



Innovative CRISPR-Cas9 Strategies for Epigenetic Modulation of HIV-1 Latency: Targeting Tissue-Specific Reservoirs with Multiplexed Approach

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Abstract

The most significant barrier to curing HIV-1 is the persistence of HIV-1 in latent reservoirs, distinct populations of long-lived, transcriptionally silent cells located in blood, lymphoid tissues, gastrointestinal (gut-associated lymphoid tissue), and central nervous system, which necessitate lifelong antiretroviral therapy but reliably fail to result in viral eradication. Maintenance of these reservoirs in an epigenetic state is achieved by DNA methylation, a repressive chromatin remodelling and histone modification and is the mechanism that evades immune detection of the integrated proviruses. Several promising advances are noted herein, such as multiplexed guide RNAs to concurrently target multiple proviral and host regulatory sites; tissue-specific delivery through viral vectors with tissue-specific promoters, ligand-decorated lipid nanoparticles, and exosome delivery; and a concept of integrating combinatorial gene editing with latency reversing agents and broadly neutralizing antibodies to increase immune-mediated eradication. Complimentary preclinical verification procedures such as humanized mouse models and skilled organoids exhibit the security, performance, and versatility of such disciplines. A paradigm shift necessitated by the evidence is a global shift to personalized, reversible epigenetic modelling of

latency to overcome heterogeneity and anatomical compartmentalization in the reservoir. In future progress in delivery, specificity, and multi-omic profiling is expected to propel faster clinical translation, not only in HIV-1 but also in other chronic viro-infections, and mark the dawn of a new age of curative genomic medicine.

Keywords: HIV-1 latency, Viral reservoirs, Epigenetic editing, CRISPR-dCas9 systems, Tissue-targeted delivery, Gene therapy.

Introduction

HIV-1 remains a world health crisis and it is estimated that 40 million people will be living with HIV 2025 [1]. These facts are highlighted by the persistent socioeconomic and geographic differences in situations and mortality [2]. Exceptional progress with antiretroviral therapy (ART) has shown HIV to be not a deadly but a chronic disease; however, suppression by ART fails to eliminate the integrated proviral DNA in host cells[3]. Continued compliance is thus essential, with risks of toxicity/development of resistance and the risk of viral rebound should treatment be discontinued [4]. HIV-1 persists in reservoirs within blood, lymphoid tissues, gut, and CNS [5]. DNA methylation, the LTR region, repressive histone finish (H3K9me2/3, H3K27me3) and chromatin-remodeling complexes that enhance the latent condition; all these mechanisms keep silencing; and tissue-specific microenvironmental factors add to that [6]. While the approaches highlighted as shock-and-kill and block-and-lock strategies pursued different dimensions of viral latency, none of the existing ones accomplishes complete reprogramming of the chromatin state in all compartments of the HIV-1 reservoir [7]. Therefore, one of the research directions concerning the pursuit of HIV-1 cure is CRISPR-based genomic editing [8]. dCas9 with epigenetic effectors enables site-specific HIV provirus modification [9]. Directed reprogramming enables both HIV latency reversal and durable silencing, addressing reservoir heterogeneity [10]. Multi-guide CRISPR enables precise, rational control of HIV latency networks [11].

In **Table 1**, the most popular strategies of HIV-1 curing were compared: shock-and-kill approach, block-and-lock approach and gene-editing approach. These approaches vary in their mechanisms, with some being reactivation and eradication of latent reservoirs, and some being permanent silencing of the viruses, and direct genetic modification of proviral DNA. Although promising, both methods still have drawbacks including a lack of clearance of the reservoir, safety issues and difficulty in clinical translation.

Table1 :- Comparison of existing HIV-1 cure strategies (shock-and-kill, block-and-lock, gene editing)

Sr no.	Strategy	Mechanism	Strengths	Key Limitations	Clinical Status	Reference
1.	Shock-and-kill	Lytic-reagent activators (LRAs) wake up latent HIV-1, induce viral expression and cause either immune-mediated or cytopathic killing of cells with latent virus.	Viral reservoirs form a critical part of viral persistence and destruction of viral reservoirs induces effective immunological clearance.	Incomplete remobilization, toxicity risk or immune activation as well as reservoir persistence.	Early/mid stage clinical trials/HDAC inhibitors/PKC agonists	[12]
2.	Block-and-lock	Epigenetic regulators that are used to enhance silencing in integrated proviruses of the HIV, ultimately aim at driving them into permanent latency.	Reduced viral rebound and little huge immune excitation.	May max out the silence and still have a reservoir	Preclinical, early clinical/Tat inhibitors,em d Launch siRNAs	[13]
3.	Gene editing	Employs gene-editing technologies (e.g. CRISPR-Cas9) to excise or inactivate proviral DNA, or sabotage host factors (such as CCR5)	The possible intervention that would sanitize or make cell resistant is a one-voyage action.	Problems with delivery and off-target; safety; scalability	Before clinical trials and currently in few clinical and in CRISPR based CCR5 knockout studies.	[14]

2. Epigenetic Mechanisms of HIV-1 Latency

Proviral chromatin condensation through histone deacetylases and DNA methylation is an outcome of HIV -1 latency. The acetyltransferase, including p300, aids in the process of relaxing chromatin to allow transcriptional activity to take place, but repression complexes maintain a silenced state. Epigenetic and tissue-specific signatures render the maintenance of viral reservoirs and consequently, allows immune avoidance and treatment [15].

2.1 Histone modifications (acetylation, methylation)

Dynamic histone modifications of the viral provirus within the host chromatin are essential to modulate HIV-1 latency formation and its maintenance [16]. There are two major classes of modifications that predominate, acetylation which is generally activating and methylation which are generally repressive [17]. Once integrated, especially with resting CD4 + T cells, repression follows, by a decrease in activating marks and an increase in the repressive ones [18]. Histone acetyltransferases open chromatin; HDACs compact and silence it [19]. Histone deacetylase inhibitors (HDACis), in particular vorinostat and romidepsin, have thus been tested in clinical studies in terms of their ability to interrupt latency [20]. H3K9- and H3K27-histone methylation depict a more long-lasting silencing mechanism. H3K9me3 and H3K27me3 recruit repressors, locking HIV untranscribed. Bivalent histone marks indicate poised latency; drugs induce lasting chromatin changes. CRISPR-dCas9 enables specific HIV-1 LTR epigenetic editing with precision [21]. As shown in Figure 1, it has been established that epigenetic alterations of the histones and chromatin can either either sustain transcriptional silence or activate the viral genome, which is the way HIV-1 latency and viral gene expression are regulated.

2.2 DNA methylation and UHRF1 role

In the response to HIV-1 infection, DNA methylation directly influences both activation and silencing in the context of DNA methylation of HIV-1 latency [21]. The 5-methylation of CpG dinucleotides in the 5Lyn prevents the recruitment of transcription factors and in parallel contributes to a more durable silencing, the maintenance of latency. DNMT1 recruited to HIV LTR via UHRF1 binding hemi-methylated CpG. In a way, UHRF1 connects both DNA and histone-dependent silencing pathways. UHRF1 depletion or drug suppression alters the localization of DNMT1 in the latency-associated lymphocytes, resulting in the reactivation of latent provirus efficiently [22]. UHRF1 also binds H3K9 histone methyltransferase G9a to further anchor H3K9 methylation to the HIV-1 LTR region [23]. In recent CRISPR-based screens, it has been confirmed that the repression or activation of UHRF1/regulating DNA methylation machinery reactivates or blocks accrual of latency, respectively, in a robust manner. DDMP5 is a key CpG site silencing HIV LTR in memory T cells [24].

2.3 Chromatin remodeling complexes (SMC5/6, NuA4 HAT, SRCAP)

The way nucleosomes are arranged at the HIV-1 long terminal repeat (LTR) is critically important to control latency, and this nucleosome arrangement is regulated by various chromatin remodeling complexes. SMC5/6 complex normally implicated in chromosomal stabilization and DNA repair factors is revealed as a direct transcriptional repressor of HIV, especially unintegrated or early integrated proviruses [25]. SMC5/6 binds HIV DNA and through NSMCE2-mediated SUMOylation of chromatin condenses and limits transcription [26]. SMC5/6 inhibition restores HIV gene expression, delaying silenced reservoirs [27]. NuA4 acetylates HIV LTR, while FACT and SRCAP remodel nucleosomes blocking transcription. CRISPR, RNAi screens showed FACT/SRCAP modulation leads to viral reactivation by disrupting latency [28].

2.4 Key Epigenetic Regulators in HIV Latency

HIV-1 latency is epigenetically regulated by DNA methylation, histone modification, and chromatin remodelling [29]. Important molecular players are histone methyltransferases (EZH2, G9a), histone deacetylases (HDACs), DNA methyltransferases (DNMT1) associated with UHRF1 and chromatin remodeling machines such as SMC5/6 that transduce chromatin compaction via their SUMOylation [30]. Increased silencing of HIV-1 through non-coding RNAs is also reinforced by the recruitment of epigenetic repressors [31]. The combined action of these interactions produces an enduring and distributable repressive environment of chromatin at viral promoter. Understanding mechanisms enables designing therapies for latency reversal or permanent silencing [32].

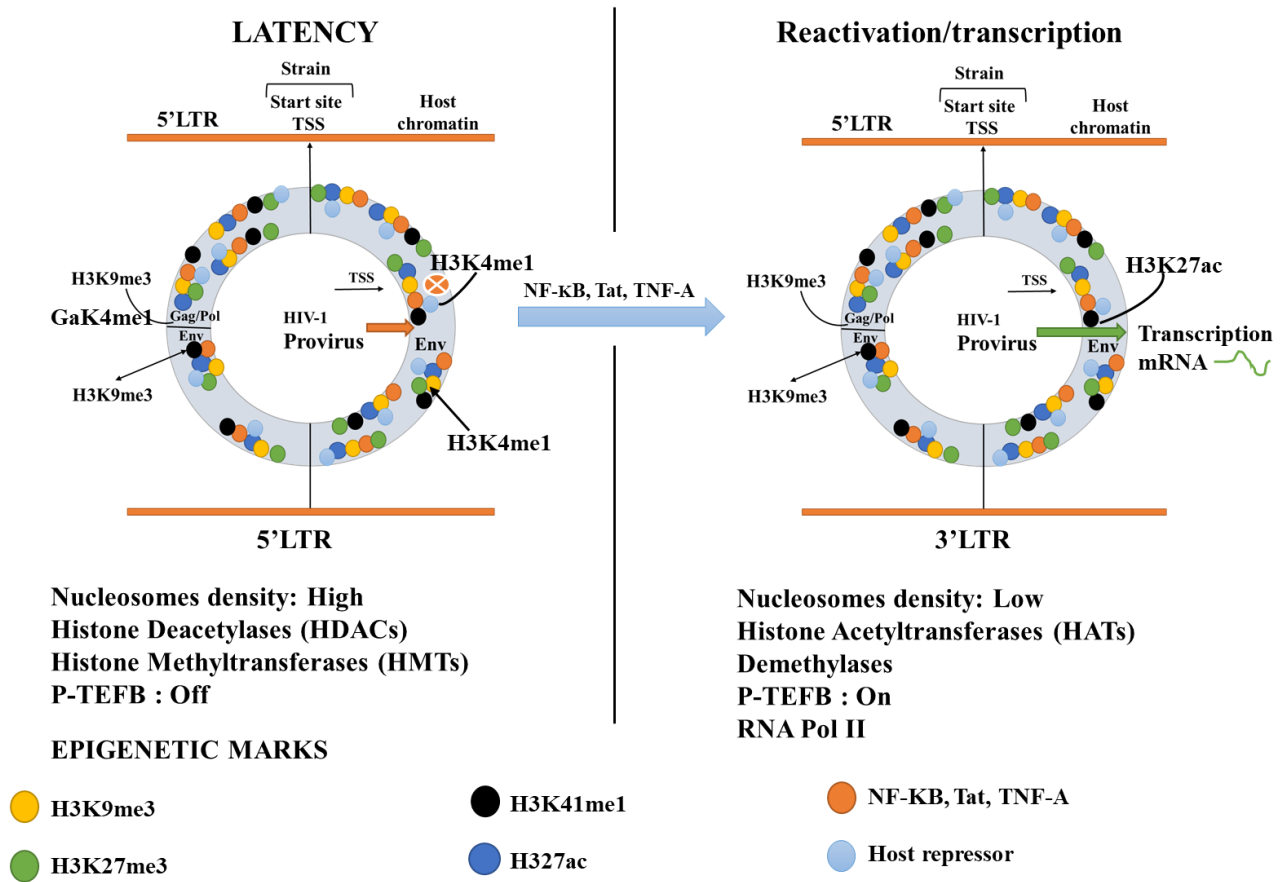


Figure 1: Multiplexed dCas9-epigenetic effector delivery schematic.

The figure shows how the changes in histone and chromatin can regulate HIV-1 proviral latency and transcriptional activity in which different epigenetic modifications can either impose transcriptional silence or activate the viral genome.

3. Tissue-Specific Features of HIV-1 Reservoirs

HIV-1 reservoirs are still evident in peripheral blood, lymphoid and gastrointestinal tissues and even in the central nervous system. These different anatomical compartments are retained in proviral latency via the distinct epigenetic repression mechanisms. Modern methods, which include controlled delivery systems and multiplexed genome editing methods, will alleviate this compartmental heterogeneity [33].

3.1 Blood vs. lymphoid tissue vs. gut-associated lymphoid tissue vs. CNS

Latent reservoirs promote the maintenance of HIV-1 and are distributed widely in the body with the distinct compartments varying in their involvement in the viral rebound and obstacles to eradication [34]. The most fully characterized reservoir is the peripheral blood reservoir, which includes predominantly resting memory CD4+ T cells in central (TCM), transitional (TTM) and effector memory (TEM) compartments; however, this is not the complete reservoir [35]. Lymph nodes and spleen as lymphoid tissues may serve as key bastions, TCM/TTM cell lodging with CXCR5 expression and somewhat immunologically protected due to sparse interstitial CD8+ activation. Recent data redefines prior beliefs in regards to T follicular helper (Tfh) cells as the main reservoir, especially among long-term people with long-standing ART suppression [36]. Instead the gastrointestinal tract seems to be the major reservoir, which is filled with most of the infected cell compartment, namely, CCR6+ and Th17-like memory CD4+ T cells capable of long-term viral preservation [37]. Genetic analyses indicate that clones of infected cells mediated by trafficking between the blood, gut and lymphoid as a mechanism by which genetic diversity is maintained and enhances local and inter-linked blood reservoirs. In myeloid cells of the central nervous system, HIV is also provided with a special reservoir, which is protected by the blood-brain barrier [38].

3.2 Cellular phenotypes and marker expression across compartments

The long-term viability of HIV-1 in various tissue reservoirs is typified by strong heterogeneity across different anatomical sites and within each site cellular repertoire. Lymph node reservoirs are GZMA-, CXCR5+, quiescent memory T cells [39]. The gut mucosa contains large amounts of CCR6+, strongly activated Th17/Th1/Th17 memory CD4+ T cells, and large amounts of latent viral DNA replicating in the presence of chronic inflammation and unique microenvironmental stimuli [40]. Microglia and astrocytes have a discrete marker profile in the central nervous system, and pathways of specialized regulation of viral persistence in the brain include chromatin topology and SUMOylation [41]. Single-cell multiomic profiling also demonstrates high inter- and intra-individual heterogeneity in immune checkpoints and activation states in the multiple immune compartments [42].

3.3 Implications for targeted therapy

There is a significant journey of therapeutic trajectories owing to anatomical and phenotypic heterogeneity of HIV-1 reservoirs. First, the spatial pattern of diseased cells requires any intervention, be it on gene editing, on latency reversal, or on immunotherapy, to target populations across tissues in order to repair a reservoir [43]. Ligand-modified nanoparticles target lymph nodes and CNS via CCR5, CX. Phenotypic matching also affects the therapeutic efficacy; central

memory in lymph nodes but more strongly activated Th17 cells in the gut could behave distinctly to latency-reversing agents [44]. Comprehensive reservoir disruption requires multiplexed and personalized interventions, using highly specific guide RNA libraries and epigenetic effectors of combinatorial combinations and sequences. Immune microenvironment, such as, cytokines (IL-7, TGF, and IL1), stromal cell support, and compartmentalization zones (e.g. CNS and germinal centers) cannot be ignored [45]. Single-cell and spatial profiling track reservoir monitoring and allow assessing clearance and adaptation in real time. Continued infected clone trafficking across tissues requires simultaneous multi-tissue targeting. A comprehensive approach to treating HIV includes multi-modal integrative approaches which are inclusive of delivery, profiling and gene editing, to enable successful cure [46].

Table 2 summarises the phenotypic or functional differences of HIV reservoir cells between the different tissues such as peripheral blood, lymphoid tissues, gut-associated lymphoid tissue, and the central nervous system. It underlines the prevalent forms of reservoir cells, their surface markers, the role of tissue-specific microenvironment in viral persistence, and latency.

Table 2:- Phenotypic and functional differences of reservoir cells by tissue.

Sr no.	Tissue	Dominant reservoir cell type	Key surface markers	Functional characteristics	Microenvironmental feature	Reference
1.	Peripheral blood	Resting Memory CD4+ T Cell (TCM& TTM &TEM)	KLRG1,PD-L1, TIGIT, BTLA, GZMA	Low frequency; latency provirus, high mobility, phenotypic diversities	circulating cytotoxines, immunomonitoring	[47]
2.	Lymphoid tissue (LNs)	TCM/TTM, Tfh CD4+ T Cells	CXCR5,PD-L1,TIGIT,BTLA	There is severe depletion of CD8+ T cells in the germinal centers further extending viral latency.	There is also promoting long-term survival, immunity concealment, by IL-7 & chemokines	[39]
3.	Gut-associated	Memory-CD4+ T cells	CCR6, IL-17+, IFN-	High activation,	Inflammatory signals, variable	[48]

	ed lymphoid	are differentiated in CCR6+ subset of CCR6+ toward Th17/Th1/Th17 phenotypes.	γ^+ , hiv dna high.	elevated reservoir density, microbial translocation influences persistence	Th17 restoration	
4.	CNS(brain)	Microglia, Astrocytes, Perivascular macrophages.	Brain-specific marker	Immunosuppressive environment; special latent control, hindered by drugs akses;	increasingly comprised by increasal corticosterone, CNS-Immuned microvasculature	[38]

4. CRISPR-dCas9 Systems for Epigenetic Modulation

CRISPR-dCas9 systems are used to specifically epigenetically regulate HIV-1 proviral genomes without causing DNA cleavage, which involves the expression of a catalytically dead Cas9 protein that is fused with a transcriptional effector, including KRAB to silence or p300 to activate. These locus-specific specific tools attack the long terminal repeat, as well as vital chromatin regulators, and this is what reverses latency or imposes long-lasting repression. Guide RNAs multiplexed further increase the therapeutic efficacy of heterogeneous viral reservoirs [49].

4.1 dCas9 fusion constructs (KRAB, p300, LSD1, DNMT3A)

CRISPR-dCas9 uses inactive Cas9 proteins (Cas9) fused with a variety of epigenetic regulators to directly control the transcription of HIV-1 without introducing any DNA cleavage. The KRAB domain, which functions as a potent transcriptional repressor, recruits the histone methyltransferase KAP1 and SETDB1 to silence transcription of the viral 5LTR by laying down repressive H3K9me3 chromatin marks thereby compacting chromatin and enhancing proviral latency [50]. Combinatory fusion constructs of dCas9-KRAB with DNA methyltransferases (e.g. DNMT3A) allow co-recruitment of both histone and DNA methylation machineries, leading to almost complete and stable silencing in humanised mouse models, reducing viral RNA >99% over

months [51]. On the other hand, dCas9 conjugated to acetyltransferase enzyme p300 induces the acetylation of H3K27, which opens chromatin, and reactivates provirus in a highly specific way-which supports shock and kill approaches [52]. A chromatin-remodeling complex containing the demethylase LSD1 is able to eliminate transcriptionally repressive H3K9me2 marks, restructuring chromatin in a way that facilitates transcriptional activation. Mutually, these modular dCas9-effector constructs offer a platform to finely tune HIV-1 transcription states to instead reactivate proviruses when desired or silence them when needed in a chronic silencing format, all based on therapeutic need [53].

4.2 Guide RNA design for epigenetic factors

Specialisation and optimisation Adaptive modifications to exert robust and specific binding places focus on gRNAs, which are central to dCas9 based genome editing systems; their design directly governs the efficiency and specificity of any given on target process [54]. In the analysis of HIV-1 latency, gRNAs are enriched on the conserved sequence of 5LTR that involve regulatory motifs, NF-HB- and Sp1 binding sites, TAR and transcriptional start sites, thus preventing viral evasion through the mutational activational process[55]. Chromatin accessibility particular to locus of interest is particularly essential: heavily repressed regions might require proximal nucleosome free regions to be targeted or use of combinatorial sets of Grna [56]. The robustness can be reinforced with multiplexed gRNA pools that can bind several provirus locations at the same time, or that bind both viral and host epigenetic elements. Via computations, guide sequences are streamlined to produce minimal off target activity, low secondary structure potential and on target high affinity [57]. Also, tissue specific delivery of gRNA libraries can be achieved by coupling of gRNA libraries with tissue specific promoters or conditional systems [58].

4.3 Multiplexed gRNA strategies and combinatorial targeting

The intricacy of the regulation of the HIV-1 latency warrants the multipronged intervention that would target various loci and epigenetic pathways in a multiple manner. The possibility of proviral reactivation or silencing as a result of delivery of several gRNAs that target different sites within LTR domains is substantially improved across the range of virus variants and integration sites obtained [59]. The use of combinatorial fusion proteins, such as paired dCas9-KRAB and dCas9-DNMT3A, or dCas9-p300, allows programmable combinatorial activation/repression: activating in some areas, and repressing others as a synergy [60]. The recent advances, combined split-dCas9 and orthogonal Cas effectors (e.g. SaCas9, Cas12a), allow conducting two or more edits at different loci in a single cell [61]. The combination of viral clearance agents (epigenetic-editing strategies) with latency-reversal agents and immune-modifying therapies has demonstrated increased viral clearance during the viral reactivation phase. Advance, multiplexed editing

counters the development of viral escape mutants, and considers tissue-specific chromatin structures that shape the maintenance of latency [62].

4.4 Off-target concerns and specificity improvement

Despite the high specificity associated with CRISPR-based epigenomic editing, this editing process is still vulnerable to off target effects, off-target binding and editing of host genomic or epigenomic sites, leading to inappropriate and dysregulated gene silencing or activation, with associated off target toxicology liabilities [63]. In response to this risk, researchers have proposed a number of countermeasures: high fidelity variants of Cas9 (HiFi Cas9 and eSpCas9) as well as truncated gRNAs have been reported to reduce nonspecific activity [64]. Broad screening tools, such as GUIDE seq, CIRCLE seq, and Digenome seq, can allow the identification of promiscuous guides and excision [65]. Epigenetic effectors, including DNMT3A and KRAB, can alter chromatin without causing DNA breaks and thus are not expected to pose as much genome instability as nuclease active Cas9. Exposure is not extensive since inducible or transient delivery systems control time [66]. Tissue specific or cell types promoters can keep editing activity within reservoirs, reducing the systemic risk of off-targets. The redundancy of gRNA and gRNA combinatorial expression can be multiplexed to further increases specificity on integrated provirus and minimize viral escape [67].

As **Figure 2** shows, it is planned to deliver multiplexed dCas9-epigenetic effectors into cells with the help of a delivery vector which is directed by a set of gRNAs. This system allows precise epigenetic alteration of individual areas of the genome to regulate the expression of genes as well as to regulate HIV-1 proviral activity.

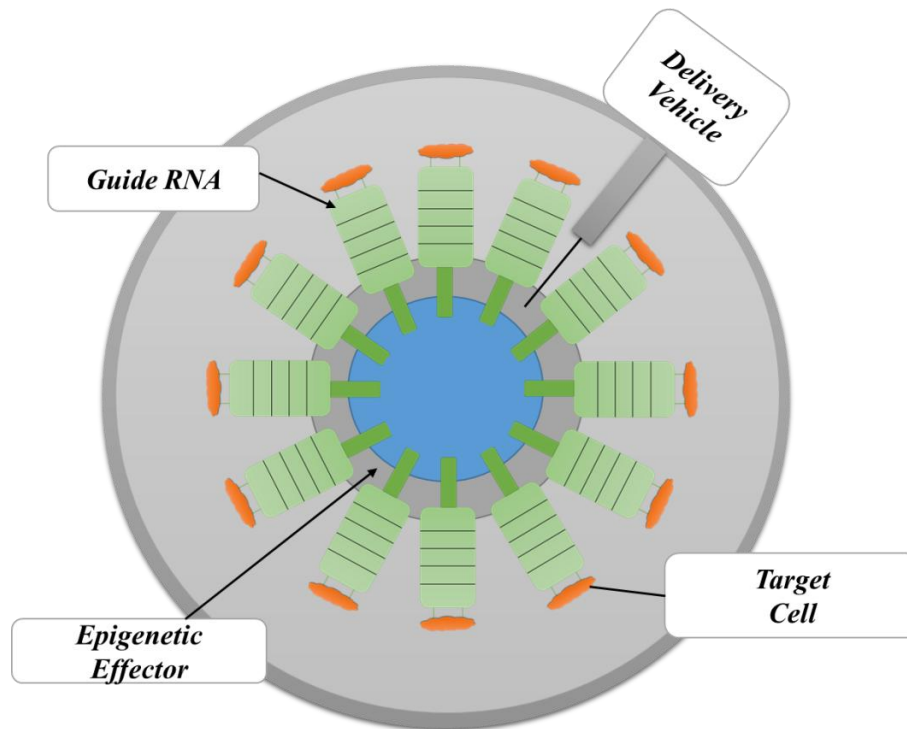


Figure 2: Multiplexed dCas9-epigenetic effector delivery schematic.

The scheme represents the layer of dCas9-epigenetic effector transmission into target cells via a particular delivery vehicle, which is controlled by a complex of gRNAs to attain specific epigenetic adjustment.

5. Novel Tissue-Targeted Delivery Platforms

It is possible to target HIV reservoirs using ligand-engineered lipid nanoparticles and exosomes to interact with chemokine receptors CXCR4 and CCR5. The use of viral vectors that have tissue specific promoters enables targeted delivery of therapeutic cargo to the systemic compartments including the blood stream, lymphoid organs, gastrointestinal tract and within the central nervous system. Through such platforms, multiplexed CRISPR-mediated genome editing is achieved, and hence overcomes the anatomical barriers to effective clearance of latent reservoirs [68].

5.1 Viral vectors with tissue-specific promoters

Recent innovations in molecular virology have placed lentiviral vectors and adeno-associated viruses (AAVs) in a favourable position as highly effective methods to shuttle gene-editing or other effective cargo to HIV-1 reservoirs efficiently. Besides, eliminating of the most conservative glycoprotein, pseudo typing with engineered tropism-enhanced envelope proteins also can

significantly increase viral entry to reservoir cells [69]. Off target effects are reduced with conditional gene expression and immune activation is mitigated. Hematopoietic stem cells and long lived reservoir cells transduction efficiencies are excellent in preclinical studies and toxicity is low [70]. Still there are challenges such as immune responses to viral proteins, risk of insertional mutagenesis and difficulty related to repeated administration methods based on the presence of host immunity. Despite these concerns, safer, more reliable and specific delivery of gene editing and epigenetic modulation reagents to the various HIV reservoirs is being honed through ongoing fusion of tissue specific promoters with enhanced vector engineering [71]. A summary of the key delivery platforms utilized in targeted therapeutic approaches, which are viral vectors, lipid nanoparticles, peptide-based delivery systems, and exosome-based delivery systems, is presented in **Table 3**. It juxtaposes their main characteristics, tissue targeting capabilities, strengths, and weaknesses concerning an effective delivery of gene and epigenetic editing.

5.2 Lipid nanoparticles and ligand-mediated targeting

Lipid nanoparticles (LNPs) are a diverse carrier platform capable of nucleic acid delivery (messenger RNA and CRISPR components) that may be used to eliminate the reservoirs of latent HIV. Conjugation of LNP surfaces with ligands or antibodies targeting HIV co-receptors (especially CCR5 and CXCR4) endows preferential LNP uptake in lymphoid and gut-associated lymphoid tissues, which has been shown to selectively increase delivery to key subsets of CD4+ T-cells and macrophages [72]. Rilpivirine-loaded LNPs further decorated with CCR5-ligand have significantly been found to enhance their biodistribution and retention in lymph nodes as well as the central nervous system in animals. LNP formulations that cross the blood-brain barrier, e.g., with focused ultrasound or receptor-mediated transcytosis, have the potential to outperform targeting of CNS reservoirs, a traditionally challenging drug delivery target [73]. These lipids have been optimized not only enable a tightly controlled drug release but also exhibit low immunogenicity and the benefit of high-scale production [74].

5.3 Peptide-based and exosome-based delivery

Carriers based on peptides combine cell-penetrating peptides (CPPs) and cell-targeting ligands to generate cell selective delivery of therapeutic payloads- nucleic acids, proteins, or other biomolecules intracellularly. Peptides facilitate translocation of the composite across biological barriers (e.g. mucosa, blood-brain barrier) by conjugating to nanoparticles or fusing directly to therapeutic proteins, and increasing delivery to target T cells and macrophages [75]. These carrier systems are low toxic, can be adjusted to tissue tropism, and have cargo loading potential. Naturally secreted extracellular vesicles, also known as exosomes, have attracted interest as an endogenous, biocompatible delivery vehicle with systemic distribution, efficient targeting,

including to the central nervous system and mucosal tissues [76]. The engineered exosomes loaded with anti-HIV gene modulators or immunoregulatory proteins have shown to have antiviral effect and immune modulation with low immunogenicity. However, poor reproducibility along with scale up manufacturing, cargo loading and complete characterization in the exosome based delivery have hindered clinical translation [77]. These injection strategies, as represented by peptides and exosomes can therefore provide complementary, versatile delivery methods, particularly in therapies needing repetitive dosing or the capacity to overcome insurmountable biological barriers [78].

5.4 Comparative efficacy and safety

A comparison of the new gene delivery platforms shows contrasting yet complementary profiles to emerge. Long term expression of genes is stable and depicts solid transductivity in target cells due to use of viral vectors, which have insertional mutagenesis or varied immunogenicity issues [79]. Lipid nanoparticles provide tunable transient delivery with low immunogenicity and enhanced using tissue distributions, such as privileged sites, including the central nervous system, but exhibit inhomogenous biodistribution, which must be optimized to maximize payload stability [80]. Peptide carriers are highly specific, uptake, and are tolerated well, however, short half life due to renal clearance after a single dose performance. Exosomes with outstanding biocompatibility and a natural ability to target cells need to be developed further to overcome manufacturing and regulatory issues [81]. The combination of these platforms by using the viral vectors to deliver long-term editing, LNPs and peptides to real time modulation, and exosomes to both systemic and CNS-direct applications represents the most promising method to eliminate HIV-1 reservoirs in a safe and efficacious manner [82].

Table 3. Delivery platforms: features, tissue tropism, advantages, limitations.

Sr no.	Delivery platform	Key feature	Tissue tropism/ targeting	Advantages	Limitations	References
1.	Viral Vectors with Tissue-Specific Promoters	Lentivirus, AAV, adenovirus; engineered promoters (Tat/Rev inducible)	CD4+ T cells, hematopoietic stem cells; viral tropism pseudotypin	Stable, long-term expression; high transduction efficiency; regulated expression	Risk of insertional mutagenesis; potential immune responses; limited re-	[83]

			g enhances specificity	reduces off-targets	administratio n	
2.	Lipid Nanoparticles (LNPs) with Ligand-Mediated Targeting	Lipid-based nanoparticles decorated with ligands (CCR5, CXCR4) or antibodies	Lymphoid tissues, gut-associated lymphoid tissue, CNS (with BBB penetration)	Biocompatible; low immunogenicity; tunable targeting; efficient mRNA and gene editing delivery	Variable tissue distribution; challenges in uniform delivery in fibrotic tissues	[83]
3.	Peptide-Based Delivery	Cell-penetrating peptides, targeting ligands attached to nanoparticles or drugs	Lymphoid cells, mucosal tissues, CNS (improved penetration)	Enhances cell entry and delivery; adaptable targeting; low toxicity	Rapid clearance; moderate targeting specificity; potential proteolytic degradation	[75]
4.	Exosome-Based Delivery	Natural vesicles carrying nucleic acids/proteins; engineered cargo loading	Broad systemic distribution including CNS and mucosal tissues	Excellent biocompatibility; low immunogenicity; natural targeting and barrier crossing	Challenges in large-scale production and standardization; cargo loading variability	[84]

6. Proposed Multiplexed Epigenetic Targeting Strategy

The engineered multiplexed CRISPR -dCas9 effector construct can selectively identify HIV proviral sequences in a tissue-specific manner. The CRISPR -dCas9 complexes are delivered in the blood, lymphoid tissues, gastrointestinal tract, and central nervous system reservoirs using ligand-

functionalized nanoparticles. Such delivery approaches are synergistic to latency-reversing agents and universal neutralization of antibodies to mediate the clearance of viral reservoirs [85].

6.1 Selection of epigenetic targets (ING3, SLTM, SMC5/6, UHRF1)

Intervention during HIV-1 latency requires targeting multiple epigenetic modulators, since repression is multilayered and provides the tolerated persistence of proviruses. ING3, part of NuA4 complex, mediates chromatin remodeling for proviral reactivation. Chromatin-associated transcription modulator SLTM has been shown through independent CRISPR screens to mediate a strong inhibitory role by outcompeting nucleosomes on the HIV-1 promoter and maintaining latency [86]. The SMC5/6 complex epigenetically silences HIV-1 by SUMOylation, inhibiting proviral transcription and latency establishment. UHRF1 connects DNA methylation and histone modification, reinforcing proviral silencing and regulating viral latency reactivation. Multiplexed targeting of acetylation, methylation, and chromatin structure tackles HIV's layered epigenetic barriers. This combined therapy recognizes latency of virus as a complex phenomenon with multiple sites requiring concomitant multifocal interference to create relevant reactivation or significant silencing [87]. The workflow of the preclinical assessment of the multiplexed epigenetic editing, as presented in **Figure 3**, includes the consecutive steps of rational design and in-vitro verification of the technology, followed by in-vivo experiments. These schematic underlines the systematic method of evaluation of the safety and therapeutic potential of the strategies of epigenetic editing.

6.2 Tissue-specific promoter and vector design

Anatomical and cellular specificity of HIV reservoirs is the key to achieve therapeutic index and avoid off-target effects by targeting gene-editing reagents to the HIV reservoirs. Reservoir-targeted promoters (e.g., CXCR5, CCR6) enable specific therapeutic delivery. Tropism is further narrowed after envelope pseudotyping to CD4+ T cell subsets or tissue-resident macrophages [88]. Polycistronic vectors are used to deliver multiple guide RNAs towards ING3, SLTM, the SMC5/6 components and UHRF1 simultaneously, with architectures that reduce promoter interference and maintain high levels of expression. Recent developments such as synthetic regulatory systems reacting to small molecules or endogenous viral proteins to give inducible control over the gene-editing activity to make it safer and flexible [89]. The tissue-specific constructs result in highly efficient transduction, long-term gene modification, and limited off-target expression as shown by transfer into preclinical humanized mouse models, providing a basis of scalable clinical delivery options [90].

6.3 Preclinical model considerations (humanized mice, organoids)

Humanized mouse models, especially BLT mouse and NSG mouse, will be invaluable tools in vivo to evaluate multiplexed epigenetics editing strategies. These animals recapitulate human architecture of immune system, vectors distributions in reservoirs, and viral latency dynamics at high levels, thus enabling longitudinal follow up of reservoir size, viral rebound, and immune response after vector delivery [91]. Simultaneously, human lymphoid, gut, and CNS organoid cultures can be used to provide scalable and physiologically relevant models of tissue-specific responses to epigenetic perturbation and the efficacy of delivery, cytotoxicity, and off-target transcriptome changes. Spatial transcriptomics in conjunction with single-cell RNA sequencing can be used to characterize and optimize multiplexed epigenetic editing protocols before implementation in the clinical setting [92].

6.4 Experimental validation pipeline

A stringent multiplexed epigenetic targeting validation starts with in vitro CRISPR screening targeting ING3, SLTM, SMC5/6, and UHRF1. Quantitative PCR, RNA sequencing and Chr-IP assays are employed to quantify alterations in viral transcription, chromatin remodeling and histone/DNA model patterns [93]. Candidate hits are progressed to organoid platforms (lymphoid, gut and CNS reservoirs) to evaluate tissue specific efficacy, cytotoxicity, and off target gene profile at single cell resolution. Humanized mouse in vivo assays test gene marking, reservoir impact, rebound kinetics, immunity. Reporter systems that can be selectively advantageous to drug selected cells can be used to enrich the potency measurement of gene-modified cells [94]. Longitudinal monitoring includes viral outgrowth, sequencing, and safety assessments. The feedback between these phases enables an iterative process of optimization of multiplexed epigenetic editing approaches to the goal of long-lasting and tissue-penetrant cure approaches against HIV [95].

WORKFLOW SCHEMATIC FOR PRECLINICAL EVALUATION OF MULTIPLEXED EPIGENETIC EDITING

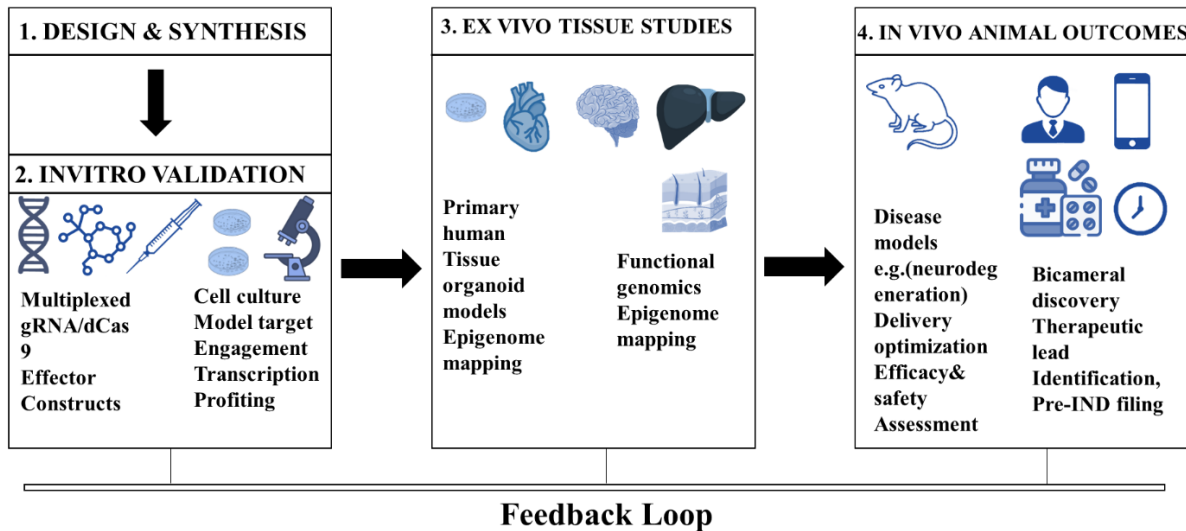


Figure 3: Workflow schematic for preclinical evaluation of multiplexed epigenetic editing.

The flowchart that shows the preclinical examination of multiplexed epigenetic editing approaches. The figure indicates the important steps involved in rational design and in-vitro testing to further in-vivo research to determine safety and therapeutic efficacy.

7. Potential Clinical Translation and Challenges

The safety is assessed by using viral outgrowth assays, sequencing, and GUIDE-seq. The major issues are the delivery efficiency, off-target effects, and immune responses. The ethical governance and scalability remains a limitation to the practice of HIV cure trials [96].

7.1 Safety and immunogenicity considerations

There are still safety considerations that are a limiting factor to clinical translation of gene-editing strategies intended to target HIV-1 reservoirs. EBT-101 CRISPR HIV trial shows good safety, mild transient effects. The absence of off target DNA damage during the monitoring process also proves the accuracy of modern CRISPR platforms of delivery [97]. However, immunological awareness, and adverse inflammatory reactions to the CRISPR-related proteins or viral treatment products discussion remain highly noteworthy and potentially require additional immunomodulatory measures [98]. Other safety concerns are that integration of the vector may cause insertional mutagenesis, even in vectors designed to have minimal effects, and long-term expression of the

gene editors/epigenetic modifiers may be cytotoxic. Long-term effects on immune cells post-reservoir editing need careful evaluation [99].

7.2 Regulatory landscape for gene editing therapies

Current regulatory agency governance on gene-editing treatment directed towards HIV-1 is still evolving at a level pace with technological advancement. Regulatory agencies, like the FDA and the EMA, currently require rigorous preclinical dossiers that provide clear evidence of therapeutic specificity, efficacy and safety, with a great emphasis on off-target activity, biodistribution and immunogenicity. However, trial designs have to incorporate precise endpoints such as viral reservoir measurement and the analytical pause to assess the potential impact of the intervention regarding functional addressing [100]. The standards in manufacturing also find themselves in close supervision whereby cGMP compliance is required to maintain reproducibility, potency, purity and absence of replication-competent contaminants [101].

7.3 Scalability and manufacturing hurdles

The transition of gene editing therapy development to clinical practice has the insurmountable challenges linked to scale up and manufacturing. Production, lentiviral and adeno associated or lipid nanoparticle, of viral vectors is a complex, capital intensive, and in many contexts prohibitively expensive process [102]. High quality control standards should ensure that each batch meets the necessary specifications of the required vector titer, purity and homogeneity, effective regulatory standards. Moreover, autologous ex vivo gene editing of patient cells, a logistical complexity that entails patient specific cell acquisition, editing, expansion, and reinvention, all of which require aseptic processes [103]. As personalized approaches have been gaining increasing interest, the field needs to establish new production lines that are less expensive and high in outputs [104].

7.4 Ethical considerations

Ethical aspects of gene editing drug treatments to pursue a possible HIV-1 cure are central. Primary concern is the protection of valid informed consent, frank disclosure of possible risks, potential benefits, and uncertainty, especially in early-phase investigations where analytic treatment interruptions are used [105]. Germline editing prohibition is a widely accepted ethical and regulatory standard reinforced by controversies. Equitable access to transformative therapies must prevent worsening health disparities. Constant ethical supervision is irreplaceable to trace unexpected long run impact, control expectations of patients, and investigate the communities in the conversation on technologies of gene-editing [106].

8. Future Perspectives and Combination Approaches

The future directions seek to combine CRISPR dCas9 mediated epigenetic editing with latency reversing agents and comprehensive neutralization of antibodies to boost clearance of the HIV-1 reservoir, which new CRISPR screening studies have discovered novel latency regulators support this. Individualized multiplexed therapies, using single cell omics data and AI optimized guide RNA libraries, can be used to target the heterogeneity of viral reservoirs, with base and prime editing techniques, which are safe in human trials already completed in 2025, potentially targeting proviral sequences with greater specificity without causing double-strand break repair. Together with ligand targeted nanoparticles and Chimeric Antigen Receptor T cell synergies, these approaches possess the potential of scalable, tissue-specific curative intervention of HIV and other persistent viruses like hepatitis b virus. Clinical trials are being continued on the need to address the dilemma of delivery efficiency and off target effect to successfully translate into therapeutic practice.

9. Conclusion

The more recent developments in HIV-1 biology outline a paradigm change to a functional cure, being the precision with which CRISPR dCas9 mediated epigenetic editing of proviral chromatin can be used using multiplexed effector proteins KRAB, p300, LSD1 and DNMT3A in order to induce targeted sustained latency reversal. The heterogeneity of tissue is alleviated through the use of multiplexed guide RNAs and optimization of delivery vectors to be circulated to the circulatory system, the lymphoid organs, the gastrointestinal tract and central nervous system reservoirs. Immunosenescence is enhanced by the concomitant administration of latency-reversal agents and broad-spectrum neutralizing antibodies. The technology of base and prime editing allows increasing accuracy and safety, allowing proviral excision and receptor knockout without breaking the two strands of DNA. Individualized therapy and operational understanding of reservoir dynamics Single-cell and spatial omics technologies enable personalized therapy and enhance understanding of the dynamics of reservoirs. Mutually these innovations have the potential to bring out flexible patient centered and minimally invasive interventions that may not only be limited to HIV but other chronic viral diseases. While the delivery efficiency, safety profiles, as well as scalability are still problematic, the strategy provides unprecedented long-term remission opportunities.

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