Polish Journal of Veterinary Sciences Vol. 26, No. 1 (2023), 163–172

DOI 10.24425/pjvs.2023.145019

Review paper

Review and future perspectives on the integration characteristics for equine lentivirus in the host genome

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Abstract

Despite over 40 years of research on the human immunodeficiency virus type 1 (HIV-1) vaccine, we still lack a considerable progress. Equine infectious anemia virus (EIAV) is a lentivirus in the Retroviridae family, akin to HIV-1 in genome structure and antigenicity. EIA is an important infectious disease in equids, characterized by anemia, persistent infection, and repeated fevers. The EIAV attenuated vaccine in China is the only lentiviral vaccine used on a large scale. Elucidating the mechanism of waning and induction of protective immunity from this attenuated vaccine strain will provide a critical theoretical basis and reference point for vaccine research, particularly in the development of lentivirus vaccines, with far-reaching scientific value and social significance. In this paper, we summarize the information related to EIAV integration site selection, particularly for the Chinese EIAV attenuated vaccine strains on the equine genome. This may improve our mechanistic understanding of EIAV virulence reduction at the host genome level. The obtained data may help elucidate the biological characteristics of EIAV, particularly the Chinese attenuated EIAV vaccine strain, and provide valuable information regarding retroviral infections, particularly lentiviral infection and associated therapeutic vectors.

Key words: lentivirus, equine infectious anemia virus, integration, long interspersed elements

Introduction

Equine infectious anemia virus (EIAV) and human immunodeficiency virus type 1 (HIV-1) are lentiviruses belonging to the Retroviridae family (Wang et al. 2018a). Other non-primate lentiviral species include ovine visna-maedi virus (VMV), caprine arthritis encephalitis virus (CAEV), bovine immunodeficiency virus (BIV), feline immunodeficiency virus (FIV), and Jembrana disease virus (JDV) (Cavalieri et al. 2018, de Pablo-Maiso et al. 2018, Munis 2020). Host genome "integration" of retroviruses, including EIAV and HIV-1, is essential in the viral life cycle completion (Coffin et al. 1997, Anderson and Maldarelli 2018). In the process of host cell retroviral infection, with retroviral membrane protein and target cell receptor

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binding, the viral envelope and cell membrane fuse, followed by viral genomic RNA release into host cells and reverse transcription into cDNA using reverse transcriptase (Coffin et al. 1997, Daniel and Smith 2008, Anderson and Maldarelli 2018). The cDNA then binds to viral integrase (IN) and other cellular and viral proteins forming pre-integration complexes (PICs), which are localized and integrated into the host chromosome under the guidance of cytokines, such as lens epithelium-derived growth factor (LEDGF/ P75) (Suzuki and Craigie 2007, Daniel and Smith 2008, Kvaratskhelia et al. 2014, Anderson and Maldarelli 2018). The formed proviruses act as transcriptional templates for effectively synthesizing progeny viral mRNA and genomic RNA and persist in host cell chromosomes for an extended period; they can enter progeny cells similarly to other genes during cell division (Suzuki and Craigie 2007, Demeulemeester et al. 2015). Proviruses can affect the transcription of host genes near their insertion sites. Conversely, the host chromosomal environment can likewise affect the transcription of proviruses (Suzuki and Craigie 2007, Demeulemeester et al. 2015). These interactions are partly responsible for the consequences following retrovirus integration (e.g., insertional mutagenesis and viral latency) (Hacein-Bey-Abina et al. 2003, Hacein-Bey-Abina et al. 2008, Rothe et al. 2013, Maldarelli et al. 2014, Wagner et al. 2014, Cohn et al. 2015, Demeulemeester et al. 2015, Anderson and Maldarelli 2018). The chromosomal environment of HIV-1 provirus integration sites demonstrably influences viral gene expression. It contributes to establishing latent or persistent infection, which likely also influences viral pathogenicity and disease progression (Anderson and Maldarelli 2018, Linden and Jones 2022).

The integrated nature of retrovirus life cycles allows for the successful use of retroviral vectors, including lentiviruses, γ-retroviruses, foamy viruses, and retrotransposons, in treating single-gene mutation diseases in humans (e.g., X-linked severe combined immunodeficiency, thalassemia, chronic granulomatous disease, adrenoleukodystrophy, adenosine deaminase deficiency, and Wiskott–Aldrich syndrome) (Yoder et al. 2021). Compared with other retroviruses, lentiviruses can infect both dividing and non-dividing cells and integrate transgenes into the host cell genome (Munis 2020). Therefore, lentiviral vectors are commonly designed based on EIAV and HIV-1, are often used as gene transfer vectors, and have been widely used in clinical gene therapy and transgenic animal studies (Cavalieri et al. 2018, de Pablo-Maiso et al. 2018, Munis 2020). The EIAV vector has been used with promising results in gene therapy treatment of hereditary retinal dystrophy using *RPE65*, *MERTK*, *REP1*, *ABCA4*, or *MYO7A* gene mutations (Stieger and Lorenz 2014, Parker et al. 2022).

Early studies suggested that retrovirus integration into host cell chromosomes is random, and the viral integration processes minimally disrupt or activate gene expression. However, in human gene therapy clinical trials, some therapeutically competent retroviral vectors were integrated near or in the proto-oncogene, causing insertion mutations and proto-oncogene activation that subsequently lead to disease manifestation in the host (i.e., tumor development) (Rothe et al. 2013, Demeulemeester et al. 2015, Anderson and Maldarelli 2018). Therefore, to develop safer gene therapy vectors and low-risk gene therapy regimens, further scientific research is required to explore the integration preferences, such as targeting DNA sequence and chromatin features, of each retrovirus in the host genome. Recently, numerous studies have been conducted to elucidate the mechanism of insertional mutagenesis and viral latency (Cavazza et al. 2013, Maldarelli et al. 2014, Demeulemeester et al. 2015, Anderson and Maldarelli 2018). The current integration site selection information for Chinese EIAV attenuated vaccine strains and EIAV vectors in the equine and human genomes are summarized to help understand the safety of equine lentivirus vectors.

Viral and biological characteristics of EIAV

EIAV pathogenicity

Equine infectious anemia (EIA) is an endemic equine zoonotic disease (horses are the most susceptible, followed by donkeys and mules) caused by EIAV, with an almost worldwide distribution (Cook et al. 2001, Leroux et al. 2004). Clinically, EIAV is characterized by a persistent viral infection and recurrent fever, accompanied by anemia, bleeding, jaundice, cardiac dysfunction, edema, and emaciation (Leroux et al. 2004, Craigo and Montelaro 2013). Pathologically, degeneration and hyperplasia of reticuloendothelial cells in the liver, spleen and lymph nodes are observed with iron metabolism disorders (Leroux et al. 2004). Active EIAV replication occurs in the spleen, liver, lung, lymph nodes, and bone marrow, while *in vivo* target cells primarily supporting EIAV infection and replication are mononuclear/macrophage cell lines (Harrold et al. 2000, Leroux et al. 2004). Macrophages support active EIAV replication in clinical and subclinical stages (Oaks et al. 1998). Vascular endothelial cells support viral replication in the acute but not subclinical phase (Oaks et al. 1998, Oaks et al. 1999). Primary cells or cell lines have been successfully used

in vitro for EIAV culture, including monocyte-derived, splenic, or bone marrow macrophages, endothelial cells, and fibrocytes (Leroux et al. 2004).

The Chinese EIAV attenuated vaccine strains

In the early 1970s, Chinese scientist Shen Rongxian successfully developed attenuated EIAV vaccines, including donkey leukocyte-attenuated vaccine strain and fetal donkey dermal (FDD) cell-attenuated vaccine strain, providing hope for further lentiviral vaccine development (Leroux et al. 2004, Wang et al. 2018a). These attenuated vaccine strains provide reasonable protection against homologous or heterologous EIAV strains and do not regain virulence (Shen et al. 1979, Feng. 1997). The development includes four steps; *in vivo* phase: horse ($EIAV_{Liao}$ [$EIAV_{LN}$], obtained from a natural EIAV wild strain isolated from Liaoning Province after 16 consecutive passages in healthy horses) and donkey virulent strains ($EIAV_{DV}$, obtained by more than 100 passages of horse virulent virus $EIAV_{LN40}$ in donkeys); *in vitro* phase: Chinese donkey leukocyte-adapted ($EIAV_{DLV,}$ obtained by over 120 subcultures of an EIAV virulent strain $EIAV_{DV117}$ in donkey leukocytes) and Chinese FDD cell-adapted attenuated EIAV strains ($EIAV_{FDDV}$, obtained by further subculturing the $EIAV_{DLV121}$ in FDD cells for 10–15 generations) (Wang et al. 2018a). The protection rate of $EIAV_{DLV121}$ (developed by 121 generations of successively passaging $EIAV_{DV117}$ in donkey monocyte-derived macrophages [MDMs]) against homologous EIAV virulent strains was 75% and 95% in horses and donkeys, respectively. Compared with the $EIAV_{DLV121}$ attenuated vaccine, the donkey FDD cell-adapted attenuated $EIAV_{FDDV13}$ strain (developed by 13 generations of passaging $EIAV_{DLV121}$ in FDD cells) provides improved protection, and production is more conveniently expanded (Dong et al. 1989).

Genomic composition of EIAV

EIAV contains two single RNA strands connected by hydrogen bonds in a dimer structure (Leroux et al. 2004, Cook et al. 2013). The 8.2 kb genome and both ends of the provirus are long terminal repeats (LTRs), which are divided into the U3, repeated R, and U5 regions (Leroux et al. 2004, Cook et al. 2013). The LTRs contain several transcription factor binding sites that interact with various cytokines to regulate virus transcription (Leroux et al. 2004, Cook et al. 2013). The EIAV genome primarily includes three structural genes: *gag*, *pol,* and *env*(Leroux et al. 2004, Cook et al. 2013). The Gag-precursor ($PR55^{gag}$) generates four major structural proteins integral to virus budding and assembly: matrix (MA; p15), capsid (CA; p26), nucleocapsid (NC; p11), and late domain proteins (p9) (Leroux et al. 2004, Cook et al. 2013). EIAV Gag-Pol precursor ($PR\ 180^{gag/pol}$) is cleaved into proteinase (PR; p12), reverse transcriptase (RT; p66), dUTPase (DU; p15) and integrase (IN; p30), and are important in viral replication, transcription, and translation (Leroux et al. 2004, Cook et al. 2013). The *env* gene primarily encodes surface (SU; gp90) and transmembrane envelope glycoproteins (TM; gp45) (Leroux et al. 2004, Cook et al. 2013). Both glycoproteins have a significant role in virus-induced immune protection (Leroux et al. 2004, Cook et al. 2013). EIAV also includes three non-structural genes, S1, S2, and S3, encoding Tat, S2, and Rev accessory proteins, respectively, which are required for viral protein expression and virus production (Leroux et al. 2004, Cook et al. 2013). In addition, a novel, fully spliced, accessory gene in EIAV is found located in the coding regions of MA and TM (Zhang et al. 2022).

EIAV integration

The EIAV life cycle is similar to retrovirus life cycles (Leroux et al. 2004, Lesbats et al. 2016). EIAV uses self-encoded reverse transcriptase to synthesize DNA using genomic RNA as a template; the genomic RNA is then degraded by reverse transcriptase and duplicates using the remaining DNA as a template to produce double-stranded (ds) DNA (Coffin et al. 1997, Leroux et al. 2004). Subsequently, viral dsDNA is transported to the nucleus and is integrated into the host chromosome. Viral DNA is then used as a template, generating messenger RNA and new RNA genomes before being translated into proteins in the cytoplasm. The capsid is subsequently formed, and two new RNA strands are packed into the capsid along with reverse transcriptase; virus particles are formed and secreted out of the cell after coating the capsid (Leroux et al. 2004, Lesbats et al. 2016). The "integration" of EIAV genome into the host genome is a critical process in the EIAV life cycle (Leroux et al. 2004). Several steps, including processing viral DNA ends, joining viral DNA ends to target DNA, and gap repair, participate in this process (Lesbats et al. 2016). The first two steps are catalyzed by viral integrase (Engelman and Kvaratskhelia 2022). The host enzyme repair system mediates the final steps. In EIAV-DNA 3′-end processing, the two nucleotides at the dsDNA 3′ end are removed, mediated by integrase, to form the PIC (Hare et al. 2012). Chain transfer reaction follows; after the PIC enters the nucleus, the integrase continues to cut the 3′-end while making a staggered incision in the host cell chromosomal DNA to open a gap to embed the EIAV-DNA (Hare et al. 2010). Finally, the integrase

Table 1. Datasets for the equine infectious anemia virus (EIAV) integration sites.

Virus or vector	Cell type	Number of integration sites	Accession numbers (GenBank)	Source of reference
EIAV vector	HEK 293T	416 (458)	DQ498348-DQ498763	Hacker et al. 2006
$EIAV_{DLV121}$[d]	Equine MDM[e]	525	KS297078–KS297602	Liu et al. 2017
$EIAV_{FDDV13}$	Equine FED	447	KO454223-KO454699	Liu et al. 2015

a: Human embryonic kidney 293T cells.
b: Actual number of sequences downloaded from the NCBI database provided by Hacker.
c: Number of sequences reported by Hacker.
d: EIAV fetal donkey dermal cell-adapted strain.
e: Fetal equine dermal cell.
EIAV donkey leukocyte-attenuated vaccine strain
Equine monocyte-derived macrophage.

binds the hydroxyl group at the end of the viral DNA to the 5′-end of the host cell DNA, joining the two fragments of DNA (Lesbats et al. 2016). As the target DNA breach fragment is joined to the viral DNA end at the viral insertion site, the viral DNA ends on both sides produce single-stranded breaks at each host-viral DNA junction, with two additional bases originating from viral DNA. Finally, the host enzyme repair system mediates the gap repair (Lesbats et al. 2016, Engelman and Kvaratskhelia 2022).

Integration characteristics of EIAV

Currently, EIAV integration site research data primarily includes the integration and localization characteristics of Chinese EIAV attenuated vaccine strains ($EIAV_{DLV121}$ and $EIAV_{FDDV13}$) in the genome of cultured cells (equine MDMs [eMDM] and fetal equine dermal [FED) cells] *in vitro* and human genome EIAV vector integration characteristics (Hacker et al. 2006, Liu et al. 2015, Liu et al. 2017) (Table 1). The provirus integration sites in the Chinese EIAV attenuated vaccine strain and the EIAV vector in the horse and human genomes were not randomly distributed (Hacker et al. 2006, Liu et al. 2015, Liu et al. 2017). The integration characteristics of Chinese EIAV attenuated vaccine strains, $EIAV_{DLV121}$ and $EIAV_{FDDV13}$, had a certain degree of commonality in eMDM and FED cells infected *in vitro* (Liu et al. 2015, Liu et al. 2017). These include: evident viral integration support of transcription units (TUs) region in horse genome; weak palindromic structure formation at the integration site; the tendency for integration processes to occur in genome AT-rich regions; the tendency for integration processes to occur in genomic regions with 11–40/2 Mb gene densities and long interspersed elements (LINEs), LINE1 particularly supported viral integration events; host genes integration by $EIAV_{DLV121}$ and $EIAV_{FDDV13}$ responded to DNA damage stimuli as determined by GO analysis, and were involved in adaptive immune response, leukocyte differentiation, apoptosis, lipid biosynthetic processes, and oxidation (Liu et al. 2015, Liu et al. 2017). The HIV-1 integration signatures do not depend on viral entry routes (HIV-1 or VSV.G envelope) in the human genome. Combining the integration signature data obtained from Chinese EIAV-weakened vaccine strains, $EIAV_{DLV121}$ and $EIAV_{FDDV13}$, (EIAV envelope) using an updated horse genome database, and the EIAV vector (VSV.G envelope) using an updated human genome database, EIAV integration signature is surmised to be independent of virus entry route (Hacker et al. 2006, Liu et al. 2015, Liu et al. 2017). The characteristics of EIAV integration site selection have similarities and extend from the human genome to the horse genome (Liu et al. 2015, Liu et al. 2017). Notably, EIAV has a unique integration feature, with significant integration events occurring in LINEs on the equine and human genomes, unlike HIV-1, which is prone to integration in short interspersed elements (SINEs) on the human genome (Hacker et al. 2006, Liu et al. 2015, Liu et al. 2017). This trend is particularly evident in the equine genome (Liu et al. 2015, Liu et al. 2017). Collectively, the integration features of EIAV proviruses in the equine genome are similar to those of HIV-1 in the human genome (Hacker et al. 2006, Liu et al. 2015, Liu et al. 2017, Singh et al. 2022).

The EIAV integration features in equine and human genomes share similar integration targeting trends with lentiviruses, including HIV-1, such as propensity to integrate within reference genes and AT-rich regions (Hacker et al. 2006, Ciuffi 2008, Liu et al. 2017). This suggests that integration mechanisms are conserved among lentiviruses (Hacker et al. 2006, Ciuffi 2008). Retroviral integration site selection is determined by viral integrase and cellular factors on the host genome (Ciuffi 2008, Bedwell and Engelman 2021). Several cytokines, including LEDGF/p75, barrier-to-autointegration factor, high mobility group protein A1,

integrase interactor 1 protein, polycomb group embryonic ectoderm development protein, Fanconi anemia (FA) effector proteins FANCD2 and FANCI (FANCI-D2), histidine-rich protein2, heat-shock protein 60, Ku autoantigen 70 kDa, and p300 acetyltransferase, that interact with HIV-1 IN and contribute to efficient integration have been identified (Craigie and Bushman 2014, Ran et al. 2019, Fu et al. 2022). In addition, viral proteins other than integrase reportedly influence HIV-1 integration site selection (Engelman 2021). Studies using HIV-1/MLV chimeric viruses demonstrated the HIV-1 capsid protein (CA), which interacted with cleavage and polyadenylation specificity factor 6 (CPSF6), nucleoporin protein 153, and E3 SUMO-protein ligase, played a role in influencing the bias of viral integration sites (Lewinski et al. 2006, Lusic and Siliciano 2017, Bedwell and Engelman 2021, Engelman 2021, Yoder et al. 2021).

Future research perspectives

Proviruses of EIAV are highly integrated into LINEs, particularly LINE1 (Liu et al. 2017). HIV-1 is integrated into SINEs, chiefly Alu (Cohn et al. 2015). Three reasons may explain the discrepancy: Firstly, the cellular or physiological factors required for proviral integration in human cells are absent in equine cells. CPSF6 had a critical role in targeting primate lentivirus (e.g. HIV-1) integration into transcriptionally active speckle-associated domains, but correspondingly failed to appreciably interact with nonprimate lentiviral capsids (Li et al. 2020). We concluded that CPSF6-dependent targeting of speckle-associated domains distinguished primate from nonprimate lentiviral integration. Secondly, the proportion of LINEs and SINEs differs in equine and human chromosomes. The proportion of LINEs in human and equine chromosomes is approximately 20%; SINEs make up approximately 13% of human and 7% of equine chromosomes (Huang et al. 2014). The third and most likely explanation for EIAV and HIV-1 repeated element integration differences is the inherent properties of the viruses. Repeated elements are not randomly distributed in the genome. For example, in the human genome, the Alu (SINE) element is present in GC-and gene-rich regions, whereas the LINEs element, containing two open-reading frames (ORF)-1 and ORF2, is present in regions with low GC content and low gene density (Richardson et al. 2015, Mueller et al. 2018). The LINE1 ORF2 fragment insertion into the green fluorescent protein (GFP) reporter gene significantly down-regulated RNA and protein amounts of target genes in mammalian cells (Han et al. 2004, Ustyugova et al. 2006). Another study demonstrated that genes with high expression levels were missing the LINE1 element, which has a strong polyA signal in the sequence capable of disrupting transcriptional elongation (Perepelitsa-Belancio and Deininger 2003, Ustyugova et al. 2006). This mechanism partially explains the presence of LINE1 in genes with decreased transcriptional activity (Perepelitsa-Belancio and Deininger 2003). LINE1 constitutes the majority of autonomous transposons in chromosomes (Perepelitsa-Belancio and Deininger 2003, Ramos et al. 2021). LINE1 reportedly causes genome instability through its autonomous transposition, indicating that LINE1 is vital in genome composition and evolution (Ramos et al. 2021). Furthermore, LINE-1 and its activity trigger innate immune activation through DNA- and RNA-sensing pathways that would suppress HIV replication (Zhao et al. 2021). Therefore, we hypothesized that LINE1 is integral in weakening the Chinese EIAV attenuated vaccine strains *in vitro*. Further investigation is required to determine the mechanism by which LINE1 affects the transcription and expression of EIAV integrated genes and viral weakening.

EIAV attenuated vaccine provides good protection, is non-pathogenic for equines, and does not return to virulence (Shen et al. 1979, Feng 1997). These strains with positive biological characteristics are crucial for studying lentiviruses, particularly their applications. Currently, the EIAV vector, like other lentiviral vectors, provides some clinical advantages in that it can infect both non-dividing and dividing cells (Munis 2020). As with other retroviruses, HIV-1 vectors can reportedly cause clonal extension and persistence of infected cells (Cesana et al. 2017, Huang et al. 2021, Yeh et al. 2021). However, inhibiting virus replication and blocking the extension of infected cells is considered crucial to curing HIV-1 infection. While HIV provirus cannot be detected in neoplastic tissues of HIV-1-infected patients, this is not well established due to the small number of integration sites inserted into oncogenes and the limitations of the corresponding experimental techniques (Anderson and Maldarelli 2018, Yeh et al. 2021). Therefore, several strategies must be designed and implemented to eliminate persistent HIV infection and improve the safety of lentiviral vectors for gene therapy in humans. Genome integration of EIAV is non-random; however, the involvement of EIAV provirus-inserted genes in replication and transcription remains unknown. Using GO analysis of all EIAV provirus genes, frequent insertion into gene clusters responsible for ion-binding molecular functions was demonstrated (Liu et al. 2017). Ions participate in multiple steps during retroviral integration (Wlodawer 1999, Engelman and Kvaratskhelia 2022). for example, the N-terminal

zinc-binding sequence of integrase binds Zn^{2+} and contributes to dimerization, trimerization, and multimerization of integrase (Engelman and Kvaratskhelia 2022). The crystal structure analysis of some integrases illustrated that Mn^{2+}, Mg^{2+}, and Cd^{2+} were bound in the catalytic core region of the integrase, which could help their correct folding (Wlodawer 1999). Furthermore, EIAV proviruses also insert functional gene clusters responsible for DNA damage stimuli responses (Liu et al. 2017). In the retrovirus life cycle, integration is crucial to complete replication (Lesbats et al. 2016). In this process, the retroviral DNA causes a single strand break in the host chromosome, forming a gap (Lesbats et al. 2016). This activates the host DNA repair system, base excision repair pathway enzymes DNA polymerase β, flap endonuclease 1, and ligase I (Skalka and Katz 2005, Lesbats et al. 2016). The attenuated vaccine strain $EIAVD_{LV121}$ also inserted functional gene clusters such as acquired immune response (Liu et al. 2017). A study found that integration of latent HIV-1 proviruses is significantly enriched in Krüppel-associated box (KRAB) domain–containing zinc finger (ZNF) genes, which are associated with repressive chromatin marks and provides a survival advantage, in clonally expanded CD4+ T cells of HIV-1 elite controllers (ECs) (Huang et al. 2021). HIV proviruses integrated in some cancer-associated genes, BACH2, STAT5B, MKL1, MKL2 and IL2RB have been previously shown to contribute to the growth and persistence of infected cells in individuals on combined antiretroviral therapy (cART) (Bedwell et al. 2021, Coffin et al. 2021). Therefore, further investigation is required to determine whether the integration of EIAV into these functional genes indicates an interaction between the pathways involved in these genes and EIAV.

Whole genome amplification (WGA), commonly used in retroviral integration site analysis, is a group of techniques that involve nonspecific amplification of the whole genome sequence to substantially increase the total DNA amount without sequence bias (Wang et al. 2017, Patro et al. 2021). Near full-length single-genome sequencing assay (NFL-SGS), the quadruplex qPCR (Q4PCR) assay, the intact proviral DNA assay (IPDA), the tat/rev induced limiting dilution assay (TILDA) are critical to WGA technology development (Procopio et al. 2015, Hiener et al. 2017, Bruner et al. 2019, Gaebler et al. 2019, Katusiime et al. 2020). These techniques have been extensively used to amplify host DNA regions flanking proviral cDNA and is reportedly effective in mapping provirus integration events of varied retrovirus genera on a genome-wide scale without causing significant bias (Procopio et al. 2015, Hiener et al. 2017, Bruner et al. 2019, Gaebler et al. 2019, Katusiime et al. 2020). However, due to the shortcomings of detecting genetically intact proviruses necessitates large amounts of donor material (e.g., blood, lymph node), which is typically unrecoverable. Multiple displacement amplification (MDA) is an isothermal method often used to amplify whole genomes, enabling multiparametric proviral characterization at the single-genome level, including proviral genome sequence, host-proviral integration sites, and phenotypic characterization of host cell lineages, such as CD4 memory cell subsets and antigen specificity (Bleier et al. 2008, Patro et al. 2021). Combined MDA and next-generation sequencing (NGS) have become the gold standard method for genome research (Wang et al. 2017). Currently, MDA is widely used for genome or transcriptome amplification; typically, MDA uses Phi29 DNA polymerase, which exhibits excellent strand displacement and exonuclease activities (Dean et al. 2002). MDA is currently recognized as the best single-cell genome amplification technology, able to perform high-fidelity uniform amplification of the whole genome, amplify 10–100 kb fragments, and provide a large number of uniform and complete whole genome sequences, which is particularly suitable for NGS library construction (Patro et al. 2021, Sun et al. 2022). MDA-based detection methods, including MIP-seq and SIP-seq, have been widely used to analyze the integration characteristics of HIV-1 in the host genome (Patro et al. 2021, Sun et al. 2022). These approaches apply to other viral diseases, such as EIA, which integrate host genetic material during their life cycle.

There are over 37 million people with HIV infection worldwide; among these, <0.5% of infected people are positive for HIV antibodies, do not require antiretroviral drugs, are sufficiently autologous to control HIV replication, and have a continuously undetectable HIV viral load in the blood (Saez-Cirion and Pancino 2013, Shahzad et al. 2022). The common features of EC include several gene mutations (such as human HLAI loci specific mutations), rapidly-produced broad neutralizing antibody (bNAb), and powerful features such as cellular immunity of the EC (Migueles et al. 2008, Saez-Cirion and Pancino 2013, McLaren and Carrington 2015, Jiang et al. 2020). Additionally, MIP-Seq technology revealed that compared with individuals receiving long-term antiretroviral therapy, complete proviral sequences from EC are preferentially integrated into Krüppel-associated box domain zinc finger genes on chromosome 19 and centriolar satellite DNA in the human genome, both of which are associated with heterochromatin features (Jiang et al. 2020). Furthermore, integration sites of intact EC proviral sequences increased distance from the host genome transcription start sites and accessible chromatin and are enriched with chromatin-repressor markers (Jiang

et al. 2020). Complete proviral sequences were not detected in an EC, despite analyzing over 1.5 billion peripheral blood mononuclear cells (Jiang et al. 2020). These characteristics must be achieved for the functional cure of HIV. By identifying these genetic mechanisms, scientists can replicate this process with a combination of gene therapies, immune vaccines, or biomedical approaches. Even in vaccinated immune-suppressed horses, the EIAV Chinese vaccine strain maintains stable low-level replication characteristics, suggesting that the host immune system is not involved in the low-level replication of the attenuated vaccine strain (Ma et al. 2009). This may be due to the weakening of the virus itself.

The relationship between the weakening of Chinese EIAV vaccine strains and chromosome integration characteristics remains unknown. The quasispecies EIAV attenuated vaccine strains, that is, heterogeneous virus groups comprised closely related but not completely identical sequences (Wang et al. 2018b, Liu et al. 2019). Viral genetic characteristics in HIV-1 studies influence integration site selection, viral pathogenicity and disease progression (Anderson and Maldarelli 2018, Linden and Jones 2022). The characteristics of various single virus integration sites within heterogeneous virus groups of vaccine strains require clarification. The EIAV strains and vectors integration process was affected by cytokines and selection pressure in cultured cells *in vitro*. Using EIAV vaccine strains with full replication ability to infect horses and studying the integration sites of EIAV-infected cell subsets *in vivo* helped discover the actual EIAV integration bias in host cells. Future studies are required to accurately characterize the proviral reservoir of horses inoculated with Chinese EIAV attenuated vaccine strains, defined as host-integrated viral DNA genomes, which do not drive viremia relapse even under immunosuppression (Liu et al. 2019). Simultaneously, these new techniques, which have been widely used in the exploration of HIV proviral reservoir studies, are essential to characterize the dynamic proviral reservoir on the equine genome after vaccination, that is, to longitudinally monitor the integration sites dynamics of these rare, genetically intact, reproducible proviruses. Likewise developing the deeper and more comprehensive integration site mapping techniques, e.g. three-dimensional (3D) chromatin structure of integrated proviruses, may give new insights into mechanisms infuencing the location of integration as well as the 3D interactions between integrated proviruses and host genes.

Acknowledgements

This study was supported by grants from the Nanchong Vocational and Technical College for basic scientific research (no. NZYBZ2002), the Nanchong High-Level Talents Special Support Program (Scientific Elite Of Ten-Thousands Talents Program), the Science and Technology Planning Project of Nanchong (no. 22XCZX0003), the Science and Technology Planning Project of Yibin (YBZYSC22BK07) and the Science and Technology Research Project of Chongqing Education Commission (KJQN202003503).

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