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EDITORIAL

RNA interference and antiviral therapy

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Abstract

RNA interference (RNAi) is an evolutionally conserved gene silencing mechanism present in a variety of eukaryotic species. RNAi uses short double-stranded RNA (dsRNA) to trigger degradation or translation repression of homologous RNA targets in a sequence-specific manner. This system can be induced effectively *in vitro* and *in vivo* by direct application of small interfering RNAs (siRNAs), or by expression of short hairpin RNA (shRNA) with non-viral and viral vectors. To date, RNAi has been extensively used as a novel and effective tool for functional genomic studies, and has displayed great potential in treating human diseases, including human genetic and acquired disorders such as cancer and viral infections. In the present review, we focus on the recent development in the use of RNAi in the prevention and treatment of viral infections. The mechanisms, strategies, hurdles and prospects of employing RNAi in the pharmaceutical industry are also discussed.

Key words: RNA interference; Short hairpin RNA; Micro RNA; Antiviral therapy; Viral infection; Human immunodeficiency virus; Hepatitis C virus; Hepatitis B virus; SARS-coronavirus

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INTRODUCTION

RNA interference (RNAi), a highly conserved gene silencing mechanism plays an important role in the regulation of gene expression. This system was examined in a broad variety of species including plants, fungi, yeasts, nematodes, flies and mammals. In fact, RNAi serves as a safeguard for the preservation of genomic integrity. It protects the host from viral infections and invasion by mobile genetic elements by degrading the exogenous genomic material (e.g., viral RNAs).

RNAi is triggered by small double-stranded RNA (dsRNA) and functions at all levels, including transcription[1], post-transcription[2] and translation[3]. The first reports on RNA-induced post-transcriptional gene silencing (PTGS) phenomena were published in the early 90s, when Napoli[4] and Van der Krol[5] described the co-suppression of both viral transgenes and their homologous endogenous genes in transgenic plants. Similar inactivation of gene expression called "Quelling" was observed in *Neurospora crassa* by transformation with homologous sequences[6]. In 1995, sense RNA was demonstrated to be as effective as antisense RNA in disrupting the expression of *par-1* in *Caenorhabditis elegans*[7]. The mechanism of action remained enigmatic until 1998, when Fire and Mello discovered that dsRNA, instead of the single-stranded sense or antisense RNA, mediated gene silencing by degrading endogenous mRNAs in a sequence-specific manner[8]. They also challenged a previous report published in 1995 claiming it to be an artificial effect of dsRNA contamination. Further studies have revealed that RNAi can occur at both the transcription and post-transcription levels. Transcriptional gene silencing involves histone H3 methylation and the formation of heterochromatin[9-11]. Post-transcriptional gene silencing includes small interfering RNA (siRNA) that mediates sequence-specific target RNA degradation, and micro RNA (miRNA) which promotes blockage of protein translation at the 3'-untranslated region (3'UTR)[12].

In recent years, RNAi has become a powerful tool to probe gene functions and to rationalize drug design. It has been employed as a prophylactic and therapeutic agent for combating a wide range of disorders, including infectious diseases, tumors and metabolic disorders. Several lethal viruses, including human immunodeficiency virus (HIV), the hepatitis C and B viruses (HCV & HBV), coronavirus, influenza A virus (IAV), human papillomavirus (HPV), have been shown to be inhibited or eliminated by RNAi. These findings have emphasized the potential of RNAi in clinical applications. In the present review, we discuss the mechanism of RNAi, and its role in the prevention and the treatment of viral infections.

Mechanisms of RNAi

Biochemical and genetic studies have revealed the detailed

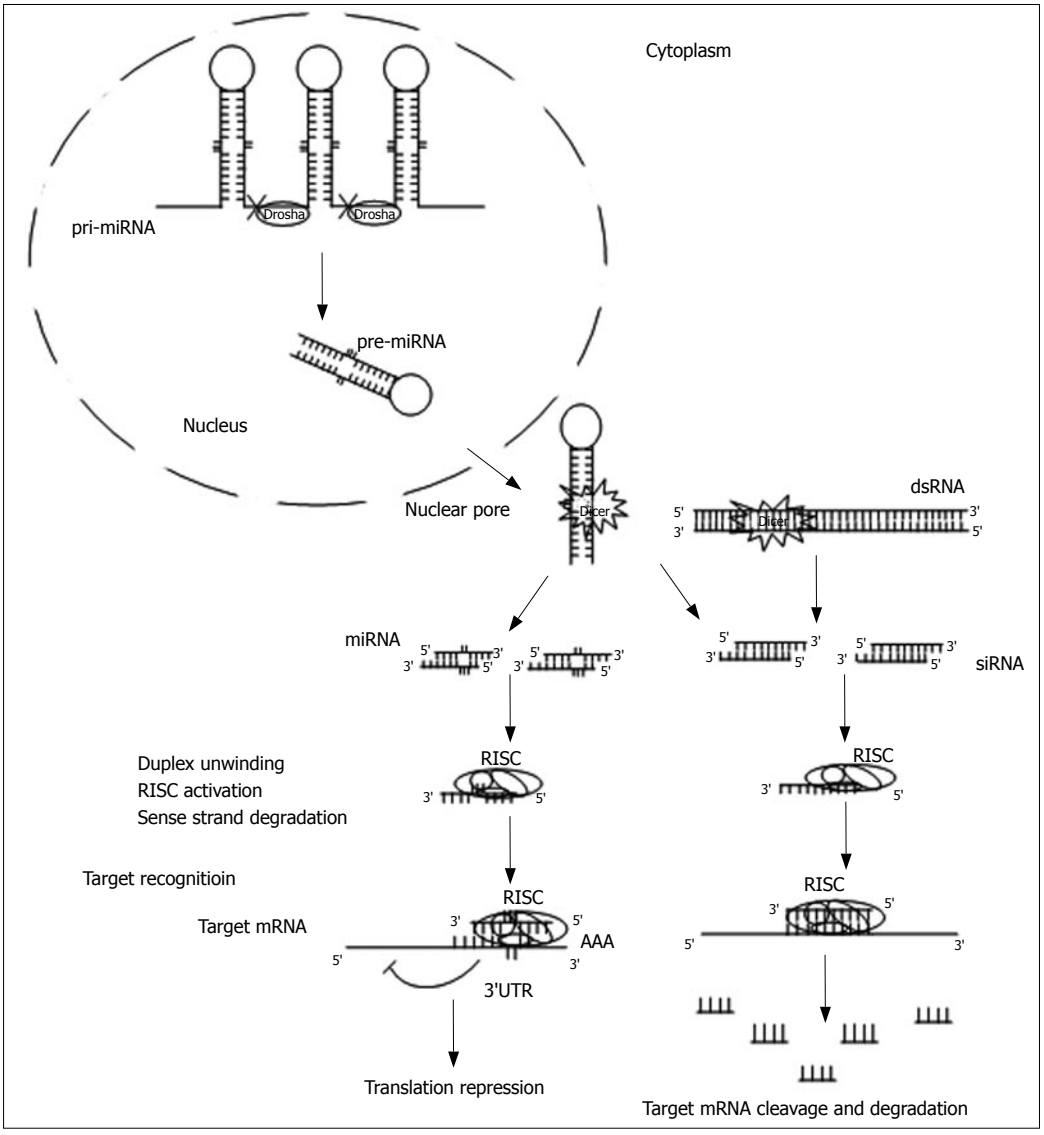

Figure 1 The RNA interference pathways.

mechanism by which dsRNA-mediated gene silencing takes place. In general, the mechanism includes two major steps: the initiator step and the effector step (Figure 1).

In the initiator step, long dsRNAs, which are produced by endogenous genes, invading viruses, transposons or experimental transgenes, are initially recognized by a dsRNA-binding protein, RDE-4/R2D2[13,14]. They are then submitted to and cleaved by the RNase Ⅲ-like nuclease Dicer[15], which generates 21-23 nucleotide duplex RNAs with overhanging 3' ends[16], called small interfering RNAs (siRNAs). The presence of highly conserved Dicer in yeast[11], plants[17,18], *C. elegans*[19], *Drosophila*[15], mice[20] and humans[21,22], suggests that the RNAi pathways share similar basic mechanisms in these organisms.

In the effector step, siRNAs are incorporated into a multicomponent nuclease complex, the RNA-induced silencing complex (RISC)[23]. The antisense strand of the duplex directs RISC to recognize and to cleave cognated target RNAs, which undergoes specific base pairing and endonucleolytic cleavage. This leads to the degradation of the unprotected and single-stranded target RNA. To date, several components of the RISC have been identified, including some conserved argonaute proteins that share the PAZ domain with Dicer family proteins[24].

The Dicer also cleaves the 60-70nt long precursor miRNA (pre-miRNAs) into miRNAs, which are of similar size as siRNAs. This pathway is referred to as miRNA-dependent gene silencing. The pre-miRNAs, whose structures are imperfectly complementary to each strand, are generated from endogenous stem loop precursors or hairpins, named primary-miRNA (pri-miRNA). The pri-miRNAs are first cleaved by Drosha RNase Ⅲ in the nucleus[25]. The resulting pre-miRNAs are then exported into the cytoplasm for further processing by Dicer. The complex of the activated RISC and miRNA binds the 3'UTR of specific mRNAs, which triggers cleavage by perfect base-pairing, or translational repression by partial base-pairing recognition[26-29].

Strategies for RNA interference

In order to study the functional genomics and biology of RNA interference, much effort has gone into the study of

artificial RNAi-inducing gene silencing. Strategies for delivery of RNAi reagents into mammalian cells can be divided into two types, the transient RNAi and the stable/inducible RNAi.

The methods commonly used in producing siRNA extraneously include chemical synthesis, *in vitro* transcription, and recombinant human Dicer/*E. coli* RNase Ⅲ digestion of long dsRNAs. These siRNAs can be transiently transfected into target cells. Alternatively, the short hairpin RNAs (shRNAs) are expressed endogenously from plasmids and viral vectors. The shRNA expression cassettes can be stably integrated into the genome of target cells, transcribed intranuclearly and processed into siRNAs by Dicer in the cytosol. In general, RNA Pol Ⅲ promoters (i.e., U6, H1 and tRNA promoters) are commonly used to drive shRNA expression in the RNAi studies. The viral vectors including retro-[30-33], lenti-[34-36], adeno- and adeno-associated viral vectors[37-39] have been demonstrated to feature high-efficiency gene delivery and can overcome the obstacles of cell-type-dependent transfection. The development of an inducible RNAi system has certainly enhanced our understanding of candidate genes' functions, as it provides an invaluable genetic switcher that allows the inducible and reversible control of specific gene's expression *in vitro*[40-42] and *in vivo*[43-45].

RNAi applications to combat viral infection

Viral infection is a serious public health, social and economic problem. More effective approaches are urgently needed to prevent viral propagation. Several studies have shown that RNAi technology has potential advantages over traditional measures such as the use of anti-viral drugs and vaccines, because of its ease of use, rapidity of action, high efficiency and specificity of activity when applied to the different stages of virus-host interactions[46]. In this section we will focus on the prospective use of RNAi in several common human pathogens such as HIV, HCV, HBV, SARS-coronavirus and influenza virus.

Human immunodeficiency virus

Human immunodeficiency virus type 1 (HIV-1) is the first primate virus shown to be inhibited by RNAi. HIV is a retrovirus that has been categorized into the subgroup of lentiviruses. Upon infection, the positive strand of the HIV's RNA genome is reversely transcripted into a linear dsDNA soon after the virus enters the host cells by receptor recognition and cell adhesion. The linear dsDNA becomes circularized, is then transported into the nucleus and integrated into the host chromosome as a provirus. By utilizing the host enzymes, HIV provirus converts viral genes into mRNAs, which are used as blueprints for the subsequent expression of viral structure proteins and enzymes. It has been suggested that the genomic RNA or the newly transcribed viral mRNAs are good targets for siRNAs intervention.

It is unclear whether RNAi can target RNA genome of HIV-1 infectious particles. Jacque reported siRNA-mediated inhibition of the early and late steps of HIV-1 replication, by targeting various regions of the HIV-1 genome and by preventing the formation of viral complementary-DNA intermediates[47]. Other workers have suggested that the incoming HIV-1 RNA genome may not be accessible to siRNAs[48,49]. To date, several viral target sequences have been identified. These include the structure proteins, Gag[48,50-52] and Env[52,53]; the reverse transcriptase Pol[48]; the regulatory proteins, Tat[54,55] and Rev[54,56], and the two accessory proteins Nef[47,57,58] and Vif[47] (Table 1). The long terminal repeats that the integrase employs to insert HIV's DNA genome into host DNA, have also been targeted[47,51].

Several studies have demonstrated that HIV may be able to escape RNAi target by mutations[58-60]. To overcome this problem, lentiviral vectors incorporated with different shRNA-expressing-cassettes, which can simultaneously target multiple sequences including conserved sequences of the HIV genome, have been constructed[61,62]. Another proposed strategy using RNAi application is the targeting of host genes. Some host genes are essential for viral replication but have a much slower mutation rate than the viral genes. These genes have been targeted by RNAi, and the results are very encouraging[63-69] (Table 1). Down-regulation of the cell surface CD4 receptor and/or one of the co-receptors CCR5 and CXCR4 by RNAi has led to dramatic reduction of viral entry into cells[34,70,71]. Compared with CD4 and CXCR4, CCR5 has been found to be a preferential target, since no immune defects or host mortality was observed on its deletion[72,73]. Therefore, careful selection of host immutable co-factors that are important for viral replication, but not for host survival, is of prime importance in the development of anti-HIV strategies. Furthermore, simultaneous targeting by RNAi of both the virus and host factors[50,74] has been shown to be more effective in inhibiting HIV-1 replication than the targeting of either virus or host factors alone.

Hepatitis C virus

Hepatitis C virus infection is a major cause of chronic liver diseases, including liver cirrhosis and hepatocellular carcinoma (HCC). The estimated number of infected individuals are about 170 million worldwide[75], which accounts for nearly 3% of the world's population. The World Health Organization (WHO) has recognized HCV infection as a global health problem.

HCV is a small, enveloped RNA virus that belongs to the Flaviviridae family. The cytoplasmic replicating virus contains a 9.6 kb RNA genome that functions as the messenger RNA and replication template. The development of anti-HCV drugs has accelerated since the replicon-based culture system was established a few years ago[76,77]. Several regions of the HCV's RNA genome, including 5'UTR and the coding sequences of Core, NS3, NS4B and NS5B, are sensitive to the action of siRNA[78-83] (Table 2). The therapeutic potential of RNAi was further emphasized by *in vivo* studies[84,85]. The administration of siRNA and shRNA to target cell surface receptor FAS[86], caspase 8[87] and NS5B[84], has resulted in the destruction of cognate mRNAs and protection of mice from liver failure. The use of multiple siRNAs against highly conserved HCV sequences with and without host cell cofactors may limit the emergence of resistant viruses as has been demonstrated in several studies[88-92] (Table 2).

Table 1 Strategies designed to inhibit HIV replication *via* RNA interference

Target gene	RNAi inducer (length)	Promoter	Vector	Cell type	Delivery methods	Inhib. of virus prod. (fold)	Reference
Viral Gene							
LTR, Vif, Nef	siRNA (21 bp)/ shRNA (19 bp)[1]	T7	Plasmid	Magi, PBLs	Transfection	> 20	[47]
Gag, Pol	siRNA (21 bp)	-	-	HOS.T4.CXCR4	Transfection	> 10	[48]
Gag, LTR	siRNA (23 bp)/ dsRNA (21nt)[2]	-	-	U87-CD4$^+$-CCR5$^+$/CXCR4$^+$, PBMC	Transfection	4	[51]
Gag, Env	dsRNA (441-531nt)[3]	-	-	COS, Hela-CD4$^+$, PBMC, ACH2	Transfection	70	[52]
Tat + Rev	siRNA (21 bp)	-	-	293T, Jurkat, PBMC	Transfection	> 15	[54]
Rev (Tat)	siRNA (21 bp)[5]	Dual U6	Plasmid	293/EcR	Transfection	10000	[56]
Nef	dsRNA (556nt)[3]	-	-	MT4-T, U937	Transfection	2.5	[57]
Env	siRNA (20 bp) /shRNA (20 bp)[4]	U6	Plasmid, Lentivirus	COS, MT-4	Transfection / Transduction	> 10	[53]
Nef	shRNA (21 bp)	H1	Retrovirus	SupT1	Transduction	> 10	[58]
Gag, Pol, Int, Vpu	shRNA (21 bp)	H1	Lentivirus	293T, Magi, GHOST hi5, CEM-A, Molt-4, PBMC	Transduction	> 20	[61]
Cellular gene							
Tsg101	siRNA (21 bp)	-	-	293T	Transfection	10-20	[63]
LEDGF/p75	siRNA (21 bp)	-	-	Hela	Transfection	NR	[64]
P-TEFb (CDK9/CyclinT1)	siRNA (21 bp)	-	-	Hela, Magi	Transfection	3-5	[65]
hRIP	siRNA (21 bp)	-	-	Hela, Jurkat, Macrophages	Transfection	-100	[66]
Emerin	siRNA (21 bp)	-	-	Hela, Macrophages	Transfection	> 10	[67]
LEDGF/p75, HRP2	siRNA (21 bp)	-	-	Hela-P4	Transfection	2-3	[68]
CXCR4	siRNA (21 bp)	-	-	HOS-CD4$^+$, HOS-CD4$^+$-CXCR4$^+$/CCR5$^+$	Transfection	3-5	[70]
Importin 7	siRNA (21 bp)	-	-	Hela, Macrophages	Transfection	-10	[69]
CXCR^{4+} CD4, CXCR^{4+} CCR5	shRNA (19/21 bp)[6]	-	-	Magi-CXCR4/CCR5, PBMC	Transfection	> 15	[71]
CCR5	shRNA (19 bp)	U6	Lentivirus	Magi-CCR5, PBLs	Transduction	3-7	[34]
Combination of viral and cellular genes							
Gag, CD4	siRNA (21 bp)	-	-	Magi-CCR5, Hela-CD4	Transfection	4-25	[50]
Tat, RT, NF-κB (p65)	siRNA (21 bp)	-	-	Magi, Jurkat	Transfection	5-500	[74]

The fold inhibition of virus production refers to the results obtained with the most potent siRNA/shRNA tested in a specific cell model. All siRNAs were prepared by chemical synthesis unless indicated otherwise. LTR: Long terminal repeat; PBLs: Peripheral blood lymphocytes; PBMC: Peripheral blood mononuclear cell; Pol: Polymerase; Env: Envelope; Tsg101: Tumor susceptibility gene 101; LEDGF/P75: Lens epithelium-derived growth factor/transcription co-activator p75; NR: Not reported; P-TEF: Positive transcription elongation factors; hRIP: Human Rev-interacting protein; HRP2: Hepatoma-derived growth factor related protein 2; RT: Reverse transcriptase; NF-κB: Nuclear factor-NF-κB. [1]shRNA expressed from a transfected plasmid under the control of a T7 promoter. [2]dsRNA produced by *in vitro* T7 promoter-mediated transcription. [3]dsRNA produced by *in vitro* SP6/T7 promoter-mediated transcription. [4]shRNA and siRNA expressed from transfected plasmids under the control of one and two U6 promoters respectively, shRNA further stably expressed from a recombinant lentiviral vector driven by a U6 promoter. [5]siRNA expressed from a transfected plasmid under the control of two U6 tandem promoters that drive the synthesis of each of the siRNA strand. [6]shRNA produced by *in vitro* T7 promoter-mediated transcription.

Hepatitis B virus

Hepatitis B virus infection is a major public health problem. It is estimated that, approximately 2 billion people are infected with HBV worldwide, and about 400 million are HBV chronic carriers[93]. HBV infection is highly prevalent in Asia and South Africa and results in over one million deaths worldwide annually.

Although the clinical symptoms caused by HBV and HCV infection are very similar, the viruses are completely unrelated[94]. HBV, the prototypical member of the Hepadnaviridae family, is one of the smallest DNA viruses (-3.2 kb), which can undergo reverse-transcription for viral replication. The HBV genome contains four overlapping open reading frames: P (polymerase-reverse transcriptase), C (core structure protein), S (surface glycoprotein) and X (HBx protein). After the uncoated nucleocapsids enter the nucleus, the HBV genome is repaired to form a covalently closed circular DNA (cccDNA), which is a template for messenger RNA transcription. The RNA intermediates-pregenomic and subgenomic RNAs, coding for viral multifunctional proteins, are transported into the cytoplasm where translation is initiated. After the pregenomic transcript is packaged into virion core particle, it is reversely transcribed by viral reverse transcriptase, thus producing a single stranded (-) DNA. Based on the structure of the (-) stranded DNA, a complementary (+) DNA strand is synthesized. Due to the lack of proofreading function of its polymerase, HBV undergoes rapid mutagenesis, with the creation of a large number of drug-resistant variants. These drug-resistant variants are further amplified under selective pressure during antiviral treatment, resulting in the elimination of the anti-viral effect and virus rebound during treatment. In severe cases, this can lead to death, even after cessation of treatment. Because of this challenge, new drugs with different targets or drug metabolism mechanisms are urgently required for better treatment outcome.

Several sites of the HBV genome including the *P, Pre*

Table 2 Strategies designed to inhibit HCV replication *via* RNA interference

Target gene	RNAi inducer (length)	Promoter	Vector	Model	Delivery methods	Inhib. of virus prod. (fold)	Reference
In vitro studies							
Viral gene							
5′-UTR	siRNA (21 bp)	-	-	5-2 cells (Huh-7)	Transfection	-6	[79]
	siRNA (21 bp)/shRNA(19 bp)[1]	U6	Plasmid	293T, Huh 7	Transfection	> 10	[78]
NS4B	siRNA (23 bp)	-	-	Huh-7.5	Transfection	-80	[80]
NS3, NS4B, NS5A, NS5B	siRNA (21 bp)	-	-	S1179I (Huh-7)	Transfection	-23	[81]
IRES, NS3, NS5B	siRNA (23bp)/shRNA (21 bp)[2]	Dual H1	Plasmid	Huh-7	Transfection	> 9	[82]
5′-UTR, C, NS4B, NS5A, NS5B	esiRNA (15-40 bp)[3]/shRNA (19bp)	H1	Mo-MuLV	Huh-7	Transfection / Transduction	-100	[88]
5′-UTR, C, NS3, NS5B	siRNA (21 bp)/shRNA (19 bp)[4]	U6	Plasmid/Lentivirus	Huh-7	Transfection / Transduction	-7	[83]
Cellular gene							
Lα, PTB, eIF2Bγ, hVAP33	shRNA (19 bp)	U6	Plasmid/Adenovirus	Huh-7	Transfection / Transduction	-13	[91]
Cyp-A,B,C	shRNA (NR)	U6	Plasmid/Retrovirus	Huh-7	Transfection / Transduction	-10	[92]
Combination of viral and cellular genes							
5′-UTR, 3′-UTR, PSMA7, HuR	shRNA (19-21 bp)	U6	Plasmid/Retrovirus	Huh-7	Transfection / Transduction	> 2	[89]
CD81, IRES, NS5B	shRNA (19-21 bp)	H1	Lentivirus	Huh-7	Transduction	> 32	[90]
In vivo studies							
NS5B	siRNA (23 bp)	-	-	Mice	Hydrodynamic transfection	3	[84]
IRES	shRNA (19-25 bp)[5]	-	-	Mice	Hydrodynamic transfection	-50	[85]

The fold inhibition of virus production represents the most potent effect caused by a specific siRNA or combinatorial siRNAs. All siRNAs were prepared by chemical synthesis unless indicated otherwise. UTR: Untranslated region; NS: Non-structural; IRES: Internal ribosomal entry site; C: Core protein; esiRNA: Endoribonuclease-prepared siRNA; Mo-MuLV: Moloney murine leukemia virus; PTB: Polypyrimidine tract-binding protein; eIF2Bγ: Subunit gamma of human eukaryotic initiation factors 2B; hVAP-33: Human VAMP-associated protein of 33 kDa; Cyp: Cyclophilin; PSMA7: Proteasome a-subunit 7; HuR: Hu antigen R; N.R: not reported. [1]stem-loop- and tandem-type siRNA expressed from DNA-based vectors driven by one and two U6 promoters respectively. [2]shRNA expressed from a transfected plasmid under the control of two H1 tandem promoters that drive the synthesis of each of the siRNA strand. [3]esiRNA generated by *in vitro* T3/T7 promoter-mediated transcription. [4]shRNA expressed from a transfected plasmid or a lentivirus vector respectively under the control of a U6 promoter. 5 shRNA generated by *in vitro* T7 promoter-mediated transcription.

C/C, PreS/S, X gene, have been employed as targets to examine the *in vitro* efficacy of RNAi[95-99] (Table 3). Some sites have also been tested in hydrodynamic HBV model and transgenic HBV model[100-104] (Table 3). Our group has successfully designed multiple shRNAs that target DR elements and regions that code for core, polymerase, PreS, S, and X proteins. These shRNA were found to potently inhibit HBV replication and showed synergistic antiviral effects with the commonly used antiviral drug, lamivudine[105]. In a recent study, we showed that simultaneous delivery of two shRNAs that target different regions, exhibited strong synergistic antiviral effects in a hydrodynamic transgenic mice model. In this study, both S and e antigens were reduced to undetectable levels, and the viral load was reduced by greater than one hundred-fold (He *et al* unpublished observations). These results clearly demonstrate the potential of RNAi application in anti-HBV therapy.

SARS-coronavirus

Severe acute respiratory syndrome (SARS) outbreak affected nearly 30 countries during the years 2002-2003. This epidemic was caused by a novel SARS-associated coronavirus (SARS-CoV)[106-108]. SARS-CoV is a large (-30 kb), enveloped, positive-stranded RNA virus and its genome is composed of replicase (*rep*), spike (*S*), envelope (*E*), membrane (*M*), and nucleocapsid (*N*) genes. The prophylactic and therapeutic efficacies of siRNAs were tested because of the absence of any effective drugs or vaccines against SARS-CoV infection. Both *in vitro* and *in vivo* applications proved satisfactory, using synthetic siRNAs as well as vector-based shRNAs against leader sequence[109,110], 3'-UTR[110], non-structural[111] and structural genes[110,112-115] of SARS-CoV (Table 4). Another recent report revealed that the siRNA-mediated depletion of the host cellular clathrin heavy chain gene, reduced the SARS-CoV infectivity[116]. Locked nucleic acid (LNA)-modified siRNAs, an RNA-like high affinity nucleotide analogue, has been found to improve the performance of gene silencing via enhancement of siRNA biostability and specialty. The improvement was clearly apparent when siRNA was transfected into Vero cells prior to a lethal SARS-CoV attack[117].

It is worth mentioning that our group was the first to demonstrate in 2003 the remarkable inhibition and replication of SARS-CoV infection by siRNAs against *rep* gene[118]. Subsequently, we designed siRNAs that could target both *rep* and structural genes. We also evaluated the antiviral effect, dose response, duration and viral kinetics of siRNAs in foetal rhesus kidney (FRhK-4) cells[119,120]. Two of the siRNAs were further evaluated for safety and antiviral efficacy in a rhesus macaque SARS model[119].

Table 3 Strategies designed to inhibit HBV replication *via* RNA interference

Target gene	RNAi inducer (length)	Promoter	Vector	Model	Delivery methods	Inhib. of virus prod. (fold)	Reference
In vitro studies							
C	siRNA (21 bp)	-	-	Huh-7, HepG2	Transfection	-4-5	[95]
	siRNA (19 bp)	-	-	HepAD38, HepAD79	Transfection	-50	[96]
C, X	shRNA (19 bp)	hH1	Plasmid	Huh-7, HepG2.2.15	Transfection	2-20	[97]
C, S, P, X, DR	shRNA (21-24 bp)	mU6	Plasmid	HepG2.2.15	Transfection	-2	[98]
	shRNA (21 bp)	hU6	Plasmid	HepG2	Transfection	> 30	[105]
S	shRNA (19 bp)	hH1	PFV, AAV	293T.HBs, HepG2.2.15	Transduction	4-9	[99]
In vivo studies							
C, S, P, X	shRNA (25 bp)	hU6	Plasmid	Immunocompetent C57BL/6J mice, Immunocompromised NOD/SCID mice	Hydrodynamic transfection[1]	3-12	[100]
C, S	siRNA (21 bp)	-	-	Male NMRI mice	High-volume injection *via* tail vein[1]	-4	[101]
S	shRNA (19 bp)	hH1, hU6	Plasmid	BALB/c mice, HBsAg-transgenic FVB/N mice	Hydrodynamic transfection[2]	-9	[102]
P, S, X	shRNA (20 bp)	hH1	Plasmid	C57BL/6 HBV-transgenic mice	Hydrodynamic transfection[1]	19-99	[103]
P, S, X	shRNA (NR)	mU6	Adenovirus	HBV-transgenic mice	Hydrodynamic transfection	> 9	[104]

The fold inhibition of virus production refers to the results obtained with the most potent siRNA/shRNA. All siRNAs were prepared by chemical synthesis unless indicated otherwise. C: Core antigen; S: Surface antigen; P: Polymerase; X: X protein; DR: Direct repeat element; PFV: Prototype foamy virus; AAV: Adeno-associated virus; mU6: Mouse U6; hU6: Human U6; hH1: Human H1. 1shRNA expression plasmid/naked siRNA coinjected with the pHBV construct. 2shRNA expression plasmid simultaneously or subsequently injected with the pHBV/pSAg construct in BALB/c mice.

Table 4 Strategies designed to inhibit SARS-CoV replication *via* RNA interference

Target gene	RNAi inducer (length)	Promoter	Vector	Model	Delivery methods	Inhib. of virus prod. (fold)	Reference
In vitro studies							
Viral gene							
Leader, TRS, 3'-UTR, S	siRNA (21 bp)	-	-	Vero E6	Transfection	9	[110]
N	shRNA (20 bp)	U6	Plasmid	293	Transfection	NR	[112]
E, M, N	siRNA (21 bp)	-	-	Vero E6	Transfection	> 4	[113]
P	shRNA (19 bp)	H1	Plasmid	Vero	Transfection	> 100	[114]
S	shRNA (22 bp)	U6	Plasmid	Vero E6, 293T	Transfection	-6	[115]
Rep	siRNA (21 bp)	-	-	FRhk-4	Transfection	> 12	[118]
Cellular gene							
CHC	siRNA (25 bp)	-	-	HepG2, COS7	Transfection	-1	[116]
In vivo studies							
S, NSP12	siRNA (21 bp)	-	-	BALB/C mouse, Rhesus macaque (*Macaca mulatta*)	*i.t.*[1] and *i.n.*[2] *administration*	3	[119]

The fold inhibition of virus production refers to the results obtained with the most potent siRNA/shRNA. All siRNAs were prepared by chemical synthesis unless indicated otherwise. TRS: Transcription-regulating sequence; UTR: Untranslated region; S: Spike protein; N: Nucleocapsid protein; NR: Not reported; E: Envelope protein; M: Membrane protein; P: RNA polymerase; Rep: Replicase; FRhk-4: Fatal Rhesus monkey kidney cells; CHC: Clathrin heavy chain; NSP: Non-structural protein; i.t.: Intratracheal; i.n.: Intranasal. [1]siRNA and target-sequence containing reporter plasmid co-administered intratracheally into mouse lungs in D5W or Infasurf solution; [2]siRNA instilled intranasally to monkey in D5W solution with different dosing regimens.

These siRNAs relieved SARS-like symptoms, and were safe for prophylaxis and therapeutic treatment. These findings greatly encouraged the clinical testing of siRNAs as an anti-SARS therapy.

Influenza virus

Influenza virus is one of the public health scourges worldwide. Three influenza epidemics have occurred in the last century and have caused tens of millions of deaths globally. Recent outbreaks of highly pathogenic avian influenza in Asia and Europe have greatly increased public awareness, and accelerated the development of measures for the prophylaxis and therapy of this infection.

Influenza viruses are enveloped, single-stranded, segmented (7-8) RNA viruses which belong to the Orthomyxoviridae family[121]. They are classified into

influenza virus types A, B, and C, based on their nucleoproteins and matrix proteins. Influenza A virus (IAV) is the most prevalent respiratory pathogen worldwide.

Since it is an RNA virus, IAV has the ability for rapid genetic changes through antigen drift[122] or antigen shift[123]. This involves the accumulation of minor mutations within the viral genome, or reassortment of RNA segments between different viruses, which results in the emergence of new viral strains. Ge *et al*[124,125] and Tompkins *et al*[126] verified the efficacy of siRNAs which specifically target the conserved regions of the influenza virus genome (nucleocapsid and acid polymerase). They confirmed that siRNAs were potent inhibitors of the influenza virus both *in vitro* and *in vivo*, and could be administered both prior to and subsequent to a lethal IAV challenge. Moreover, Ge developed an unconventional delivery system, administering small volumes of siRNAs or DNA vectors encoding shRNA in complex with polyethyleneimine (PEI) by slow intravenous infusion[127]. This system was effective in reducing virus production in infected mice and provided helpful suggestions for future clinical application of siRNAs.

Progress of RNAi for clinical application

Since RNAi was found to have antiviral activity in transgenic plants, much evidence has emerged with regard to its pivotal role in antiviral therapeutic applications. Numerous investigations have reported successful inhibition of viral replication in cultured cells and in murine/nonhuman primate models using both transient transfection of synthetic siRNA and stable expression of shRNA. To harness the full potential of RNAi for therapeutic applications, pharmaceutical companies are actively engaged in clinical trials. In 2004, Acuity Pharmaceuticals initiated a clinical trial using RNAi in the treatment of macular degeneration; encouraging results have been obtained in the Phase Ⅰ/Ⅱ studies[128]. In 2006, Alnylam Pharmaceuticals launched a Phase I clinical trial in the U.S. of an inhaled formulation of ALN-RSVO1 (an RNAi-based drug) to combat respiratory syncytial virus (RSV) infection[129]. Other potential indications for RNAi use include asthma, Huntington's disease, spinocerebellar ataxia, and HIV, HAV, HBV and influenza virus infections, and clinical trials are under consideration in many of these conditions[130].

Challenges and perspectives

Despite the rapid progress in RNAi use, its clinical application still poses several challenges. These include target specificity, biostability, biosafety, and delivery efficacy of the RNAi system in various diseases. Recent studies have indicated off-target effects associated with the use of siRNA[131-133]. In order to improve the power of gene silencing and to avoid undesirable adverse effects induced by siRNAs, such as nonspecific gene silencing and immunoactivation[134,135], great effort has been made to improve siRNA design, including its sequence[136], size[137] and structure[138]. However, the poor pharmacokinetic properties of siRNAs have added another hurdle in the development of RNAi-based therapies. Multiple chemical modifications at different positions of the siRNA duplexes, including sugars[117,139-141], backbones[142,143], and bases of oligonucleotides[144,145] have been found to prolong siRNA half-life in serum. Conjugation of one or both strands of siRNAs with lipids[146,147] and peptides[148,149], has been shown to enhance nuclease stability and improve cellular uptake.

The systematic and site-specific deliveries of siRNA also need to be addressed. Non-viral vectors, such as cationic lipids[150-152] and polymers[153-156], have been widely used for *in vitro* and *in vivo* siRNA delivery. It has been reported that siRNAs encapsulated into stable nucleic acid lipid particles (SNALPs) improve the potency, lengthen the half-life, lower the effective dose and reduce the dosing frequency. This was observed in a study comparing unformulated siRNAs in rodents challenged with replicating virus[157,158] and non-human primates[159]. Besides, Song *et al* designed a protamine-antibody fusion protein to deliver siRNA to HIV-infected or envelope-transfected cells. This study established a systemic, cell-type specific, antibody-mediated *in vivo* delivery system of siRNAs *via* cell surface receptors[160]. The current advances have brought siRNA close to the era of clinical trials and real-life therapeutic applications in infected human subjects.

However, before RNAi-based clinical trials can be carried out, the toxicity and side-effects of RNAi, and the harmful potential of viral vectors need careful attention. It has been shown that over-expression of shRNA by double-stranded AAV8 viral vectors resulted in severe hepatic toxicity and even death. Moreover, it has been observed that over-expressed shRNA can saturate the miRNA pathway[161]. Our studies have shown that simultaneous delivery of two shRNAs using a weaker expressing viral vector (AAV2) did not produce any obvious liver toxicity or side-effects (He *et al* unpublished). Therefore, it is essential to use safer vectors and in this respect we believe that inducible viral vectors may be good candidates for future clinical studies.

Scientists in different fields, including geneticists, biochemists, pharmacologists, chemists and materials scientists, have supported the use of RNAi in clinical applications. As a part of the research force, our team while being cautious, is optimistic regarding the use of RNAi in human diseases.

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