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Human Gene Therapy Vectors Derived from Feline Lentiviruses

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Summary

Lentiviral vectors are useful for gene transfer to dividing and non-dividing cells. Feline immunodeficiency virus (FIV) vectors transduce most human cell types with good efficiency and may have advantages for clinical gene therapy applications. This article reviews significant progress in the development and refinement of FIV vector systems.

Keywords

gene therapy; retroviral vectors; feline immunodeficiency virus; innate immunity

Introduction

Several years after HIV-1 was described as the cause of human AIDS, a feline lentivirus was shown to cause a similar immunodeficiency in domestic cats (Pedersen et al., 1987). Like HIV-1, FIV is also T-lymphotropic, but displays broader tropism, since it infects CD8+ T cells and B cells as well as CD4+ T cells and macrophages. Many other non-domestic feline species are infected with highly related feline lentiviruses. Although some immune abnormalities have been reported (Roelke et al., 2006), non-domestic feline species rarely if ever suffer detectable illness (Troyer et al., 2005). This situation parallels the record in primates, where Old World monkeys remain healthy despite highly productive infection with lentiviruses (Beer et al., 1998; Broussard et al., 2001; Chakrabarti, 2004; Chakrabarti et al., 2000). No lentiviruses are transmitted across primate-human-ungulate barriers and FIV is no exception: the virus has not been transmitted to humans despite presumably widespread exposure via the same efficient mechanism operative in inter-feline transmission (biting) (Pedersen, 1993) and despite use of similar chemokine co-receptors (Willett et al., 1997a; Willett et al., 1997b; Poeschla and Looney, 1998). The primary receptor, analogous to CD4, is CD134 (Shimojima et al., 2004).

Lentiviral vectors were first demonstrated in 1995 (Parolin and Sodroski, 1995). Here and subsequently, the basic concept followed that of previous retroviral vectors: place the cis-acting viral nucleic acid signals needed for packaging, reverse transcription and integration into a “transfer vector” that also contains the non-viral gene or genes to be transferred. Then supply the viral particle proteins in trans to this artificial genome. The resulting integrated transfer vector will lack any viral protein encoding genes and will be replication-defective. Because these initial lentiviral vectors used the native HIV-1 envelope glycoprotein, they were restricted to targeting CD4+ T cells and the achievable titer was too low to be useful practically (Parolin

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and Sodroski, 1995; Parolin et al., 1996). At the same time, others were establishing that alternative envelope glycoproteins, e.g. from rhabdoviruses, could replace the native envelope protein in retroviral particles and dramatically enhance both infectivity and host range (Burns et al., 1993; Yee et al., 1994). Adapting this envelope “pseudotyping” strategy to HIV vectors, along with their design of a more optimally configured transfer vector and viral protein expression (packaging) construct, Naldini and colleagues first demonstrated effective transduction of nondividing cells in vitro and in vivo (Naldini et al., 1996a; Naldini et al., 1996b). The potential of nonprimate lentiviral vectors was at that point uncertain since the parental lentiviruses were much less studied and none can replicate in human cells. The realization that the block to FIV particle production in human cells was essentially only transcriptional and could be completely overcome with promoter (U3 element) substitution (Poeschla and Looney, 1998) enabled the first FIV vector, which was shown to transduce dividing and nondividing human, feline and murine cells (Poeschla et al., 1998). Iterative refinements have further streamlined the systems. The general design of a current three component FIV vector system is shown in Fig. 1 and the molecular events that occur during vector particle production are shown in Fig. 2. The concept of using non-primate lentivirus vectors for gene transfer to human cells was further validated using a lentivirus in the ungulate group, equine infectious anemia virus (Olsen, 1998; Mitrophanous et al., 1999). So far, other ungulate lentiviruses have been more difficult to adapt into high-titer vector systems.

Thus, when re-engineered into a replication-defective vector, FIV retains in human cells the core feature that has motivated lentiviral vector development – the ability to translocate its pre-integration complex across the nuclear envelope and complete integration in nondividing cells (Poeschla et al., 1998). The mechanism of this process, which has fascinated retrovirologists for decades and sharply differentiates these vectors from gammaretroviral vectors (e.g., murine leukemia virus vectors), remains poorly understood at the cell biological level. As noted above, the negligible transcriptional activity of the FIV long terminal repeat (LTR) in human cells was an initial obstacle to producing such vectors in well-characterized and highly transfectable adherent human cell lines. The problem was circumvented by replacing the 5' U3 element with a heterologous promoter, a step that revealed that the remainder of the productive phase proceeds with high efficiency in human producer cells. Since the U3 element is regenerated at the upstream end of the integrated vector, the FIV U3 inactivity may be considered to provide a first level protection against unwanted promoter interference or activation of human cellular genes, (e.g., oncogenes). To eliminate such potential completely, U3-deleted FIV vectors have been constructed (Khare et al., 2007) following principles well-established for other retroviral vectors (Yu et al., 1986).

Although in our view there are no substantial grounds to doubt that HIV-1 can be adapted to clinical vector use safely, certain potential advantages of FIV vectors have been noted: (i) there is a substantial epidemiologic record of clinically uneventful human exposure to FIV; (ii) FIV particles do not induce HIV-cross-reactive immune responses in humans; and (iii) the possibility that a non-HIV system may result in greater patient acceptance. A fourth consideration – the built-in impediment to potential replication-competent retrovirus (RCR) propagation afforded by saturable intrinsic immunity mechanisms -- is discussed below. Initial experiments showed that FIV vectors could efficiently transduce various human cell lines, primary neurons and macrophages (Poeschla et al., 1998). These results have been confirmed with this system and with independently-derived similar systems in other laboratories (Johnston et al., 1999; Curran et al., 2000; Song et al., 2003). FIV vectors have now been applied with favorable results in the brain, eye, ear, airway, liver, muscle, pancreas and hematopoietic system (Poeschla et al., 1998; Johnston et al., 1999; Wang et al., 1999; Alisky et al., 2000; Curran et al., 2000; Loewen et al., 2001; Stein et al., 2001; Brooks et al., 2002; Curran et al., 2002; Curran and Nolan, 2002a, b; Derksen et al., 2002; Djalilian et al., 2002; Hughes et al., 2002; Kang et al., 2002; Loewen et al., 2002; Lotery et al., 2002; Price et al.,

2002; Stein and Davidson, 2002; Loewen et al., 2003a; Loewen et al., 2003b; Haskell et al., 2003; Sinn et al., 2003; Loewen et al., 2004; Saenz and Poeschla, 2004; Kang et al., 2005; Harper et al., 2006; Saenz et al., 2006; Khare et al., 2007). In addition to animal models, success has been evident in explanted human organs (Wang et al., 1999; Loewen et al., 2001; Loewen et al., 2002).

For reviews and detailed protocols for FIV vector production, see (Loewen et al., 2003a; Poeschla, 2003; Saenz and Poeschla, 2004; Loewen and Poeschla, 2005; Saenz et al., 2006). These and other lentiviral vectors are generally produced by high efficiency transfection of three components: (i) a transfer vector (encodes the minimal genome RNA having the transgene, which is generally internally promoted), (ii) a packaging construct (encodes viral structural proteins and Rev in *trans*, and is engineered to lack encapsidation signals), and (iii) a non-lentiviral envelope glycoprotein construct for pseudotyping. This three-component system (Fig. 1) permits the production of replication-incompetent FIV viral particles containing two copies of plus-stranded RNA transfer vector. Thus, viral protein encoding sequences are not transferred to the target cell, ensuring replication-defectiveness. Existing FIV vector systems have been constructed from FIV molecular clone 34TF10 (Talbot et al., 1989).

FIV vector system overview

Advancements in basic FIV virology have continued to contribute to engineering improved vectors. The FIV vector system currently utilized in our laboratory (illustrated in Fig. 1), is described, and further details of the production components and protocols are referenced here (Loewen et al., 2003a; Saenz and Poeschla, 2004; Saenz et al., 2006;).

Transfer vector

An optimal transfer vector should exclude all native viral sequences except for the encapsidation signal and elements necessary for reverse transcription and integration (Fig. 1). Currently, standard transfer vectors incorporate these native elements and two others: the FIV rev responsive element (RRE) to facilitate efficient nuclear export of unspliced transfer vector RNA, and the central polypurine tract and central termination sequences (or cPPT-CTS) to facilitate reverse transcription and, possibly, efficient nuclear import. The encapsidation signal permits selective packaging of the transfer vector RNA. Most other elements in transfer vectors are derived from other viruses. For example, internal promoters, such as the human cytomegalovirus immediate early gene promoter (CMV) or EF1 α promoter drive transgene expression. Other strong promoters or tissue-specific promoters may be exchanged. A picornavirus internal ribosomal entry site (IRES) is frequently used to allow simultaneous expression of two transgenes from a single internally-promoted mRNA. The woodchuck hepatitis virus posttranscriptional regulatory element (WPRE) enhances transgene mRNA expression (Donello et al., 1998). A marker gene, such as GFP, can be used in such a vector to evaluate transduction and titrate vector stocks.

Packaging construct

The packaging construct serves as a source of FIV *gag* and *pol* genes required for necessary virion structural and enzymatic proteins. It also encodes the viral protein Rev to rescue the RRE-containing transfer vector RNA (Phillips et al., 1992). Elimination of most or all sequence homology with the transfer vector is important to reduce the risk of replication-competent retrovirus (RCR) production. Our current packaging construct, pFP93 (Fig. 1) contains sequence overlap with the transfer vector only at a short region in the 5' end of *gag* (discussed below), and the RRE (Loewen et al., 2003a; Saenz and Poeschla, 2004). Compared to the initial version (Poeschla et al., 1998), *vif*, *orf 2*, additional *env* sequence, and the 3' LTR have been removed. In addition, except for 11 nt containing the major splice donor, all sequences in the

viral mRNA upstream of the *gag* ORF have been eradicated (Poeschla et al., 1998; Loewen et al., 2003a).

Pseudotyping

Pseudotyping constructs provide heterologous glycoproteins in trans to expand or re-direct cellular tropism, increase infectivity, and add stability to vector particles. Vesicular stomatitis virus glycoprotein (VSV-G) remains the most widely used glycoprotein for this purpose (Burns et al., 1993; Yee et al., 1994). Alternative glycoproteins may be used for pseudotyping lentiviral vectors including glycoproteins from rabies virus, mokola virus, gammaretroviruses, and other viruses (Cronin et al., 2005).

Progress in basic FIV virology

Encapsidation

Encapsidation is the process whereby a virion selectively packages its genomic RNA in lieu of other RNA species. The process is not completely understood, but the signals contained within the genomic RNA have been systematically mapped. Kemler and colleagues utilized RNase protection assays to demonstrate that FIV encapsidation requires two discrete RNA segments, one in R-U5 region and one in the proximal 230 nucleotides of *gag* (Kemler et al., 2002; Kemler et al., 2004). The location of the latter element just downstream of the major splice donor provides a mechanism for selectively packaging full length RNA into virions. Maintenance of these encapsidation elements in the transfer vector yields wild-type levels of packaging (Kemler et al., 2004).

The feature of lentiviral vectors that most distinguishes them from conventional gammaretroviral (e.g., murine type C) vectors is their ability to transduce non-dividing cells. The pre-integration complex -- the product of reverse transcription and 3' end processing by integrase -- is more than twice the maximum diameter of the nuclear pore complex (Cullen, 2001). Thus nuclear import cannot occur by passive diffusion. However, the mechanism of import remains controversial despite years of effort and numerous discarded hypotheses (Fouchier and Malim, 1999; Stevenson, 2000; Cullen, 2001; Vodicka, 2001). A number of peptide signals in virion proteins have been implicated as has the central DNA flap, a triple-stranded DNA structure that results at the center of the genome from a second locus of plus strand initiation (Zennou et al., 2000; Cullen, 2001). Inclusion of the HIV-1 central DNA flap elements (cPPT and CTS) augments the efficiency of HIV-1 vectors (Follenzi et al., 2000; Sirven et al., 2000; Dardalhon et al., 2001; Van Maele et al., 2003). Whitwam et al. identified and characterized the cPPT and CTS (Whitwam et al., 2001). Unlike the situation in HIV-1, the FIV cPPT and FIV 3' PPT are not identical in sequence, which suggests the cPPT could be a partially degenerate element in FIV; alternatively, coding constraints in integrase prevent identity with the 3' PPT. Flap formation occurs both in the context of the full length FIV and in replication-defective FIV vectors, but appears to happen less efficiently per round of replication than does flap formation in HIV-1, and a major contribution to vector efficiency has so far not been evident (Whitwam T., and Poeschla E., unpublished data).

Integration

Integration is catalyzed by the viral integrase protein (IN). Mutations at certain IN residues that participate in the catalytic center of the enzyme (e.g., D64 and D116 in HIV-1 integrase, D66 and D118 in FIV) result in "class I" phenotypes -- only integration catalysis is blocked (Engelman and Craigie, 1992; Leavitt et al., 1993; Saenz et al., 2004). Many other point mutations and virtually all wholesale deletions so far studied produce class II IN phenotypes, in which various other early and late life cycle events are defective. These complex phenotypes arise in part because integrase initially is expressed as the C-terminal portion of the Gag/Pol

precursor. Class I HIV-1 and FIV mutant vectors are capable of high-level transgene expression in growth-arrested or non-dividing cells, but not dividing cells, provided an internal promoter is used (Loewen et al., 2003b; Saenz et al., 2004). These vectors illustrate the potential for transgene expression by unintegrated DNA. Indeed, this property has been utilized for retinal gene transfer with HIV-1 vectors (Yanez-Munoz et al., 2006). While such long-term durability of unintegrated lentiviral vector DNA in diverse situations was not evident in all studies so far (Naldini et al., 1996a; Loewen et al., 2003b), intensive focus on this as a distinct goal may identify more roles for non-integrating lentiviral vectors in avoiding consequences of insertional mutagenesis.

The transcriptional coactivator LEDGF/p75 was recently established to be a cellular cofactor for lentiviral integration (Llano et al., 2006; Vandekerckhove et al., 2006; Shun et al., 2007). This appears to be a lentiviral-specific role since HIV-1 and FIV, but not a gammaretrovirus, MLV, were affected by LEDGF/p75 knockdown. Lentiviruses integrate preferentially into actively transcribed genes (Schroder et al., 2002; Hacker et al., 2006; Kang et al., 2006;). Genome-wide integration site analysis showed that LEDGF/p75 also plays a significant role in determining this property (Ciuffi et al., 2005). Further understanding of the role of LEDGF/p75 could allow progress in targeting of lentiviral vector integration.

Restriction

Lentiviruses and other retroviruses display distinctive species-specific tropisms. While entry blocks (i.e., specific receptors) are one important factor, it is also clear that species-specific post-entry blocks (or restrictions) to infection occur. The so-called Ref1 and Lv1 post-entry restrictions in human and non-human primate cells act at a step at or immediately after reverse transcription in target cells, which is similar in many respects to a previously described pattern (Fv1) for gammaretroviruses (Bieniasz, 2003). Recently, the tripartite motif protein TRIM5 α was determined to account for Ref1 and Lv1 restriction activities (Stremlau et al., 2004). Briefly, TRIM5 α is a host factor that appears to bind to viral capsid protein trimers and disable incoming viral particles. A key caveat is that this factor is saturable, i.e., moderate to high multiplicity of infection permits efficient transduction even in a restricted cell. Saturable TRIM5 α mediated manner restriction has been shown for EIAV and FIV (Saenz et al., 2005; Saenz et al., 2006). For FIV, the restricting effect of TRIM5 α is fairly substantial in macaque cells, but is rather mild in human cells. In some situations, e.g., very low multiplicity transduction, post-entry restriction may reduce the efficiency of FIV vectors in human targets. Such restriction may be overcome by increased dosage or simply by adding genome-less pseudotyped viral particles since cytoplasmic availability of fully mature capsids to TRIM5 α is the determinant of saturation. However, in many if not most gene therapy situations, particularly those involving focal deposition of vector, particle concentrations are well above those needed to saturate Ref1/TRIM5 α activity. In such common situations, this kind of restriction could provide a net advantage by providing an innate barrier to systemic propagation of any replication-competent retroviruses (RCR) that might theoretically arise.

Conclusion

Understanding of basic FIV molecular virology is increasing, providing a basis for efficient and safe vector design. These vectors are highly useful for genetically modifying dividing and nondividing cells *ex vivo* or *in vivo*. Further discoveries of molecular events that underlie key steps in the lentiviral life cycle are likely to foster vectors with enhanced capabilities.

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Fig. 1. Overview of a three-component FIV vector system

FIV-derived elements are shown in green. Normal long terminal repeats (LTRs) contain U3 (3' unique element), R (repeat element) and U5 (5' unique element) in that order. The transfer vector illustrated here lacks any U3 element (either 5' or 3'), so that the viral promoter has been deleted from the system entirely. In the integrated vector, the only active promoter is the internal non-lentiviral one that drives transgene expression. The packaging construct expresses only Gag, Pol and Rev (*vif*, *env*, *orf2* are abolished, as are cis-acting sequences such as both LTRs, the 5' leader sequence, and encapsidation signal). CMV: human cytomegalovirus immediate early gene promoter. □□ packaging elements. RRE: Rev response element. WPRE: woodchuck hepatitis virus posttranscriptional regulatory element. cPPT-CTS: central polypurine tract – central termination sequence (central DNA flap). Δ *gag*: 230 proximal nt of *gag*, which is part of the FIV encapsidation signal.

Fig. 2. Overview of events occurring during vector particle production

The packaging construct (red circle in nucleus) is the source of Rev (blue semi-circular shape) and Gag/Pol-derived proteins (viral structural proteins and enzymes such as reverse transcriptase and integrase); the latter are shown as red cylinders. The transfer vector 513 construct (green circle in nucleus) is exported and packaged in trans. The envelope 514 glycoprotein (the expression construct is the yellow circle in the nucleus) is transported to 515 the plasma membrane and incorporated into particles.