the treatment of melanoma was carried out. In this, a retrovirus encoding the α- and β-chains of a melanoma antigen (MART-1)-specific T-cell receptor (TCR) was engineered.

This TCR was cloned from a patient who showed regression after adoptive transfer therapy. Peripheral blood lymphocytes were transduced with this retrovirus and re-administered, leading to complete regression in two of fifteen patients (Morgan et al. 2006). This was the first demonstration that immune cells could be genetically modified to fight cancer in a human gene therapy trial.

Nowadays we are reliving exciting times. During the last decade, immense scientific leaps have been made, from somatic cloning (Wilmut et al. 1997) and completion of the human genome project (Venter et al. 2001) to the discovery of microRNA-based gene regulatory systems (Lombardo et al. 2007). It is in this context that lentivectors have been developed with the promise of becoming the substitutes of retroviral vectors, the first gene carriers leading to long-term full correction of human genetic diseases.

Retrovirus Biology and Basic Engineering of Lentivectors

Viruses are intracellular obligate parasites and they have evolved as efficient vehicles for the delivery of DNA or RNA to target cells. A large number of viruses with unique characteristics useful for gene therapy have been identified. This has led to the application of recombinant viruses such as adenoviruses, adeno-associated viruses, herpes viruses, poxviruses, retroviruses, and, more recently, lentiviruses, both in the laboratory and clinic. Vectors based on retroviruses such as MLV were amongst the first to be developed (Mann et al. 1983) and to be "successfully" used in gene therapy trials (Anderson et al. 1990; Blaese et al. 1993; Levine and Friedmann 1991). Currently, these vectors are used in about 21.2% of clinical trials (http://www.wiley.co.uk/genmed/clinical/ [July 2009]).

In recent years, research has focused on the use of lentivectors, which, like their simple retrovirus counterparts, are devoid of viral proteins, are free of replication-competent virus, and additionally able to transduce non-dividing cells (Bukrinsky et al. 1993). This characteristic is advantageous in many gene therapeutic applications targeting post-mitotic, highly differentiated cells. Currently, these lentivectors are applied in about 1.4% of clinical trials (http://www.wiley.co.uk/genmed/clinical/ [July 2009]).

The Retroviral Particle

Retroviridae is a family of single-stranded (ss) RNA spherical viruses of around 80–120 nm in diameter (Vogt and Simon 1999). The retroviral particle contains two copies of positive-strand RNA which are, together with the enzymes reverse transcriptase, integrase, and protease, complexed with nucleocapsid protein. A second protein shell, formed by capsid protein, encloses the nucleocapsid and delimits the viral core (Jones and Morikawa 1998). Matrix proteins form a layer outside the core and interact with a cell-derived lipid envelope which incorporates viral envelope glycoproteins (env), responsible for the interaction with specific cellular receptors. Two units form these glycoproteins: the transmembrane, which anchors the protein into the lipid bilayer, and the surface, which binds to the cellular receptor (Fig. 1).

The Retroviral Genome

Based on their genome organization, the *Retroviridae* are divided in simple and complex retroviruses. Examples are oncoretroviruses, such as MLV, and lentiviruses, such as human immunodeficiency virus (HIV)-1, respectively.

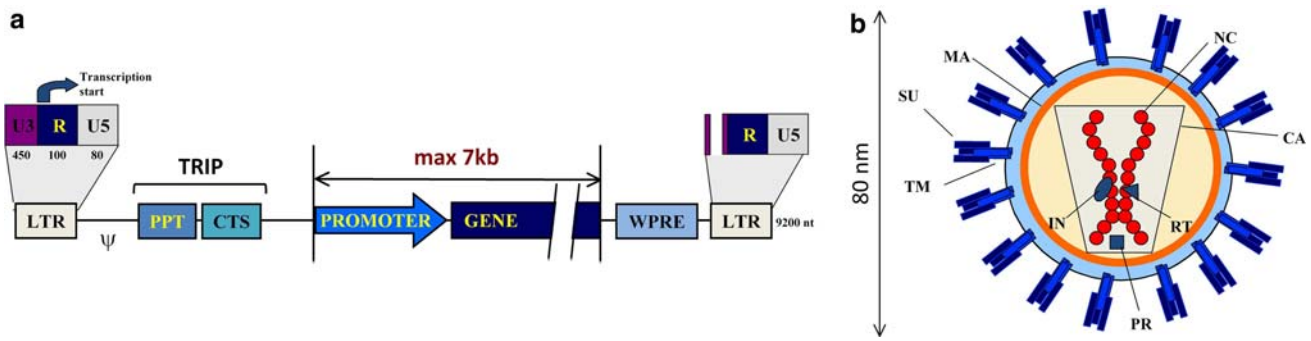

Fig. 1 Basic engineering of lentivectors. The self-inactivating (SIN) lentiviral transfer vector (**a**) and the resulting lentivector (**b**). The SIN vector contains a modified U3 region within the 3′ LTR, in which the enhancer/promoter sequences have been deleted as shown in the transfer vector. Abbreviations: *CA* capsid, *CTS* central termination site, *IN* integrase, *LTR* long terminal repeat, *MA* matrix, *NC* nucleocapsid, *PPT* polypurine tract, *PR* protease, *RT* reverse transcriptase, *SU* surface, *TM* transmembrane, *TRIP* triple helix, *WPRE* woodchuck hepatitis B posttranscriptional element

Lentiviruses include primate and non-primate retroviruses. Examples of the former are HIV and SIV (simian immunodeficiency virus) and of the latter FIV (feline immunodeficiency virus), BIV (bovine immunodeficiency virus), CAEV (caprine arthritis-encephalitis virus), EIAV (equine infectious anemia virus), and visnavirus. Their genome is organized in the gag, pol, and env genes. Gag encodes the structural proteins, whereas the pol gene encodes the enzymes that accompany the ssRNA. Of these, reverse transcriptase carries out reverse transcription of the viral RNA to DNA, integrase catalyses the integration of the proviral DNA into the host genome, and protease is involved in gag-pol cleavage and virion maturation (Katz and Skalka 1994). Env encodes the viral envelope. In addition, complex retroviruses have accessory genes whose concerted action regulates viral gene expression, assembly, and replication (Coil and Miller 2004). Moreover, the retroviral genome contains *cis*-acting sequences, such as two long terminal repeats (LTR), with elements required for gene expression, reverse transcription, and integration into the host chromosomes. Other important components are the packaging signal (psi or ψ), required for the specific RNA packaging into newly formed virions (Watanabe and Temin 1982), and the polypurine tract (PPT), which is the site of the initiation of positive-strand DNA synthesis during reverse transcription (Charneau et al. 1992; Rattray and Champoux 1989).

The Retroviral Life Cycle

The retroviral life cycle can be broken down into several steps, starting with the binding of the viral envelope to its receptor (i) and fusion of the viral envelope with the cell membrane (ii). Subsequently, the particle is uncoated and the viral core released into the cytoplasm (iii). The ssRNA is reverse-transcribed into dsDNA within the core (iv), which is transported to the nucleus (v) upon cell division for oncoretroviruses (Lewis and Emerman 1994; Ryser et al. 2007) or through active transport in the case of lentiviruses (Bukrinsky et al. 1993). Herein lies the major advantage of lentiviruses over oncoretroviruses: their ability to transduce non-dividing cells. Once in the nucleus, the viral DNA is integrated into the host DNA (provirus) (vi), resulting in long-term expression of viral genes which are transcribed (vii) and spliced (viii) during the life of the infected cell. The full-length viral RNA as well as the RNA encoding all the viral proteins are transported to the cytoplasm (ix), where they are translated (x). The unspliced full-length viral RNA is packaged and a viral particle assembled (xi). Virion maturation occurs together with budding of the particle from the cell (xii), as such resulting in infectious virions (Palu et al. 2000).

Basic Engineering of Retrovirus-Based Vectors

The use of gene delivery vectors based on retroviruses was introduced in the early 1980s (Mann et al. 1983). The most commonly used retroviral vectors are based on Moloney MLV and have a simple genome. From this genome, the polyproteins gag, pol, and env are required *in trans* for viral replication and packaging. Required *in cis* are the 5′ and 3′ LTR, the sequences for packaging the viral RNA, as well as the tRNA binding site and sequences involved in reverse transcription and integration of the provirus. The gag, pol, and env genes are replaced with an expression cassette containing the gene of interest, which is under the control of a chosen promoter (see "Cell- and Tissue-Specific Promoters" and "Regulatable Promoters").

The major advantages of oncoretrovirus-based vectors are: (i) their lack of viral proteins, which renders them replication deficient and less immunogenic, in the sense that they do not elicit anti-vector immune responses (see Sect. 5.1), and (ii) their ability to integrate into the host genome, leading to persistent gene expression. However, there are some important limitations, such as: (i) instability of the viral particle (Andreadis et al. 1997), (ii) low viral titers (Le Doux et al. 1999), (iii) inability to transduce non-dividing cells (Lewis and Emerman 1994; Ryser et al. 2007), and (iv) insertional mutagenesis. To overcome these shortcomings, vectors based on lentiviruses were developed (Naldini et al. 1996b). Lentivectors can also induce insertional mutagenesis, however at lower frequencies than their γ-retroviral counterparts, possibly due to different integration patterns (Hematti et al. 2004; Modlich et al. 2009).

Lentivectors are capable of transducing quiescent cells (Lewis and Emerman 1994). For HIV, this process is facilitated by (i) the integrase protein (Naldini et al. 1996b), (ii) the matrix protein (Naldini et al. 1996b), (iii) vpr (Heinzinger et al. 1994), and (iv) the PPT (Vanden-Driessche et al. 2002). As a result of their capacity to transduce quiescent cells, although progression through the cell cycle is necessary for some cell types, (Breckpot et al. 2004; Korin and Zack 1998), lentivector development has received much interest. As with oncoretrovirus vectors, the design of lentivectors is based on the separation of *cis*- and *trans*-acting sequences.

Development of Lentivectors (Fig. 2)

In general, lentivector particles are generated by the co-transfection of three plasmids in human embryonic kidney (HEK) 293T cells (Naldini et al. 1996a): (i) a packaging plasmid, (ii) a transfer plasmid (Fig. 1), and (iii) an envelope-encoding plasmid. In the latest-generation

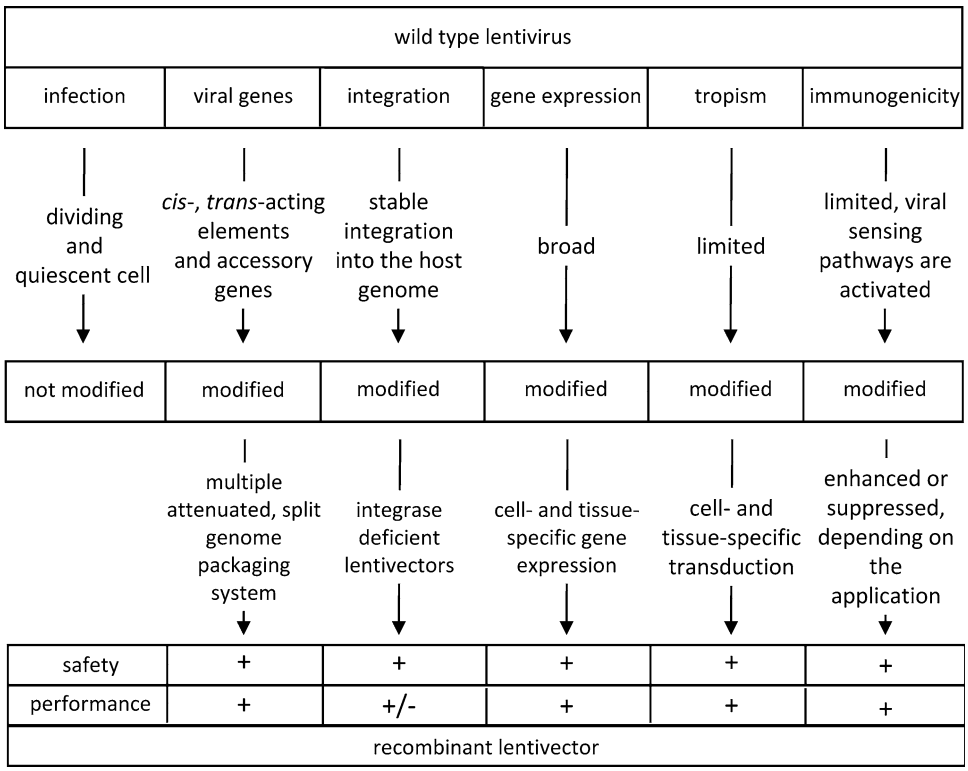

Fig. 2 Characteristics of lentiviruses and the development of improved recombinant lentivectors

vectors, the rev and tat genes are not longer included in the packaging plasmid, but provided in a fourth plasmid. The development of lentivector systems is reviewed elsewhere (Breckpot et al. 2007a; Breckpot et al. 2008).

The Different Generations of Lentivectors

The lentiviral particles are divided into "generations" according to the packaging plasmid used for production. The first-generation packaging plasmid provided all gag and pol sequences, the viral regulatory genes tat and rev, and the accessory genes vif, vpr, vpu, and nef. Identification of the HIV genes disposable for transfer of the genetic cargo allowed the engineering of the multiple-attenuated second-generation packaging systems (Gruber et al. 2000; Zufferey et al. 1997). In these, the four accessory genes, vif, vpr, vpu, and nef, were removed without negative effects on vector yield or infection efficiency, and as such improving lentivector safety since any replication-competent lentivirus would be devoid of all virulence factors. Safety was further improved in the third-generation packaging systems, consisting of a split-genome packaging system in which the rev gene is expressed from a separate plasmid and the 5′ LTR from the transfer vector replaced by a strong tat-independent constitutive promoter (Dull et al. 1998).

Improving Vector Performance and Safety

Modification of the Packaging Plasmid: Integrase-Deficient Lentivectors

Long-term stable transgene expression as a result of lentivector integration is very useful for diseases in which permanent cell correction is sought. However, as already mentioned, insertion of the γ-retrovirus led to oncogene transactivation and leukemia in some of the X-SCID children treated by gene therapy. Therefore the development of non-integrating lentivectors (NILVs) has attracted much attention. Accordingly, selected mutations within the integrase-coding region of the packaging plasmid have been exploited to generate integrase-deficient lentivectors. These mutations eliminate integrase activity without affecting reverse transcription and transport of the pre-integration complex to the nucleus. Then, the lentivector DNA remains in the nucleus as an episome, leading to sustained expression in post-mitotic cells and tissues such as retina, brain, and muscle (Apolonia et al. 2007; Philippe et al. 2006; Yanez-Munoz et al. 2006). NILVs with multiple mutations either in the integrase itself or in the integrase attachment sites have been shown to be as effective as standard lentivectors in a lymphoma model when dendritic cell (DC) constitutive activators were co-expressed with a surrogate tumor antigen (Karwacz et al. 2009). NILVs

could be applied in cases where post-mitotic tissue is the target or in applications that do not require persistent antigen expression, such as vaccination. However, background integration by recombination can still occur (Apolonia et al. 2007). Recently, NILVs have been used as donor DNA sequences for the activity of zinc-finger nucleases (Brown et al. 2007a; Cornu and Cathomen 2007). This has allowed the targeted correction of gene mutations even in human stem cells, circumventing the drawback of random integration and insertional mutagenesis (Brown et al. 2007a).

Modification of the Transfer Plasmid (Fig. 1)

The transfer plasmid consists of an expression cassette and the HIV *cis*-acting factors necessary for packaging, reverse transcription, and integration and can be modified to augment lentivector performance and safety. Nuclear import of the transfer construct was improved by including the PPT and its central termination sequence, together forming a triple helix, resulting in higher vector titers and enhanced transgene expression (Sirven et al. 2000). Addition of other elements such as the woodchuck hepatitis B posttranscriptional regulatory element improves the lentiviral vector design by increasing gene expression by modification of polyadenylation, RNA export, or translation (Zufferey et al. 1999). Engineering of self-inactivating transfer vectors by deleting the enhancer/promoter unit in the U3 region of the 3′ LTR (Miyoshi et al. 1998; Zufferey et al. 1998) minimized the risk of replication-competent lentiviruses and decreased promoter interference (Ginn et al. 2003), which brings us to the topic of promoter engineering, another strategy to improve both performance and safety.

Cell- and Tissue-Specific Promoters Cell-specific promoters are advantageous since they are less sensitive to promoter inactivation and less likely to activate the host-cell defense machinery (Liu et al. 2004). As such, improved stability and longevity of gene expression can be expected. Therefore it is not surprising that much effort has been put in research on this topic. To date, specific gene expression has been described for several cell types, including erythroid (Moreau-Gaudry et al. 2001), endothelial (De Palma et al. 2003), central nervous system (Gascon et al. 2008; Greenberg et al. 2007; Kuroda et al. 2008; Lai and Brady 2002; Liu et al. 2008), retinal (Miyoshi et al. 1997; Semple-Rowland et al. 2007), liver (Oertel et al. 2003; VandenDriessche et al. 2002), and cancer cells (Morgan et al. 2006; Seo et al. 2009; Uch et al. 2003; Yu et al. 2001). In addition, gene-specific expression has been achieved for immune cells, in particular DCs, that can contribute to the induction (antitumor gene therapy) or suppression (auto-immune and graft-versus-host disease) of immune responses (Cui et al. 2002; Lopes et al. 2008; Zhang et al. 2009).

As an example, the cellular diversity within the central nervous system underscores the importance of restricting transgene expression to target cells (Costantini et al. 2000). Several promoters have been tested to drive gene expression in neurons, cells of the hypocampus, and glial cells. Amongst these are the enolase promoter (Lai and Brady 2002), SYN promoter (Gascon et al. 2008; Liu et al. 2008), synapsin 1 promoter (Kuroda et al. 2008), CD44 promoter, glial fibrillary acidic protein promoter, and vimentin promoter (Greenberg et al. 2007; Liu et al. 2008). Some of these have been shown to drive high transgene expression (Kuroda et al. 2008; Lai and Brady 2002), for example the CD44 promoter, which allows high and sustained GFP expression in Muller glia cells for over 6 months. In contrast, the glial fibrillary acidic protein promoter and vimentin promoter were less efficient (Greenberg et al. 2007), demonstrating the need for the testing of candidate promoters and strategies to enhance their activity, such as transcriptional activation, in which activity is enhanced by the use of two promoter copies, or by artificial transcriptional activators. In this regard, Liu et al. demonstrated an increase in transgene expression driven by a modified SYN promoter or glial fibrillary acidic protein promoter in neurons and glia cells, respectively (Liu et al. 2008).

Several promoters have been tested in view of curing retinal diseases. Amongst these is the rhodopsin promoter, which results in high levels of photoreceptor-specific expression (Miyoshi et al. 1997). In another study, two photoreceptor promoters, the interphotoreceptor retinoid-binding protein and guanylate cyclase-activating protein promoters, were evaluated alongside the rhodopsin promoter to express two proteins in a retinal cell-specific manner (Semple-Rowland et al. 2007). The ability to engineer one lentivector that targets the expression of multiple genes to single (cone cells) or multiple cells (cone cells and rod cells) in vivo was demonstrated. This type of application should prove useful in the development and delivery of complex combination therapies.

Another proof of the benefits of cell-specific gene expression was shown in liver using the albumin promoter, leading to long-term transgene expression in rat liver, in contrast to cytomegalovirus, which was rapidly silenced (Oertel et al. 2003; VandenDriessche et al. 2002).

For anticancer gene therapy, envisaging the delivery of cell death-inducing genes, it is important to restrict transgene expression to cancer cells. Human hepatocarcinoma cells have been transduced with lentivectors containing a suicide gene under the control of the α-fetoprotein promoter, resulting in specific destruction of these malignant cells (Uch et al. 2003). Furthermore, a patient-derived prostate-specific antigen (PSA) promoter inserted into a

lentivector has driven efficient activity in prostate cells with satisfactory efficacy and specificity (Yu et al. 2001). More recently, a PSA promoter-based lentivector has been used to deliver the diphtheria toxin A gene into prostate cancer cells, resulting in specific eradication of cancer cells in cell culture and in a mouse tumor model (Morgan et al. 2006). Another example is the metalloproteinase (MMP2) promoter, which has been applied to introduce the pro-apoptotic genes Bax and tBID, resulting in cell death in MMP2-expressing cancer cell lines, but not healthy $MMP2^+$ cells (Seo et al. 2009).

For "gene immunotherapy", a specialized form of gene therapy, DCs constitute an interesting target. The development and function of DCs as well as DC targeting by lentivectors are reviewed in Breckpot et al. (2007a, 2008). Briefly, DCs are specialized antigen-presenting cells involved in modulating immune responses and can be exploited to fight cancer and infectious diseases or to reestablish tolerance in view of auto-immunity and transplantation. To achieve antigen-presenting cell-specific gene expression, Cui et al. (2002) exploited the selective and high expression of MHC class II on antigen-presenting cells. Using the non-obese diabetic/SCID mouse engraftment model, they showed specific expression in MHC class II^+ human cells upon transduction with HLA-DRα promoter-harboring lentivectors. Other promoters, i.e. the dectin-2 and CD11c promoters, have been used in view of antitumor immunotherapy (Lopes et al. 2008) and for the generation of DC-specific transgenic mice (Zhang et al. 2009), respectively.

Taken together, there is a growing list of cell-specific promoters that are being applied successfully and that improve both the safety and performance of lentivectors as gene transfer vehicles.

Regulatable Promoters The potential to regulate transgene expression is appealing for many gene therapy applications, as in the case of genetic diseases in which the gene expression levels or the timing of expression would be desirable, for example diabetes. Therefore, several groups have focused their research on the use of inducible promoters. Many variations of each system have been developed, and the basic systems are briefly explained.

The tetracycline-based induction system is probably among the most widely used (Blomer et al. 1997; Gascon et al. 2008; Reiser et al. 2000; Seo et al. 2009; Vigna et al. 2002). For gene therapy, an inducible vector dependent on the delivery of the antibiotic (tet-on) is preferred over one based on gene silencing (tet-off), which necessitates constant administration of antibiotics unless transgene expression is required. With the exception of some reports (Blomer et al. 1997; Gascon et al. 2008; Johansen et al. 2002), all the tetracycline-inducible lentivectors listed in the literature were constructed using the tet-on system (Farson et al. 2001; Georgievska et al. 2004; Reiser et al. 2000; Seo et al. 2009; Vigna et al. 2002). The authors showed that this system in lentivectors allows dose-dependent rapid inducible expression. Other inducible systems have also been adapted to lentivectors, such as the drosophila ecdysone receptor (Galimi et al. 2005), which is based on: (i) a chimeric protein made of the herpes simplex virus protein VP16 activation domain and an ecdysone receptor (VgEcR), (ii) the retinoid X receptor (RXR), and (iii) the inducible promoter. In this system, ecdysone (or synthetic analogs) binds to the VgEcR-RXR heterodimer, which then binds to the inducible promoter driving gene transcription (Saez et al. 2000). As a drawback, multiple lentivector components have to be administrated simultaneously (Galimi et al. 2005). This multicomponent problem can be overcome by appropriate vector design, as with the tet-on/tet-off system in a single lentivector backbone using a fusion protein between the tetracycline repressor with the Kruppel-associated Box domain repressor as the regulator (Szulc et al. 2006).

This system has been used for tightly regulated conditional transgene expression in the brain, gene silencing in hematopoietic cells, and for the generation of drug-inducible transgenic mice (Szulc et al. 2006). Other systems adapted to lentivectors include glucocorticoid-inducible promoters (Parker et al. 2009) and mifepristone-inducible systems (Sirin and Park 2003).

Alteration of the Viral Envelope Targeting lentivectors to specific cell types: Lentivector gene delivery requires entry into the cell of interest. The tropism of lentivectors is determined by their viral envelope glycoproteins which, upon interaction with their receptors, trigger fusion of the viral envelope with the cell membrane. Since wild-type HIV glycoproteins have restricted tropism and do not allow the production of high-titer lentivector preparations, heterologous glycoproteins are used for lentivector production. This process is termed "pseudotyping". Lentivectors are often pseudotyped with the envelope of vesicular stomatitis virus (VSV G), a glycoprotein which interacts with a ubiquitous receptor, or a phospholipid component of the cell membrane (Coil and Miller 2004). VSV G endows a broad host-cell range and confers high vector particle stability (Burns et al. 1993), two attractive properties in terms of *ex vivo* gene modification. However, restricting infection to specific cells, known as "transductional targeting", is critical when it comes to efficient and safe in vivo gene delivery and is key to enhancing therapeutic effects, reducing side effects, and possibly lowering the amounts of vectors required. To achieve this goal, two methods can be used: (i) taking advantage of the natural properties of existing viral proteins and (ii) using genetic

engineering to retain, abolish, or extend the original tropism of vectors.

There is an ever-growing list of glycoproteins that have been successfully used for pseudotyping lentivectors, each with its (dis)advantages. Examples are glycoproteins from *Retroviridae*, *Rhabdoviridae*, *Arenaviridae*, *Flaviviridae*, *Paramyxoviridae*, *Baculoviridae*, and *Filoviridae* (reviewed by Bouard et al. (2009)). Although each of these glycoproteins preferentially interacts with specific cell types, and can thus be used to restrict transgene expression, finding a natural envelope for cell-specific targeting may be challenging.

Modification of the viral surface by genetic engineering is an alternative to pseudotyping with existing envelopes. The aim is to alter the receptor attachment function in the glycoprotein without hampering membrane fusion. For some viral envelope proteins, such as that of Sindbis virus, which use more than one receptor for cell entry, this task was relatively straightforward. Mutation of the most important contact residues prevented binding to its ubiquitously expressed receptor (heparan sulfate), resulting in an envelope protein variant that only bound to DC-SIGN. This is a DC-specific surface molecule, thus allowing DC-specific gene transfer (Morgan et al. 2006). However, engineering of re-targeted envelope proteins by fusion to natural ligands has proven to be difficult, and these strategies were at first applied with limited success (Waehler et al. 2007). Although binding of the vector to target cells was achieved, the inclusion of the ligand inhibited viral entry, except in one study. In that case the pH-dependent glycoprotein from influenza virus was fused with epidermal growth factor (EGF), demonstrating specific infection of EGF receptor$^+$ target cells (Hatziioannou et al. 1999). Alternative strategies have been developed based on specific requirements, such as the expression of specific proteases on the surface of target cells that can release the vector from the bound receptor (Szecsi et al. 2006). Such protease-activatable vectors were applied for the targeted infection of tumor cells that express MMP (Duerner et al. 2008; Springfeld et al. 2006).

Recently described targeted lentivectors exploit envelope proteins such as those of Sindbis virus and measles virus in which the binding and fusion functions are provided by separate proteins. The Sindbis virus envelope consists of the E1 and E2 protein, which harbor the fusion peptide and receptor binding sites, respectively. Together, the two proteins mediate pH-dependent cell entry. The targeting strategy involves mutation of the natural receptor binding sites of E2 and provision of an alternative binding method. Thus far, several studies have been published targeting tumor (Morizono et al. 2005; Pariente et al. 2007), endothelial (Pariente et al. 2008), and B cells (Morgan et al. 2006). Targeting is achieved by either covalent conjugation of a cell-specific antibody (Morizono et al. 2005; Pariente et al. 2008; Pariente et al. 2007) or its incorporation into the viral envelope alongside the engineered Sindbis virus glycoprotein (Morgan et al. 2006). However, this strategy requires endocytosis for the pH-dependent membrane fusion. In contrast, the measles virus proteins F and H mediate pH-independent membrane fusion and thus allow direct entry at the cell-membrane level. Similar to the approach for the Sindbis virus envelope, binding residues present in H were mutated and target ligands, such as EGF and a single-chain antibody against CD20, were fused to the mutated H protein. These lentivectors efficiently and specifically transduced EGF receptor$^+$ and CD20$^+$ cells, respectively (Funke et al. 2008), demonstrating the feasibility of targeting specific cell types. Moreover, transductional targeting has been combined with cell-specific promoters (transcriptional targeting) (Pariente et al. 2008; Pariente et al. 2007), as such further impacting on lentivector safety and performance.

Application of Lentivectors in Gene Therapy

Insertional mutagenesis has been a major drawback for the use of γ-retroviral vectors in human gene therapy. Lentivectors also exhibit insertional mutagenesis in animal models and cell-based systems prone to oncogenesis (Bokhoven et al. 2009), although overall they seem to be less mutagenic than their oncoretrovirus counterparts (Hematti et al. 2004; Montini et al. 2009). In fact, in the first approved human clinical trial using lentivectors for the treatment of HIV, no abnormal cell expansion or enrichment of integration sites near proto-oncogenes has been detected so far (Manilla et al. 2005; Themis et al. 2005).

Lentivectors have been tested for some time in many gene therapy animal models for metabolic diseases, such as β-thalassemia (Zhao et al. 2009), SCID (Mortellaro et al. 2006; Throm et al. 2009), Wiskott-Aldrich syndrome (Mantovani et al. 2009; Marangoni et al. 2009), hemophilia (Brown et al. 2007a), metachromatic leukodystrophy (Biffi and Naldini 2007), Fanconi anemia (Jacome et al. 2009), and liver diseases in nonhuman primates (Menzel et al. 2009). The list of gene therapy models in which lentivectors are being used is increasing and it is hoped that the last lentivector generation will exhibit the safety and therapeutic efficacy that we have been waiting for.

Gene Therapy of the Immune System

The proof-of-principle of the therapeutic efficacy of gene therapy targeting the immune system was established by

the "successful" clinical trials for the treatment of melanoma and X-SCID (Cavazzana-Calvo et al. 2000; Gottlieb 2006; Moreno-Carranza et al. 2009). At the present time, lentivectors are being tested for gene therapy of the immune system, and the main strategies are briefly summarized below.

Cancer Immunotherapy

A very promising approach for "gene immunotherapy" is specific lentivector targeting to DCs, the professional antigen-presenting cells of the immune system that regulate both immunity and tolerance. For cancer immunotherapy, the major goal is to break tolerance of tumor-associated antigens, since they are mostly either self or quasi-self antigens. To drive an efficient antitumor immune response, antigens have to be presented to specific CD4 and CD8 T cells by mature immunogenic DCs, which are characterized by high expression levels of co-stimulatory molecules and pro-inflammatory cytokines. To meet these requirements, DC-specific transgene expression (Sects. 3.2.2.1. and 3.3) and the induction of efficient DC maturation have to be achieved (Breckpot and Escors 2009). Antigen presentation by mature DCs will eventually result in the expansion of tumor-specific T cells and the inactivation of tolerogenic mechanisms.

Lentiviral vectors have been extensively used to transduce *ex vivo* generated monocyte-derived DCs, demonstrating that these DCs can be further activated and can present tumor-derived peptides to both CD4 and CD8 T cells (Breckpot et al. 2003). DC-based vaccines are patient specific. Their generation requires specialized expertise and facilities and is time consuming. Therefore, researchers have evaluated the direct use of lentivectors as off-the-shelf anticancer vaccines (Dullaers et al. 2006). It has been demonstrated that lentivectors do not provoke immunological tolerance but instead elicit powerful cytotoxic T-lymphocyte responses against transgene-encoded proteins (reviewed in Breckpot et al. (2007a, 2008)). This suggests that lentivectors or components present in virus preparations activate innate viral-sensing pathways leading to strong adaptive immune responses. Many studies in human and mice have shown that this is in part mediated by plasmacytoid DCs, a DC subset specialized in sensing viral infection. Although it has been shown that human conventional DCs are activated by lentivectors (Breckpot et al. 2007b; Tan et al. 2005), the in vivo contribution of conventional DCs, the subset believed to orchestrate the antitumor immune response, remains elusive.

Nevertheless, many strategies have been developed to further enhance DC activation. These include: (i) the delivery of activation signals, such as constitutive active Toll-like receptor 4 (Xu et al. 2007) and CD40 ligand (Koya et al. 2003), (ii) overexpression of adaptor molecules involved in DC-activating innate intracellular signaling, such as TRIF and MyD88 (Akazawa et al. 2007a, b), (iii) overexpression of viral molecules that interact with cellular signaling, such as vFLIP (Rowe et al. 2009), (iv) introduction of constitutive active forms of DC activators (Escors et al. 2008), as well as (v) removal of inhibitory mechanisms by RNA interference (Song et al. 2008). These strategies are extensively reviewed in (Breckpot and Escors 2009).

Immune Silencing by Gene Therapy

Without any doubt, a major drawback of viral-based gene therapy is anti-vector and anti-transgene immune responses, leading to clearance of corrected cells by the immune system. In addition, lentivectors are relatively immunogenic, especially when the transgene is expressed in antigen-presenting cells (Brown et al. 2007c). Although this is an advantage in the case of cancer immunotherapy or vaccination, it is a major drawback in the gene therapy of metabolic diseases. Several approaches have been undertaken to prevent this problem. In a very elegant work, Brown and coworkers (Brown et al. 2007b) incorporated a target for the endogenous microRNA mir 142-3p present in cells of hematopoietic origin within the mRNA encoding the transgene. Transgene expression was strongly silenced by the endogenous microRNA only in antigen-presenting cells, leading to long-term expression in immunocompetent mice. A second approach to silence transgene-specific immune responses involved lentivector expression of ERK and IRF3 constitutive activators together with the transgene (Escors et al. 2008). Constitutive ERK activation resulted in immature DCs with down-regulated CD40 and expression of TGF-β, while IRF3 activation led to high-level secretion of IL-10. Activation of these pathways suppressed antigen-specific immune responses and expanded $Foxp3^+$ regulatory T cells. These approaches can also be applied for the treatment of diseases with an autoimmune etiology, such as diabetes, multiple sclerosis, and rheumatoid arthritis.

Conclusions and Future Perspectives

For the first time, several human gene therapy clinical trials have been successful, at least from a therapeutic point of view. These have been mainly carried out using onco-retroviruses and, regrettably, insertional mutagenesis has proven to be a major complication. Meanwhile, lentivectors have arisen with the promise to become the substitutes of onco-retroviruses due to their improved performance and, possibly, enhanced biosafety. Lentivectors have been successfully applied in gene therapy models, the generation

of transgenic animals, and gene silencing by combination with small interfering RNA- and microRNA-based technologies. Overall, lentivectors offer greater advantages than their γ-retrovirus counterparts, and the first human clinical trials using them have already started. For the first time, gene therapy has become a reality and lentivectors may be the gene carriers we have been waiting for.

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