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Gene Therapy for HIV and Chronic Infections

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Preface

This book volume deals with gene therapy and gene transfer approaches to prevent or treat chronic virus infections. The focus of many chapters is on the Big Three: human immunodeficiency virus (HIV)-1, hepatitis B virus (HBV), and hepatitis C virus (HCV). HIV continues to be a major global public health concern, having claimed more than 25 million lives due to AIDS over the past three decades. In 2013, there were approximately 35 million people living with HIV. Sub-Saharan Africa is the most affected region, with nearly one carrier in every 20 adults. According to the World Health Organization (WHO), two billion persons (approximately 5 % of the world's population) have been infected with HBV, of which more than 350 million have a chronic HBV infection. It has been estimated that up to 3 % of the world's population is infected with HCV, of which 170 million people are chronically infected, and an additional three to four million people are newly infected each year. Long-term chronic infection with one or both of these hepatitis viruses is the most common cause of liver fibrosis and cirrhosis, leading to liver failure and hepatocellular carcinoma.

There is no cure for HIV infection, but effective treatment with antiretroviral drugs has dramatically improved the life span and quality of life of infected individuals. A similar trend can already be recognized for HBV and HCV: the development of multiple (directly acting) antiviral drugs and plans to control or even cure the infection. However, approaches that help prevent infection or which provide long-lasting treatment (such as a cure) remain important clinical goals. Gene therapy can theoretically provide a means to obtain durable virus suppression in the absence of medication.

Gene therapy applications for clearance of chronic virus infections have been discussed since the early 1990s. In case of persisting infections with CMV, a herpes virus, vaccination such as with a DNA vaccine has shown to lower viremia, which may be beneficial to reduce CMV-mediated disease prior to immunosuppression of transplant recipients. Whereas a true cure seems difficult to achieve for HIV due to its intrinsic property to deposit its genome into that of the host, such attempts may be within reach for HCV where spontaneous viral clearance occurs in a small percentage of the infected individuals. But even the more difficult HIV scenario has

recently been spurred by the first (and thus far only) functional cure reported for the "Berlin HIV patient." In this unique case, a bone marrow transplant was performed because of leukemia and the selection of a CCR5-negative donor, whose cells lack expression of the major coreceptor used by HIV for cell entry, has resulted in the apparent disappearance of the virus from blood. It is clear that such a transplant remains a high-risk procedure, but it provides an important proof of principle that could hopefully be mimicked by gene therapy approaches.

A diversity of anti-HIV gene therapies have been proposed and several of these strategies also hold potential for anti-HBV or anti-HCV approaches. In Chap. 6 by Cornu et al., the multitude of editing approaches against the CCR5 target suggests a very positive pipeline of options for anti-HIV gene therapy. There are alternative ways to reach the same end point by, for example, silencing of CCR5 expression posttranscriptionally or ablating its expression by targeting the CCR5 genomic locus for excision or epigenetic shutdown. Herrera Carrillo and Berkhout (Chap. 4) discuss RNA interference (RNAi)-mediated gene silencing approaches against HIV, thereby targeting either host cell cofactors or the viral RNA genome. The particular focus is on combinatorial RNAi strategies to prevent viral escape. Takahashi et al. (Chap. 11) describe how siRNAs targeted to HIV targets as well as to host genes can be delivered by cell-internalizing aptamers, a strategy that represents a novel cell-specific delivery method, allowing systemic application of naked RNA to infected patients. Scarborough and Gatignol (Chap. 5) describe ribozymes, catalytic RNA molecules and conjugates thereof with other inhibitory moieties. Ribozymes can be designed in a sequence-specific manner to cleave other RNA molecules, e.g., the HIV-1 RNA genome. Egerer et al. (Chap. 10) describe the conversion of known antiviral peptide drugs that block the membrane fusion process into transgene constructs that express potent antiviral proteins, either on the cell surface or in a secreted form. Blazquez and Fortes (Chap. 3) describe the unique antiviral properties of the modified U1 small nuclear ribonucleoprotein (snRNP), which is particularly suited for applications against HIV and HBV whose viral genomes express mRNAs that must be polyadenylated.

Verstegen et al. (Chap. 1) focus on the history of anti-HCV therapeutics, from interferon to directly acting antiviral drugs and the prospects for gene therapeutic strategies. The prospects of a gene therapy for HBV are described by Bloom et al. (Chap. 2). Wu et al. (Chap. 7) deals with CMV that infects up to 60–95 % of adults. Although primary infections are in general benign, CMV establishes a latent infection and has been linked to fatal disease in immunocompromised patients and to chronic disease such as inflammatory diseases, cancer, and heart diseases in individuals with an intact immune system.

Prophylactic vaccines are not yet available for HIV, HCV, or CMV and correlates of protection remain ill-defined. For HIV it is assumed that virus-neutralizing antibodies may prevent virus acquisition. Nevertheless, as the envelope of HIV-1, the target for neutralizing antibodies is extremely variable, immunogens that elicit those that are broadly cross-reactive remain elusive. Schnepp and Johnson (Chap. 8) describe an alternative genetic vaccination approach by intramuscular gene transfer

of adeno-associated virus vectors encoding an HIV-1-specific broadly neutralizing antibody for prevention of infection.

More recently a lot of attention has been placed on the role and importance of noncoding RNAs (ncRNAs) in viral infections, especially with regard to the maintenance of cellular transcriptional regulation. Saayman et al. (Chap. 9) discuss the role of viral and cellular ncRNAs with respect to strategies aimed at affecting HIV latency.

Most early results have been described in appropriate in vitro models, but some studies have progressed towards preclinical animal models and a few antiviral gene therapies have progressed towards clinical trials. This book provides a thorough overview of this rapidly progressing field.

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Hildegund C.J. Ertl is a Professor at the Wistar Institute where she serves as the Director of the Wistar Vaccine Center. She is an Adjunct Professor of Pathology at the University of Pennsylvania and an Adjunct Professor of Pediatrics at the Children's Hospital of Philadelphia. She obtained her M.D. in 1977 from the George-August University in Göttingen, Germany, and did her postdoctoral studies at the John Curtin School in Canberra, Australia. She was an Assistant Professor at the Dana-Farber Cancer Institute in Boston, MA, and then moved as an Associate Professor to the Wistar Institute, Philadelphia, PA. Her research focuses on viral immunology with emphasis on development of viral vector vaccines and immunological consequences of virus-mediated gene transfer. She has over 220 publications and serves as an Editor on several journals.

Marc S. Weinberg is a molecular biologist and is currently Associate Professor at the University of the Witwatersrand and a Visiting Professor at the Scripps Research Institute, USA. His research focuses on the therapeutic functions of ribonucleic acid (RNA). He graduated with a Ph.D. from the University of the Witwatersrand, South Africa, in 2002 and was a postdoctoral fellow at City of Hope Medical Center, CA, USA, where he studied fast-cleaving hammerhead ribozymes and RNA-based transcriptional modulators against HIV. Thereafter, his work turned to small RNAs that attenuate viral gene function, and he was one of the first to show that small single-stranded RNAs can switch genes off directly using components of a nuclear RNA interference pathway. Dr. Weinberg has made important contributions to the development of novel RNA structures that are processed into safe and useful antiviral inhibitors, specifically against HIV and HBV. Lately, his work focuses on new ways to modulate noncoding RNA genes using cell-selected aptamers, CRISPR/Cas9 modulators, and mobilization lentiviral vectors as delivery vehicles, with specific emphasis on how these approaches can be used against latent HIV infection. Dr. Weinberg has over 50 publications and holds six patents for RNA applications in biopharma and agriculture. He has supervised 20 graduate students to completion. He is on the editorial board of several respected journals.

Gene Therapies for Hepatitis C Virus

Monique M.A. Verstegen, Qiuwei Pan, and Luc J.W. van der Laan

Abstract Hepatitis C virus (HCV) is a leading cause of chronic hepatitis and infects approximately three to four million people per year, about 170 million infected people in total, making it one of the major global health problems. In a minority of cases HCV is cleared spontaneously, but in most of the infected individuals infection progresses to a chronic state associated with high risk to develop liver cirrhosis, hepatocellular cancer, or liver failure. The treatment of HCV infection has evolved over the years. Interferon (IFN)-α in combination with ribavirin has been used for decades as standard therapy. More recently, a new standard-of-care treatment has been approved based on a triple combination with either HCV protease inhibitor telaprevir or boceprevir. In addition, various options for all-oral, IFN-free regimens are currently being evaluated. Despite substantial improvement of sustained virological response rates, some intrinsic limitations of these new direct-acting antivirals, including serious side effects, the risk of resistance development and high cost, urge the development of alternative or additional therapeutic strategies. Gene therapy represents a feasible alternative treatment. Small RNA technology, including RNA interference (RNAi) techniques and antisense approaches, is one of the potentially promising ways to investigate viral and host cell factors that are involved in HCV infection and replication. With this, newly developed gene therapy regimens will be provided to treat HCV. In this chapter, a comprehensive overview guides you through the current developments and applications of RNAi and microRNA-based gene therapy strategies in HCV treatment.

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Abbreviations

A1AT	Alpha-1 anti-trypsin
AAV	Adeno-associated viruses
DAA	Directly acting antiviral agents
HCC	Hepatocellular cancer
HCV	Hepatitis C virus
IFN-α	Interferon α
IRES	Internal ribosome entry site
LDL-R	Low-density lipoprotein receptor
LNA	Locked nucleic acid
miRNA	micro RNA
NS	Non-structural
ORF	Open reading frame
RdRp	RNA-dependent RNA polymerase
RISC	RNA induced silencing complex
RNAi	RNA interference
scAAV	Self-complementary adeno-associated viruses
shRNA	Short hairpin RNA
siRNA	Short interference RNA
SVR	Sustained virological response
UTR	Untranslated region

Introduction

Hepatitis C virus (HCV) is a hepatotropic member of the *Flaviviridae* that causes acute and chronic hepatitis. With more than three million new infections per year, hepatitis C virus (HCV) is a major health issue. Worldwide, an estimated 170 million people are chronically infected with the virus and over 350,000 patients die every year from HCV-related liver disease. HCV is contagious and most-likely spreads via exposure to infectious blood. As such, preventive measures against HCV, including development of vaccines and neutralizing antibodies are a priority among researchers in the field [1–3]. Moreover, 55–75 % of the infected individuals fail to clear the virus [4, 5] which leads to a chronic infection state that in turn may lead to development of liver cirrhosis, hepatocellular cancer (HCC), and/or liver failure [6]. The progression of chronic infection is not linear in time, possibly due to the many cofactors involved in the development of fibrosis, cirrhosis and HCC. It has been demonstrated that patients that achieve sustained virological response (SVR), which is the closets one can get to a cure and the goal for HCV treatment, have a clear prognostic advantage over those who do not [7, 8].

The majority of the infected persons do not show any symptoms and the diagnosis of acute infection is therefore often missed. It is needless to say that an early

diagnosis can prevent many problems with regard to treatment of the patient as well as keeping transmission confined. Although efforts are made to develop and produce vaccines and neutralizing antibodies against HCV [1, 3], there is currently no effective vaccine or antibody available for the prevention of infection, which is partly due to the genetic heterogeneity of the virus. As there are seven genotypes of the virus [9] that may all respond differently to treatment, careful screening is of utmost importance to establish the best treatment regimen. The current standard treatment of HCV, a combination of PEG-interferon and ribavirin or the recently available direct-acting antiviral agents, does not necessarily eliminate the HCV. However, it can suppress the virus to undetectable levels for an extended period of time, i.e., 6 months after treatment. Due to the accumulation of basic knowledge on viral and host cellular factors involved in the HCV life cycle, and recent technical advances in viral cell culture techniques, new options for the development of novel therapeutic strategies have become apparent. Small-molecule inhibitors, immune modulators, antibodies, antisense RNA and other agents are studied in (pre)clinical trials [10–15]. Among the new approaches to tackle HCV efficiently, RNA interference (RNAi) is one of the promising methods for the development of antiviral therapies. RNAi acts by the sequence-specific inhibition of gene expression at the posttranscriptional level [16] and has led to several important discoveries on HCV biology, providing new therapeutic targets for treatment and infection prevention.

Molecular Biology of HCV

The HCV genome is a positive-sense, single-stranded RNA genome of the *Flaviviridae* family and is approximately 9,600 nucleotides (nt) in length. It contains a 5′ untranslated region (5′UTR) of 341 nt, an open reading frame (ORF) that encodes a polyprotein precursor of approximately 3,000 amino acids, and a 3′ untranslated region (3′UTR) of about 27 nt [17]. The 5′UTR contains the internal ribosome entry site (IRES) that mediates the cap-independent translation initiation of the viral polyprotein [18]. The polyprotein precursor is processed by cellular and viral proteases to yield ten mature proteins: C, E1, E2, p7, NS2, NS3, NS4A, NS4B, NS5A, and NS5B [19, 20] of which six are non-structural (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) [21–24]. The structural region encodes the core protein (C) [25], two envelope proteins (E1 and E2) [26], and the small integral membrane protein (p7) [27, 28] that are cleaved into individual proteins by host signal peptidases [29, 30]. The non-structural proteins (NS) are processed by two viral proteases, NS2 and NS3/4A that are involved in the intracellular processes of the viral life cycle [31, 32]. The positive-sense RNA genome provides a template for the synthesis of a negative-stranded RNA replication intermediate by the viral RNA-depended RNA polymerase, NS5B. This newly synthesized negative strand acts as a template for de novo synthesis of positive-stranded RNAs that are packaged in newly formed HCV virions during viral assembly. The viral structure and life cycle are summarized in Fig. 1.

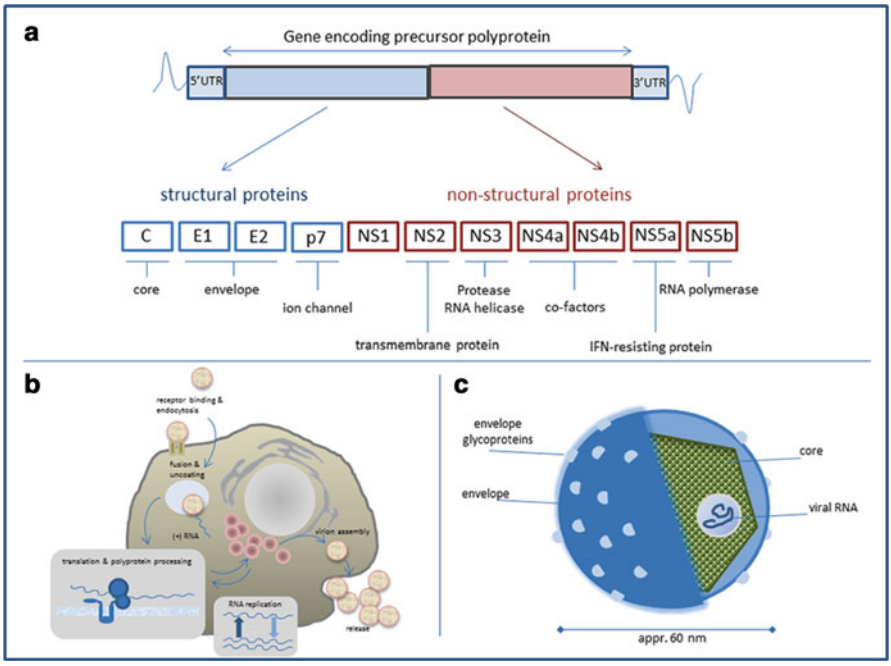

Fig. 1 Viral structure and life cycle. (**a**) The HCV genome. HCV is a single-stranded RNA virus (~9,600 nt) that consists of a single open reading frame between the 3′ and 5′UTR, the latter including the IRES sequence that mediates the translation of the polyprotein. The structural region contains the core protein (c), two envelope proteins (E1 and E2) and the small integral membrane protein (p7) that are cleaved into individual proteins by host signal peptidases. The non-structural proteins (NS) are processed by two viral proteases, NS2 and NS3/4A. (**b**) Virus cell entry and replication in the host cell and (**c**) schematic representation of a HCV virion

HCV isolates, collected from over the world, show substantial nucleotide sequence variability throughout the viral genome, leading to the classification of at least seven major genotypes [33], numerous subtypes and even within a given patient, a myriad of different variants, the so-called quasispecies [34] (Table 1). Sequence variability is distributed equally throughout the viral genome, except for the highly conserved 5′UTR, and the highly similar amino acid sequence of the core protein [35]. The genomic sequences of HCV isolates can vary by as much as 35 % [36].

The similarity of sequences encoding the viral envelope is low (hypervariable) between the subgroups, making it difficult to develop broadly active immunoglobulins or vaccines against it [37–39]. Patients infected with HCV mount a humoral immune response to epitopes of this hypervariable region. However, sequential changes in the consensus sequence during infection result in the generation of variants (quasispecies) that are not recognized by preexisting antibodies. This might represent a mechanism by which HCV evades host immune surveillance and establishes a persistent infection.

Table 1 HCV genotype classification

Genotype	Classification	Distribution	% Nucleotide similarity
Major	1	World wide	60–70
	2	World wide	
	3	World wide	
	4	Middle East, Africa	
	5	Southeast Asia	
	6	Canada	
	7		
Subtype	1a, 1b, 1c, 1g		75–85
	2a, 2b, 2c, 2i, 2k		
	3a, 3b, 3i, 3k		
	4a, 4b, 4c, 4d, 4f, 4g, 4k, 4l, 4m, 4n, 4o, 4p, 4p, 4q, 4r, 4t		
	5a		
	6a, 6b, 6c, 6d, 63, 6f, 6g, 6h, 6i, 6j, 6k, 6m, 6n, 6o, 6p, 6q, 6r, 6s, 6s, 6t, 6u		
	7a		
Quasispecies	numerous, continuously changing		90–98

HCV is classified on the basis of similarity of nucleotide sequence into major genetic groups designated genotypes. HCV genotypes are numbered in the order of discovery. The closely related strains within the major types are designated subtypes, also in the order of their discovery. The complex of genetic variants found within an individual isolate is termed quasispecies and results from the accumulation of mutation during viral replication in the host. Adapted from Nakano T, et al., 2012, Simmonds P, et al., 2005 and Zein & Nizar N, 2000 [9, 33, 218]

Viral Life Cycle

Host Cell Entry

The way a HCV virion enters its target cell, the hepatocyte, has been elucidated to a large extent. It is known that cell entry is a multistep process where the readily available factors that are present on the basolateral surface of hepatocytes are first used, followed by the tight junction components that reside in physically difficult to reach cell surface locations.

HCV entry contributes to tissue tropism and species specificity of the virus. Only human and chimpanzee hepatocytes can be infected by it. The viral factors that are involved in binding and entering a target cell are the envelope glycoproteins E1 and E2. Cell culture systems, such as the HCV pseudotyped particle (HCVpp) [40] system, have been developed to study viral binding and entry. In the HCVpp system, infectious pseudoparticles have been assembled by displaying unmodified and functional HCV E1 and E2 glycoproteins onto retroviral and lentiviral core particles.

This study showed that E1 and E2 are involved in separate steps of viral entry and that both proteins are essential.

HCV cell entry requires at least four host-cellular factors, namely, CD81 [41], scavenger receptor class B type 1 (SR-BI) and the tight junction proteins claudin-1 (CLDN1) and occludin (OCLN). CD81 is a transmembrane protein of the tetraspanin family. The extracellular loop of CD81 binds the viral E2 protein. However, CD81 is not exclusively expressed by hepatocytes and non-hepatic cells that do express CD81, are not susceptible to HCV infection. Further studies revealed that HCV entry is dependent on cooperation between CD81 and SR-BI in the presence of cholesterol [42]. Also glycosaminoglycans [43], low-density lipoprotein receptor [44], DC-SIGN [45], and L-SIGN [46] have been suggested to be additional host factors for CD81. In addition, CLDN1, a tight junction component that is highly expressed in the liver, acts late in the entry process, after virus binding and interaction with the co-receptor CD81 [47]. However, several cell lines remain resistant to HCVpp infection, even upon the ectopic expression of CLDN1, which led to the assumption that CLDN1 functions as a co-receptor rather than a primary receptor. Additional Claudin-family members CLDN6 and CLDN9 also appear to be co-receptors for HCV and are thought to be substitutes for lack of CLDN1 during HCV infection [48, 49]. OCLN is, like CLDN1, a four-transmembrane protein that regulates para-cellular permeability and confers cell adhesiveness [50]. OCLN also seems to be required late in the viral entry process, but how it is actually involved remains unclear [51]. Studies show that both OCLN and CLDN1 are downregulated during infection and thereby prevent superinfection [52, 53]. In addition, host-delivered receptors, the Niemann–Pick C1-like 1 (NPC1L1) cholesterol uptake receptor [54] and the receptor tyrosine kinases (such as the epidermal growth factor receptor, EGF-R and Ephrin receptor A2, EphA2) [55] are known to be involved in HCV entry, but their exact role remains unclear. A fraction of the HCV particles is naturally taken up by the liver though an LDL-receptor-dependent mechanism. This does, however, not establish a productive infection [44, 56].

Replication, Assembly, and Release

Similar to viral entry, HCV replication requires both viral and host cellular factors. After viral entry in the host cell, HCV replication starts through translation of the viral polyprotein from the genomic RNA. This is mediated by the internal ribosome entry site (IRES) located at the 5′UTR [18]. The IRES initiates the first step of translation in a cap-independent manner by directly binding the 40S ribosomal subunit to form a stable pre-initiation complex [18, 57]. The 40S subunit interacts with the eukaryotic initiation factor (eIF) eIF3, followed by eIF2, guanosine triphosphate, and the initiator transfer RNA to form a 48S-ribosomal RNA complex. Finally, this RNA-complex is converted into a functional 80S ribosome that initiates viral protein synthesis [58, 59].

Also the non-structural proteins of HCV; NS3, NS4a, NS4b, NS5a, and NS5b appear to be essential for replication [24]. NS3 is a bifunctional molecule that exhibits serine protease activity that is essential, in combination with cofactor NS4a, to process other domains of the respective NS proteins [60]. NS3 is a nucleoside triphosphatase-RNA helicase that belongs to the superfamily of class II helicases [61]. About NS4b not much is known, other than its hydrophobic nature. Studies suggest that it plays an important role in the development of HCC [62]. In addition, NS4b facilitates, like NS5a, viral replication by modeling the endoplasmic reticulum (ER) membrane [63]. NS5a is furthermore involved in interferon resistance [64, 65]. NS5b protein functions as an RNA-dependent RNA polymerase (RdRp) and is an integral membrane protein [66]. This RdRp is involved in replication of the viral genome in a membrane-bound RNA replication complex [67].

Many cellular components that are important for HCV replication have been identified. Apart from the cell's machinery such as the ER and its ribosomes, cellular components have been found to interact with (non-)structural proteins of HCV or act as modulators during translation and/or RNA synthesis [68–73]. As reviewed by Bode et al. [74], the lipid and vesicle-associated membrane proteins FBL-2 [75], VAP-A and VAP-B [76, 77], the chaperone proteins cyclophilins CyPA [78] and CyPB [79], heat shock proteins (HSP90), and FKBP8 [80] are known to be involved in viral replication.

Although the mechanism is not fully elucidated, the hepatocyte-abundant microRNA (miRNA) miRNA-122 (miR-122) is also a crucial positive regulator of HCV replication [81], as described in subsequent sections. Recently, Blackham and McGarvey elucidated the role of another cellular factor, Staufen1, in HCV replication. Silencing experiments indicated that this double stranded RNA-binding protein that is involved in the regulation of translation, trafficking, and degradation of cellular RNA, also plays a role in the translation, replication or trafficking of the HCV genome [82].

Upon cellular entry, the viral proteins mediate replication of the viral RNA via a negative strand-intermediate in membrane-bound replication complexes containing HCV non-structural proteins, HCV RNA, ER membranes, and lipid droplets [63, 83], a process that is induced by NS4b in combination with NS5a [84]. The positive strand RNA genome acts as a template to generate the negative strand replicative intermediate that is used for further production of positive sense genomes. These are either translated into new viral proteins or assembled in novel infectious virions. Virus assembly is initiated when the HCV core associates with cytosolic lipid droplets. NS5A, or more specifically, subdomain DIII of NS5a, is a prerequisite for HCV particle production via its interaction with core proteins [85]. DIII and core proteins accumulate on the surface of lipid droplets. Deletions in DIII abrogate infectious particle formation and lead to an enhanced accumulation of core protein on the surface of lipid droplets identifying NS5A as a factor modulating HCV assembly [86]. Cellular components involved in this process includes the clathrin adaptor (AP2M1) [87] and group IVA phospholipase A2 (PLA2G4A) [88]. Next, although the exact process is not

fully elucidated, viral envelope proteins E1 and E2 are added to nucleocapsids and combine with the very low-density lipoprotein (VLDL) assembly pathway (microsomal triglyceride transfer protein [89] and the apolipoproteins, apoB and apoE) [90, 91] to yield lipoviroparticles that are released from the host cell [92]. Viral structural protein p7 also appears to be an essential player in the assembly of virions [93]. The process of budding and release of the virus remains largely unclear. There is evidence that HCV virion release requires the functional endosomal sorting complex required for transport III (ESCRT-III) and the AAA ATPase, Vps4, which are needed for the biogenesis of the multivesicular body (MVB), which is a late endosomal compartment [94, 95]. Likely the MVB also plays a role in the loading of HCV into exosome-like microvesicles, which have recently been reported to contain a minor subset of viruses released from infected cells [96]. Although little is known about the role of late endosomes in the budding of HCV, they are known to play a role in the life cycle of several other viruses, including hepatitis B virus [97], retroviruses, arenaviruses, and rhabdoviruses [98–103]. Lai et al. studied the involvement of subcellular components in HCV assembly and release [104]. A schematic presentation of the host factors involved in the HCV life cycle is shown in Fig. 2.

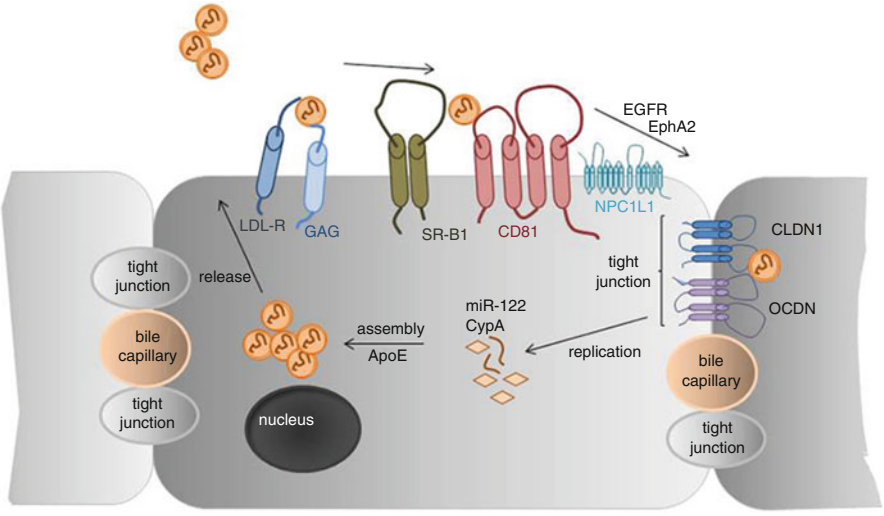

Fig. 2 Host factors involved in HCV life cycle. Viral entry is a complex, multistep mechanism in which many host factors are involved. Membrane-bound LDL-R, Gag, SR-BI, CD81, and NPC1L1, in combination with receptor tyrosine kinases EGFR and EphA2, followed by the tight junction components CLDN1 and OCLN all play a role in the entrance of HCV. Following entry, replication is initiated by the virus' IRES sequence. The cellular components involved are, amongst others, miR-122 and the cyclophilins CypA and CypB. Also the cell's heat-shock proteins HSP90 and FKBP8 are known for their role in replication. Assembly & release (Figure adapted from Shulla and Randall, 2012 [217])

Current HCV Treatment

Patients infected with HCV do not always need treatment as the infection can lead to a mild illness lasting a few weeks. However, HCV infection may also cause a serious, life long, chronic illness. HCV is one of the few viruses for which complete viral remission can be achieved, partially due to the absence of integration of the viral sequence into the cellular genome. Depending on the HCV genotype, around 15–45 % of the infected people spontaneously clear the virus. Unfortunately, the remaining population gets chronically infected [105]. Chronic HCV infection is associated with an increasing risk to develop liver cirrhosis (10–20 %) or HCC (1–5 %). Progression to liver fibrosis, development of cirrhosis and subsequent mortality are associated with patient age at the time of infection, duration of the infection, the HCV genotype, HIV co-infection, alcohol (ab)use, and gender [105]. As there are seven major genotypes, that all respond differently to treatment, careful screening is important to determine the most optimal treatment regimen. Prior to the discovery of the infectious replicon system [106], HCV has been notoriously difficult to study in cell culture and in vivo models (reviewed by Dustin and Rice [107]), which has hampered the development of more tolerable and effective therapies.

Current Therapies

The conventional combination treatment of pegylated-interferon alpha (peg-IFN) and ribavirin has achieved substantial success [108]. Newly developed drugs aim for antiviral resistance and virological breakthroughs and are so-called “directly acting antiviral agents” (DAA). Telaprevir and boceprevir are DAA that act as NS3 serine protease inhibitors [109]. Combinations of the DAA and the classic peg-IFN/ribavirin treatments are highly effective in ~75 % of the patients infected with HCV genotype 1. Unfortunately, mainly due to high costs, these therapies are not available globally [110–112]. In addition, serious side effects frequently occur when using boceprevir/peg-IFN/ribavirin, include anemia, neutropenia, and dysgeusia (altered taste sensation) [113, 114]. The combination of telaprevir/peg-IFN/ribavirin may lead to anemia, rash, and anorectal discomfort [110, 115].

Liver transplantation is the only treatment for patients with end-stage liver disease caused by HCV and in selected patients with HCV-induced HCC. The course of reinfection of the transplanted liver is more aggravated and often resistant to antiviral therapy [116]. It is known that the viral load increases rapidly directly after liver transplantation, and a significant viral quasispecies is formed [117], making treatment even more challenging.

Gene Therapy Strategies for HCV Infection

Due to gain of basic knowledge on HCV biology, several promising therapies have been developed that are currently tested in clinical trials. Among these are the use of new viral enzyme inhibitors, immune modulators, and monoclonal and polyclonal antibodies (reviewed by De Bruijne et al. and Scheel and Troels [118, 119]). Experimental therapies, in particular gene therapy using small RNA-based technology represents another promising approach [120, 121]. As HCV replicates in the cytoplasm of hepatocytes without integration into the host cell genome, and because the HCV genome is a single-stranded RNA that functions as a messenger (mRNA) and as replication template, targeted destruction of HCV RNA could potentially eliminate not only virus-driven protein synthesis but also viral replication. In addition, as replication depends on a negative RNA template as a replication intermediate, both positive and negative strands are possible targets for RNAi.

The RNAi Mechanism

RNAi is a sequence-specific process that is based on posttranscriptional gene silencing by using double stranded RNA that is homologous to the mRNA of interest. This double stranded RNA is an exogenous small interfering RNA (siRNA) that can be of viral or synthetic origin. When designed well, it will have perfect base pairing complementary with the targeted mRNA. In fact, many of the processes and key players in HCV entry and replication (reviewed above) have been unraveled by using RNAi technology [16]. RNAi is triggered by a 21-nucleotide-long siRNA that can be directly introduced into target cells by transfection or indirectly via delivery vehicles such as (viral)vectors. The siRNA is generated from a gene cassette that drives the expression of a short hairpin RNA (shRNA) transcript that is processed by the RNAi machinery [122, 123] into two single stranded RNAs, a guide strand and a passenger strand. The latter is usually degraded and the guide strand is incorporated into the RNA induced silencing complex (RISC) that facilitates the interaction with complementary mRNA molecules. The binding of the siRNA-programmed RISC complex to a complementary mRNA will block the ribosomal translation into protein and can tag the mRNA for degradation [124] by the Argonaute-2 protein with slicer activity that is part of the RISC complex [125, 126] (Fig. 3).

RNAi Delivery

RNAi can be mediated in a transient or stable manner. For transient interference, synthetic siRNAs can be designed and introduced directly into the target cell by electroporation. A brief, powerful electric pulse results in the temporary loss of the semipermeability of the cell membrane that leads to escape of intracellular content

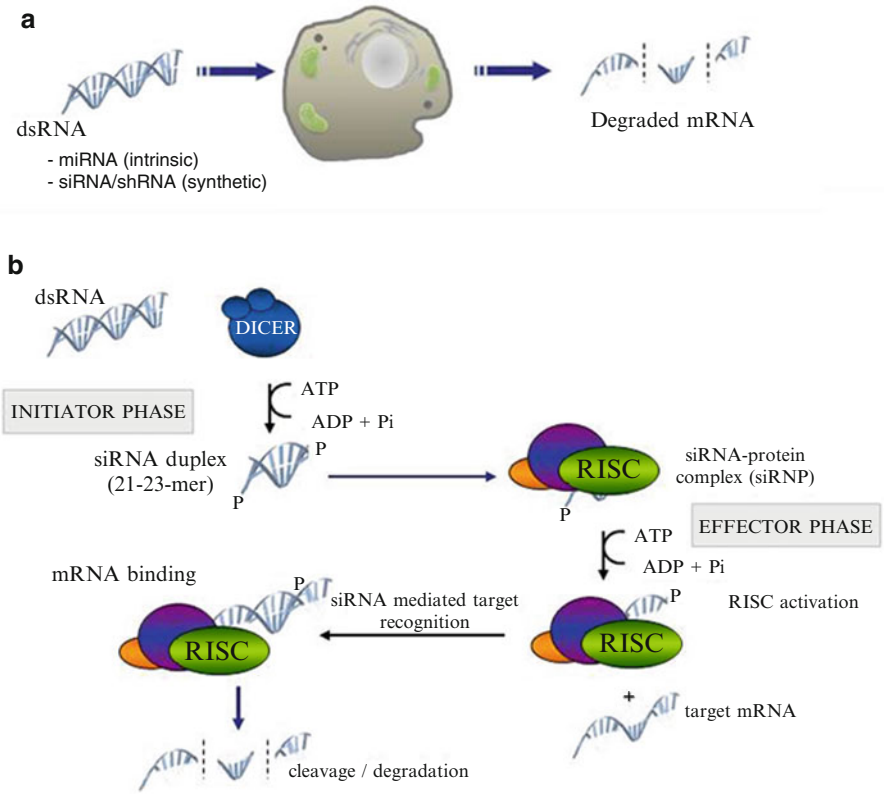

Fig. 3 RNAi mechanism. (**a**) Sequence-specific, posttranscriptional gene silencing; RNA interference (RNAi). Two types of small RNA molecules—microRNA (miRNA; present in eukaryotic cells) and small interfering RNA (siRNA; synthetic) play a key role in RNAi. These small RNAs can bind to specific mRNA molecules and influence their activity (either increase or decrease), thereby regulating protein production and gene expression. The RNAi pathway is initiated by the endonuclease Dicer which cleaves long double stranded RNA (dsRNA) into short double stranded fragments of ~20 nucleotides; siRNA. This phase is called the initiator phase. Each siRNA is then denatured into two single stranded (ss) RNAs; the passenger strand which is degraded and the guide strand that is incorporated into the RNA-induced silencing complex (RISC). This is the effector phase. This complex binds to a complementary sequence in an mRNA molecule where it induces cleavage and thereby degradation (**b**)

but also simultaneous uptake of nucleic acids. The high mortality rate of the permeabilized cells and the fact that the siRNA is only transiently available makes this method only feasible in vitro and incompetent for gene therapy purposes [127].

In addition to electroporation, other transfection methods may deliver the siRNA into the hepatocyte. For this, the siRNA or a vector containing the so-called shRNA sequence (the precursor of siRNA) can be complexed with a carrier that allows traversing of the cell membranes. Typically, this involves packaging into liposomes, or synthetic cationic polymer-based nanoparticles. Also addition

of cationic cell-penetrating peptides can be used. Although this method succeeds in delivering the siRNA into the host cell, the transfection efficiency might be quite low and one should realize that cell division may dilute the siRNA [128]. Even direct injection of nucleic acids, in particular shRNAs, into the blood of rodents via a hydrodynamic tail vein procedure has shown some, albeit transient, efficacy for delivery to the liver [129–131].

For use as an effective treatment for chronic infectious disease such as HCV infection, stable, long-term expression of the antiviral shRNA is needed. Stable expression of shRNAs in host cells can be accomplished by delivery through viral vectors, i.e., retroviral, lentiviral, adenoviral, or adeno-associated vectors (AAV). Several clinical trials have been set up using virus-mediated gene transfer for different diseases. Gene therapy has gained particular attention since the first successful clinical trial in 1990 for the treatment of a young girl suffering from ADA-SCID (severe combined immunodeficiency caused by a mutation in the adenosine deaminase gene, ADA) using retroviral vectors [132]. However, a tragic setback occurred when an 18-year-old boy died when he was participating in a gene therapy trial for congenital ornithine carbamoyltransferase deficiency [133]. The latter trial was done with AV-based vectors. In 2003 several patients that participated in a retroviral-based gene therapy trial for treatment of X-linked SCID developed leukemia 3 years after successful treatment. As a result, gene therapy trials were put on hold and vector construction and the use of (replication incompetent) virus was reconsidered (reviewed by Baum et al. [134]). To date, over 1,800 gene therapy clinical trials have been completed or have been approved worldwide [135]. Amongst these trials are two studies for HCV treatment. A Phase I study using the modified vaccinia Ankara vector (MVA) to target the HCV RNA coding for non-structural proteins and a Phase I/II study in which AAV vectors are used to deliver shRNA into infected hepatocytes. With the use of tissue-specific promoters, the shRNA can be delivered and expressed in a relatively safe way, as putative toxic effects will be limited to the target cells.

Delivery of transgenes into mammalian hepatocytes can be achieved by ex vivo [136, 137] and in vivo [138–141] methods. Chowdhury [136] and Kay [137] have achieved reasonably high (ex vivo) expression of transgenes encoding the low-density lipoprotein receptor (LDL-R) and human α1-antitrypsin (hAAT), respectively. However, disadvantages of ex vivo gene therapy include the potential microbial contamination during hepatocyte culture, limitation of the number of hepatocytes that can be reintroduced due to possible venous obstruction, and the need for a partial hepatectomy to harvest the cells for transduction. Therefore, different routes for in vivo delivery of vectors have been explored, including intravenous injection, intraperitoneal injection [142], intraportal injection [139, 143], asanguineous (isolated from blood circulation) liver perfusion [140], and hepatic inflow occlusion/portal vein injections [141, 144]. In the following paragraphs an overview of different vector types is given.

1. *Adenovirus-based vectors*: Curiel et al. demonstrated in 1991 that replication-incompetent adenovirus can enhance the transfer of DNA into HeLa cells by 2,000-fold [145]. Since then, adenoviruses have been widely used in hepatic gene transfer studies [146]. Adenoviruses are non-enveloped viruses of the

Adenoviridae family that contain a dsDNA genome. The use of adenoviruses as an endosomal lysis agent, along with the formation of DNA–protein complexes, results in great enhancement of gene delivery into hepatocytes [146]. Coupling the adenovirus directly to the DNA-protein complex an even more efficient uptake into the cell and transgene expression is accomplished [147]. Adenovirus-mediated gene transfer does not lead to integration of the transgene into the host cell genome and the vector is slowly lost during cell division, which limits their use in lifelong gene therapy. Next to this, adenoviruses commonly infect humans and may therefore trigger an immune response when used in gene therapy [148]. Despite these drawbacks, adenoviruses have been successfully used in delivery of shRNA cassettes to liver cells. Using an adenovirus vector that expresses an anti-hepatitis B virus (HBV) siRNA, specific inhibition of HBV gene expression was observed [149]. More recently, intravenous delivery of an adenovirus expressing HCV targeted shRNA also efficiently and specifically suppressed HCV genomic RNA and protein synthesis in the liver of mice transgenic for the HCV structural proteins (CN2-29 mice) [150]. The immunogenicity of adenoviral vectors can be suppressed by polyethylene glycol (PEG)-modification of the virus [151]. Adenovirus express small noncoding RNAs that suppress the RNAi pathway by acting as competitive substrates for the microRNA/siRNA machinery, which may further limit its use for RNAi delivery [152, 153]. Recently, third-generation adenoviral vector-mediated shRNA expression resulted in inhibition of target gene expression in the liver [154]. High level shRNA expression resulted in activation of the interferon response [155].

2. *Adeno-associated virus vectors*: Adeno-associated virus (AAV) belongs to the *Parvoviridae* family, which are non-enveloped viruses with a linear single stranded DNA genome. AAV is further distinguished from other human viruses by its requirement for a helper virus to complete its life cycle in the host cell. The helper virus, typically an adenovirus or a herpes virus, provides RNA or protein factors that either stimulate gene expression from the AAV promoters, enhance transport or splicing of the AAV pre-mRNAs and, in case of herpes simplex virus type 1, support replication of the AAV genome [156]. AAV vectors have a broad tropism and can infect both dividing and non-dividing cells and although the size of the transgene is generally limited to <5 kilobases (kb) this is obviously enough space for delivery of the smaller shRNA sequences. Many serotypes of AAV have been described (reviewed by Grimm and Kay [157]), of which AAV serotype 2 was the first to be used in gene transfer experiments. Despite its non-pathogenic character in humans, preexisting immunity for AAV2 is prevalent in humans, which may explain the failure to obtain long-term gene expression in a hemophilia B gene therapy trial [158]. AAV8, a serotype that was isolated from rhesus monkeys, seems much more efficient with a 10–100-fold increase in transduction efficiency in mouse liver cells [159], likely because the preexisting antibodies in humans do not recognize this serotype [84]. This makes AAV8 a good candidate for shRNA delivery into hepatocytes.

   Dimeric, or self-complementary AAV vectors (scAAV) have been specially designed for gene therapy. The ssDNA in these vectors forms an extended hairpin structure by intra-molecular base pairing. This results in a higher transduction

efficiency and efficient replication and transcription [160]. Consequently, scAAV vectors can accommodate only half of the insert size that a regular AAV vector can hold; ~2.5–3.3 kb.

3. *Retrovirus-based vectors* (*gamma-retrovirus and lentivirus*): Gene transfer vectors derived from replication-incompetent gamma-retroviruses or lentiviruses are most robust and reliable tools to achieve sustainable and long-term shRNA expression in cells. Gamma-retroviruses and lentiviruses belong to the family of *Retroviridae*, which are enveloped viruses containing an RNA genome that is reverse transcribed into DNA in the infected cell. Retroviral vectors maintain the integration preferences of the original virus, which has significant implications for their biosafety. The gamma-retroviruses need replication of the host cell to enter the nucleus and to integrate the DNA genome into that of the host, which makes them less suitable for gene transfer of hepatocytes. Lentivirus is capable to integrate into non-dividing cells, which makes this vector system more useful for gene therapy of all cell types, including senescent cells. The use of retroviruses for gene transfer revealed undesired effects caused by insertional deregulation of cellular gene expression at the transcriptional or posttranscriptional level. Severe adverse events in several clinical trials involving the transplantation of hematopoietic stem cells genetically corrected with retroviral vectors [161] lead to a more careful redesign of these vectors. The retroviral vectors were made safer by pseudotyping the vector envelope, the development of U3 deleted self-inactivating (SIN) vectors, carefully choosing internal promoters, and adjustment of the viral backbone. However, the gamma-retroviruses will likely continue to integrate in the actively transcribed parts of the host cell's genome, which needs to be carefully monitored (reviewed by Maetzig et al. [162]). The use of lentiviral vectors rather than gamma-retroviral vectors further reduced the potential genotoxicity as integration usually occurs away from regulatory elements [163, 164]. Although lentivirus-mediated shRNA delivery has been widely used for therapeutic applications, only few reports refer to HCV therapy [165–168].

Hepatocyte-Specific Promoter Elements

Gene regulatory elements can be positioned relatively far away from the gene, but are usually concentrated upstream of the promoter. Smith et al., have identified previously characterized motifs for transcription factors known to play tissue-specific regulatory roles [169, 170]. To increase gene expression in retrovirus or other viral vectors, strong viral promoters are frequently used. Despite this, transgenes are usually poorly expressed in hepatocytes in vivo. To overcome this limitation, viral promoters have been replaced by tissue-specific mammalian promoter elements. Several liver-specific promoters are known and include those of the apolipoprotein A-1 [171], transthyretin (mTTR.hUGT1A1) [172], albumin [173], and α1-antitrypsin (hAAT) gene [174]. The liver-specific promoter of the thyroxin-binding globulin (TBG) gene may display slightly less activity than the widely used CMV

(cytomegalovirus) and EF1α (elongation factor 1α) promoters, but TBG has proven to direct long-term exogenous gene expression in the liver [175, 176]. In addition to liver-specific promoters, also enhancers such as the one of the hepatocyte nuclear factor-3 (HNF-3) gene can boost the transcriptional activity [177]. Adding trimers of the DNA binding sequence for HNF-3 increased the expression of the hAAT reporter gene in vitro [177] and stabilized long-term serum hAAT protein expression in vivo [174].

In addition to classical transcriptional regulation, miRNAs are new key players in (tissue-specific) mammalian gene regulation at the posttranscriptional level. MiRNAs are short (21–23 nt) noncoding RNA molecules and have been exploited in the control of transgene expression. The liver-enriched miR-122 stimulates differentiation of hepatocytes [178] and contributes to hepatic functions such as cholesterol synthesis and fatty-acid metabolism [179]. With regard to viral hepatitis, miR-122 is shown to inhibit HBV replication [180], but for HCV this miRNA is an important host factor that stimulates viral protein synthesis and replication [181]. Modulation of hepatocyte miRNAs, including miR-122, has already proven to be a promising strategy for targeting HCV [182].

Placing a therapeutic gene under the control of a tissue-specific promoter does not guarantee that its expression will be restricted to the target tissue. Even toxic side effects in other tissue and cell types may be the result of low level background gene expression, depending of course, on which transgene is expressed [183]. Attempts to improve the specificity of AAV mediated gene expression have led to the development of AAV serotype 9 capsid (AAV9) vectors with microRNA-regulated expression [184, 185]. For this, the liver-specific target sequence for miR-122 was incorporated in the AAV vector such that AAV gene expression was blocked in the liver and cardiac myocytes. The AAV9 has a broad cell tropism and can mediate gene transfer to a variety of target tissues including heart, skeletal muscle, liver, and lung following systemic administration [186, 187]. Although the AAV capsid exhibits a high natural affinity for certain target tissues such as liver, strategies aimed at further restricting AAV9 gene transfer include the engineering of a modified tropism [188, 189], tissue-specific promoters [190] and the use of localized gene delivery methods such as direct cardiac versus systemic injection [191]. In addition, methods to increase and control gene expression such as dimerization-inducible systems (reviewed by Pollock and Clackson [192]) have resulted in dose-dependent, reversible, reproducible, and tissue-specific regulation in liver and heart in vivo when combined with tissue-specific promoters [193].

Potential Targets for RNAi Therapy

In the past decade, RNAi-mediated knockdown of cellular components has led to a better understanding of the HCV life cycle and the potential targets for HCV therapy. A major step forward was the application of RNAi libraries with which functional genome-wide screening can be achieved. Using these RNAi libraries, a

number of known host genes that are involved in HCV replication and infectious virus production can be screened and analyzed. This may lead to the identification of new important cellular players that interact with HCV (non)structural genes [68, 194, 195]. Results from screenings should however be interpreted with caution and the right controls need to be included. Recently, our group reported that two widely used lentiviral shRNA libraries exhibited unexpected effects on HCV replication due to a disturbance of endogenous miRNA production by the high level of exogenous shRNAs [196]—A problem that could be solved by modulating shRNA levels. Several studies indicate the involvement of miRNAs in HCV replication. For instance, knockdown of hVAP33, a vesicular membrane protein, inhibits the interaction with NS5b and NS5a and thus interferes with HCV RNA replication [84]. Knockdown experiments revealed that the stress granule proteins T-cell-restricted intracellular antigen-1 (TIA-1), TIA-1-related protein (TIAR), and the RasGAP-SH3 domain binding protein-1 (G3BP1) are required for efficient HCV RNA and protein accumulation at early time points in the infected cell. Moreover, G3BP1 and TIA-1 are required for intracellular and extracellular production of infectious virus, suggesting that these cofactors are required for virus assembly [197, 198].

Host cellular proteins that are known to be involved in HCV replication include VAMP-associated protein (hVAP-A), La-antigen, and polypyrimidine-tract binding protein (PTB) may provide an alternative approach for blocking HCV replication [199]. In addition, cellular NF/NFAR proteins, a group of predominantly nuclear proteins including NF90/NFAR-1, NF110/NFAR-2, NF45, and RNA helicase A (RHA) are also known to be involved in HCV replication by recruitment to the viral replication complexes in the cytoplasm. By reducing the level of RHA in the cell with a shRNA, HCV replication was inhibited [200]. These and many more cellular components such as FUSE binding protein [201], and the known entry factors including OCLN have been silenced using RNAi techniques. This did not only result in a better understanding of HCV entry, the life cycle, and replication but also provided new strategies to treat HCV.

MiRNA Blocking with Antisense Technology

A new molecular biology approach that is a promising tool to treat HCV is the use of antisense technology, which originally employed RNA transcripts to bind to an mRNA and thereby preventing its translation in a sequence-specific manner. Also miRNAs can be efficiently blocked by this approach. Locked nucleic acid (LNA) is a nucleic acid analog that, as a short oligonucleotide, possesses high affinity for complementary DNA or RNA, and can be used as antisense molecules in vitro and in vivo (reviewed by Stenvang et al. [202]). The ribose ring in these molecules is "locked" by a methylene bridge connecting the 2′-O atom with the 4′-atom. As a consequence, LNA molecules are constrained in the ideal confirmation for Watson–Crick base pairing, thus facilitating the interaction with complementary molecules. In addition to improve annealing kinetics, the stability of the resulting duplex is increased [203].

El-Awady et al. showed a complete arrest of intracellular replication of HCV type 4 by antisense oligonucleotides against sequences that encode the N-terminus of the viral polyprotein precursor. These experiments were done in an HCV cell line model and showed the potential to use antisense technology for HCV (type 4) treatment in patients [204]. By packing the antisense RNA, directed at the 5′UTR and IRES sequences of HCV, in self-assembling MS2 bacteriophage capsids, the oligonucleotides were protected against degradation in vitro and in vivo. However, the high level of antisense oligonucleotides needed to inhibit HCV replication was toxic to the cells [205]. A more promising approach may use miravirsen, a 15-nt oligonucleotide that is complementary to miR-122 and thus interferes with HCV replication. After successful animal studies in which the plasma and liver HCV RNA in infected chimpanzees decreased remarkably by antisense technology against miR-122 [206], this technique was studied in a Phase II study. Also in the HCV patients enrolled in this study (36 included that were treated with three doses of miravirsen or placebo), HCV RNA decreased in a dose-dependent fashion, although in three of the patients a relapse occurred 4–5 weeks after follow-up [207, 208]. Although short-term administration of miravirsen did not result in any complications, long-term use could be problematic as miR-122 is postulated to have tumor-suppressor effects for hepatocellular carcinoma. Mice that lack the miR-122 gene have a high risk of fatty liver, fibrosis, and hepatocellular carcinoma [209, 210]. Because these conditions are also side effects of HCV infection, safety monitoring of this approach remains of critical importance [211].

Combinations of RNAi and Other Antivirals

In spite of the great potential of RNAi technology to silence essential genes needed for HCV replication, it has also been shown that HCV via its high mutation rate has the potential to escape from RNAi-mediated inhibition. As with antiviral monotherapy, RNAi with a single shRNA inhibitor may only work transiently because of mutational escape and the selection of resistant virus variants. An obvious answer to this problem is the combinatorial use of multiple antivirals, e.g., IFN-α with the new RNAi approach. As IFN-α exhibits antiviral activity indirectly through stimulation of genes that trigger an antiviral response, the combination of antivirals will increase the antiviral activity and the genetic threshold for the development of resistance. Pan et al. reported that lentivirus-mediated RNAi and IFN-α act independently on HCV replication resulting in pronounced antiviral activity without cross-sensitive drug interference [120]. One could also combine RNAi attack to silence specific host and viral genes. It is also possible to use RNAi for boosting of the IFN-α response by silencing the ubiquitin-specific protease 18 (USP18) because upregulated USP18 expression is associated with a poor response to IFN-therapy [212].

As HCV mutates rapidly [213], effective RNA interference-based therapies might require the use of combinatorial RNAi (coRNAi) to target multiple regions within the HCV RNA genome. For this, shRNAs are delivered simultaneously to

individual cells while maintaining high expression levels needed to suppress viral replication. The use of host cell factors that are genetically stabler than the viral genome may represent good therapeutic targets for coRNAi to prevent resistance development [214]. The drawback of combining viral and cellular targets could be the potential deleterious effects by impeding normal cellular functions, and, in addition, viruses can still develop resistance as was shown for inhibition of host cyclophilins [215]. By applying coRNAi, one can impart either a protective strategy or a long-term treatment postinfection without the eventuality of mutational outgrowth due to incomplete selection pressure [167].

In the near future more therapeutic combinations such as coRNAi and RNAi with ribavirin, ribozymes, DAAs, and others will be further analyzed.

Concluding Remarks

Many studies have demonstrated that gene therapy, to be more precise the use of RNAi and LNA techniques, beholds a very promising approach not only to treat HCV but to also to gain a better understanding of the factors involved in the HCV life cycle. Identification of host factors that are involved in the different stages of the HCV life cycle either by single target or genome-wide RNAi screening, has provided new insights into viral and host factors and their interactions. This will further result in novel treatment regimens for HCV infected patients in the near future. In particular, the discovery of the host cellular miR-122 as a modulator of HCV replication has led to many new insights in this disease. In addition, humanized mouse models in which the complete HCV life cycle can be studied, have been established [216]. Persistently infected humanized mice produce de novo infectious particles that are inhibited with directly acting antiviral drug treatment. This provides evidence for the completion of the entire HCV life cycle in inbred mice which opens new opportunities to dissect genetically HCV infection in vivo. With this an important preclinical platform is established for testing and prioritizing drug candidates to treat HCV infection.

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Recent Advances in Use of Gene Therapy to Treat Hepatitis B Virus Infection

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Abstract Chronic infection with hepatitis B virus (HBV) occurs in approximately 5 % of the world's human population and persistence of the virus is associated with serious complications of cirrhosis and liver cancer. Currently available treatments are modestly effective and advancing novel therapeutic strategies is a medical priority. Stability of the viral cccDNA replication intermediate is a major factor that has impeded the development of therapies that are capable of eliminating chronic infection. Recent advances that employ gene therapy strategies offer useful advantages over current therapeutics. Silencing of HBV gene expression by harnessing the RNA interference pathway has been shown to be highly effective in cell culture and in vivo. However, a potential limitation of this approach is that the post-transcriptional mechanism of gene silencing does not disable cccDNA. Early results using designer transcription activator-like effector nucleases (TALENs) and repressor TALEs (rTALEs) have shown potential as a mode of inactivating cccDNA. In this article, we review the recent advances that have been made in HBV gene therapy, with a particular emphasis on the potential anti-HBV therapeutic utility of designed sequence-specific DNA binding proteins and their derivatives.

Hepatitis B Virus Epidemiology

Conservative estimates of the global prevalence of chronic hepatitis B virus (HBV) infection place the number of affected individuals in excess of 240 million worldwide [1, 2]. Although a third of the world's population has been exposed to the virus [1, 2], most acute infections are cleared spontaneously. Infections that are not

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cleared progress to chronicity and it is this persistent HBV infection that is associated with serious sequelae, such as cirrhosis and liver cancer (hepatocellular carcinoma or HCC). Annually 600,000 deaths are attributable to complications that arise from chronic HBV infection. HBV alone accounts for 53 % of new HCC cases worldwide and the hepatitis C virus (HCV) is estimated to account for 25 % of new cases [3]. HCC is a particularly aggressive cancer that has a high mortality. In 2008, 92 % of new HCC cases were fatal [4]. Although vaccination effectively prevents HBV infection, the incidence of liver cancer has not changed significantly in the past 10 years. Moreover, modest curative efficacy of currently available treatment regimens is unlikely to prevent complications arising in those already infected with the virus. Liver cancer remains the sixth most common cancer worldwide and is still ranked as the third most common cause of cancer-related deaths [4, 5]. HBV is hyperendemic to sub-Saharan Africa, East and South-East Asia as well as the western Pacific islands. These regions, largely comprising developing countries, are also the most severely affected by HCC. HBV itself is a non-cytopathic virus; however, the increased risks of cirrhosis and HCC associated with chronic viral infection makes the disease a global priority.

Evidence from early intervention programmes have shown that decreasing the incidence of HBV has a positive impact on the incidence of cirrhosis and liver cancer [6, 7]. In 1984 Taiwan implemented the first anti-HBV vaccination programme and the most recent data from 2004 demonstrate that, with a 97 % vaccination coverage rate, HBV seroprevalence in children has decreased from 9.8 % to 0.6 % [7]. The decrease in HBV prevalence has been accompanied by a decrease in the incidence of HCC [7]. However, vaccination failure may occur and has been attributed to emergence of viral vaccine escape mutants. Nevertheless, these cases represent just a small number of individuals, only 33 in total. A second limitation of vaccination is that it is prophylactic and not therapeutic. As a consequence vaccination has little therapeutic benefit in cases where chronic HBV infection has already been established. Seven drugs are currently licensed for treatment of chronic HBV infection (reviewed in ref. [8]). These are broadly divided into two groups: (1) immunomodulators (interferon alpha (IFN-α) and pegylated IFN-α) and (2) nucleoside and nucleotide analogues (lamivudine, telbivudine, adefovir dipivoxil, tenofovir disoproxil fumarate and entecavir), which act by inhibiting viral reverse transcriptase. The efficacy of immunomodulators is limited as side effects are common, there are several contraindications and cure occurs in only a small subset of chronic HBV carriers. Nucleoside and nucleotide analogues exhibit a number of advantages over immunomodulators, which include ease of use (oral administration route) and better patient tolerance to the drugs. However, the first generation drugs exhibit a low barrier to resistance and viral escape rates have been reported to range from 29 % to 80 % [9–11]. Treatment with lamivudine in particular is complicated by high rates of viral escape by mutation [9, 10]. Newer drugs exhibit improved viral suppression and also limit development of viral escape (low in the case of entecavir and none reported for tenofovir (for review see ref. [8])). Although these data are promising, nucleoside and nucleotide analogues rarely eliminate HBV completely from infected hepatocytes. Stability of the covalently closed circular DNA (cccDNA)

replication intermediate of HBV, and inability of available therapies to disable this viral transcription template, are mainly responsible for the limitations of available HBV treatments.

HBV Biology

HBV is an enveloped DNA virus belonging to the Hepadnaviridae family of viruses (reviewed in ref. [12]). The DNA genome is contained within an icosahedral capsid which in turn is enveloped in a lipid bilayer. The genome of HBV exists in a partly double-stranded, circular conformation called the relaxed circular DNA or rcDNA [13]. The minus strand encompasses the entire genome and comprises approximately 3,200 bases. Until recently the receptor used by HBV to enter liver cells has remained elusive. Yan and colleagues identified the sodium taurocholate cotransporting peptide (NTCP) as the receptor employed by the virus to infect hepatocytes [14]. A basic overview of hepatocyte infection and viral replication is illustrated in Fig. 1. Upon

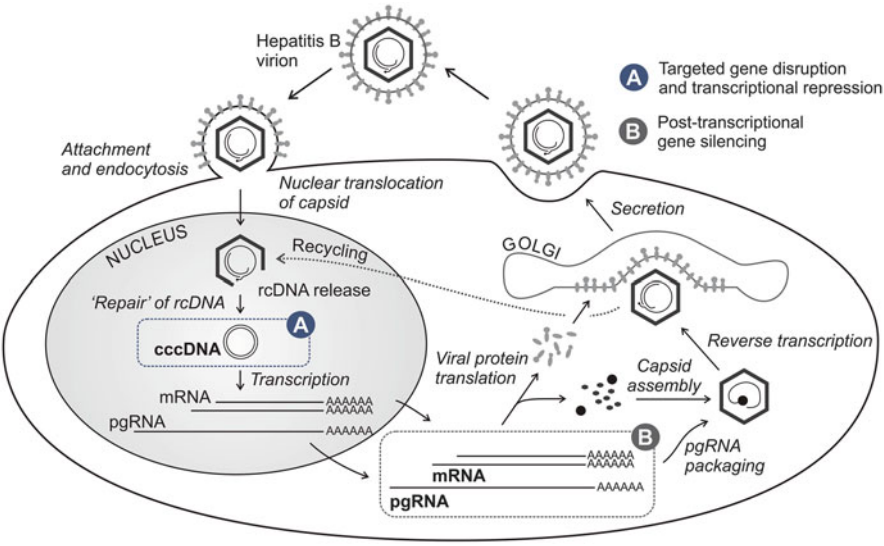

Fig. 1 Hepatitis B virus replication cycle. Following attachment to the hepatocyte cell membrane, the capsid is released into the cytoplasm and is then translocated to the nucleus. Here the rcDNA viral genome is released and repaired to form cccDNA. The cccDNA acts as the primary replication intermediate from which the pgRNA and viral subgenomic mRNAs are transcribed. These viral RNAs are transported to the cytoplasm where the subgenomic mRNAs are translated into proteins required for virion packaging and assembly. The pgRNA associates with polymerase and new core proteins to assemble the new capsid. Within the capsid, the pgRNA is reverse transcribed to form rcDNA. Within the endoplasmic reticulum, surface proteins surround the capsid to form new virions before secretion from the infected hepatocyte. Current gene therapy targets include the episomal cccDNA (**A**) for targeted gene disruption and transcriptional repression, and the cytoplasmic viral mRNAs (**B**) for a post-transcriptional gene silencing approach

infection the rcDNA, still contained within the capsid, is transported to the nucleus where it is released and "repaired" by cellular enzymes to form cccDNA [15]. The cccDNA serves as template for transcription of viral RNAs. These include the greater-than-genome length pregenomic RNA (pgRNA) which is reverse transcribed to form the viral rcDNA genome. The cccDNA exists episomally as a stable mini-chromosome [15, 16]. Since viral replication may be initiated from this stable mini-chromosome, an effective cure for chronic HBV infection requires that cccDNA be inactivated or eliminated.

HBV produces new virion DNA through error-prone reverse transcription of the pgRNA template. Although numerous mutations may be introduced during this reverse transcription step, the highly compact genome limits sequence plasticity and emergence of mutant strains. The four viral open reading frames (ORFs), which code for seven viral proteins, overlap with one another. Furthermore, the viral ORFs cover the entire genome of HBV and all viral regulatory elements are contained within protein coding regions (reviewed in ref. [17]). Since most regions of the HBV genome have dual use, mutations at one site commonly affect more than one genetic function to compromise viral fitness severely. The HBV genome is therefore more stable than genomes of other viruses that employ a reverse transcriptase during replication. This feature makes HBV an ideal target for gene therapy based on sequence-specific DNA recognition.

Until recently the technology required for efficient targeted disruption of specific DNA sequences was not readily available. Gene therapy using cccDNA-targeting engineered proteins has shown promise as a means of disabling HBV replication. Discovery of gene silencing by RNA interference (RNAi) was a major development in gene therapy. Several studies have reported that harnessing this pathway can be used successfully to inhibit HBV replication. In this chapter we summarise recent advances in HBV gene therapy, with particular emphasis on progress in adapting sequence-specific DNA binding proteins to counter the viral infection.

RNAi Against HBV

Activation of RNAi by microRNAs (miRNAs) is the prototypic mechanism by which endogenous post-transcriptional silencing of target genes is achieved [18]. The pathway occurs in metazoan cells and naturally involves stepwise processing of RNAs containing hairpin motifs. This leads to production of mature miRNAs, comprising hairpin-derived duplexes of approximately 23 bp. Post-transcriptional gene silencing by the guide strand from a mature miRNA is effected by the RNA induced silencing complex (RISC) and involves hybridisation of the guide to complementary sequences in the 3′ untranslated region of target genes. Efficient gene silencing may be achieved by introducing artificial RNA mimics of intermediates of the RNAi pathway into cells. Both synthetic [19–23] and expressed [24–31] exogenous activators of the RNAi pathway have been used to silence HBV replication in vitro and in vivo. Studies using synthetic RNAi activators against HBV were amongst the first to demonstrate the utility of harnessing RNAi in vivo [19,

21]. An important finding into the mechanistic aspect of these therapeutic sequences was that RNAi-mediated silencing did not require viral replication [19]. This is in contrast to the mechanism of action of nucleotide and nucleoside analogues, which need to be incorporated into a growing DNA chain to exert their inhibitory effects. RNAi-based activators interfere with gene expression at a post-transcriptional level and therefore act at a later stage of HBV replication than do nucleoside and nucleotide analogues. Since these first reports demonstrating potential utility of RNAi inducers, significant progress has been made using synthetic as wells as expressed anti-HBV effectors. Various chemical modifications have been introduced into synthetic anti-HBV sequences [32–35]. These have been formulated in non-viral vectors and shown to be effective inhibitors of HBV replication. Anti-HBV expression cassettes, which generate precursor miRNA (pre-miRNA) and primary miRNA (pri-miRNA) sequences transcribed from RNA polymerase (Pol) II and Pol III transcription regulatory elements, have also been found to be effective against the virus [36–42]. Some of these cassettes have been incorporated into recombinant adeno- and adeno-associated viral vectors, which have been effective against the virus in murine models of HBV replication. However, as with nucleoside and nucleotide analogues, RNAi-based therapy does not completely eliminate the stable pool of cccDNA. Consequently RNAi-based approaches are unlikely to cure HBV infection. This was demonstrated by Starkey et al. [43], who showed that expressed anti-HBV RNAi activators inhibited new formation of cccDNA in cultured hepatocyte-derived cells. However, concentrations of established cccDNA were unaffected by the HBV-targeting short hairpin RNAs (shRNAs). These results were not entirely unexpected and confirmed that RNAi-based anti-HBV agents function by a post-transcriptional mechanism to inhibit gene expression from the cccDNA template. Viral RNA, viral protein and new virion formation are subsequently suppressed but cccDNA levels remain unaffected. To have therapeutic utility against HBV, RNAi activators would need to be administered repeatedly or produced in a sustained manner from stable intrahepatic DNA expression cassettes. Both strategies pose challenges and highlight the need to develop strategies for directly disabling nuclear cccDNA.

Targeting cccDNA Using Engineered Sequence-Specific DNA Binding Proteins

During the past decade, engineered Zinc Finger Proteins (ZFPs) have been widely used to regulate expression of genes for therapeutic gain [44]. These DNA binding proteins, which occur naturally as eukaryotic transcription factors, may be designed to target specific DNA sequences. Since each zinc finger is capable of binding a triplet of nucleotides, the sequential arrangement of an array of six “fingers” enables site-specific targeting of up to 18 nucleotides by ZFPs (Fig. 2a) [45]. Although these DNA binding proteins have been engineered primarily for endogenous gene

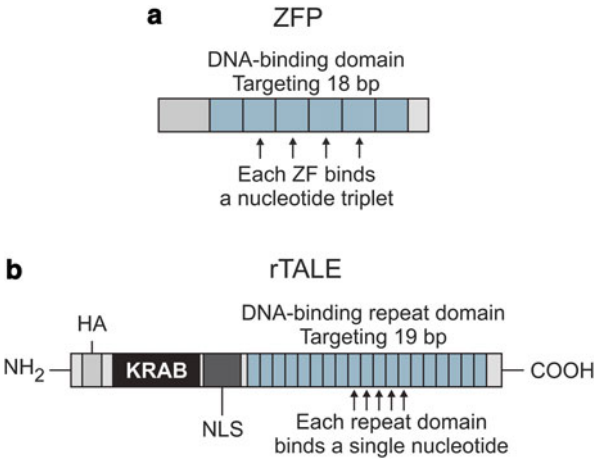

Fig. 2 Proteins that bind specific DNA sequences and which have been used to inhibit HBV gene transcription. (**a**) Polydactyl ZFPs include multiple zinc fingers, each targeting a predefined nucleotide triplet. The length of the DNA binding region can be adjusted to target an 18 base pair sequence by adding up to six consecutive fingers within an array. (**b**) Repressor TALEs (rTALEs) are generated by fusing KRAB repressor domains to the N-terminal of the DNA binding TALE proteins. The repeat domain comprises 19 TALE modules, each with a nucleotide specific RVD to enable sequence-specific DNA binding. The nuclear localisation signal (NLS) facilitates efficient trafficking of the protein into the cell nucleus, whilst the haemagglutinin epitope (HA) sequence acts as a tag for convenient protein detection

regulation, the chromosome-like structure of HBV cccDNA is likely to be amenable to similar modes of transcriptional manipulation. This was initially investigated by Zimmerman and colleagues who generated polydactyl ZFPs to manipulate duck hepatitis B virus (DHBV) gene expression [46]. They demonstrated that ZFPs designed to bind either 9 or 18 base pair sequences within the enhancer region of DHBV cccDNA reduced pgRNA expression by 41.6 %. Decreases in the DHBV surface and core proteins, as well as viral particle equivalents, were also observed. As the enhancer region includes multiple transcription factor binding sites, it is likely that these ZFPs functioned by competitively obstructing binding of transcription factors to *cis* elements of the cccDNA. However, as these ZFPs do not exert a permanent effect on their HBV targets, effectiveness is likely to be transient and dependent on the duration of their expression. Nevertheless, demonstration of an effect on cccDNA was a significant observation. This paved the way for investigating the utility of functionally enhanced DNA binding proteins that target cccDNA in human liver cells. Coupling transcriptional repressor domains such as the Krüppel associated box (KRAB) domain to DNA sequence-specific binding domains of Transcription Activator-Like Effectors (TALEs) to achieve more durable silencing may be preferable (Fig. 2b) (discussed later).

Designer nucleases have been developed by engineering DNA binding proteins to enable introduction of double stranded breaks (DSBs) at specific target sites

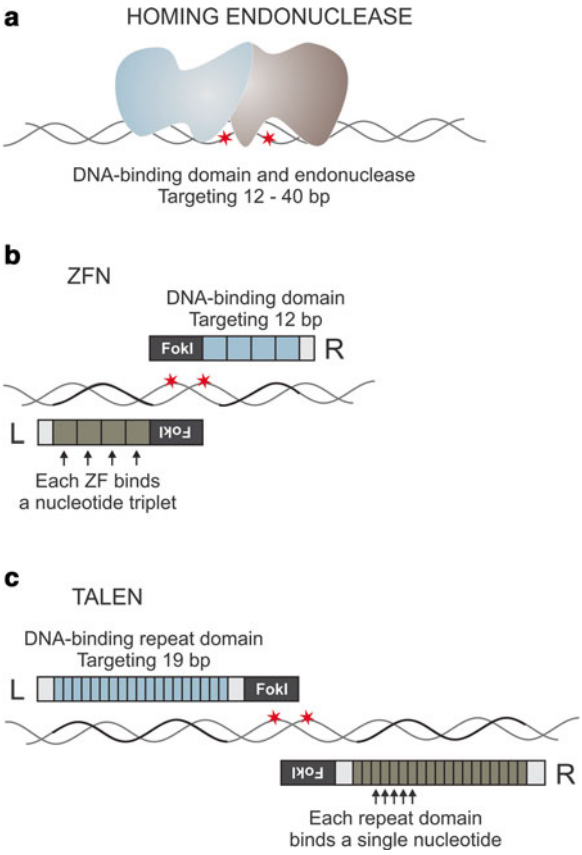

Fig. 3 Designer nucleases used to disable HBV sequences. The three different engineered nucleases currently being investigated as potential anti-HBV therapies are illustrated schematically. (**a**) Homing endonucleases or meganucleases are found naturally as endonucleases which target DNA sequences between 12 and 40 bp in length. (**b**) ZFNs are engineered as pairs with each ZF in the array binding to a specific nucleotide triplet. When a polydactyl ZFP array consists of four ZFs, each left (L) or right (R) subunit targets a 12 bp sequence. (**c**) TALENs are engineered as pairs with each left (L) or right (R) subunit DNA-binding domain targeting a 19 bp sequence. Each subunit is assembled from single TALE repeats which confer single nucleotide specificity. Both ZFNs and TALENs cleave target DNA with *Fok*I endonuclease domains to introduce double strand breaks (DSBs) indicated by the *asterisks*

(Fig. 3). DSBs are typically repaired by non-homologous end-joining (NHEJ) or homology directed repair (HDR) (Fig. 4) (reviewed in refs. [47, 48]). The NHEJ pathway is intrinsically error prone and may cause combinations of insertions, deletions and substitutions at sites of DSBs. By introducing DSBs designer nucleases may therefore be used to introduce disabling mutations at an intended target. When homologous sequences are introduced into target cells together with DSB-inducing nucleases, HDR may be used to restore gene function, as has been applied to the

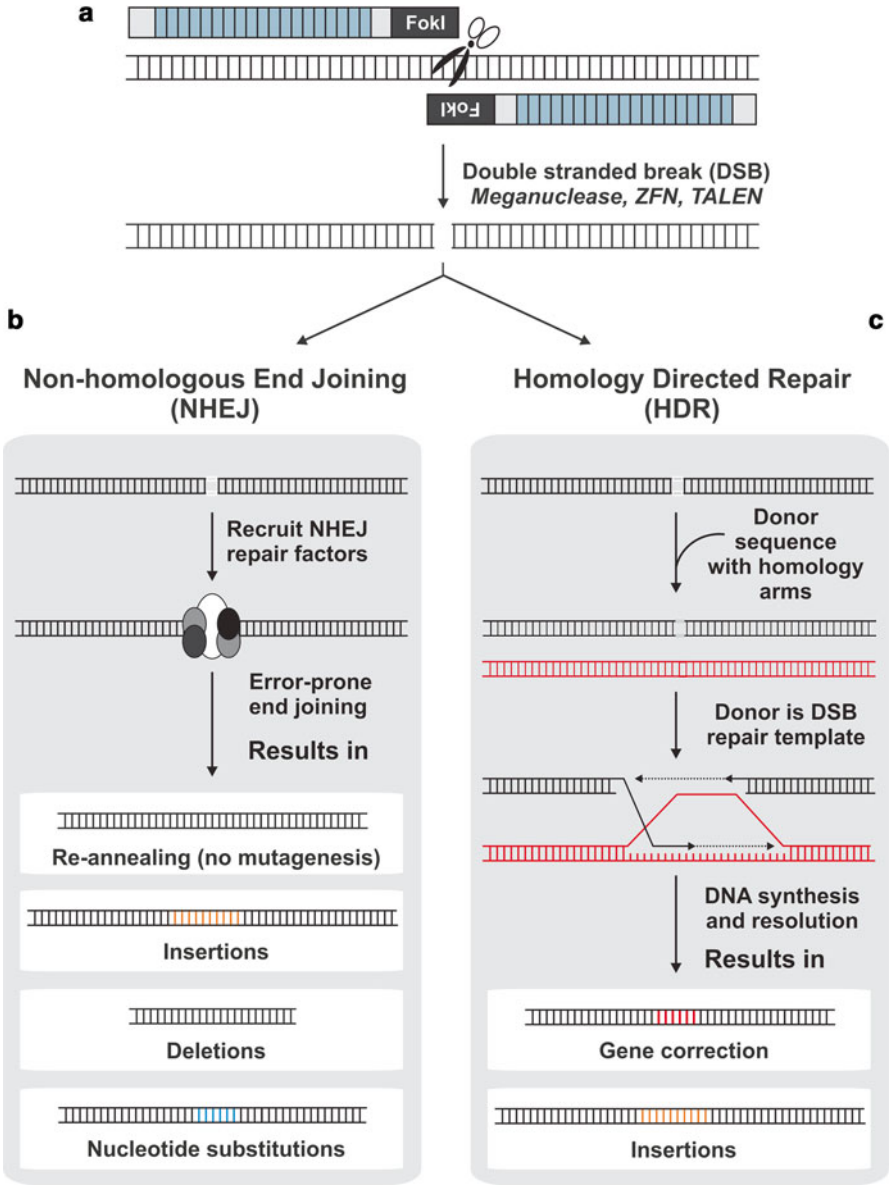

Fig. 4 Cellular repair pathways used during nuclease-mediated mutagenesis or gene correction. (**a**) TALEN dimerisation, depicted here, leads to cleavage at the DNA target sites and formation of DSBs, which are typically repaired by non-homologous end joining (NHEJ) (**b**) or homology-directed repair (HDR) (**c**). Meganucleases or ZFNs may also cleave double stranded DNA to initiate NHEJ or HDR. When NHEJ is triggered, DSB repair factors are recruited to the site. Repair may lead to restoration of the wild-type sequence or introduction of mutations. Abbreviations are spelled out in the legend to the figure to maintain consistency with the other figures. (insertions, deletions or substitutions), which occurs commonly when repeated cleavage occurs at a target site. If donor sequences with homologous regions flanking the DSB site are introduced, the HDR pathway may be triggered to repair or insert a sequence at the DSB

treatment of monogenic inherited diseases [49]. Gene correction, particularly for the treatment of monogenic disorders, has been a major focus of designer nuclease research [50]. However, the intentional disruption of pathology-causing viral DNA has generated considerable enthusiasm for this approach to viral gene therapy (reviewed in ref. [51]). Viruses that are capable of establishing latent infections, such as herpes simplex virus (HSV), human immunodeficiency virus (HIV) and HBV, are prime candidates for nuclease-mediated targeted disruption. Since HBV has a small compact genome arrangement with restricted sequence plasticity, it is suited to this gene therapy approach. Currently there are three different types of designer nucleases that have been used to target HBV cccDNA: homing endonucleases, otherwise known as meganucleases (Fig. 3a); zinc finger nucleases (ZFNs) (Fig. 3b) and TALE nucleases (TALENs) (Fig. 3c). Recent research on protein-RNA-based CRISPR (Clustered Regulatory Interspaced Short Palindromic Repeats) and CRISPR-associated (Cas) nucleases [52, 53] to modify genomic targets has been particularly vigorous. CRISPR/Cas nucleases have, however, not yet been used against HBV, but this topic is no doubt under investigation.

Homing endonucleases, also known as meganucleases, are DNA-specific cleavage proteins that were identified within mobile genetic elements of yeast (Fig. 3a) [54, 55]. They target DNA sequences ranging from 12 to 40 nucleotides in length, and have successfully been used to trigger HDR in eukaryotic cells [56]. Although homing endonucleases may be engineered to cleave defined DNA targets, the number of possible binding sites is limited by constraints of the effectiveness of motifs targeting these predefined targets. Despite this, homing endonucleases have recently been shown by Cellectis Bioresearch (Paris, France) to have therapeutic potential for treatment of HSV infection [57]. This group has also patented anti-HBV meganucleases (WO/2010/136981 and US2012/0171191A1) but published results of these studies are not yet available. Whilst meganucleases have natural endonuclease activity, ZFNs and TALENs are generated by fusing a *Fok*I nuclease domain to the C-terminus of a ZFP or a TALE array (Fig. 3b, c) [58, 59]. The *Fok*I enzyme exists as a monomer but requires interactions between the catalytic domains of two monomers to enable cleavage of double stranded DNA [60, 61]. For this reason, ZFNs and TALENs are designed in pairs comprising so-called left and right subunits. Although both subunits are required for efficient cleavage, the catalytic activity of the *Fok*I nuclease domain itself may function in a potentially genotoxic sequence-independent fashion.

ZFN DNA-binding domains typically include ZFPs, with left and right subunits that each target sequences of between 9 and 12 base pairs [45]. The DNA binding sites are separated by a six base pair spacer region to enable *Fok*I dimerisation and induction of DSBs at intended target sites. This configuration has been shown to cleave endogenous human genes efficiently [62, 63]. In an assessment of antiviral efficacy, Cradick and colleagues investigated the ability of engineered ZFNs to cleave HBV DNA targets in vitro [64]. The ZFNs were designed to bind to two 9 base pair target sequences within the *core* ORF that overlapped with the common polyadenylation site. Disruption of viral DNA targets was verified following co-transfection of Huh7 cells with plasmids encoding ZFN pairs and an

HBV replication competent plasmid. In addition to inhibiting pgRNA transcription, the anti-HBV ZFNs cleaved 36 % of DNA targets. Sequencing indicated that site-directed mutations occurred in 6 % of HBV targets, of which 81 % produced frameshift mutations. Although the activity of these ZFNs on episomal cccDNA was not determined, this important study was the first to describe targeted disruption of HBV DNA as a potential gene therapy.

Recently designer nucleases have been re-engineered by substituting ZFPs with the plant bacterial TALEs as an alternative DNA binding domain (Fig. 3c). TALEs, which are transcription activators of host avirulence genes, are natural pro-survival proteins produced by the *Xanthomonas* plant pathogens [65]. Unlike ZFPs, which require specific context-dependent arrangement of each protein subunit for effective DNA binding [66, 67], a single monomer within the TALE DNA binding domain confers individual nucleotide specificity with little effect of neighbouring monomers [68, 69]. As a result, TALE monomers can be conveniently concatamerised to form sequence-specific DNA-binding domains that may be fused to the *Fok*I nuclease domain [70]. Individual TALE monomers comprise approximately 34 amino acid polypeptide chains and TALE arrays are made up of tandem repeats of these monomers. Each monomer varies at amino acid positions 12 and 13, and these repeat variable diresidues (RVDs) confer specificity of binding by the TALE sequences to their DNA targets [68, 69, 71]. TALEN subunits are typically designed to target 19 base pair sequences. This confers higher sequence specificity than ZFNs, and may also account for their improved activity in vitro [72]. There are several commercial and publically available techniques currently used to assemble designer TALE (dTALE) arrays. Two commercial companies, Cellectis Bioresearch (Paris, France) and Life Technologies (New York, USA), offer design and synthesis services whilst several publically available methods have successfully been used to generate TALE arrays and TALENs [70]. As with the ZFNs, TALENs have been used to target multiple endogenous genes [73–75; however, the antiviral efficacy of TALENs has only recently been described [76, 77].

Disrupting the HBV cccDNA Minichromosome

Building on the success of the anti-HBV ZFNs [64], HBV-specific TALENs were investigated for their efficacy against viral DNA [76]. TALENs were engineered to target multifunctional sites within the *surface* (S), *core* (C), and *polymerase* (P1 and P2) ORFs. Co-transfection experiments, conducted using cultured liver-derived Huh7 cells, showed that the S-TALEN inhibited HBsAg secretion by 80 %. This result was corroborated by studies on the HepG2.2.15 cell line, which stably and constitutively produces HBV. In these cells, the S-TALEN-encoding sequences mediated inhibition of HBsAg secretion by approximately 60 % and caused targeted disruption of 31–35 % of cccDNA copies. The C-TALEN caused 12 % targeted disruption of cccDNA and predictably did not inhibit HBsAg secretion. Although TALENs provide an efficient means of disrupting HBV DNA sequences,

cytotoxicity resulting from non-specific targeting is possible and may cause off target mutagenesis and cell death [78, 79]. A genome wide analysis confirmed that no potential off-target binding sites for P1-, P2-, S- and C-TALENs were found in either the mouse (*Mus musculus*) or human genomes. This finding is in line with the lack of cytotoxicity that was observed when treating cells with the panel of HBV-directed TALENs [76]. Moreover, multiple alignments of 26 different HBV genotypes and sub-genotypes showed high sequence homology at the selected TALEN target sites. This is particularly true for the S TALEN, and suggests that the HBV-targeting nucleases may be effective across HBV genotypes.

To establish whether TALENs were capable of inactivating HBV replication in vivo, the anti-HBV efficacy of the S and C TALENs were investigated in a murine model of HBV replication [76]. This was achieved by hydrodynamic tail vein injection of both TALEN and HBV-encoding plasmids. Importantly, murine hepatocytes do not support cccDNA formation [80] and the TALEN effects were a result of their action on the co-injected HBV replication-competent plasmid. In vivo, the S-TALEN inhibited HBsAg secretion by 95 % and induced disruption in 58–68 % of intrahepatic HBV DNA targets [76]. The C-TALEN inhibited HBcAg expression and induced disruption in 62–87 % of intrahepatic HBV DNA targets. Serological analysis showed a reduction in circulating virions and no apparent liver toxicity. Deep sequencing at the S- and C-TALEN binding sites showed targeted mutation of HBV that was specific to mice that had been treated with anti-HBV TALENs. As expected, deletions were predominantly detected at both the S- and C-TALEN target sites [81]. This effect is distinct from that of ZFNs, which may give rise to a combination of insertions, deletions and substitutions.

The therapeutic potential of anti-HBV TALENs has recently been corroborated by Chen and colleagues [77]. By engineering TALENs to bind to the *core* ORF and the RNaseH region of the *polymerase* ORF, significant knockdown of HBeAg, HBsAg and pgRNA levels in Huh7 cells could be achieved. This antiviral effect was observed for genotype B, C and D isolates, supporting the notion that TALENs designed to target conserved regions of HBV may be effective against several genotypes. Efficacy was also confirmed in vivo when using murine hydrodynamic injection. Importantly, this study also showed a synergistic antiviral effect when combining TALENs with INF-α in Huh7 cell cultures. As INF-α is a licensed HBV therapeutic, using TALENs in combination with other drugs is an interesting approach to improving treatment efficacy.

Transcriptional Gene Silencing with rTALEs

The endonuclease domains of TALENs and ZFNs may also be substituted with other effector proteins to enable gene-specific transcriptional regulation. Naturally, *Xanthomonas* TALEs contain activation domains, which increases transcription from specific plant gene promoters through association with the DNA-targeting repeat domains. After secretion from the bacteria, the TALEs enable survival of the

bacteria through transcriptional activation of otherwise silent genes [65, 82–84]. As the TALE array can be engineered to bind to any target sequence, these transcriptional activators have been used in mammalian cells to trigger human gene expression [85–88]. As an alternative, TALE DNA binding arrays may be fused to gene repressors. These repressor TALEs (rTALEs) have recently been shown to be potent inhibitors of endogenous mammalian gene transcription [86, 87]. Different repressor domains have been fused to DNA-binding TALEs. Amongst the inhibitors of gene expression, the mSin interaction domain (SID) and KRAB domain silenced gene activity most efficiently [86, 87]. KRAB repressors occur naturally as zinc finger fusions [89], and are the largest group of mammalian transcriptional regulators (Fig. 2b). Although the exact mechanism of KRAB transcriptional repression has not been completely elucidated, KRAB-ZF protein binding results in the recruitment of heterochromatin forming complexes, which lead to gene silencing [90].

When studying anti-HBV efficacy of TALENs, it was found that one of the nucleases under investigation, the P1-TALEN, inhibited markers of viral replication without causing detectable mutation at the target site [76]. Since the HBV enhancer I sequence overlaps with the P1-TALEN target, it is likely that this TALEN inhibits viral replication through transcription inhibition rather than by mutating protein-coding sequences. In the same way that ZFPs were shown to inhibit DHBV transcription [46], transient transcriptional repression with TALENs is likely to involve competitive binding at the target site without any endonuclease cleavage. To investigate this further, we generated KRAB-rTALEs from the P1 and P2 left and right TALE DNA binding arrays (unpublished data). As with naturally occurring repressor proteins containing the KRAB domain, these rTALEs were designed with the repressor domain at the N-terminus (Fig. 2b). Individually, the left and right P1 rTALEs inhibited HBsAg expression in Huh7 cells following co-transfections with an HBV replication competent plasmid. Furthermore, *surface* and *core* mRNA concentrations were reduced by up to 80 %, suggesting the anti-HBV effect is a result of transcriptional inhibition. The HBV Enhancer I sequence regulates viral protein expression through binding of key hepatocyte transcription factors (reviewed in [17, 91, 92]). The P1 left rTALE is likely to inhibit the binding of retinoic acid response element (RARE) or regulatory factor X1 (RFX1), which in turn may prevent the cooperative binding of hepatocyte nuclear factor 4 (HNF-4) and retinoid X receptor alpha/peroxisome proliferator-activated receptor (RXRα/PPAR) heterodimers. The P1 right rTALE subunit, however, targets a region of the LSR element of Enhancer I motif that is essential for HBV gene expression. This sequence contains *cis*-elements that bind several hepatocyte transcription factors and is also involved in activation of *HBx* gene expression [93]. The Enhancer I motif operates in conjunction with Enhancer II, to increase surface protein expression [94]. As both the P1 left and P1 right rTALEs inhibit HBsAg expression, it is likely that these proteins are obstructing RARE and RFX1 binding or LSR regulation, and consequently inactivating the Enhancer I motif. Moreover, rTALE-induced formation of heterochromatin on the cccDNA may disable viral transcription permanently. Although TALENs are a promising therapeutic for the targeted inactivation of cccDNA, unwanted mutations and chromosome translocations are a concern [95]. HBV DNA

is frequently integrated into the genomes of carriers of the virus, and TALENs acting at these sites may cause unwanted genotoxicity. By using transcriptional repression with rTALEs, instead of introducing targeted DSBs with TALENs, the likelihood of introducing undesirable mutagenic events may be diminished.

Conclusions

To date treatment with IFN-α or its pegylated derivatives has been the only intervention capable of eradicating HBV from infected cells [96]. The treatment is, however, only effective in a small subset of HBV carriers. Moreover use of IFN-α may be complicated by side effects, is contraindicated in several cases, and is expensive. Nucleoside and nucleotide analogues may effectively suppress viral replication but do not eliminate cccDNA and treatment withdrawal is associated with reactivation of HBV replication. With the advent of technology enabling the engineering of designer nucleases and transcriptional repressors, methods of efficiently silencing HBV replication by inactivating cccDNA have been added to the arsenal of potential anti-HBV therapies. Currently, the use of TALENs against HBV appears to be the most efficient. Although not yet reported, information on the utility of HBV-targeting CRISPR-Cas derivatives will be an interesting development.

Studies on viral resistance in cultured cell have been hampered by the lack of convenient models that simulate all steps of viral replication. Most reports describing anti-HBV therapy have employed replication competent plasmids or cell lines with an integrated copy of the viral genome. These strategies rely on assessing silencing of a single viral sequence and consequently provide little information on viral resistance. The discovery by Yan and colleagues that HBV uses the NTCP to infect hepatocytes may address this problem [14]. Cells ectopically expressing NTCP are permissive for HBV infection and should allow for analysis of emergence of resistance in response to treatment. Furthermore, as NTCP-expressing human cultured cells support cccDNA formation they will provide a useful model to assess the effect of anti-HBV therapeutics on cccDNA. Unfortunately, since mice do not synthesise cccDNA [80], NTCP-transgenic murine models will not recapitulate the entire HBV replication cycle.

Although results from testing anti-HBV nucleases and repressors have generated significant enthusiasm, significant hurdles need to be overcome before they are used in a therapeutic context. Safe and efficient delivery of sequences encoding anti-HBV nucleases is particularly challenging. The size of sequences encoding individual TALEN subunits (approximately 4 kb) is at the limit of the transgene capacity of single stranded adeno-associated viral (AAV) vectors. Delivery of complete TALENs with these favoured vectors would therefore require simultaneous administration of two recombinant viruses. Although constrained by the compact nature of its genome, selection of HBV escape mutants may be possible. It remains to be determined whether mutations within the HBV genome can confer resistance to TALEN- or rTALE-mediated silencing and if so, whether resistance can be prevented

by simultaneously targeting the virus with multiple engineered DNA-binding proteins. The duration of expression of anti-HBV TALENs or rTALEs that is required to disable cccDNA completely is not yet known. Also, off target and long-term effects of expressing TALENs or rTALEs on hepatocytes need to be characterised. Thorough analysis of these topics will be required before this powerful technology is implemented as a therapeutic modality. Despite the unanswered questions, use of derivatives of engineered sequence-specific DNA binding proteins is an interesting novel strategy. Used alone or in combination with other antiviral approaches, such as RNAi and existing licensed therapies, they have the potential to improve the management of chronic carriers significantly.

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U1 interference (U1i) for Antiviral Approaches

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Abstract U1 snRNP (U1 small nuclear ribonucleoprotein) is an essential component of the splicing machinery. U1 snRNP also plays an additional role in 3′-end mRNA processing when it binds close to polyadenylation sites (PAS). Cotranscriptionally, U1 snRNP binding close to putative PAS prevents premature cleavage and polyadenylation and consequently safeguards pre-mRNA transcripts and defines promoter directionality. At the 3′-end of mRNAs, U1 snRNP binding to putative PAS may regulate mRNA length or inhibit polyadenylation and, therefore, gene expression. U1 interference (U1i) is a technique to inhibit gene expression based on the property of U1 snRNP to inhibit polyadenylation. It requires the expression of a modified U1 snRNP, which interacts with a target gene upstream of its PAS and inhibits target gene expression. U1i has been used to inhibit the expression of reporter or endogenous genes both in tissue culture and in animal models. In addition, U1i combination with RNA interference (RNAi), another RNA-based gene silencing technology, results in a synergistic increased inhibition. This is of special interest for antiviral therapy, where strong inhibitions may be required to decrease the expression of replicative viral RNAs and impact the replication cycle. Furthermore, the combination of U1i and RNAi-based inhibitors should prevent the appearance of viral variants resistant to the treatment and allows the dose of inhibitors to be decreased and a functional inhibition to be obtained with fewer off target effects. In fact, U1i has been used to inhibit the expression of HIV-1 and HBV, whose viral genomes express mRNAs that must be polyadenylated by the nuclear polyadenylation machinery. In the case of HBV, antiviral U1i has been combined with RNAi to demonstrate a strong inhibition of expression from HBV sequences in vivo. This shows that, although several aspects of U1i technology remain to be addressed, U1i and U1i combined with RNAi have great potential as antivirals.

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Introduction: U1 Structure and Function

U1 small nuclear ribonucleoprotein (U1 snRNP) is an essential component of the spliceosome, the large complex that catalyzes precursor mRNA (pre-mRNA) splicing. U1 snRNP is composed of a 164 nucleotide (nt) long U snRNA molecule bound by a set of seven Sm proteins (B, D1, D2, D3, E, F, and G) and three U1-specific proteins (U1-70K, U1A, and U1C) (Fig. 1). The U1 snRNA structure consists of four stem-loops (I–IV) and a short helix (H). U1 snRNP biogenesis starts with U1 snRNA transcription by RNA polymerase II as a snRNA precursor that moves to the cytoplasm, where it interacts with the Sm proteins, modifies the 5′ cap and undergoes 3′-end maturation. The U1 snRNP is then imported into the nucleus where it is internally modified and associates with the particle specific proteins [1].

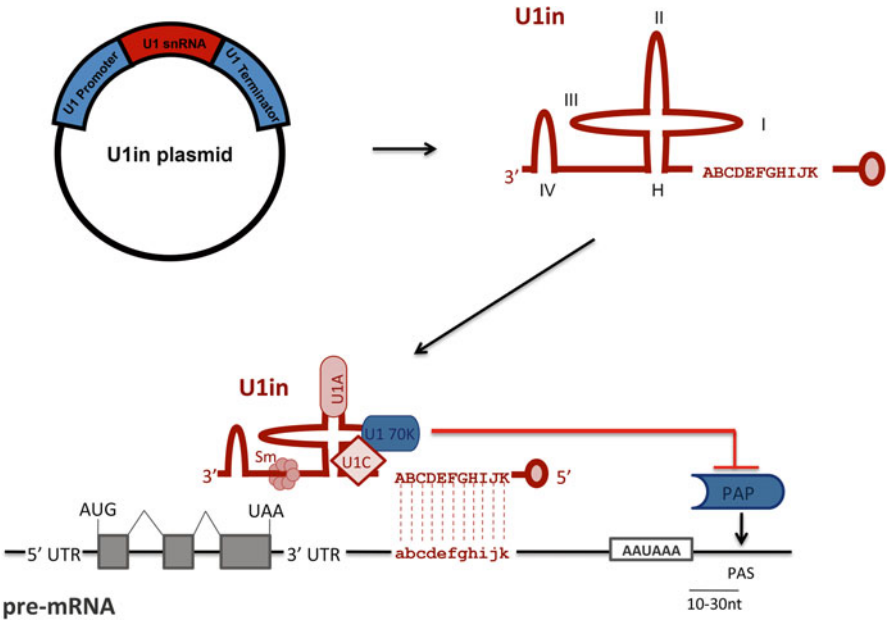

Fig. 1 U1i mechanism of action. Pre-mRNA polyadenylation involves the cleavage of nascent pre-mRNA in the polyadenylation site (PAS) and the addition of a poly-A tail to the cleaved 3′ end of the pre-mRNA molecule. The 3′UTR of the pre-mRNA contains several cis-acting RNA elements required for correct polyadenylation. In mammals, A(A/U)UAAA element is located 10–30 nt upstream of the PAS. In U1i, an exogenous U1 inhibitor (U1in) with a modified 5′-end is expressed from an U1in plasmid. The U1in RNA molecule, similar in structure to endogenous U1 snRNA (stem loops I, II, III, and IV and helix H) is bound by U1 snRNP proteins (U1-70K, U1A, U1C, and Sm) during cellular processing. U1in interacts with its target sequence located in the 3′ terminal exon of the pre-mRNA upstream of the PAS. Cleavage in the PAS occurs correctly. However, the U1-70K component of U1 snRNP binds to the carboxy-terminal region of mammalian poly-A polymerase (PAP) and inhibits polyadenylation. Non-polyadenylated targets are unstable and are rapidly degraded, leading to gene expression inhibition

U1 snRNP plays an essential role in defining the 5′ splice-sites (5′ss) of pre-mRNAs in the splicing reaction. 5′ss are recognized through RNA: RNA interaction with the 5′ end of U1 snRNA. The modest complementarity of U1 snRNA with the natural 5′ss sequences results in a mild affinity which is enhanced by the action of several proteins, including the U1-70K, U1-C and members of the SR (serine-arginine-rich) protein superfamily [2, 3]. U1 snRNP binding is followed by the formation of the catalytic core of the spliceosome.

In humans, the gene that transcribes for U1 snRNA is present in multiple copies within the genome [4] and is expressed at an estimated copy number of ~10^6 molecules per HeLa cell [5]. Although all the snRNPs in the spliceosomal complex come together in 1:1 stoichiometry [6], U1 snRNP is much more abundant than the other snRNPs in higher eukaryotes [5]. This suggests that U1 snRNP may have other functions besides its role in splicing. Indeed, U1 snRNP plays relevant roles in other processes that regulate gene expression such as transcription and 3′ end processing. It has been described that splicing could promote transcription by enhancing the recruitment of general transcription factors to promoter-proximal 5′ splice-sites [7, 8]. In addition, U1 snRNA, but not other U snRNAs, might modulate transcription efficiency independently of splicing, through its association with transcription factor TFIIH and stimulation of TFIIH-dependent reinitiation of productive transcription [9].

U1 snRNP also regulates 3′-end processing, a posttranscriptional modification of pre-mRNA which is performed together with 5′ capping and splicing in the nucleus. 3′-end processing involves the cleavage of nascent pre-mRNA at the polyadenylation site (PAS) and the addition of a poly-A tail to the cleaved 3′-end of the pre-mRNA. U1 snRNP can inhibit 3′ end processing by binding to cis-elements located upstream or downstream of a PAS. This mechanism was first described in bovine papillomavirus type 1 (BPV-1), where U1 snRNP binding to a consensus 5′ ss upstream of the PAS posttranscriptionally inhibits the expression of viral late transcripts at early times post-infection. The inhibition is mediated by the SR domain of U1-70K, which inhibits mammalian Poly-A-polymerase (PAP), the enzyme that catalyzes poly-A tail addition. Viral pre-mRNAs are cleaved but not polyadenylated and are therefore unstable [10, 11]. Other viruses also use U1 snRNP binding to control 3′-end processing. In Human papillomavirus type 16 (HPV-16) U1 snRNP binding together with CUG-BP1 upstream of the PAS inhibits late gene expression soon after infection [10, 12]. In HIV-1, U1 binding downstream of a PAS located at the 5′LTR inhibits pre-mRNA cleavage and thus allows synthesis of the full-length genomic RNA, essential for viral replication [13]. U1 snRNP binding downstream of PAS also inhibits cleavage rather than polyadenylation in vitro [14], but to date the mechanism by which U1 snRNP inhibits cleavage remains unknown.

U1 snRNP binding also represses 3′-end processing in eukaryotic genes resulting in an overall protection of the transcriptome from premature cleavage and polyadenylation (PCPA), independently of its role in splicing [15]. U1 snRNP binds cotranscriptionally to consensus or cryptic 5′ss located throughout pre-mRNAs (PAS are found every ~1,000 nts and frequently in introns) and inhibits PCPA.

This is essential for the proper processing of most cellular mRNAs and indeed, U1 snRNP depletion results in an accumulation of prematurely cleaved transcripts that terminate only few (1–5) kb away from the start of transcription [15]. Moreover, a partial decrease of U1 snRNP levels by only 10–50 %, which could occur naturally after certain stimuli, results in the selection of premature 3′-end processing sites and in the generation of shorter mRNA isoforms [16]. These shorter isoforms are expected to bind to fewer regulatory factors and, in some cases, encode for proteins with different carboxy-termini. Interestingly, such short isoforms have been described in activated neuronal and immune cells, stem cells and cancer [16]. Furthermore, U1 snRNP inhibition of premature cleavage also defines promoter directionality at divergent promoters [17]. RNAPII complex undergoes the initial phase of elongation in both sense and antisense direction but transcription in the antisense direction terminates early. It has been recently reported that sense genes are enriched in U1 snRNP binding sites whereas the antisense regions contain many cleavage signals [17, 18]. Thus, antisense transcripts are frequently terminated by PAS-directed cleavage shortly after initiation whereas U1 snRNP protects sense RNA from PCPA in promoter-proximal regions [17]. It has also been described that when U1 snRNP regulated cryptic PASs located proximal to promoters (<500 base pairs) are activated, there is a negative impact on transcription rates [19].

Finally, some cellular mRNAs contain conserved U1 snRNP binding sites in their 3′ UTRs, according to bioinformatic analyses [20]. The best hit is U1A mRNA, where U1 snRNP and U1A bind synergistically to inhibit U1A mRNA 3′ end processing [21]. Interestingly, it has been recently described that a point mutation that creates a functional 5′ ss in the 3′ UTR of the gene encoding *p14/robld3* is the cause of a complex immunodeficiency syndrome [22]. U1 snRNP binding at this new site inhibits p14/robld3 mRNA 3′ end processing showing that U1 snRNP activity can be deleterious if misled. The exact mechanism of how U1 snRNP interferes with 3′ end processing remains unclear, but U1 snRNP seems to interfere with cleavage or PAS selection instead of directly inhibiting PAP, as previously reported [15].

U1 Interference (U1i)

U1 interference (U1i) is a technique to inhibit gene expression based on the property of U1 snRNP to inhibit 3′ end processing. To achieve inhibition of a given gene, a target sequence located in the 3′ terminal exon of the gene of interest must be chosen. Then, the 5′ end of the U1 snRNA, which is used in 5′ss recognition, should be replaced by a sequence complementary to the chosen target sequence [23, 24] (Fig. 1). This is performed by standard cloning in a plasmid expressing U1 snRNA gene. The 5′-end modified U1 snRNA, herein referred to as U1 inhibitor (U1in), has to be expressed in the cell, where it matures in the same way as endogenous U1 snRNP and localizes to the nucleus. Upon standard transfection of a plasmid expressing the U1in, exogenous U1 represents 1–8 % of the endogenous U1 snRNP [23]. Finally, the U1in should interact with the target sequence and inhibit

polyadenylation (Fig. 1). Cleavage in the PAS of transcripts targeted by U1ins occurs correctly [23], but there is an increase in the relative accumulation of non-polyadenylated targets [25]. The inhibitory mechanism involves the blockage of PAP by U1-70K. Indeed, mutations in the U1 snRNA binding site for U1-70K abolish inhibitory activity [23, 24, 26]. The non-polyadenylated targets are unstable, but the level of target RNAs decreases in both the nuclear and cytoplasmic compartments, since targeted mRNAs could be exported to the cytoplasm [23]. In all cases tested, U1ins neither activate aberrant splicing nor alternative polyadenylation, since no aberrant target transcripts are visible by protection assay, Northern-blot or RT-PCR analysis [23, 25, 27].

U1snRNP as a Therapeutic Tool

U1ins have been shown to inhibit both endogenous and exogenous genes in tissue culture (when expressed either stably or transiently) and in mice. Therefore, U1i can be applied to inhibit genes of therapeutic relevance.

In tissue culture, the inhibitory activity of U1i was first tested using cellular U1 snRNP and luciferase reporter genes with U1-complementary sequences in their 3′ UTR. This resulted in a 15- to 30-fold decrease of luciferase activity compared to controls. The decrease was observed in a variety of mammalian cell lines, from 12 to 72 h after transfection and even when the amount of transfected reporter gene varied over a 1,000-fold range [24, 25]. Likewise, several reporter genes (such as β-gal, CAT, GFP, or *Renilla* or *Firefly* luciferases) have been targeted with exogenous U1ins expressed from a plasmid [23–25, 27]. U1i can also inhibit the expression of endogenous genes. U1ins designed to target a 10-nt long sequence in the 3′ terminal exon of endogenous mRNAs such as aryl sulfatase A or human chorionic gonadotropin decreased gene expression two to eightfold when transiently expressed [25, 28]. In these cases, the degree of downregulation observed varies with the potency of the U1in tested but also with the amount of inhibitor expressed, the transfection efficiency and the stability of the evaluated protein. Moreover, the stable expression of U1ins that target endogenous genes leads to a permanent reduction in the expression of these genes. For instance, stable expression of U1ins targeting osteocalcin and collagen 1a1 decreased the mRNA levels of these cellular genes for long-term 15- to 30-fold, respectively [25].

Suppression of reporter or endogenous genes by U1i is also feasible in vivo [29, 30]. Expression of U1ins could be achieved by hydrodynamic injection of U1in plasmids which allow a transient expression of U1ins in mouse liver [29, 30]. For reporter genes, hydrodynamic co-injection of U1in plasmid with a firefly luciferase reporter plasmid has shown a 25 % decrease in luciferase activity in mouse liver [29]. Hydrodynamic injection was also employed to evaluate the inhibitory activity of U1ins that target Notch 1 endogenous gene. Since hydrodynamic injections only transduce 10–30 % of the hepatocytes, Notch 1 downregulation was evaluated by measuring the expression of a luciferase reporter whose expression depends on

Notch 1 [31]. Four days after co-injection of reporter and U1in plasmids, Notch 1-dependent luciferase activity decreased to 56.5 %, demonstrating that U1i also inhibits expression of endogenous genes in vivo [30]. The evaluation of long-term inhibition by U1i requires the use of viral vectors to express U1ins. Among viruses, recombinant adeno-associated virus (AAV) vectors have been the vector of choice for long term transgene expression in several in vivo studies. Therefore, AAV vectors have been used to express U1ins that target luciferase reporter gene in murine muscle. The results show exogenous U1 snRNA expression in transduced muscle at 8 weeks post-transduction without a significant decrease in the luciferase targeted gene [29]. The lack of efficacy might be due to the low potency of the U1in employed in these experiments [29]. Importantly, long-term U1in expression in vivo did not lead to detectable toxicity and therefore, these results can be considered as the first proof of principle for the in vivo use of U1i.

Rules for U1i

To achieve a powerful inhibition with U1i several aspects should be taken into consideration:

1. U1i only inhibits the expression of RNAs polyadenylated by the nuclear polyadenylation machinery [25].
2. The U1in binding site must be located in the 3′ terminal exon upstream of the PAS. When the U1in target site is upstream of an intron, inhibition is lost for unknown reasons [25, 27]. The distance between the PAS and the upstream U1in binding site can be as large as 1,000 nt [16, 25]. When the U1in target site is downstream of the PAS, cleavage instead of poly-A tail addition could be inhibited. U1 induced cleavage inhibition can also lead to the inhibition of gene expression or to the selection of a PAS located downstream. This has not been studied in detail with U1in.
3. Although silencing can be detected with target sites which are only 8 nt long, the best inhibitions are obtained with 10–11 nt long target sites. Increases in the length up to 16 nt have slight effects on the efficiency of the inhibition [24, 25, 27].
4. In the U1in, the target binding site must be in nucleotides 1–2 to 11. Expansion of the hybridized region into the first stem of U1 snRNA abrogates the inhibitory effect. Similarly, U1ins whose 5′ ends have been extended to increase the binding size, mature properly and are expressed to similar levels as standard U1ins, but they are poor inhibitors [24, 25, 27]. The U1in is expressed from a plasmid that contains the U1 snRNA gene under a U1 snRNA promoter and termination sequences (Fig. 1). The U1 snRNA gene in the U1in plasmid is identical to the cellular human U1 snRNA gene except for two cytosines that base pair with two guanines in the stem III of U1 snRNA, which have been interchanged to allow proper quantification of the U1in [23, 30]. Replacement of U1 promoter by standard polymerase II promoters results in inactive U1ins [32].

5. Target sequences should be accessible. U1in binding sites located in a secondary stem structure are not functional for inhibition [25]. Accessible target sequences can be identified with the help of some bioinformatic tools. For instance, Mfold Web Server gives direct evidence of accessibility [33], whereas others such as Spectral Repeat Finder (SRF) [34], prediction programs that identify target sites for miRNAs or the rules described by McQuisten and Peek to identify sequence motifs associated with antisense activity [35] are indirect indicators of good accessibility and U1i potency.
6. Target sequences should not be cryptic donor splicing sites, since this might induce removal of the U1in during spliceosome formation. To avoid this, target sequences should not contain A, G, and T at positions 2, 8, and 9, respectively [24, 32].
7. Target neighbor sequences may affect inhibition. Nearby sequences that recruit factors which increase U1 binding (such as U-rich sequences downstream of U1in sites that bind TIA-1), may increase U1i specificity. On the contrary, although rare, SR binding sites near the U1in site should be avoided, since they impede binding of U1-70K to PAP [11, 24].
8. Target sequences should be specific for the 3′ terminal exon of the target transcript to avoid off-target effects. To circumvent redundancy, the presence of the target sites in the 3′ terminal exon of unrelated genes should be checked using Basic Local Alignment Search Tool (BLAST) [36].
9. Duplicated U1in binding sites might give increased synergistic inhibitions [24, 25, 37]. Likewise, synergistic inhibitions might also be obtained if two different U1ins that target different sequences in the same 3′ terminal exon are used [25].

U1i Specificity

Based on the results of the experiments performed to date, U1i seems to be a specific gene expression inhibition technique. Thus, single mutations abrogate U1in binding and therefore target inhibition [24]. In vivo, the exchange of the two central nucleotides of a functional U1in binding site inhibits gene silencing [30]. In addition, U1ins are able to distinguish between two target sequences that differ by one or two nucleotides. Therefore U1i can potentially be applied for allele-specific inhibition of a transcript encoding a dominant negative or a gain-of-function mutation [27]. However, not all the point mutations in the target sequence affect the efficiency of U1i equally well. Extensive mutagenesis of a 10 nt-long target sequence in a reporter gene for endogenous U1 snRNP showed that any single mismatch different from GU at the central core of the U1in target sequence (positions 3–8) destroys silencing. Therefore, it is important to avoid G and U nucleotides in internal positions of the U1in binding site, since they would lower U1i specificity. However, mismatches within the 2 nt-long lateral sequences give partial silencing [24]. Although these results suggest that off-target inhibition could occur, the fact that the target site has to be in unstructured regions of the 3′ terminal exon decreases this possibility.

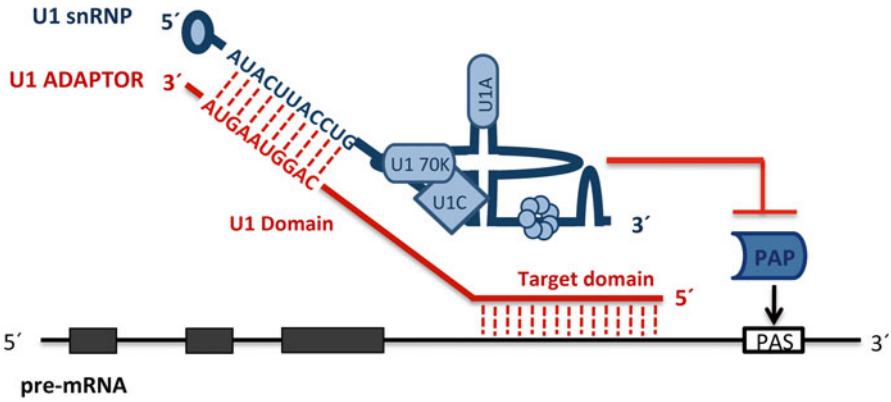

Fig. 2 U1 adaptors. U1 adaptors are bifunctional synthetic oligonucleotides with a target domain that hybridizes with the 3′ UTR of the target transcript and a U1 domain that binds endogenous U1 snRNA. U1 adaptors tether U1 snRNP to the terminal exon of the target pre-mRNA, promoting U1-70K mediated inhibition of nuclear PAP. Inhibition of poly-A tail addition at PAS suppresses pre-mRNA maturation and induces target destabilization

To date, several cells lines that stably express different U1ins have been generated [25, 38]. These cells have normal growth parameters and normal morphology. Furthermore, short-term expression of several U1ins does not lead to an increase in the expression of interferon stimulated genes or to a modification in the cellular level of endogenous U1 snRNP (Abad et al., unpublished results). Finally, U1ins have been expressed in mouse liver over the long-term without detectable toxicity [29]. Therefore, U1i seems to be a specific technique. However, high-throughput experiments are still required to analyze the overall specificity of U1i, since U1ins may affect the expression of non-target genes by unspecific target recognition, or by affecting transcription, mRNA length or mRNA stability [8, 9, 16, 39].

Other U1ins

Besides U1ins, other modified-U1 snRNPs have been designed to modulate gene expression. 5′ end modified U1 molecules can be used to selectively disturb splicing of target gene transcripts and thus induce a decrease in gene expression [40, 41]. This is different from the use of 5′-end modified U1 snRNAs that restore correct pre-mRNA splicing to overcome disease-generating mutations [42, 43]. To avoid the inconvenience of preparing custom U1i expressing plasmids, a class of synthetic oligonucleotides named U1 adaptors, has been developed [20]. As they are synthetic molecules, they can be modified to increase affinity, specificity, stability, biodistribution or efficacy. U1 adaptors are bifunctional oligonucleotides with a “target domain” complementary to a site in the target gene and a “U1 domain” that binds to the U1 snRNA (Fig. 2). Thus, U1 snRNP is tethered to the target pre-mRNA by the

U1 adaptor and inhibits poly-A tail addition. Although the use of U1 Adaptors might lead to "off-target" silencing effects as a result of sequestration of endogenous U1 snRNP when used at high doses [44], recent results demonstrate that very low doses of two U1 Adaptors targeting BCL2 and GRM1 suppress growth of human melanoma xenografts in vivo with little toxicity [45].

U1i Combination with RNAi

As previously explained, U1i specificity still needs to be addressed in detail using high throughput experiments, similar to what has been done with RNA interference. In the case of RNAi, it has been described that siRNAs lead to dose-dependent off-target effects [46–48]. Since RNAi and U1i processing machineries are independent of each other, both techniques could be combined to obtain increased inhibition and allow a decrease in the dose of the inhibitors and thus reduce the risk of off-target effects. This was first tested in cell culture for the inhibition of reporter genes or endogenous genes. In both cases, co-expression of U1ins and shRNAs resulted in stronger inhibition of the target gene than the expression of the highest dose of either of the inhibitors on their own [38]. The increase in inhibition represents a synergic effect, based on a synergy index which is calculated with the fold-inhibition values obtained when inhibitors are used on their own or combined [38]. Moreover, synergism is also observed in vivo [30]. Synergism requires the combination of two inhibitors with different knockdown mechanisms, since the combination of two functional U1ins or two functional shRNAs does not result in synergistic inhibitions [30, 38].

Antiviral Use of U1i

U1ins can be used as antivirals. Since U1ins are expressed and function in the nucleus, viral target mRNAs should be processed in the nucleus using the cellular polyadenylation machinery. Indeed, the efficacy of U1ins as antivirals has already been explored for the inhibition of HIV-1 and HBV [26, 30, 37]. Otherwise, when the virus does not replicate in the nucleus, antiviral U1ins could target a nonessential cellular gene crucial for virus replication. The efficacy of RNAi and U1i combination has also been explored to target a replicative viral RNA, where a high inhibition seems mandatory [30].

HIV-1

Human Immunodeficiency Virus type 1 (HIV-1) is the cause of the acquired immunodeficiency syndrome (AIDS), a chronic disease of human immune system which leads to opportunistic infections. Approximately 34 million people lived with HIV across

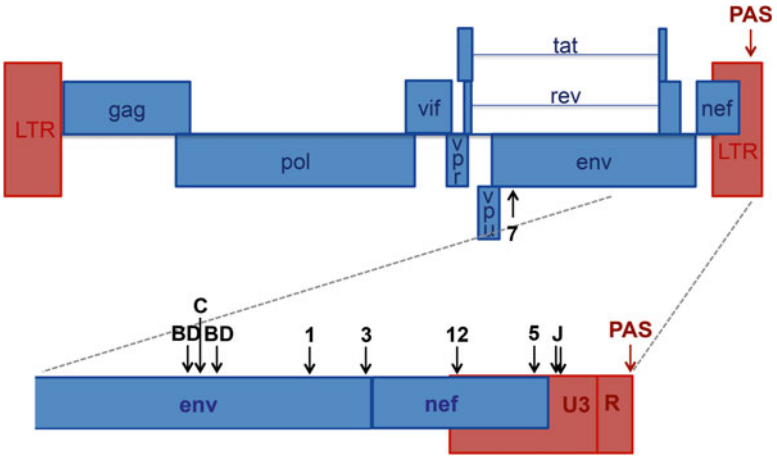

Fig. 3 Target sites of anti-HIV-1 U1ins. Schematic of HIV-1 genome. The anti HIV-1 U1in target sites are indicated with *arrows*. PAS marks the position of the polyadenylation site

the world by the end of 2012; 2.5 million of them newly infected during the same year [49]. HIV-1 is a single-stranded RNA virus that infects vital cells in the human immune system. The genomic RNA is converted into double-stranded DNA by HIV-1 reverse transcriptase. Viral DNA integrates in the host cell genome and transcribes a single 9 kb viral transcript (Fig. 3). The viral RNA undergoes extensive and complex alternative splicing to generate over 40 viral mRNAs and expresses viral proteins [50].

AIDS treatment with HIV-1 antiretroviral therapy can slow the progress of the disease, but unfortunately it is not curative and interruption of the treatment results in a rapid rebound of viremia. In addition, the virus may eventually acquire resistance against current pharmacological treatments and therefore, new therapeutic strategies are needed. U1i can be applied to treat HIV-1 infection, since viral RNA is polyadenylated by the nuclear polyadenylation machinery. To date, two studies have evaluated the use of U1ins that target HIV-1 in cell culture [26, 37]. Other works have also used modified U1 snRNAs to suppress HIV-1 gene expression, but as vehicles of antisense molecules that bind cis-elements in viral RNA to selectively increase HIV-1 splicing and decrease unspliced viral RNA levels [41, 51].

Sajic and colleagues [26] selected target sites within highly conserved regions of the HIV-1 genome by performing multiple sequence alignment of all HIV-1 strains. These sequences were further scanned against the human genome using BLAST to improve the specificity of target sites. Fifteen different U1ins that target a 10 nt sequence in the *env* or *nef* region present in the 3′-terminal exon of all HIV-1 transcripts were designed (Fig. 3; 1, 3, 5, 7, and 12). U1 snRNA expression plasmids were co-transfected in 293T cells with a plasmid that contains a HIV-1 provirus without the RT and IN genes. 48 hours post-transfection the effect of U1in on HIV-1 gene expression was monitored by western blot. One out of three U1ins yielded a significant reduction in viral expression, which was not attributable to gross toxicity in the cell as U1in expression did not downregulate a non-target RNA (secreted alkaline phosphatase, SEAP) which was co-expressed from a plasmid. Two potent

U1ins were selected to further demonstrate that suppression of HIV-1 protein expression coincides with loss of viral RNA. Importantly, the potent U1ins could be combined to achieve increased inhibitions. The authors also demonstrate that activity of U1ins was dependent upon proper assembly of U1ins in a ribonucleoprotein (RNP) complex since mutations which affected any component of the RNP abolished inhibition. Finally, to mimic an in vivo situation, cells that stably express U1ins were challenged with virus. Although stable expression of the U1in anti-HIV-1 reduced viral replication, the inhibition was lower than that observed in transient assays, probably due to a lower expression of the U1in molecules in stable cells.

In an independent study, 14 new anti-HIV-1 U1i molecules were extensively tested in various in vitro models and in lentivirus-transduced CD4 + T cells to study the impact of U1in on HIV-1 replication [37]. U1i molecules were designed according to previously proposed rules [24, 25] and were 100 % complementary to at least 70 % of all HIV-1 sequences deposited in Los Alamos database (http://www.hiv.lanl.gov). The U1i action was first evaluated by knockdown of p24 viral protein production in 293T cells transfected with HIV-1 and U1in plasmids. Viral p24 values were normalized for Renilla expression, which was co-transfected in cells as a non-target transfection control. Three out of 14 U1ins inhibited HIV-1 gene expression (Fig. 3; C, J and BD). The most efficient U1in, named J, also demonstrated its robustness in titration experiments. The inhibitory effect of U1ins was also studied more quantitatively using a Renilla luciferase reporter which contains the HIV-1 3′terminal exon. Again, the J inhibitor exhibited the strongest potency with a reduction of protein expression to 8 %. However, U1ins also decreased the expression of Firefly luciferase reporter, a non-target RNA used as a transfection control in the experiment. The nonspecific repression of J inhibitor was further confirmed using a construct that contains wild-type or mutated versions of J site downstream Renilla reporter gene. Although nonspecific inhibition was only observed when reporter genes were expressed from SV40 promoters, further studies are needed to address the specificity of these anti-HIV U1i molecules. Finally, T-cell lines stably expressing U1i molecules were generated by infection with a lentiviral vector which contains the U1i cassette. Stable cells were challenged with HIV-1 virus, but viral replication kinetics was similar in control and in U1i transduced T cells. The lack of effect could be due to the fact that cells were transduced at a low multiplicity of infection (MOI) and therefore only integrated a single copy of the U1in gene. Indeed, the ratio between exogenous and endogenous U1 snRNAs was much lower in stably versus transiently transfected cells [37]. These results suggest that, while a single copy of a potent antiviral shRNA cassette is sufficient, a single copy of the U1i gene is insufficient to prevent the spread of HIV-1 [52].

HBV

Hepatitis B Virus is a non-cytopathic enveloped virus that predominantly infects the liver. The viral genome is a partly double stranded DNA genome, which upon hepatocyte infection is converted to a covalently closed circular DNA (cccDNA).

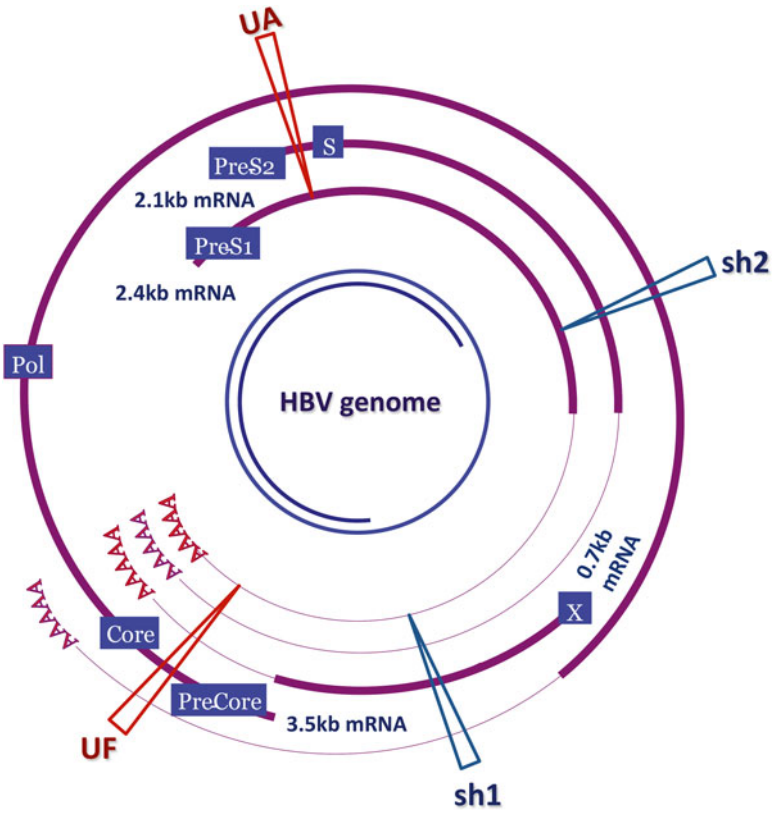

Fig. 4 HBV genome organization. The diagram shows the partly double-stranded HBV DNA genome (*inner blue circles*). The genome serves as a template for the four HBV transcripts, which share the same polyadenylation site. Target sites for functional U1ins and shRNAs are shown in *red* and *blue* respectively

From the cccDNA four HBV transcripts are produced (Fig. 4). The 3.5 kb transcript is the pregenomic RNA that is retrotranscribed to generate new HBV DNA molecules and translated to yield the viral core and polymerase proteins. The 2.4 kb and 2.1 kb mRNAs encode for the viral envelope proteins. The 0.7 kb mRNA encodes for the viral X protein. Newly formed viral genomes can travel to the endoplasmatic reticulum for assembly of envelope proteins and budding out of the cell or can move to the nucleus to increase the levels of viral cccDNA [53].

HBV infection can counteract the immune response and lead to chronic HBV infection, which may progress to chronic inflammation, liver cirrhosis, and hepatocellular carcinoma [54]. Although there is an effective vaccine against HBV, it is only useful for the prevention of viral infection. Nevertheless, approximately two billion people are already infected with HBV and it is estimated that 400 million people are chronic HBV carriers [55]. Currently, the two main treatments for antiviral therapy of chronic HBV infection are nucleoside/nucleotide analogues, such

as entecavir or lamivudine, and interferon α [56]. Nucleoside/nucleotide analogues decrease pregenomic RNA retrotranscription to DNA. Thus, they lead to a drastic decrease in viral particles in serum, but in the majority of patients the infection is not cured as the nuclear cccDNA is very stable and is not affected by the therapy. Interruption of the treatment normally leads to a rebound in viral particle production. Likewise, interferon-α has limited efficacy due to side effects and its contraindication profile [57]. Therefore, novel therapies are required for HBV treatment and the use of RNA silencing technologies could be a promising option. Unlike nucleoside/nucleotide analogues, these techniques could downregulate surface antigen expression, the major cause of liver damage and immune suppression.

RNAi is the most widely studied gene-silencing technology to target HBV [58]. However, U1i has also been used to target HBV [30]. The HBV genome is so compact that all viral transcripts have overlapping sequences and share the same polyadenylation site (Fig. 4). This allows the design of U1i molecules that inhibit several transcripts at the same time by binding to the same sequence. To inhibit HBV, 6 different U1ins were designed following previously proposed rules [32]. Thus, target sites for U1ins were 10–11 nt-long conserved sequences of the HBV genome. In addition, they were scanned to be accessible sequences according to several bioinformatics programs and redundancy of target site was evaluated using BLAST. Finally, certain bases at specific positions of the target site were avoided to impede the recognition of target sequences as cryptic donor splicing sites. First of all, the inhibitory activity of U1ins was evaluated in cell culture. Therapies against HBV infections can be tested in vitro in any of the several tissue culture cells that support viral replication, such as HepG2.2.15. Moreover, assays can be performed in cell lines transfected with plasmids that express a little more than the complete HBV genome, which drive viral replication. However, the inhibition in HBV expression can be evaluated more quantitatively using plasmids that contain HBV sequences modified to include reporter genes such as luciferase. Thus, anti-HBV U1ins were evaluated in cells expressing pCH-Fluc plasmid, which contains all the sequences from HBV pCH-9/3090 but the S sequence has been replaced by the Firefly luciferase ORF [59]. The inhibitory activity of U1ins was measured in HuH7 cells co-transfected with pCH-Fluc and U1in plasmids. 72 hours post-transfection all U1ins decreased luciferase activity from HBV sequences by 50–75 % [30]. In vivo there are several HBV mouse models [58], but following the strategy used in cell culture the U1ins that target HBV genome were studied in mice that express HBV-dependent luciferase in mouse liver after hydrodynamic injection of pCH-Fluc plasmid. Surprisingly, only two out of the six functional U1ins in cell culture inhibited luciferase expression from HBV sequences in vivo [30] (Fig. 5). However, the inhibition obtained with the functional U1ins in vivo was higher than in vitro (92–97 % vs. 50–75 %). Regarding the inhibitory mechanism, quantification of HBV transcripts that encode for luciferase determined that, as expected, U1i decreased luciferase activity by decreasing the stability of the target mRNA. In vivo, HBV inhibition by U1i was specific as inhibition was not observed in other genes which lack a target sequence, such as SEAP. In addition, mutated versions of functional U1ins failed to inhibit HBV-dependent luciferase activity in mouse liver [30].

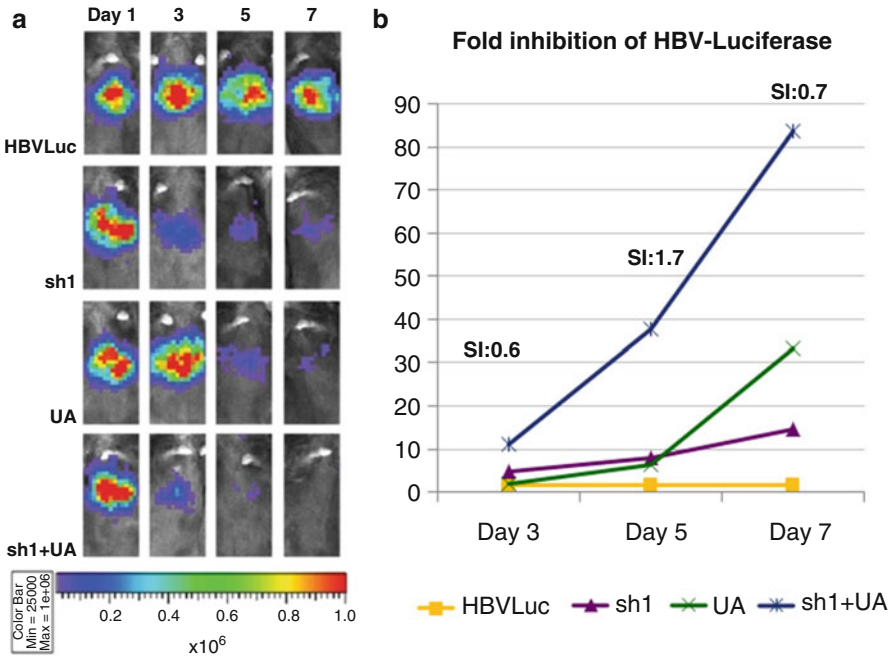

Fig. 5 Analysis of inhibition of luciferase expression from HBV by RNAi and U1i in mice. (**a**) Representative pictures of HBV-dependent luciferase activity in living mice obtained with a CCD camera. *Rows* represent groups injected with pCH-Fluc plasmid (HBVLuc) combined or not with plasmids expressing UA, sh1, or both. *Columns* represent time post-injection (in days). The *color scale* is shown at the *bottom*. (**b**) Luciferase activity was quantified and used to calculate fold-inhibitions (FI) or synergy indexes (SI)

Further characterization of non-functional U1ins in vivo demonstrated that two of them were not functional probably because they accumulated to very low levels in mouse liver. The reason for this is unclear since in tissue culture the expression level of all U1ins was similar and in mouse liver, the DNA levels of the plasmids that express U1ins were similar for all the inhibitors. Therefore, transcription or stability of these two non-functional U1ins could be lower in mouse liver for unknown reasons. The third non-functional inhibitor accumulated to similar levels as the functional U1ins. Therefore, it is possible that the U1in target site is not accessible in mouse liver, although it is in cell culture. This strengthens the need for animal studies to validate results obtained in tissue culture.

Since strong inhibitions are desirable to block the expression of viral genes for therapeutic applications, anti-HBV U1i therapy was combined with RNAi. To that end, two different shRNAs that target HBV genome were used. Both shRNAs were more efficient than U1ins in cell culture, as previously reported [29, 37, 38]. But interestingly, the inhibitions obtained with shRNAs in mice were similar to those obtained with U1ins (15- to 33 and 13- to 33-fold respectively) [30] (Fig. 5). Combination of U1ins and shRNAs in cell culture led to increased synergistic

inhibitions of HBV-dependent luciferase activity in most cases. In vivo, those U1ins which have been functional in mice also gave synergistic inhibitions when combined with shRNAs (64- to 84-fold vs. 15- to 33-fold when used independently) [30] (Fig. 5). Combination of two inhibitors of the same kind; that is two shRNAs or two U1ins, did not result in synergistic inhibitions in cell culture or in mice. The molecular mechanism that allows synergistic inhibitions upon RNAi and U1i combination is unknown. Synergism could result from a nuclear decrease in target RNA by U1i that could help RNAi efficiency in the cytoplasm. However, siRNAs act catalytically. After target cleavage the siRNA complex is released to act on another target molecule [60], and therefore RNAi efficacy should not be sensitive to target concentration. Synergism is neither caused because U1ins affect shRNA processing or vice versa, as shRNA or U1in accumulation is not altered when both RNAi and U1i are combined [30]. An alternative explanation could be that the synergism results from the formation of an mRNA complex where both inhibitors are bound at the same time, leading to an increased efficiency of one or both inhibitors. However, further experiments are required to verify whether this is the case and address the molecular mechanism of synergy.

Therefore, U1i in combination with RNAi leads to increased inhibition of the expression of HBV viral genes. Moreover, this combination can also reduce viral escape from the therapy since two different HBV sequences are targeted simultaneously. In addition, the combination of U1i and RNAi is of great therapeutic interest as with this strategy good inhibition can be obtained with lower doses of U1i or RNAi-based inhibitors, thus minimizing toxicity.

Conclusion

Although few in number, the studies performed to date with U1i have shown that it could be used to generate new antiviral molecules. Several viral infections can be considered as U1i targets. A clear target for U1i is any RNA intermediate that is required for viral cycle and that is polyadenylated by the nuclear polyadenylation machinery. This may be a viral or a cellular RNA. In principle, the design of U1i molecules only requires knowledge of the sequence of the viral genome or the cellular RNA required for viral replication. However, the selection of good target sites is a challenge that generally decides the success of U1i. Although several rules could be followed to increase its success (see "Rules for U1i"), U1in design requires further development. Currently, three to five U1ins should be designed to have a high chance of obtaining one or more functional inhibitors in cell culture. In addition, users of this technology should move to in vivo models with at least three U1in functional in cell culture, since the results obtained in culture may not help to guarantee the outcome in animal models. While some functional U1ins in cell culture might not work in vivo, others can be even more efficient in vivo than in vitro. Regarding U1i specificity, U1i seems to be a specific technique as functional inhibitors can be expressed in tissue culture and in vivo over the long-term without

detectable toxicity or morphological changes. However, high-throughput experiments are required to analyze the overall specificity of U1i in detail and to address dose dependent off-target effects.

At present, more studies that evaluate U1i to harness viral infections are needed. And in those viruses which have already been targeted with U1i, experiments with animal models are required before moving to the clinic. In vivo delivery of U1ins might also pose a challenge for the therapeutic application of U1i, as it is for RNAi. Both viral and nonviral vectors might be useful for U1in delivery and the decision regarding the best vehicle will crucially depend on the viral infection which is being targeted. In acute infections, nonviral particles could efficiently deliver U1ins. However, for those viruses that establish chronic infections, recombinant viruses that allow long-term expression of the inhibitor might be a better choice. As for the expression level of inhibitors, integration of a single copy of an U1in gene may not be sufficient for inhibition, whilst this is the case for a shRNA gene. Functional inhibition might therefore require higher relative levels of U1ins than siRNAs. Finally, U1in could be combined with other therapeutic applications to prevent the appearance of escape mutants resistant to the treatment. In addition, when U1i is combined with RNAi, increased synergistic inhibitions are obtained, which is of special interest when targeting a replicative viral RNA, where high inhibitions are mandatory. Future progress in all these aspects will help U1i to occupy a place in clinical practice in the coming years.

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Gene Therapy Strategies to Block HIV-1 Replication by RNA Interference

Elena Herrera-Carrillo and Ben Berkhout

Abstract The cellular mechanism of RNA interference (RNAi) plays an antiviral role in many organisms and can be used for the development of therapeutic strategies against viral pathogens. Persistent infections like the one caused by the human immunodeficiency virus type 1 (HIV-1) likely require a durable gene therapy approach. The continuous expression of the inhibitory RNA molecules in T cells is needed to effectively block HIV-1 replication. We discuss here several issues, ranging from the choice of RNAi inhibitor and vector system, finding the best target in the HIV-1 RNA genome, alternatively by targeting host mRNAs that encode important viral cofactors, to the setup of appropriate preclinical test systems. Finally, we briefly discuss the relevance of this topic for other viral pathogens that cause a chronic infection in humans.

Introduction on the RNAi Mechanism

Noncoding RNA plays an important role in the regulation of cellular gene expression, most notably via the RNA interference (RNAi) mechanism, which is evolutionary conserved among eukaryotes. RNAi triggers the sequence-specific inactivation of one or multiple complementary mRNAs. At least three small RNA classes can be distinguished that participate in RNAi mechanisms: microRNAs (miRNAs) [1, 2], endogenous small interfering RNAs (endo-siRNAs) [3], and PIWI-associated RNAs (piRNAs) [4]. The latter two classes are mainly implicated in the suppression of transposons. The miRNAs are involved in the regulated expression of many cellular genes at the posttranscriptional level [5, 6]. miRNA-mediated gene regulation plays an important role in cell metabolism, cellular developmental and differentiation processes in mammals.

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More than 1,000 human miRNAs have been identified, which are involved in the regulated expression of at least 30 % of human genes [7]. Many efforts have been made to elucidate the biological function of these miRNAs by identification of their target mRNAs. This miRNA–mRNA matching remains a formidable task in the absence of good algorithms for target site prediction and is complicated by the fact that many mRNAs may be influenced by a single miRNA. The design of man-made miRNA mimics like short hairpin RNAs (shRNAs) provided new tools for the control of introduced transgenes in therapeutic applications. The development of vector-mediated RNAi allowed the establishment of durable gene silencing approaches, in particular for retroviral and lentiviral vector systems that are stably inherited by integration into the host cell genome [8–10]. We describe our research line on the development of an RNAi-based gene therapy against HIV-1, which was initiated around 2003. The scientific discoveries and practical hurdles that we came across along this translational track towards the clinic are described.

RNAi: From Natural Mechanism to Therapeutic Approach

The endogenous pathway for miRNA biosynthesis is widely conserved among vertebrates and invertebrates. First, a primary miRNA transcript (pri-miRNA) is made, of which a hairpin-like RNA structure is processed by the "Microprocessor" complex, which consists of the Drosha nuclease and its dsRNA-binding partner DGCR8 (Fig. 1, endogenous RNAi pathway). Microprocessor recognizes pairing in the stem and multiple primary sequence elements in the single-stranded flanks and loop region [11, 12]. The resulting pre-miRNA is cleaved near the terminal loop by the Dicer nuclease in collaboration with the trans-activation response RNA-binding protein (TRBP) and protein activator of PKR (PACT) cofactors [13]. This miRNA pathway yields the mature RNA duplex, of which one strand of approximately 22-nucleotides is preferentially loaded into the Argonaute (Ago) enzyme as part of the RNA-induced silencing complex (RISC). The miRNA-loaded RISC complex targets mRNA transcripts, usually with multiple partially complementary targets, for translational repression. In exception cases there is full complementary of the miRNA with the mRNA causing degradation of the latter.

Short hairpin RNAs (shRNAs) are perfectly base paired minimal miRNA mimics that are synthesized from man-made gene cassettes. These hairpins enter halfway the RNAi pathway as Drosha cleavage is not needed to remove flanking sequences (Fig. 1, exogenous RNAi inducer). The loop of the hairpin is cleaved off by Dicer to produce the small interfering RNA (siRNA) with an active guide strand and an inactive passenger strand. Synthetic siRNAs and shRNA genes are usually designed with full base pairing complementarity with the intended target mRNA. Only a single target sequence is needed to trigger sequence-specific cleavage and inactivation of the mRNA. This specificity is the greatest asset for turning the RNAi mechanism into a therapeutic mode, but it also creates problems when dealing with genetically flexible microorganisms as escape mutations may frustrate the therapy. In general, the idea to develop an RNAi-based antiviral therapy is supported by the recent finding that RNAi represents an antiviral defense mechanism in mammalian cells [14, 15].

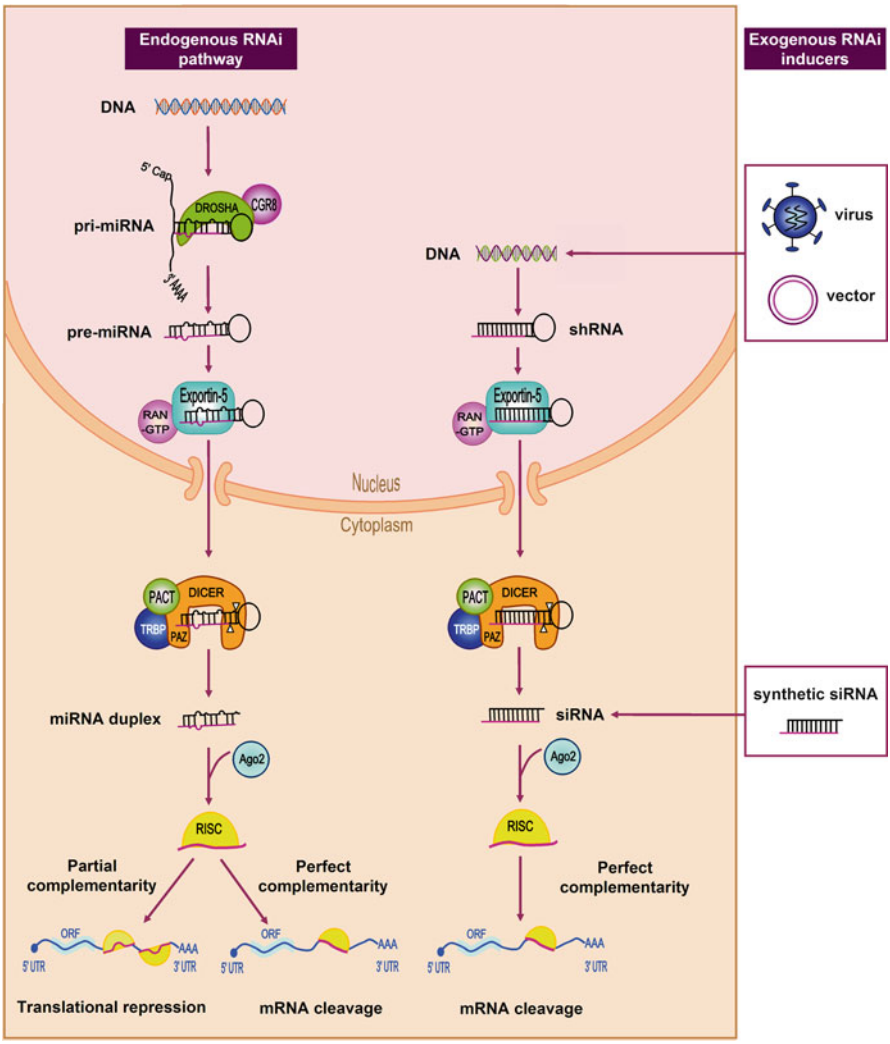

Fig. 1 The RNAi pathway. The endogenous RNAi pathway of mammalian cells is plotted on the left hand side. Host pri-miRNAs are transcribed in the nucleus, processed by Drosha and DGCR8 into a pre-miRNA and transported by Exportin-5 to the cytoplasm. The pre-miRNA is processed by the Dicer/TRBP/PACT endonuclease complex to yield the miRNA duplex. One strand (the mature miRNA) is loaded into the Ago2 enzyme, thus forming the RNA-induced silencing complex (RISC) that induces gene silencing by translational repression or cleavage of the mRNA target. The exogenous RNAi cascade is shown on the right hand side. Man-made shRNA gene cassettes are expressed in the nucleus and enter the RNAi pathway at the level of Exportin-5. Synthetic siRNA is transfected into the cell and will directly instruct RISC for mRNA silencing. The man-made shRNAs and siRNAs direct mRNA cleavage by perfect base pairing complementarity

HIV-1 as RNAi Target

We previously reviewed the ins and outs of using the RNAi machinery for a specific and durable attack on HIV-1 [16, 17]. We list the most significant points to consider. A relevant question is whether one can target the "incoming" viral RNA genome that is introduced into the cell by virus infection (Fig. 2a, route a). We demonstrated that an early attack on the incoming RNA is impaired because the RNA genome is protected by the viral nucleocapsid protein as part of the virion core structure [18]. Alternatively, transcriptional gene silencing may be induced at the viral LTR promoter much later in the replication cycle after the integrated DNA provirus is established [19, 20] (Fig. 2a, route b). Most likely, the newly produced viral transcripts will be targeted (Fig. 2a, route c).

It is important to screen many shRNA candidates for their potential to knock down HIV-1 gene expression, but some general rules can be applied. First, it is important to target conserved HIV-1 sequences such that many different isolates and subtypes are sensitive to the therapy (Fig. 2b). Second, but related to the first rule, one should target sequences that are essential for HIV-1, most notably sequences that encode critical domains of the essential viral proteins. These sequences are not only well conserved (rule 1), but will also avoid mutations that are needed to escape from specific RNAi pressure [21]. Third, it is important to consider the complex HIV-1 splicing pattern that generates many different mRNA targets [22]. The best target sequences may be located in the extreme 5′ and 3′ ends of HIV-1 RNA, which are present on each mRNA splice variant (Fig. 2c, shaded areas). The first three rules are not mutually exclusive as some of the 5′ and 3′ end sequences, particularly those overlapping with the regulatory long-terminal repeat (LTR) and critical RNA signals, are fairly well conserved, although not coding for protein [23]. Fourth, it seems important to target sequences that are accessible in the HIV-1 RNA structure for the shRNA-programmed RISC complex [24, 25]. The importance of this rule was underlined by the description of an RNAi-escape variant with an altered local RNA conformation [26]. Fifth, one could argue that it is favorable to interrupt the replication cycle by targeting the early viral functions like the Tat, Rev and Nef proteins that are translated from the early wave of multiply spliced transcripts (Fig. 2c). Mathematical modelling predicted that HIV-1 decay dynamics depend on the stage of the viral replication cycle that is attacked, much more so than the actual drug efficacy [27]. Tat may be a particularly good target because this protein has been proposed to exhibit RNAi suppressor activity [28, 29].

When talking about early and late protein function, we should realize that the actual target for RNAi attack is the RNA molecule. For instance, sequences involving the 3′ end of the Nef gene are present on all HIV-1 transcripts, thus forming an ideal target according to the third rule (Fig. 2c). However, the first selection rule remains of overwhelming importance as some of the Nef sequences are not well conserved among HIV-1 isolates and different subtypes. In addition, the Nef gene is not an essential part of the HIV-1 genome and can even be inactivated by deletions under specific RNAi pressure (see "HIV-1 Escape").

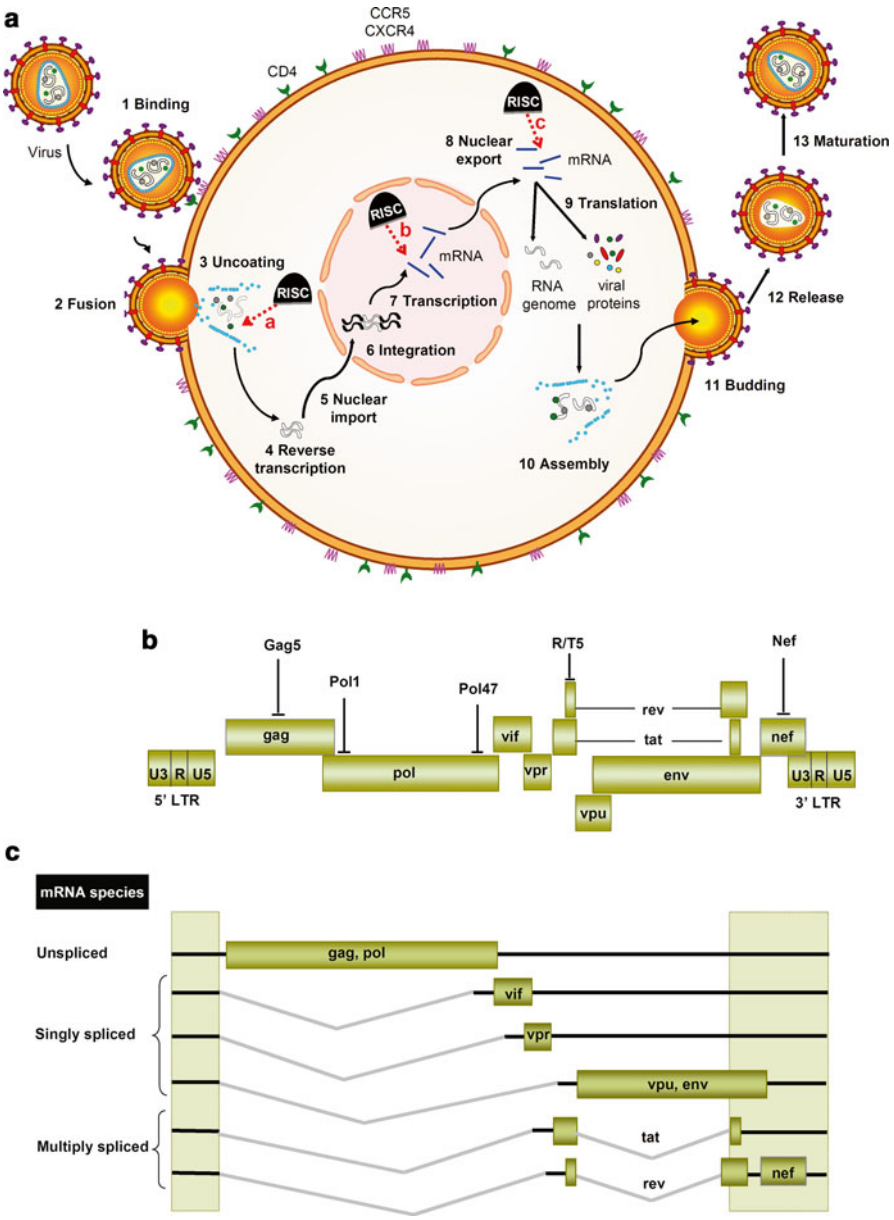

Fig. 2 Where and how to attack HIV-1 with RNAi-based inhibitors? (**a**) The HIV-1 replication cycle and three RNAi attack possibilities. Upon virus entry into the cell, the viral core with the RNA genome is released into the cytoplasm. This incoming RNA genome represents an attractive target (attack a). HIV-1 RNA is reverse-transcribed and the DNA is integrated into the host cell chromosome. New viral mRNAs are subsequently transcribed from the integrated provirus. Viral mRNA production in the nucleus can be blocked by transcriptional silencing (attack b). RISC-mediated silencing can occur in the cytoplasm (attack c). Attack route c seems the major mechanism of HIV-1 inhibition; see the text for further details. (**b**) The HIV-1 DNA genome map with some shRNA targets. The shRNAs Gag5, Pol1, Pol47, and R/T5 target conserved and essential HIV-1 sequences. The Gag5 shRNA was subsequently removed from the combinatorial RNAi cocktail because of toxicity (see Figs. 8 and 9). The shRNA Nef was used for viral escape studies (see Fig. 3). (**c**) Alternative splicing of the HIV-1 primary transcript is illustrated. The *shaded terminal boxes* mark genome segments (mostly noncoding) that are present in all mRNA forms and thus form ideal shRNA targets

Despite all these rules for the selection of candidate target sites, it remains important to construct and test the activity of a significant number of distinct shRNAs as only a few will have potent activity [30]. Perhaps we will be able to raise the overall success rate in the future by improved shRNA design algorithms. Anyhow, a large screen will allow one to identify potent inhibitors. Simple co-transfection experiments can be performed to score the silencing efficiency on matching luciferase-HIV reporter constructs. As this may require the comparison between different reporters, each with a different piece of the HIV-1 genome attached, the alternative test system becomes more attractive. Co-transfection of the shRNA construct with a full-length HIV-1 molecular clone can be performed, with silencing scored as a reduction in virus production, which can be measured by different means (e.g., CA-p24 Elisa or Reverse Transcriptase activity). The antiviral potency should subsequently be tested in stably lentivirus-transduced T cells against a spreading HIV-1 infection, preferentially using diverse virus isolates. Subsequent tests will include preclinical studies in vitro and in vivo, e.g., in the humanized mouse model, to confirm the antiviral efficacy and to test for adverse effects (see "Preclinical Efficacy Tests," "Preclinical Safety Tests," "The Humanized Mouse Model").

HIV-1 Escape

Prolonged in vitro HIV-1 replication studies revealed how this virus can escape from the pressure of an antiviral shRNA. We reported an detailed escape study for the shNef inhibitor that targets sequences encoding the Nef gene [31]. As said, this may be a good position for shRNA attack because these sequences are present in all HIV-1 RNAs, full-length or spliced. On the other hand, Nef is not an essential viral function and thus may facilitate "easy" escape. Although robust and reproducible virus suppression was obtained in a T cell line that was transfected with the lentiviral vector encoding shNef, viral escape was eventually observed. Multiple parallel HIV-1 escape experiments with a single shRNA inhibitor are shown in Fig. 3a. To confirm the escape phenotype, the virus was collected at the peak of infection and used to infect a fresh batch of shNef-expressing cells, causing a rapid spreading infection (Fig. 3b).

The next step is to determine the sequence changes that underlie the escape phenotype. For shNef, we sequenced the target site and flanking sequences of these escape viruses and documented a wide range of escape options (Fig. 3c). Point mutations were selected in the target site of some cultures (C1, F, I), demonstrating the exquisite sequence specificity of RNAi action. Over time the C1 culture evolved a second point mutation (C2), indicating that the single change does not provide full RNAi-resistance. In other cultures, HIV-1 acquired a deletion in the target site, either removing it partially (B, D, E) or completely (A, G). This result confirms the absence of a replicative function for the Nef protein in this in vitro culture system. As predicted, such deletion-mediated escape was not observed when more critical domains of the HIV-1 RNA genome were targeted [30].

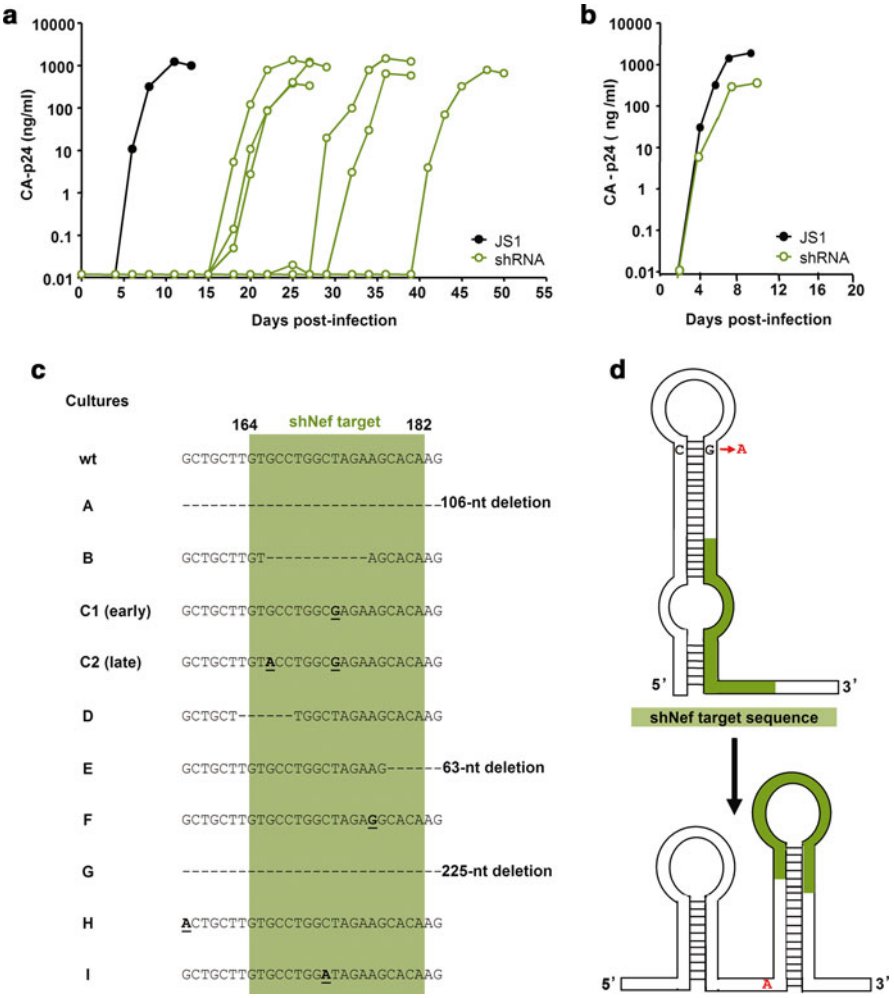

Fig. 3 HIV-1 escape options. (**a**) Long-term shRNA inhibition study with replication-competent HIV-1. T cells expressing an individual shRNA were infected with HIV-1 and virus replication monitored by measuring CA-p24 production. Control cells with the empty lentiviral vector JS1 show rapid virus spread as measured by CA-p24 ELISA in the culture supernatant. Robust HIV-1 inhibition is observed in all shRNA cultures, but breakthrough replication is apparent at variable times. (**b**) The virus-containing supernatant from an escape culture was transferred to fresh T cells, demonstrating immediate spread on shRNA and control JS1 cells, demonstrating phenotypic viral escape. (**c**) An example of HIV-1 escape mutations under pressure of the shNef inhibitor. The shNef target sequence is highlighted in *green*. Escape is possible by a single or multiple point mutations in the target sequence, partial or complete deletion of the target or a mutation outside the target. (**d**) Rearrangement of the local RNA structure due to a point mutation causes RNAi-resistance by occlusion of the 3′ end of the target sequence, which forms the annealing site for the shRNA inhibitor. See the text for further details. Panel C was modified from [26]

In fact, when important protein domains were targeted, e.g., in the Protease or Integrase enzymes, we observed a preference for mutations that represent "silent" codon changes [32]. These results indicate that HIV-1 prefers not to change the encoded protein function, and RNAi-escape variants do in fact mimic the sequences, present in natural HIV-1 variants [21].

Culture H is unique and remained unexplained as there was no mutation inside the target site. Follow-up studies demonstrated that the point mutation that is located seven nucleotides upstream of the target site does cause RNAi-resistance by a different mechanism. This mutation destabilizes a local hairpin structure in the HIV-1 RNA genome such that an alternative folding is induced (Fig. 3d). Annealing of the shRNA inhibitor is initiated at the 3′ end of the target site, which is accessible in the original structure, but occluded in the induced structure. Several other studies highlighted the importance of target RNA structure on the efficiency of RNAi attack [25, 26, 33–36]. Knowledge on the actual structure of the HIV-1 RNA genome can thus be used for the selection of improved shRNA reagents [24, 37].

Targeting Cellular Cofactors

If targeting of the virus causes so much escape problems, targeting of a host cell cofactor may represent a better antiviral option. The number of candidate cellular cofactors has increased considerably based on RNAi knockdown screens [38–40]. As these candidate cofactors were obtained in transient assays with reporter genes in non-T cells, which is remote from the physiological setting, they first need to be confirmed in regular HIV-1 infection experiments. Some candidates were subsequently knocked down to test for the antiviral activity in T cells [41]. Targeting of cellular cofactors imposes specific advantages and shortcomings. It may obviously cause cytotoxicity, but may have a dual advantage concerning viral escape. Inhibition of an important cofactor will be effective against all viral variants in an infected individual and likely all HIV-1 strains and subtypes that circulate worldwide. Additionally, viral escape would seem possible only through adaptation to an alternative cellular cofactor. Thus, it is important to target components of cellular pathways that lack redundancy [41].

As discussed, RNAi does not allow an early attack on the RNA genome of the infecting virus particle [18]. However, one could target cellular entry factors that facilitate virus–cell contact and entry. The chemokine receptor 5 (CCR5) is the most important HIV-1 receptor and represents a promising target because this protein is not important for human physiology as demonstrated by individuals with a homozygous gene deletion that interrupts CCR5 protein expression [42]. A proof of concept for this concept was obtained by bone marrow transplantation from such a CCR5-minus donor in the "Berlin" HIV-1 patient who subsequently did not need antiviral drugs to maintain an undetectable viral load [43, 44]. This functional cure has spurred a search for alternative cofactors that are vital for HIV-1 replication, yet without an important role in human physiology. It has even been proposed to remove

the CCR5 gene with a tailored nuclease that excises the CCR5 gene [45, 46]. When silencing or removing the CCR5 function, a possibility is that HIV-1 escapes by switching to CXCR4 as alternative receptor and such CXCR4-using HIV-1 variants may be more pathogenic [47].

Combinatorial RNAi Approaches

Promising anti-escape approaches include targeting of highly conserved and evolutionary restrained regions of the viral RNA genome, but HIV-1 is still likely to escape from a potent shRNA by selecting a mutation in the target sequence [48]. In case only a few HIV-1 escape routes are observed, it can be proposed to develop modified shRNAs that specifically target these escape variants. These modified shRNAs should be combined with the original inhibitor to prevent viral escape. We used this approach to skew the evolution of resistance, but it became apparent that virus evolution could not be blocked completely as HIV-1 started using new escape routes.

It makes sense that combinatorial RNAi approaches should mimic the action of combinatorial drug regimens that are very successful in the durable control of HIV-1 in patients [30, 49]. In other words, one should simultaneously express multiple shRNA inhibitors that target different parts of the HIV-1 genome or important cofactors [30, 50]. Besides additive inhibition, one will raise the genetic threshold for the development of resistant viruses as multiple mutational hits will be required in multiple target sites.

Combinatorial RNAi can be achieved with multiple shRNA cassettes introduced into the same lentiviral vector [50], but alternative scenarios have been tried with variable success [51, 52]. The different methods are listed in Fig. 4 with some of the major advantages and disadvantages. Multiple inhibitors can be generated from polycistronic miRNA transcripts [53, 54]. Stacking of two shRNAs on top of each other leads to the so called extended shRNA (e-shRNA) design, but most silencing activity is lost upon further extension as in long hairpin RNAs (lhRNAs) [51, 55–57]. The RNAi inhibitors can be combined with other RNA-based inhibitors [49]. One could even create hybrid molecules that combine siRNA and other antiviral activities, e.g., an RNA aptamer that binds to and neutralizes the viral Envelope protein [58, 59].

To further explore the power of combinatorial approaches, we tested the influence of RNAi-mediated knockdown on the activity of conventional antiretroviral drugs (fusion, RT, Integrase and Protease inhibitors). We compared the fold-change in IC_{50} ($FCIC_{50}$) of these drugs in cell lines stably expressing anti-HIV and anti-host shRNAs and measured increased values for some combinations [60]. Additive or synergistic anti-HIV effects were observed with combinations of shRNAs and small-molecule drugs. The multiplication of inhibitors that target a single replication step yielded some prominent inhibitory effects. Leonard et al. reported that a combination of RNAi attack with antiretroviral drug did enhance the antiviral activity [61]. We recently demonstrated that second-generation shRNAs can be combined with

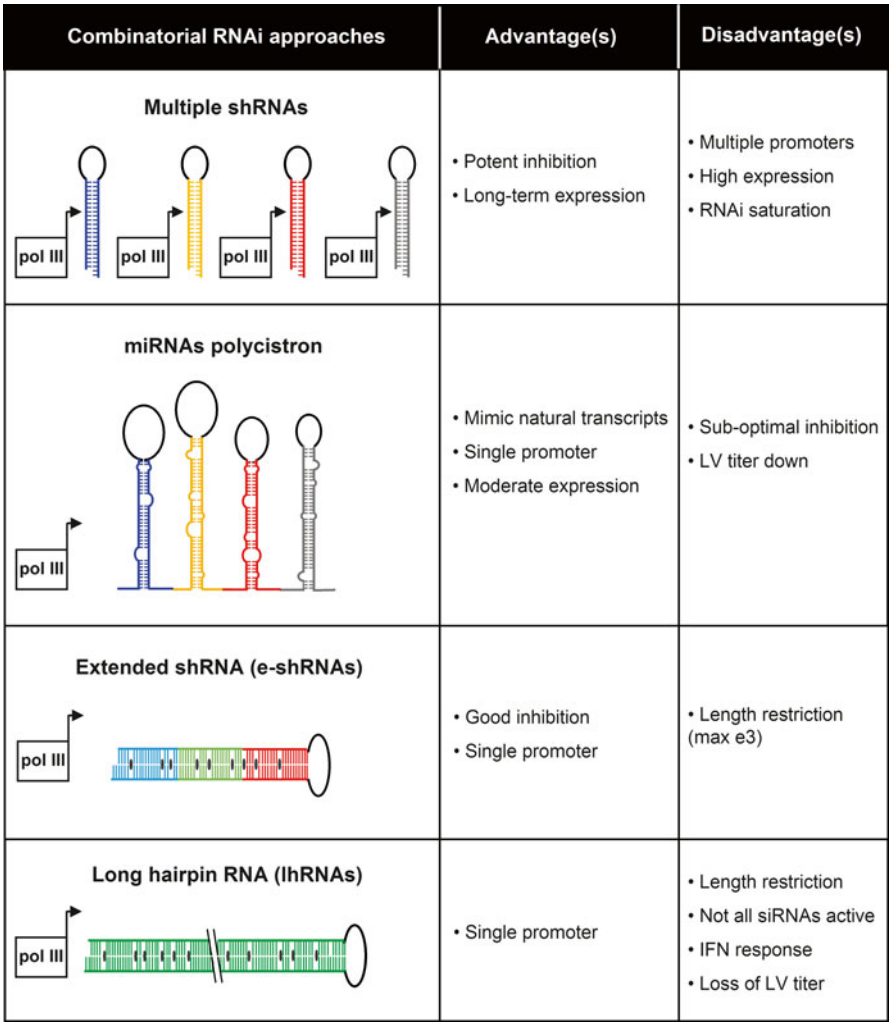

Fig. 4 Combinatorial RNAi strategies. Four inhibitory scenarios are plotted. Expression of multiple shRNAs from independent cassettes with RNA polymerase III, a single transcript composed of multiple miRNAs under control of an RNA polymerase II promoter, processing of multiple shRNAs from e-shRNA transcripts made by RNA polymerase III, or the expression of many siRNAs from long hairpin RNAs (lhRNAs)

Protease inhibitors to skew virus evolution, imposing an evolution block or triggering the selection of less fit virus variants [62]. The combination of two siRNAs against the viral Gag mRNA and the cellular CCR5 mRNA provided additive inhibition [63]. These combined results confirm that a high degree of anti-HIV cooperativity between shRNAs, targeting the virus or cellular cofactors, and drugs can be achieved. As previously discussed [64–66], this result supports the therapeutic interest in shRNA-drug combinatorial approaches.

Improved shRNAs and the Novel AgoshRNA Design

Several attempts to improve the shRNA hairpin design have been reported, with a particular focus on the loop segment [8, 67–73]. It is important to note that design algorithms for siRNAs cannot be applied to shRNA design [74]. Some confusion was created by the presentation of the original pSuper system as hairpins with a 9-nucleotide loop [8], which likely do allow the formation of two additional base pairs and consequently a 5-nucleotide loop [71]. The loop sequence may have an effect on shRNA processing, e.g., Dicer recognition and/or cleavage [67, 75]. miRNA-derived loops may interact with specific cell proteins to facilitate processing by Drosha and/or Dicer [76–78]. Recent evidence indicates that one could create shRNAs with a grossly different design due to Dicer-independent processing [79].

The first evidence for Dicer-independent shRNA processing came from studies on synthetic shRNAs [10, 75, 80]. A subclass of short shRNAs (sshRNAs) was described with a short stem of only 16–19 base pairs [81]. Whereas regular shRNAs are Dicer substrates, sshRNAs cannot be cleaved by Dicer in vitro [81, 82]. Yet these sshRNAs are active via RNAi-mediated target RNA cleavage [81], and a cellular endonuclease of unknown origin was suggested to execute the processing [10]. Ago2 involvement was suggested based on modification of the putative Ago2 cleavage site in the middle of the base paired stem [83]. A peculiar feature of sshRNAs that also needed to be explained is their "handedness." The active guide strand switches to the other side of the hairpin when compared to regular shRNA molecules [81, 83].

Similar results were described for shRNA molecules that are synthesized inside the cell from gene expression cassettes. Early studies described the effect of the shRNA loop sequence [71, 84–86] and stem length [57, 82] as important determinants for regular Dicer processing. We recently identified a specific shRNA design with a short stem length and small loop that triggers an alternative processing route [87]. As described above, we observed a strand switch such that the passenger strand is effectively converted into guide strand. Sequencing indicated that cleavage occurred half-way the 3′ side of the duplex, suggesting a role for Ago2 that is predicted to cleave between base pair 10 and 11 (Fig. 5). Production of the typical approximately 30-nucleotides RNA fragments was abolished with a catalytically defective Ago2 mutant. This new design was termed AgoshRNA because the short shRNAs of 17–19 base pairs are too small to be recognized by Dicer and consequently end up in Ago2 for alternative processing. Ago2 has a dual role in AgoshRNA processing and subsequent target RNA cleavage. It is likely that these two processes are functionally coupled and executed by the same Ago2 molecule. If processed AgoshRNAs leave Ago2 prematurely, they are less likely to be bound again because of the disrupted RNA structure. Furthermore, "pre-sliced" sshRNAs molecules are inactive, which is consistent with a coupled two-step mechanism [83].

We discussed the advantages of the AgoshRNA design over regular shRNAs [87] and these differences are listed in Fig. 5. AgoshRNAs produce only a single RNAi-active guide strand, which is an important feature to restrict RNAi-induced off target effects due to the passenger strands. AgoshRNAs will be the silencing method of choice for cells that lack a significant amount of Dicer, including monocytes [88].

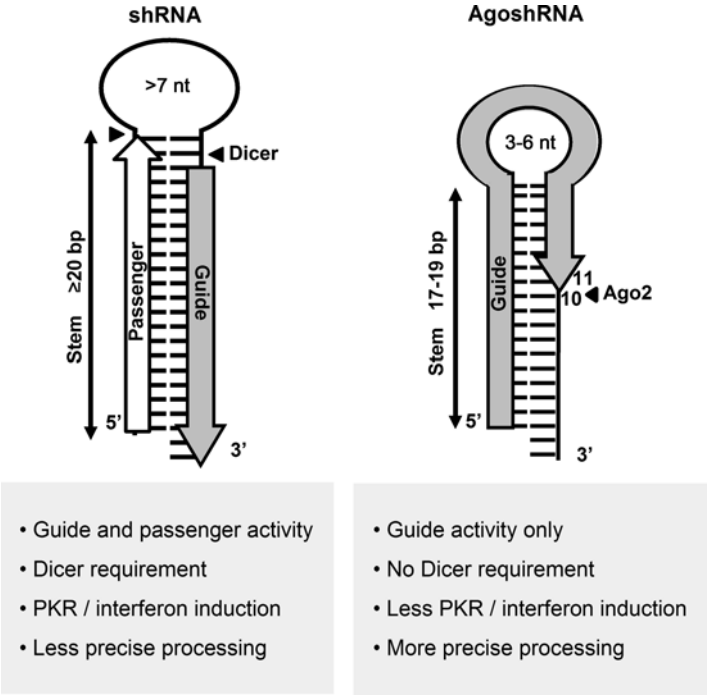

Fig. 5 Characteristics of the traditional shRNA and novel AgoshRNA design. The regular shRNA is processed by Dicer. The shorter AgoshRNA duplex is not recognized by Dicer and is processed by Ago2, triggering a switch in guide strand from the 3′ to the 5′ arm of the duplex. See the text for further details

AgoshRNAs may be safer than regular shRNAs for several reasons: saturation of Dicer as critical component of the cellular RNAi pathway is less likely and innate immunity mechanisms will be triggered less likely by these short RNA duplexes [89]. Ago2-mediated processing of shRNAs may yield more precise ends compared to Dicer processing, which is notoriously inaccurate [90]. Finally, AgoshRNAs may mimic the Dicer-independent cellular miR-451 that is loaded exclusively into Ago2, thus avoiding off target effects via Ago1, 3, and 4 [91]. Nevertheless, it is too early to tell whether the regular shRNA or special AgoshRNA design yields more RNAi-active molecules as this requires the testing of many more molecules in similar experimental settings.

Vector Issues

Although promising effects have been reported for a transient siRNA treatment in an HIV mouse model [92], long-term control of the virus would require a gene therapy approach to modify the target cells such that they can resist virus infection [93].

The idea is to develop a gene therapy with a durable effect that lasts the life span of the infected individual. To achieve such a durable effect, the optimal delivery system is based on the lentiviral vector that stably integrates in one of the chromosomes. This HIV-based vector system has recently been demonstrated to be safe in vivo, also when used to transduce hematopoietic stem cells [94–96].

A schematic of the lentiviral vector components is presented in Fig. 6a. Basically this represents the third-generation self-inactivating lentiviral vector [97–99]. The vector plasmid termed JS1 encodes the transgene from a promoter (P), which could be a Polymerase III unit encoding a shRNA transcript. The HIV-1 sequences that ensure packaging of the encoded RNA transcript into virion particles, reverse transcription and subsequent integration into the chromosomes of the target cell are marked by black boxes. The Green Fluorescence protein (GFP) reporter is made from the PGK promoter. A helper plasmid is needed to synthesize the structural Gag subunits for assembly of virion particles and the Pol enzymes that execute critical replication steps. A second helper plasmid encodes the viral Rev protein that is needed for Gag-Pol and vector gene expression through interaction with the RRE responsive element. Finally, the VSV-G glycoprotein is expressed to provide the virion particles with a broad target cell specificity. These four plasmids are co-transfected into 293T cells to initiate the production of lentiviral vector particles with the vector RNA genome, which can stably transduce a wide variety of target cells (Fig. 6b).

Because the lentiviral vector is actually based on the HIV-1 genome, one may expect some problems when anti-HIV shRNAs are introduced. We previously discussed these potential problems and presented protocols to use lentiviral vectors for an RNAi-based attack on HIV-1 [100–102]. Briefly, it is important to avoid the targeting of HIV-1 sequences that are also present in the lentiviral vector system. This is relatively easy because all HIV-derived sequence elements in the four components of this system (marked in black in Fig. 6a) have been codon-optimized such that they lose similarity to the HIV-1 genome. Other potential issues are self-targeting of the vector construct, which seems to be suppressed by the hairpin structure of shRNA constructs [103]. Constructs with miRNA-like antiviral inserts may face other specific problems like Drosha-mediated cleavage and inactivation of the vector RNA genome. We discussed strategies to avoid such adverse effects [104, 105].

Preclinical Efficacy Tests

It is important to realize that a gene therapy will likely reach only a fraction of the T cells in the human body. It is thus pivotal to study viral escape in the presence of unprotected T cells that will support ongoing HIV-1 replication and thus potentiate the risk of viral escape [106, 107]. Unhindered HIV-1 replication in unmodified T cells will generate many HIV-1 variants or a viral quasispecies, e.g., variants that acquire resistance to the shRNA inhibitor by a point mutation in the target sequence (Fig. 7). We studied virus inhibition and evolution in pure cultures of shRNA-expressing cells versus mixed cell cultures of protected and unprotected T cells [106].

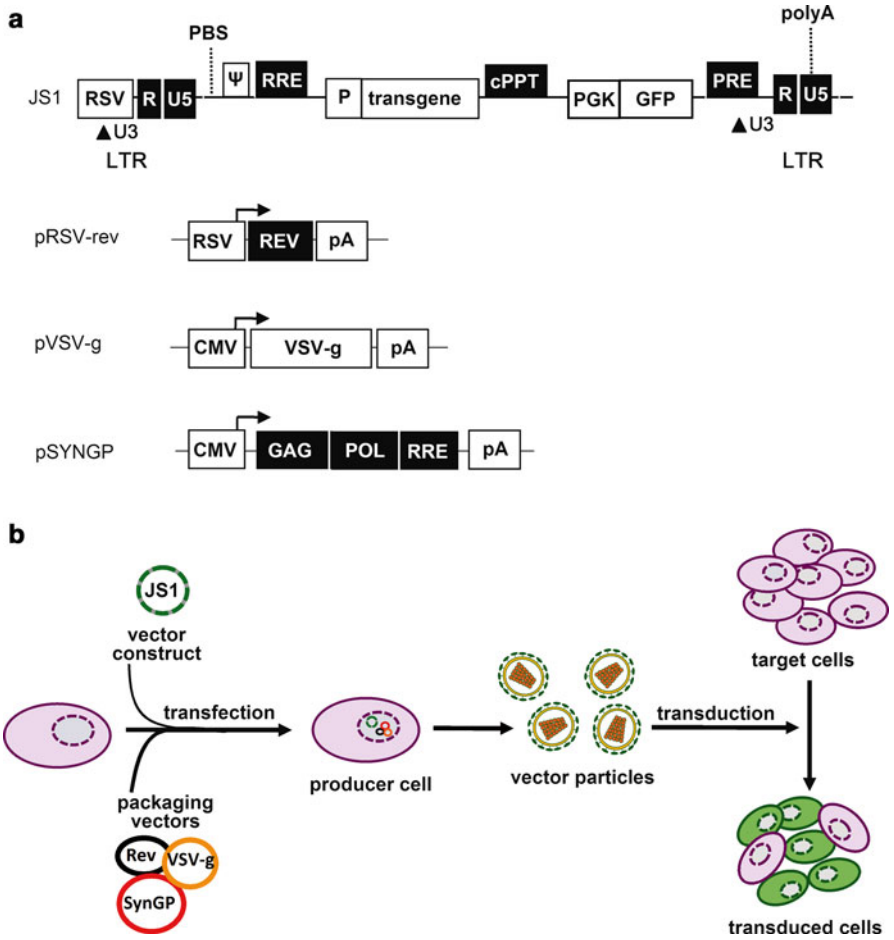

Fig. 6 Lentiviral vectors for stable shRNA expression. (**a**) The four plasmids needed for lentiviral vector production. The vector genome is expressed from the Rous Sarcoma Virus (RSV) promoter. Transcripts start with the HIV-1 R and U5 regions, the packaging signal (ψ). The deletion introduced in the U3 region of the 3′LTR will be copied into the 5′ LTR promoter during reverse transcription, resulting in self-inactivation. To improve transduction efficiency and transgene expression in target cells, the HIV-1 central PPT (cPPT) was inserted upstream of the transgene promoter and the posttranscriptional regulatory element (PRE) was inserted downstream of the transgene, respectively. The enhanced green fluorescent protein (GFP) reporter is expressed from the phosphoglycerate kinase promoter (PGK). Transcription of the vector genome and the GFP reporter terminates at the HIV-1 polyA signal within the 3′LTR. The HIV-1 Gag-Pol gene was expressed from pSYNGP as a human codon-optimized sequence without RRE. The Rev protein was expressed from pRSV-rev. The viral vector was pseudotyped with the vesicular stomatitis virus G protein (VSV-g) expressed from pVSV-g. (**b**) Scheme of lentiviral vector transduction by co-transfection of 293T cells with the four plasmids. Vector particles are then used to transduce target cells, in which the vector genome is stably integrated into the DNA genome

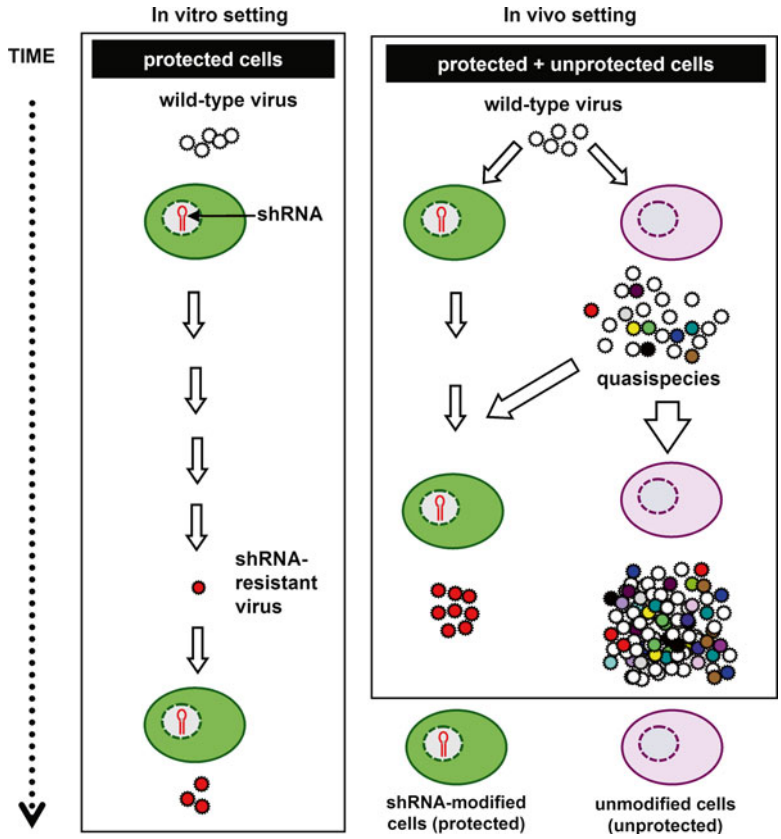

Fig. 7 HIV-1 escape facilitated by unmodified bystander cells. In vitro *setting*: pure cultures of shRNA-expressing cells (*green*) are protected against HIV-1 infection. HIV-1 variants will be generated at a very low rate and only shRNA-resistant variants (*red* virus) will be able to spread. In vivo *setting*: mixed cell cultures of protected (*green*) and unprotected (*pink*) cells will allow virus replication in the unprotected cells, leading to the rapid generation of a viral quasispecies by spontaneously acquired mutations. This quasispecies may also contain one or more shRNA-resistant variants that can replicate in the protected cells. Modified from ref. [106]

The addition of the unprotected T cells indeed accelerated HIV-1 evolution and consequently triggered viral escape from a gene therapy with a single shRNA inhibitor. But the expression of three antiviral shRNAs from a single lentiviral vector prevented escape, also in the presence of unprotected cells. These results confirm the increased inhibitory capacity and more durable effects of a combinatorial RNAi approach against HIV-1.

It is also important to test the combinatorial RNAi regimen against a wide range of HIV-1 variants, including all circulating subtypes. Please note that the shRNA targets were selected based on sequence conservation among the different subtypes,

but this obviously does not result in 100 % coverage due to the considerable genetic variability among HIV-1 strains [30]. The survey of clinical HIV-1 isolates should include drug-resistant HIV-1 variants, but we tried to avoid targets that correspond to protein domains in which drug-resistance mutations are located [16]. We have recently performed such tests, which indicate the broad effectiveness of the triple shRNA regimen [108].

Preclinical Safety Tests

Gene silencing by means of RNAi can have adverse effects on cell physiology, metabolism, and growth. The shRNAs can affect unintended mRNAs in addition to the intended target mRNA. Although complete base pairing complementary with such secondary mRNA targets can be avoided using in silico screens, silencing may occur with partial sequence complementarity [109]. Overexpression of the shRNA from powerful Polymerase III systems may trigger saturation of RNAi components like Exportin-5, Dicer, or Ago2 [110] or trigger innate immune responses [111, 112]. As mentioned, silencing of a cellular cofactor can affect cell growth. Although there are several possibilities to score cell growth over time, we realized the need for a simple assay to score subtle cell growth effects and developed the Competitive Cell Growth (CGG assay) [113]. This method is based on the difference in proliferation rate of transduced (GFP-positive) and untransduced cells in the same culture. One only has to maintain the transduced cell culture, that is the mixture of transduced and untransduced cells, and perform fluorescence-assisted cell sorting (FACS) staining for the percentage of GFP-positive cells. This percentage will go down if the transgene has a negative impact on the cell (Fig. 8). This internally controlled assay is able to detect very small differences in cell replicative capacity.

The Humanized Mouse Model

The safety and efficacy of a gene therapy protocol can be tested in a humanized mouse model (Fig. 9a). In particular, we used the BRG-HIS mouse model in which immunodeficient newborn mice are injected with human hematopoietic progenitor cells. These mice build a fairly complete human immune system consisting of different cell lineages, including mature T cells. This complex process of hematopoiesis can be monitored to screen for a negative impact of the gene therapy on cell development. For instance, we tested the impact of lentivirus-transduced anti-HIV shRNAs cells and initially reported the absence of severe adverse effects [114]. Using the very sensitive cell competition concept as described above [113], we noticed a transient negative effect of one of the four shRNAs on T cell development (Fig. 9b), which allowed us to reformulate the shRNA cocktail [115].

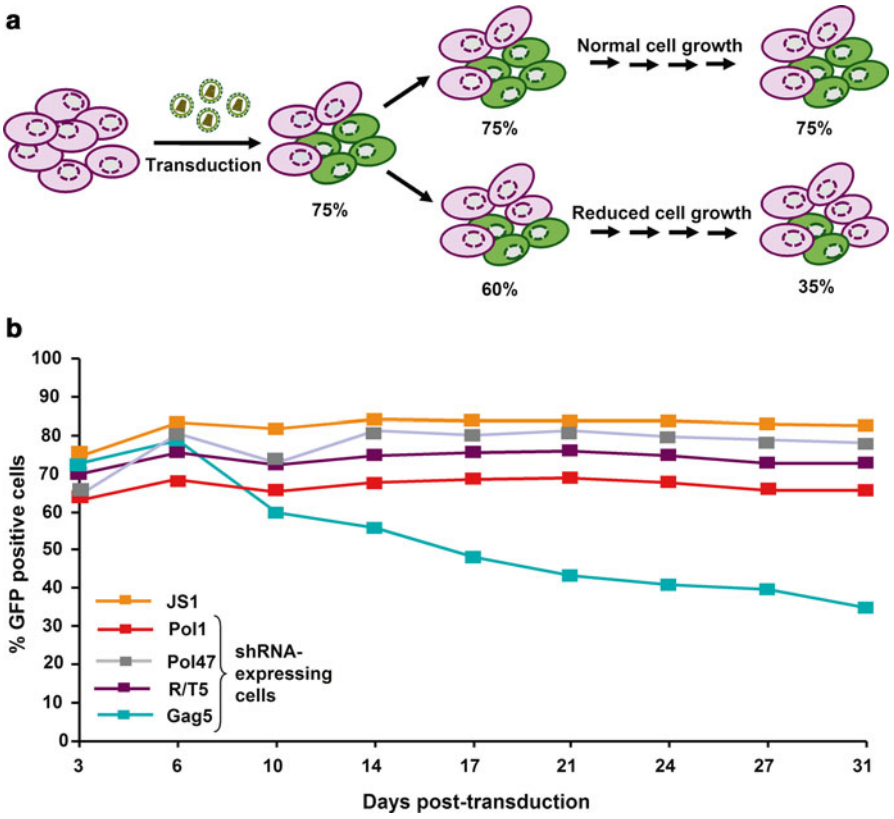

Fig. 8 The competitive cell growth (CCG) assay. (**a**) Percentage of transduced GFP positive cells (*green*) among unmodified cells (*pink*) as a function of the culturing time. This culture can simply be initiated with the transduction mixture, followed by regular FACS analysis. (**b**) A T cell line was transduced with a shRNA-encoding lentiviral vector and passaged. The Gag5-transduced cells are gradually lost, indicating a growth deficit

Gene Therapy Strategies for HIV-AIDS

We previously discussed the potential dangers of a gene therapy based on a lentiviral vector [100]. Although the number of treated patients is still relatively low, there is growing evidence that these vectors can be used safely for the ex vivo transduction of hematopoietic stem cells [94–96]. In fact, safety was demonstrated in an anti-HIV trial with a third-generation lentiviral vector encoding a triple RNA payload of anti-HIV shRNA, ribozyme against the CCR5 mRNA and TAR RNA decoy [49]. Similarly, a first-generation retroviral vector was safely used to deliver an anti-HIV ribozyme [116]. Thus far no therapeutic effects were scored in these trials, perhaps because of the low number of cells that were transduced in these initial studies.

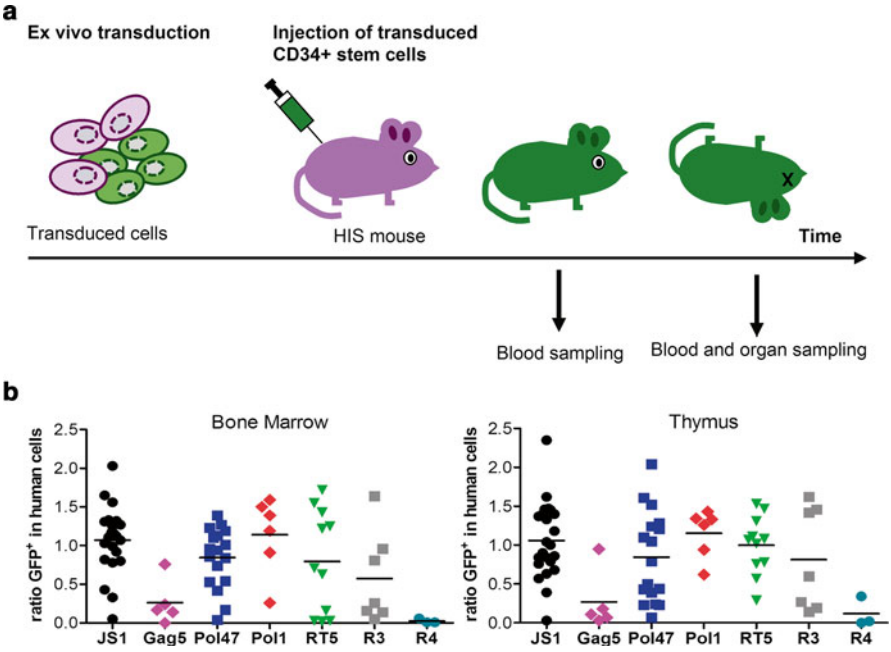

Fig. 9 In vivo safety studies in the humanized immune system (HIS) mouse model. (**a**) Human CD34+ stem cells are transduced with a lentiviral vector expressing the indicated single or combinatorial shRNA(s) against HIV-1. The mixture of transduced cells (*green*) and unmodified cells (*pink*) are injected into newborn immunodeficient mice, followed by hematopoietic development for two months and subsequent cell analysis. (**b**) The bone marrow and thymus were analyzed by FACS for the percentage of transduced GFP+ human cells, which was compared to that of the input cell mixture. A ratio around 1.0 indicates that transduced cells have no growth deficit or benefit over unmodified cells. Each dot represents an individual mouse. The Gag5 shRNA and the R4 combinatorial regimen (Gag5, Pol47, Pol1, and RT5) show a detrimental effect on cell recovery. Removal of Gag5 from R4 to create R3 restored cell recovery. Modified from data in ref. [115]

We propose to develop an ex vivo gene therapy as illustrated in Fig. 10. The therapy will likely be offered to HIV-infected individuals that fail on regular drug regimens. The patient will be pretreated with granulocyte colony stimulatory factor (GCSF) to mobilize the hematopoietic precursor cells from the bone marrow into the periphery. These stem cells will be purified from the blood and transduced ex vivo with the lentiviral vector that encodes the anti-HIV RNAi arsenal. Upon infusion back into the patient, the modified cells should resist productive HIV-1 infection and thus preferentially survive over the unmodified cells that are infected by HIV-1 and removed by the immune system. This should lead to a repair of the immune system, which may be a slow and partial process, but with a durable impact.

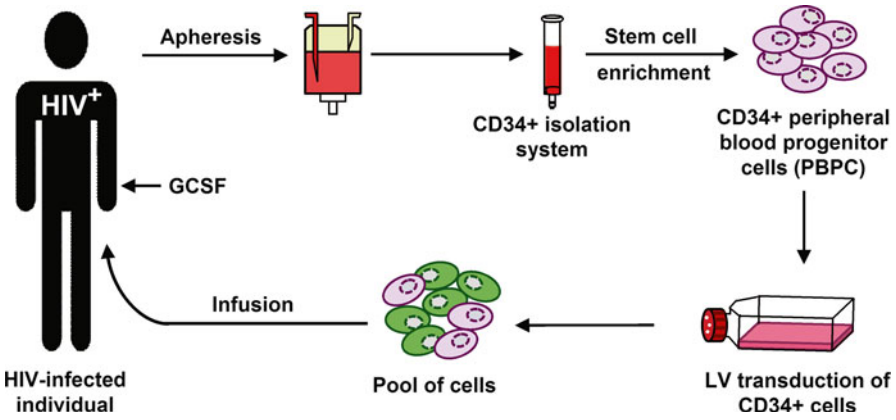

Fig. 10 RNAi gene therapy against HIV-1. An HIV-infected patient that fails on regular drug therapy will undergo apheresis for the collection of hematopoietic stem cells after pretreatment with granulocyte colony stimulatory factor (GCSF). The hematopoietic stem cells will be purified and transduced ex vivo with the therapeutic lentiviral construct. Transduced cells will be infused back into the patient and the antiviral shRNA(s) will protect these cells against HIV-1. The HIV-resistant immune cells will survive preferentially and should prevent disease progression

RNAi Against Other Chronic Infections

Other chronic virus infections may be targeted by RNAi-mediated gene therapy [54, 117–123]. Virus eradication may be easier to achieve for the hepatitis B and C viruses (HBV, HCV) because chromosomal integration is not an intrinsic part of their replication cycle. In fact, recent trials with novel anti-HCV drugs indicated that complete viral clearance can be achieved fairly easily [124, 125]. This contrasts with the HIV-1 situation, where powerful combinatorial drug regimen can nearly completely suppress replicating virus, but drug treatment cannot be stopped because the viral reservoirs will reignite HIV-1 spread. This reservoir is established in different cell types, mostly resting T cells [126], but possibly also activated T cells [127]. There are likely multiple molecular mechanisms to impose HIV-1 latency, but the stably integrated HIV-1 DNA genome is central in most scenarios [128]. Much attention is given to the formulation of therapeutic strategies that will lead to a complete cure [126]. The best current option would be to start therapy as early as possible to avoid establishment of the viral reservoir, such that the immune system can control the virus once therapy is stopped [129, 130].

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HIV and Ribozymes

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Abstract Ribozymes are structured RNA molecules that act as catalysts in different biological reactions. From simple genome cleaving activities in satellite RNAs to more complex functions in cellular protein synthesis and gene regulation, ribozymes play important roles in all forms of life. Several naturally existing ribozymes have been modified for use as therapeutics in different conditions, with HIV-1 infection being one of the most studied. This chapter summarizes data from different preclinical and clinical studies conducted to evaluate the potential of ribozymes to be used in HIV-1 therapies. The different ribozyme motifs that have been modified, as well as their target sites and expression strategies, are described. RNA conjugations used to enhance the antiviral effect of ribozymes are also presented and the results from clinical trials conducted to date are summarized. Studies on anti-HIV-1 ribozymes have provided valuable information on the optimal expression strategies and clinical protocols for RNA gene therapy and remain competitive candidates for future therapy.

Abbreviations

bRN-P	Bacterial RNase P
CMV	Cytomegalovirus
DIS	Dimerization initiation signal
EGS	External guide strand
HCV	Hepatitis C virus
HDV	Hepatitis delta virus
HH	Hammerhead
HIV	Human immunodeficiency virus
Hp	Hairpin
hRN-P	Human RNase P
HSC	Hematopoietic stem cell

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LTR	Long terminal repeat
MMLV	Moloney murine leukemia virus
MSCV	Mouse stem cell virus
Pol	Polymerase
pre	Precursor
RNase P	Ribonuclease P
RRE	Rev response element
Rz(s)	Ribozyme(s)
shRNA	Short hairpin RNA
SLII	Stem loop II
snoRNA	Small nucleolar RNA
snRNA	Small nuclear RNA
SV40	Simian virus 40
TAR	Transactivation response
Tk	Thymidine kinase
U	Uridine
UTR	Untranslated region
VA	Virus associated

Introduction

The first demonstration that an RNA molecule could function as an enzyme was provided in 1982 by Kruger and colleagues [1]. They showed that a segment of Tetrahymena ribosomal precursor (pre) mRNA mediated the RNA cleavage and ligation reactions involved in intron self-splicing. These catalytic RNAs were called ribozymes (Rzs) and several self-splicing group I and II intron Rzs have since been identified in the genomes of diverse species [2]. In 1983, a second class of Rzs were identified, when it was shown that the RNA component of bacterial ribonuclease P (RNase P) complexes was responsible for mediating the cleavage of pre-tRNAs [3]. It has since been confirmed that this Rz is the catalytic moiety in all RNase P complexes and a variety of substrates in addition to pre-tRNAs have been identified [4]. Another ubiquitous Rz is found in the ribosome, where ribosomal RNA catalyzes the peptidyl transferase reaction required to link amino acids together during protein synthesis [5]. The most diverse group of Rzs identified to date is the small self-cleaving Rzs [6]. Rzs from this group include hammerhead (HH) and hairpin (Hp) Rzs, initially identified in the tobacco ringspot virus satellite RNA, and the hepatitis delta virus (HDV) Rz, identified in the HDV satellite RNA of the hepatitis B virus [7]. Both HH [8, 9] and HDV-like [10, 11] Rzs have since been identified in the genomes of a wide range of organisms where they mediate diverse functions in RNA biogenesis and regulation. Additional small self-cleaving Rzs that have been described include the varakud satellite [12], glmS [13], CoTC [14], and the recently identified twister [15] Rzs. Shortly after their discovery, it was proposed that small self-cleaving Rzs could be used as therapeutic agents, and several have been

designed as potential therapies for different conditions including cancer, hepatitis C virus (HCV), and human immunodeficiency virus (HIV) infections [16].

In this chapter we first describe the different Rz motifs used to design anti-HIV RNAs (section "Design of Anti-HIV-1 Ribozymes"). We next provide a summary of their target sites (section "Anti HIV-1 Ribozyme Target Sites"), followed by expression strategies that enabled their development as gene therapy agents (section "Ribozyme Expression Strategies"). Experiments carried out to evaluate the potential for different RNA conjugations to improve the antiviral effect of Rzs are described in section "Ribozyme-RNA Conjugates" and results from clinical studies conducted to date are summarized in section "Anti-HIV-1 Ribozymes in Clinical Trials." Studies on anti-HIV Rzs have provided a wealth of information that will be useful for the future design and evaluation of other Rz reagents. The information presented in this chapter is intended to provide guidance for researchers and clinicians pursuing the development of therapeutic Rzs against HIV-1 infection or other human diseases.

Design of Anti-HIV-1 Ribozymes

Among the first Rzs designed to target HIV-1 replication were a HH [17] and Hp [18] Rz targeting HIV-1 RNA. As the natural motifs are self-cleaving (in cis), modifications were made so that they can specifically and efficiently cleave a target RNA in trans [19–21] (Fig. 1a, b). The vast majority of anti-HIV Rzs have been based on the HH and Hp motifs and only Rzs from these groups have reached clinical trials (section "Anti-HIV-1 Ribozymes in Clinical Trials"). Another small self-cleaving Rz motif that has proven amenable to the development of therapeutic Rzs is the HDV Rz [22–26] (Fig. 1c) and our group has shown that they can be designed to target HIV-1 RNA [27, 28]. In addition to small self-cleaving Rzs, the endogenous activity of human RNase P (hRN-P) has been exploited to inhibit HIV-1 replication by introducing a sequence-specific antisense RNA (external guide sequence, EGS) that binds to its target in such a way that it resembles RNase P substrates [29–31] (Fig. 1d). A modification to this approach has also been used to express bacterial RNase P (bRN-P) linked to an EGS to cleave a target in HIV-1 RNA [32]. The potential for using Group II intron Rzs for targeted intron insertion into HIV-1 DNA has also been explored [33, 34], and future studies may identify new approaches to harness the activity of natural Rz motifs to inhibit HIV-1 replication.

Anti HIV-1 Ribozyme Target Sites

The first target site exploited for the design of anti-HIV Rzs was a sequence in HIV-1 RNA coding for the Gag polyprotein [17]. This landmark study demonstrated that a trans-cleaving Rz could be used to inhibit HIV-1 gene expression when delivered from a DNA vector and it launched a number of efforts to identify Rzs suitable

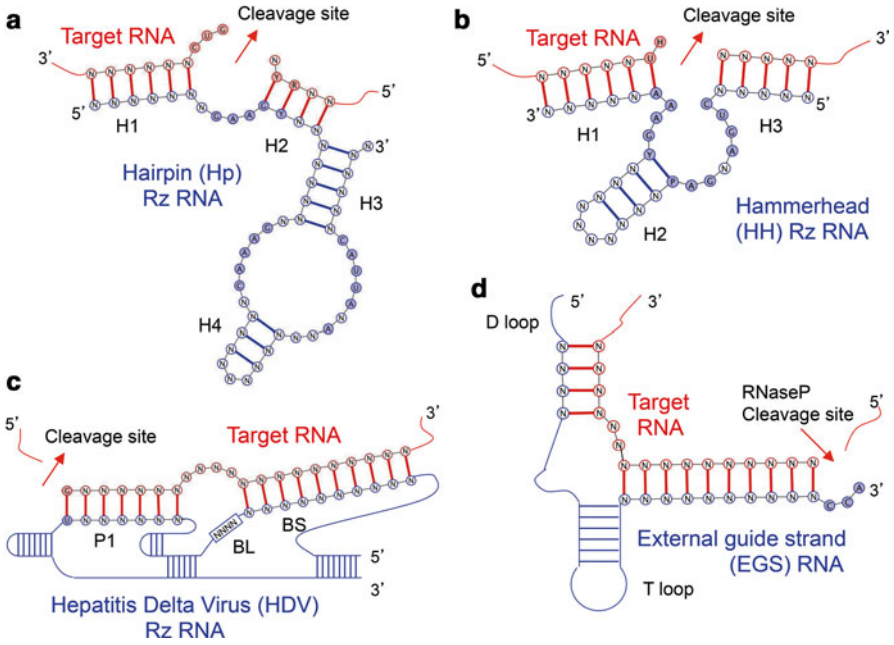

Fig. 1 Schematic of ribozymes designed to target HIV-1 replication: Base pairing between a ribozyme (Rz) or external guide strand (EGS) with its target RNA is shown as *red bars*, internal Rz or EGS base pairing is shown as *blue bars*. Nucleotides in the target RNA, Rz or EGS that are commonly conserved are *shaded* and named according to IUPAC conventions for nucleotides. The cleavage site in the target RNA is indicated with a *filled red arrow*. RNA secondary structures were drawn using VARNA [119]. (**a**) Hairpin (Hp) Rz: In complex with its target RNA, a typical Hp Rz forms four helices (H), H1 can be lengthened to increase specificity [21]. (**b**) Hammerhead (HH) Rz: In complex with its target RNA, a typical HH Rz forms three helices (H). H1 and H3 can be lengthened to increase specificity [21]. (**c**) Hepatitis delta virus (HDV) Rz: In complex with its target RNA, the specific on/off adaptor (SOFA) HDV Rz forms the P1 stem of the HDV Rz and an additional biosensor (BS) stem. The BS stem can be lengthened to increase specificity and the length of the spacer (SP) in the target RNA can be changed to facilitate Rz design. An internal blocker (BL) sequence can replace the target RNA in the P1 stem, preventing it from nonspecifically cleaving unintended 7 base targets [47, 120]. (**d**) RNase P EGS: The T loop and D loop which resemble the corresponding structures in a tRNA are shown, the EGS directs cleavage by RNase P at the indicated position in the target [29]

for gene therapy clinical trials. The first to reach the clinic were two Hp Rzs targeting sequences in the 5′ untranslated region (UTR) and Pol coding sequence of HIV-1 RNA [35]. Originally described in 1992 [18], the target site in the 5′UTR was attractive because of its high sequence conservation, its importance in HIV-1 replication and its presence in all spliced and unspliced HIV-1 transcripts (Fig. 2). As HIV-1 RNA is structured [36], many potential Rz target sites are inaccessible and several approaches have been described to identify optimal cleavage sites. In one of the first studies to screen HIV-1 RNA for accessible Rz target sites, Bramlage and colleagues probed the 5′UTR using HH Rzs with randomized binding arms [37].

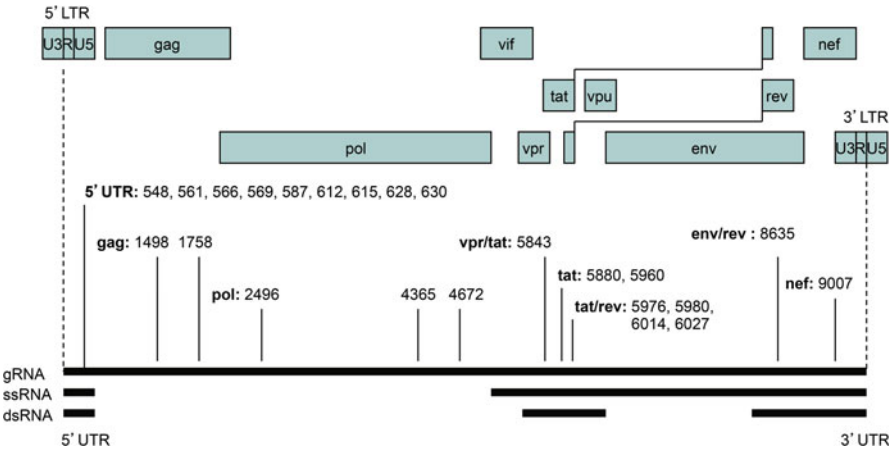

Fig. 2 Positions of ribozyme target sites in HIV-1 RNA. Untranslated and protein coding regions (*light blue boxes*) of HIV-1 are shown above the three groups of HIV-1 RNA transcripts (*black lines*). Rz target sites are illustrated based on their position directly after their cleavage site in reference strain HXB2 (GenBank: K03455). Approximate locations with respect to the full length genomic (g) RNA are shown. The regions that include all singly spliced (ss) and doubly spliced (ds) transcripts are illustrated below the gRNA

Using an in vitro screen, two target sites were identified with cleavage sites upstream of genome positions 569 and 615 according to the numbering in the HIV-1 strain HXB2 (GenBank K03455). The 569 target remains one of the most popular sites for HH Rzs, as well as for Hp Rzs with a cleavage site upstream of position 566 (Fig. 2 and Table 1). While a good correlation between the in vitro cleavage of the HIV-1 5′UTR and its accessibility in a cellular environment was reported [37], this is not the case for many other HIV-1 RNA sequences [38, 39], making cellular screens more appropriate for target site identification.

The first cellular screen, conducted to identify target sites in HIV-1 RNA that were accessible to inhibition by Rzs, used a randomized library based on small nucleolar RNA (snoRNA) linked HH Rzs [40]. Two novel target sites were identified in the HIV-1 5′UTR and Pol coding sequence (positions 548 and 4365, Fig. 2 and Table 1) and the corresponding Rzs were able to strongly inhibit both HIV-1 production in an adherent cell line and virus replication in transduced $CD4^+$ T cells [41]. For other antisense molecules, several cellular screens have been conducted to identify the best antiviral candidates from libraries of molecules targeting highly conserved sequences in HIV-1 RNA [42–45]. Using this approach, Müller-Kuller and colleagues identified a target site in the Pol coding sequence of HIV-1 RNA (position 4672, Fig. 2 and Table 1) that was particularly accessible to the antiviral activity of a HH Rz [46]. Our lab has also used this approach to identify a target site in the Gag coding sequence of HIV-1 RNA (position 1498, Fig. 2 and Table 1) [47] that was accessible to the antiviral activities of both an HDV-Rz and a short-hairpin RNA (shRNA) [28]. While several target sites in HIV-1 RNA have been identified as being accessible to different Rzs (summarized in Table 1), cellular screens have

Table 1 Study results for different ribozyme target sites in HIV-1 RNA

Target[a]	Rz	RNA conj.	Prom.	~ % Inhibition of HIV-1 expression[b]	Activity on HIV-1 replication[c]	Rz effect[d]	Ref.
UTR: 548	HH	sno-RNA	U6	~100 % in 293T cells	Partial in CEM cells (pHIV7)	Strong	[41]
UTR: 561	hRNaseP	–	$tRNA^{Val}$	ND	Complete in M4C8 cells (stbl. transf.)	Strong	[29]
				ND	Partial in M4C8 cells (pLNL6)	Strong	[30]
UTR: 566 (Hp) 569 (HH)	Hp	–	β-actin	~70 % in HeLa cells	ND	Strong	[18]
			$tRNA^{Val}$ VA1	~95 % in HeLa cells ~85 % in HeLa cells	ND ND	Strong Strong	[100]
			$tRNA^{Val}$, VA1	ND	Partial in HSC derived macrophages (pLNL6), VA1-Rz > $tRNA^{Val}$-Rz	ND	[101]
			$tRNA^{Val}$	ND	Partial in Jurkat cells (pLNL6)	ND	[102]
				ND	Partial in PBMCs (pLNL6)	ND	[103]
				ND	Partial in PBMC derived CD4+ T cells (pLNL6)	ND	[104]
		RRE-SLII	$tRNA^{Val}$	ND	Partial in Molt 4/8 cells (stbl. transf.)	ND	[73]
				ND	Partial in Molt 4/8 cells (pLNL6), Hp-SLII > Hp	ND	[105]
	HH	–	U6	~40 % in 293 cells	ND	Weak	[66]
		sno-RNA	U6	ND	Complete in CEM cells (pBabe)	Strong	[40]
		α-TAR	U6	ND	Partial in U87-CD4-CXCR4 cells (co-transf.)	Weak	[74]
	HH, Hp	3′ cisRz	CMV	~40 % (HH), 50 % (Hp) in HeLa cells	Complete in HUT78 cells (stbl. transf.), HH = Hp	ND	[59]
		α-DIS	U6	~90 % (HH), 60 % (Hp) in 293 T cells	ND	ND	[76]
UTR: 587	HH	–	Tk-TAR	ND	Partial in MT-4 cells (pMoTN)	ND	[60]
UTR: 612 (HH)	Hp	α–TAR	U6	ND	Complete in U87-CD4-CXCR4 cells (co-transf.)	Weak	[74]
615 (Hp)	HH, Hp	α-DIS, DIS	U6	~55 % (HH-αDIS), 80 % (Hp-DIS) in 293 T cells	ND	ND	[76]
UTR: 628	HH	$tRNA^{Lys3}$	U6	No effect in 293 cells, strong effect on infectivity of produced virus	ND	Weak	[79, 80]
UTR: 630	HH	3′ cisRz	CMV	~10 % in Hela cells (stbl. transf.)	ND	ND	[59]
Gag: 1498	HDV	–	H1	~50 % in 293 T cells	Partial in Jurkat cells (stbl. transf.)	Strong	[28]
Gag: 1758	HH	–	β-actin	ND	Partial in Hela CD4+ cells (stbl. transf.)	ND	[17]

Pol: 2496	Hp	–	VA1, MMLV-LTR	ND	Partial in Jurkat cells (pLNL6), VA1 > MMLV LTR	ND	[106]
Pol: 4365		sno RNA	U6	~100 % in 293T cells	Partial in CEM cells (pHIV7)	Strong	[41]
Pol: 4672	HH	3′ cisRz	CMV	~50 % in Hela cells (stbl. transfection)	ND	ND	[59]
		–	MMLV-LTR	ND	Partial in HuT78 cells (pLN-LCo)	Strong	[46]
Vpr/Tat: 5843	HH	–	SV40	ND	Complete in SupT1 cells (stbl. transf.)	ND	[107]
		–	MMLV-LTR	ND	Partial in PBLs (pLNL6)	ND	[108]
		–	MMLV-LTR	ND	Partial in CEM T4 cells (pLNL6)	Strong	[109]
	HDV	–	CMV	~60 % in Hela cells	ND	ND	[27]
Tat: 5880	HH	–	MMLV-LTR	ND	Partial in Jurkat cells (pBNSP1)	None	[110]
		U1sn RNA	U1	ND	Partial in Jurkat cells (stabl trans)	Strong	[77]
Tat: 5880 Tat/Rev: 6027	HH	–	MMLV-LTR	ND	Partial in CEM cells for both Rzs (pLN)	Strong	[111]
	HH×2	–	MMLV-LTR, CMV, $tRNA^{Met}$	ND	Complete in CEM for all promoters (pLN)	Strong	[112]
		–	MMLV-LTR	ND	Partial in bone marrow cultures (pLN)	ND	[113]
Tat/Rev:6014	bRNase P	–	U6	~90 % in 293T cells	Partial in H9 cells (pLXSN)	Strong	[32]
Tat/Rev: 5960, 5976, 5980	HDV	–	CMV	~60 % for all in Hela cells	ND	ND	[27]
Env/Rev: 8635	Hp	–	$tRNA^{Val}$	ND	Complete in Molt 4/8 cells (pLNL6)	Strong	[114]
		RRE SLII	$tRNA^{Val}$		Partial in Molt 4/8 cells (pLNL6)	ND	[105]
Nef: 9007	HH	3′ cisRz	CMV (3′cis HH Rz)	ND	Partial in HUT78 cells (stabl. transf.)	ND	[58]

Conj. RNA conjugate, *prom.* promoter, *ref.* reference, *ND* not determined

[a]The HIV-1 RNA region (illustrated in Fig. 2) followed by the nt position directly after the Rz cleavage site in HIV-1 reference strain HXB2 (GenBank K03455) is given

[b]Results for studies with Rzs co-transfected with an HIV-1 molecular clone into cells that support expression of HIV-1, but not infection. Studies with cells stably transfected with Rz DNA are indicated (stbl. transf.)

[c]Results are reported for Rzs in cells that support HIV-1 infection. Methods of expressing Rzs are indicated in brackets, viral vectors used to transduce cells are indicated by the gene transfer plasmid used (i.e., pHIV7)

[d]The Rz effect is reported for studies that compared inhibition by the Rz to an inactivated Rz or antisense control

been useful in identifying better target sites for previously described HH [41, 46] and HDV [28] Rzs, and are likely to play an important role in the identification of new candidates.

In addition to HIV-1 RNA, the mRNAs for cellular factors that assist in HIV-1 replication have been the target for several antisense technologies and one Rz screen has been used to identify novel target genes [48]. Most attention has been given to the β-chemokine receptor, CCR5 (R5), which serves as the co-receptor for the commonly transmitted R5-tropic HIV-1 strains. Individuals with a homozygous 32 base pair deletion in the CCR5 gene are resistant to HIV-1 infection with no apparent health problems, making CCR5 an ideal target for both drug and gene therapies [49]. Part of the reason why this gene may be dispensable is because other β-chemokine receptors can respond to the same set of chemokines. For the design of Rzs, it is therefore very important that they exclusively target the CCR5 gene and not the other highly similar β-chemokine receptor genes. The first Rzs designed to target this gene were two HH Rzs with cleavage occurring upstream of positions 77 [50] and 359 [51] according to a published sequence (GenBank U54994.1) (Table 2). Using sequence alignments, the cleavage position 77 was shown to be highly specific for the CCR5 gene and used to design a HH Rz with activity against HIV-1 replication [52, 53]. In combination with an shRNA and an HIV-1 trans-activation response (TAR) RNA decoy, this Rz has been evaluated in advanced preclinical studies [54, 55] and in the first clinical study [56]. Potential Rz target sites have been identified in other regions of the CCR5 gene (summarized in Table 2) and, with the characterization of new cellular cofactors of HIV-1 replication [57], may soon be identified in other host genes.

Ribozyme Expression Strategies

The first Rz designed to target HIV-1 replication in human cells was expressed from the human RNA polymerase II (Pol II) β-actin promoter [17]. Other RNA Pol II promoters that have been used include elements from the simian virus 40 (SV40), cytomegalovirus (CMV) and the long terminal repeat (LTR) of moloney murine leukemia virus (MMLV) or mouse stem cell virus (MSCV) (Tables 1 and 2). One limitation of RNA Pol II promoters is that the Rz must be expressed within a larger transcript and this may affect both its ability to bind and cleave its target. Nevertheless, several HH and Hp Rzs have been shown to be catalytically active when expressed from these promoters as a larger transcript (Table 1) and most clinical trials have used them to express Rzs in patient cells (section "Anti-HIV-1 Ribozymes in Clinical Trials"). Because they are expressed within an RNA transcript with both a 5′ cap and a 3′ poly(A) tail (Fig. 3a), Rzs expressed from RNA Pol II promoters are transported to the cytoplasm. In the cytoplasm, Rzs can target "incoming" virion RNA, new HIV-1 transcripts made from proviral DNA or the mRNAs for cellular factors involved in viral replication [21]. To reduce the effect of steric interference by the poly(A) tail, Rz expression vectors have been designed with a self-cleaving HH Rz

Table 2 Study results for different ribozyme target sites in CCR5 mRNA

Target[a]	Rz	Prom.	~ % Inhibition of cell surface CCR5 expression[b]	Inhibition of HIV-1 replication[c]	Rz effect[d]	Ref.
69, 445, 952	Hp	$tRNA^{Val}$	~80 % in PM1 cells (pAMFT), target 69	Complete in PM1 cells (pAMFT), all targets	Strong	[115]
72, 208, 304	HH×3	MMLV-LTR	Reduced in HOS-R5 cells in tandem (pLN)	ND	ND	[116]
77	HH	CMV	~60 % from pCCR5 in HEK293 cells (co-transf.)	ND	Strong	[50]
	HH	VA1	~70 % in HOS-R5 cells (stbl transf.)	Partial in PM-1 cells (pBabe) and HOS-R5 cells (stbl transf.)	Weak	[52]
			~50 % in HOS-R5 cells (pGINa)	Partial in HSC derived macrophages (pG1Na)	Weak	[53]
			Reduced in SupT1, PM1 and monocyte derived macrophages (pSV)	Partial in PM-1, SupT1, macrophage and microglial cells (pSV)	ND	[117]
			ND	Partial in HSC derived monocytes (pHIV-7: sh-Rz, sh-Rz-TAR)	ND	[54]
			ND	Partial in HSC derived thymocytes (pHIV-7: sh-Rz-TAR)	ND	[55]
359	HH	In vitro	ND	ND	ND	[51]
Seven sites	HH×7	MSCV-LTR	~90 % in PM1 cells	Complete in PM1 cells (pMGIN)	ND	[118]

Prom. promoter, *ref.* reference, *ND* not determined

[a]Target sites are grouped by their nt position directly after their cleavage site in the CCR5 gene (GenBank: U54994.1)

[b]Results are reported for reduction in CCR5 expression relative to control cells. Methods of expressing Rzs are indicated in brackets, viral vectors used to transduce cells are indicated by the gene transfer plasmid used (i.e., pAMFT)

[c]Results are reported for inhibition of HIV-1 replication, methods of expressing Rzs are given as in[b]

[d]The Rz effect is reported for studies that compared inhibition by the Rz to an inactivated Rz or antisense control

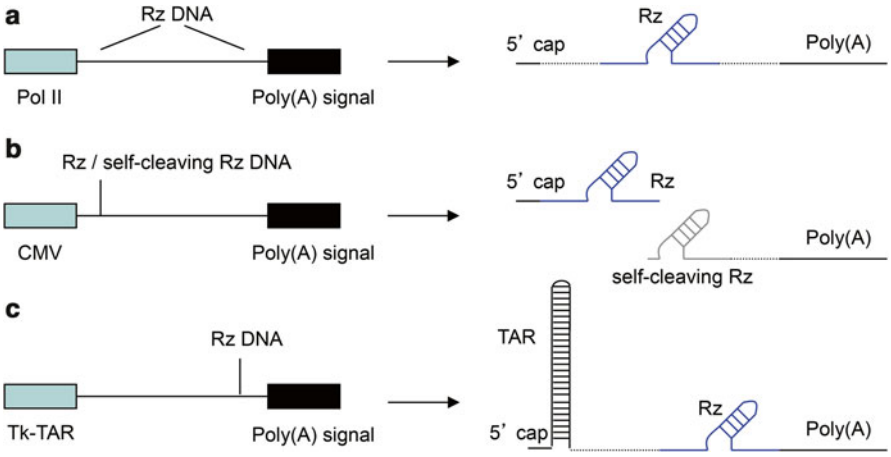

Fig. 3 RNA Polymerase II promoter strategies for anti-HIV riboyzmes. Promoters and poly(A) signals are shown as *light blue* and *black boxes* respectively. Rz containing transcripts are shown with a 5′ methylated cap and 3′ poly(A) tail. Major double stranded regions are illustrated, not to scale. (**a**) General Pol II promoter: The Rz DNA can be inserted anywhere between the promoter and poly(A) signal. The resulting Rz transcript (*blue*) will be embedded in the Pol II transcript (*dotted line*). (**b**) Pol II–3′ self-cleaving Rz: The addition of a self-cleaving Rz (*grey*) directly after the intended Rz (*blue*) can produce a Pol II promoted transcript with a defined 3′ end. A CMV promoter strategy is illustrated [59]. (**c**) Pol II–TAR: Modification of Pol II promoters using the HIV-1 TAR RNA enable high levels of transcription in the presence of the HIV-1 protein Tat. A modified thymidine kinase (tk) TAR RNA promoter strategy is illustrated [60]

positioned at the 3′ end of the HIV-1 specific Rz [58, 59] (Fig. 3b). Rzs expressed in this manner were able to inhibit both HIV-1 gene expression as well as viral integration into the host cell genome [59], suggesting that the poly(A) tail is dispensable for their ability to target "incoming" HIV-1 RNA in the cytoplasm. To design an expression vector that responds to the presence of HIV-1 infection, a herpes thymidine kinase (Tk) RNA Pol II promoter has been engineered to express the HIV-1 TAR RNA element [60] (Fig. 3c). In the HIV-1 LTR, this structure binds to the HIV-1 Tat protein and enhances the recruitment of RNA Pol II transcription elongation factors [61]. Due to enhanced Rz expression in cells infected by HIV-1, a HH Rz expressed from the Tk-TAR promoter was more effective at inhibiting HIV-1 replication compared to the same Rz expressed from the Tk, CMV or SV40 promoters [60]. Similar HIV-1 specific expression strategies have been used for other anti-HIV RNAs including shRNAs [62] and micro RNAs [63] using either the HIV-1 LTR itself, or the TAR RNA expressed in the context of another RNA Pol II promoter.

RNA Pol III promoters exclusively express small noncoding RNAs and can be grouped in three main types according to the promoter structure [64]. Type II promoters share structurally related intragenic A and B boxes and include the virus associated (VA) 1 RNA promoter as well as most tRNA promoters [65]. Both tRNA and VA1 promoters have been used to express anti-HIV Rzs appended to the 3′ ends of the RNA transcripts and some VA1 promoters have been designed to express Rzs

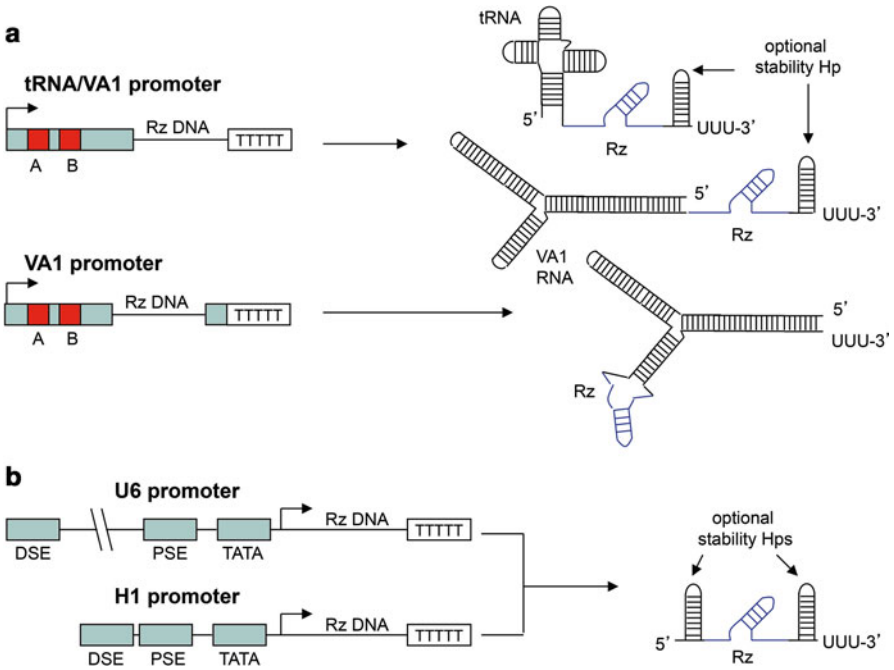

Fig. 4 RNA Polymerase III promoter strategies for anti-HIV ribozymes. Promoters are shown as *light blue boxes* and the Pol III termination sequence of five thymidines (T) is outlined. Examples of Rz containing transcripts are shown with predominantly double stranded regions illustrated, not to scale. Optional stability hairpins (Hp) are shown in some transcripts. These can be added at the transcription initiation site and/or directly before the termination signal, to protect single stranded ends from degradation by exonucleases. (**a**) Type II promoters: tRNA or VA1 promoters with conserved A and B boxes highlighted in *red* are shown [65]. Examples of Rz transcripts (*blue*) expressed from these promoters are illustrated with respect to their placement in a tRNA or VA1 gene. In the bottom transcript, the Rz is expressed within VA1 RNA at the end of a stem loop [52]. (**b**) Type III promoters: Distal and proximal sequence elements (DSE, PSE) as well as TATA boxes are shown for the human U6 [64] and H1 [121] promoters. The first 27 nt of the U6 transcript have been used to serve as a stability hairpin at the 5′ end (called U6+27 promoter) [66]

within the VA1 RNA (Fig. 4a). As with RNA Pol II promoters, an advantage of the type II RNA Pol III promoters is that the RNA transcript may be localized to the cytoplasm through tRNA and VA1 RNA export pathways. Several effective anti-HIV Rzs expressed from tRNA promoters have been described (Tables 1 and 2) and one has been tested in a clinical trial [35]. The design of these promoters can affect the localization of Rzs. Some studies showed a predominantly nuclear distribution ($tRNA^{Met}$) [66, 67] and others a predominantly cytoplasmic distribution ($tRNA^{Val}$) [68]. Depending on the desired cellular localization, careful design of these promoters may be required to achieve an optimal therapeutic effect. As VA1 RNA transcripts are predominantly cytoplasmic [69, 70], Rzs expressed together with the VA1 RNA are expected to have a cytoplasmic distribution. To limit cellular toxicity

of the VA1 RNA, Rzs expressed within the VA1 transcript have been inserted to replace stem loop IV [52] (Fig. 4a, bottom transcript), which is known to bind the cellular p68 kinase PKR [71]. Although careful consideration of the potential VA1 RNA mediated effects is warranted, several effective Rzs have been designed using this promoter (Tables 1 and 2) with one advancing to clinical trials [56].

Type III RNA Pol III promoters do not include intragenic regions and can be used to express any RNA with the addition of 4-6 uridines (U) at the 3′ end (Fig. 4b). Examples of type III promoters include the human U6 small nuclear (sn) and H1 RNase P RNA promoters [64]. Advantages of the U6 and H1 promoters for the expression of anti-HIV-1 RNAs include their precise transcription start and end sites, high transcriptional activity in different cell types and small size [72]. U6 or H1 promoters have been used to express HDV Rzs [28], RNase P-EGS Rzs [32] and several HH and Hp Rzs with different RNA conjugates added to enhance their inhibition of HIV-1 expression or replication (Table 1).

Ribozyme–RNA Conjugates

Several studies have explored the possibility of conjugating Rzs to different RNA structures in order to enhance their antiviral activity. The first conjugation used for this purpose was a Hp Rz fused to stem loop II (SLII) of the HIV-1 Rev response element (RRE) [73] (Fig. 5a). The rationale behind this approach was that the SLII-RRE could serve both as a decoy RNA for the HIV-1 Rev protein and as a means to localize the Rz to singly spliced and unspliced HIV-1 transcripts, which must interact with Rev to exit the nucleus. Using a similar approach, the TAR RNA has been conjugated to HH and Hp Rzs to provide an additional decoy effect for the HIV-1 Tat protein and to localize Rzs to their target sites in the 5′UTR of HIV-1 RNA [74, 75]. The HIV-1 dimerization initiation signal (DIS) has also been used to localize Rzs to the 5′UTR, while also acting to directly inhibit the process of HIV-1 RNA dimerization [76] (Fig. 5b).

Human RNA motifs have also been used to enhance the antiviral effects of Rzs. One of the first human RNAs used for this purpose was the spliceosomal U1 snRNA (Fig. 5c), modified to target the HIV-1 Rev 5′ splice site and to localize a HH Rz to an adjacent region [77]. The $tRNA^{Lys3}$ molecule, which binds to the 5′UTR and serves as the primer for HIV-1 reverse transcription [78] has also been used to localize Rzs to HIV-1 RNA. Rzs targeting a site adjacent to the primer binding site (position 628, Fig. 2 and Table 1) and conjugated to $tRNA^{Lys3}$ (Fig. 5d) were not able to inhibit HIV-1 expression, but reduced the infectivity of HIV-1 virions, suggesting that they localize with HIV-1 RNA in viral particles [79, 80]. HH Rzs conjugated to the human U16 snoRNA (Fig. 5e) have been shown to localize to the nucleolus [40] and provide strong antiviral effects against both HIV-1 production and replication [40, 41]. Based on these results, it has been suggested that HIV-1 RNA traffics through the nucleolus [40] and co-localization in this compartment may be particularly beneficial for Rzs targeting HIV-1 RNA.

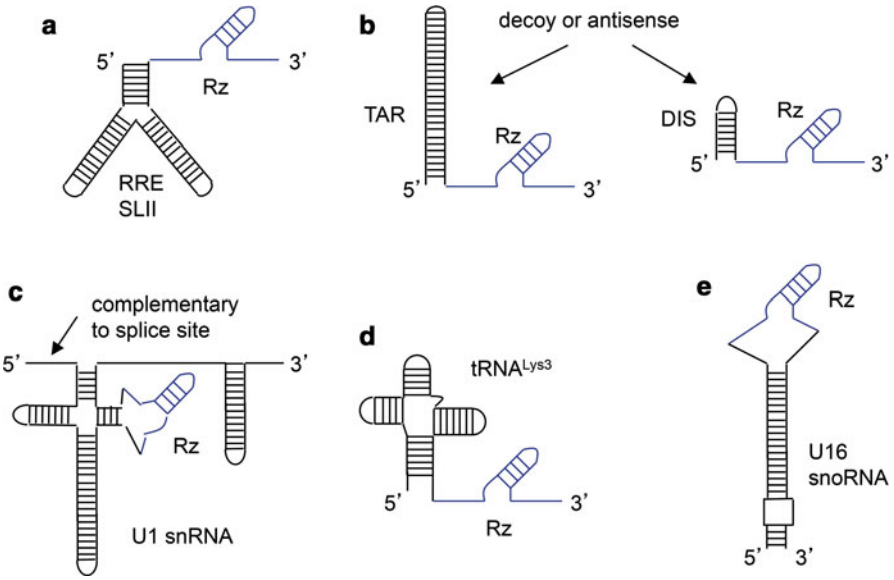

Fig. 5 Ribozyme RNA conjugates. Predominantly double stranded regions of Rzs conjugated to different RNA structures are illustrated (not to scale). Depending on the Rz and its expression strategy, different RNA may be appended to the 5′ or 3′ ends. (**a**) HIV-1 Rev Response Element (RRE) stem loop II (SLII): Acts as a decoy for HIV-1 Rev and localizes its Rz conjugate to HIV-1 transcripts that bind to Rev [73]. (**b**) HIV-1 trans activation response (TAR) and dimerization initiation signal (DIS): Act as decoys for HIV-1 Tat and HIV-1 RNA dimerization, respectively. Through hybridization with their corresponding structures in HIV-1 RNA, both sense and antisense molecules can localize their Rzs to targets in the 5′UTR [74, 76]. (**c**) Human U1 small nuclear (sn) RNA: Can be modified to recruit the splicing machinery to a complementary target sequence in HIV-1 RNA and localizes a Rz to that splice site [77]. (**d**) $tRNA^{Lys3}$: Localizes a Rz to the $tRNA^{Lys3}$ primer binding site located within the 5′UTR [80]. (**e**) U16 small nucleolar (snon RNA: Localizes a Rz to the nucleolus [40])

Anti-HIV-1 Ribozymes in Clinical Trials

The first trial to evaluate the clinical potential of anti-HIV Rzs used two Hp Rzs targeting the 5′UTR and Pol coding sequence of HIV-1 RNA (positions 569 and 2469, Fig. 2 and Table 1) [35]. The Rzs were inserted into a single MMLV vector (LNL6) and expressed from the vector LTR (position 2469) or a $tRNA^{Val}$ promoter (position 569). Following ex vivo transduction of patient derived $CD4^+$ T cells, Rz expressing cells could be detected for a short term post-infusion in a single patient [81]. Only the Rz expressed from the $tRNA^{Val}$ promoter could be detected in this patient, leading to speculation that the $tRNA^{Val}$ promoter may be better suited for sustained Rz expression compared to the MMLV LTR in a clinical setting [82]. A similar clinical protocol used the MMLV LTR to express a single HH Rz targeting the overlapping vpr/tat coding sequence (position 5843, Fig. 2 and Table 1).

In this trial, cells were isolated from HIV-1 negative donors and transplanted into their sero-discordant, HIV-1 positive identical twins [83]. Four pairs of sero-discordant twins were evaluated and the recipients were followed from 29 to 44 months post-transplantation. Although expression of the Rz was detected in cells from all patients throughout the study period, the number of Rz expressing cells was low and the study was not set up to evaluate the antiviral efficacy of the Rz [84].

In addition to $CD4^+$ T cells, hematopoietic stem cells (HSC) have been used in an effort to express Rzs and other anti-HIV-1 RNAs in all major HIV-1 target cells [85]. The first Rzs evaluated for use in HSC transplant were two HH Rzs targeting the tat and overlapping tat/rev coding sequences of HIV-1 RNA (positions 5880 and 6027, Fig. 2 and Table 1). The Rzs were expressed in tandem from the MMLV LTR and transduced ex vivo into patient derived HSCs. Two trials were conducted with and without marrow ablation prior to autologous transplant [86]. Although Rz expression could be observed in patient cells, it did not occur at a high enough frequency to evaluate an antiviral effect in either trial. A similar trial using autologous HSC transplant without marrow ablation [87, 88] was conducted for the tat/vpr Rz vector evaluated for use in $CD4^+$ T cell transplant [83, 84] (position 5843, Fig. 2 and Table 1). A phase II clinical study was next performed with this Rz and for the first time a moderate antiviral effect for an anti-HIV Rz was observed [89]. Although improvements in the efficacy of the Rz therapy are needed, results from this trial have so far shown that expression of an anti-HIV Rz is safe and the study remains active for follow-up evaluation (ClinicalTrials.gov NCT01177059). Recently, a HH Rz targeting the HIV-1 entry co-receptor gene CCR5 (position 77, Table 2), has advanced to clinical trials. This Rz has been introduced into patient HSCs, in combination with an shRNA and snoRNA-linked TAR decoy, using a lentiviral vector (HIV-7) [56]. A safety/efficacy study to further evaluate this therapy is in preparation (ClinicalTrials.gov NCT01961063).

Conclusions/Perspectives

Rzs were among the first gene therapy agents to reach clinical trials, and remain the only agent tested in a phase II, vector-controlled trial for HIV-1 infection [90]. Although a successful therapy has not yet been identified, the ability of Rzs to cleave their target RNAs without the assistance of cellular proteins makes them attractive candidates for therapeutic applications [91]. Data from clinical trials conducted to date have so far suggested that anti-HIV-1 Rzs can be expressed safely in patients undergoing both T cell transplant [81, 84] and HSC transplant [56, 86, 88, 89]. Advances that have been made in the identification of new Rzs and different expression and RNA conjugation strategies should lead to improved antiviral efficacy in future clinical trials. The use of new gene therapy vectors and clinical protocols [92–94] will also help towards the goal of developing safe and effective Rzs for the treatment of HIV-1 infection.

Advances in nonviral delivery methods have resulted in more than 50 RNA-based therapeutics entering clinical trials [16]. Although there has not yet been a clinical trial for HIV-1 infection, exogenous delivery of small interfering RNAs to HIV-1 infected mice has been achieved [95, 96]. With recent advances in RNA delivery technologies [97–99], a clinical application for pre-synthesized Rzs may be achieved in the near future. While the delivery vehicles used for Rz therapeutics are likely to change, results from studies designed to identify ideal target sites, expression strategies and Rz-RNA conjugates will remain useful in future studies to identify new Rzs for HIV-1 gene or drug therapy.

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Editing *CCR5*: A Novel Approach to HIV Gene Therapy

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Abstract Acquired immunodeficiency syndrome (AIDS) is a life-threatening disorder caused by infection of individuals with the human immunodeficiency virus (HIV). Entry of HIV-1 into target cells depends on the presence of two surface proteins on the cell membrane: CD4, which serves as the main receptor, and either CCR5 or CXCR4 as a co-receptor. A limited number of people harbor a genomic 32-bp deletion in the *CCR5* gene (*CCR5*Δ*32*), leading to expression of a truncated gene product that provides resistance to HIV-1 infection in individuals homozygous for this mutation. Moreover, allogeneic hematopoietic stem cell (HSC) transplantation with *CCR5*Δ*32* donor cells seems to confer HIV-1 resistance to the recipient as well. However, since *Δ32* donors are scarce and allogeneic HSC transplantation is not exempt from risks, the development of gene editing tools to knockout *CCR5* in the genome of autologous cells is highly warranted. Targeted gene editing can be accomplished with designer nucleases, which essentially are engineered restriction enzymes that can be designed to cleave DNA at specific sites. During repair of these breaks, the cellular repair pathway often introduces small mutations at the break site, which makes it possible to disrupt the ability of the targeted locus to express a functional protein, in this case CCR5. Here, we review the current promise and limitations of *CCR5* gene editing with engineered nucleases, including factors affecting the efficiency of gene disruption and potential off-target effects.

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Introduction

As HIV research continues to identify novel druggable viral and host factors that promote virulence and latency, the long-term clinical management and survival of HIV-positive individuals has improved considerably. Combination therapies, like highly active antiretroviral therapy (HAART), continuously suppress HIV replication while attenuating the development of escape mutants. However, because HAART is unable to clear latent viral reservoirs [1, 2], patients require lifelong treatment, which not only is expensive but has been associated with multiple adverse side effects and the development of drug-induced diseases [3–5]. The sustained antiviral efficacy of these therapeutic regimens is also strongly influenced by the compliance of each patient, which remains a key factor in managing not only the HIV infection but also the development of any accompanying disease [6]. Ideally, a therapy aimed at eliminating both the replicating and latent viral populations would provide a long awaited cure.

HIV-1 fusion with the cell membrane and ensuing virus entry is an intricate process that requires the expression of both the CD4 transmembrane glycoprotein as well as an associated seven-pass G-protein coupled chemokine co-receptor, CCR5 or CXCR4 (Fig. 1), a receptor combination typically found on CD4+ T cells, macrophages and dendritic cells [7]. Virus attachment is mediated by gp120, a viral surface glycoprotein located in the lipid membrane of the HIV-1 virion. Initially gp120 binds to CD4, which then facilitates the sequential attachment of gp120 to either the CCR5 or CXCR4 co-receptors. R5-tropic viruses, most prominently detected during the early stages of HIV-1 infection, bind to the CCR5 co-receptor, whilst X4-tropic viruses bind to CXCR4. The subsequent conformational change of the viral envelope protein exposes the viral gp41 glycoprotein, which mediates fusion with the target cell membrane. The resulting formation of a transmembrane pore enables the delivery of the viral capsid, which initiates viral integration and replication. Whereas the majority of the population is susceptible to infection, a small percentage of individuals are protected from infection with particular HIV strains. This resistance to HIV infection has been linked to naturally occurring genetic variations, including polymorphisms within the locus encoding the CCR5 co-receptor [8–14]. As a consequence, rational design of novel therapeutic strategies has also focused on blocking viral entry with small molecule drugs or genetic engineering to generate HIV-resistant T cells.

CCR5 as a Target for HIV Antiretroviral Therapy

CCR5 was first identified as the prominent co-receptor for R5-tropic viruses following the discovery that three chemokines, RANTES (CCL5), MIP-1α (CCL3), and MIP-1β (CCL4), impede HIV-1 binding [15]. Ever since, pharmaceutical companies have focused heavily on the development of HIV antiretroviral therapies based on entry and fusion inhibitors. One such drug, Maraviroc, binds to the

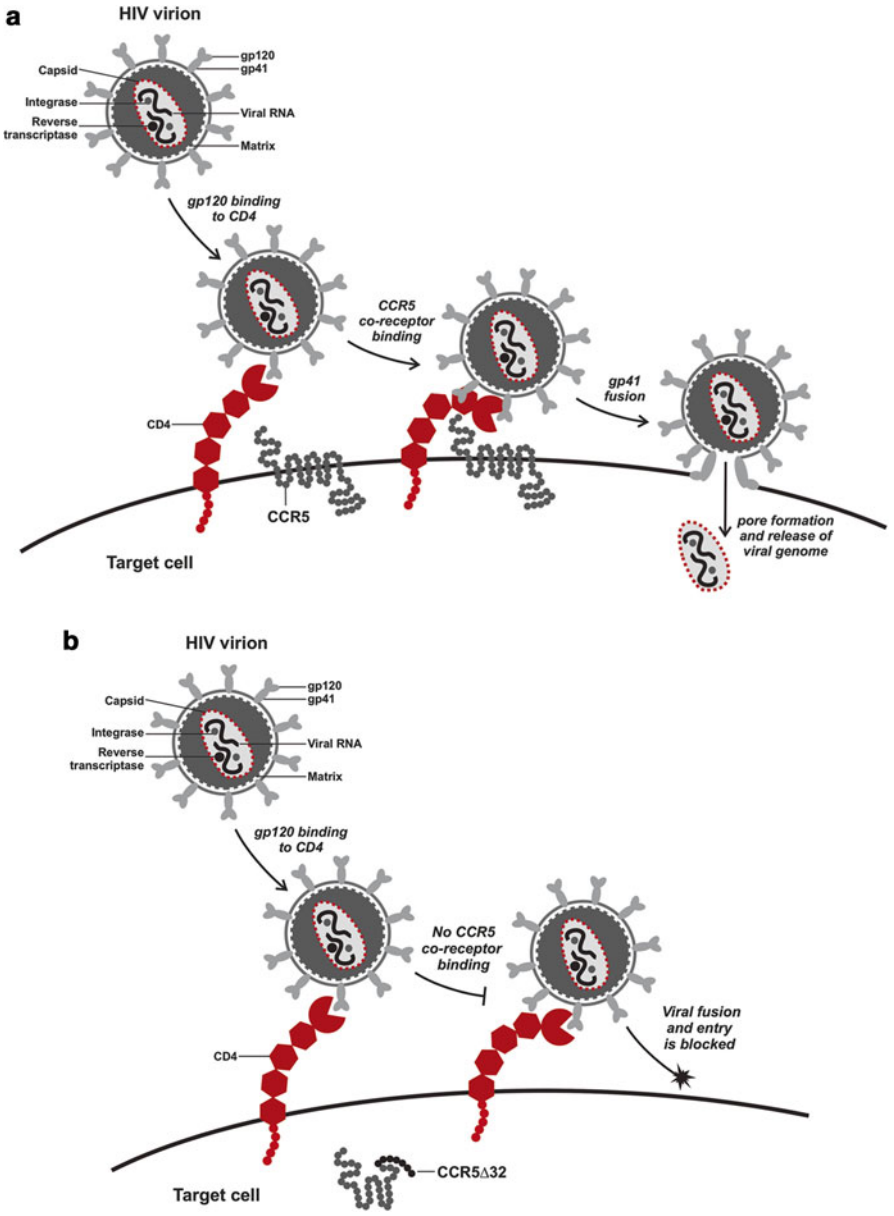

Fig. 1 Attachment and entry of R5-tropic HIV-1. (**a**) The HIV virion initially binds to target cells through interactions between the viral gp120 surface glycoprotein and the CD4 receptor. The CD4 receptor then draws the virion closer to the target cell, facilitating the interaction between the CCR5 co-receptor and gp120. This triggers a conformational change, allowing the gp41 glycoprotein to fuse to the cell membrane in order to create a transmembrane pore. The viral capsid, which contains the HIV RNA, integrase and reverse transcriptase, is then released into the target cell. (**b**) Initial binding of the HIV-1 virion occurs as described above; however, the CCR5Δ32 mutant form of this co-receptor is severely truncated and remains cytosolic, ultimately eliminating the gp120 binding site. As there is no co-receptor binding, the conformational change required to expose the gp41 protein is blocked, preventing viral fusion and entry

transmembrane domains of CCR5, ultimately preventing viral attachment and fusion [16]. Based on successful clinical trials, Maraviroc has been approved for HIV-1 treatment in both Europe and the USA. However, as with most currently available HIV therapies, viral escape mutants have been isolated [17–19]. Furthermore, as Maraviroc binds to the CCR5 co-receptor, it is not effective against X4-tropic viral infections.

Moving away from traditional HAART therapy, the adoptive transfer of synergistic T cells and allogeneic stem cells has been investigated as potential curative treatments. Initially, the efficacy of synergistic or autologous transplantation of hematopoietic stem/progenitor cells (HSPCs) was described in HIV-positive patients that had developed lymphomas [20–23]. Whilst patients remained on antiviral therapy, the myeloablative conditioning required prior to transplantation facilitated the reconstitution of the T cell compartment. In 2007, Hoffmann and colleagues reported that the adoptive transfer of T cells between HIV-1 discordant twins resulted in improved $CD4^+$ T cell counts [24]. The patients remain on antiretroviral therapy and required a total of 12 transfers to achieve a sustained expansion of $CD4^+$ cells. Since myeloablative conditioning was not performed before adoptive transfers, these results suggest that HLA-matched T cells could help reprise aspects of the immune system, provided that HIV viral loads are continuously repressed. Nonetheless, neither approach is curative, as patients still require continuous antiretroviral therapy post-transplantation.

In contrast, an allogeneic HSPC transplantation from a donor homozygous for the *CCR5Δ32* mutation has given rise to the first described permanent "cure" for HIV [25–27]. The *CCR5Δ32* mutation was originally identified in a small group of people who, despite being repeatedly exposed to HIV, did not contract the disease [12, 13]. This 32 base pair deletion in the *CCR5* gene induces a frameshift mutation and the resulting truncated protein does not support gp120 binding, ultimately preventing HIV-1 infection (Fig. 1). Although individuals who are homozygous for this mutation are resistant to R5-tropic HIV-1 infection, they remain susceptible to X4-tropic strains [28]. A number of studies showed that HIV-positive patients, who are heterozygous for the *CCR5Δ32* mutation, have reduced disease progression and better overall prognosis than patients who are homozygous for the wild-type *CCR5* gene [10, 11, 29, 30]. In 2009, Hütter and colleagues described the first curative allogeneic HSPC transplantation using an HLA-matched donor who was homozygous for the *CCR5Δ32* mutation [26]. Timothy Brown (alternatively referred to as the "Berlin patient"), an HIV-positive patient on HAART therapy, received the initial HSPC transplant after developing acute myeloid leukemia (AML), which was refractory to induction and consolidation chemotherapy. As his AML relapsed, a second HSPC transplantation from the same homozygous *CCR5Δ32* donor was performed. To date, the patient remains cancer-free and HIV negative in the absence of HAART [25–27], suggesting that homozygous *CCR5Δ32* HSPC transplantation could be used to cure not only the blood-related malignancy but also HIV-1 infection. Although this presents an idealistic approach, the number of homozygous *CCR5Δ32* donors is low, since only approximately 1 % of the Caucasian population has this HIV-1-resistant genotype [30, 31]. Accordingly, much research has focused on engineering homozygous *CCR5Δ32*-like mutations in patient-derived HSPCs and T cells using designer nucleases.

Gene Editing with Designer Nucleases

Designer nucleases are engineered enzymes that are comprised of a DNA binding domain, tailored to bind to a specific target sequence, and a DNA cleavage domain (Fig. 2). Binding of the engineered nuclease to a defined genomic target site results in the formation of a DNA double stranded break (DSB) which, in turn, elicits cellular DNA repair mechanisms that can be exploited to achieve targeted and permanent genetic modifications. Mammalian cells rely on two major DSB repair pathways: non-homologous end joining (NHEJ), which is active throughout the cell cycle, and homologous recombination (HR) based repair, which is restricted to the S/G2 phase.

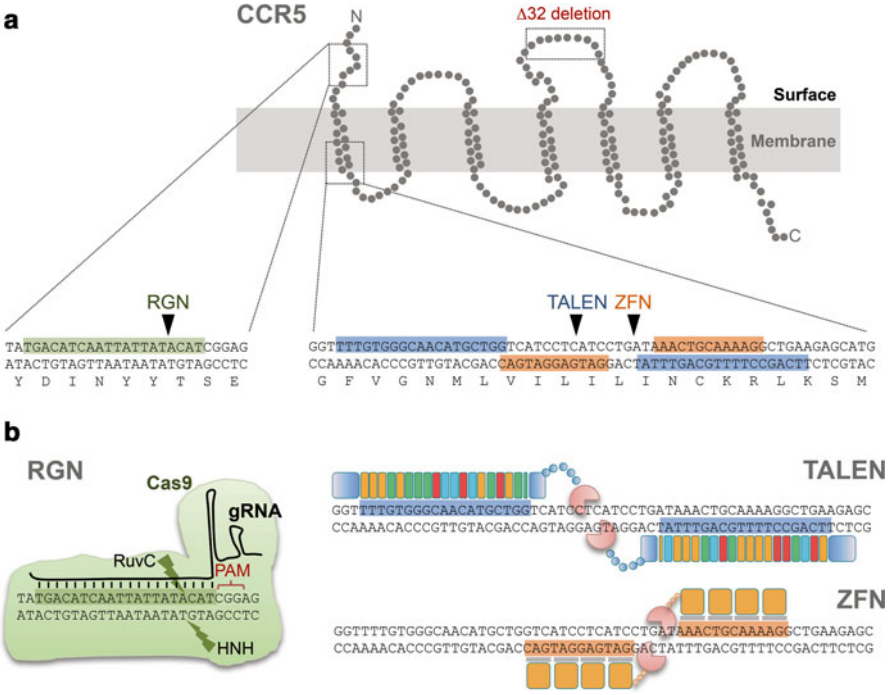

Fig. 2 Designer nucleases to disrupt *CCR5*. (**a**) Schematic of the CCR5 protein localized to the cellular membrane. The *dotted boxes* indicate the corresponding regions of the genomic locus targeted by designer nucleases as well as the location of the Δ*32* deletion. Three different designer nuclease platforms have been efficiently engineered to knock out *CCR5* and the corresponding DNA target sites are indicated in *green* (RGN), *light blue* (TALEN), and *orange* (ZFN). The putative cleavage sites are indicated (*black triangles*) (**b**) Designer nucleases. RGENs are composed of the Cas9 nuclease and a guide RNA (gRNA) that directs the enzyme to the target site. The protospacer adjacent motive (PAM) required by the Cas9 enzyme to recognize and cleave the target site is indicated in *red*. The two nuclease domains within the Cas9 protein (RuvC and HNH) are highlighted. TALEN or ZFN monomers include a modular DNA binding domain that is engineered to recognize a specific DNA target sequence. Each TALE module specifically recognizes one nucleotide in the target subsite, while a ZF module binds to a nucleotide triplet. A short linker connects the respective DNA binding domain to the cleavage domain of the *Fok*I restriction enzyme (*light red*), which cuts the DNA upon dimerization of the two monomers at the target site

As compared to HR, NHEJ is an error-prone pathway, which can be harnessed to insert small insertion/deletion (indel) mutations at the DNA break in order to inactivate a target gene, such as *CCR5*. Conversely, HR relies on the genetic information contained in the sister chromatid for the accurate repair of a DSB. For gene editing, this pathway can be exploited by including a donor DNA template with specific sequence homology during the generation of nuclease-mediated DSBs [32, 33]. In this setting, the genetic information is transferred from the donor DNA to the target locus, thus allowing precise genomic modifications.

Dimeric zinc-finger nucleases (ZFNs) have been traditionally used for genetic modifications [34]. The DNA binding domain is comprised of multiple zinc-finger modules, each recognizing three to four nucleotides in a sequence-specific manner. However, generating highly active ZFNs with novel specificities is challenging and cumbersome, as context-dependent interactions between individual modules within the zinc-finger array affect the overall binding efficiency [35]. In the last 15 years, ZFNs have been successfully used in basic research to study gene function [36–44] and to correct genetic defects underlying human disorders for therapeutic purposes [45–47] in preclinical settings. Their relatively small size has allowed ZFNs to be delivered using the most common viral and non-viral platforms as well as a direct protein delivery [48].

For therapeutic applications, a high specificity of the designer nuclease is of utmost importance, as off-target cleavage activity poses obvious concerns with regard to genotoxicity. Two studies assessing the genome-wide specificity of the *CCR5*-specific ZFN pair revealed a considerable level of non-specific off-target activity [49, 50]. In view of the complexity of generating highly specific ZFNs, the discovery of a novel modular DNA binding domain identified in transcription activator-like effectors (TALEs) of plant pathogens has provided new momentum to the genome engineering field. TALE-based nucleases (TALENs) can be easily customized to target any given sequence (Fig. 2) due to their simple recognition code in which a TALE module specifically recognizes one nucleotide [51–53]. When compared to an existing ZFN, some *CCR5*-specific TALENs showed similar activity but lower cytotoxicity [54, 55]. While more work needs to be invested to dissect the specificity signature of designer nucleases, initial results suggest that TALENs seem to harbor a rather high specificity [55–57]. TALENs have hence evolved as a valid alternative for the generation of transplantable HIV-resistant T cells. Unlike ZFNs, TALENs are relatively large proteins with a highly repetitive structure. While adenoviral vectors can be used to deliver single TALEN monomers, lentiviral vectors have failed to transfer intact TALEN encoding expression cassettes [58]. As a consequence, many labs have relied on in vitro transcribed mRNA or plasmid DNA to deliver the TALENs.

The newest addition to the toolbox for genome engineers is of bacterial origin as well. The clustered regularly interspaced palindromic repeats (CRISPR)/Cas9 system is used by prokaryotes to defend themselves against invading DNA [59]. It consists of the Cas9 cleavage enzyme complexed to a guide RNA strand that directs the enzyme to a 20-nucleotide long target site [60, 61]. Exchanging a specific portion of the gRNA molecule allows researchers to redirect the Cas9 cleavage activity

to a user-defined target sequence (Fig. 2). This versatile platform, also known as RNA-guided nuclease (RGN) technology, holds many advantages over both ZFNs and TALENs. The most obvious one is the simplicity to customize the enzyme to target any sequence of choice by simple molecular cloning techniques [62, 63]. Moreover, delivering the Cas9 protein with more than one gRNA molecule allows multiplexing, i.e., to target several sites simultaneously [64]. Although RGNs have been shown to target *CCR5* efficiently [65, 66], concerns regarding their specificity have been raised [67–69]. On the other hand, further advances, such as Cas9 nickases [70], the use of truncated guide RNAs [71], and dimeric RNA-guided *Fok*I nucleases [72], have shown promise to generate more specific RGNs.

Target Cells

Two potential cellular targets have been envisioned for a *CCR5* disruption-based HIV therapy: CD4+ T cells, which are the mature lymphocytes infected by HIV, or CD34+ HSPCs, which would give rise to HIV-resistant T cells and macrophages.

In the first scenario, patient derived CD4+ T cells will be collected by apheresis and modified ex vivo using designer nucleases [47]. Modified cells will then be amplified in vitro and subsequently reintroduced in the patient (Fig. 3). For the therapy to be effective, a large number of cells are required to retain proficient proliferative and effector functions. Consequently, patients enrolled in such trials should have a CD4+ T cell count above a set threshold that allows collection of enough CD4+ T cells to be genetically altered and subsequently expanded ex vivo. Transfer of the *CCR5* modified T cells will at least temporarily restore T cell immunity of the patients. Discontinuation of antiretroviral medication would allow the virus to infect

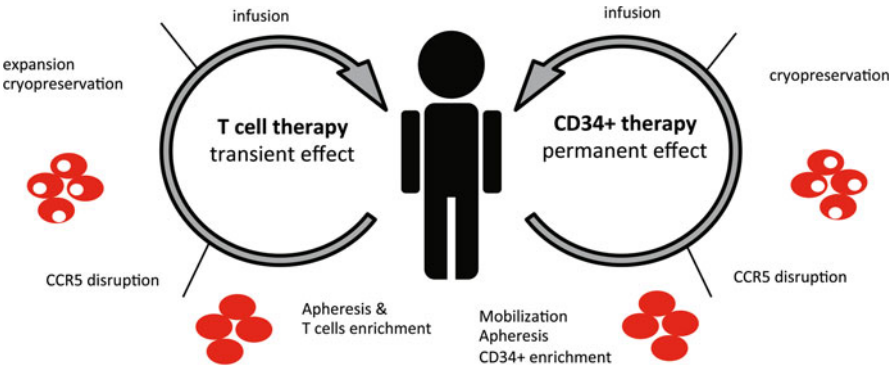

Fig. 3 Clinical application of modified T cells and CD34+ cells. After collection of cells by apheresis, CD4+ T cells or CD34+ hematopoietic stem cells are enriched and *CCR5* disruption is accomplished by expression of designer nuclease. T cells are expanded ex vivo before adaptive transfer. In case of CD34+ cells, chemotherapy of AIDS lymphoma patients will assist the engraftment of the modified cells

and replicate in susceptible cells. Over time, only cells devoid of CCR5 will be able to expand in presence of the virus. Once the modified pool of T cells is depleted as a result of cellular senescence, the transfer of modified T cells can be repeated. Of note, the *CCR5* disrupted cells remain susceptible to CXCR4-tropic strains and discontinuing HAART could result in a flare of these X4-tropic strains. Importantly, however, a viral rebound was not observed in the "Berlin patient" although he was positive for CXCR4-tropic strains [26, 73]. Nonetheless, to overcome this potential limitation, a simultaneous disruption of *CCR5* and *CXCR4* has been reported in primary CD4+ T cells, and protection from both R5 and X4-tropic virus was verified in a mouse model [74].

The second approach is directed towards the targeting and manipulation of CD34+ HSPCs [45]. The main advantage of this strategy when compared to CD4+ T cell targeting is the ability of modified CD34+ cells to engraft and produce a long-lasting effect. HSPCs continuously differentiate in all the hematopoietic lineages, including T cells and macrophages that can be infected by HIV. The downside is that stem cells are difficult to manipulate and tend to lose their differentiation potential when cultured ex vivo. In addition, transplantation of HSPCs requires a mild preconditioning regimen to provide adequate space in the bone marrow for engraftment of the modified HSPCs. In this setting, leukopoiesis will occur from both modified and non-modified CD34+ cells, and the survival advantage in the presence of replicating HIV will occur on the level of CD4+ T cells and macrophages.

The advantages of the two approaches are apparent: since the genetic modification is performed in autologous cells, there is no need for HLA matching, which significantly decreases the risk of developing graft-versus-host-disease or graft rejection. Additionally, there is no need for post-transplantation immunosuppressive therapy. The patients will be provided with an autologous pool of HIV-resistant cells, which restores the immune system either transiently or permanently. An open question is whether active clearance of HIV reservoirs will occur in an autologous setting where the graft-versus-host effect is not present.

Applying Designer Nucleases for HIV Gene Therapy

Many HIV gene therapy trials based on the ex vivo modification of CD4+ T cells or HSPCs have used ribozymes, aptamers, and siRNAs [75]. Although none of these studies have reported clinical benefit in terms of decreased viral load or protection from HIV replication so far, they showed promising outcomes in terms of safety, long-term engraftment and survival of modified peripheral cells [76, 77], including maintenance of the genetic modification in mature myeloid and T cells [77, 78]. These positive aspects were the basis for the clinical trials aimed at disrupting the *CCR5* co-receptor gene with designer nucleases. This strategy has a major advantage over conventional knockdown approaches using RNA interference, since it permits the generation of HIV-resistant cells after a single treatment. Indeed, when *CCR5*-specific ZFNs were delivered to primary human T cells by adenoviral

transduction, a population of HIV-resistant T cells was observed in vivo 50 days after transplantation in a murine HIV infection model [47]. A similar approach was applied to human CD34+ HSPCs by nucleofection of DNA expression plasmids encoding *CCR5*-specific ZFNs. Following transplantation in a humanized HIV mouse model, *CCR5* disrupted cells showed selective survival after challenge with HIV [45]. However, nucleofection of plasmid DNA into primary cells, and in particular into stem cells, can be associated with considerable cytotoxicity. This drawback has been recently overcome by delivering ZFNs in the form of in vitro transcribed mRNA [79].

Based on these preclinical accomplishments, the use of ZFNs as an HIV gene therapy for the generation of transplantable autologous HIV-resistant T cells has entered phase I/II clinical trials. The protocol was similar in all studies (Fig. 3): CD4+ T cells were isolated from HIV patients and transduced with an adenoviral vector expressing a ZFN pair targeted to *CCR5*. After ex vivo expansion, the cells were reinfused into the patients. In the first published study [80], 12 patients were recruited and received one infusion of 10 billion CD4 T cells. Six patients underwent a 12-week treatment interruption 4 weeks after infusion. The primary objective was the assessment of safety, while secondary objectives included the evaluation of increased CD4+ T cell counts, the trafficking of *CCR5*-modified cells to the gut mucosa, and a decrease in viral load. The modified CD4+ T cells engrafted and were detected in the patients up to 42 months after transfer. Moreover, modified cells were detected in all biopsies of the rectal mucosa, revealing successful trafficking. Treatment was prematurely discontinued and HAART reinitiated in two patients because of a rise in HIV RNA levels above the threshold. In four patients who completed the 12-week HAART interruption, a relative survival advantage of the modified cells was observed. The decrease in virus load correlated with the number of circulating cells carrying biallelic modifications at the *CCR5* locus. Actually, the one patient with undetectable HIV load after treatment interruption was found to be heterozygous for the *CCR5Δ32* allele. In summary, this first-in-human application of ZFN designer nucleases showed infusion of *CCR5*-modified T cells to be safe and well tolerated, and led to reduced virus loads in some patients. However, complete eradication of HIV could not be achieved, probably due to suboptimal engraftment and the low number of cells carrying a biallelic disruption. It will be interesting to learn what further safety evaluations involving a larger sample size and a long-term follow-up will reveal.

Based on these promising results, more studies have been initiated, including one which specifically enrolled ten patients heterozygous for *CCR5Δ32* (NCT01044654). As expected, the biallelic modification frequency in the *CCR5Δ32* cohort was doubled as compared to normal, and three out of eight subjects with high levels of engraftment had virus loads below detection limit up to 20 weeks following interruption of HAART (Sangamo Biosciences Inc., Richmond, CA: press release on Dec. 6, 2013). To improve engraftment and increase of CD4+ T cell counts, another study involving 12 patients has evaluated the use of escalating doses of cyclophosphamide (NCT01543152), a drug used for non-myeloablative lymphodepletion to enhance adoptive T cell transfer [81]. Conditioning with cyclophosphamide was

reported to be safe and well tolerated, and a dose-dependent increase was observed for both normal and modified CD4+ T cells (Sangamo Biosciences Inc., Richmond, CA: press release on Dec. 6, 2013).

Since HIV can also use the *CXCR4* co-receptor for viral entry, an alternative strategy for HIV treatment using *CXCR4*-specific ZFNs delivered by adenoviral vectors has been investigated [82]. However, while *CCR5* disruption seems to be well tolerated by the immune system, the CXCR4 receptor plays an important role in immune regulation, especially in B cell development [83], and its disruption raises concerns of potential deleterious effects. ZFNs have not only been used to create HIV-resistant cells but novel strategies have also been developed to eradicate the provirus from infected cells [84]. While promising, this approach may be limited by the difficulties associated with targeting the integrated provirus, especially in rare cells like resting T cells or latently infected cells.

Concluding Remarks

The presented clinical results are encouraging and validate the *CCR5* knockout strategy as an important development in fighting HIV infection. Furthermore, the data underline the number of T cells with biallelic *CCR5* disruption to be a key factor for clinical success. On the other hand, off-target cleavage of designer nucleases is a major concern. This is especially true if applied in multipotent stem cells predestined to be transplanted in patients, as the potentially mutagenic events could prompt a malignant phenotype. Hence, specificity of engineered nucleases will be the second key factor required to pave the road for this new line of gene therapy into the clinic.

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Synthetic DNA Approach to Cytomegalovirus Vaccine/Immune Therapy

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Abstract There is no licensed vaccine or cure for human cytomegalovirus (CMV), a ubiquitous β-herpes virus that infects 60–95 % of adults worldwide. Infection is a major cause of congenital abnormalities in newborns, contributes to development of childhood cerebral palsy and medulloblastoma, can result in severe disease in immunocompromised patients, and is a major impediment during successful organ transplantation. While CMV has been increasingly associated with numerous inflammatory diseases and cancers, only recently has it been correlated with increased risk of heart disease in adults, the number-one killer in the USA. These data, among others, suggest that subclinical CMV infection, or microinfection, in healthy individuals may play more of a causative role than an epiphenomenon in development of CMV-associated pathologies. Due to the myriad of diseases and complications associated with CMV, an efficacious vaccine would be highly valuable in reducing human morbidity and mortality as well as saving billions of dollars in annual health-care costs and disability adjusted life years (DALY) in the developing world. Therefore, the development of a safe efficacious CMV vaccine or immune therapy is paramount to the public health. This review aims to provide a brief overview on aspects of CMV infection and disease and focuses on current vaccine strategies. The use of new synthetic DNA vaccines might offer one such approach to this difficult problem.

Introduction

Human cytomegalovirus (CMV) is a ubiquitous β-human herpes virus, also known as human herpes virus type 5, with broad clinical implications in both the developing and developed world. It is the largest member of the human herpes viruses with a linear, double-stranded DNA genome of ~230 kbp coding for 200–250 open reading frames (ORF)s [1]. It is highly seroprevalent in the human population and

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establishes lifelong latency within the host with periodic reactivation. Reports of seropositivity in the USA range from 36.3 % in 6–11 year old children to 90.8 % in those aged ≥80 years [2]. Worldwide annual seroconversion rates among pregnant women and health-care workers were found to be around 2.3 % and 8.5 %, respectively [3]. CMV is transmitted primarily via saliva, placental transfer, breast-feeding, blood transfusion, sexual contact, solid-organ transplantation (SOT), or hematopoietic stem cell transplantation (HSCT) [4]. While acquired CMV infection is asymptomatic in the vast majority of immunocompetent hosts, the consequences of infection in fetuses and immunocompromised patients make CMV an important public health concern [5]. Furthermore, infection is a major impediment to successful organ transplantation [6–8]. Despite over 50 years of clinical research, there is no vaccine or cure available.

Overt Danger: CMV Infection and Its Burden to Public Health

CMV is estimated to infect 60–95 % of adults worldwide. The most common overt CMV-related disease is congenital CMV, which is a major cause of neurological and sensory impairments in children [9]. Newborns may develop cytomegalic inclusion body disease, a severe disease characterized by jaundice, petechiae, hepatosplenomegaly, microcephaly, motor disability, chorioretinitis, cerebral calcification, and multiple organ involvement [10]. Permanent physical sequelae include microcephaly, hearing loss, vision loss, and mental retardation. Furthermore, there is evidence that intrauterine CMV infection is significantly associated with cerebral palsy [11]. Between 20,000 and 40,000 children are born with congenital CMV infections in the USA each year, resulting in 100–200 deaths and 4,000–8,000 individuals developing permanent neurological sequelae [12, 13]. Sensorineural hearing loss is the most common symptom of CMV infection, occurring in 10–15 % of symptomatic children.

Immunocompromised adults including AIDS and transplant patients are also at major risk for CMV disease. In AIDS patients, viral disease is most commonly manifested as retinitis during which CMV causes a complete-thickness infection of retinal cells. If left without treatment, this infection results in subacute progressive retinal destruction and permanent blindness [14]. CMV disease can also less commonly involve other organ systems, including the central nervous system (resulting in polyradiculopathy and ventriculoencephalitis) and the respiratory system (causing pneumonitis) [13].

Along with the potential for significant morbidity and mortality, CMV disease, in addition to medical consequences, also places an extraordinarily high economic burden on the US health-care system. The economic burden of congenital disease alone exceeds $2 billion annually in the USA [12]. In liver transplant recipients, CMV disease is associated with a roughly 49 % increase in medical charges [15]. Congenital CMV is a significant contributor to the lifetime costs associated with

mental retardation, hearing loss and vision impairment, estimated to be $51.2 billion, $2.1 billion, and $2.5 billion respectively [16]. A severely affected, CMV-infected child has been estimated to have additional lifetime health-care costs of ~1 million dollars [17]. All told, overt CMV disease is estimated to cost the US health system at least $4 billion annually [18]. Therefore, CMV morbidity and mortality among immune-compromised patients (such as those infected with HIV), solid-organ and HSCT patients, as well as fetuses and newborns, calls for the development of an efficacious vaccine to combat this infectious disease.

CMV Microinfection: The Silent Threat

While it was widely held that latent or asymptomatic CMV infection was virtually benign in healthy individuals, it has now become increasingly clear that subclinical infection with CMV may play a greater role in a variety of diseases. This low-grade "microinfection" has been only recently detectable through the use of advanced techniques [19, 20] and has been implicated as a causative factor rather than an epiphenomenon in certain cancers, inflammatory, and hypertensive and pulmonary diseases [20–24]. This may be due to CMV's polytrophic nature, large proteome and immunomodulatory activity, allowing CMV to exert significant effects in a variety of organ systems. Recently, CMV microinfection has been correlated to increased risk of essential hypertension. Through quantitative reverse-transcription polymerase chain reaction, Li et al. have identified the presence of CMV microRNA in individuals with hypertension, finding a significant correlation between the presence of CMV DNA and diagnosed hypertension [20]. Essential hypertension is a prevalent risk factor for a variety of cardiovascular diseases including stroke, coronary heart disease and renal and heart failure, affecting >1 billion adults worldwide.

Low-grade CMV infection has also recently been associated with various forms of cancer, including medulloblastoma [24], colon cancer, malignant glioblastoma, EBV-negative Hodgkin's lymphoma, prostatic carcinoma, and breast cancer [25]. In medulloblastomas, which are the most frequent malignant brain tumors in children, inhibition of CMV replication activity with the antiviral drug valganciclovir was reported to reduce tumor growth both in vitro and in vivo [24]. The molecular basis for such oncogenesis and "oncomodulation" has been described in broad terms. Several CMV-encoded gene products have been shown to control cellular pathways that may be involved in oncogenesis, including cellular differentiation, cell cycle regulation, DNA damage and repair, epigenetic functions, apoptosis, cellular migration, angiogenesis, and immune evasion [26].

CMV microinfections have also been implicated in a number of inflammatory diseases. Studies have found that approximately 90 % of patients with inflammatory bowel diseases have an active CMV infection in their bowel [27]. While infected cells were rare, they were present in the deep mucosa of the bowel and only in inflamed areas. CMV reactivation has also been seen in the inflamed, but not non-inflamed, tissues of patients with rheumatoid arthritis (RA), Sjögren's syndrome,

dermatomyositis and polymyositis, psoriasis, Wegener's granulomatosis, ulcerative colitis, and Crohn's disease [25]. These viral microinfections were thus invariably associated with disease pathology and inflammation. This association may in part be the result of elicitation of $CD4^+CD28^-$ T cell populations, which have only been described in CMV-infected individuals. In patients with RA, only CMV-seropositive patients, which constitute the majority of all RA patients, carried $CD4^+CD28^-$ T cells. These $CD28^-$ T cells were found to be enriched in RA patients, as well as in patients with dermatomyositis and polymyositis, but found in a lower frequency in healthy CMV-seropositive controls [28–30]. Indeed, in patients with myositis, 60–90 % of all infiltrating T cells in inflamed muscle were $CD28^-$. These T cells responded in vitro to CMV antigen (Ag) stimulation, suggesting that CMV may drive the accumulation of such $CD28^-$ T cells in inflamed tissues during the course of an inflammatory disease [25]. Thus, a link between low-grade CMV infection and numerous inflammatory diseases has emerged in recent studies.

While the costs of overt CMV disease are substantial, the recent interest in microinfections in a variety of other disease outcomes has broadly expanded the potential economic implications of CMV infections. In the USA, cardiovascular diseases are estimated to have cost $444 billion in 2010, with treatments accounting for 1/6 of all health-care expenditures (http://www.cdc.gov/chronicdisease/resources/publications/AAG/dhdsp.htm). Total direct medical costs of cardiovascular disease are projected to triple from $273 billion in 2010 to $818 billion in 2030. Real indirect costs due to lost productivity for all cardiovascular disease are estimated to increase from $172 billion in 2010 to $276 billion in 2030, a 61 % increase [31]. A recent model estimated that life-time costs of patients diagnosed with prostate cancer are $110,520 per patient, with prostate-cancer related costs estimated to be $34,432 or roughly 31 % of total costs [32]. With over 660,000 new cases diagnosed each year, including 186,300 in the USA alone, prostate carcinoma represents a significant economic burden on the health-care system [33]. Furthermore, the potential role of latent CMV infection in inflammatory bowel disease represents significant costs to the health-care system. A study by Feagan et al. found that the median annual costs for Crohn's disease patients was $3,668 per patient with the subset of hospitalized patients having a median annual cost of $21,671 per patient [34]. As the CDC estimates that as many as 1.4 million persons in the USA suffer from Crohn's disease or ulcerative colitis, both thus cause significant costs to the health-care system and patients [35]. Thus, when considering the impact of CMV microinfection to CMV-associated diseases, development of an efficacious vaccine is of the utmost importance and has the potential to dramatically reduce associated health-care costs.

Quest for a CMV Vaccine/Immune Therapy

Due to significant human and economic costs, the need for an effective vaccine against CMV has been ranked as of the highest priority by the US Institute of Medicine. Further emphasizing the need for an effective vaccine is the emerging

evidence implicating CMV microinfection in a number of other diseases, including cancers and inflammatory conditions. While numerous attempts have been made for over 5 decades in this regard, there is no currently licensed CMV vaccine or cure. However, the ability of the immune response to suppress virus for long intervals of time during CMV infection provides evidence of protective immune correlates and suggests that the development of a CMV vaccine may be feasible. Therefore, the understanding of immunological markers that can predict protection from CMV along with the identification of immunogenic CMV antigen targets may be essential for improving future vaccine immunogenicity and duration of protection.

CMV Immunology

A better understanding of protective immune responses against CMV is pivotal in the quest for a CMV vaccine. Suppression of CMV within otherwise healthy individuals is an active process mediated by antiviral CMV-specific immune responses. Both promising clinical [36] and preclinical [37] data suggest that both neutralizing antibodies (NAbs) and cell-mediated immunity contribute to protection against CMV disease [38]. Therefore, a vaccine should aim to elicit both CMV-specific NAbs and cell-mediated immunity.

CMV induces a strong humoral response, which serves to restrict viral dissemination and limit disease severity. Glycoprotein B (gB), which is involved in cell attachment and penetration, has been found to be a major target for NAbs and is responsible for at least 50 % of the NAbs in CMV-infected individuals [39, 40]. Glycoprotein H (gH), which is involved in the fusion of the viral envelope with the host cell membrane, also has been found to induce potent NAbs [41]. This antibody (Ab) response is likely to be important in controlling infection, as transfer of Abs from CMV-seropositive mothers to newborn infants was shown to be protective against CMV infection from seropositive blood transfusion [42].

While humoral responses are an important part of the adaptive immune response against CMV, T-cell-mediated immune responses are considered the predominant mechanism by which CMV replication is controlled. $CD8^+$ and/or $CD4^+$ T cells are directed toward more than 70 % of the CMV proteins, indicating the importance of T cell responses in controlling CMV infection [43, 44]. Mature and functional fetal $CD8^+$ cytotoxic T lymphocytes (CTL) in humans expand in utero in response to primary CMV infection [45]. In patients with AIDS, IFN-γ CMV-specific $CD8^+$ T cells are protective against CMV-associated retinitis [46]. Similarly, in bone marrow transplant patients, the development of CMV-specific $CD8^+$ T cell responses was correlated with protection and recovery from CMV disease [47, 48]. Furthermore, infusion of donor-derived CMV-specific $CD8^+$ T cells effectively restored Ag-specific cellular immunity in allogeneic bone marrow transplant recipients and protected from CMV-associated complications [49]. This correlation between CMV-specific CTL responses and protection against CMV disease has also been demonstrated in solid organ transplant (SOT) patients. CMV-specific $CD8^+$ T cells

make up a huge proportion of all $CD8^+$ T cells in adult infected humans populations, with a median of 10 % of $CD8^+$ T cells in the peripheral blood of healthy virus carriers and up to 40 % in elderly individuals devoted to the anti-CMV response [4, 44]. Furthermore, the relative contributions of reactivation and reinfection to CMV disease are not yet clear, and the role of antibody or cellular immunity in preventing them still needs to be elucidated. A more comprehensive literature review on cell-mediated immunity on CMV is addressed in the following reviews [50–52].

The importance of $CD4^+$ T cells in controlling CMV has become increasingly evident. Low levels of CMV-specific $CD4^+$ T cells have been found to be significantly correlated with susceptibility to infectious complications with CMV in lung and renal transplant recipients, as well as prolonged viral urinary and salivary shedding in otherwise healthy children [53, 54]. In bone marrow transplant recipients, a detectable $CD4^+$ T-helper response has been shown to correlate with protection from CMV disease [55]. Additionally, the adoptive transfer of $CD4^+$ CMV-specific T cell lines dramatically reduced CMV viral load in allogeneic HSCT recipients [56]. As with $CD8^+$ T cell populations, anti-CMV immunity occupies a significant proportion of the total $CD4^+$ T cell population in healthy seropositive individuals, with individuals devoting a median of 9.1 % of their circulating $CD4^+$ memory T-cell population to control CMV [44]. Most frequently detected in healthy individuals is a large proportion of the $CD4^+$ CTL response specific for highly conserved regions of the gB and gH proteins [57]. Overall, currently it is assumed that CD4 T cells, CTL, and Nabs are essential for the control of CMV disease. Nevertheless, a better understanding of how the immune system keeps CMV under control will eventually lead to identification of established immune correlates for protection. The correlates perhaps will only be identified from the evaluation of potential vaccine candidates in future clinical trials.

Vaccine Target Selection

One major limitation to development of a successful CMV vaccine has been the lack of relevant animal models, which are typically proven crucial in the development of new vaccines. Unlike many other viruses, the cytomegaloviruses are highly species-specific, and CMV's specificity to humans and low infectivity in other species present a significant challenge to vaccine development. Although in vitro models may be useful, animal studies ultimately are required to determine vaccine efficacy. Currently, mouse, guinea pigs, and rhesus macaques and their corresponding, species-specific viruses serve as the model systems in which CMV vaccine immunogenicity is studied [58]. Of these, guinea pigs and guinea pig CMV (gpCMV) are believed to be the most clinically relevant models as gpCMV, similar to CMV, crosses the placenta in utero and causes infection through vertical transmission [37]. Species-specific model viruses provide some utility as challenge models, but fundamental differences in the structure and biology between CMV and these viruses limit their predictive power when assessing potential efficacy of a

human vaccine. Thus, regarding the development of CMV vaccines, the lack of reliable CMV infection mouse models has limited progress in the field of CMV vaccines. However, this issue will benefit strongly from studies aimed at developing better small animal models of human CMV infection. In addition, a better understanding of CMV structure, replication cycle, and specific mechanisms of immune suppression may be critical to identifying viable targets for vaccine development.

The CMV virion consists of an icosahedral capsid, tegument, and cellular lipid layer [59]. The major capsid protein, pUL86, forms the penton and hexons of the icosahedral capsid and is the most abundant protein component of the capsid [60, 61]. In the tegument, ppUL53 and ppUL83 (pp65) are expressed in the nucleus of host cells early after infection but become localized primarily in the cytoplasm later in the replicative cycle of CMV [62]. While the structural functions of these tegument proteins are poorly defined, pp65 is believed to inhibit the expression of genes associated with induction of interferon responses [1, 63]. It has also been shown to elicit strong T cell responses and is a major component of many current CMV vaccine strategies [37]. The lipid membrane is comprised by a number of envelope glycoproteins including gB, gH, gL, gM, gN, and gO, among others. These more abundant CMV glycoproteins have been shown to exist as disulfide-linked complexes within the virion as gCI (gB homodimer), gCII (gM/gN), and gCIII (gH/gL/gO) [1]. In terms of composition, gM/gN have been shown using mass spectroscopy to be the most abundant, followed by gB and gH/gL/gO [1]. Since the envelope glycoproteins are anchored to the surface of the virion and exposed to binding by Abs they are attractive vaccine targets for induction of NAbs, which are considered more likely to prevent or attenuate primary infection. Moreover, since these antigens could also elicit cell-mediated responses (essential to mediate lifelong control of virus replication after infection has established) they are considered key targets for future CMV vaccines.

Glycoproteins M and N

As one of the most abundant glycoproteins in CMV, gM, the product of *UL100*, appears to exhibit very little amino acid variation among different strains of CMV and may therefore be a good candidate for vaccine target selection. While its structure has not yet been defined, this conservation of amino acid sequence suggests that either there is little selective pressure on this viral envelope protein or that it is structurally constrained such that it cannot tolerate significant amino acid variation with major loss of function [1]. In contrast, the *UL73* product gN displays a high degree of amino acid sequence variability, although the total number of O-linked carbohydrate modification sites appears to be relatively conserved [1]. The variation in gN's primary structure may indicate positive selective pressure during the evasion of the Ab response by CMV. The extensive glycosylation of gN, then, may serve to shield this protein from Ab recognition in a similar fashion to that shown for the envelope protein of HIV-1 [64]. gM forms a heterodimeric infectivity

complex with gN in the endoplasmic reticulum through a network of covalent disulfide bonds and non-covalent interactions [65]. Complex formation is required for the native folding and intracellular transport of both gM and gN and studies show that infectious virus cannot be recovered from viral genomes with deletions in either *UL100* or *UL73* [65, 66]. Encouragingly, this gM-gN infectivity complex has been shown to elicit binding Abs during natural human infection [67]. These anti-gM/gN Abs appear to react specifically with the gM/gN complex and were found to efficiently neutralize infectious CMV in vitro [67].

Glycoprotein B

gB is an integral membrane protein that homodimerizes to form a type 1 membrane protein. This homodimer is expressed on the surface of both infected cells and virions [68]. Posttranslational modifications of gB have been shown to enable this glycoprotein to interact with components of the endosomal recycling system, particularly phosphofurin acidic cluster sorting protein-I (PACS-I). These interactions between PACS-I and gB may result in the retention of gB in the trans-Golgi network, a possible site of virion envelopment [69]. gB has been observed to play a crucial role in the initial virion-tethering, attachment and fusion, necessary for cell entry [70]. Importantly, gB is a major target for NAbs and has been the subject of intense investigation as a core component of CMV prophylactic vaccine strategies [37, 71, 72].

Glycoproteins H, L, O

The gCIII complex is formed by gH, gL, and gO. Similarly to gM/gN, gH requires coexpression of gL for intracellular transport and terminal carbohydrate modification [73]. In the absence of gH, gL remains localized in the endoplasmic reticulum. These virion surface proteins are crucial for viral entry into host cells. Recent reports demonstrate that a complex formed by gene products UL128, UL130, and UL131A, along with gH and gL is required for viral entry into endothelial and epithelial cells [74]. By contrast, a gH/gL/gO complex has been implicated in viral entry into fibroblasts [74]. Importantly, gH appears to function in a post-attachment event during infection such as membrane fusion or virus penetration [75, 76]. gH is a significant target of NAbs, which seem to block this function. Interestingly, the primary structure of gH is more than 95 % conserved between CMV strains and anti-gH monoclonal Abs are broadly reactive. To evade these NAbs, CMV can modulate gH expression and, under Ab selection, infectious virion containing limiting amounts of gH could be positively selected [77, 78]. Deletion of the gO gene does not prevent assembly and release of infectious virus, but does appear to impair growth [66].

In conclusion, numerous CMV gene products including several glycoproteins and non-structural proteins have been identified as B- and T-cell targets, although protective Ab levels have not been established [18]. While gB is a major target of NAbs, gH and glycoprotein M-glycoprotein N (gM-gN) have also been identified as important Ab targets along with pp65, IE1, pp150, pp28, pp71, and pp52, which are targets of cell-mediated immunity. The most immunodominant Ags to which CMV-specific $CD8^+$ T cells are directed have been identified as IE-1, IE-2, and pp65, although it is unclear whether magnitude of responses directly correlate with efficacy in restricting CMV replication [4]. In particular, pp65, IE-1, IE-2, gH, gL, gM, gN, gO, and gB were found to be recognized at high frequency by both $CD4^+$ and $CD8^+$ T cells, making these particularly tempting vaccine targets [44, 79].

The Road So Far: Vaccine Platforms Under Development

Viral Vaccines

Several attenuated CMV vaccines have been studied. The Towne strain of CMV, a strain passaged 125 times in WI-38 human diploid fibroblasts, has been the most extensively studied of these replicating, attenuated vaccines. Intramuscular injection of Towne has been shown to result in seroconversion of seronegative adults and the elicitation of NAbs. These Ab levels, however, waned over the course of a year [80]. Towne vaccination has also been shown to elicit CMV-specific $CD4^+$ and $CD8^+$ T cell responses in immunocompetent individuals [18]. Challenge studies (using a less passaged CMV strain, Toledo) showed that Towne afforded some protection against infection, but this protection was inferior to natural infection. Additionally, Towne failed to protect seronegative women with children in daycare (a population at high risk of CMV exposure) against CMV infection while natural infection was highly protective against reinfection with CMV [81]. The lack of protective efficacy afforded by Towne has led to the development of genetic recombinants attempting to achieve a level of attenuation between the Towne strain and wild-type virus. Various Towne/Toledo chimeras have been produced and tested in double-blind, placebo controlled clinical trials and found to be safe, well-tolerated, and appear attenuated [18]. This phase 1 trail is currently in progress.

More recently, a potential CMV vaccine option is based on noninfectious subviral particles of HCMV termed dense bodies (DB). DB are derived by the infection of cultured fibroblasts which then leads to the production of not only infectious virions, but also defective noninfectious particles [82]. These noninfectious DB particles contain enveloped structures consisting of viral tegument proteins and glycoproteins but lacking a capsid, and noninfectious enveloped particles, which resemble normal virions, but lack infectious DNA. This strategy in HLA-A2 transgenic mice was found to yield high virus neutralization titers and developed Abs against a variety of CMV Ags, including gB, gH, pp65, and pp150 when immunized with these dense bodies [83–85]. Interestingly, dense bodies have also been

shown to *elicit* high levels of CMV-specific CTLs in mice. Further evaluation, development, and optimization of this potential CMV vaccine approach are currently ongoing [85].

Nonviral Vaccines

Subunit vaccines in which select proteins are used in combination with an immune adjuvant to augment immunity, has also been explored extensively for CMV. The most potential promising subunit CMV vaccine targets the CMV gB, a highly conserved CMV antigen that induces potent neutralizing antibodies. In healthy seronegative adults, CMV gB with MF59 (an oil and water adjuvant) was found to elicit levels of binding and NAbs comparable to those induced by natural CMV infection with anti-gB IgG and IgA evident in saliva or nasal washes of subjects [86]. NAb titers fell rapidly following vaccination, possibly due to an insufficient CD4+ T cell response, but rebounded significantly following a boosting dose of vaccine [87, 88]. Furthermore, vaccination with gB/MF59 induced strong anti-gB and anti-CMV lymphocyte proliferative responses which persisted for the year following vaccination [88]. A gB vaccine with MF59 adjuvant recently completed a Phase 2 study and has been found to be safe in seronegative women within 1 year after giving birth. The vaccine was found to be 50 % efficacious in this population. Immunized patients did not experience significant differences in adverse event frequency or severity [89].

DNA Vaccines

DNA vaccines, which involves the direct injection of purified DNA encoding specific Ags has been shown to induce levels of protective immunity especially in small animals. Although poor immunogenicity of "first-generation" DNA vaccines in animal models tended to compromise the potential uses for DNA as a vaccine platform, the development of new optimization and delivery strategies, however, have revived DNA vaccines as a viable vaccine vector [90]. These improvements have significantly boosted DNA vaccine immunogenicity and efficacy far beyond "first-generation DNA vaccines." As such, these improved platforms are collectively termed "second-generation DNA vaccines." Gene-level optimization such as codon-optimization to improve RNA stability, and transcriptional and translational efficiency have significantly boosted DNA vaccine immunogenicity against a variety of Ags through increased in vivo expression. Furthermore, Ag design has improved the breadth of protection to target highly variable pathogens such as CMV. These optimized immunogenic sequences can be created based on a collection of target Ag protein sequences. In response to polymorphism, likely due to spontaneous mutations or immune selective pressure [79], immunity can be altered

to target multiple circulating strains by "consensus-engineering" of the amino acid sequence of the DNA vaccine immunogens [91]. Finally, a cocktail of DNA constructs could be used to drive the immune response against a plethora of variable antigens.

Furthermore, the development of new delivery methods to increase transfection efficiency has dramatically improved DNA vaccine immunogenicity. The delivery of Ag-encoding plasmids adsorbed to gold beads using gene guns has been shown to be efficacious in inducing NAbs against the gM and gN proteins of CMV [92]. Delivery of DNA plasmid with adjuvants such as aluminum salts has been shown to increase Ab responses in mice. In particular, a DNA vaccine containing the CMV gB gene and administered with aluminum phosphate gel and CpG oligodeoxynucleotides was found to elicit a significantly higher Ab response and greater NAb titers compared to DNA alone [93]. The use of molecular adjuvants has also been shown to boost DNA vaccine efficacy. Mice co-immunized with the MCMV gB and type I interferon genes exhibited enhanced protection against MCMV challenge compared to mice immunized with the MCMV gB gene alone [94]. Finally, the use of in vivo electroporation with DNA vaccination has been shown to significantly increase antigen-specific immune responses in a variety of animal models against a wide array of pathogens [95–98]. The electroporation process makes use of probes that deliver square-wave pulses after inoculation with DNA plasmid. This electroporation and inoculation procedure can be administered intramuscularly, subcutaneously, or intradermally. This delivery method has been shown to dramatically improve both humoral and cellular immunogenicity of DNA vaccines. As a result of the "second-generation" DNA platform optimizations, DNA vaccines have been shown to been potently immunogenic against a variety of CMV proteins [79].

In addition, the advantages of DNA vaccines extend far beyond their immunogenic potential. Since DNA vaccines are DNA plasmids whose function is not dependent on thermodynamically stabilized secondary, tertiary, and quaternary structures, they are more temperature-stable and do not require the same cold-chain transportation that is essential for protein-based vaccines (viral-vectored vaccines, recombinant protein vaccines). This consideration reduces transportation costs and is particularly important for vaccine delivery to developing countries, where electricity and proper refrigeration may not be readily available. As these nations are often the most affected by epidemics, ease of distribution is a crucial factor in the success of any vaccine.

Finally, DNA vaccines have been shown to have favorable safety profiles in the preclinical and clinical settings. As of 2011, 43 clinical trials were underway to evaluate the effectiveness of DNA vaccines against various viral and nonviral diseases [91]. These vaccine targets include HIV, various cancers, influenza, hepatitis B and C, HPV, and malaria [91]. In addition, an important anti-CMV DNA vaccine currently undergoing clinical trials is the TransVax vaccine by Vical, a vaccine consisting of plasmids encoding CMV gB and pp65 formulated with poloxamer CRL1005 and benzalkonium chloride [36]. TransVax is being tested as a CMV therapeutic DNA vaccine. In a recently completed Phase 2 double-blind, placebo-controlled, parallel group trial, the TransVax or placebo were given to

CMV seropositive recipients undergoing allogeneic HSCT, a population at high-risk for CMV reactivation or reinfection. Safety of the vaccine compared to placebo as well as rates of CMV viremia resulting in initiation of cytomegalovirus-specific antiviral therapy were assessed as primary endpoints. The immunogenicity of vaccine compared with placebo was measured using interferon-γ enzyme-linked immunosorbent spot (ELISPOT) responses to pp65 and gB and gB-specific Ab concentrations measured in an indirect binding IgG ELISA against full-length gB protein [36]. The TransVax vaccine was well-tolerated by patients, with only mild adverse reactions and one allergic reaction reported, indicating favorable safety for the DNA vaccine [36]. Although the randomized Phase 2 study was not designed to demonstrate potential effects on CMV diseases, the TransVax vaccine elicited gB and pp65 cell-mediated immunity responses and reduced the rate of viremia in CMV-seropositive HSCT recipients [36]. Furthermore, the number of pp65 interferon-γ-producing T cells was increased in the TransVax group compared to placebo group at all time points following HSCT. Additionally, the longitudinal anti-pp65 T-cell responses were higher in the TransVax group. However, anti-gB T-cell responses were the same at all time points between the TransVax and placebo groups while no significant increase in anti-gB IgG concentrations were observed in TransVax group compared to the placebo group [36].

Overall, the TransVax DNA favorable safety profile is indicative of the safety of an anti-CMV DNA vaccine. Nevertheless, through genetic optimization, improved delivery methods such as electroporation, and the use of different molecular adjuvants, the efficacy of DNA vaccines can likely be significantly improved while maintaining a similar safety profile to TransVax. While TransVax was not highly immunogenic, its ability to elicit anti-pp65T-cell responses indicates that DNA vaccines can induce cellular responses against a plasmid-encoded Ag. This is likely to be an important factor in the success of any CMV vaccine, especially in a therapeutic vaccine, given the importance of cellular immunity in natural control of CMV infection and reactivation in healthy seropositive individuals. However, promising clinical and preclinical data support that an effective vaccine will need to induce both humoral and cellular immune responses. Thus, DNA vaccines are an extremely promising platform for the future development of both therapeutic and prophylactic vaccines against CMV. Given DNA vaccines' safety profile in clinical settings and their ability to drive both humoral and CMI, which are considered essential for CMV immunity, makes DNA a suitable platform for use in immunocompromised populations. This platform is germane for CMV, since immunocompromised patients comprise the vast majority of the at-risk population for CMV disease and would be the target population for a CMV vaccine.

Conclusion

The development of a CMV vaccine would be highly effective to reduce congenital diseases, to improve longevity of transplant patients, and to address the significant unmet public health issues caused by CMV infections. However, CMV's

Table 1 Most advanced CMV vaccines currently in clinical trials

Vaccine	Vaccine platform	Viral antigens	Stage
Towne	Attenuated	Whole virus	Phase 2
gB/MF59	Subunit protein	gB	Phase 2
TransVax	DNA vaccine	gB, pp65	Phase 2

sophisticated mechanisms of immune evasion, the relative complexity of its genome, its numerous glycoproteins associated with cell tropism, and due to the lack of identified CMV immunogens has stunted CMV vaccine development. However, the identification of new target CMV immunogens and further studies of our understanding of immune responses to CMV should inevitably lead to the establishment of immunological correlates that could aid future rational vaccine design. The results of the most currently advanced ongoing clinical trials (Table 1) should identify correlates of protection for revolutionizing the next generation of CMV vaccines.

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Vector-Mediated Antibody Gene Transfer for Infectious Diseases

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Abstract This chapter discusses the emerging field of vector-mediated antibody gene transfer as an alternative vaccine for infectious disease, with a specific focus on HIV. However, this methodology need not be confined to HIV-1; the general strategy of vector-mediated antibody gene transfer can be applied to other difficult vaccine targets like hepatitis C virus, malaria, respiratory syncytial virus, and tuberculosis. This approach is an improvement over classical passive immunization strategies that administer antibody proteins to the host to provide protection from infection. With vector-mediated gene transfer, the antibody *gene* is delivered to the host, via a recombinant adeno-associated virus (rAAV) vector; this in turn results in long-term endogenous antibody expression from the injected muscle that confers protective immunity. Vector-mediated antibody gene transfer can rapidly move existing, potent broadly cross-neutralizing HIV-1-specific antibodies into the clinic. The gene transfer products demonstrate a potency and breadth identical to the original product. This strategy eliminates the need for immunogen design and interaction with the adaptive immune system to generate protection, a strategy that so far has shown limited promise.

Introduction

Monoclonal antibodies as therapeutics have rapidly become a powerful new class of biologically based drugs. There are at least 25 antibodies that are approved for clinical use, with approximately ten times that number in development [1]. The essentially infinite number of possible antibody targets has given rise to antibody products

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for treatment of autoimmunity, cancer, inflammation, and infectious diseases, to name a few. Antibody therapy involves injecting highly purified antibody proteins to provide an immediate treatment for illnesses and diseases. Specific antibodies can also be passively administered to healthy people in the form of a vaccine, or immunoprophylactic. This strategy essentially bypasses the adaptive immune response by engendering the host with protective antibodies to prevent infection. While these methods hold tremendous promise for the treatment of many diseases, they are less applicable for a large-scale human prophylactic vaccine approach. Injections of antibodies every few weeks to potentially millions of people are not practical or cost effective. In this chapter, we will discuss the emerging field of vector-mediated antibody gene transfer as an alternative vaccine for infectious disease, with a specific focus on HIV-1. However, this methodology need not be confined to HIV-1. The general strategy of vector-mediated antibody gene transfer can be applied to other difficult vaccine targets like hepatitis C virus, malaria, respiratory syncytial virus, and tuberculosis. This approach is an improvement over classical passive immunization strategies that administer antibody proteins to the host to provide protection from infection. With vector-mediated gene transfer, the antibody *gene* is delivered to the host resulting in long-term endogenous antibody expression from the injected muscle that confers protective immunity.

Finding Potent Broadly Neutralizing Antibodies for HIV-1

The need for a safe and effective HIV-1 vaccine is undisputed. In 2012 alone, 1.6 million people died from AIDS related causes, while 2.3 million people were newly infected with HIV [2]. Two HIV-1 Envelope (Env) subunit vaccines tested in Phase 3 clinical trials (Vax003 and Vax004) failed to protect vaccine recipients from infection, and neither diminished viral replication after infection [3, 4]. A similar lack of efficacy was also seen from the Step Study, which used recombinant adenovirus vectors (rAd) that expressed multiple HIV-1 proteins [5, 6]. The RV144 trial in Thailand tested a canary pox vector prime/Env protein boost strategy and showed modest efficacy (31 %) [7]. Detailed analyses of the RV144 study results revealed two significant correlations with infection among vaccine recipients. The presence of IgG antibodies against V1/V2 loop of Env may have contributed to protection against HIV-1 infection, whereas high levels of Env-specific IgA antibodies correlated virus acquisition [8]. More recently, the HVTN 505 trial was stopped for futility, dealing yet another blow to HIV vaccine efforts [9]. The HVTN 505 trial, which used a DNA prime/rAd boost, showed no difference in HIV-1 infections between those recipients who received the vaccine and those receiving placebo [10]. Vaccine recipients did generate IgG antibodies to Env; however, the majority were non-neutralizing with low reactivity to the V1/V2 loop [10]. These observations underscore the tremendous hurdles that must be overcome to develop an effective HIV-1 vaccine. Foremost is figuring out how to induce antibodies that neutralize a wide array of HIV-1 field isolates. It was initially believed that potent, broadly

neutralizing antibodies (bNAbs) to HIV-1 were extremely rare and difficult to elicit. In fact for some time, only four such antibodies had been identified, known as b12, 2F5, 2G12, and 4E10 [11–14]. These antibodies provided valuable information as to what regions of HIV-1 envelope were potentially sensitive to neutralization, which could aid in better vaccine antigen design. More recently, a large number of new, significantly more potent bNAbs have been identified using improved screening and sequencing techniques. These newer antibodies were isolated by high throughput screening of sera from healthy HIV-1-infected individuals categorized as "elite neutralizers" based on their neutralization breadth and potency [15–23]. Detailed analyses of these antibodies indicated they are approximately 10- to 100-fold more potent and have an increased breadth compared to the original four isolates. Furthermore, this new class of antibodies can neutralize HIV-1 through binding to a variety of envelope domains including the CD4 binding site (VRC01, NIH45-46, and PGV04) [18, 21, 24], glycan containing regions in the variable loops (PG9, PG16, PGT121, and PGT128) [16, 17], and the membrane-proximal external region (MPER) on gp41 (10E8) [19].

Epitope mapping of these new, potent antibodies has invigorated the vaccine field by providing precise regions to target when designing new protein or subunit vaccine antigens to induce bNAbs [25]. For example, highly stable Env trimers have been generated that bind to most of the known neutralizing antibodies, but generally do not bind non-neutralizing antibodies, and could potentially be used as a next-generation immunogen [26]. However, even with this new wealth of information at hand, generating bNAbs with improved, redesigned antigens may still prove to be problematic. Extensive sequence analysis of these potent, broadly neutralizing antibodies reveal that high levels of somatic mutations (as much as 30 %) can occur in the generation of the mature antibody [16, 18, 21, 23, 27]. Furthermore, the maturation may have involved repeated rounds of antibody selection through interactions with the HIV-1 antigen. In light of this, several groups have developed novel immunogens, such as glycopeptides or computation-derived multimerized nanoparticles that are designed to induce bNAbs [28, 29]. These immunogens can bind to both mature bNAbs as well as the receptors on their germ-line (naïve) B-cells, which can trigger their activation and maturation into plasma cells that are able to produce the bNAb.

Passive Immunization Strategies for HIV

While induction of bNAbs by various next-generation immunization strategies holds promise, the question remains as to the best use the human monoclonals that have already been isolated and characterized. One obvious option is passive immunization. Passive immunization using neutralizing monoclonal antibodies has protected monkeys from simian–human immunodeficiency virus (SHIV) challenge infections [30–36]. In a recent study by Moldt et al. [36] they showed that passively administered PGT121 can mediate sterilizing immunity against SHIV in monkeys at serum concentrations that were significantly lower than those observed in previous studies. Sterilizing immunity was achieved in all animals administered 5 and 1 mg/kg and

three of five animals administered 0.2 mg/kg PGT121, with corresponding average antibody serum concentrations of 95 μg/mL, 15 μg/mL, and 1.8 μg/mL, respectively. The results suggested that a protective serum concentration for PGT121 was in the single-digit μg/mL amount. While this study demonstrates the potential for passive immunization with the new class of bNAbs, unfortunately, an injection of antibodies every few weeks is not practical or cost effective as a large-scale human prophylactic vaccine approach.

Vector-Mediated Antibody Gene Transfer to Bypass Adaptive Immune System

Given the difficulties of using the classic concept of passive immunization as a vaccine, we developed a second option: isolate the representative antibody gene and use gene transfer technology to endow a target host with the gene. In this way, the antibody gene directs endogenous expression of the antibody molecule, and the host (in theory) will now have the antibody in its circulation. Thus, after a single injection, the muscle now serves as a depot to synthesize the bNAbs that are passively distributed through the circulatory system (Fig. 1). The host is now armed with a

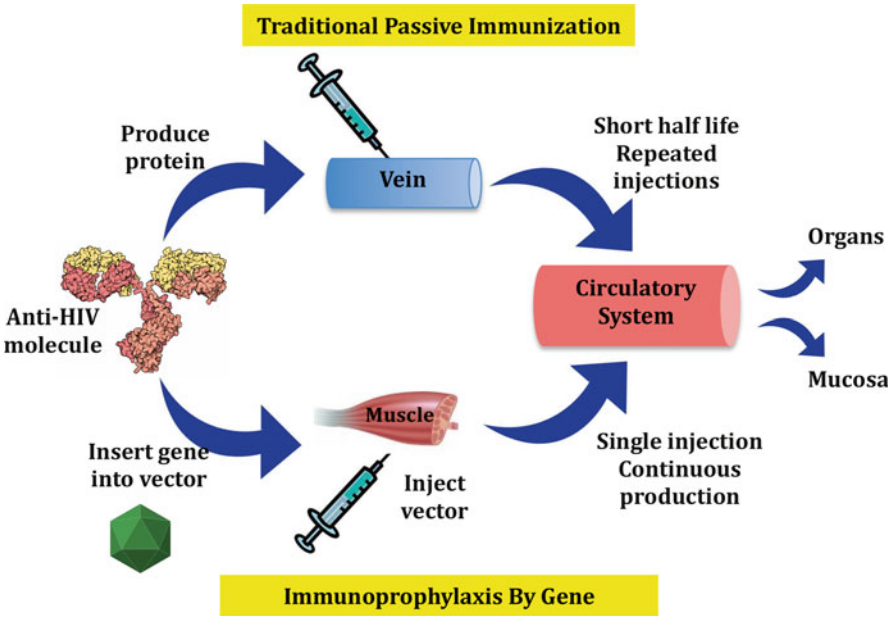

Fig. 1 Immunoprophylaxis by antibody gene transfer. Passive immunization involves intravenous delivery of purified antibodies to engender the host with short-lived immunity in serum and mucosa. In contrast, vector mediated antibody gene transfer uses a viral vector to deliver the antibody *gene* to the host via intramuscular injection. The antibody is produced endogenously in the muscle and secreted into the circulatory system and mucosa providing long-term protection from infection

potent bNAb against HIV-1 that effectively bypasses the adaptive immune system. This is in contrast to the traditional idea of passive immunization whereby the purified antibodies are injected intravenously into the host to provide protection from infection. However, due to the antibody half-life (approximately 6 days for PGT121 [36]), the levels decline requiring repeated injections. The obvious advantage is that antibody gene transfer engenders the host with long-term antibody persistence from a single injection due to endogenous antibody expression.

Choosing the Right Gene Transfer Vector

A popular antibody gene delivery vector is the recombinant adeno-associated virus (rAAV) vector, which is derived from wild-type AAV. AAV is a *dependovirus* with a 4.7 kb single strand DNA genome that contains only two genes (*rep* and *cap*) flanked by inverted terminal repeats (ITRs). AAV natural infection is common and has not been associated with any disease. Multiple AAV serotypes have been identified with different transduction efficiencies in different tissues, offering flexibility for gene transfer targets such as muscle or liver [37]. rAAV vectors have an established record of high-efficiency gene transfer in a variety of model systems [38, 39]. Following injection, the rAAV vector genome can form stable non-integrating circular episomes that can persist in non-dividing cells [40–42]. Because of these features, rAAV vectors have become popular gene delivery vehicles for use in clinical studies for the treatment of diseases such as alpha1-antitrypsin deficiency, cystic fibrosis, hemophilia B, Leber's congenital amaurosis, lipoprotein lipase (LPL) deficiency, Parkinson's disease, and muscular dystrophy [43].

rAAV gene transfer vectors are devoid of the endogenous *rep* and *cap* genes, and consist of the antibody gene expression cassette flanked by the AAV ITRs (Fig. 2). The ITRs (145 bp each), which are necessary for rAAV vector genome replication and packaging, are the only part of the AAV genome present in the rAAV vectors. One method for antibody expression utilizes a two-promoter system whereby the heavy and light chain genes are transcribed independently using two different promoters and polyadenylation signals within the same rAAV vector genome (Fig. 2) [44]. Another method uses a single promoter for expression of both the heavy and light chains, which are separated by the foot-and-mouth-disease virus (FMDV) 2A peptide, which undergoes self-cleavage to produce separate heavy and light chain proteins (Fig. 2) [45]. The advantage of this system is that the heavy and light chains can potentially be expressed in a 1:1 ratio using a single promoter, which may translate to more efficient expression. However, a potential disadvantage is that the FMDV-2A peptide is derived from a viral sequence and may be immunogenic in the host causing immune clearance of cells expressing the antibody.

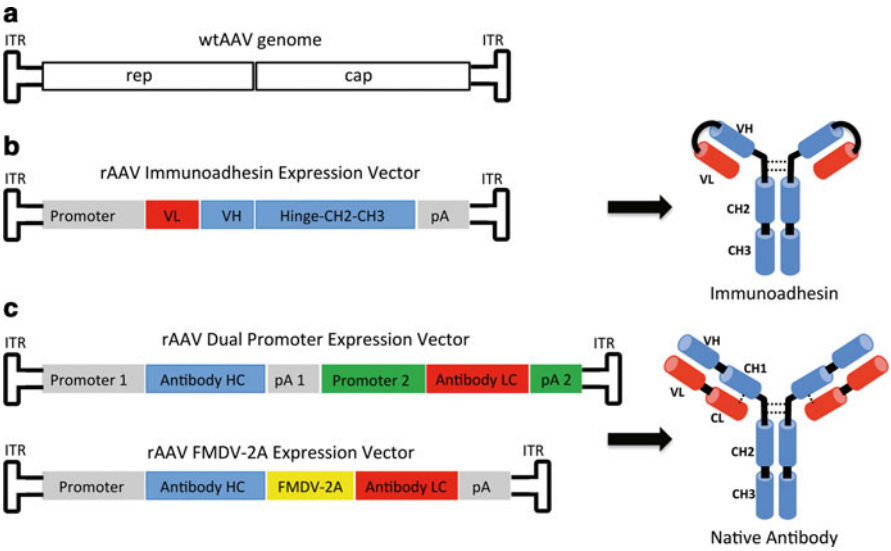

Fig. 2 rAAV vectors for antibody gene transfer. (**a**) The wild-type AAV (wtAAV) genome consists of the rep and cap genes flanked by inverted terminal repeats (ITR). For rAAV vectors, the rep and cap genes are removed and replaced by an antibody expression cassette flanked by ITRs, which are necessary for rAAV vector genome replication and packaging. (**b**) Immunoadhesins contain the antibody variable domains (*VL* variable light, *VH* variable heavy) usually joined by a flexible protein linker. The variable domains are connected to the hinge and constant heavy chain domains (CH2 and CH3). The immunoadhesins can form dimers through disulfide bonding in the hinge region. (**c**) Full antibodies can be expressed using either a dual promoter of single promoter system. For dual promoter expression, the antibody heavy and light chains are each expressed separately from their own promoter. For the single promoter system, the heavy and light chains are expressed as a single polypeptide separated by the foot-and-mouth-disease virus 2A peptide (FMDV-2A). The FMDV-2A peptide can undergo self-cleavage to give rise to separate heavy and light chains

Antibody Gene Transfer for an HIV-1 Vaccine

We first tested the concept of rAAV-mediated antibody gene transfer in animals by using one of the first bNAb isolated, IgG1b12. The human monoclonal IgG1b12 heavy and light chains were cloned independently into an rAAV genome using the two promoter system. The resulting vector was injected into the quadriceps muscles of immunodeficient mice (to avoid immune responses to human IgG). IgG1b12 was expressed in mouse muscle (confirmed by histochemical staining), and biologically active antibody was found in sera for over 6 months [44]. Characteristic biologic activity was determined by HIV-1 neutralization assays against IgG1b12 sensitive/resistant viruses. This study provided the first evidence that: (1) rAAV vectors transferred antibody genes to muscle; (2) myofibers produced antibodies; (3) antibodies were distributed to the circulation; and (4) such antibodies were biologically active.

Our next objective was to test the gene transfer concept in monkeys in a challenge study. In pilot experiments using the rAAV-IgG1b12 vector, macaques developed antibody responses to the human-derived transgene that effectively shut down expression. We turned to using rhesus-derived antibodies by taking advantage of native macaque SIV gp120-specific Fab molecular clones that had been derived directly from SIV-infected macaques [46]. When designing the antibody gene transfer vectors, we chose to express the Fabs as immunoadhesins, which in pilot experiments in mice were superior to single-chain (scFv) or whole-antibody (IgG) molecules with respect to steady-state serum concentrations (unpublished data). Immunoadhesins are chimeric, antibody-like molecules that combine the functional domain of a binding protein like a scFv or CD4 extracellular domains 1 and 2 (D1D2) with an immunoglobulin constant domain [47] (Fig. 2). They have been shown to be effective in disease models including HIV, SIV, and influenza [48–50]. A typical immunoadhesin lacks the constant light chain domain and the constant heavy domain 1 (CH1); however, it can be expressed as a single polypeptide from a single promoter, and forms dimers through disulfide bonding in the hinge region. While immunoadhesins have many attractive features such as efficient expression/secretion in vivo, they also have some drawbacks. Immunoadhesins may not exhibit the same neutralization breadth and potency as the native antibody. While we have seen cases where a specific immunoadhesin functions identically to its native antibody counterpart, we have also seen an immunoadhesin become tenfold less potent at neutralizing HIV-1 (unpublished observation). Thus, each immunoadhesin must be fully characterized and compared to the native antibody from which they were derived before it can be considered as a vaccine. Another drawback to using immunoadhesins is possible immunogenicity. Immunoadhesins are not naturally occurring proteins and may contain amino acid linkers connecting the variable domains (Fig. 2), which could trigger an immune response leading to loss of expression. However, it should be noted that Enbrel (etanercept), an immunoadhesin consisting of the TNF receptor fused to IgG1-Fc, was well tolerated in patients for long-term treatment (10 years) of rheumatoid arthritis [51].

For the macaque experiments, we constructed immunoadhesins derived from two different SIV Fab fragments (4L6 and 5L7), as well as a third immunoadhesin containing the rhesus CD4 D1D2, which was modeled after CD4-Ig fusion proteins [52]. All of the constructs neutralized in vitro the proposed SIV challenge stock (SIVmac316), indicating that the immunoadhesins were functioning like the original Fab clones [49]. The three immunoadhesins were injected into three monkeys each (for nine total), followed by an intravenous SIVmac316 challenge 4 weeks later, including six naïve controls. Immunoadhesin expression levels were as high as 190 μg/mL at the time of challenge (4 weeks post injection) and peaked around 6 months with levels reaching 400 μg/mL in some animals [49]. Overall, six of the nine monkeys receiving the immunoadhesins were completely protected from SIV infection while all six naïve controls became infected. Analysis of the three monkeys from the immunoadhesin group that became infected revealed that these specific animals had developed an immune response to the immunoadhesin by 3 weeks post injection, suggesting a correlation between

an immune response to the immunoadhesin and failure to protect from infection. We have performed longitudinal studies of the protected monkeys, which are now over 6 years post injection. Immunoadhesin levels dropped to a stable level of approximately 20 μg/mL, which has persisted for over the last 4 years. The monkeys have remained negative for SIV infection and have not developed an immune response to the immunoadhesins (unpublished observation). Thus, this crucial study was instrumental in proving the concept of vector mediated gene transfer as a viable HIV vaccine.

More recently other investigators performed rAAV vector-mediated gene transfer expression/challenge studies, which they called vectored immunoprophylaxis (VIP) [53]. They expressed the native, full antibodies of 2G12, IgG1b12, 2F5, 4E10, and VRC01 using the single promoter FMDV-2A system. Following intramuscular rAAV injection in mice, antibody expression levels greater than 100 μg/mL were observed for at least 12 months. Using a humanized mouse model, they further showed that these rAAV vectors provided protection following HIV-1 challenge, with antibody serum levels as low as 8.3 μg/mL (antibody VRC01). These encouraging results reinforce the efficacy of the antibody gene transfer approach, especially when potent antibodies such as VRC01 are used. Taken together, these murine and primate studies show that vector-mediated antibody gene transfer can bypass the adaptive immune response and engender the host with antibodies that provide protection from infection. Furthermore, antibody expression can persist several years following a single injection, suggesting long-term protection is possible.

Antibody Gene Transfer for HIV-1 Therapy

While antibody gene transfer shows great promise for providing protection from HIV-1 infection, one obvious question is whether this strategy can also be used for antibody therapy in HIV-1 positive individuals. Several recent studies have demonstrated that it may be possible. One group of experiments was done using a humanized mouse model [54, 55]. Humanized mice were generated by injecting immunodeficient mice with human fetal liver-derived $CD34^+$ hematopoietic stem cells, resulting in mice with a complete human immune system. The advantage here is that the mice will not generate an immune response to the presence of human antibodies, which is a problem when trying to express human antibodies in nonhuman primates.

For these studies, HIV-1-infected humanized mice were passively administered several bNAbs either singly or in combination. The mice showed sustained decreases in plasma viral loads starting a few days after antibody delivery. Furthermore, these mice also exhibited reduced cell-associated viral DNA, suggesting the antibodies played a role in killing infected cells expressing HIV gp120 on their surface through antibody-dependent cell-mediated cytotoxicity (ADCC). Viral loads tended to rebound over time, which correlated with the loss of the bNAbs due to the antibody half-life in the serum. Not surprisingly, some mice developed viral escape mutants.

These escape mutants, predominantly from groups that were passively administered a single bNAb, were further analyzed and, as one would predict, they had developed mutations at sites that conferred resistance to the respective antibody. The level of viral escape mutants was dramatically reduced in mice that were passively administered multiple bNAbs at once. These antibody mixtures targeted different regions of gp120 and suggested that the viruses were unable to escape the broad selective pressure provided by using antibodies targeting multiple epitopes.

To answer the question as to whether vector mediated antibody gene transfer could be used for HIV-1 therapy, one of these studies [54] included a group of mice that received an intravenous injection of a rAAV (serotype 8) vector expressing bNAb 10-1074, which targets the base of the V3 stem of gp120 [56]. These mice maintained a high level of antibody 10-1074 expression of around 200 μg/mL for the entire length of the 67-day observation period. During this time, six of the seven mice in the group were able to control HIV-1 plasma viral loads, whereas one mouse exhibited viral escape. As seen with the escape mutants from the passive immunization studies, sequence analysis of the gp120 of these escape virus revealed mutations in the 10-1074 binding site that conferred resistance to the antibody. It remains to be seen if simultaneously administering rAAV vectors expressing multiple bNAbs could dramatically reduce or even possibly eliminate the generation of escape mutants. Furthermore, long-term studies will be required to see if escape mutants could arise over time, even in the presence of multiple antibodies.

Similar passive antibody therapy studies have been performed in SHIV-infected rhesus monkeys [57, 58]. SHIV is a virus composed of both HIV and SIV, and allows researchers to study antiviral compounds against HIV-1 Env in a nonhuman primate model. As seen in the humanized mice studies, SHIV-infected monkeys demonstrated a dramatic drop in plasma viral load as well as reduced proviral DNA in peripheral blood following intravenous infusions of bNAbs, either administered alone, or in combination. SHIV virus levels rebounded when serum bNAb titers declined to undetectable levels at around day 60. However, some animals maintained long-term SHIV control (>100 days) in the absence of additional bNAb infusions [57]. Some groups of monkeys that received only a single infused bNAb went on to develop neutralization-resistant escape mutant variants, which was also observed in the humanized mouse studies. In contrast, monkeys receiving only the single bNAb PGT121 [57] did not give rise to escape mutants, which may be reflective of the overall potency and breadth of the bNAb used in the studies, or may be due to duration of the bNAb in the serum. One caveat here is that long-term studies with repeated infusions of these human monoclonal antibodies in monkeys are not possible. The monkeys will recognize the human antibodies as foreign and generate anti-human IgG immune responses to eliminate the infused antibodies. Thus, the effects of long-term selective pressure on the generation of escape mutants cannot be properly addressed here.

Neither of these immunotherapy studies in monkeys used vector-mediated gene transfer to deliver the bNAbs to SHIV infected monkeys. However, for reasons described above, the human antibodies would very likely not be expressed long-term in these monkeys due to species incompatibility with a human antibody. A study using bNAbs isolated from monkeys would be required, as was done in the

nonhuman primate immunoprophylactic study [49]. Those issues notwithstanding, the antibody immunotherapy studies done in humanized mice and monkeys suggest that using vector-mediated gene transfer to deliver bNAbs to HIV-1 infected individuals could be a viable option, possibly even used in conjunction with standard antiretroviral therapy (ART). An overriding theme is that multiple bNAbs would be required to provide the selective pressure to avoid viral escape mutants. Multiple antibodies could target different gp120 domains such as the exterior loops, CD4 binding site and MPER. Furthermore, multiple antibodies could be used that target different stages of viral entry including CD4 binding, CCR5 binding, and membrane fusion. Of course this strategy of the simultaneous use of multiple antibodies against multiple viral targets or stages of entry could also be applicable and in a prophylactic vaccine approach for maximum efficacy.

Antibody Gene Transfer for Respiratory Tract Infections

The use of vector-mediated antibody gene transfer has not been limited to just HIV-1 (Table 1). Respiratory syncytial virus (RSV) is a major cause severe respiratory infection in high-risk populations (such as infants) for which a vaccine is not

Table 1 Summary of rAAV vector-mediated antibody gene transfer studies

Application	rAAV serotype	Antibody	Animal model	Route of administration	Reference
HIV vaccine	2	b12	Mice	Intramuscular	Lewis [44]
HIV vaccine	1	SIV immunoadhesins	Rhesus Macaque	Intramuscular	Johnson [49]
HIV vaccine	8	4E10, 2G12, 2F5, b12, VRC01	Mice	Intramuscular	Balazs [53]
HIV vaccine	1	PG9	Human	Intramuscular	Clinical Trial in 2014[a]
HIV therapy	8	10-1074	Mice	Intravenous (liver)	Horwitz [54]
RSV vaccine	rh.10	Palivizumab	Mice	Intranasal and intrapleural (lung)	Skaricic [59]
Influenza vaccine	9	FI6	Mice and Ferrets	Intranasal	Limberis [50]
Influenza vaccine	8	F10, CR6261	Mice	Intramuscular	Balazs [62]
Nicotine addiction	rh.10	NIC9D9	Mice	Intravenous (liver)	Hicks [65]
Cocaine addiction	rh.10	GNC92H2	Mice	Intravenous (liver)	Rosenberg [66]
Cancer therapy	8	DC101	Mice	Intravenous (liver)	Fang [45]

[a]The first clinical trial using rAAV vector mediated antibody gene transfer is scheduled to begin in 2014 as a result of collaboration between The Children's Hospital of Philadelphia, The International AIDS Vaccine Initiative, and Division of AIDS (DAIDS)

yet available. Currently, the only way to prevent infection is through the passive administration of anti-RSV antibodies, such as palivizumab (also known as Synagis, manufactured by MedImmune). This antibody can be administered intramuscularly once each month during the RSV season (winter and spring) to prevent RSV infection. While this treatment is effective, it is costly and limited to high-risk individuals, which are attributes that make it a prime candidate for antibody gene transfer. Instead of repeated monthly injections of the purified antibody, the antibody could be endogenously expressed from a single injection using antibody gene transfer and provide a constant level of protective anti-RSV antibodies in the host.

The study used different vector systems to deliver antibodies against RSV infection [59]. They compared expression and efficacy of a mouse version of palivizumab in a mouse model system when delivered by either a rAAV vector (serotype rh.10) or adenovirus (Ad) vector. Adenovirus vectors have the capacity for high-level gene transfer with rapid and robust transgene expression. However, Ad vectors are highly immunogenic, and transduced cells are quickly cleared by the immune system resulting in rapid loss of transgene expression. In contrast, rAAV vectors have very low immunogenicity and can give rise to long-term gene (antibody) expression for potentially the life of the individual. The Ad-palivizumab vector was administered intravenously, with palivizumab detected in the lungs by day 3 post-administration. Following an intranasal RSV challenge 7 days post administration, the mice showed >5-fold decrease in RSV titers in the lung compared to control animals. Long-term antibody expression and challenge studies were done using the rAAVrh.10-palivizumab vector via intrapleural administration. Palivizumab was detected in the serum of these animals by 8 weeks post administration that started to peak by week 20. These rAAVrh.10-palivizumab mice were intranasally challenged with RSV at 7 and 21 weeks post-administration. They showed a 14.3-fold and 10.6-fold lower numbers of RSV pfu in the lungs, indicating that protection against RSV infection can be sustained at least 21 weeks post delivery of a rAAV vector.

Antibody gene transfer studies using rAAV vectors have also been done to prevent influenza. Although traditional vaccination strategies for influenza are quite effective, they may not be adequate for a possible zoonotic strain that could lead to a pandemic (such as the 2009 H1N1). In this case, the time needed to develop a traditional vaccine may not be rapid enough. The rationale is that vector-mediated antibody gene transfer could quickly deliver a bNAb that is effective against multiple strains of influenza that would provide protection against a pandemic. One study looked at delivering the bNAb antibody FI6 [60] as an immunoadhesin using rAAV serotype 9 via intranasal delivery in mice and ferrets [50]. FI6 immunoadhesin expression was detected in the nasal and lung lavage fluids of mice 14 days post vector administration at concentrations ranging from 0.5 to 2.0 μg/mL. Animals challenged as early as 3 days after rAAV9-FI6 administration could be protected. Furthermore, this strategy was able to protect both mice and ferrets from exposure of lethal doses of various clinical isolates of H5N1 and H1N1. An additional study [61] also demonstrated that rAAV9-FI6 administration showed partial efficacy in mice challenged with the newly emergent avian H7N9, which is believed to be transmitted from poultry to humans.

A separate study used a similar strategy but with intramuscular administration of the rAAV antibody vector in mice [62]. They expressed antibodies F10 [63] and CR6261 [64] in a rAAV serotype 8 vector using the FMDV-2A expression system. Antibody expression levels in the serum reached 200 μg/mL at 5 weeks post intramuscular injection, with levels still around 10 μg/mL out to at least 11 months after a single injection. These treated mice were protected from diverse strains of H1N1 influenza when challenged at either of these time points (5 weeks and 11 months), demonstrating once again the incredible potential for this strategy as a vaccine. The results from both the intranasal [50] and intramuscular [62] routes of vector administration reinforce the flexibility of vector-mediated gene transfer and provide important proof-of-concept studies that could lead to translation into humans.

Other Applications for Antibody Gene Transfer

Up to this point, we have discussed the use of vector-mediated antibody gene transfer for the prevention and possible treatment of infectious diseases such as HIV-1, RSV, and influenza. However, this strategy can be expanded for use in noninfectious disease applications where antibodies still play a critical role, such as cancer treatment. In a study by Fang et al. [45], they examined the efficacy of an antitumor antibody to reduce tumor growth in a mouse model system. They used a rAAV8 vector that expressed antibody DC101 by the FMDV-2A system. Antibody DC101 is an antiangiogenic monoclonal antibody that targets vascular endothelial cell growth factor receptor-2 (VEGFR2). Mice given an intravenous injection of rAAV8-DC101 could express high levels (>1 mg/mL) of the antibody in the serum for the length of the 5-month monitoring period. Mice receiving this rAAV vector exhibited shrinkage of tumors and prolonged survival time compared to untreated control animals. These encouraging results set the stage for combining antibody gene transfer technology with the ever increasing number of antibody-based therapies for cancers that include such antibodies as Herceptin and Avastin (Genentech), to name a few.

Perhaps a less conventional use of vector-mediated antibody gene transfer is a potential role in the treatment of substance addiction. Antibodies exist that can bind to these substances in the blood and prevent their transfer to the brain, which leads to their addictive properties. Antibody therapy for addiction would require routine, costly injections, which once again makes this a prime candidate for antibody gene transfer. NIC9D9 is an anti-nicotine antibody that was delivered intravenously (targeting the liver) to mice using a rAAVrh.10 vector [65]. NIC9D9 antibody was detected in the serum for the length of the 18-week study. Following intravenously nicotine delivery, the rAAV-NIC9D9 mice had 83 % of the nicotine bound to the NIC9D9 antibody in serum, which drastically reduced the amount of serum delivered to the brain. Furthermore, these mice had reduced cardiovascular effects compared to control animals. These results indicate that this strategy may hold promise as an effective preventative therapy for nicotine addiction.

Along the same line, GNC92H2 is a mouse monoclonal antibody with high affinity for cocaine. This antibody was also delivered to mice using the rAAVrh.10 vector via intravenous injection [66]. GNC92H2 was detected in the serum for the entire duration of the 24-week study. The GNC92H2 antibody was able to sequester intravenously administered cocaine in the blood, thereby protecting the brain from the effects of cocaine. Furthermore, these mice showed suppressed cocaine-induced hyperactivity derived from weekly cocaine exposure (12–17 weeks post rAAVrh.10 vector administration). These findings offer an alternative intervention to cocaine addiction therapy. High affinity cocaine antibodies could be maintained long-term in the serum following a single administration. This strategy could be coupled with traditional behavioral therapies for a combined approach for the treatment of cocaine addiction.

Limitations of rAAV Gene Transfer

When using rAAV vectors to deliver antibodies, there are several factors that could limit their effectiveness. These considerations pertain to the antibody transgene, as well as the rAAV vector itself. Given the fact that AAV infection is common in the human population, plus AAV capsid sequences contain highly conserved regions, many people have circulating neutralizing antibodies to AAV capsids of multiple serotypes [67]. The presence of preexisting neutralizing antibodies, with titers as low as 1:5 to 1:10, can have a negative impact on vector transduction [68–70]. Furthermore, neutralizing antibodies may prevent repeated administrations [69], which would impede rAAV delivery of potentially more potent antibodies at a later time. Potential solutions to the AAV neutralization conundrum involve using rare AAV capsids, or capsids that have been reengineered to remove or alter neutralization epitopes [37].

Another immune obstacle to rAAV vectors is capsid-specific T cell responses. The leading hypothesis is that prior exposure to AAV gives rise to AAV-specific memory T cells that are activated following rAAV vector gene transfer [71]. Results from clinical trials using both hepatic and muscular delivery indicate T cell responses occur within 30 days after administration, which is associated with reduced or lost transgene expression [72, 73]. A more recent clinical study using intramuscular delivery of a rAAV vector for α-1 antitrypsin (AAT) deficiency did observe an early capsid-specific T cell response and associated decline in transgene expression [74]. However, AAT transgene expression did not completely disappear. In fact, AAT levels persisted for more than a year after administration despite the persistence of anti capsid T cells [74]. Clearly more studies will be required to elucidate the mechanism behind T cell responses and their effects on transgene expression.

With regard to limitations of the antibody transgene, essentially any therapeutic or immunoprophylactic protein can be expressed using rAAV vector gene transfer, as long as it fits within the vector packaging limit. However, one must be careful that the expressed protein is not immunogenic in the host. However, this is also the same

concern for all exogenously (passively) administered proteins, including monoclonal antibodies and other biologics. Most, if not all, of the 25 monoclonal antibodies that have been approved as therapeutics have exhibited some level of immunogenicity [75, 76]. Several factors may contribute to immunogenicity including antibody structure, dosing regime, and the recipient's genetic background. Also, it remains to be determined if an antibody that was endogenously expressed in the host via gene transfer will be more or less immunogenic than when passively administered as an exogenously produced protein. The big question is what effect would an immune response to the transgene have in the host? In the simplest scenario, as was seen in the nonhuman primate studies [49], the appearance of anti-antibody responses would limit the vaccine efficiency through loss of transgene expression, with no adverse events observed. Ultimately, at this stage it is difficult to predict with any certainty, which, if any, of the candidates would be immunogenic, and what the consequences would be. Human clinical trials will be the best predictor.

Perhaps of greater concern is the risk that the antibody will bind off target causing an unanticipated adverse event. Preclinical testing, such as passive administration and GLP human tissue binding studies, can help avert most of these issues. However, if off-target effects occur in vivo, there is currently no efficient method to stop antibody gene expression. As the data shows from animal models, antibodies are expressed for potentially the life of the host following a single intramuscular administration. A few studies have attempted to regulate gene expression from rAAV gene transfer vectors in mice and monkeys [77–79], but these schemes are transient and require continuous exogenous drug administration to maintain a constant level of gene expression. Clearly, identifying an efficient method to permanently eliminate antibody gene expression in the host is a top priority if rAAV vector-mediated antibody gene transfer is to become applicable for wide-scale use.

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HIV Latency and the Noncoding RNA Therapeutic Landscape

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Abstract The Human Immunodeficiency Virus (HIV) belongs to the subfamily of lentiviruses that are characterized by long incubation periods and chronic, persistent infection. The virus integrates into the genome of infected CD4+ cells and, in a subpopulation of cells, adopts a transcriptionally silent state, a process referred to a viral latency. This property makes it exceedingly difficult to therapeutically target the virus and eradicate infection. If left untreated, the inexorable demise of the infected individual's immune system ensues, a causal result of Acquired Immunodeficiency Syndrome (AIDS). Latently infected cells provide a reservoir that maintains viral infection indefinitely. In this chapter we explore the role of noncoding RNAs in HIV infection and in the establishment and maintenance of viral latency. Both short and long noncoding RNAs are endogenous modulators of epigenetic regulation in human cells and play an active role in gene expression. Lastly, we explore therapeutic modalities based on expressed RNAs that are capable of countering infection, transcriptionally regulating the virus, and suppressing or activating the latent state.

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Chronic HIV Infection and the Search for Novel Therapies

HIV/AIDS persists as a global health problem with little hope in the near future for an efficacious vaccine. Despite this, combinations of antiretroviral therapies (ART) have massively reduced the morbidity, mortality and transmission of HIV-related illness [1], resulting ultimately in a slow turning of the tide, and newfound optimism, in the fight against this chronic viral disease. However, current treatment regimens have significant limitations. These include drug toxicities, resistance to ART and the inability to eradicate latent viral infection. Moreover, the daily medication burden has made it difficult to ensure adequate patient compliance with treatment and the costs associated with lifelong treatment and monitoring remain a concern (particularly in developing countries). Nevertheless, the most significant limitation of ART is the inability to eliminate HIV reservoirs, which persist under lifelong treatment. At present, it is not yet feasible to discontinue ART. Latent viral reservoirs within resting memory CD4+ T cells are extremely long-lived and can persist for as long as 60 years for patients receiving ART [2]. In this chapter we explore the noncoding RNA landscape associated with HIV infection and in the establishment and maintenance of viral latency. We focus on gene therapy strategies based on RNA that are capable of transcriptionally regulating the virus, thus ultimately approaching the goal of a functional cure for HIV/AIDS.

HIV Life Cycle

HIV infects cells of the immune system, which express the relevant surface receptors necessary for interaction with the HIV transmembrane Env protein (Fig. 1). HIV enters its target cells, which include CD4+ T cells; macrophages; monocytes; dendritic cells and microglia [3–6], through interaction of the viral trimeric glycoprotein initially with the primary cellular CD4 receptor, and subsequently with the CCR5 or CXCR4 cellular chemokine co-receptors (reviewed in [7]). The gp120 surface subunit of the Env protein initially engages with the cellular CD4 receptor leading to a conformational change in gp120 which allows it to bind to a co-receptor [8]. Viral tropism is largely determined by the co-receptor to which it binds. R5 strains use the CCR5 co-receptor and are able to infect CD4+ T cells, macrophages and dendritic cells. X4 strains bind the CXCR4 co-receptor and only infect CD4+ T cells [9]. Co-receptor binding then triggers the interaction of the gp41 transmembrane subunit of the Env protein with the host cell membrane. Fusion of the cellular and viral membranes ensues [10] and the viral core is released into the cellular environment and uncoated, releasing the viral genome [11]. The viral RNA genome is reverse transcribed into cDNA in the cytoplasm by the viral Reverse Transcriptase (RT) [12] and is then transported within the pre-integration complex consisting of viral cDNA; viral RT; matrix protein; integrase and Vpr, to the nucleus. Nuclear localization signals on Vpr, integrase and matrix mediate import into the nucleus in

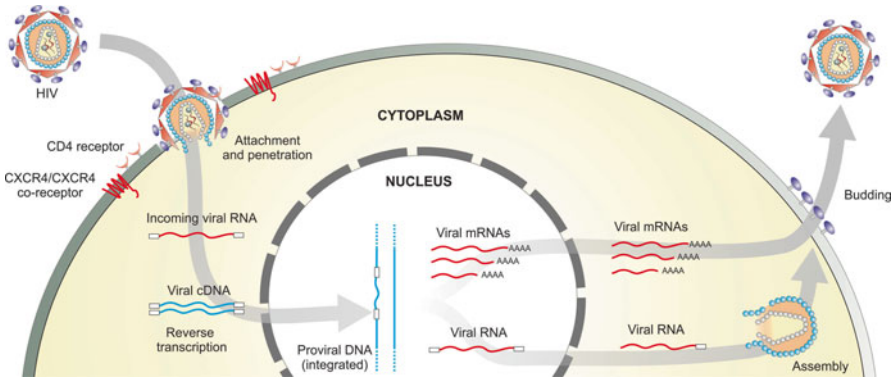

Fig. 1 HIV infection cycle. HIV infects cells and enters target CD4+ cells, through the cellular CD4 receptor, and subsequently with the CCR5 or CXCR4 cellular chemokine co-receptors. Once inside the cell, virus uncoats, releasing the viral RNA genome, which is reverse transcribed into cDNA and transported to the nucleus. Viral cDNA is subsequently integrated into the host genome to form a provirus. Tat-dependence ensures the synthesis of a full-length viral pregenomic transcript and mRNAs. Viral proteins, together with two copies of the viral genome, assemble into immature progeny virions and are released from infected cells through budding

both actively dividing and quiescent cells through the nuclear pores [13–15]. Viral cDNA is subsequently integrated into the host genome, usually within active euchromatin [16], to form the provirus. The provirus is flanked by the 5′ LTR which serves as a promoter for transcription and the 3′ LTR which provides the termination site. The synthesis of full-length transcripts requires the interaction between the regulatory Tat protein and the Transactivating Response (TAR) loop. In the absence of Tat, only short attenuated RNA transcripts are produced [17]. Tat is a multiply spliced protein synthesized early in the viral life cycle. The interaction between Tat and the regulatory TAR loop is enhanced by the positive elongation factor (P-TEFb). Tat binds to the cyclin T1 subunit of P-TEFb and thereby recruits the cyclin dependent kinase 9 (CDK9) subunit to the LTR. CDK9 phosphorylates RNA Pol II, enabling the transition of initiation to elongation and the consequent synthesis of full-length viral transcripts (reviewed in [18, 19]).

Early phase transcripts encoding the Tat, Rev and Nef proteins are completely spliced and are exported from the nucleus. Unspliced transcripts including genomic RNA, Gag-Pol precursors, and incompletely spliced mRNAs encoding Env, Vif, Vpr, and Vpu, require the interaction between the regulatory Rev protein and the Rev responsive element present within these transcripts for nuclear export (reviewed in [20]). Following translation, viral proteins together with two copies of the viral genome assemble into immature progeny virions within lipid rafts localized at the cell membrane. Env proteins are processed into their respective subunits, which also accumulate at the cell membrane. Virions are released from infected cells through a budding process which results in virus particles coated with the host cell membrane embedded with trimeric Env glycoproteins. Maturation of progeny virions occurs following extracellular processing of the Gag and Gag-Pol polyprotein precursors [21].

Viral Latency

HIV-1 infection consists of an initial acute phase followed by a chronic phase. The acute phase is characterized by an increase in viral RNA in the blood (viral load) and the consequent decline in CD4+ T cells [22]. The activation of the immune system subsequently results in the suppression of viremia to a low steady state level termed the viral set point, and an increase in CD4+ T cells. During the chronic phase, viral loads as well as the number of CD4+ T cells may remain constant for several years with the patient remaining largely asymptomatic. During the chronic phase of viral infection, viruses may also enter into a dormant latent state.

Total eradication of HIV continues to present a serious challenge in the struggle against infection with this virus. Cocktails of chemically synthesized antiretroviral drugs, ART, have been successfully used to control viremia during the acute phase of infection. However, despite the significant decrease in morbidity and mortality offered by ART, problems associated with these treatment regimens persist. Although viral replication may be effectively suppressed, current drug regimens are incapable of completely eradicating the virus. Latent reservoirs are characterized by a complete lack of viral gene expression. The underlying molecular mechanisms of latency however are extremely complex and poorly understood, and the exact locations of these latent viral pools are still disputed (reviewed in [23]). There are currently two dominant theories that help to explain what drives latency. One view suggests that viruses are capable of infecting CD4+ T cells that are transitioning into a memory state from a previously active state (reviewed by Siliciano and Greene [24]). Memory CD4+ T cells are a well-defined reservoir and are thought to be the main source of residual viremia [2, 25, 26]. Therefore, viral latency is simply a consequence of natural heterochromatin-mediated epigenetic silencing of transcription in these cells. Another view suggests that latency is the product of stochastic transcriptional "noise" [27]. Since viral transcription is dependent on a Tat-mediated positive feedback circuit, variance in cellular levels of Tat and other transcriptional regulators can greatly impact viral replication in any specific cell [28]. This latter theory has gained much more prominence with new data suggesting that the latent reservoir, comprising replication-competent non-induced provirus, is much larger than previously thought [29]. Even during maximum T cell activation, proviral activation remains largely stochastic [29].

HIV Self-Mediated Transcription Regulation

Growing evidence suggests that an intricate relationship naturally exists between viral infection and the endogenous RNA interference (RNAi) pathway [30–34]. Intriguing data have shown that a number of viruses, including HIV-1, may either exploit the RNAi pathway to their advantage or, alternatively, activate mechanisms which repress the pathway. Furthermore, the expression levels of both viral and host derived microRNAs (miRNAs) may be modulated during viral infection for host or viral gene regulation [35]. In addition, it has been proposed that the

interaction between HIV and the RNAi pathway may also contribute to the multifaceted mechanism underlying viral latency [36, 37]. It is clear that an RNAi-based HIV-host relationship exists, yet a better understanding of this interplay at the molecular level is imperative for the development of novel therapeutics, which exploit this relationship.

Several studies suggest that once integrated into the host genome, the provirus is subject to transcriptional regulation by the host epigenetic regulatory machinery. The activation of latent virus has been well characterized and requires the recruitment of histone acetyltransferases as well as other chromatin remodeling proteins to the activated viral promoter/LTR [38, 39]. Conversely, enrichment of silent state chromatin marks (such as histone 3 lysine 9 trimethylation) and HDAC-1 have been observed at the LTR of transcriptionally inactive proviruses [40–45]. These reports suggest a distinct role for chromatin remodeling, and thus the host epigenetic response, in viral latency. However, the cellular factors responsible for guiding host epigenetic complexes to specific loci are currently unknown.

Noncoding RNAs and Gene Regulation

Recent advances in transcriptomic studies have revealed that a much larger portion of the genome is transcribed than previously anticipated. Studies from the ENCODE consortium have demonstrated that a large proportion of the human genome is transcriptionally active [46]. In addition to protein coding messenger RNA (mRNA) and the well-studied noncoding RNAs (ncRNAs) such as transfer RNA (tRNA); ribosomal RNA (rRNA); small nuclear RNA (snRNA); small nucleolar RNA (snoRNA), many other ncRNAs, in particular long noncoding RNAs (lncRNAs) greater than 200 bp have recently been annotated. Although the functionality of many of these ncRNAs is still fiercely debated [47], it is becoming clear that certain ncRNAs can play a crucial role in gene regulation via multiple mechanisms [48]. Furthermore, these ncRNAs may represent diagnostic biomarkers as well as novel therapeutic targets, thus adding a layer of complexity to the role of ncRNAs in different disease states.

Noncoding RNA has been shown to guide epigenetic complexes to targeted gene promoters resulting in transcriptional gene silencing (TGS) of the targeted gene (reviewed in [49]). Noncoding RNAs antisense to low-copy promoter-associated RNA are able to direct transcriptional silencing complexes containing HDAC1, Ago1 and DNMT3a to a targeted promoter leading to histone modifications and heterochromatin formation [50–56]. This suggests that antisense noncoding RNAs are actively involved in the epigenetic regulation of gene expression (Fig. 2). A growing body of evidence indicates that HIV-1 expresses noncoding RNAs in both the sense and antisense orientation to the viral LTR and viral mRNA [57–59]. It is therefore likely that these RNA species may guide epigenetic silencing complexes to the LTR and thus play a crucial role in the establishment and maintenance of latency.

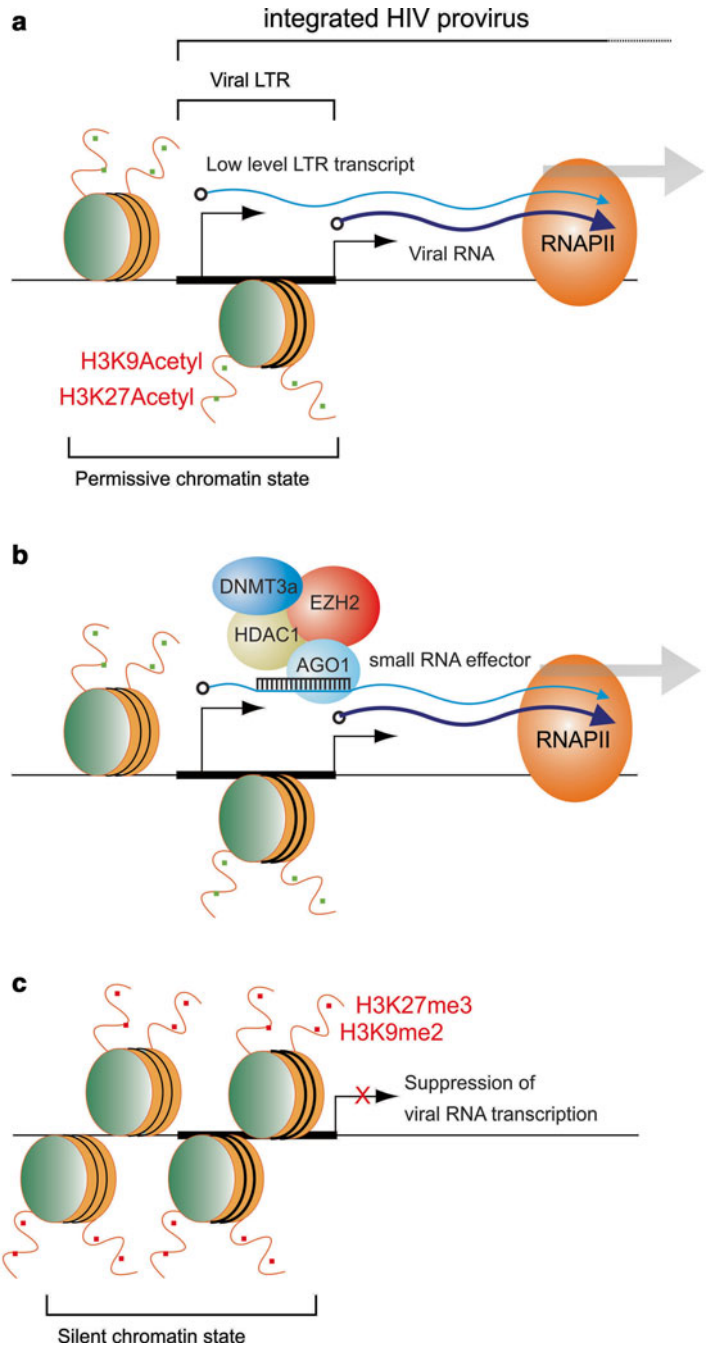

Fig. 2 Inhibition of HIV by transcriptional gene silencing. (**a**) The 5′ LTR of the integrated HIV provirus drives transcription of downstream viral RNAs and is associated with active histone modifications (*green circles*). Additionally, low levels of transcription also occur across the LTR region itself. (**b**) A small RNA effector guides AGO1 to the low copy LTR transcript and thereby recruits the histone deacetylase HDAC1, histone methyltransferase EZH2 and DNA methyltransferase DNMT3A to the LTR chromatin. (**c**) Replacement of active chromatin marks with silent modifications (*red circles*) leads to heterochromatinization and inhibition of viral transcription

Noncoding RNAs Associated with HIV Infection

Noncoding RNAs derived from both the human host as well as from the HIV virus itself are emerging as important regulatory elements of host cellular functions, viral replication, pathogenesis, disease progression and host-pathogen interactions. These regulatory roles are fulfilled by various types of ncRNAs including well-characterized small ncRNAs such as miRNAs, siRNAs, and asRNAs.

Until now, three approaches existed for inhibiting HIV-replication using gene therapy modalities and included directly targeting the viral genome, targeting host dependency factors or targeting newly synthesized viral messenger RNA. Each mature virion contains two copies of the viral RNA genome, which, after fusion, are released into the cytoplasm for reverse transcription as described above. Inhibition of the incoming viral genomic RNA is an attractive target because synthesis of cDNA and establishment of the provirus is prevented, thus inhibiting the replicative cycle of HIV at a very early stage. The second possible strategy for the inhibition of HIV replication and an alternative approach to the prevention of viral entry is to suppress host cellular genes essential for the replicative cycle of HIV. In addition, inhibiting host dependency factors has the added advantage of being refractory to the emergence of viral escape mutants. The third and most obvious category of gene therapy-susceptible targets in HIV is the newly synthesized viral mRNA transcripts, which are exported to the cytoplasm from the nucleus for translation. Every possible HIV transcript has been targeted for silencing using various RNAi effectors with varying degrees of efficacy, often achieving >90 % inhibition of viral replication. The drawback of this strategy is associated with the extraordinarily high rate of error of the viral RT enzyme. HIV is a rapidly evolving virus, and in any infection a pool of viral variants exists. It is thus essential to target conserved regions within viral genes to limit the emergence of viral mutants, which are refractory to RNAi-mediated gene silencing. However, even when targeting conserved sites within the viral genome, mutations may arise under selective pressure, rendering the RNAi effector ineffective. To circumvent this problem, multiple sites should be targeted simultaneously using combinatorial RNAi. Three major platforms currently exist for the simultaneous suppression of multiple gene targets, and all three strategies have been exploited for the inhibition of HIV. These approaches include multiple shRNA expression cassettes; polycistronic miRNA shuttles and long hairpin RNAs (lhRNAs) [60–65]. Given the recent advances in transcriptome analysis, the role of noncoding RNAs in viral infection is fast becoming realized and these noncoding RNAs could thus potentially represent novel therapeutic targets.

HIV-Derived Noncoding RNAs

Small Noncoding RNAs

Small noncoding RNAs (sncRNAs) have been clearly shown to play a role in the replicative life cycle of HIV-1. Although a small number of virally derived sncRNAs have been identified and characterized which are discussed below, evidence suggests that many more low abundance HIV-1 sncRNAs may exist which can be detected with improved selection and screening protocols [66, 67]. Enrichment of low abundant sncRNAs using hybridization capture techniques showed that HIV encodes many sncRNAs of varying lengths spread throughout the viral genome [66]. Deep sequencing technologies have also allowed for a more sensitive method to detect virus-derived small RNAs [67] and together these results suggest that numerous small virus-derived RNAs are produced in HIV infected cells which may potentially play a role in viral replication.

MicroRNAs

MicroRNAs (miRNAs) are small RNA duplexes 21–24 bp in length that regulate gene expression at the posttranscriptional level. miRNAs are derived from the sequential processing of imperfect RNA stem-loop structures by the Drosha/DGCR8 microprocessor complex and by Dicer. The majority of HIV-derived small RNAs are found throughout the sense strand of the viral genome [67]. These small RNAs are thought to be processed from hairpin-like secondary structures within the sense RNA strand by Drosha and Dicer in a similar manner to endogenous miRNAs to generate virus-derived miRNAs.

The first HIV-derived miRNA was described in 2004 [31]. It was proposed that HIV encoded a miRNA precursor within its nef gene. The encoded miR-N367 was reported to target a site at the 3′ end of nef, which overlaps the U3 region of the viral LTR and was thought to play a role in transcriptional regulation of the virus [31, 33].

The Transactivating Response (TAR) element of the HIV genome is a commonly known source of HIV-derived miRNAs [30, 68–71]. TAR is a structured RNA approximately 50 nt in length found at the 5′ end of all HIV mRNA transcripts. The TAR element has been reported to be the source of two functional miRNAs; however, the regulatory role of these TAR-derived miRNAs was initially unclear [68, 69]. Klase et al. identified two host cellular genes as TAR miRNA targets: Excision repair cross complementing-group 1 (ERCC1) and Intermediate early response 3 (IER3). These genes are involved in apoptosis and cell survival and the downregulation of their expression leads to the protection of HIV-infected cells from apoptosis [70]. Another recent study has shown that these TAR-derived miRNAs utilize the same RNAi machinery as host endogenous miRNAs and in addition, further endogenous target genes were identified resulting in the elucidation of their

role in viral infection. In this study, TAR-derived miRNAs were found to target multiple additional genes related to apoptosis including Caspase 8, Aiolos, and Ikaros [71]. The TAR-derived miRNAs therefore play an important role in HIV disease progression by regulating cellular apoptosis and promoting cell survival to ensure the persistence of viral infection.

Small Interfering RNAs

Studies to identify small RNA species in HIV-infected cells have identified noncoding RNAs derived from the viral genome. An initial study looking at the potential of HIV-1 to elicit an RNAi response identified a perfectly duplexed 19 bp Dicer substrate within the HIV-1 genome. This HIV-derived short interfering RNA (siRNA) was able to specifically target its complementary transcript within the Env gene leading to a reduction in both Env mRNA and protein levels which was reversible upon inhibition of the siRNA [32]. Since then a small number of antisense RNAs emanating from the 3′ LTR region have been identified as well. These antisense RNAs may be transcribed from an internal promoter within the HIV genome or from a host promoter downstream of the integrated provirus. Either way, it is thought that these antisense transcripts may form double stranded RNA intermediates with HIV mRNA, which are able to be processed by Dicer to generate viral-derived siRNAs. These siRNAs appear to function via the endogenous RNAi pathway and are capable of modulating viral production. HIV-derived siRNAs identified in a deep sequencing study of HIV-infected cells were found to inhibit virus production by mediating cleavage of the viral transcript at the target site [67]. Furthermore, inhibition of the viral-derived siRNAs by LNA antagomirs reversed the inhibitory effect of the siRNAs on viral production resulting in increased HIV production.

Long Noncoding RNAs

The abundance of long noncoding RNAs (lncRNAs) and their wide range of functional roles in human cells are fast becoming realized. Importantly, lncRNAs have been identified as epigenetic modulators and consequently play a pivotal role in the regulation of gene expression. lncRNAs are able to guide epigenetic silencing complexes to targeted loci in the promoters of protein coding genes resulting in gene silencing in human cells and it is thus becoming apparent that antisense lncRNAs may act as endogenous effector molecules capable of utilizing RNA-based transcriptional silencing pathways in human cells [55, 72–74].

Antisense ncRNAs emanating from the HIV genome during viral infection have also been described [57–59, 75]. Promoter activity has clearly been demonstrated in the reverse orientation with transcription start sites identified in the U3 region of the 3′ LTR as well as in the nef gene sequence. Such transcripts are localized within the

nucleus and are capable of suppressing HIV gene expression over extended periods [59]. Suppression of this antisense lncRNA using small single stranded asRNAs targeted both to the lncRNA transcript as well as to the promoter driving expression of the lncRNA in cell-lines and primary CD4+ T-cells has been shown to result in the activation of viral gene expression [75]. The observed increase in viral gene expression and replication correlated to a loss of silent state epigenetic marks at the viral promoter. This HIV expressed lncRNA was found to localize to the 5′ LTR directly with DNA methyltransferase 3A (DNMT3a) and to usurp components of endogenous cellular pathways that are involved in ncRNA directed epigenetic gene silencing. Collectively, it was shown that the HIV expressed antisense lncRNA is involved in modulating HIV gene expression and that this regulatory effect is due to an alteration in the epigenetic landscape at the viral promoter [75]. This work reflects one of the mechanisms by which HIV regulates its own gene expression. HIV-derived lncRNAs may therefore play an active role in the regulation of viral transcription providing novel insight into the replicative cycle of HIV.

Host-Derived Noncoding RNAs

A subset of host cellular genes have been identified as essential in the replicative cycle of HIV and a variety of these host factors have been considered for downregulation to inhibit HIV infection. In addition to the host proteins encoded by these genes, noncoding RNAs transcribed from some of these genes may play a pivotal role in the course of infection. Furthermore, the expression levels of host derived noncoding RNAs may be modulated during viral infection for host or viral gene regulation [35].

MicroRNAs

Host cell miRNAs have in recent years been implicated in the regulation of HIV infection and the differential expression of these miRNAs has been found to play crucial roles in viral replication, pathogenesis and disease progression. One of the major clues pointing to this miRNA based regulation was uncovered when it was observed that the inhibition of Drosha and Dicer, two key enzymes involved in miRNA biogenesis, resulted in increased viral replication [76, 77]. Subsequently, a plethora of endogenous host miRNAs have been identified which are capable of regulating infection by directly targeting the virus, or by targeting host factors which play a role in the viral replicative cycle, thereby indirectly restricting viral replication.

Two cellular miRNAs, miR-29a and miR-29b, are involved in the regulation of viral replication by directly targeting the viral genome [77, 78]. These two miRNAs

target a conserved site within the viral nef gene [78] and miR-29a has been shown to suppress HIV replication through accumulation of viral mRNA in P-bodies [77]. More recent studies have also confirmed the ability of miR-29a and miR-29b to inhibit viral replication [79]. Further studies have demonstrated the presences of up to 22 target sites within the HIV genome for host cellular miRNAs and at least 5 of these synthetically generated miRNAs are capable of decreasing viral replication [80]. Taken together, the data certainly implies that host cellular miRNAs may act as a cellular defense mechanism against HIV infection.

It has also been proposed that the interaction between HIV and the RNAi pathway may contribute to the multifaceted mechanism underlying viral latency. In resting CD4+ T cells harboring latent HIV, a cluster of five host miRNAs (miR-28, miR-125b, miR-150, miR-223 and miR-382) have been shown to be upregulated [36]. These enriched cellular miRNAs inhibit HIV-1 protein translation through interactions with the 3′ end of viral mRNA transcripts and therefore appear to play a pivotal role in HIV latency. The use of a panel of miRNA inhibitors effectively facilitated viral production in resting T cells and may potentially be used to aid in the purging of latent reservoirs [36, 37].

Host cellular factors are also regulated by endogenous miRNAs as in the case of the polycistronic miRNA cluster miR-17/92, which is downregulated in HIV-infected cells. The histone acetyltransferase P300/CBP-associated factor (PCAF) is a cofactor for Tat and plays a role in procession of viral transcription. Intriguingly, PCAF is a target for miR-17-5p and miR-20a, which are two miRNA components of the miR-17/92 cluster, suggesting an intricate interplay between HIV replication and miRNA-mediated gene regulation of host factors to benefit the virus [76].

Cyclin T1 is another host factor targeted by host-derived miRNAs during HIV infection. As described above, cyclin T1 is a subunit of p-TEFb to which TAT binds for transactivation of HIV transcription. miR-198 targets the 3′ UTR of cyclin T1 mRNA and overexpression of this miRNA leads to inhibition of viral replication [81]. Furthermore, miR-198 was found to be highly expressed in monocytes and downregulated upon differentiation to macrophages suggesting a mechanism for the natural restriction of HIV replication in monocytes [81].

In addition to endogenous host miRNAs having an inhibitory effect on viral replication, host miRNAs may also enhance viral replication depending on the functional role of the miRNA target being modulated. For example, miR-132 is highly upregulated in activated CD4+ T cells and appears to augment viral replication, suggesting that a miR-132 target is a host factor with natural antiviral effects [82].

Long Noncoding RNAs

While the role of cellular miRNAs in the HIV-1 life cycle has been broadly studied in recent years, the relationship between cellular long noncoding RNAs and HIV-1 infection has not yet been fully explored. One study looked at the differential

expression of lncRNAs in HIV-infected cells and identified NEAT1 as a lncRNA that is upregulated during viral infection [83]. Further characterization of NEAT1 showed that it regulates unspliced HIV transcripts at the posttranscriptional level. The depletion of NEAT1 led to increased HIV-1 expression and also resulted in a significant reduction in the number of paraspeckle bodies suggesting that NEAT1 plays a scaffolding role in paraspeckle bodies to which unspliced and singly spliced HIV transcripts containing cis-acting instability elements (INS) bind. Additionally, it was shown that knockdown of NEAT1 leads to an increase in nuclear cytoplasmic export of unspliced Rev-dependent HIV-1 INS-containing transcripts [83].

Noncoding RNA-Based and Therapeutic Targeting of HIV Latency

miRNA Antagonism

The diverse repertoire of noncoding RNAs involved in HIV replication and viral latency present exciting opportunities for therapeutic intervention [84]. Technologies for inhibiting miRNAs are highly developed. The most advanced miRNA therapeutic (a first-in-class pharmaceutical compound) is Miravirsen, developed by Santaris Pharma A/S for treating chronic Hepatitis C Virus (HCV). Miravirsen is a phosphorothioate/locked nucleic acid mixmer antisense oligonucleotide inhibitor of miR-122, a miRNA which is required for effective HCV replication [85]. Miravirsen is currently in late stage clinical trials and has shown promise in preclinical studies in chronically infected chimpanzees [86]. It remains to be seen if miRNA inhibitors will show efficacy in inhibiting HIV replication. For example, TAR-derived miRNAs promote the survival of HIV-infected cells by inhibiting apoptosis [70] Other miRNAs therefore represent potential targets for anti-miRNA therapeutic strategies.

Small-RNA-Induced Transcriptional Gene Silencing of HIV

Exogenous small RNAs complementary to target gene promoters can induce transcriptional gene silencing (TGS) [87] by inducing silent state chromatin formation [51, 56], and promoter DNA methylation [56, 87–90]. TGS is mediated by the RNA induced transcriptional (RITS) complex which contains an argonaute protein (AGO1) [91], and is dependent on the presence of a sense-orientation promoter-associated noncoding RNA [52]. Similarly, miRNAs have also been shown to induce TGS [92–96] and RNAi components have been found to be present, and functional, in the nucleus [97, 98]. The targeted induction of epigenetic alterations at a specific promoter is particularly promising as a therapeutic approach to treating chronic viral infections. Given that HIV viral latency is believed to be mediated by

epigenetic alterations at the proviral DNA, TGS presents the opportunity to effectively 'lock' the virus in an inert, pseudo-latent state.

The effects of conventional RNAi are transient, typically reaching maximal effect within 4–7 days and therefore require frequent repeat dosing. In contrast, TGS has been shown to induce long-term inhibition of the target promoter [56, 99, 100]. As a result, multiple studies have focused on the 5′ LTR of HIV-1 (and also the E6/E7 promoter of Human Papilloma Virus [100]). Two groups of researchers have used small RNAs to target the NF-κB binding motif doublet in the U3 region of the 5′ LTR of HIV-1. Suzuki et al. demonstrated silencing of HIV transcription using small interfering RNAs (siRNAs) in chronically infected MAGIC-5 cells [101]. Silencing was accompanied by methylation of proviral DNA CpG dinucleotides and lasted for 30 days post transfection. The silencing effect was accompanied by the formation of a closed chromatin structure at the viral promoter as evidenced by nucleosome repositioning and enrichment of the silent state mark H3K9me2 [54]. Additionally, AGO1 and the histone deacetylase 1 (HDAC1) were also enriched at the HIV promoter following treatment [54]. Similarly, siRNAs targeted against the simian immunodeficiency virus (SIV) were also shown to induce TGS in MAGIC-5 and CEMx174 cells [102].

When the HIV-1 promoter-targeting sequence was delivered as a short hairpin RNA (shRNA) using a retroviral vector, viral suppression was observed for at least 1 year post-transduction in an HIV infected T-cell line [99]. Silencing was accompanied by enrichment of H3K9me2 and H3K27me3 silent state chromatin modification. TGS of HIV was subsequently demonstrated to be highly specific as (a) mismatched shRNAs were unable to induce TGS, (b) HIV-1 targeting shRNAs were unable to silence HIV-2, (c) NF-κB regulated genes were unaffected by treatment with the on-target shRNA, (d) viral entry-related proteins were not affected by the treatment, and (e) no evidence of interferon induction was observed following shRNA transfections [103]. Recently, the same group demonstrated the *in vivo* potential of TGS approaches to HIV-1 inhibition [104]. Human peripheral blood mononuclear cells (PBMCs) were transduced with a retrovirus carrying the HIV-1 promoter-targeting shRNA. The transduced PBMCs were then transplanted into nonobese diabetic (NOD)/SCID/Janus kinase 3 knockout (NOJ) humanized mice and antiviral activity demonstrated [104].

In parallel, we have also targeted the NF-κB doublet of the HIV-1 5′ LTR (the target site being only four nucleotides off-set relative to that targeted by Suzuki and co-workers). This study showed that the antisense strand of a siRNA alone was sufficient to induce transcriptional silencing of a luciferase cassette driven by the HIV-1 5′ LTR [51]. Subsequently, LTR-targeting antisense RNAs (asRNAs) delivered by mobilization-competent lentiviral vectors were shown to repress viral replication for up to 1 month post-transduction with no evidence to suggest the occurrence of viral escape mutations [90]. Silencing was concurrent with enrichment of silent state chromatin modifications (H3K27me3), recruitment of AGO1, and loss of NF-κB occupancy at the 5′ LTR. Pretreatment of cultures with siRNAs demonstrated that the TGS effect was dependent on expression of HDAC1, AGO1, and the

de novo DNA methyltransferase DNMT3A. A follow-up study also demonstrated the efficacy of the LTR-targeting asRNA in primary human CD4⁺ T lymphocytes [105]. Alternatively, TGS approaches may also be an effective means of silencing HIV host factors. For example, the HIV co-receptor *CCR5* has been shown to be amenable to TGS [52, 91]. CCR5 is a particularly promising target as it is both required for viral entry and is dispensable as evidenced by the prevalence of the inactivating CCR5-Δ32 mutation in certain northern European populations [106].

Small-RNA-Induced Transcriptional Activation of HIV

Latent viral reservoirs within resting memory CD4+ T cells are extremely long-lived and can persist for as long as 60 years for patients receiving ART [2]. One promising strategy is to purge the pool of latently infected cells in the presence of ART by reactivating dormant virus. Reactivation of latent HIV purges infected cells directly (via active viral replication), or indirectly via the host immune system; ARTs can then act to prevent new infection from the released virus to extinguish the reservoir [107]. A seminal clinical study using the histone acetylase (HDAC) inhibitor vorinostat resulted in viral reactivation, but it remains uncertain whether only partial transcriptional reactivation was induced in memory CD4+ T cells [108]. Since activation from latency is largely driven by stochastic events in both active and resting memory T cells [29], HDAC inhibitors and cell-reactivation strategies alone are unlikely to reverse the mechanisms of latency for the entire reservoir [29, 109]. Novel approaches are therefore needed to target the latently infected provirus specifically for activation or suppression/elimination.

Small RNAs targeting noncoding regions can also induce epigenetic activation [110] by targeting antisense lncRNAs [72, 111] (Fig. 3). While the functions of many lncRNAs are currently unknown, some common themes are starting to emerge (reviewed in [112]). Given that the HIV-1 genome encodes both sense and antisense noncoding RNAs [57–59] it is possible that these transcripts act to epigenetically regulate the establishment and maintenance of viral latency. Indeed recent observations suggest that those antisense lncRNAs emanating from the HIV 3′ LTR [57–59] are indeed active epigenetic modulators of HIV expression [75]. The HIV expressed antisense lncRNA appeared to interact with and be involved in the recruitment of DNMT3a, EZH2, and HDAC1 to the 5′ LTR, resulting in epigenetic modulation of HIV transcription, similar to observations with endogenous lncRNAs in human cells [74]. Collectively, these recent observations suggest that HIV, once integrated functions synonymously to endogenous genes and is under lncRNA directed epigenetic regulation. Such insights suggest that it is possible to control viral latency, possibly inhibiting the viruses ability to enter latency by suppressing the expression of the HIV expressed antisense lncRNA (Fig. 3).

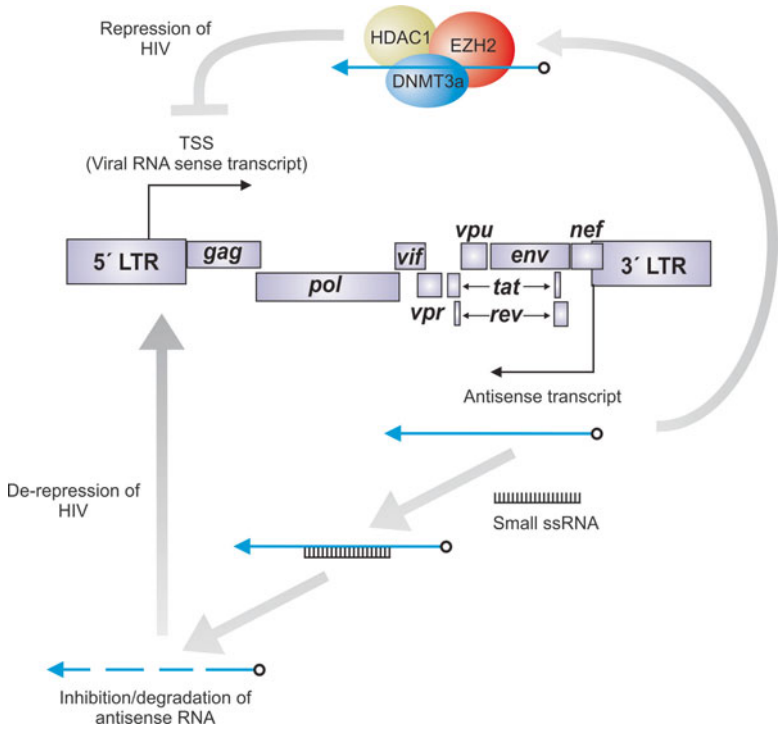

Fig. 3 Model for HIV-encoded antisense lncRNA mediated regulation of viral transcription. A long noncoding RNA (lncRNA) antisense to the viral genome is transcribed from a putative promoter within the nef gene. The lncRNA recruits chromatin remodeling proteins DNMT3a, EZH2, and HDAC-1 and guides these proteins to the viral promoter (5′ LTR). The localization of the chromatin remodeling complex at the 5′ LTR results in the formation of heterochromatin. This alteration of the chromatin state at the viral promoter leads to transcriptional shutdown and an epigenetically silenced virus. The HIV-encoded lncRNA may be inhibited by small single stranded RNAs targeted to the promoter driving expression of the ncRNA via transcriptional gene silencing, or by small RNAs targeted to the ncRNA transcript via posttranscriptional gene silencing (shown). The inhibition of the antisense lncRNA by small RNAs prevents the recruitment of chromatin remodeling proteins to the viral promoter which remains in a euchromatin state, free of silent state epigenetic marks and ultimately resulting in elevated viral gene expression

Conclusions

The success of ART cocktails has meant that the tide is now turning against a once intractable disease. However, the latent HIV reservoir, which harbors transcriptionally dormant virus, represents a unique challenge to efforts aimed at eradicating infection. The fact that current ART cocktails cannot eliminate infection makes it imperative that new therapies are sought, especially those that focus on targeting latent virus and the mechanisms that maintain viral latency. Since viral latency reactivation is stochastic, affecting the positive feedback circuit of viral activation, it is

likely that general factors associated with transcriptional and posttranscriptional control can modulate this process. Already, significant evidence points to both long and short ncRNAs as positive and negative effectors of HIV infection. Moreover, using gene therapy approaches that target these ncRNAs represents a viable approach to eradicating infection. Already a multitude of studies have demonstrated the feasibility of TGS approaches for suppressing HIV-1 replication. Also, the effectiveness of small RNA/oligonucleotide-mediated epigenetic modulation has been demonstrated in vivo [100, 104, 113–116]. This suggests that these approaches may be viable antiviral therapies in the future. Moreover, a new understanding of host and viral-derived ncRNA control of transcription has yielded novel approaches for transcriptionally activating viral gene expression. The latter represents an intriguing therapeutic possibility whereby modalities that interfere or "de-repress" the regulatory ncRNAs result in target-specific viral transcriptional activation. Both these two pathways could be utilized in combination in order to induce enhanced viral suppression or activation of latent infection, thereby working together to act as a "functional cure" of infection.

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C Peptides as Entry Inhibitors for Gene Therapy

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Abstract Peptides derived from the C-terminal heptad repeat 2 region of the HIV-1 gp41 envelope glycoprotein, so-called C peptides, are very potent HIV-1 fusion inhibitors. Antiviral genes encoding either membrane-anchored (ma) or secreted (iSAVE) C peptides have been engineered and allow direct in vivo production of the therapeutic peptides by genetically modified host cells. Membrane-anchored C peptides expressed in the HIV-1 target cells by T-cell or hematopoietic stem cell gene therapy efficiently prevent virus entry into the modified cells. Such gene-protection confers a selective survival advantage and allows accumulation of the genetically modified cells. Membrane-anchored C peptides have been successfully tested in a nonhuman primate model of AIDS and were found to be safe in a phase I clinical trial in AIDS patients transplanted with autologous gene-modified T-cells. Secreted C peptides have the crucial advantage of not only protecting genetically modified cells from HIV-1 infection, but also neighboring cells, thus suppressing virus replication even if only a small fraction of cells is genetically modified. Accordingly, various cell types can be considered as potential in vivo producer cells for iSAVE-based gene therapeutics, which could even be modified by direct in vivo gene delivery in future. In conclusion, C peptide gene therapeutics may provide a strong benefit to AIDS patients and could present an effective alternative to current antiretroviral drug regimens.

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Abbreviations

AAV	Adeno-associated virus
BCNU	Bis-chloroethylnitrosourea
HIV-1	Human immunodeficiency virus type 1
HPC	Hematopoietic stem/progenitor cells
HR	Heptad repeat
IC_{50}	Inhibitory concentration 50
iSAVE	In vivo secreted antiviral entry inhibitor
ma	Membrane-anchored
MGMT	O6-Methylguanine-DNA Methyltransferase
O6-BG	O6-benzylguanine
SIN	Self-inactivating
SIV	Simian immunodeficiency virus
wPRE	Woodchuck hepatitis virus posttranscriptional regulatory element

Introduction

The HIV-1 Entry Process

The human immunodeficiency virus type 1 (HIV-1) enters cells in a multistep process mediated by the viral surface glycoproteins gp120 and gp41 (Fig. 1) [1]. Gp120 is linked non-covalently to gp41, a class I transmembrane protein that anchors the gp120/gp41 heterodimer to the cell or viral surface. The glycoproteins form spikes consisting of three gp120 and three gp41 subunits each. The outer surface protein gp120 is required for the attachment to target cells. Interaction with its cellular receptor CD4 triggers structural changes within gp120 uncovering the binding sites for a co-receptor of the chemokine receptor family, usually CXCR4 or CCR5 [2, 3].

The gp41 protein plays a pivotal role in fusion of the viral and cellular membranes. Receptor engagement results in major conformational changes in the gp41 subunit inducing transformation into a fusion-active state, the so-called pre-hairpin structure. The hydrophobic fusion peptide at the N-terminus of gp41 is exposed and penetrates into the plasma membrane of the target cell [4, 5]. Subsequently, the N-terminal (HR1) and C-terminal heptad repeat domains (HR2) of gp41 assemble into a six-helix bundle. The six-helix bundle is a trimer of hairpins, in which the three HR1-helices form a parallel, coiled-coil core and the HR2 helices pack in the grooves of the coiled-coil core in an antiparallel manner [6, 7]. Six-helix bundle formation brings the viral and cellular membranes into close proximity, enabling fusion pore formation. As the fusion pore widens, the nucleocapsid of HIV is introduced into the cytoplasm [8, 9].

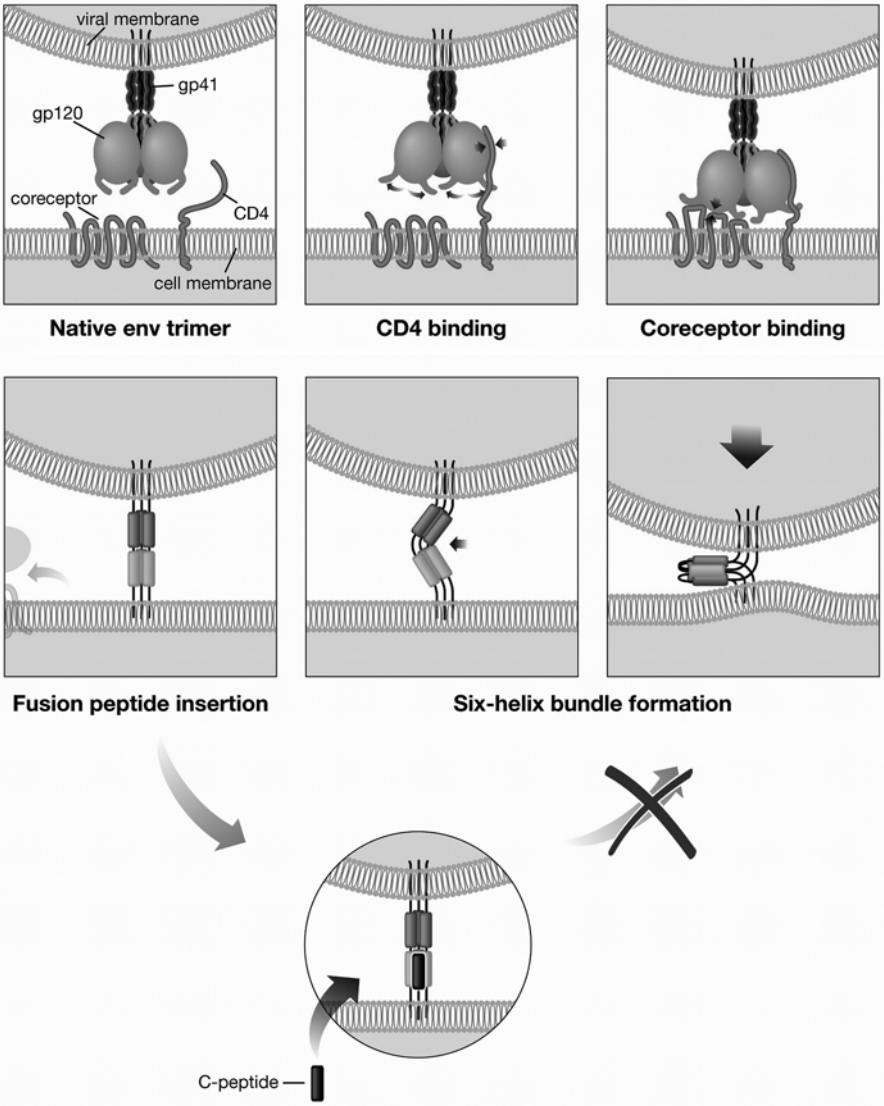

Fig. 1 The HIV-1 entry process. HIV-1 entry into target cells is mediated by the envelope glycoproteins gp120 and gp41 upon binding to the CD4 molecule and a co-receptor on the target cell plasma membrane. Subsequent conformational changes in gp41 bring the viral and cellular membranes in close proximity and finally allow fusion pore formation. C peptides derived from the C-terminal HR2 of gp41 block membrane fusion by binding to the coiled-coil of HR1 helices and thus inhibit six-helix bundle formation (see *circle*). (Figure from: Groner, B. (Ed.): Peptides as Drugs. Discovery and Development. p. 76. 2009. Copyright Wiley-VCH Verlag GmbH & Co. KGaA. Reproduced with permission)

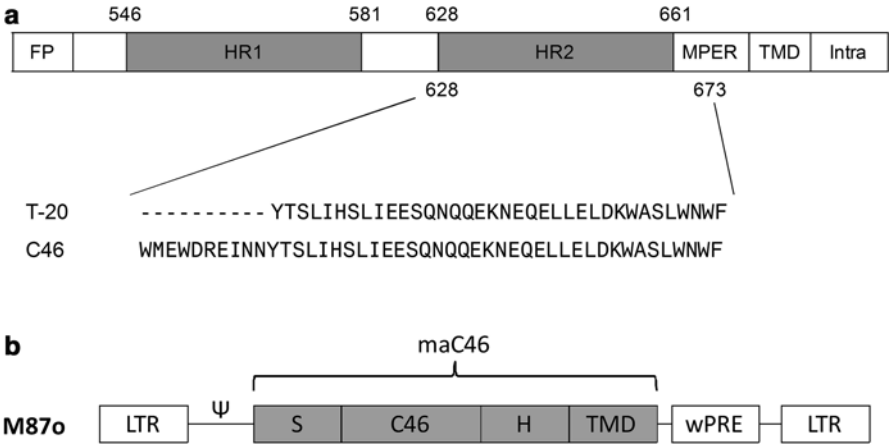

Fig. 2 Structure of gp41 and the vector M87o encoding the HR2 derived C peptide maC46. (**a**) The functional regions of the gp41 molecule are an N-terminal fusion peptide (FP), two leucin-zipper like heptad repeat regions (HR1 and HR2), and the transmembrane domain (TMD). The amino acid numbering of the individual regions is according to the HIV-1_{HxB2} envelope protein. Inhibitory C peptides, such as T-20 and C46, are derived from the C-terminal heptad repeat. (**b**) The gammaretroviral vector M87o encodes membrane-anchored C46 (maC46) as a therapeutic transgene. maC46 consists of a signal peptide (S), the fusion inhibitor C46, a flexible linker (H) and a transmembrane domain (TMD). Packaging of vector RNA into virus particles is mediated by the Ψ element, while the long-terminal repeats (LTR) are required for integration of the transgene into the target cell genome. The 3′ untranslated region contains a woodchuck hepatitis virus post-transcriptional regulatory element (wPRE)

C Peptides as HIV-1 Fusion Inhibitors

C peptides are HIV fusion inhibitors derived from the highly conserved amino acid sequence of the C-terminal HR2 domain of gp41 (Fig. 2a). Antiviral C peptides were first described in 1992 by C. Wild and colleagues [10]. During the fusion process C peptides interact with the coiled-coil formed by the N-terminal hydrophobic helices of the pre-hairpin structure [11]. Thus, the binding sites for HR2 helices are competitively blocked, and six-helix bundle formation and subsequent membrane fusion are prevented (Fig. 1, circle) [7].

C peptides have been shown to potently and broadly neutralize not only various primary and laboratory adapted HIV isolates with inhibitory concentration 50 (IC_{50}) values in the low nanomolar range [12–14], but also simian immunodeficiency virus (SIV) strains [15, 16].

Of the several C peptides described, T-20 (DP178, Enfuvirtide or Fuzeon®) made it into clinical application as a licensed drug. T-20 is a synthetically produced soluble peptide of 36 amino acids representing a portion of the natural sequence of the gp41 HR2 of the HIV-1_{LAI} strain (corresponding to amino acids 638–673 of HIV-1_{HxB2} envelope protein) [17]. In cell culture T-20 inhibits the entry of both HIV-1 clinical and laboratory isolates with IC_{50} values in the low nanomolar range [17]. In clinical phase III trials an optimal dose of 100 mg Enfuvirtide twice daily resulted in a clearly reduced viral load (two logs) and an increase of the patients' CD4 T-cell counts [13, 18].

To date Enfuvirtide (T-20) is the only fusion inhibitor approved for treatment of HIV-1 infection. In 2003, the US Food and Drug Administration approved the agent as a salvage therapy in patients with multidrug resistant HIV [19]. Enfuvirtide was a much anticipated first-in-class therapeutic with a truly novel mechanism of action. However, regardless of its premium price—$20,000 for a year's supply in the beginning and around $35,000 by now—Enfuvirtide did not become the expected blockbuster drug. The reason is that T-20 is not orally bioavailable and thus must be injected subcutaneously and that due to a very short serum half-life of only 2–4 h, a high peptide dose must be administered every 12 h [13]. Finally, Enfuvirtide frequently induced local reactions at the injection sites. Mainly due to this inconvenient application Enfuvirtide never enjoyed much popularity among physicians and patients. Unfortunately, yet another major problem with Enfuvirtide treatment has been the rapid emergence of resistant virus variants [20]. Several novel C peptides are active also against viruses that are resistant to Enfuvirtide. However, synthesis of many of these peptides is too complex and expensive to turn them into standard peptide therapeutics and the need for frequent injections involving local reactions remains.

Gene Therapy Approaches Involving C Peptides

C Peptides as Antiviral Genes

All of the described shortcomings of therapeutic C peptides could be overcome by a gene therapeutic approach, where antiviral C peptides are produced by genetically modified patient cells. Gene therapy has the potential to not only suppress virus replication and prevent CD4 T-cell depletion, but might even be used to eradicate the virus after a few treatments.

Two basic gene therapeutic strategies involving C peptides have been developed. Firstly, a membrane-anchor has been added to the active C peptide sequence and the resultant gene expressed in the HIV target cells. The membrane-bound peptide is produced in the cell and presented on the cell surface directly at the site of action, protecting the cell from virus entry. Secondly, antiviral genes coding for secreted C peptides have been engineered. Cells genetically modified to express such genes release the fusion inhibitory peptides into the extracellular space. The mode of action of secreted C peptides is expected to resemble that of the injected synthetic peptide drug Enfuvirtide (T-20). Once secreted, the C peptides will prevent infection of both gene modified and non-modified HIV target cells. Both strategies have assets and drawbacks and are discussed in detail below.

On the whole, C peptides are among the most promising antiviral gene products as they interfere with an early step of viral replication and prevent infection of the cells. Antiviral genes that inhibit HIV replication prior to virus integration have previously been categorized as class I genes [21]. Class I comprises genes encoding entry inhibitors and inhibitors of the viral enzymes reverse transcriptase and integrase. Antiviral genes that allow integration of the proviral DNA into the host cell genome but inhibit subsequent viral protein expression and reproduction of the viral

genome are grouped into class II, while class III genes encode late inhibitors that impede virus assembly and release [21]. For antiviral genes that protect the gene-modified cells from HIV infection, class I genes are expected to lead to selection of gene modified T-cells that are not infected with HIV, class II genes support the selection of T-cells harboring an HIV provirus, while class III genes do not confer a selective advantage to the gene-modified T-cells in the HIV infected individual.

Mathematical modeling predicts that the stage of the viral life cycle that is inhibited by an antiviral gene product significantly influences therapeutic efficacy and that class I genes will have the highest impact on virus and T-cell dynamics [21]. Consequently, C peptide-based gene therapy strategies for HIV infection are a promising alternative to the injection of synthetically produced peptides.

Membrane-Anchored C Peptides

Intracellular Immunization and Selective Survival Advantage

Expression of membrane-anchored C peptides (maC peptides) on the surface of gene-modified cells is expected to interfere with six-helix bundle formation and thus inhibit infection of the cell with HIV. Gene therapeutic strategies involving protection of the HIV target cells by expression of antiviral genes have been termed "intracellular immunization" [22]. Intracellular immunization strategies for HIV have two potential modes of action: First, if sufficient levels of gene protection can be reached, T-cell counts normalize and viral replication declines due to a lack of permissive target cells. A second antiviral effect will be achieved if sufficient T-helper cell clones specific for HIV antigens are protected against viral infection. These gene-protected helper cells could support the immunologic control of viral replication, without the risk of infection or virus-induced cytotoxicity, both of which are enhanced by HIV antigen activation.

The basic problem of all intracellular antiviral strategies for HIV is that the total number of target cells for the virus in the patient is large, more than 10^{11}, so that direct genetic modification of the entire cell population, whether by T-cell or stem-cell targeting, will not be feasible in the foreseeable future. Sole application of cells containing an antiviral gene is therefore not expected to lead to a substantial level of gene protection, with a significant reduction of susceptible target cells, unless the genetically protected cells have a selective advantage over the non-modified cells and accumulate with time [23]. Indeed, mathematical models indicate that class I antiviral gene products, such as membrane-anchored C peptides that effectively suppress virus infection and protect the cells from the associated cytopathic effect, confer such a selective advantage and lead to the accumulation of gene-modified, non-infected cells. Post-integration inhibitors, on the other hand, support the accumulation of cells carrying an integrated provirus, ultimately resulting in an accumulation of HIV-1-infected cells that counteracts the antiviral effect [21].

Membrane-Anchored C46

A membrane-anchored version of the 46 amino acid C peptide C46 (maC46) has been developed in the laboratory of Dorothee von Laer several years ago [24, 25]. The antiviral component C46 is an elongated version of T-20, which contains ten additional amino acids at the N-terminus and can therefore interact with the highly conserved hydrophobic pocket at the C-terminus of the HR1 coiled-coil core. The maC46 peptide is a fusion protein with an N-terminal signal peptide to mediate transport through the endoplasmic reticulum to the plasma membrane, followed by the C46 sequence, a flexible linker derived from human IgG2 and the membrane anchor from human CD34 (Fig. 2b).

In the initial design, the maC46 protein was encoded by the gammaretroviral vector M87o. The vector provides the long-terminal repeat sequences required for integration of the transgene into the target cell genome as well as the Ψ element for packaging of the vector RNA into retroviral particles. The cis-acting RNA element wPRE (woodchuck hepatitis virus posttranscriptional regulatory element) is located in the 3′ untranslated region and increases virus titers and gene expression (Fig. 2b).

Transduction of T lymphocytes with the M87o vector resulted in the expression of high levels of maC46 on the cell surface. Replication of a broad range of laboratory adapted and primary HIV-1 isolates (different clades B, D, AE or group O) and even HIV-2 was effectively inhibited in transduced bulk cultures of T helper cell lines and primary T-cells [25]. The predicted mode of action of maC46 at the level of virus entry was confirmed by single-round infections with different replication-incompetent lentiviral vector HIV–Env pseudotypes [26, 27]. Here, cells expressing maC46 showed a more than 10,000-fold inhibition of HIV-1 Env-mediated viral entry [28]. This strong antiviral activity conferred a sufficient selective survival advantage to the maC46 expressing T-cells to gain prevalence in HIV-1-infected mixed populations of M87o-transduced and untransduced cells [25]. Here, gene-modified cells were protected from infection, while non-modified cells were lost due to virus-induced cell death. Thus, in mixed cultures containing as few as 1 % gene-modified cells at the time of HIV infection, these protected cells rapidly accumulated to 100 % and eliminated the virus from the culture [29].

Moreover, the effect of maC46 on viral inhibition and the ability to confer a selective advantage following HIV-1 infection were evaluated in a xenotransplant mouse model. Immunodeficient mice were transplanted with gene-modified human $CD4^+$ T-cells expressing the fusion inhibitor from a lentiviral vector and infected with HIV-1 [29]. MaC46-expressing human $CD4^+$ T-cells showed a preferential survival and marked expansion relative to untransduced $CD4^+$ T-cells after HIV-1-challenge in this in vivo setting. However, the gene-protected cells in the infected mice did not proliferate and expand at the rate seen in uninfected mice suggesting that T-cell regeneration may be disturbed in general in HIV-1-infected mice.

Interestingly, maC46 was found to be highly active also against virus strains resistant to T-20. Hermann and coworkers selected an HIV-1 strain with reduced sensitivity to maC46 [30]. After over 200 days of passaging on suboptimal and slowly increasing concentrations of maC46, a virus strain with a tenfold-reduced sensitivity

to maC46 emerged. This virus had five mutations at highly conserved positions in the viral envelope, three in gp120, and one each in the HR1 and HR2 of gp41. No mutations developed in the domain around the GIV motif in HR1, generally associated with resistance to C peptide fusion inhibitors. The GIV mutation is thought to reduce binding affinity of T-20 to the gp41 HR1 coiled-coil. In contrast, the mutations that reduced sensitivity of HIV to maC46 were not found to reduce binding affinity to C46, but either enhanced intramolecular binding affinity between gp41 HR1 and HR2 or accelerate the entry process. Thus, resistance to maC46 does not readily develop and requires multiple cooperating mutations at highly conserved positions of the viral envelope glycoproteins gp120 and gp41. This finding is extremely interesting, as the reason for this "resistance to resistance" for maC46 is most likely the large interaction surface between the viral envelope gp41 HR1 and the C46 peptide. Interacting domains tend to be much smaller for low molecular weight antiviral compounds and even for most neutralizing monoclonal antibodies, to which HIV-1 rapidly develops resistance when applied as a monotherapy.

Secreted C Peptides

Gene therapeutic regimens aiming at in vivo secretion of therapeutic peptides are an alternative to the protection of HIV target cells by the expression of membrane-anchored C peptides. Cells genetically modified to express in vivo secreted antiviral entry inhibitors (iSAVE) will release the fusion inhibitory peptides into the extracellular space. Thus, iSAVE peptides have the crucial advantage of protecting not only the gene-modified cells but also non-modified neighboring cells. Due to this bystander effect secreted peptides have the potential to suppress viral replication in the patient even at relatively low levels of gene modification. This is in contrast to the maC peptides, for which, to be therapeutically effective, the gene-protected cells must accumulate to high levels at which the target cell availability for HIV is reduced to a level that effectively reduces viral replication and the viral load.

The modification of either T-cells or hematopoietic progenitor cells with iSAVE genes has great therapeutic potential, as gene-modified cells would be expected to home to lymphatic tissues, which are the major sites of viral replication and the desired site of antiviral drug activity [31]. The fact that serum levels of Enfuvirtide of more than 1 μg/ml are required for therapeutic efficacy, three orders of magnitude above effective in vitro concentrations, and the low steady state volume of distribution of 5–7 L indicate that Enfuvirtide only inefficiently penetrates the lymphatic tissue. Secretion of therapeutic iSAVE peptides from gene-modified cells directly in the lymphatic tissue is expected to lead to high and much more stable local effective concentrations of peptide.

The major challenge for the development of a C peptide-based iSAVE strategy is the size requirement of at least 50–80 amino acids for efficient entry of a protein into the secretory pathway [32, 33]. The therapeutic peptide sequence must therefore be linked to a scaffold for efficient secretion. We engineered an antiviral gene encoding an iSAVE peptide by linking of two therapeutic C46 peptides via a

cleavage site recognized by the cellular protein convertase furin [34]. These C46-concatemers were efficiently transferred into the endoplasmic reticulum and processed into monomers by furin protease cleavage within the secretory pathway. In cell culture, the secreted peptides mediated a substantial protective bystander effect on non-modified cells, thus suppressing virus replication even if only a small fraction of 3 % of cells was genetically modified. In addition, secreted C peptides were highly stable with a half-life of more than 30 h. This is expected to correspond to a longer and more stable availability of the active peptide in vivo [34].

Target Cells and Gene Transfer Systems for C Peptides

For gene therapy of HIV-1 infection, the maC46 gene should be selectively transferred to the major target cells of HIV-1. The highest level of replication occurs in CD4 T-cells, especially in the lymphatic tissue, where the follicular helper CD4 cells have the highest infection level. Thus, protection of CD4 T lymphocytes by maC46 would be expected to reduce the viral load. However, in clinical trials, including the maC46 trial described below, the transfer of gene-modified T-cells has rarely achieved primary marking levels of more than 1 % [35, 36]. Clinical trials on adoptive T-cell transfer for cancer treatment suggest that higher levels may be achieved with lymphotoxic or even myelotoxic pretreatment [37]. However, to our knowledge, this has not been tested yet for the transfer of gene-protected T-cells in HIV-infected individuals. Efficient in vivo selection of maC46-expressing cells would be required to reach near 100 % marking levels, which would lead to reduction of target cell availability and viral load. However, no significant increase of gene-protected cells has so far been reported in clinical T-cell gene therapy trials for HIV infection, including the maC46 trial. The reason is most likely that advanced HIV-infected patients lack intact lymphatic tissue and regenerative capacity of T lymphocytes, which is expected to massively reduce the ability of maC46-protected T-cells to accumulate and prevail [21]. In addition, the T-cell repertoire is considerably narrowed in HIV-infected individuals and cannot be regenerated by an autologous T-cell transfer. T-cell precursors in the thymus, which could regenerate the repertoire, are not protected from HIV-infection and killing in a purely T-cell-based gene therapy. Finally, transfer of an antiviral gene to T-cells only will not protect other cellular reservoirs such as macrophages and microglia.

In contrast, efficient gene transfer of maC46 to hematopoietic stem/progenitor cells (HPC) could generate gene-protected T-cells and macrophages and regenerate the T-cell repertoire in the HIV-infected individual. Here, HIV-specific CD4 T-cells could be generated that could mediate the immune control of HIV replication. However, one disadvantage of a stem cell approach is that some level of myelotoxic preconditioning is required to enable engraftment of gene-modified stem cells. The second disadvantage is derived from the fact that proliferating cells such as T-cell and hematopoietic stem cells so far can only be stably gene-modified with integrating vectors. Here, retroviral (generally gammaretroviral or lentiviral) vectors are used. However, insertional mutagenesis by retroviral vectors has been

Table 1 Target cells for gene therapy approaches for maC peptides

Target cells	T-cells	Stem cells
Conditioning	Improves marking	Essential
Risk of leukemia, insertional mutagenesis	Not described	Described in mice and humans
Regenerative capacity	Intermediate	High
Regeneration of T-cell repertoire	Not possible	Possible
Protection of macrophages	No	Yes

shown to have a considerable risk to cause myelodysplasia or leukemia by integrating in or near oncogenes in several clinical trials [38–40]. In contrast, development of lymphomas has never been described after retroviral gene transfer to mature T lymphocytes [41], which seem to have a natural resistance to transformation [42]. This limited the use of maC46 to T lymphocytes in the first-in-man study in the early 2000s described below. Advantages and disadvantages of T lymphocytes and HPC for gene therapy are summarized in Table 1.

In the past years, vectors with improved safety profile, at least in mouse models, have been developed. Here, lentiviral self-inactivating (SIN) vectors are currently most widely used. However, increased safety of SIN lentiviral vectors relative to the originally used gammaretroviral vectors has not been formally proven in man, yet, but is highly likely. Recent clinical trials using lentiviral gene transfer to treat inherited immunodeficiencies indicate safety and efficacy of SIN lentiviral vectors, although extended follow-up will be necessary to prove long-term safety [43, 44]. Thus, current gene therapy trials, including those involving maC46 gene transfer for HIV-infection (NCT01734850), generally use lentiviral vectors for gene transfer.

The secreted C peptides (iSAVE) are less restricted concerning the target cell for gene transfer and do not even necessarily require ex vivo gene transfer. In previous studies with other secreted antiviral proteins, intramuscular expression from an adeno-associated virus (AAV) vector was sufficient to achieve protein levels protective against HIV infection [45]. Alternatively, systemic application of an AAV vector targeting primarily liver tissue has been shown to support therapeutic levels of a secreted protein such as erythropoietin [46]. Both approaches for in vivo gene delivery are currently under investigation.

Next-Generation C Peptides

Since the development of T-20 in the early 1990s several next-generation C peptides have been engineered as peptide therapeutics: variants with improved and broadened antiviral activity, with enhanced stability or reduced immunogenicity have been designed (Table 2). Some of the novel C peptides are elongated compared to T-20 making manufacturing and formulation even more challenging; however, many of them are attractive antiviral peptides for gene therapy approaches.

Table 2 Overview of C peptide sequences

Peptide	Sequence	Amino acids	Reference
T-20	----------YTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF	36	[10]
C34	--WMEWDREINNYTSLIHSLIEESQNQQEKNEQELL	34	[52]
C46	--WMEWDREINNYTSLIHSLIEESQNQQEKNEQELLELDKWASLWNWF	46	[25]
T-1249	--WQEWEQKITA------LLEQAQIQQEKNEYELQKLDKWASLWEWF	39	[53]
C34-EHO	--WQQWERQVRFLDANITKLLEEAQIQQEKNMYELQ	34	[57]
C46-EHO	--WQQWERQVRFLDANITKLLEEAQIQQEKNMYELQELDKWASLWNWF	46	[63]
V2o	--WQTWERQVDNITQTISKALEEAQIQNEKNMYELQKLNQWDIFSNWF	46	[63]
Sifuvirtide	-SWETWEREIENYTRQIYRILEESQEQQDRNERDLLE	36	[61]
SC35EK	--WEEWDKKIEEYTKKIEELIKKSEEQQKKNEEELKK	35	[60]
MT-SC22EK	MTWEEWDKKIEEYTKKIEELIKKS	24	[56]

Elongated C Peptides

Many next-generation C peptides are amino-terminally elongated compared with T-20 and therefore bind a highly conserved hydrophobic groove at the C-terminus of the central HR1 coiled-coil structure. The interaction with this conserved pocket improves the inhibitory activity of peptides and also blocks the entry of HIV-1 strains resistant to T-20 [47–49]. Moreover, the elongation also delays the development of viral escape mutants [50, 51]. The hydrophobic amino acids W628, W631, and I635 (numbering according to the HIV-1_{HxB2} Env protein) within the elongated C peptides are the major determinants for interaction with the conserved binding pocket [6] and can for instance be found in C34 [52], maC46 [25], or the second-generation C peptide T-1249, which is derived from HIV-1, HIV-2, and simian immunodeficiency virus (SIV) sequences [53].

Chong and coworkers recently found that the amino acid residues methionine M626 and threonine T627 preceding the C peptide pocket-binding domain adopt a unique hook-like structure [54, 55]. Addition of the M-T-hook to the N terminus of poorly active short C peptide variants dramatically improved the antiviral activity and thermostability [56].

C Peptides with HIV-2 and SIV Activity

A panel of 34-mer peptides derived from various strains of HIV-1, HIV-2, and SIV was analyzed in vitro for anti-HIV and anti-SIV activity in the lab of G. M. Clore [57]. Interestingly, the C34 peptide derived from HIV-2_{EHO} was found to be a highly potent inhibitor not only of HIV-1, but also of SIV Env-mediated cell fusion. Inhibition of SIV is a useful characteristic, as it permits preclinical efficacy testing of C peptides in the rhesus macaque model. In addition, the breadth of antiviral activity predicts that resistance may not emerge readily.

Borrego and colleagues recently described the 34 amino acid peptide “P3” derived from ancestral HIV-2/SIV HR2 sequences [58]. In cell culture experiments, this peptide was active against both HIV-1 (including variants resistant to T-20) and HIV-2 in the low nanomolar range. Moreover, the P3 peptide was very stable and HIV-1 infected individuals had fewer preexisting antibodies to the peptide than to T-20.

C Peptides with Enhanced Helix Stability

Using rational design, C peptides with a greatly enhanced helical structure were engineered, e.g., T-2635 [59], SC35EK [60], or sifuvirtide [61]. These peptides have superior affinity to HR1, thus significantly improved bundle stability, antiviral activity and pharmacokinetics. Moreover, HIV-1 has major problems to gain resistance against these third generation peptides, which always comes at the expense of reduced fitness [62].

Sifuvirtide was designed by Y. He and coworkers after a series of alterations starting from the gp41 sequence of the HIV-1 subtype AE [61]. Compared to

T-20, sifuvirtide shows improved affinity to HR1 and thus higher bundle stability. The peptide demonstrates superior inhibitory activities against a wide variety of primary and laboratory-adapted HIV-1 isolates, including non-subtype AE viruses and also T-20-resistant virus strains. The safety of sifuvirtide was proven in a clinical Phase Ia trial. In clinical pharmacokinetics studies, the half-life in humans was found to be 26 h, compared to 3.8 h for T-20, making sifuvirtide a potent candidate for future treatment of HIV/AIDS patients.

C Peptides with Reduced Immunogenicity

Immune responses against antiviral peptides produced directly in vivo from gene-modified cells may significantly impair clinical efficacy and pose safety risks to patients. Consequently, reducing or even eliminating antigenicity and immunogenicity of C peptides (while retaining full function) may significantly promote safety and antiviral activity. We recently described a novel C peptide, V2o, with greatly reduced immunogenicity and excellent antiviral activity [63]. V2o is based on the chimeric C peptide C46-EHO, which is derived from the HR2 regions of HIV-2_{EHO} and HIV-1_{HxB2} and has broad anti-HIV and anti-SIV activity. Antibody and MHC class I epitopes within the C46-EHO peptide sequence were identified by in silico and in vitro analyses. Using rational design, we removed these epitopes by amino acid substitutions and thus minimized antigenicity and immunogenicity considerably. At the same time, antiviral activity of the "de-immunized" peptide V2o was preserved or even enhanced compared to the parental C46-EHO peptide [63]. Thus, V2o is an excellent candidate for novel gene therapeutic approaches for HIV infection.

C Peptide Gene Therapy in Nonhuman Primates

As mentioned in section "Target cells and gene transfer systems for C peptides" above, genetic modification of hematopoietic stem/progenitor cells (HPC) with maC46 is expected to have a greater therapeutic effect than T-cell based gene therapies. The protected T lymphocytes derived from the gene-modified HPC could replenish the T-cell repertoire and are expected to have a higher regenerative potential than directly modified mature T-cells in HIV-infected patients, which show signs of T-cell exhaustion over time [64]. To test this prediction, a stem cell gene therapy study with maC46 expressed from a lentiviral vector was performed in two macaques [65]. In this trial, the initial level of gene marking was improved by including an in vivo selectable gene expressing a O6-Methylguanine-DNA Methyltransferase (MGMT) mutant, which confers resistance to the cytotoxic effects of the nitrosoureas anticancer drugs [66].

Pigtail macaques underwent identical transplants and SHIV challenge procedures with the only variation between control and maC46 macaques being the inclusion of the fusion-inhibitor expression cassette. Gene modified HPC were selected by treating the animals with bis-chloroethylnitrosourea (BCNU) and

O6-benzylguanine (O6-BG) chemotherapy to reach a level of gene marking of 20 % in the two control animals and in one of the maC46 treated animals. The other macaque had an maC46 marking level of around 55 % in CD4 T-cells. Following SHIV-challenge, maC46 macaques, but not control macaques, showed a positive selection of gene-modified $CD4^+$ T-cells in peripheral blood, gastrointestinal tract and lymph nodes accounting for >90 % of the total $CD4^+$ T-cell population. maC46 macaques also maintained high frequencies of SHIV-specific, gene-modified $CD4^+$ T-cells, an increase in non-modified $CD4^+$ T-cells, enhanced cytotoxic T lymphocyte function and antibody responses. This nonhuman primate study proved for the first time that in vivo selection of genetically protected T-cells in the presence of HIV replication is possible in primates and thus potentially in man. These results have fueled further development of maC46 stem cell gene therapy of HIV-infection.

Clinical Trials

In the years 2003–2005 the first clinical gene therapy trial for HIV-1 infection using the maC46 encoding gene was performed. MaC46 was expressed from the classical gammaretroviral vector M87o. Initially, for the above mentioned reasons, a stem cell gene therapy trial had been planned. However, the first cases of leukemia caused by retroviral genotoxicity were reported in a stem cell gene therapy trial for X-linked SCID in children during preparation of the maC46 trial [67]. Thus, based on safety considerations, T lymphocytes were chosen as the target for the first-in-man study.

MaC46 gene-modified autologous T-cells were infused into ten HIV-infected patients with advanced disease and multidrug-resistant virus during antiretroviral combination therapy. Cell infusions were tolerated well with no severe side effects. A significant increase of CD4 counts was observed after infusion. At the end of the 1-year follow-up, the CD4 counts of all patients were still around or even above baseline. This effect is often seen in patients treated with ex vivo activated T-cells and is most likely attributed at least partially to the production of IL-2 from the infused T-cells.

Gene-modified cells could be detected in peripheral blood, lymph nodes, and bone marrow throughout the 1-year follow-up, and marking levels correlated with the cell dose (Fig. 3). Now, 10 years after treatment the patients still show a low level of maC46-gene positive cells in peripheral blood (<0.1 %). No accumulation of gene marked cells was observed during follow-up. This finding is in accordance with the predictions from previous mathematical modeling studies [21, 68] that in patients with reduced proliferative capacity of T-cells, the gene-protected cells cannot accumulate and prevail under the selective pressure of HIV-1 replication.

Fig. 3 (continued) macaque represents non-modified $CD4^+$ T-cells. As indicated, 70 % of $CD4^+$ T-cells at day 140 post-SHIV challenge are non-modified (Figure originally published in [62] and reproduced here with the kind permission of the journal Blood). (**c**) 0.3 to 1×10^{10} autologous $CD4^+$ T-cells expressing maC46 were infused into patients with advanced HIV-infection. The M87o vector copy number per 100 cells (genomes) of total peripheral blood leukocytes was determined by quantitative polymerase chain reaction regularly during the first year of follow-up [36]

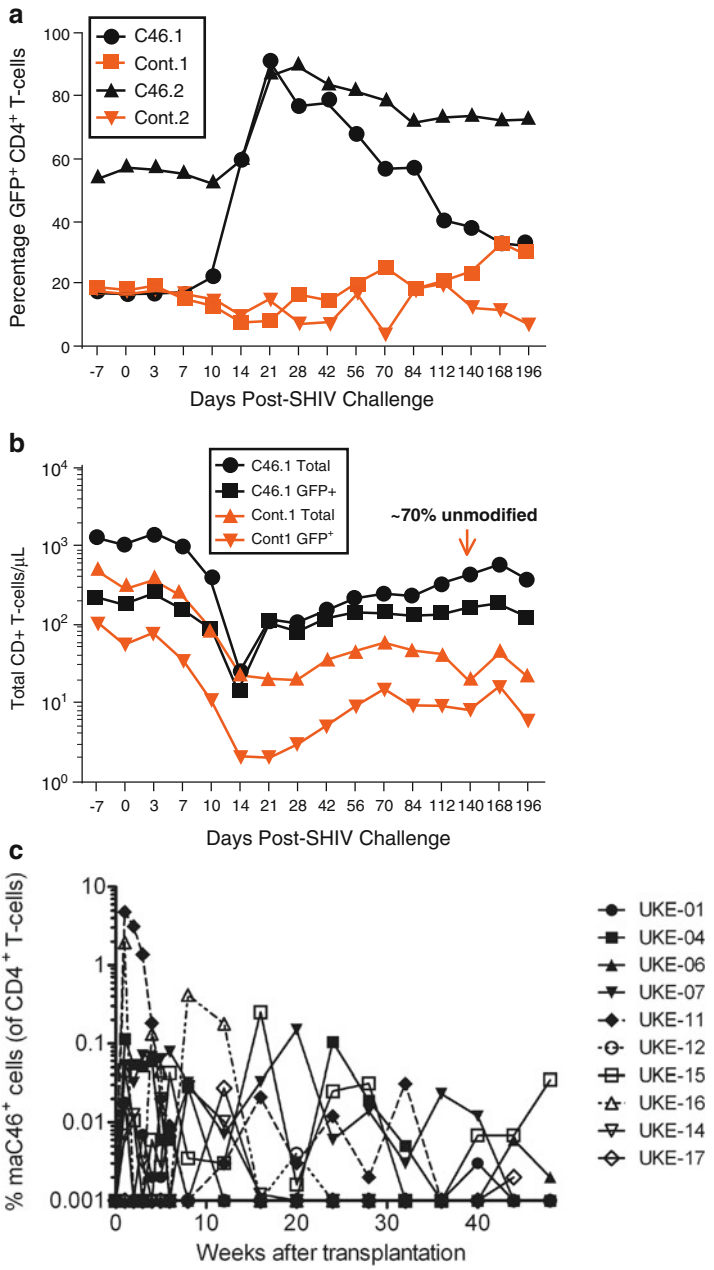

Fig. 3 In vivo selection of maC46+ T-cells in SHIV-infected nonhuman primates transplanted with gene-modified HPC but not in HIV-infected patients treated with gene-modified autologous T-cells. (**a**) Macaques were treated with autologous HPC expressing maC46-GFP fusion (maC46) and MGMT or GFP (control) and MGMT. After in vivo selection of gene-modified HPC with BCNU and O6-BG, animals were challenged with SHIV. The percentage of gene-modified CD4+ T-cells in peripheral blood was determined by flow cytometry analysis based on GFP expression in the control (*squares* and *diamonds*) and maC46-macaques (*circles* and *triangles*). (**b**) The absolute number of CD4+CD3+ (*circles* and *triangles*) and total CD4+CD3+GFP+ T-cells (*squares* and *diamonds*) was determined by FACS analysis for control and maC46-macaque set 1. The area between the respective lines of each

No significant changes of viral load were observed during the first 4 months. Four of the seven patients who changed their antiviral drug regimen thereafter responded with a significant decline in plasma viral load. Eight patients are still alive more than 10 years after treatment, one died of B-cell lymphoma, another was lost to follow-up. Taken together the transfer of gene-modified cells expressing maC46 was safe, led to sustained levels of gene marking, and may have improved immune competence in HIV-infected patients with advanced disease and multidrug-resistant virus.

As discussed in section "Target cells and gene transfer systems for C peptides," targeting hematopoietic stem cells with an antiviral gene such as maC46 may well lead to stronger therapeutic effects than seen in the clinical T cell gene therapy trial. The maC46-gene modified HPC could generate new T cell clones expressing maC46 directed against new target antigens including HIV-antigens. Thus an maC46-protected T cell repertoire could be generated increasing the immunocompetence of the patient and suppressing HIV-replication. This prediction has been confirmed in nonhuman primates as described above and is now being tested in a stem cell gene therapy trial. In this trial a lentiviral vector co-expressing a CCR5 shRNA with maC46 is used. The first patients have been treated (clinicaltrials.gov identifier: NCT01734850). If the results from the nonhuman primate model can be confirmed in man, stem cell-derived maC46-modified T-helper cells may well have the potential to suppress HIV-replication, possibly even long-term. Whether co-expression of an in vivo selectable marker such as MGMT used in the nonhuman primate study will be required to initially increase the level of maC46 gene-modified HPC to a therapeutic level must be demonstrated. If the results from the nonhuman primate study can be reproduced in HIV-1-infected patients, stem cell gene therapy using potent entry inhibitory genes such as maC46 could turn an HIV-infected patient with active viral replication into an Elite controller.

Conclusions and Outlook

The holy grail of gene therapy for HIV-infection has been the in vivo selection of gene-protected cells to a level that leads to a lack of target cell availability for HIV, reduction of virus replication and immunologic reconstitution of the patient. For few anti-HIV genes selection of gene-protected cells was reported in cell culture and in humanized mice infected with HIV. As expected, only early-acting antiviral genes that hinder permanent integration of HIV have shown this capacity (e.g., entry inhibitors and Tre-recombinase [25, 29, 69]). For maC46, efficient in vivo selection of gene-protected cells has also been reported in a nonhuman primate model. The ongoing stem cell gene therapy trial with the entry inhibitors CCR5 shRNA and maC46 should demonstrate whether potent entry inhibitors are therapeutically effective in patients with HIV infection.

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Aptamer–siRNA Chimeras for HIV

Mayumi Takahashi, John C. Burnett, and John J. Rossi

Abstract Since 1980s, HIV/AIDS has escalated into a global pandemic. Although combinatorial antiretroviral therapy (cART) regimens can suppress plasma virus levels to below the detection limit and the survival rate of HIV-1 infected patients has been improving, long-term cART holds the potential to cause a number of chronic diseases. RNA interference (RNAi) is considered as a powerful method for developing new generation of therapeutics. Discovery of small interfering RNAs (siRNAs) shed light on limitations of targets that are "undruggable" with current technologies. However, delivery remains a major hurdle of siRNA-based therapy. Recent progress in technology of engineering nucleic acid enables a targeted delivery of siRNAs using aptamers, which, as often regarded as nucleic acid "antibodies," can recognize/bind to multiple different proteins and small-molecule targets by forming scaffolds for molecular interactions. SELEX technology enabled to isolate highly target specific aptamers from a random sequence oligonucleotide library. A number of aptamers for HIV-1 proteins as well as host proteins that interact with HIV-1 have been developed and some of them have potent viral neutralization ability and inhibition of HIV-1 infectivity. The availability of these aptamers has given an idea of using aptamers for targeting delivery of siRNAs. So far, aptamers against either HIV-1 gp120 or CD4 have been eagerly evaluated as the aptamer portion of the aptamer–siRNA chimeras for the treatment or prevention of HIV-1. In this chapter, we highlight the development and therapeutic potential of aptamer–siRNA chimeras for HIV-1.

HIV-1

Since the first recognition of AIDS in the United States in 1981 [1] and the discovery of the human immunodeficiency virus (HIV) that causes AIDS [2], HIV/AIDS has escalated into a global pandemic that has infected an estimated 75 million

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people worldwide [3]. Upon the clinical approval of azidothymidine (AZT) in 1987 [4] and subsequent introduction of other classes of antiretroviral drugs in the 1990s and 2000s, combinatorial antiretroviral therapy (cART) regimens can suppress plasma virus levels to below the detection limit. Owing to the considerable advance in HIV-1 treatment over the past decades, the survival rate of HIV-1 infected patients has been drastically improved, including many who maintain a normal life by taking daily cART medication. According to UNAIDS report on the global AIDS epidemic 2013 [3], an estimated 35.3 million people were living with HIV in 2012, and because of an increase in the number of people receiving cART, the number of new infections and AIDS-related deaths are declining from previous years. However recent studies suggested that long-term cART may cause a number of chronic diseases, such as cardiovascular disorders, diabetes, mitochondrial dysfunction and many kinds of cancer [5]. For example, mitochondrial toxicity is recognized as a major adverse effect of nucleoside reverse transcriptase inhibitors (NRTIs) which can cause lactic acidosis and hepatic steatosis [6]. NRTIs have high affinity for the viral reverse transcriptase, but it can also be incorporated into human DNA polymerase as well as mitochondrial DNA polymerase, which can inhibit the replication of mitochondrial DNA, leading mitochondrial dysfunction. Lipoatrophy, lipohypertrophy, and hyperlipidemia are also reported to the adverse effects of NRTIs [7, 5]. Protease inhibitors are known to alter lipid metabolism, cause lipid abnormalities, e.g., hyperlipidemia, peripheral lipoatrophy, and central fat accumulation that can be associated with atherosclerotic disease [8, 9]. Because most protease inhibitors are metabolized by the cytochrome P450 (CYP450) resulting drug-drug interactions [8], one should carefully choose statins for treatment of HIV-1 patients these who have lipid disorders. Other drugs like fusion inhibitors and integrase inhibitors are reported to have some adverse effects as well [5]. Although these adverse effects of antiretroviral drugs are remarkably reduced in the current cART regimens, these still should not be ignored.

Considerable research on HIV-1 lifecycle has revealed that HIV-1 proteins and host cellular ligands are fundamental for virus replication [10–12]. The HIV-1 virion is composed of two copies of the positive sense genomic viral RNA, cellular $tRNA^{Lys3}$ molecules to prime cDNA synthesis, the viral envelope (Env) protein, the Gag polyprotein, and the three viral enzymes: protease (PR), reverse transcriptase (RT), and integrase (IN) [13] (Fig. 1). The viral genomic RNAs are enclosed by a viral capsid Gag protein p24, forming a core of the viral particle. The HIV matrix Gag protein (p17) is positioned between the virus core and the lipid membrane envelope. The viral envelope is composed of the lipid bilayer derived from the infected cell membrane and HIV-1 envelope glycoproteins, gp120 and gp41. The extracellular gp120 and transmembrane gp41 are bounds by non-covalent interactions and are associated as a trimer on the cell surface. The entry of HIV-1 to a target cell begins with the interaction between gp120 and the cell surface glycoprotein cluster of differentiation 4 (CD4) [14, 15], which is stabilized by one of two co-receptors, C-C chemokine receptor 5 (CCR5) and CXC chemokine receptor 4 (CXCR4) [16–20], both of which have seven transmembrane domains. The process triggers viral membrane fusion to the host cell plasma membrane [21] and releases viral

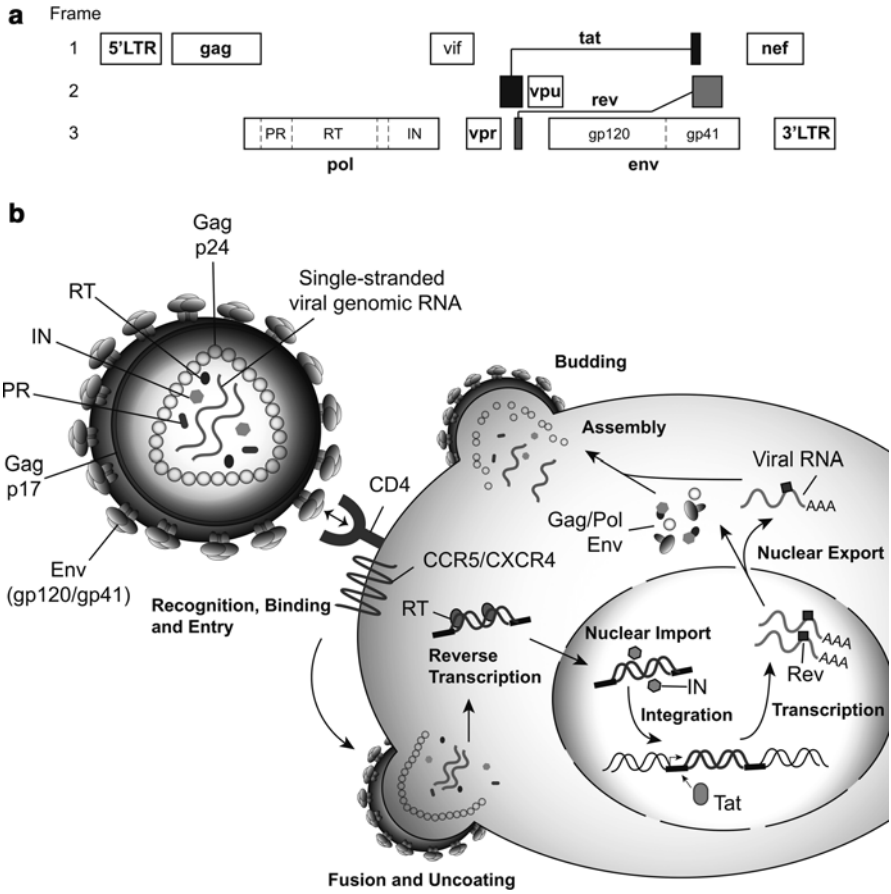

Fig. 1 (**a**) Schematic structure of the HIV-1 complete genome. HIV-1 genome encodes viral proteins that are essential for viral replication process. These viral proteins can be classified in three groups: structural proteins (*gag, pol, env*), essential regulatory elements (*tat, rev*), and accessory proteins (*nef, vpr, vif, vpu*). Multiple RNA splicing pattern enables to give nine different gene products from a less than 10 kb viral genome. (**b**) HIV-1 structure and viral replication pathway in a host cell. HIV-1 virion is a diameter of 100–120 nm spherical shape. The viral core that is surrounded by capsid protein, p24, contains two copies of viral genomic RNAs and some structural proteins such as reverse transcriptase (RT), integrase (IN) and protease (PR). The viral envelop is composed of lipid bilayer derived from a host cell and viral env glycoproteins, gp120 and gp41. HIV matrix protein, p17 lies between the viral core and the viral envelope. HIV-1 recognizes target cell through interaction between the gp120 and CD4 receptors expressed in host cell surface. This event is supported by either CCR5 or CXCR4 endogenous co-receptors. After fusion and uncoating, the viral genomic RNA undergoes reverse transcription, giving a cDNA that encodes viral genes. The viral cDNA is transported to the nucleus and integrated in the host genome by the viral IN. the viral genome is transcribed by the host RNA polymerase, which can be promoted by Tat. The viral transcripts are exported from nucleus to cytoplasm where the viral RNAs are translated. All viral components including the viral proteins and genomic RNAs are assembled to make the de novo virus which undergoes budding from the host cell

components to cellular cytoplasm. Here, viral RNAs are reverse-transcribed to generate viral DNA that is transported across the nucleus and integrated into host cell chromosomes by the IN. The binding of the cellular transcription preinitiation complex to enhancer elements in the promoter in the 5′ long terminal repeat (LTR) that induce transcription factors, such as NF-κB, Sp1, AP-1, and NFAT which activate transcription machinery and promote the RNA polymerase binding to the TATA box to initiate transcription [22–24]. The virus transcription factor, Tat, also enhances HIV-1 gene expression by binding the TAR RNA element and recruiting the positive transcription elongation factor b (P-TEFb) [25]. The transcribed viral RNA goes through processing, nuclear export and splicing regulated by viral regulatory protein Rev [26, 27]. All viral components assemble on the cell surface and form a new immature viral particle that buds off the cell, and mature through processing of HIV proteins by protease [13]. Most drugs used in cART target different steps of this viral replication cycle [28, 5]. The combination of at least three drugs makes the therapy powerful and also prevents drug resistance resulting from extremely rapid mutation of HIV-1. On the other hand, there are disadvantages and limitations associated with cART, such as potential adverse effects, its inability to eradicate the virus, frequent doses and unfavorable cost. Therefore, a new strategy for anti-HIV therapy is required and has been sought after by many researchers.

RNA interference (RNAi) [29] offers a powerful method for developing new generation of therapeutics. Small interfering RNAs (siRNAs) [30] that cause RNAi have attracted attention over the years because of their ability to inhibit expression of virtually any gene including targets that have been considered to be "undruggable" with current technologies [31]. Indeed, due to limitations and challenges of the current antiretroviral therapy, siRNAs have been evaluated as a new strategy in anti-HIV-1 therapy [32–36]. However, delivery remains a major hurdle of siRNA-based therapy [37, 38]. Recent progress in technology of engineering nucleic acid enables a targeted delivery of siRNAs using aptamers. In this chapter, we overview the current progress in anti-HIV-1 RNAi based therapy and the development of technology to deliver siRNAs using aptamers.

Targeting HIV-1 by siRNAs

RNAi Mechanism

The emergence of RNAi shed light on the challenge of overcoming the limitations of cART. RNAi is an endogenous phenomenon discovered by Fire et al. in 1998, where short double-stranded RNAs specifically regulate gene expression [29]. Soon thereafter, Elbashir et al. first reported that exogenously introduced synthetic siRNA could achieve sequence-specific gene silencing in mammalian cell lines without sequence nonspecific silencing [30]. Since then, siRNA has been recognized as a potential novel therapeutic intervention and the study of RNAi based therapy has been accelerated. Indeed, wide varieties of RNAi strategies intended for use in treatment of human disease have been introduced [39, 40].

Double-stranded stranded siRNAs, which are typically 21–23 nucleotides long, are processed by the RNA induced silencing complex (RISC), which includes a member of the Argonaute protein family as its core component [41] (Fig. 2). In RISC, one strand of siRNA (passenger strand) is cleaved and released from the complex. The other strand (guide strand) remains in RISC and recognizes its target messenger RNA (mRNA) in a sequence specific manner. RISC binding promotes Argonaute 2 cleavage of the target mRNA, usually between the nucleotides that are matched to the position of 9 and 10 of the passenger strand from 5′ end [42, 43]. Alternatively, RNAi can be induced by transfection of either plasmid or viral based vector that encodes short-hairpin RNA (shRNA), which is transcribed in the nucleus and exported by Exportin-5 to the cytoplasm. The shRNAs are then processed into double-stranded siRNAs by an RNase III-like enzyme called Dicer and loaded into the RISC.

Recent studies achieved dramatic advances in developing RNAi based therapy and clinical benefit and potential gene silencing have been shown in recent Phase I, II and III clinical studies [44]. However, despite these advantages, obstacles are also

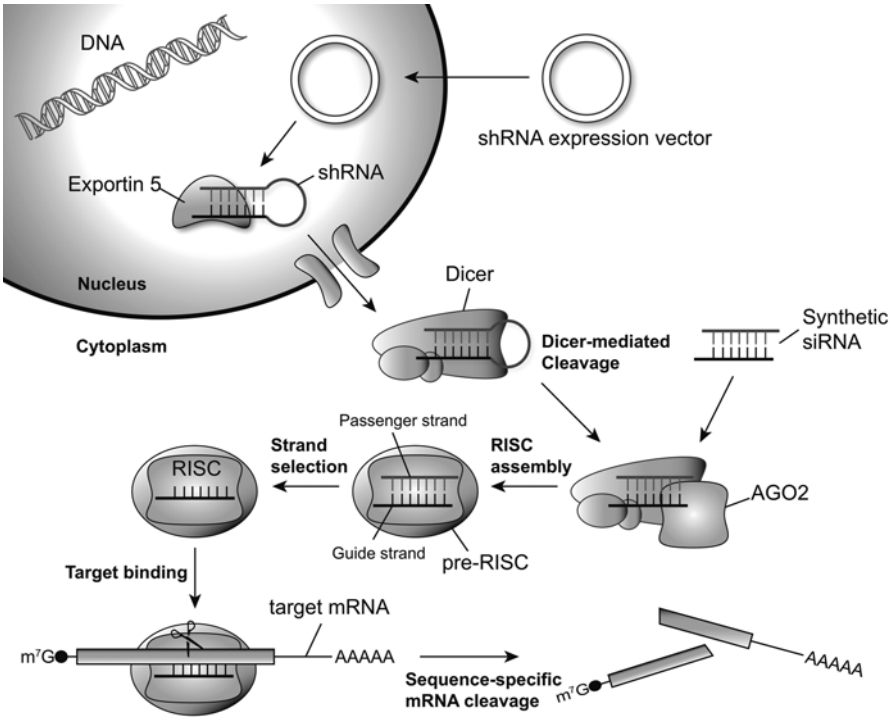

Fig. 2 siRNA/shRNA pathways of RNAi in mammals. siRNAs are provided either by the shRNA expression vectors or transcription of the synthetic siRNAs. After transcription in nucleus, shRNAs are exported to cytoplasm by exportin 5 and processed by Dicer into siRNAs. Within pre-RISC complex, an AGO2 cleaves the passenger strand of the siRNA. Then, the RISC complex containing guide strand binds and cleaves a target mRNA in sequence specific manner

revealed at the same time. For instance poor nuclease resistance in biological fluids and off-target effects have been reported. Nonspecific innate immune activation through Toll-like receptors (TLR-3, TLR-7/8) and RIG-I is also a possible undesired effect of siRNA [45]. It has been shown that not only a long double-stranded RNA (>30 nt) but also a standard 21-mer siRNA have the potential to induce severe innate immune responses in vivo. However, these can be overcome by chemical modifications on the sugar (2′-*O*-methyl, 2′-*O*-methoxyethyl, 2′-fluoro, or LNA), the phosphate backbone (phosphorothioate, boranophosphate), or the nucleobase (4-thiouracil, 2-thiouracil, diaminopurine) moiety of siRNAs [46–51]. In case of anti-HIV-1 siRNA, the emergence of resistant virus is also anticipated.

siRNAs Against HIV-1

Theoretically, RNAi can target any known mRNA sequence, assuming that the mRNA target is accessible to interact with RISC. In addition to targeting HIV genes with RNAi, host genes that play essential roles in the HIV-1 replication cycle can be potential therapeutic targets [52].

Many studies have reported that several HIV-1 gene expression and replication could be inhibited by RNAi against HIV-1 genes including *gag* [53–55], *pol* [56, 57], *rev* [58–60], *vif* [61], *nef* [62] and LTR [53, 63]. However, HIV-1 RT lacks proofreading ability and has an extremely high error rate with the rate of 10^{-4} to 10^{-5} mutations per nucleotide per cycle of replication [64]. As the RNAi mechanism requires perfect or near-perfect complementarity between the siRNA and mRNA sequences, it is likely that the virus can escape from RNAi mediated gene silencing through single or multiple mutations or deletions [65–67], resulting in the emergence of resistant virus variants, which raises a serious challenge for designing RNAi based anti-HIV-1 strategy. Targeting highly conserved sequences is one of the possible strategies and may give effective RNAi for the various mutants. A large number of conserved sequences in the HIV-1 genome have been identified for targets of RNAi and successful anti-HIV-1 RNAi effects have been observed in many studies. For example, Lee et al. tested three different viral target of RNAi: *rev* and *gag*, the sequences of which were conserved only among clade (subtype) B isolate, and *vif*, highly conserved across clades (subtypes). This study revealed successful suppression of five different viral clades when targeting a conserved *vif* sequence; on the other hand, targeting *rev* and *gag* showed inhibition of only clade B isolates [61]. Another study by ter Brake et al., in which authors have identified highly conserved sequences within HIV-1 genome and tested 86 shRNAs targeting the conserved regions [59]. As a result, they observed sequence specific inhibition of HIV-1 production with 21 of the 86 shRNAs. Furthermore, the authors showed that three different shRNAs targeting *gag* and *pol* expressed from a single lentiviral vector under U6 promoters resulted in similar levels of inhibition per shRNA compared to single shRNAs. Thus, targeting multiple conserved regions of HIV-1 gene by a combination of siRNA/shRNAs is, like cART, also a potential approach to inhibit

HIV at several stages of its replication cycle as well as to avoid drug resistance of the virus [62, 68–70, 60, 55].

Host cellular factors involved in the HIV-1 replication cycle can also be potential targets for RNAi [71]. To date, over 1,000 candidate host factors that potentially support HIV-1 replication have been identified by recent large scale siRNA and shRNA screens [52], in which many new potential cellular factors required for HIV-1 replication. Many such host factors are important in the initial phase of HIV infection, so targeting these could prevent viral entry into the cells or proviral integration of the HIV genome. To block viral fusion and infection, the CD4 receptor [72, 54, 73], the co-receptors CCR5 [74, 72, 75, 76] and CXCR4 [74, 72, 77] have been targeted in many studies. Other cellular targets for anti-HIV siRNA include transcriptional factors (e.g., NF-κB [78], cyclin T1 and cyclin-dependent kinase 9 [79]), the retrograde Golgi transport proteins (e.g., Rab-6, Vps53) [80], and transportin 3 (TNPO3) [80, 73]. However, these factors also have some important cellular functions, and one should keep in mind that targeting the cellular factors might harm host cells. Blocking CD4 may cause depression of immunity. In this regard, CCR5 is found to be a potential target because it is known that a 32-base pair homozygous deletion mutation of the CCR5 gene effectively protects cells from HIV-1 infection as in the case of the "Berlin patient" [81].

As mentioned above, targeting multiple genes simultaneously may increase the overall antiviral potency, and decrease the potential for mutational escape. Indeed, a pilot feasibility clinical study has been carried out with a combination lentiviral construct composed of three anti-HIV small RNAs (pHIV7-shI-TAR-CR5RZ) [82]. In this study, autologous CD34+ hematopoietic stem and progenitor cells (HSPCs) were transduced with the vector that encoded a shRNA against the overlapping reading frames of viral genes *tat* and *rev*, a nucleolar-localizing mimic of the viral RNA hairpin TAR that serves as a decoy that binds and sequesters the Tat protein, and a ribozyme targeting the mRNA of the endogenous CCR5 co-receptor [83]. The clinical study examined the safety and feasibility of expressing three RNA-based anti-HIV genes in autologous CD34+ HSPCs. Four patients with AIDS-related lymphoma received transplantations of both HIV-resistant and unmodified HSPCs. Two of these patients exhibited prolonged expression of the antiviral genes up to 18 and 24 months, respectively. The study showed a promise of RNA-based anti-HIV-1 therapy [82, 84].

Current siRNA Delivery Approaches for HIV-1

One of the major hurdles for RNAi based therapy is in vivo delivery to target cells/tissues. Viral vectors such as adenoviruses, adeno-associated viruses (AAV), retroviruses and lentivirus can be used for shRNA expression. However, because of undesired pharmacokinetic properties—including large molecular weight (>13 kDa), inability to cross cell membrane and lack of specific targeting—safe and efficient nonviral delivery system is required for in vivo delivery of siRNAs [44].

To date, a number of potential delivery systems have been developed including nanoparticles, lipids, peptides, polymers, antibodies and aptamers, some of which are tested in clinical trials [44]. Generally, lymphocytes and other primary blood cells are refractory to most traditional delivery methods for siRNA, making it difficult to prevent or treat HIV-1 infection. Hence, an efficient in vivo siRNA delivery system for targeting of these HIV-1 susceptible cells has to be developed for therapeutic use of anti-HIV siRNAs.

Song et al. took advantage of the nucleic acid binding properties of protamine to deliver siRNA via an antibody Fab fragment-protamine fusion protein, where a Fab antibody fragment directed against HIV-1 envelope was fused to protamine [85]. Using the siRNA-bound protein, they successfully observed efficient targeted delivery of siRNA to gp120-expressing primary T cells and inhibition of HIV-1 expression in vitro. Moreover, systemic administration in vivo resulted in specific delivery of siRNA by the fusion protein and target gene silencing in mice. This study demonstrated the possibility of targeting specific cell surface proteins for siRNA delivery. Meanwhile, Kumar et al. used a single chain antibody variable fragment to the CD7 receptor conjugated to a nucleic acid-binding nonamer arginine peptide (scFvCD7-9R) for T cell specific delivery of siRNA [86]. In vivo systemic delivery of antiviral siRNAs (combination of siRNAs against *vif*, *tat*, and CCR5) in HIV-infected NOD/SCID/IL2rγ$^{-/-}$ mice reconstituted with human lymphocytes (Hu-PBL) or CD34+ hematopoietic stem cells (Hu-HSC) using scFvCD7-9R showed suppression of HIV-1 replication and prevention of CD4+ T cell depletion. Because CD7 is expressed on the surface of the majority of human T cells and is rapidly internalized after antibody binding, it has been well studied for targeted delivery of some monoclonal antibodies to target toxins to T cell lymphomas and leukemia in clinical trials. Another study of antibody-protamine delivery has been demonstrated by Peer et al. [87], in which the fusion protein directed to the integrin lymphocyte function associated antigen-1, which is expressed on all leukocytes, efficiently delivered siRNAs and silenced the target gene in immune cells such as primary lymphocytes, monocytes and dendritic cells. Recently, this LFA-1 antibody has been utilized with stabilized nanoparticles (LFA-1 I-tsNPs) for targeted delivery of anti-HIV-1 siRNA [88]. The study demonstrated that systemic administration of the LFA-1 I-tsNPs selectively delivered siRNA to LFA-1 expressing human leukocytes including human T cells, B cells and monocytes in a humanized mouse model. Furthermore, anti-CCR5 siRNA/LFA-1 I-tsNPs treatment HIV-1 infected humanized mice resulted in the reduction in viral load and CD4 T cell depletion. The advantage of LFA-1 antibody over CD7 scFv is that LFA-1 antibody would be able to target a broad spectrum of HIV-1 susceptible cell types.

Despite these many advantages and possibilities, antibodies are often immunogenic, chemically unstable, and large size molecule that limits bioavailability, they have a high cost for manufacturing and difficult to synthesize on a large scale. These obstacles limit the therapeutic use of antibody-based delivery of siRNAs. Several strategies other than non-antibody-based siRNA delivery have also been reported. Eguchi et al. have developed a novel siRNA delivery system in which a peptide

transduction domain (PTD) from HIV-1 TAT protein is fused to a single double-stranded RNA-binding domain (DRBD), which is known to bind siRNA with high avidity (K_d ~10^9) [89]. This PTD-DRBD fusion protein was shown to deliver siRNAs efficiently to primary murine T cells and induced targeted gene silencing in vitro as well as in vivo without any cytotoxicity, off-target effect and innate immune stimulation. Recently, aptamers have emerged as excellent in vivo delivery tools due to their specificity, non-immunogenicity, and their ability to be chemically synthesized, and tolerability of chemical modifications.

Targeting HIV-1 by Aptamer–siRNA Chimera

Aptamers Overview

Among a vast number of siRNA delivery approaches that have been investigated so far, synthetic single-stranded nucleic acid ligands, aptamers, have emerged as one of the most promising tools for targeted delivery [90].

As often regarded as nucleic acid "antibodies," aptamers can recognize/bind to multiple different proteins and small-molecule targets by forming scaffolds for molecular interactions. The aptamer (from the Latin, *apto* that means "to fit") selection process was first introduced in 1990 from three separate labs, in which the technology of in vitro selection of functional nucleic acid, also known as SELEX (Systemic Evolution of Ligands by Exponential enrichment), was described [91, 92]. In the SELEX process, aptamers were selected from a random sequence oligonucleotide library, which is typically a pool of 20–40 long oligonucleotides containing 10^{13} to 10^{16} different sequences flanked by fixed regions containing binding sequences for reverse transcriptase and polymerase chain reaction (PCR) primers, a promoter sequence for T7 RNA polymerase, and restriction endonuclease sites for cloning, through cycles of sequential steps. For more information about SELEX procedure, see ref. [93]. A number of technological improvements made in the SELEX process in the past decades enabled researchers to isolate aptamers from not only a soluble and pure protein target but a complex mixture including cell-surface proteins [94] and human plasma [95]. Multiple groups have recently reported isolation of cell- and receptor-specific aptamers using living cells [96–101, 94, 102]. Moreover, Giangrande's group recently reported a novel cell based selection strategy referred as cell-internalization SELEX [103, 104], in which aptamers capable of recognizing their target in cell membrane and of undergoing cell-specific uptake can be enriched. One of the most important advantages of the cell-based SELEX is that aptamers can be selected to the native state cell-surface proteins. Consequently, the technology opened the door to exploit aptamers for targeted delivery of a variety of therapeutic agent into the cell [105]. A number of aptamers for a broad class of protein families including proteases, kinases, cell-surface receptors, and cytokines, as well as small chemical compounds, have been identified.

Like monoclonal antibodies, aptamers are potentially useful as therapeutics for multiple human diseases. As mentioned above, wild-type RNA and DNA molecules are extremely unstable in biological fluids, have poor PK properties and potency of innate immune activation. In this regard, most aptamers currently under clinical investigation are chemically modified in some manner [106]. Aptamers can be modified "in SELEX" and "post-SELEX." A variety of chemically modified nucleoside triphosphates (NTPs) have been tested and successfully incorporated in aptamers during the SELEX process including 2′-fluoro pyrimidines, 2′-amino pyrimidines, 2′-*O*-methyl nucleotides, 4′-thio pyrimidines. However, in general, wild-type polymerases such as T7 RNA polymerase and Taq DNA polymerase, which are usually used in SELEX, have very high specificity for natural NTPs. Therefore considerable efforts have been made to identify polymerases that more flexibly incorporate modified NTPs. Y639F and Y639F/H784A mutants of T7 RNA polymerase discovered by Sousa et al. are known to be able to polymerize 2′-modified NTPs including 2′-fluoro and 2′-amino pyrimidines [107–109]. The Y639F mutant is now commercially available and widely used for in vitro selection of 2′-fluoro modified aptamers. Another T7 RNA mutant, created by Chelliserrykattil and Ellington, can accept NTPs with more bulky 2′-*O*-methyl group [110]. In terms of immune recognition and nuclease resistance, 2′-*O*-methyl is an extremely desirable modification [111]. Although some challenges have been done to use 2′-*O*-methyl NTPs in SELEX, there are still some difficulties. Therefore, this modification is usually incorporated post-SELEX. Polyethylene glycol (PEG) is another type of chemical modification that is often used with aptamer and other oligonucleotides as well to improve their PK and bioavailability [112]. The presence of the modification stabilizes aptamers in biological fluid as well as improves their target affinities that could reach the K_d values of single-digit nanomolar to picomolar range. For more information about chemical modification of aptamer, see ref. [113].

In 2005, the first therapeutic aptamer, Macugen (pegaptanib) was approved by the US FDA for treatment of age related macular degeneration [112]. Also, several aptamers are currently undergoing clinical trials [114]. These clinical studies will provide more accurate information and a better understanding of the possibilities and limitations of the clinical use of the aptamers.

Aptamers for HIV-1

Aptamers that target various HIV-1 proteins have been isolated and shown to specifically bind targets and effectively suppress viral replication [115]. For example, HIV-1 RT which is a key protein that initiates viral replication process has been considered as an ideal therapeutic target for HIV/AIDS. Hence, many nucleoside and non-nucleoside RT inhibitors have been developed and are currently in widespread clinical use. HIV-1 RT can also be a potential target of aptamers. To date, a number of RNA and DNA aptamers that bind HIV-1 RT with a range of affinities and specificities have been identified [116]. Among those aptamers, pseudoknot

RNA aptamers, in which there is an intramolecular base pairing of the loop sequences of a RNA hairpin to sequences either 5′ or 3′ to that hairpin have been shown to bind the viral RT specifically with nanomolar affinity and efficiently inhibit HIV-1 replication [117–126]. HIV-1 IN is another potential target in HIV/AIDS therapeutics. It catalyzes the integration of reverse transcribed proviral DNA into host genome. Several aptamers to IN have been identified, most of which form G-quadruplex structure which consists of short and stable G-rich sequence. For instance, 93del and 112del which were derived from longer aptamer sequences originally selected as inhibitors of HIV-1 RNase H activity associated with RT showed strong inhibition on 3′-end processing activity of IN in the presence of 100 mM KCl with IC_{50} values of 42 nM and 9 nM, respectively. Furthermore, 93del and 112del inhibited HIV-1 infectivity in vitro with an IC_{50} around 20 nM [127–130]. Recently, 93del has been shown to inhibit entry and other intracellular early steps of HIV-1 replication such as RT and IN [131]. T30695 and its unmodified version, T30923 which have a repetitive motif of $d(GGGT)_4$ and form a parallel-strand G-quadruplex are also identified as potential HIV-1 IN inhibitors [132–134]. Magbanua et al. recently reported that a G-quadruplex forming 16-DNA aptamer, AD-1, which turned out to be identical to T30923, has binding affinity to interleukin-6 receptor ($K_d = 209$ nM) as well as HIV-1 IN ($K_d = 15$ nM) [135]. The authors also mentioned that an all-parallel quadruplex structure may be required for HIV-1 IN inhibition.

There are some potential aptamers available for HIV-1 EN glycoprotein gp120. In 2002, James et al. demonstrated the isolation and structural characterization of 2′-fluoro substituted RNA aptamers for gp120 of CXCR4-tropic (X4) HIV-1 strain, HXB2 ($HIV\text{-}1_{HXB2}$) [136]. The aptamers were highly specific to X4 strain. These were shown neither to neutralize the infectivity of the virus, nor to bind to the gp120 of clinically relevant CCR5-tropic (R5) HIV-1 strains. In the following study, the authors isolated 2′-fluoro substituted RNA aptamers that bind specifically to the gp120 of the R5 strain, Ba-L ($HIV\text{-}1_{Ba\text{-}L}$). These aptamers potently neutralize HIV-1 infectivity in human peripheral blood mononuclear cells (PBMC) as well as the infectivity of different subtypes of various R5 clinical isolates [137]. Subsequently, the authors identified the minimal region of the aptamer essential to bind R5 gp120 and successfully obtained a 77-nucleotide truncated aptamer, B40t77 without losing the binding affinity and viral neutralization potency [138–140]. Khati et al. predicted a three-dimensional model of B40t77 alone and in complex with gp120 by molecular modeling [141]. The B40t77-gp120 modeled structure and site-directed mutagenesis on gp120 of the predicted model indicated that B40t77 may make direct contact with at least four conserved core residues on gp120 within the CCR5 binding site. Furthermore, B40t77 was found to significantly reduce the level of binding of gp120 to monoclonal antibodies B6, B12, and B2G12, none of which have overlapped binding site with B40t77, suggesting that B40t77 may induce distant conformational changes in gp120 that disrupt its association with host cells. In further study, chemical modifications such as an inverted thymidine at the 3′-end and a dimethoxyltrityloxy-(CH2)6-SS-(CH2)6-phospho linker at the 5′-end were introduced by solid-phase synthesis on B40t77 in order to increase the stability of

the RNA to degradation by nucleases and the viral neutralization ability of modified B40t77 (UCLA1) was tested in PBMCs and blood monocyte-derived macrophages (BDMs) and found to be comparable with that of the parental aptamer [138]. The UCLA1 aptamer was further examined by Khati et al. against a large panel of HIV-1 subtype C [142]. UCLA1 showed strong neutralization of the HIV-1 isolates with IC_{50} in the nanomolar range. The aptamer was also shown to have synergistic effects with T20, a gp41 fusion inhibitor, and IgGb12 (b12), and anti-CD4 binding site monoclonal antibody.

In 2009, Zhou et al. demonstrated selection of 2′-fluoro modified RNA aptamers for R5 gp120 (HIV-$1_{Ba\text{-}L}$), resulting in isolation of two potential aptamers with high binding affinity to the R5 gp120 ptotein (K_d = 52 and 97 nM) [143]. The authors found that the isolated aptamers specifically bound and were internalized into CHO-gp160 cells that stably express the HIV gp160. Thus, the aptamers were subsequently applied to aptamer mediated siRNA delivery as described below in detail.

Aptamer–siRNA Chimeras for HIV-1

As mentioned above, HIV-1 uses host cellular factors including cell surface receptors CD4, CCR5, and CXCR4 and expresses specific components that are required for viral assembly. Thus, in theory, targeting factors that play an important role in the viral replication cycle could suppress viral replication. In this regard, many potential aptamers against HIV-1 and host factors have been introduced [116], and the availability of these aptamers encouraged researchers to develop therapeutic aptamer–siRNA chimeras for HIV-1. So far, aptamers against either HIV-1 gp120 or CD4 have been eagerly evaluated as the aptamer portion of the chimeras for the treatment or prevention of HIV-1. In this section, we overview development of these chimeras from the design and optimization to in vivo evaluation.

Anti-gp120 Aptamer–siRNA

The first gp120 aptamer–siRNA chimera consisted of the R5 neutralizing aptamer against gp120 and siRNA targeting the HIV-1 *tat/rev* common exon sequence [144] (Fig. 3). The aptamer portion and passenger-strand portion was synthesized by in vitro transcription using 2′-fluoropyrimidine triphosphates, so that the resulting RNAs were stable in cell culture and in vivo. The chimeras were constructed by annealing this aptamer–passenger strand RNAs with equimolar amounts of an unmodified guide strand RNA. This design utilized Dicer substrate 27-mer siRNA (dsiRNA), which have been shown by the same group to enhance RNAi potency and efficiency [145]. Moreover, a 4-nt (CUCU) linker was inserted between the aptamer and siRNA portions to minimize steric interference of the aptamer portion with Dicer. The authors first examined whether the gp120 aptamer–siRNA chimeras bind and were internalized by gp120 expressing cells [144]. Using flow cytometry

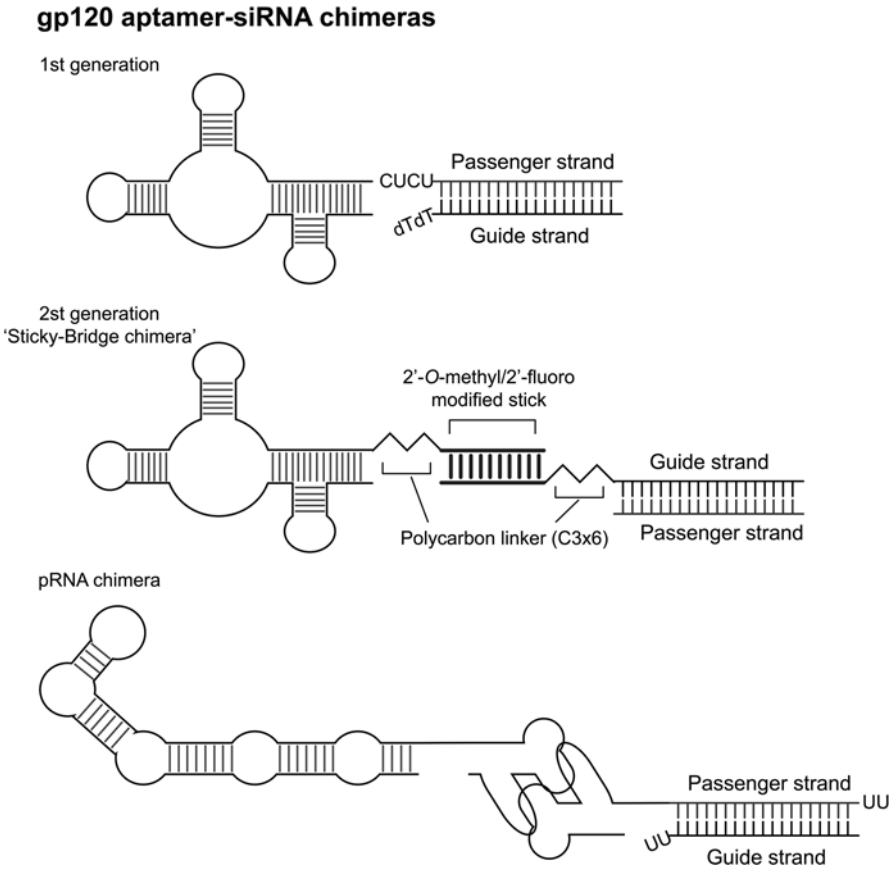

Fig. 3 Aptamer–siRNA chimera architectures

analysis, the authors observed binding of the fluorescently labeled chimeras to cells with surface expression of HIV-1 gp120 (CHO-gp160). Furthermore, selective uptake and cellular internalization of the chimeras in CHO-gp160 cells was observed in z-axis confocal microscopy and three-dimensional image reconstruction. The dsiRNA was shown to be processed by Dicer, resulting in silencing of siRNA target *tat/rev* mRNA in HIV-1 infected T cells (CEM) and inhibition of viral replication. This work first showed a dual functioning aptamer–siRNA chimera in which both the aptamer (virus neutralization) and the siRNA (viral gene silencing) have potent anti-HIV-1 activities.

In a subsequent study by the same group, the novel neutralizing 2′-fluoropyrimidine modified RNA aptamers against gp120 were isolated from a RNA library by the SELEX procedure [143]. Using these aptamers, the authors introduced a "sticky-bridge" where the aptamer and siRNA are linked via "stick" sequences consisting of 16-nt at the aptamer 3′-end, which are complementary to 16 bases on one of the

two siRNA strand. Seven three carbon atoms (C3) as linker was put between the aptamer and the stick sequence in order to provide molecular flexibility (Fig. 3). In this design, all components can be chemically synthesized, so that it suits large-scale synthesis. In addition, this "sticky-bridge" approach enables to attach any siRNA sequence to a single design of aptamer, by simply adding the 16-nt sequence to the desired siRNA. In this regard, this design is considered to be beneficial in avoiding the viral mutations.

An in vivo study of the gp120 aptamer–siRNA chimeras was reported in 2011 [146], in which the first evidence for the therapeutic efficacy of the dual-function aptamer–siRNA chimera was described. Thus, the gp120 aptamer–*tat/rev* siRNA chimera was administrated intravenously by five weekly injections of 0.38 mg/kg in HIV-1 infected humanized Rag2$^{-/-}\gamma c^{-/-}$ (RAG-hu) mice. The viral loads in treated mice suppressed to below detectable levels within a week of the last injection, and this suppression persisted throughout the treatment period. In addition, the chimera treatment prevented HIV-1-induced helper CD4+ T cell depletion, which is a major characteristic of HIV-1 infection during the acute stage of infection. The authors also confirmed siRNA internalization and target gene silencing in PBMC collected from the treated mice. Importantly, the chimera did not stimulate immune response in vivo, possibly due to 2′-fluoro chemical modifications. Thus, this first in vivo study of the dual-function aptamer–siRNA chimera demonstrated the therapeutic potency of the strategy for treatment of HIV-1 infection.

Subsequently, in vivo efficacy of their second-generation sticky-bridge chimera was evaluated [147]. In this experiment, the authors demonstrated the advantage of the sticky-bridge approach, which facilitates the interchange of different dsiRNAs with a given aptamer. Thus, three different dsiRNAs that target HIV-1 *tat/rev*, and two HIV-1 host dependency factors, CD4 and Transportin-3, respectively, were linked to the gp120 aptamer via the stick sequence. Systemic administration of the cocktail of the three sticky-bridge gp120 aptamer–siRNA chimeras in HIV-1 infected Rag-hu mice by five weekly injections of 0.38 mg/kg showed prolonged suppression of the viral load and protection from CD4+ T cell depletion, while there was no suppression of viral load in the animals treated with dsiRNAs alone. Furthermore, reduction of the three target transcripts in blood cells was observed in the treated mice. The study highlighted a power of this technology that allows in vivo delivery of multiplexes anti-HIV-1 dsiRNAs via a chemically synthesized aptamer. Overall studies by our group showed well-designed experimental approach in a drug development and significantly contribute to therapeutic advance in the aptamer–siRNA technology.

Using a unique technology called packaging RNA (pRNA) scaffold, the same authors assembled chimerical RNA nanoparticles for aptamer targeted siRNA delivery [148]. pRNA is a component of the bacteriophage phi29 DNA-packaging motor, which has been developed and manipulated to produce chimeric RNAs that form dimers via interlocking right- and left-hand loops (Fig. 3). The authors took advantages of the gp120 aptamer binding ability for HIV-1 infected cells and the ability of pRNA to form dimers to explore the potential use of chimeric pRNA aptamers for delivery of anti-HIV-1 siRNAs into HIV-1 infected cells. Thus, a

pRNA–gp120 aptamer portion and pRNA–tat/rev siRNA portion were coupled via a pRNA loop–loop interaction. The resulting pRNA chimera molecule showed a specific binding and internalization into cells expressing HIV-1 gp120. The authors also confirmed virus neutralization effect of pRNA–gp120 aptamer chimera. This study showed potential use of the gp120 aptamers in different scaffold of siRNA targeted delivery.

Recently, the same group developed another anti-HIV-1 aptamer against CCR5, which has already shown to deliver dsiRNAs via the sticky bridge technology and elicit anti-HIV-1 effect. Details of these results will be published elsewhere.

Anti-CD4 Aptamer–siRNA

One of the causes of the continued spread of the HIV-1 epidemic is sexual transmission of the virus. Topical vaginal microbicide is considered to be an effective strategy for preventing HIV-1 transmission. Many studies of development of the therapeutic aptamer–siRNA technology have aimed at treatment of established disease. Wheeler et al. recently described prophylactic approach for HIV-1 infection using the aptamer–siRNA strategy [149]. In this design, the aptamer targeted the human CD4 receptor and three different siRNAs that target HIV-1 *gag* and *vif*, or HIV-1 host dependency factors, CCR5 were conjugated to the CD4 aptamer. The resulting anti-CD4 aptamer–siRNA chimeras were designed to inhibit de novo infection of uninfected CD4+ cells (e.g., CD4+ T cells, macrophages) or to deliver the anti-HIV siRNAs to block HIV infection and replication. In course of the study, the authors observed that the cocktails of CD4 aptamer–siRNA chimeras efficiently blocked HIV-1 transmission in intact vaginal tissue. While the CD4 aptamer itself inhibited HIV transmission, the CD4 aptamer–siRNA chimeras showed about twofold to fourfold more potent inhibition than the aptamer itself, which suggested the contribution of siRNA gene silencing. When applied to humanized NOD/SCID *Il2rg*$^{-/-}$ (NSG)-BLT mice (~0.2 mg/kg), the CD4 aptamer–siRNA showed efficient protection against HIV-1 infection where none of the CD4 aptamer–siRNA chimera treated mice developed detectable viral load up to 12 weeks. In addition, no CD4+ T cell depletion was observed in the treated mice. Importantly, the CD4 aptamer–siRNA chimeras did not appear to alter CD4 cell surface expression or other immune receptors that are sensitive indicators of immune activation. Although some issues remained, such as a complicated dosing schedule and the stability of the chimera, the study introduced a new concept for a potential therapeutic application of the aptamer–siRNA chimeras.

In a subsequent study by the same group, with a clinical use in mind, the authors examined the ability of the CD4 aptamer–siRNA to protect HIV-1 transmission using a more practical dosing schedule and RNAs formulation in a gel [150]. Thus, intravaginal administration of the CD4 aptamer–siRNA chimera targeting against CCR5 or CD4 twice 24 h apart in NSG-BLT mice showed stable target gene silencing for 2 weeks. Repeated administration of drugs is often problematic in clinical practice in terms of unintended toxicity and patient compliance. Therefore, durable

efficacy has important implications in a therapeutic viewpoint. Because a liquid microbicide is not practical for clinical use, the authors used a hydroxyethyl cellulose (HEC) gel, which is an FDA-approved nonionic, nontoxic, chemically stable, water-soluble polymer to formulate the CD4 aptamer–siRNA chimeras. The HEC gel formulation can stabilize the CD4 aptamer–siRNA chimeras and enhance cellular without affecting the siRNA gene silencing efficiency. It is worth noting that intravaginal administration of the HEC gel formulated CD4 aptamer–siRNA chimeras protected 100 % treated mice from HIV-1 infection. However, while complete protection by the HEC gel formulated CD4 aptamer–siRNA chimeras could be achieved and last several weeks, it was found to be effective only when the chimeras were administrated in ~4 days before HIV-1 infection. Interestingly, no protection was observed with 4 days delayed HIV-1 infection after treatment of the unformulated CD4 aptamer–siRNA chimeras. Although there are several possible reasons why gel formulation improved protection, such as improved stability, longer interface time, more uniform coverage of the luminal surface or better toleration without inflammation of the epithelial tissue when RNAs were incorporated into a gel, the precise reason remains unknown. The overall study showed the promising approach for developing the aptamer–siRNA technology as a microbicide for protection from not only HIV-1 infection but also various sexually transmitted diseases.

For the aptamer–siRNA chimera, RNA aptamers have been used in majority of studies reported so far. On the other hand, as DNA is more chemically stable and resistant to nuclease degradation, several studies took advantage of these properties of DNA for the aptamer–siRNA delivery technology. Zhu et al. described a DNA aptamer–siRNA chimera for HIV-1 infection [151]. In this study, the authors obtained a DNA aptamer targeting CD4 through the direct conversion of a known RNA aptamer. Although DNA forms different conformation from RNA and it might lose the affinity to target molecule, and thus this does not always work, the 39-nt DNA aptamer used in this study was able to form similar secondary structure to the original RNA aptamer and it seemed to retain the affinity to the target CD4 receptor. Therefore, the CD4 DNA aptamer was then conjugated to the siRNA against HIV-1 protease. The resulting DNA aptamer chimera was tested in CD4+ T cells expressing HIV-1 protease along with the equivalent RNA aptamer–siRNA chimera, which resulted in efficient gene silencing specifically in CD4+ T cells and it appeared to be more effective than that of the RNA aptamer–siRNA chimera. This study provides the potency of the DNA aptamer–siRNA chimera for HIV-1 infection. However, more studies including efficacy in HIV-1 infected cells, toxicity, and in vivo efficacy are required for future therapeutic use of this chimera.

Conclusion and Future Perspective

In this chapter, we have overviewed therapeutic strategies against HIV-1 focusing on current development of targeted oligonucleotide based therapeutic, aptamer mediated siRNA delivery. Although a number of aptamers for HIV-1 are currently

available, not all aptamers can be used for siRNA delivery. Aptamers that can specifically bind to target of the cell surface and be internalized into the cell cytoplasm are considered to be suitable for delivery. Current progress of SELEX technology enables to identify these aptamers and some of them have successfully been applied in siRNA delivery. For HIV-1 infection, gp120 and CD4 aptamers have shown great potential as siRNA delivery vehicles. However, there are still concerns with these approaches. For example, HIV-1 gp120 is known to have highly flexible structure and undergo the large conformational change when binding to CD4. Besides, most of the gp120 protein consists of negatively charged carbohydrate and hyper-variable, flexible loops, adapted to shielding the essential receptor-binding sites from recognition by host immune system [152, 153]. Targeting a host protein that helps maintain homeostasis might cause unfavorable effects. For example, targeting CD4, which helps the T cell receptor in recognizing an antigen-presenting cell in the human immune system might perturb CD4 expression or alter other immune receptors that are sensitive indicators of immune activation. Identifying the minimal sequence and selective binding region may help to overcome these issues. Recent studies demonstrated that molecular modeling can help prediction of binding fashion between an aptamer and target protein and design highly functional aptamer molecules [141, 154].

In terms of specificity, structure simplicity and ease of synthesis, the aptamer–siRNA approach has great advantage over other delivery technologies. To bring this technology to clinical use, several challenges must be overcome; (1) large-scale GMP grade chemical synthesis of aptamers, (2) pharmacokinetics, bioavailability and biodistribution in human body, (3) stability in serum, (4) endosomal escape, and (5) nonspecific immune stimulation. Since a number of oligonucleotide therapeutics have been clinically investigated, considerable improvement regarding these issues has been made. The continued development and improvement of aptamer–siRNA chimera technology promises that potent antiviral therapeutics will become available in the near future.

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