別紙4　　平成17年度研究成果の刊行に関する一覧表

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Gene Silencing of Virus Replication by RNA Interference

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Abstract Small interfering RNAs (siRNAs) are as effective as long double-stranded RNAs (dsRNAs) at targeting and silencing genes by RNA interference (RNAi). siRNAs are widely used for assessing gene function in cultured mammalian cells or early developing vertebrate embryos. They are also promising reagents for developing gene-specific therapeutics. The specific inhibition of viral replication is particularly well suited to RNAi, as several stages of the viral life cycle and many viral and cellular genes can be targeted. The future success of this approach will depend on the recent advances in siRNA-based clinical trials.

Keywords RNA interference · Gene silencing · Virus · Toll-like receptors · Virus escape

1 General Mechanism of RNAi

Eukaryotes have evolved a cellular defense system that responds to double-stranded (ds)RNAs and protects their genomes against these invading foreign elements. dsRNA delivery into cells has been used to elucidate the role of cellular genes that are homologous in sequence to the introduced dsRNAs by means of sequence-specific gene silencing (Fire et al. 1998). RNA interference (RNAi)-based reverse genetic analysis now provides a rapid link between se-

quence data and biological function. RNAi is particularly useful for the analysis of gene function in *Caenorhabditis elegans* (for reviews, see Hope 2001; Kim 2001). Effective gene silencing typically requires long dsRNAs (Parrish et al. 2000; Elbashir et al. 2001c). However, its application in vertebrates, including mammals, has proved to be difficult because of the presence of additional dsRNA-triggered pathways that mediate the non-specific suppression of gene expression (Caplen et al. 2000; Nakano et al. 2000; Oates et al. 2000; Zhao et al. 2001). These non-specific responses to long dsRNAs are not, however, triggered by small interfering RNAs (siRNAs) (Bitko and Barik 2001; Caplen et al. 2001; Elbashir et al. 2001a; Zhou et al. 2002). siRNAs can target genes as effectively as long dsRNAs (Elbashir et al. 2001c) and are widely used for assessing gene function in cultured mammalian cells or early developing vertebrate embryos (Harborth et al. 2001; Elbashir et al. 2002; Zhou et al. 2002). siRNAs are also promising reagents for developing gene-specific therapeutics (Tuschl and Borkhardt 2002). However, another major problem for using RNAi as a tool to inhibit viral replication is predicting the effectiveness of a specific siRNA. The difficulty lies in making siRNAs trigger silencing in a gene-specific manner without causing non-target-related biological effects or the emergence of escape variants by foreign siRNAs. Work over the past 2 years has allowed investigators to meet this challenge, and the siRNA approach has now been adopted as a standard methodology for sequence-specific silencing in mammalian cells. This review focuses on RNAi as it relates to mammalian systems and the application of siRNAs for targeting genes that are expressed in virus-infected cell lines.

Studies in plants and *Drosophila* have provided fundamental insights into the mechanism of RNAi, following the demonstration that RNAi was activated by dsRNAs and the suggestion that it might involve a derivative of dsRNAs (Fire et al. 1998; Fig. 1).

Biochemical characterization has shown that siRNAs are 21- to 23-nt dsRNA duplexes with symmetric 2- to 3-nt 3′ overhangs, and 5′-phosphate and 3′-hydroxyl groups (Elbashir et al. 2001b; Fig. 1). This structure is characteristic of an RNase III-like enzymatic cleavage pattern, which led to the identification of the highly conserved Dicer family of RNase III enzymes as the mediators of dsRNA cleavage (Bernstein et al. 2001; Billy et al. 2001; Ketting et al. 2001).

Extensive biochemical and genetic evidence has allowed a better understanding of how long dsRNAs trigger degradation of the target messenger RNAs (mRNAs) (Fig. 1; for recent reviews, see Sharp 2001; Hannon 2002; McManus and Sharp 2002; Zamore 2002). Several studies have shown that this process is restricted to the cytoplasm (Hutvagner and Zamore 2002; Zeng and Cullen 2002; Kawasaki and Taira 2003). In the first step, Dicer cleaves long dsRNAs to produce siRNAs, which are incorporated into a multiprotein RNA-inducing silencing complex (RISC). There is a strict requirement for the siRNAs to be 5′ phosphorylated in order to enter into the RISC (Nykanen et al. 2001; Schwarz et al. 2002). siRNAs that lack a 5′ phosphate are rapidly phosphorylated by

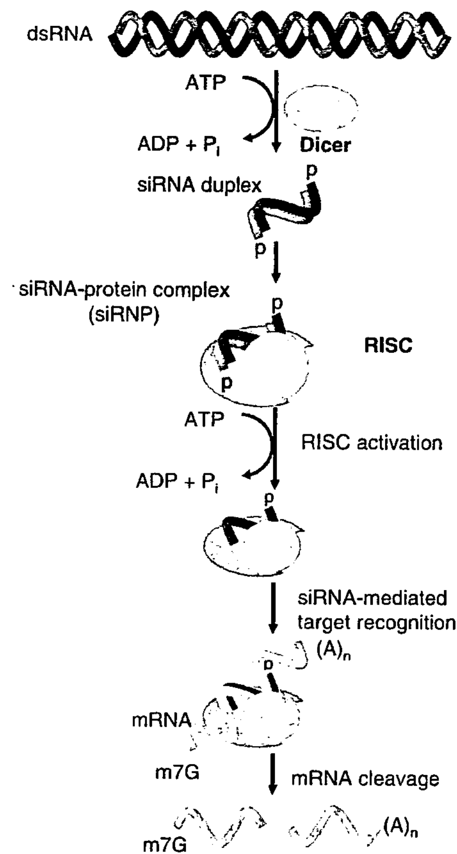

Fig. 1 Model for RNA-mediated interference and silencing. The cellular RNase III enzyme Dicer processes double-stranded RNA (*dsRNA*) to 21- to 23-nt short-interfering RNA (*siRNA*) duplexes in an ATP-dependent manner. The siRNAs are incorporated into a siRNA–ribonucleoprotein complex (*siRNP*), which uses ATP to rearrange itself into the RNA-induced silencing complex (*RISC*) by unwinding the siRNA duplex. Once unwound, the single-stranded antisense siRNA guides the RISC to mRNA with a complementary sequence, causing endonucleolytic cleavage of the target mRNA. The mRNA-cleavage products are then released and the RISC can be reactivated for another round of catalytic target RNA cleavage

an endogenous kinase (Schwarz et al. 2002). The duplex siRNA is unwound, leaving the antisense strand to guide the RISC to its homologous target mRNA for endonucleolytic cleavage. The target mRNA is cleaved at a single site in the center of the duplex region between the guide siRNA and the target mRNA, 10 nt from the 5′ end of the siRNA (Elbashir et al. 2001a,b).

Interestingly, endogenously expressed siRNAs have not been found in mammals. However, related microRNAs (miRNAs) have been cloned from various organisms and cell types (Pasquinelli 2002; Fig. 2). These short (22 nt) RNA

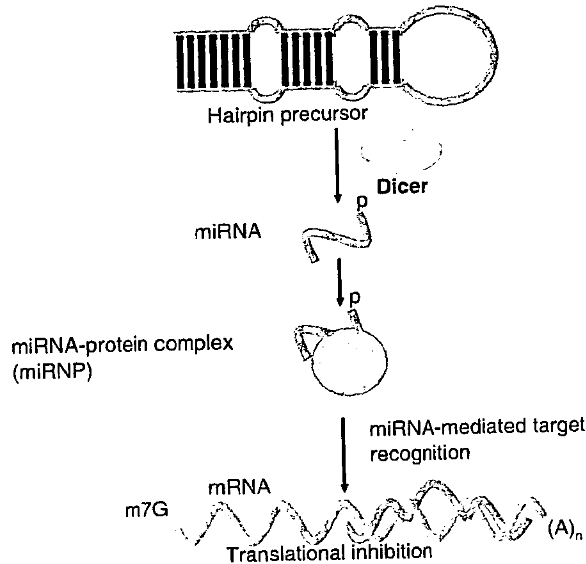

Fig. 2 MicroRNAs might use the same RNA-processing complex to direct silencing. Processing of the microRNA (*miRNA*) precursor hairpin (~70 nt) or long double-stranded RNA (dsRNA) would lead to single-stranded 21- to 23-nt RNA that is associated with the miRNA-protein complex (*miRNP*). This complex might direct either mRNA translation repression or mRNA target cleavage, depending on the degree of complementarity between the 21- to 23-nt RNA and the mRNA

species are produced by Dicer cleavage of longer (70 nt) endogenous precursors with imperfect hairpin RNA structures (Fig. 2). The miRNAs are believed to bind to sites that have partial sequence complementarity in the 3′ untranslated region (UTR) of their target mRNAs, causing the repression of translation and the inhibition of protein synthesis (Pasquinelli and Ruvken 2002). More recently, Zeng et al. (2003) demonstrated that an endogenously encoded human miRNA was able to cleave an mRNA bearing fully complementary target sites, whereas an exogenously supplied siRNA could inhibit the expression of an mRNA bearing partially complementary sequences without inducing detectable RNA cleavage. These data suggest that miRNAs and siRNAs can use similar mechanisms to repress mRNA expression, and that the choice of mechanism might be largely, or entirely, determined by the degree of complementary of the RNA target. In addition to Dicer, other PAZ/PIWI domain proteins (PPD), including eukaryotic translation-initiation factor 2C2 (eIF2C2), are likely to function in both pathways (Grishok et al. 2001; Hutvagner and Zamore 2002; Mourelatos et al. 2002).

Although the apparent lack of RNAi in mammalian cell culture was unexpected, yet RNAi has been found in mouse oocytes and early embryos (Svoboda et al. 2000; Wianny and Zernicka-Goetz 2000). Similarly, RNAi-related

transgenic-mediated co-suppression has been observed in cultured Rat-1 fibroblasts (Bahramian and Zarbl 1999). Notably, dsRNAs in the cytoplasm of mammalian cells have been reported to trigger profound physiological reactions that lead to the induction of interferon (IFN) synthesis (Lengyel 1987; Stark et al. 1998; Barber 2001). In the IFN response, dsRNAs that are longer than 30 bp bind and activate the protein kinase PKR and 2′,5′-oligoadenylate synthetase (2′,5′-AS) (Minks et al. 1979; Manche et al. 1992). Activated PKR suppresses translation by phosphorylating the translation-initiation factor eIF2α, while activated 2′,5′-AS causes mRNA degradation by stimulating RNase L. These responses are intrinsically sequence-non-specific with respect to the inducing dsRNAs.

2
Synthetic siRNAs

RNAi mediated by siRNAs is a powerful tool for dissecting gene function and drug-target validation. siRNAs can be synthesized in large quantities and thus can be used to analyze large numbers of sequences emerging from genome projects in a cost-effective manner. However, the phenomenon might reflect an incorrect sequence of RNAi, poor penetration of the mammalian cells by the nucleotides, or insufficient knowledge of the protein in question. siRNAs for gene-targeting experiments have only been introduced into cells via classic gene-transfer methods, such as liposome-mediated transfection, electroporation, and microinjection, all of which require the chemical or enzymatic synthesis of siRNAs (Donze and Picard 2002). Synthetic siRNA duplexes can be incubated with lipid formulations to generate liposomes containing siRNAs. In such formulations, cationic lipids bind to oligoribonucleotides through anion–cation and hydrophobic interactions. The efficiency of siRNA uptake is dependent upon the cell type. As high concentrations of cationic liposomes can be toxic, their application must be optimized for each type of target cell (Felgner et al. 1994). In particular, the transfection efficiency into suspension cells, such as T-cell lines and primary cells, using cationic liposomes is often less than 10%. Most of the cationic lipid reagents that are currently used for siRNAs are formulated as liposomes (lipofectamine) containing two lipid species: the polycationic lipid 2,3-diolexyoloxy-*N*-[2(spermine-carboxamido)ethyl] *N*,*N*-dimethyl-1-propanaminium trifluoroacetate (DOSPA) and the neutral lipid dioleoylphosphatidylethanolamine (DOPE) (3:1 w/w). The efficient delivery of siRNAs by the lipofectamine reagent has been reported for siRNA-mediated RNAi in cultured mammalian cells (Caplen et al. 2001; Elbashir et al. 2001a; Garrus et al. 2001; Paul et al. 2002) and siRNA-mediated anti-acquired immunodeficiency syndrome (AIDS) therapeutics (Gitlin et al. 2002; Jacque et al. 2002; Novina et al. 2002; Park et al. 2002, 2003). The further development of liposomes might enhance their ability to deliver siRNAs to a broader range of target cells.

3 DNA Vector-Mediated siRNAs or Short Hairpin RNAs

siRNA-directed silencing by transfection is limited in mammals by its transient nature. To overcome some of the shortcomings of transfecting chemically synthesized siRNAs into cells, several groups have developed DNA vector-mediated mechanisms to express substrates that can be converted into siRNAs in vivo (Kennerdell and Carthew 2000; Tavernarakis et al. 2000; Svoboda et al. 2001; Brummelkamp et al. 2002a; Lee et al. 2002; McManus et al. 2002; Miyagishi and Taira 2002; Paddison et al. 2002a,b; Paul et al. 2002; Sui et al. 2002; Yu et al. 2002; Kawasaki and Taira 2003) (Fig. 3). Alternatively, small RNA molecules might also be expressed in cells following the cloning of siRNA templates into RNA polymerase III (pol III) transcription units, which are based on the sequences of the natural transcription units of the small-nuclear RNA (snRNA) U6 or the human RNase P RNA H1 (Medina and Joshi 1999; Paule and White 2000; Myslinski et al. 2001).

Two approaches have been used to express siRNA species through constructs that are driven by RNA pol III. In the first approach, the sense and antisense strands of the siRNA duplex are expressed from different, usually tandem, promoters (Fig. 3) (Lee et al. 2002; Miyagishi and Taira, 2002; Yu et al. 2002). In vivo, these strands come together to form a 19-nt duplex with 4-nt overhangs from the pol III-termination signal. The second approach uses Dicer to express and process short hairpin (sh)RNAs into siRNAs (Fig. 1). Dicer is required for the processing of pre-*let7* RNA—which is a structured, approximately 70-nt hairpin—into the mature, 22-nt active miRNA species (Reinhart et al. 2000; Grishok et al. 2001; Hutvagner et al. 2001; Knight and Bass 2001; Hutvagner and Zamore 2002). H1 RNA–pol III-based shRNA expression vector has been used to produce hairpin RNA with a 19-nt stem and a short loop (Brummelkamp et al. 2002a; Fig. 3). This system was used to inhibit the expression of E-cadherin (CDH1) and p53 with a comparable efficiency to siRNA transfection. Using RNA based on the *let7* precursor, *luciferase* mRNA has been targeted for degradation by including a 32-nt *luciferase*-complementary sequence in the stem of the hairpin (Paddison et al. 2002b). When transfected into *Drosophila* S2 cells, they found that although *let7*-basedpre-*let7* RNA structures could target the *luciferase* mRNA, the most effective inhibitors had a simple hairpin structure with full complementarity in the stem. To express hairpin RNA in mammalian cells, they developed a U6 RNA–pol III-based expression system, which used a 29-nt sequence complementary to the luciferase gene and an 8-nt loop.

Although most expression systems use either the U6 or H1 promoter, an expression system that uses the transfer (t)RNA^{Val} promoter was described. shRNAs that have been generated using this expression system show a strong cytoplasmic localization and are efficiently processed by Dicer into siRNAs (Kawasaki and Taira 2003).

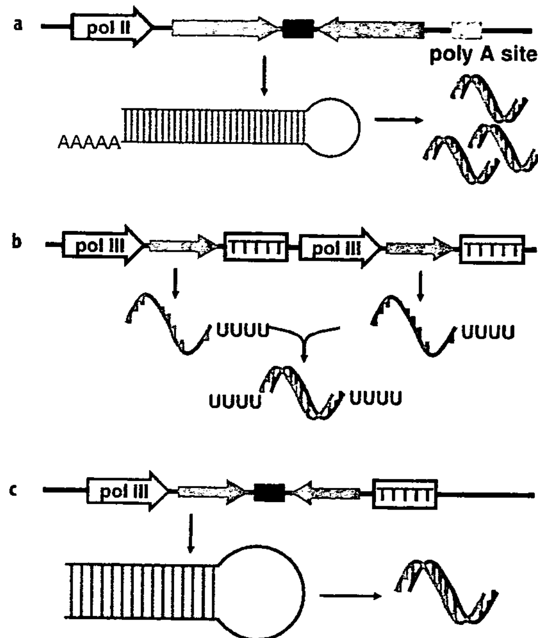

Fig. 3a–c Endogenous expression of short-interfering RNAs (siRNAs). **a** Long hairpin RNA expressed from an RNA polymerase (pol II) promoter yields a population of siRNAs with various sequence specificities. **b** An expression cassette for sense and antisense siRNAs using the tandem pol III small-nuclear RNA (snRNA) promoter. The preferred target site, which has been selected for optimal vector design, is indicated at the *bottom*. **c** A single pol III cassette for expressing hairpin RNAs that are subsequently processed to siRNAs. In this case, transcript synthesis is initiated with a +1 guanosine, and the 3′ end of the sense strand is joined by short oligonucleotide loops with the antisense strand

Some investigators have employed viral vectors in order to facilitate the introduction of siRNA-expressing cassettes into cells. Human immunodeficiency virus type-1 (HIV-1)-based lentivirus vectors have attracted particular attention in this regard. These vectors exploit the ability of HIV-1 to infect non-dividing cells (Weinberg et al. 1991; Bukrinsky et al. 1992; Lewis et al. 2002). HIV-1-based lentivirus vectors retain this central characteristic and, as such, are particularly suitable for the transduction of non-dividing cells, such as neurons and hematopoietic progenitor cells (Naldini et al. 1996).

Lentivirus vectors expressing shRNAs have been shown to promote specific gene silencing in primary dendritic cells (Stewart et al. 2003), while CD8-specific shRNAs expressed from an HIV-1-based vector were capable of silencing CD8 expression both in vitro and in vivo (Rubinson et al. 2003). Collectively, these studies illustrate the broad utility of RNAi for the silencing of viral and cellular processes in vitro and in vivo.

4 Inhibition of HIV-1 Replication by RNAi

The introduction of combination antiretroviral therapy has resulted in a remarkable improvement of the life expectancy of individuals infected with HIV and has significantly reduced their likelihood of developing AIDS. However, despite this progress, HIV infection remains incurable. Toxicity problems associated with current drug therapies and the emergence of drug resistance clearly indicate the need for alternative therapeutic approaches. Retroviral infection with HIV results in the stable integration of proviral DNA into the genome of target cells, and can therefore be viewed as an acquired genetic disease. Thus, the modulation of HIV replication by the expression of antiviral genes might be a therapeutic option for HIV infection. Baltimore (1988) was the first to suggest the concept of gene therapy as an intracellular immunization against HIV. Recently, numerous anti-HIV gene-therapy approaches have been developed and tested in clinical trials. These strategies can be divided into two main categories: first, the genetic modification of HIV target cells or their progeny in order to inhibit HIV replication and second, the genetic modification of cells in order to generate an immune response against HIV or HIV-infected cells. The latter category can be viewed as gene therapy-based immunotherapy and will not be discussed further in this review.

The inhibition of HIV replication involves the transfer of genetic material into HIV-1 target cells or their progenitors ($CD4^+$ T cells or hematopoietic stem cells). A typical gene-therapy approach for HIV-1 infection is schematically depicted in Fig. 4. HIV-1 is well suited for target RNAi because dsRNAs act at multiple steps during the HIV-1 replication cycle (Fig. 4). The inhibitory proteins that are used against HIV act intracellularly and include antibody fragments, single-chain variable fragments, transdominant negative HIV, and cellular proteins. Most of these approaches target viral RNA or proteins. Additional cellular factors that are prerequisites for HIV infection or replication are also potential targets for anti-HIV gene therapy. A number of studies have reported that the transient transfection of siRNAs directed to several HIV-1 genes (*HIV-1 LTR*, *gag*, *vif*, *nef*, *tat* and *rev*) induced pre-integrated HIV-1 RNA degradation and consequently reduced HIV-1 antigen production by infected cells (Brummelkamp et al. 2002a; Capodici et al. 2002; Coburn and Cullen 2002; Jacque et al. 2002; Lee et al. 2002; Lewis et al. 2002; Novina et al. 2002; Paul et al. 2002; Surabhi and Gaynor 2002; Yamamoto et al. 2002; Yu et al. 2002; Song et al. 2003a). Lee and colleagues, and Banerjea and co-workers, demonstrated that a psiRNA approach can be used to inhibit the expression of HIV-1 *rev* and/or *tat* transcripts in transient transfections (Lee et al. 2002) or from lentiviral-transduced hematopoietic progenitor cells (Banerjea et al. 2003). In this approach, the vectors contain two tandem human U6 snRNA promoters followed by 21-mers encoding sense and antisense siRNAs. In co-transfection experiments, psiRNAs that were co-transfected with the HIV-1 pNL4-3 provi-

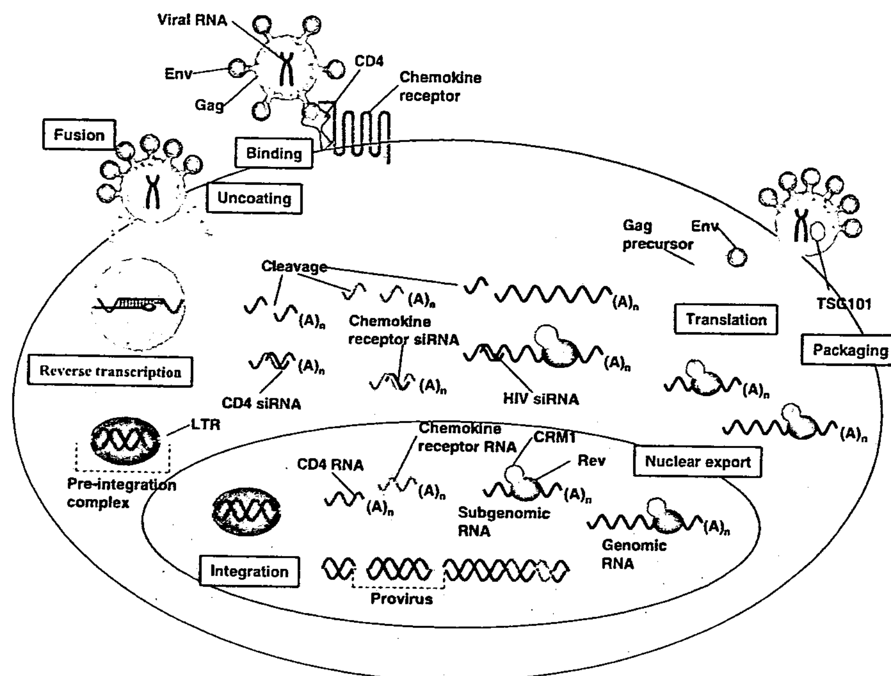

Fig. 4 RNA interference (RNAi) target sites in the human immunodeficiency virus type-1 (HIV-1) replication cycle. Short-interfering RNAs (siRNAs) that target HIV-1 RNA might induce the cleavage of pre-integrated RNA or interfere with post-integration HIV-1 RNA transcripts and block progeny virus production. siRNAs targeting CD4, CXCR4, or CCR5 RNA transcripts inhibit virus attachment to the CD4 receptor or chemokine receptor-mediated HIV-1 fusion and entry. As cleavage of the messenger RNA (mRNA) target requires a high degree of complementarity between the siRNA and its target sequence, heterogeneity in the virus population might prevent efficient silencing of some virus variants by specific siRNAs

ral DNA inhibited HIV-1 p24 antigen expression by up to 4 logs (Lee et al. 2002). This strong inhibition was achieved by simultaneously targeting two essential sites (*rev* and *tat*). Synthetic siRNAs targeted to HIV-1 *rev* and *tat* mRNAs were also shown to inhibit HIV-1 gene expression and replication in both human T cell lines and primary lymphocytes (Coburn and Cullen 2002).

Additional studies have demonstrated that siRNAs act at a later stage of the HIV-1 life cycle, causing post-integration degradation of HIV-1 RNA transcripts (Jacque et al. 2002; Lee et al. 2002; Novina et al. 2002).

The HIV-1 gag gene is expressed during the later steps of HIV-1 replication and encodes the gag-precursor protein, which is proteolytically cleaved into p24 and other polypeptides. p24 forms the HIV-1 core and functions by uncoating and packaging viral RNA. Novina et al. (2002) transfected cells with anti-gag siRNAs, exposed them to HIV-1, and observed a decrease in the in vitro production of p24. Co-transfections by Jacque et al. (2002) of a proviral

HIV-1 clone, 19-bp stem siRNAs directed against other HIV-1 genes (such as *vif* and LTR-*TAR*), and insertational mutagenesis of *nef* by a green fluorescent protein (GFP) gene, showed a significant suppression of virus production compared with non-transfected cells. Such siRNA- or shRNA-expression systems, if stable, might allow long-term target-gene suppression in cells (Naldini et al. 1996; Brummelkamp et al. 2002a; Lewis et al. 2002; Paddison et al. 2002b; Paul et al. 2002; Yu et al. 2002).

We demonstrated previously that dsRNAs specifically suppress the expression of HIV-1 genes (Park et al. 2002). In order to study dsRNA-mediated gene interference in HIV-1 infected cells, six long dsRNAs were designed to target the HIV-1 *gag* and *env* genes. HIV-1 replication was suppressed in a sequence-specific manner by these dsRNAs in infected cells. In particular, the E2 dsRNA, containing the major CD4 binding-domain sequence of gp 120 to target the HIV-1 env gene, dramatically inhibited the expression of the HIV-1 p24 antigen in peripheral blood mononuclear cells (PBMCs) for 2 weeks. More effective inhibition of HIV-1 replication was achieved using four siRNAs that were targeted to several regions of the HIV-1 env genes (Park et al. 2003). The mRNA targets for the siRNAs were selected from the middle of the *env* regions in the HIV-1 genome, as we previously showed that 531-bp (7.070–7.600) E2 dsRNAs complementary to the *env* mRNA-containing V3 loop and the major CD4 binding-domain sequence of gp 120 were more effective inhibitors than those targeted to the *gag* gene. Furthermore, the envelope protein (Env) of HIV-1 mediates functions that are critical to the viral life cycle, including viral attachment to target cells, and fusion of the viral and cellular membranes. We also showed the inhibition of HIV-1 replication in T cells using E2 shRNA directly from a lentivirus vector (Hayafune et al. 2005). On the other hand, we have shown that vif shRNA specifically suppresses the expression of HIV-1 (Barnor et al. in press). The HIV-1-encoded vif protein is essential for viral replication, virion production, and pathogenicity. HIV-1 vif interacts with the endogenous human APOBEC3G protein (an mRNA editor) in target cells to prevent its virions from encapsidation. Previous studies have established targets within the HIV-1 vif gene that are important for its biological function; however, it is important to determine effective therapeutic targets within *vif* because of its critical role in HIV-1 vif-dependent infectivity and pathogenicity. vif shRNAs increased the inhibition of HIV-1 replication in a long-term culture assay.

Rather than targeting the viral RNA, an alternative way of inhibiting virus replication by RNAi is to silence the expression of cellular genes that are critically involved in viral replication. For HIV-1, these targets include the mRNAs encoding the CD4 receptor and the CCR5 or CXCR4 co-receptors. These receptors are essential for attachment of the HIV-1 particle to the cell and for subsequent viral entry. RNAi against the viral RNA does not protect the cell against viral entry. By silencing these receptors, the HIV-1 particle will be unable to attach to, and enter, the cell, thus yielding a form of HIV-1 resistance.

Novina et al. (2002) showed that specific siRNAs that were directed against either CD4 or gag genes were able to prevent HIV-1 replication in MAGI and H9 cells. siRNA targeting rendered the receptor unavailable for virus attachment, thereby inhibiting HIV-1 entry and virus production. However, CD4 targeting might not be a feasible therapeutic approach because of its importance in immune function. By contrast, CCR5, which is the major HIV-1 co-receptor for viral entry into macrophages, might be a potentially useful cellular target, as a 32-bp homozygous deletion of the gene abolishes its function without deleterious immunological consequences and provides protection from HIV-1 infection (Martinez et al. 2002; Qin et al. 2003; Song et al. 2003a).

In this regard, RNAi is a powerful tool with which to determine the role of cellular co-factors in HIV-1 replication. Indeed, the first study to use RNAi in HIV-1 research silenced the expression of TSG-101, which is a component of the class E vacuolar protein-sorting pathway, by means of siRNAs (Garrus et al. 2001). This revealed a critical role for TSG-101 in the budding of HIV-1 virions. Moreover, when a lentivirus-based vector system was used to introduce shRNAs against CCR5 into peripheral blood T lymphocytes, the expression of CCR5 on the cell surface was reduced tenfold, resulting in a three- to sevenfold decrease in the number of infected cells (Qin et al. 2003). Lee et al. (2003) also showed inhibition of HIV-1 replication in macrophages using tat or CCR5 directly from a lentivirus vector. Similarly, siRNAs directed against CXCR4 co-receptors blocked HIV-1 entry, and protected cells from infection and delayed virus replication (Anderson et al. 2003). Another host factor that is important for HIV-1 replication is the transcription factor nuclear factor (NF)-κB to motifs in the long terminal repeat (LTR) promoter of the integrated provirus is required for viral transcription (Surabhi and Gaynor 2002). However, targeting NF-κB is not an appropriate therapeutic option, owing to the important role of NF-κB in cells.

5
siRNA Agents Work as Ligands for Toll-Like Receptors

Surprisingly, recent studies have indicated that siRNAs can induce global up-regulation of the expression of IFN-stimulated genes (Bridge et al. 2003; Jackson et al. 2003; Sledz et al. 2003; Kariko et al. 2004; Persengiev et al. 2004; Fig. 5). This effect was detected with synthetic siRNAs that were transfected into cells, and with siRNAs that were produced within cells by the expression of shRNAs. Both of these papers documented significant non-specific changes in gene expression as a consequence of the delivery of siRNAs. Sledz et al. (2003) observed a 2-fold induction of 52 out of 850 putative IFN-stimulated genes using synthetic siRNAs. By contrast, Bridge et al. (2003) observed a 50-fold induction of the IFN-stimulated gene *OAS1* with one siRNA vector alone, and a 500-fold induction when two vectors were used simultaneously. These results suggest

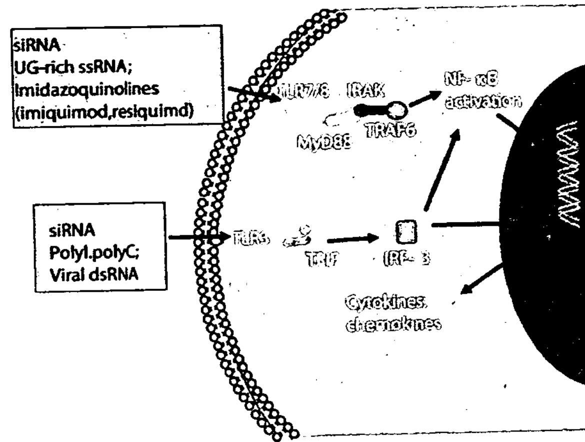

Fig. 5 Toll-like receptor (TLR) signaling pathways. TLRs recognize molecular patterns associated with bacterial pathogens, double-stranded RNA and siRNA for TLR3; siRNA, UG-rich ssRNA, imidazoquinoline and its derivatives for TLR7/8. TLR signaling pathways are separated into two groups: (1) A MyD88-dependent pathway that leads to the production of pro-inflammatory cytokines with quick activation of NF-κB and MAPK; and (2) TRIF, which may exist downstream of TLR3/4, and IKKε and TBK1 to mediate the MyD88-independent pathways leading to production of IFN-β and IFN-inducible genes

that the ability to induce the IFN system depends on both the siRNA sequence and the method of delivery. Both groups pointed out that increasing the quantity of the siRNAs enhanced the effect. Furthermore, two recent studies have indicated that the mechanism of the IFN response might include recognition of the siRNAs by Toll-like receptor 3 (TLR3) (Heidel et al. 2004; Kariko 2004). One simple method for limiting the risk of inducing an IFN response is to use the lowest effective dose of shRNA vector, as advocated by Bridge et al. (2003).

Recently, Kim et al. (2004) showed that siRNAs synthesized using the T7 RNA polymerase system can trigger the apotent induction of IFN-α and -β in a variety of cells. The mediators of this response revealed that an initiating 5′-triphosphate was required for IFN induction. These findings have led to the development of an improved method for bacteriophage polymerase-mediated siRNA synthesis that incorporates two 3′ adenosines in order to prevent base-pairing with the initiating Gs, thereby allowing RNase T1 and calf intestine alkaline phosphatase (CIP) to remove the initiating 5′ nucleotides and triphosphates of the transcripts.

6
Virus Escape from RNAi

When profound inhibition of virus replication is obtained by means of RNAi technology, the possibility of viral escape must be considered. This potential