Research News

RNA interference of HIV replication

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Double-stranded RNA-mediated interference (RNAi) induces sequence-specific post-transcriptional gene silencing and has emerged as a powerful tool to silence gene expression in multiple organisms. In mammalian cells, duplexes of 21 nucleotide RNAs, known as short-interfering RNAs (siRNAs), efficiently inhibit gene expression. Recent research demonstrates the general use of siRNAs to specifically inhibit HIV-1 replication by targeting viral or cellular genes. Importantly, RNAi opens a new avenue for gene-based therapeutics.

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Therapeutic options for combating HIV continue to expand with the development of new drugs and new strategies for their use. The management of HIV-1 infected patients has become increasingly complex. The emergence of drug resistance and the growing recognition of the long-term toxicity of anti-retroviral agents justify a continued effort to develop new antiviral strategies. Intracellular immunization against HIV aims to inhibit virus replication by introducing antiviral genes into the target cell that will then become resistant to infection. Recent findings provide evidence for RNA-mediated interference (RNAi) as a seeding strategy to activate an intracellular host defense mechanism against HIV.

RNA interference: the genome's immune system

The phenomenon of RNAi, also referred to as a mechanism of RNA-dependent gene silencing, parallels the immune system because its natural function is the protection of the genome against invasion by mobile genetic elements, such as transposons and viruses (see Forsdyke opinion in this issue). RNAi is usually described as a post-transcriptional gene-silencing (PTGS) phenomenon, in which double-stranded RNA (dsRNA) triggers degradation of homologous mRNA in the cytoplasm. Although this system was first discovered in higher plants [1,2] and later found in the nematode *Caenorhabditis elegans* [3] it was also observed subsequently in insects, fungi and vertebrates [4], suggesting an ancient evolutionary origin. RNAi has not been found in *Archea* and prokaryotes and is consequently probably an eukaryotic innovation.

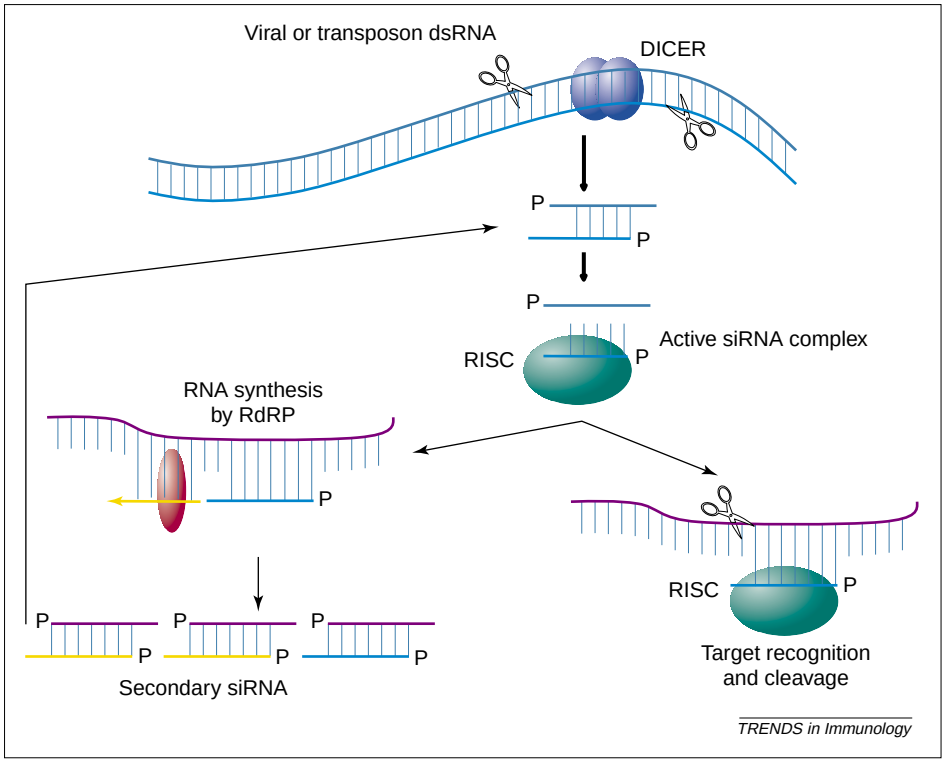

Fig. 1. Model for RNA-mediated interference (RNAi) and silencing. In the RNAi reaction, the cellular RNase III enzyme DICER cleaves the double-stranded RNA (dsRNA) silencing trigger into 21–25-nt RNAs called siRNAs (short-interfering RNA). The siRNAs are then incorporated into a multi-component nuclease, dsRNA-inducing silencing complex (RISC). The antisence siRNA pairs with its cognate mRNA, leading to degradation of target mRNA. Amplification of the silencing signal might be accomplished by siRNAs priming RNA-directed RNA polymerase (RdRP). The RdRP amplification has only been found in *Caenorhabditis elegans* and plants [29].

The relevance of RNAi as a cellular defense mechanism against intruders was demonstrated by the discovery of plant and insect viruses that encode proteins, which disable PTGS by preventing dsRNA cleavage [2,5]. Whether RNAi has a role in the protection against vertebrate viruses remains to be determined. Similarly, it is unclear if mammalian viruses have developed mechanisms to suppress RNA silencing. Nevertheless, RNAi does not only target dsRNA but also the single-stranded RNAs identical in sequence to the initiator dsRNA, suggesting that RNAi might also act in the regulation of host genes.

Mechanism of RNA interference

Current data reveal that dsRNA that is homologous in sequence to the silenced gene, serves as the initial trigger of the RNAi mechanism [3] (Fig. 1). In *Drosophila*, the initial dsRNA is processed by the DICER RNase, a member of the RNase III family of dsRNA-specific endonucleases, into short fragments of 21–25 nts (nucleotides) in length that have 2 or 3 nt 3′ overhangs [1]. These short interfering RNAs (siRNAs) are then incorporated into a dsRNA-inducing silencing complex (RISC) to guide cycles of specific RNA degradation. The RISC contains an endoribonuclease that is probably distinct from DICER [6]. This endoribonuclease uses the sequence encoded by the antisense siRNA strand to find and destroy mRNAs of complementary sequence (Fig. 1). The siRNA, thus, acts as a guide, restricting the ribonuclease to cleavage of only RNAs complementary to

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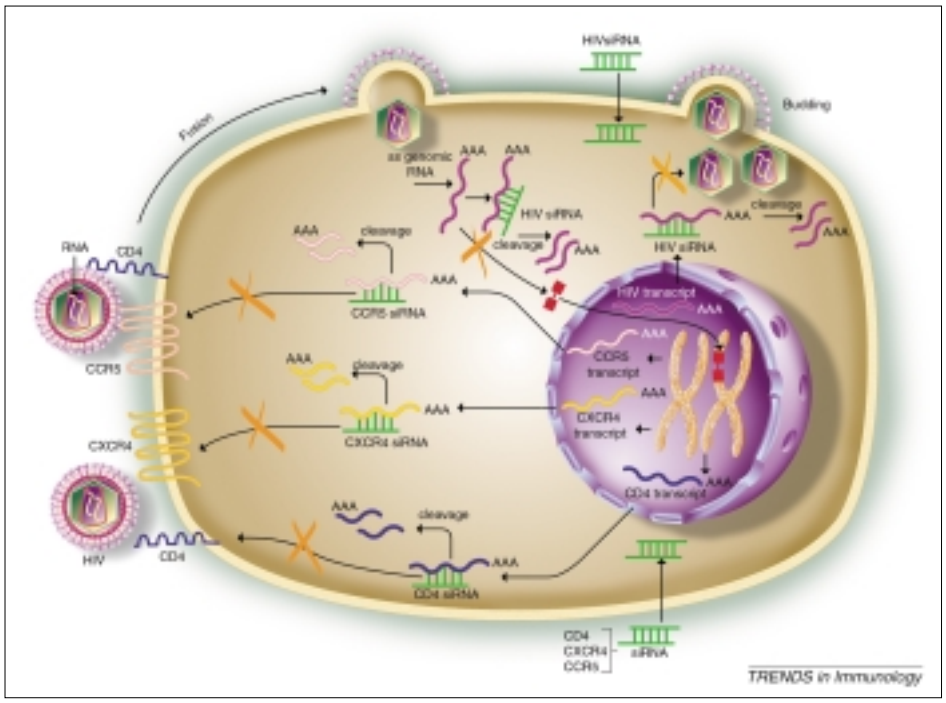

Fig. 2. Multiple sites for short-interfering RNA (siRNA) interference of HIV-1 replication. siRNA that targets HIV RNA might induce the cleavage of pre-integrated RNA or interfere with post-integration HIV-1 RNA transcripts and block progeny virus production. siRNA targeting CD4, CXCR4 or CCR5 RNA transcripts inhibit virus attachment to the CD4 receptor or chemokine receptor-mediated HIV-1 fusion and entry.

one of the siRNA strands. Therefore, the specificity of this defense mechanism is based on nucleic acid base pairing between siRNA and its target RNA. Similar to the vertebrate immune system, the RNAi machinery mounts an initial response by recognizing molecular parasites but also stabilizes and amplifies this response [3]. In *C. elegans* and plants, siRNAs can function as primers for an RNA-dependent RNA polymerase (RdRP) that synthesizes additional dsRNA, which is further processed into siRNAs [7]. Thus, cycles of dicing, new priming and amplification can occur.

The discovery that duplexes of 21 nt siRNAs can mediate RNAi in a sequence-specific manner in cultured mammalian cells has further expanded the use of RNAi. Elbashir *et al.* [8] demonstrated that transfection of synthetic 21 nt siRNA duplexes into mammalian cells inhibited endogenous genes in a sequence-specific manner. These siRNAs were too short [<30 base pairs (bps)] to trigger the nonspecific dsRNA responses, such as the RNA-dependent protein kinase (PKR) pathway, which responds to dsRNA by phosphorylating eIF-2α (initiation factor-2α) and nonspecifically arresting translation. The ability to apply RNAi in mammalian cells will undoubtedly accelerate the study of gene function in mammals and might also be used as gene-specific therapeutics.

Targeting essential genes for viral replication

Recently, four independent studies have shown the capacity of chemically synthesized siRNAs to specifically inhibit HIV-1 replication and virus production [9–12]. These studies demonstrate the anti-HIV-1 activity of various siRNAs targeted to early or late viral genes or cellular genes that are required for infection (Fig. 2). HIV is an RNA virus and requires RNA intermediates, therefore, with the demonstration that RNAi could function in mammalian cells, targeting HIV replication by RNAi at multiple steps of the HIV life cycle became the next step. Several groups [10–12] have shown that transient transfection of siRNA directed to several HIV genes (*p24*, the HIV long terminal repeat, *vif*, *nef*, *tat* and *rev*) induced pre-integrated HIV-1 RNA degradation and a consequent reduction of HIV-1 antigen production by infected cells. Novina *et al.* [10] and Jacque *et al.* [11], together with a parallel publication by Lee *et al.* [9], also demonstrated that siRNA had an effect at a later step in the HIV life cycle, namely a post-integration degradation of HIV RNA transcripts. Jaque *et al.* [11] moved on by showing that cotransfection of permissive cells with a proviral HIV clone and T7 transcripts forming a 19 bp stem of self-complementary Vif sequences suppressed virus production to a 20–30-fold increase relative to non-transfected cells [11]. Alternatively, Lee *et al.* [9] generated a mammalian Pol III promoter system capable of expressing functional double-stranded siRNAs following transfection into human cells. Cotransfection with the HIV-1 pNL4-3 proviral DNA and the *rev* siRNA-producing constructs induced a marked reduction ($\leq 4 \log_{10}$) in virus production [9]. These or alternative systems for stable expression of siRNA duplexes within cells from recombinant DNA constructs should enable long-term target-gene suppression in cells and potentially in whole organisms [13–15]. Altogether, these studies suggest that RNAi could be an interesting gene therapy approach for the treatment of HIV-1 infection.

To confirm the specificity of RNAi, Jacque *et al.* [11] evaluated the activity of siRNA that contained one or more mismatches relative to the target RNA sequence and showed that a mismatch might be sufficient to reduce the silencing effect, suggesting that activation of PKR and an unspecific antiviral response induced by short RNA was not involved in the silencing effect. Unfortunately, these results also suggest that HIV variability, through its error prone reverse transcriptase, could lead to the emergence of mutations in the gene being targeted and a rapid escape from the siRNA. Similar results have been observed with RNAi of a poliovirus infection [16]. To counteract this weakness, co-expression of multiple siRNAs that target conserved RNA sequences ould reduce the emergence of single siRNA-resistant virus with a comparable effect to that achieved by three to four anti-HIV drug combinations, commonly known as highly active anti-retroviral treatment (HAART). Delivery of combinations of multiple siRNA could induce highly active anti-retroviral gene silencing (HAAGS).

It is unclear if vertebrate viruses have developed mechanisms to suppress gene silencing, therefore, cellular genes could emerge as more attractive alternatives for targeting virus replication. Novina *et al.* [10] showed that specific siRNA for the CD4 receptor gene reduced by eightfold the cell-surface expression of CD4. This receptor was unavailable for virus attachment, and HIV entry and virus

production was also inhibited [10]. Most importantly, this work is a demonstration that cell-surface receptor genes could be targeted to block cell receptor function through RNAi. One important point that stems from their experiments is that protein half-life is an important determinant when evaluating the efficacy of gene suppression by RNAi. For instance, transfected CD4 siRNA reduced CD4 expression in ~75% of cells at day three post-transfection [10]. For *in vitro* experiments, proteins already present in the cell surface might continue to respond to stimuli or act as HIV receptors despite mRNA suppression until all the protein has been depleted from the cell surface in the absence of *de novo* protein synthesis.

In vivo suppression of genes, such as CD4, could be limited by their role in normal immune function, making HIV-1 coreceptors more attractive alternatives for targeting host proteins [10,17,18]. In particular, CCR5 might be the preferred coreceptor target because a homozygous mutation in CCR5 effectively confers protection from HIV-1 without any serious deleterious effects in immune function in humans. Indeed, we have shown that siRNAs that target chemokine receptors CXCR4 and CCR5 effectively impeded cell-surface protein expression and their consequent function as HIV-1 coreceptors in a gene specific manner [19]. Similar to that shown with the CD4 receptor, siRNA directed to HIV coreceptors blocked HIV entry, protected cells from infection and delayed virus replication.

Concluding remarks

Future developments will not be restricted to the field of HIV research. RNAi has already been evaluated as a means to block poliovirus infection [16], respiratory syncytial virus (RSV) infection [20] and human papilloma virus (HPV) [21] and could be easily extended to other human viruses [16]. Effective blockade of receptors that are constitutively expressed in the cell surface, represents a novel strategy that brings closer the possibility of generating intracellular immunization to receptor-based malignances. Good examples are chemokine receptors, which, as well as their role in HIV infection [22], are involved in a number of pathological processes, including inflammation, and allergic and immunoregulatory disorders. Overexpression of CXCR4 has been associated with a number of malignant disorders, such as metastasis of breast carcinoma cells [23], angiogenesis of normal and tumor tissue or B cell chronic lymphocytic leukaemia [24]. Potent antagonists of CXCR4 [25] are being evaluated in Phase I clinical trials for their capacity to mobilize stem cells and are thought potential candidates for anti-HIV intervention, suggesting that CXCR4 is an important therapeutic target.

The involvement of CCR5 in inflammatory processes and rheumatoid arthritis is well documented [26]. Low molecular weight compounds and novel strategies to block CCR5 coreceptor function are also being investigated [18]. Selective viral vectors containing siRNAs directed to CXCR4 and CCR5 in specifically targeted cells or after *ex vivo* manipulation of stem cells [1] could be employed to alter the role of chemokine receptors in disease. Recently, transgene expression in adult mice has been suppressed by siRNAs [27,28]. RNAi is a fast evolving field of research. Silencing of viral and cellular genes constitutes a major breakthrough with resonance beyond antiviral research.

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