Lentiviral-mediated delivery of siRNAs for antiviral therapy

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Lentiviral vectors portend a promising system to deliver antiviral genes for treating viral infections such as HIV-1 as they are capable of stably transducing both dividing and nondividing cells. Recently, small interfering RNAs (siRNAs) have been shown to be quite efficacious in silencing target genes. RNA interference is a natural mechanism, conserved in nature from Yeast to Humans, by which siRNAs operate to specifically and potently downregulate the expression of a target gene either transcriptionally (targeted to DNA) or post-transcriptionally (targeted to mRNA). The specificity and relative simplicity of siRNA design insinuate that siRNAs will prove to be favorable therapeutic agents. Since siRNAs are a small nucleic acid reagents, they are unlikely to elicit an immune response and genes encoding these siRNAs can be easily manipulated and delivered by lentiviral vectors to target cells. As such, lentiviral vectors expressing siRNAs represent a potential therapeutic approach for the treatment of viral infections such as HIV-1. This review will focus on the development, lentiviral based delivery, and the potential therapeutic use of siRNAs in treating viral infections.

Keywords: RNAi; siRNA; lentiviral vectors; HIV-1; siRNA and HIV-1

Introduction (RNAi:PTGS vs TGS)

RNA interference (RNAi) was first described in plants and is a process in which small double-stranded RNAs induce homology dependent degradation of mRNA.1-3 The small interfering double-stranded RNAs (siRNAs) 21–22 bp in length with 3' overhanging ends used in RNAi can induce a homology dependent degradation of cognate mRNA.2 The generation of siRNAs is the result of a multistep process that involves the action of an RNase III family endonuclease called Dicer4 (Figure 1). The ~22 bp siRNAs processed by Dicer provide much of the specificity in the silencing process. In human cells, following the action of Dicer, the ~22 bp siRNAs along with TAR RNA-binding protein (TRBP)5,6 are incorporated into the RNA-induced silencing complex (RISC). The RISC complex can then identify target mRNAs that contain complementary sequences to the antisense strand of the Dicer processed siRNAs (Figure 1). Once recognition of the target mRNA is initiated by RISC in the cellular P-bodies,7 Argonaute 28 mediated slicing of the target mRNA can occur (Figure 1). The requirement for double stranded siRNAs appears to be important for maximum potency, but nevertheless, it has been demonstrated that single stranded antisense siRNAs can guide target RNA cleavage with reduced efficiency.9 The specificity juxtaposed with potent suppression of target genes by siRNA has led to the adoption of RNAi as a standard methodology for silencing specific gene expression in mammalian cells. Paradoxically, the great specificity of RNAi is somewhat tempered by the reality that single base pair mismatches relative to the target mRNA on the antisense strand can significantly reduce siRNA mediated message degradation.10

RNAi can suppress gene expression via two distinct pathways: post-transcriptional (PTGS) and transcriptional (TGS) gene silencing.11,12 PTGS involves siRNAs targeted to mRNA or pre-mRNA13 whereas TGS involves siRNAs targeted to cellular promoters (i.e. PTGS = mRNA targeting, TGS = DNA targeting).14-17 TGS was only recently reported to be operable in mammalian cells and relied on the direct nuclear delivery of the siRNA. However, the strict requirements of nuclear delivery may not be necessary if temporal factors are included in the analysis.14 The observed TGS in mammalian cells appears to involve both histone and DNA methylation (reviewed in Morris et al.16 and Kawasaki et al.17) (Figure 1).

siRNA selection

The first requirement in the determination of which target sites are suitable targets for siRNA mediated silencing depend primarily on which form of RNAi is desired, that is, PTGS or TGS. Owing to the more established rules for PTGS, most investigators have chosen this methodology for silencing target genes. The first step in this process is to identify a potent siRNA/target combination. There are many commercially avail-
able reagents as well as PCR-based methodologies for use in the generation of siRNAs. Indeed the usefulness of first generating and testing siRNA on a particular target prior to construction and generation of vector systems for the delivery and expression of a particular siRNA species cannot be overstated. Specific targeting of siRNAs is extremely important as slight positional changes in the siRNA relative to the mRNA can have drastic effects on silencing. Undeniably not all siRNAs are functional and a computational design or algorithm that provides >90% successful selection of efficacious siRNAs has not yet to our knowledge been developed. Nonetheless there is a set of common rules that have begun to emerge from the plethora of studies performed so far. siRNAs in which the helix at the 5' end of the antisense strand has a lower thermodynamic stability than the 3' end are generally more effective than those with the opposite arrangement. A biochemical basis for the thermodynamic arrangement of effective siRNAs was provided by biochemical studies of the mRNA cleavage complex (RISC) in Drosophila embryo extracts, which demonstrated unequal incorporation of the two strands of the siRNA into RISC. Strand biases could be manipulated by altering the thermodynamic stability of the terminal nucleotides in a way that precisely matched the rules that were derived from empirical studies. Finally, an examination of microRNAs (miRNAs), most of which produce RISC-like complexes containing only one strand of the precursor, showed the same pattern of thermodynamic asymmetry as did effective siRNAs (reviewed in Meister and Tuschl).

Another important factor in siRNA-mediated silencing of target genes is based on cell type. Actively dividing siRNA transfected cells lose silencing over...
~96 h when they are treated with synthetic siRNAs,23,24 most likely a consequence of cell division and subsequent loss of the required template mRNA.20 In nondividing cells, siRNA silencing has been retained long-term and correlates well with the presence of the mRNA target.25 Consequently, successful targeting of a desired transcript should involve prior attempts to model the siRNA accessibility to the template mRNA, similar to approaches employed with ribozyme and antisense RNA targeting.20 Furthermore, when targeting the RNA of a virus, conserved regions should be preferentially selected, but ultimately multiplexing (siRNAs to multiple conserved regions) may be required to completely suppress the emergence of resistant viral variants. Certainly the sequence specific ability of siRNA to inhibit gene expression suggests broad applications, including targeting of viral infections such as HIV-1 and HCV. However, the sensitivity of siRNA to single base pair mismatches, coupled with extant data on the rapidity of HIV-1 to evolve drug resistance,27 might limit the usefulness of some siRNAs in targeting viral infections such as HIV-1.

Diversity of viral targets
Targeted suppression of HIV-1 has been achieved through siRNAs directed against HIV-1 tat and rev transcripts,23,28–30 reverse transcriptase,31,32 TAR and the 3′ UTR,31,33 as well as gag and the HIV-1 coreceptor CDR23 and coreceptor CCR5.32 Viruses other than HIV-1 have also been successfully targeted by siRNAs in vitro with some success including Semliki forest virus (SFV), poliovirus, dengue virus, influenza virus, and hepatitis C virus and many others (reviewed in Radhakrishnan et al.33). The fact that such a wide array of viruses can be successfully targeted by siRNAs suggests that these nucleic acid molecules can be used to target virtually any emerging or present day infectious agent. However, despite the excitement and the early proofs-of-principle in the literature, there are important issues and concerns about therapeutic applications of this technology. These concerns include difficulties with efficient delivery, uncertainty about potential toxicity, and the emergence of siRNA resistant viruses. In particular certain viruses encode proteins that block one or more steps in the RNAi pathway.34–39 Indeed resistance to siRNA occurs rather rapidly and may be contingent upon a single nucleotide substitution.40 More recently HIV-1 has been shown to elude siRNA targeting by the evolution of alternative splice variants for the siRNA targeted transcripts.41 Possible ways to circumvent resistance in therapeutic applications include; (1) designing siRNAs to best fit targets from an extensive data base of the variants in the particular target virus and/or, (2) to incorporate these best fit siRNAs into a multiple antiviral siRNA expressing transgene vector. Undeniably, the multiplexing of different siRNAs targeting different sites in the HIV-1 genome along with nonessential cellular targets such as CCR5 should be utilized to harness the full potential of this mechanism in treating HIV-1 with siRNA technology. Alternatively, siRNAs designed to more conserved regions such as to target viral intron/exon splice junctions might also prove more resistant to the emergence of viral variants.

Delivery of siRNAs to target cells
Once an siRNA or multiple siRNAs targeting viral RNAs have been designed and tested in vitro, it may prove necessary to express the siRNA from the context of the cell. There are innumerable methodologies to expressing siRNAs from the context of the cell including transient transfection of the synthesized or plasmid expressed siRNA, or stable expression of the particular siRNA by lentiviral vector delivery.42 Importantly, the expression of siRNAs from a lentiviral-based vector system can be driven by either inducible or constitutive promoters.43 Moreover, the siRNAs can be expressed either from two separate promoters (one promoter for sense and one for the antisense) or from one promoter expressing the sense a loop and the antisense, that is, a short hairpin (shRNAs, Figure 1). The two promoter based approach to expressing siRNAs could prove more useful with approaches where transcriptional gene silencing is desired as the siRNAs tend to be retained in the nucleus until the sense and antisense are paired together. Whereas the single promoter shRNA approach for expressing shRNAs is more useful when post-transcriptional targeting is desired as the shRNA is directed to Dicer via interactions in the nucleus with Drosha and/or Exportin 5 (Figure 1). Lentiviral vectors are probably the best choice currently available for delivering and stably expressing shRNAs or siRNAs in target cells. Lentiviruses, unlike retroviruses such as MoMLV, tend to integrate distally from promoters in introns, potentially limiting their overall oncogenicity.44 In addition, lentiviral based vectors are capable of transducing nondividing cells and specifically targeting the nucleus.45 Lentiviral vectors derived from HIV-1, HIV-2/SIV, or Feline Immunodeficiency Virus (FIV) are generally produced by cotransfecting the packaging constructs (gag, pol, env and required accessory protein encoding genes with the lentiviral into appropriate cells and collecting the supernatants that contain the resultant packaged vector. The lentiviral vectors are capable of stably transducing many cell types, including hematopoietic stem cells,47,48 and integrating and expressing desired transgenes.49–51 Recently, lentiviruses have been shown to cross-package one another.52–54 This observation has been carried over experimentally with HIV-1 and HIV-2 vectors being cross-packaged by FIV and capable of stably transducing and protecting human primary blood mononuclear cells from HIV-1 infection.55 The cross-packaging of lentiviral vectors such as HIV-1 with an FIV packaging system offers a unique and possibly safer method for delivering antiviral vectors to target cells in HIV-1 infected individuals. For instance, FIV packaged HIV-1 or HIV-2 vectors reduce the likelihood of immune recognition, or seroconversion, due to exposure to HIV-1 structural proteins. Finally, lentiviral vectors can be pseudotyped to target specific cell types,56,57 or alternatively designed with a receptor-ligand bridge to target specific cell types.58

Even a relatively poor siRNA can silence its target provided that sufficient quantities are delivered. However, overloading the system with a high-concentration of siRNAs is likely to lead to undesired effects including off-target suppression as well as the induction of a PKR response.59–61 Consequently, a viable basic therapeutic approach which employs an siRNA or shRNA expres-
sing lentiviral based vector to treat HIV-1 infection would most likely depend on a method where first T cells are isolated from patients, followed by transduction, expansion of the transduced cells, and re-infusion (Figure 2). A different approach is to transduce isolated hematopoietic progenitor or stem cells with lentiviral vectors harboring the therapeutic genes (siRNA or shRNA expression cassettes). These cells would then be expected to give rise to all of the hematopoietic cells capable of being infected by HIV-1. Hematopoietic stem cells are mobilized from the patients and transduced ex vivo prior to re-infusion. Two clinical trials where retroviral vectors expressing ribozymes were transduced into hematopoietic stem cells have demonstrated the feasibility of this approach. Since RNAi is more potent than ribozyme or antisense approaches, movement of this technology to a human clinical trial for HIV treatment is expected to take place within the next year or two.

In the context of an experimental setting direct delivery of particular siRNAs are required for an initial assessment of a particular siRNA’s suppressive effects, either PTGS or TGS based. Cationic lipid based complexes have proven remarkably useful for this purpose especially when determining the efficacious nature of a particular siRNA directed to a target mRNA (i.e. a PTGS based mode of suppression) (Figure 1). Importantly, peptide based siRNA delivery systems have also been shown to be important in the nuclear delivery of siRNAs designed to specifically target gene promoters. Two particular nuclear specific peptides have proven useful in nuclear delivery of siRNAs and induction of transcriptional gene silencing, MPG and NLSV404. While others have found oligofectamine to prove useful in promoter specific siRNA targeting. Indeed the initial determination of the susceptibility of a particular promoter to siRNA mediated TGS will generally rely on transient based transfection procedures.

Therapeutically, the use of stable integrating vector systems may not always prove useful as would be expected in the application of siRNAs to treat transient infections such as influenza or SARS. One alternative is the use of cationic lipid complexes to systemically or locally deliver the viral or disease specific shRNA or siRNAs to the infected individual. Systemic delivery of siRNAs has been demonstrated in mice and could be conceptualized to be a useful therapeutic strategy used to aid or augment the immune response during times of duress.

Figure 2 Lentiviral vector delivery and expression of shRNAs targeting HIV-1. Lentiviral vectors can stably transduce target cells (1) integrate and constitutively express anti-HIV-1 siRNAs or shRNAs (shRNAs are shown) (2). The anti-HIV-1 shRNAs are exported via the Exportin 5 pathway subsequently delivering the shRNA to Dicer (3) where the loop is removed by Dicer processing and the correctly processed shRNA, specifically the antisense strand loaded into RISC, where the HIV-1 mRNA is targeted and degraded (4). The lentiviral vector transduced cells can be used to either protect a cell from viral infection (A–C, depicts viral entry-integration while (D) depicts viral mRNA production from the provirus) or therapeutically to reduce overall viral burden on a cell that has already been infected.
siRNA challenges

One of the advantages of using siRNAs to treat emerging infectious agents such as viral infections is the relative ease of design, construction, and testing. The emerging field of RNAi and siRNAs in particular provide a potentially cost-effective and relatively quick methodology for treating some of the world’s most deadly emerging viral infections such as Ebola, SARS. Moreover, RNAi technology can also be used beyond the scope of human disease to treat agricultural, horticultural, and wildlife diseases. However, there are two important issues currently facing RNAi mediated technologies that must be circumvented prior to the realization of RNAi in human therapeutics. These two constraints are the avoidance of off-target effects and the delivery of the siRNA to the target cell.

Steady progress has been made with regards to gene therapy based delivery systems, specifically lentiviral based vector systems; however, delivery of therapeutic agents to target cells is still inefficient. Regarding off-target effects, the use of siRNAs to target specific cellular or viral transcripts relies essentially on hijacking the endogenous RNAi machinery, of which we know very little, that is, such as the potential for saturating the RNAi pathway. Consequently, endogenous RNAi pathways may be susceptible to high concentrations of exogenous siRNA, suggesting that it will probably be imperative to not only quantitate siRNA mediated silencing but also to monitor other genes in siRNA treated cultures for untoward off-target effects as well. Indeed a thorough understanding of the mechanism leading to nonspecific off-target effects as the result of siRNA treatment is essential before RNAi can become realized in human therapies to treat viral infections.

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