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HUMAN IMMUNODEFICIENCY LENTIVIRAL VERSUS INTEGRASE-DEFICIENT LENTIVIRAL: A COMPARISON FOR SAFER CLINICAL APPLICATIONS

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ABSTRACT

Finding ways to exploits viral persistency to achieve efficient and safe long-term transgene expression will be a major achievement for their application in regenerative medicine including gene therapy. To date, most studies have used viral vectors such as lentivirus, retrovirus and adenovirus for the introduction of these genes with raising potential safety. Despite the robust advantages of using retroviral vector systems, the inability of these vectors to infect non-dividing cells is one of the major drawbacks that limit the development of retrovirus transduction systems. Lentiviruses, a genus of retroviruses which include HIV, have been extensively analysed and used for clinical gene therapy applications. This paper reviews the advantages of using lentiviral vector transduction system in comparison to other viral vectors. Alternatively, the development of integrase-deficient lentiviral vector for successful transplantation, engraftment and differentiation for safer therapeutic applications are also discussed.

1.0 The Basic Biology of Lentivirus

The lentivirus genome consists of 2 identical single stranded RNA molecules, which are packaged by the gag protein and surrounded by the viral envelope, made up of host cells membrane and virus encoded envelope glycoproteins. Virus replication enzymes are contained within a viral protein core. In brief, infection begins when the virus enters the target cell through the interaction of viral surface glycoproteins with receptors on the cell surface, or by endocytosis (Fig. 1). This process will then release the virus core including the RNA into the cell cytoplasm, undergoes reverse transcription mediated by the viral reverse transcription complex (RTC) into blunt-ended double-stranded linear viral DNA (a substrate for viral integration) and is transported to the host genome as a part of the pre-integration complex (PIC). The viral DNA is permanently integrated into the host genome and hereafter referred to as the provirus. Pro-viral DNA is transcribed into RNA and again transported to the cytoplasm, where it can be translated into viral proteins. Later, with viral RNA, replication enzymes assemble with structural proteins and fold with envelope proteins as they bud from the cellular membrane to form mature, infectious progeny virus particles. However, lentivirus has a more complex genome and replication cycle as compared to simple retroviruses.

The basic genomic organisation of HIV-1 is similar to simple retroviruses with the addition of accessory genes (Tat and Rev proteins are crucial in the virus replication cycle) (Fig. 2). Tat activates the promoter in the HIV 5' Long terminal repeat (LTR) for efficient production of viral RNA [1]. Rev interacts with Rev responsive element (RRE) within intron-containing HIV RNAs and promotes the transport of viral RNA from the cell nucleus to cytoplasm [2]. All retroviruses encode gag, pol and env genes. Gag (encoding viral core proteins) and pol (encoding viral replication enzymes) genes are initially expressed as a Gag-pol fusion polyprotein. The env, such as the vesicular stomatitis virus G-protein (VSV-G), encodes the viral envelope protein, which is cleaved by cellular proteases to the external envelope glycoprotein. Lentivirus replication is also assisted by several viral proteins; the matrix protein from Gag [3], the integrase

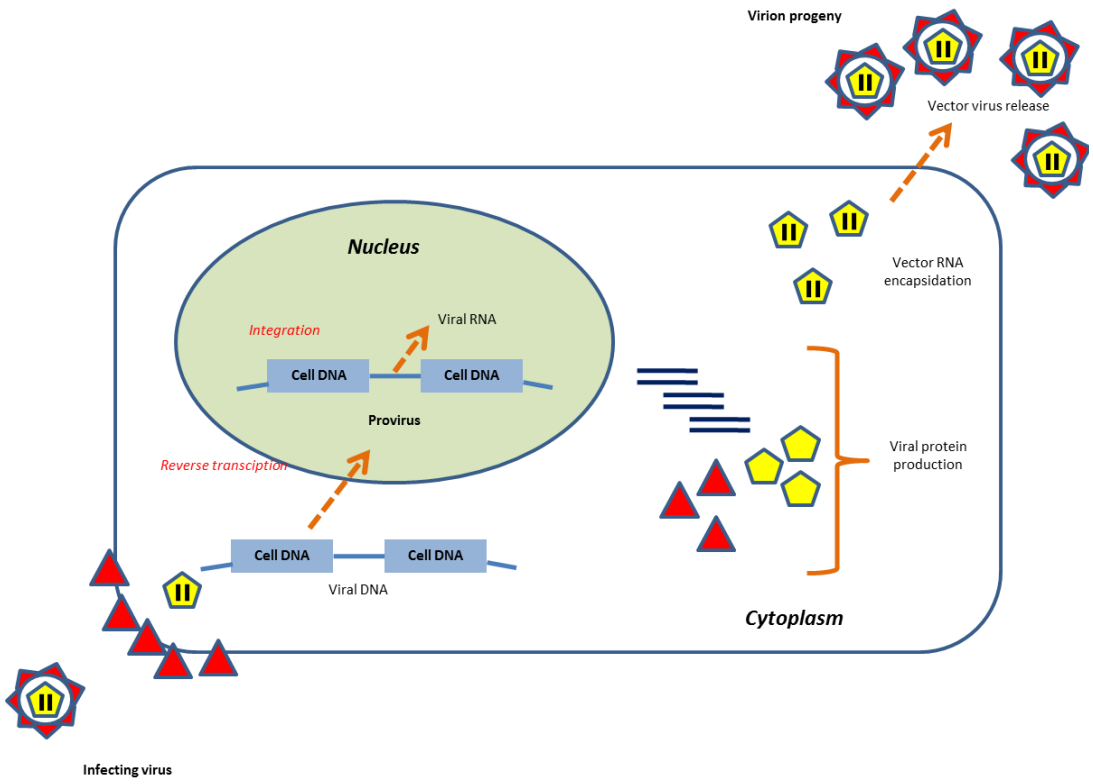

Fig. 1: Viral replication cycle. Infection begins when the virus envelope glycoprotein fuses to the cell membrane, with subsequent release of virus core into the cytoplasm. The viral RNA is transcribed into double-stranded DNA and transported into the nucleus. The viral DNA now known as provirus, will integrate into the host genome and then stably maintained, replicated during DNA synthesis and passed to progeny cells. Viral RNA is produced and translated into various viral proteins which facilitate RNA encapsulation. The mature virion containing envelope glycoprotein is released from the cells. This virion is capable of infecting new cells. [7,49].

protein [4], and the accessory protein Vpr [5]. Vpr binds directly to the nuclear core complex [6] and is required for efficient viral replication in non-dividing cells [5]. Both the matrix protein and Gag contain signal peptides for nuclear localisation. The context of co-interaction between these elements in directing viral entry nuclear transport, transcription and production of virion progeny, still remains to be elucidated.

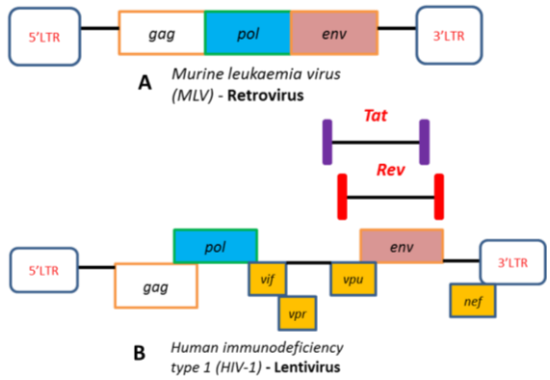

Fig. 2: Genome of a simple retrovirus and a lentivirus. A) Murine leukaemia virus (MLV), an oncogenic retrovirus and B) Human immunodeficiency type-1 (HIV-1), a lentivirus, share similar viral genes: *gag*, *pol* and *env*. Gag and pol are expressed together as a polyprotein that encodes the viral core and replication enzymes which are expressed directly from the viral promoter in the 5'LTR. The *env* gene encodes the envelope glycoprotein and is expressed from a single spliced mRNA. *Tat* and *Rev* are additional accessory genes of lentivirus. These genes are essential for efficient viral gene expression in HIV-1. [7,49].

Little is known about the functions of many other HIV accessory genes in relation to virus production and also infection of target cells.

1.1 Lentiviral Vector Production

In gene therapy for human disease, lentiviral vectors have been used to introduce foreign genes of interest into target cells. Lentivirus vectors are virus derivatives typically engineered to be replication defective. These viruses are capable of infecting cells and integrating a foreign gene into target cells, but are unable to multiply, and infect other cells. Vectors are created by replacing the viral genes with other foreign genes of interest, leaving only the cis-acting sites (regions recognised by viral proteins for transcription) (Fig. 3). In most vectors, marker genes such as genes that confer drug resistance are used to monitor the vector infection of target cells. Vectors can also be constructed such that they carry 2 or more foreign genes through additional internal viral promoters or internal ribosome entry sites (IRES) [7].

DNA constructs encoding these vectors can be propagated in cultured cells with addition of viral proteins also known as the helper DNA plasmid constructs that encoded Gag-pol and env proteins (Fig. 4). These helper DNA plasmid constructs are provided in trans. Co-transfection of these DNA plasmids either by chemical or electrical means into cultured cells result in vector propagation. The helper

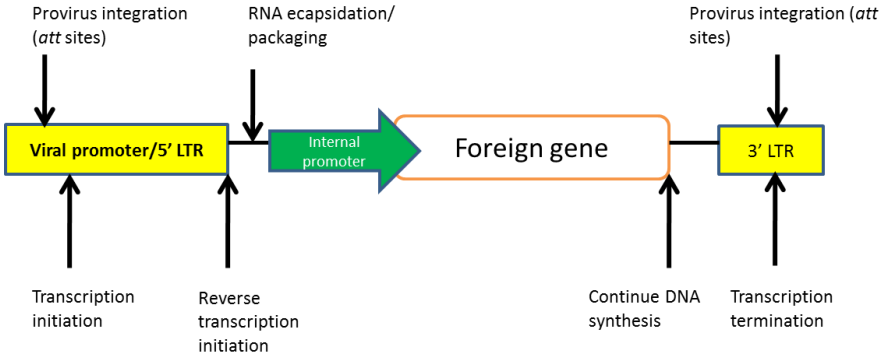

Fig. 3: Cis-acting elements of lentiviral vector. All elements necessary for lentivirus propagation are shown and roles of the various sites labelled. The viral protein sequences such as *gag*, *pol* and *env* have been deleted and replaced with a foreign gene. This foreign gene can be expressed from an internal promoter, engineered to be a part of the viral vector. [7,49].

DNA plasmid constructs do not contain the cis-acting viral sequence; therefore, the vector propagation is achieved in producer cells which contain these factors and the target cells infected with the viral vectors do not propagate the virus as they lack the presence of these essential viral gene products (gag, pol, and env). Unlike retrovirus vectors, lentivirus vectors do not have a packaging cell line that allows stable and high titre virus production. Thus, the vector production is dependent on co-transfection of vector and helper DNA plasmid constructs into cells that then transiently produce the vector.

In addition to deficiency in replication, different generations of lentiviral vector systems have been developed to counteract the safety concerns regarding HIV-1 pathogenicity. Currently, the third-generation vector system is commonly used clinically or in research, which consists of four plasmid systems: three helper plasmids and one transfecting vector plasmid. The first-generation plasmids consist of HIV-1 accessory genes (e.g. vif, vpr, vpu and ref), which are subsequently removed in second and third generation. Moreover, the U3 promoter of the 5 ' -long terminal repeats in transfection vector is replaced by constitutively active promoter sequences such as cytomegalovirus or Rous Sarcoma Virus. This renders the transacting ability of regulatory tat gene dispensable, and hence is removed in third generation plasmids [8].

1.2 Cis-Acting Elements in Modern Lentiviral Vectors

In order to improve virus titre, target specificity and transgenes expression, some modifications have been made in HIV-based vectors. These include the addition of rev responsive element (RRE), Woodchuck post-regulatory element (WPRE), and central polypurine tract/central termination sequence (cPPT/cTS) [9,10,11]. Rev gene product, a regulatory protein, binds to RRE, an important requirement for efficient transport of unsliced viral RNA genomes from the nucleus into the cytoplasm [12]. WPRE has been used to enhance transgenes expression [10,13]. In most vector constructs, WPRE is normally placed downstream of the transgene, hence, causing post-transcriptional increase in transgene expression [14,15]. WPRE also induces cytoplasmic transport of nuclear-retained RNA molecules [16]. A human cell co-cultured with lentiviral was found to increase CMV promoter driven transgene expression up to 8-fold [14,17]. The cPPT/cTS element is reported to enhance the efficiency of viral transduction in monocytes and T-lymphocytes [18].

1.3 Advantages and Disadvantages

The lentiviral vectors have several advantageous properties over other viral vectors: (i) large packaging capacity (~8 kb transgene cassette in addition to all the required cis-acting signals) [19,20]; (ii) ability to transfect different cell types including quiescent cells [21]; (iii) stable gene expression [10]; (iv) reduced immunogenicity upon in vivo administration [21]; and (v) similar transduction efficiency to adeno-associated virus vectors [10].

Despite all the advantages of using lentiviral vectors in clinical gene therapy, there are still some problems that limit their use and development for gene transfer [22,23,24,25]. These problems are (i) a variation in gene expression between cells, (ii) possible gene silencing, (iii) inability to target the vector to specific cells, (iv) insufficient vector titres, (v) decreasing levels of foreign gene expression over time, (vi) the possibility of generating wild-type virus (replication competent retrovirus) [26], vii) different restriction factors (RFs) inhibiting viral replication inside the cells, viii) activation of innate immune sensors during cell transduction [27], and ix) most importantly insertional mutagenesis, which can lead to malignant transformation [28,29]. Therefore, the non-integrating lentiviral vectors (IDLVs) or also known as integrase-deficient lentiviral vectors (IDLV) have been developed to overcome these problems. These IDLVs still maintain the positive attributes associated with the integrating lentiviral gene transfer except that the non-integrating lentiviral DNA accumulates in non-dividing cells [30].

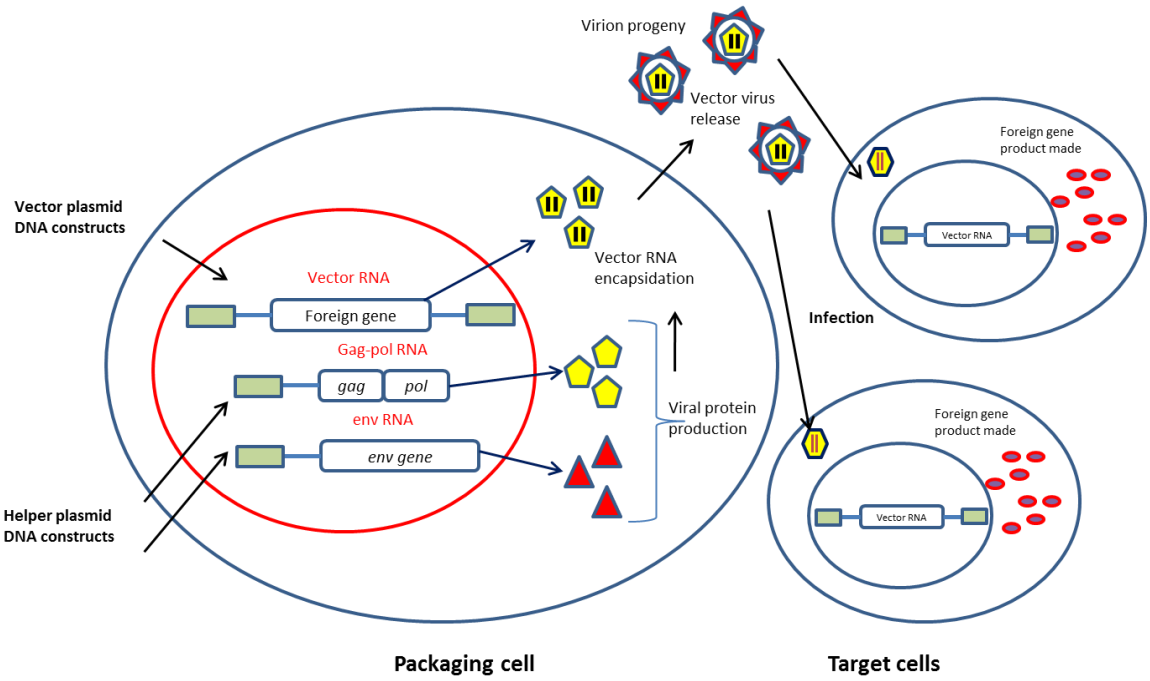

Fig. 4: Viral packaging cell line and viral vector production. Vector plasmid DNA constructs are introduced into packaging cells in *trans*, where they produce viral structure, enzymatic proteins and foreign gene copies (vector RNA). The vector RNA can be packed, resulting in the production of virions (virus progeny) containing the vector genome. These can be harvested and used to infect target cells to express the foreign gene in target cells. The helper plasmid DNA constructs that were introduced in packaging cells lack cis-acting sequences. The target cells do not express viral protein, thus the vector will not be propagated further. [7,49].

1.4 Integrase-deficient lentiviral vectors (IDLVs)

Episomal DNA, also known as extra-chromosomal lentiviral DNA is also generated during natural HIV-1 infection and exists in three forms: (i) double-stranded linear, (ii) circular with a single LTR, and (iii) circular with two LTRs (Fig. 5). The linear episomal DNA is the key precursor for wild type virus integration in the host genome [31], while the two circular DNA molecules are present in the cells, as by products of integration with very low transcriptional activity. Since episomes are not integrated into the host genome and lack an origin of replication (ORI), they do not persist in dividing cells. However, this episomal DNA accumulates in non-dividing cells if integration is blocked [30]. Several studies have reported the efficient gene expression in vitro and in vivo using IDLVs with a lower risk of causing insertional mutagenesis, thus being a better alternative for gene therapy intervention [10,32].

1.5 IDLV Mutations

Viral genome integration into host genome is mediated by the virus encoded enzyme integrase (IN), a 32-45 kDa protein [33]. This enzyme is encoded by the pol gene and translated as part of the Gag-pol polyprotein [10]. IN consists of three functional protein domains: (i) N-terminal domain containing the zinc-binding site that primarily binds viral DNA, (ii) the catalytic core domain containing the D-D35E amino acids, and (iii) the C-terminal domain containing a DNA-binding site that binds non-specific target DNA [31,34] (Fig. 6).

It is possible to introduce point mutations into IN to interrupt its normal function but it cannot be deleted completely because it is involved in the reverse transcription of viral RNA and nuclear transport [4]. There are two types of IN mutations, Class I mutations result in the vector being defective for integration but leaving other viral processes intact. Class II mutations result in reverse transcription defects or impair multiple stages of the viral life cycles. Most studies favour Class I over Class II mutations because the pleotropic effects (where a single gene influences multiple phenotype traits) make Class II mutations unsuitable for vector transgene expression [35].

Mutations of catalytic core domain (D-D35E amino acid) are commonly used to establish IDLVs. This domain comprises amino acids D64, D116 and E152 [34] (Fig. 6). Alterations including point mutations within this catalytic core domain inactivate the IN protein functions. Amongst all, D64 amino acid point mutations are most commonly used [36,37,38] followed by D116 amino acid mutations [39,40].

1.6 The Basic Biology of IDLV

A number of studies have reported the differences in transgene expression and the level of integration deficiency in integrating and integrase-deficient lentivirus vectors. Although IDLV could be an alternative vector because of

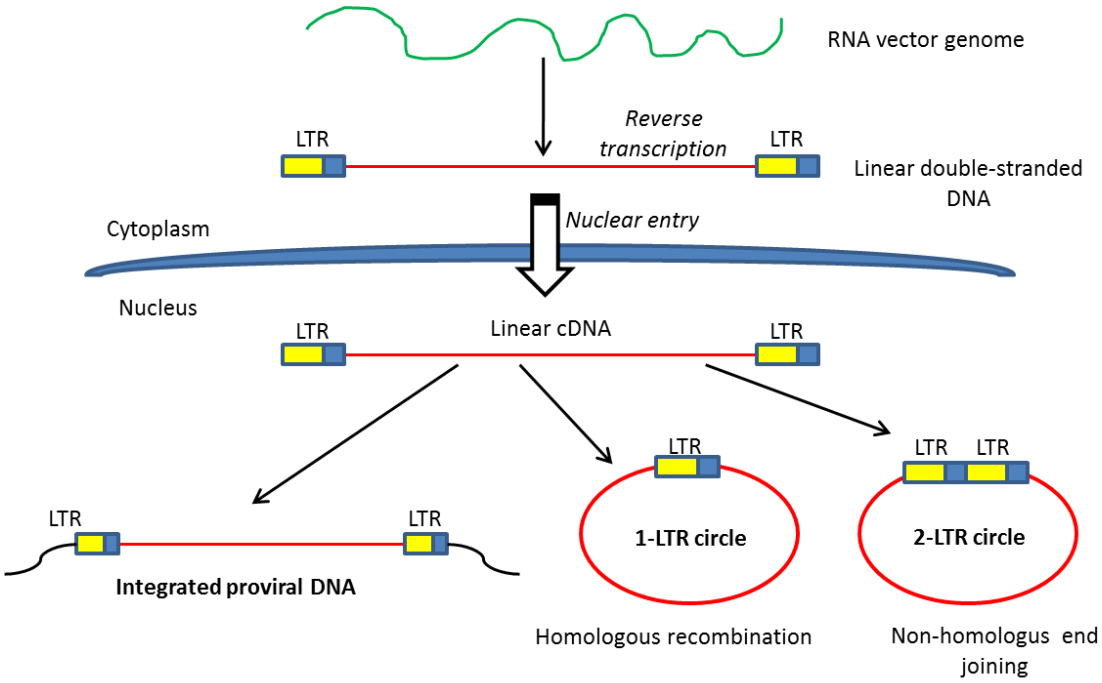

Fig. 5: The various forms of episomal DNA molecules generated in retroviruses infected cells. The product of vector reverse transcription is linear double-stranded DNA with addition of LTRs at both ends. This transcribed DNA is imported into the nucleus as part of the pre-integration complex (PIC). The linear double-stranded DNA is the precursor for viral integration in the host genome. There is also 1-LTR circle or 2-LTR circular episomal DNA. These episomal DNA accumulate in the non-dividing cells and are transcribed, but gene expression is less efficient compared to the integrated proviral DNA. [49,50].

the safety advantages over the integrating vectors, IDLVs are less effective at transgene expression in terms of the percentage of cells transduced and GFP mean fluorescence intensity (MFI) [41]. The Vargas et al. study reported long term GFP expression in IDLV (with insertion of SV40 promoter) transduced 293 and 293T cell lines whilst obtaining similar levels of GFP expression during transient vector expression stage in both 293T and 293 cell lines. This study suggests that the insertion of SV40 promoter in the lentiviral vectors allow the sustained expression of GFP when Tag is provided in trans. Using a similar approach, it has been confirmed that while IDLV transduced cells can have similar transduction percentages, the level of transgene expression suppressed [38,42]. It has been suggested that the level of transgene expression can be stable in non-dividing cells, but rapidly decreasing in dividing cells [38]. This is because episomes are not persevered in dividing cells as they are not integrated and lack an ORI sequence.

In addition, both integrating LVs and IDLVs can have similar vector titres as analysed by p24 ELISA assay [36,43], and by RT-PCR based quantification of the viral genome copy number [40], and by transducing units as determined by a visual marker [36,38]. However, other studies have reported low vector titres in IDLVs as compared to integrating LVs. One study reported ~25% reduction in GFP expression in IDLV transduced cells as compared to integrating LVs [41], while, a 10-fold and 5-fold decrease in TU/ng p24 ELISA has been reported elsewhere [42,44].

Despite having reduced transgene expression and low vector titres, IDLVs could be a useful alternative for therapeutic applications. IDLVs still preserve some benefits of integrating LVs, but can achieve gene expression without viral integration, thus reducing the potentially detrimental risk of insertional mutagenesis. However, further development is required to increase episomal gene expression.

2.0 Future Directions

2.1 Clinical applications of LV and IDLV

The ability of lentiviruses to transduce non-dividing cells, predisposes them to clinical applications involving gene therapy. To date, there have been successful clinical trials using both types of lentiviruses for the purpose of treating several disorders, suggesting their potential for clinical application [45]. In 2006, Levine et al. [46] used 3rd generation lentiviral vectors encoding an antisense gene for the HIV envelope, to treat HIV patients who were irresponsive to other antiviral treatments. 80% of the patients showed an improved immune function, with no evidence of insertional mutagenesis [46]. Another successful clinical trial involved treatment of β -thalassemia using lentiviral expressing β -globin gene [47]. The patient dependent on monthly β E/ β 0-thalassaemia transfusions, became independent of them after 33 months of lentiviral gene therapy [47]. On the other hand, integrase-deficient lentiviral vectors mostly undergone successful preclinical trials [48] which support

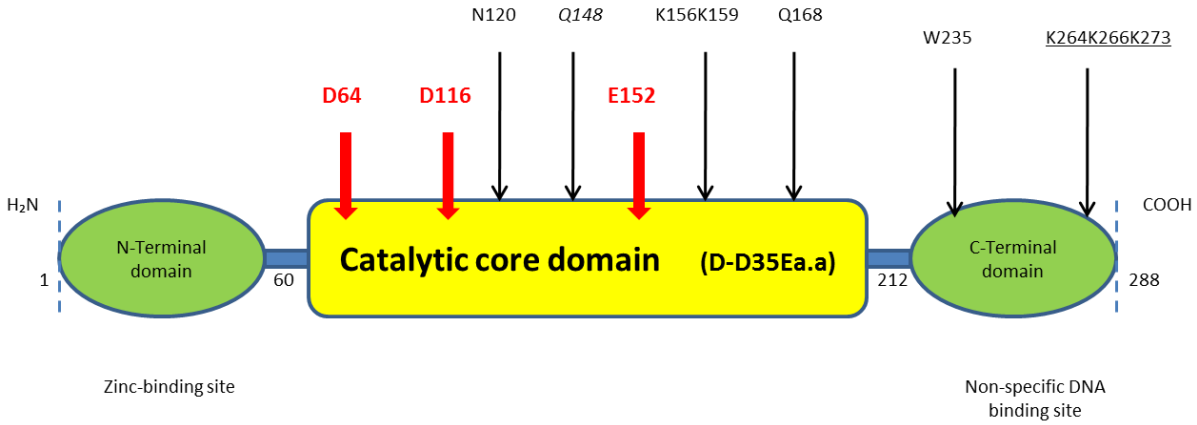

Figure 6: A schematic representation of HIV-1 integrase (IN). Structure of HIV-1 IN comprises three functional domains. Sites of IDLV mutations are indicated by arrows showing the amino acids (a.a) that give rise to the Class I integrase mutations. Mutations affecting the catalytic domain are in red bold. Mutations affecting genomic binding are underlined. Other IDLV mutations are italicised. [49].

their use clinically. Due to the lack of integration ability, IDLV are ideal for transient expressions. Studies show potential for IDLVs as vaccines, and several preclinical trials demonstrate immune responses in diseases such as HIV-1 and malaria [49, 50, 51]. In non-dividing cells, such as brain, skeletal muscle and liver, studies have also shown stable transgene expression via IDLV [52, 53].

2.2 Lentiviral-based Transduction Systems

The generation of human iPSCs by viral vectors encoding a number of pluripotency factors including KLF4, Oct-3/4, Sox2, and c-Myc, is well established [54,55,56]. Although the viral-based transduction systems have proven to be very efficient, the permanent viral genome integration in target cells results in the risk of tumourigenesis. Despite its major drawback, these iPSCs may be useful for understanding disease mechanisms, drug screening and toxicology. In addition, DNA microarray analyses detected differences between human iPSCs and human embryonic stem cells (hES), which make them non identical [57]. Therefore, further studies are necessary to determine whether human iPSCs can replace hES in medical applications. Until the safety issues are overcome, human iPSCs can at best be useful only in regenerative medicine applications that require patient-and disease-specific pluripotent stem cells for life threatening conditions.

2.3 Integrase-deficient Lentiviral-based (IDLV) Transduction Systems

As research into the mechanisms of viral genome integration advances, the development of new IDLV to overcome the risk of tumourigenesis is in progress. Previous studies have confirmed that IDLVs are efficient delivery vectors, have very low genomic integration frequencies (in the range described for DNA plasmid transfection), and importantly, have much lower risk of insertional mutagenesis [58] and replication-competent retrovirus (RCR) generation than integrating lentiviral vectors [48], thus eliminating the high risk of tumourigenesis.

Despite, the safety offered by IDLVs, more optimisations are needed to be performed before this system can be fully applied in gene therapy. For example, identifying which multiple integrase mutation sites will allow higher viral titre and minimise the potential problem of reversion back to an integrating phenotype. Moreover, identification of trans- and cis-acting factors for development of lentiviral vectors that are able to increase transduction and transgene expression efficiency in dividing cells would be beneficial for clinical applications. In addition, increased in vivo testing of IDLVs should also be considered to assess the differences in physiological changes and the side effects that may occur after IDLV administration of a single vector dose. This is crucial to show the utility of these techniques before they can be applied clinically.

Disclosure Statement

The authors declare no conflict of interest.

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