Available online at www.sciencedirect.com
SCIENCE DIRECT®
Virus Research 102 (2004) 59–64
Virus Research
www.elsevier.com/locate/virusres

RNA interference against retroviruses

Wen-Yuan Hu, Frederic D. Bushman*, Amara C. Siva

Infectious Disease Laboratory, The Salk Institute, 10010 North Torrey Pines Rd., La Jolla, CA 92037, USA

Abstract

Bang and Ellerman, and later Peyton Rous, reported the first identification of transmissible cancer-causing agents, which later turned out to be avian retroviruses. Today avian retroviruses are important models for study of retrovirus replication and pathogenesis, and also important pathogens of domestic fowl. Here we describe the use of RNA interference (RNAi) in live chick embryos to block replication of an avian retrovirus. We also describe inhibition of ASLV and HIV replication in cell culture with RNAi.

Keywords: Retrovirus; RNA interference; siRNA; ASLV; HIV

1. Introduction

1.1. RNAi background

RNA interference (RNAi) is a process in which double-stranded RNA (dsRNA) triggers the silencing of gene expression in a sequence-specific manner. RNAi has been observed in organisms as diverse as plants, protozoa, nematodes, fungi, insects, and mammals (Caplen et al., 2000, 2001; Cogoni and Macino, 1999; Elbashir et al., 2001b; Fire et al., 1998; Hamilton and Baulcombe, 1999; Hammond et al., 2000; Jorgensen, 1990; Ngo et al., 1998; Romano and Macino, 1992). Some of the components of the RNAi machinery are also involved in the processing and function of microRNAs (miRNAs; also called small temporal RNAs), a class of small endogenous noncoding RNA molecules that carry out developmental gene control via translational repression (Doench et al., 2003; Palatnik et al., 2003; Zeng et al., 2000, 2003).

RNAi can be induced by introduction of dsRNA into cells in a variety of ways. These include endogenous synthesis of complementary RNA strands, by infection with viruses that produce dsRNA, and injection or transfection of dsRNAs. Long dsRNA are processed to small interfering RNAs (siRNAs) of about 21–23 nt by the RNase III enzyme Dicer (for recent reviews of the RNAi mechanism, see Dykxhoorn et al., 2003; Pickford and Cogoni, 2003). Alternatively, RNAi can also be induced by direct transfection of siRNAs, short RNAs mimicking the product of Dicer processing.

The siRNAs are incorporated into a multicomponent RNA induced silencing complex (RISC), and act as guides for the targeting of mRNAs for cleavage and degradation (Martinez et al., 2002a; Schwarz et al., 2002). The identities of all RISC components have not been characterized to date. At least one of the members of the Argonaute gene family is a component of this complex (for review, see Carmell et al., 2002), and a micrococcal nuclease family member has been also been identified that may be the enzyme responsible for mRNA degradation (Caudy et al., 2003).

In invertebrates, the RNA silencing machinery appears to be able to amplify the degradation signal. A strand from an siRNA bound to the target mRNA can apparently function as a primer that extends a complementary strand along the targeted mRNA by an RNA-dependent RNA polymerase (RdRP). Further processing of the dsRNA generated results in generation of new siRNAs (Lipardi et al., 2001; Plasterk, 2002; Sijen et al., 2001). However, such amplification has not been observed in mammalian cells to date.

1.2. RNAi in vertebrate cells

Prior to 2001, there appeared to be little chance that RNAi could be employed to inhibit the replication of vertebrate viruses. Vertebrate cells were known to respond to double-stranded RNA by inducing the interferon response. Cells respond to interferon combined with a double-stranded RNA inducer by shutting down translation and other

* Corresponding author.
E-mail address: bushman@salk.edu (F.D. Bushman).

0168-1702/$ – see front matter © 2004 Elsevier B.V. All rights reserved.
doi:10.1016/j.virusres.2004.01.016

responses designed to block viral replication and so block spread in the organism. However, key findings in the laboratories of Tuschl and Fire revealed that the length of the double-stranded RNA determined the nature of the response (Caplen et al., 2001; Elbashir et al., 2001a). Long dsRNA induced the interferon response, while short dsRNAs induced RNAi. This opened the door to studies of RNAi against vertebrate viruses.

RNAi has been found to act as an antiviral response in plants. Plants inhibited for the RNAi response show exacerbated symptoms upon infection with some viruses. Furthermore, several plant viruses have been shown to encode proteins that antagonize the RNAi system, providing strong evidence that RNAi is part of the normal plant response to infection (Al-Kaff et al., 1998; Marathe et al., 2000; Ratcliff et al., 1999). Recently a protein inhibiting RNAi has been detected in Flock House Virus (FHV), a nodavirus that infects insects, implicating RNAi in defense against viruses in insects as well (Li et al., 2002). The FHV B2 protein, the inhibitor of RNAi, has been found to block RNA silencing in both plant and invertebrate cells, and can functionally replace the suppressor of RNA silencing 2b protein of cucumber mosaic virus (Li et al., 2002).

Is RNAi a component of the normal vertebrate response to viral infection? This question is still unanswered. We and others have investigated this question by modeling inhibition of viral replication by RNAi using retroviral models. This review focuses on studies of avian retroviruses, for which we have an in vivo model. Many publications have also reported studies of inhibition of HIV. Below we first summarize the HIV work (see also Bushman, 2003, and the article by Lee and Rossi in this volume), then studies of RNAi against avian retroviruses. These studies show that RNAi can inhibit viral replication in vivo and in cell culture, but leave the question of its normal role unanswered. Regardless of its normal function, it seems likely that RNAi can be adapted to mitigate viral pathogenesis in vertebrates.

2. RNA interference against HIV in cell culture

Inhibition of HIV-1 replication in cell culture was first reported by Garrus et al. (2001). In this study, Tsg101, a protein involved in vacuolar protein sorting, was shown to be required for HIV-1 budding through the targeting of Tsg101 mRNA by RNAi (Garrus et al., 2001). This beautiful investigation launched an explosion of studies using RNAi to inhibit HIV replication (summarized in Table 1).

Many positions in the HIV genomic RNA have been targeted by siRNAs, including the *gag* and *pol* regions (encoding viral structural proteins), and the genes for the regulatory proteins *tat* and *rev*. Cellular factors required for viral replication in addition to the *Tsg101* coding region have also been targeted, specifically the HIV receptor *CD4* and the coreceptors *CXCR4* and *CCR5* (references in Table 1).

Several methods were used to initiate RNAi in these studies. Some of these techniques in fact were first used in the HIV model system, and each suggests a possible approach for using RNAi to treat HIV infection. Transfection of cells with chemically synthesized siRNA was first used to deliver RNAi, but disadvantages of this method include that the RNAi effect is transient, and many primary cells are not

Table 1
Studies reporting inhibition of retroviral replication by RNAi

Target gene	Means of inducing RNAi	Cell line or tissue	Reference
Viral gene			
HIV-1 Tat, Rev	siRNA transfection, siRNA electroporation	293T, Jurkat, PBMC	Coburn and Cullen (2002)
HIV-1 Vif	siRNA transfection	Magi cells	Jacque et al. (2002)
Nef, LTR TAR	T7-shRNA (vector) transfection	PBL	
HIV-1 Gag, Pol	siRNA transfection	HOS	Hu et al. (2002)
HIV-1 Gag, LTR	siRNA (synthetic and transcribed from T7 in vitro) transfection	293T, U87, PBMC	Capodici et al. (2002)
HIV-1 Rev, Tat	siRNA transfection, tandem U6-siRNA (vector) transfection	293/EcR	Lee et al. (2002)
HIV-1 Rev	Tandem U6-siRNA (vector) transfection	$CD34^+$ hematopoetic progenitor cells	Banerjea et al. (2003)
HIV-1 Gag, Env	Long dsRNA transfection	COS, HeLa, PBMC	Park et al. (2002)
HIV-1 Tat, RT, human NF-κB p65 subunit	siRNA transfection	Magi cells, Jurkat cells	Surabhi and Gaynor (2002)
HIV-1 Gag, human CD4	siRNA transfection	Magi cells	Novina et al. (2002)
ASLV Gag	siRNA transfection, siRNA in ovo electroporation	DF1, chicken embryo	Hu et al. (2002)
Host genes			
Human TSG101	siRNA transfection	293T	Garrus et al. (2001)
Human CCR5	U6-shRNA on lentiviral vector transduction	PBMC	Li et al. (2003)
Human CCR5	U6-shRNA on lentiviral vector transduction	Magi cells, PBL	Qin et al. (2003)
Human CXCR4, CCR5	siRNA transfection	U87, HeLa	Martinez et al. (2002b)

readily transfected. RNAi can also be delivered via a DNA vector encoding a short hairpin RNA expressed under the control of an RNA Pol III promoter (e.g. H1 and U6) or RNA Pol II promoter (e.g. CMV). Use of viral vectors has also been reported, including vectors based on adeno-associated virus, retroviruses, and lentiviruses. These three viruses have the advantage of integrating the RNAi-initiating construct in the host genome, thereby allowing inhibition to persist during cell division.

3. RNA interference against avian retroviruses in cell culture

3.1. ASLV history

Ellerman and Bang (1908) demonstrated that a filterable agent was responsible for causing chicken leukosis, a type of leukemia/lymphoma. The cell-free transmission of solid tumors in chickens was reported by Rous (1911) at the Rockefeller Institute in New York. It was subsequently observed that inoculation of RSV onto the chorioallantonic membrane of the chick embryo resulted in the appearance of individual small tumors whose numbers could be correlated with viral concentrations (Keogh, 1938). Temin and Rubin (1958) demonstrated the oncogenic transformation of chick embryo fibroblasts in culture by Rous sarcoma virus (RSV), and that single viral particles induced discrete foci of transformed cells. Temin later used an avian retrovirus in his demonstration that retroviral particles contain reverse transcriptase activity (Temin and Mizutani, 1970). Studies of the RSV also led to the identification of cellular homologs of the oncogenes transduced by the acute transforming retroviruses (Bishop, 1991). Today avian retroviruses are important models for retroviral replication and significant pathogens affecting the poultry industry.

3.2. RNAi against ASLV replication

We have used avian retroviruses as a model to study RNAi against retroviral replication. In these studies, siRNAs matching two sequences in the *gag* gene of Avian Sarcoma Leucosis Virus (ASLV) were introduced into cells by transfection, one matching the p19 matrix (MA) coding region and the other matching the p12 nucleocapsid (NC) coding region. As a model, cultured chicken DF-1 cells were studied, and the RCAS version of ASLV was used. RCAS is a replication-competent retroviral vector derived from RSV by removal of the *src* oncogene so as to allow introduction of new genetic information in its place (Federspiel and Hughes, 1997; Hughes et al., 1987).

RCAS virus was introduced either by infecting DF-1 cells or by transfecting them with RCAS proviral DNA. The siRNAs were introduced by cotransfection concurrently. In the presence of specific siRNA, the production of the RCAS virus in culture supernatants was dramatically reduced (90%) 2 days after transfection as monitored by accumulation of the p19 matrix protein (Hu et al., 2002). Analysis of provirus formation in a spreading infection also revealed that the two siGAG RNAs inhibited RCAS replication. As controls, cultures were treated with siRNAs against irrelevant proteins or mock transfected, and these showed no significant inhibition. These data document that RNAi can efficiently inhibit RCAS replication.

3.3. Inhibition early or late?

There are two parts of the retroviral life cycle that could potentially be targeted by RNAi. The incoming viral RNA might be degraded by RNAi prior to completing reverse transcription, or the late viral mRNA and genomic RNA transcribed from the integrated proviral DNA might also be cleaved. Several groups have investigated the viral RNAs attacked by RNAi, with somewhat different pictures emerging.

We have analyzed this issue for both ASLV and HIV. To model late expression from integrated proviruses, we transfected DNA copies of the ASLV or HIV genomes along with siRNAs against the retroviral genome or with control siRNAs. Inhibition was found to be efficient for both viruses. Thus RNAi worked efficiently against the viral mRNAs and genomic RNAs expressed from a DNA copy, modeling activity against the late viral RNAs produced by transcription.

What about RNAi against the viral RNA genome introduced into cells in the early steps of infection, i.e. initial fusion of the viral and cellular membranes? To study this step, cells were infected with ASLV or HIV and the production of early reverse transcription products measured. Because the viral RNA genome serves as the template for DNA synthesis, we reasoned that degradation of the incoming viral RNA would result in reduced accumulation of viral DNA. However, no significant difference was seen in either ASLV or HIV. Thus we concluded that the incoming genome was not a substrate for RNAi.

This conclusion has not been reached by all investigators that examined this issue. Cullen and coworkers found that the incoming genome could be a substrate for RNAi, though not utilized as efficiently as the newly synthesized RNAs (Coburn and Cullen, 2002). Stevenson and coworkers did not detect much difference in sensitivity to RNAi in the early and late RNAs (Jacque et al., 2002). The reasons for these differences remain to be determined. Possibly different segments of the early RNAs are differentially exposed in the viral core, so that the choice of RNA sequence to target determines whether or not they are sensitive. Perhaps the detailed differences in experimental protocols somehow dictate the outcome. It will be interesting to work out these differences with further experimentation.

Why would the incoming viral RNA genome be less sensitive to RNAi? One possibility is that the incoming genomic RNA may be protected by viral proteins, thereby blocking

access of the RNAi machinery. Another possibility is that incoming genomic RNA does not traffic through a cellular compartment that harbors the RNAi machinery. For example, if the RNAi machinery resides at the ribosome or the nuclear pore, the incoming genomic RNA may not encounter RNAi factors. In support of this, the RNAi machinery is reported to be associated with the translational factor eIF2c, suggesting ribosomal association (Doi et al., 2003; Sasaki et al., 2003; Tabara et al., 1999).

4. RNA interference against chicken retroviruses in an embryo electroporation model

4.1. RNAi in chicken embryos

In ovo electroporation of chick embryos provides a simple and convenient system for studying gene activity during development (Nakamura and Funahashi, 2001). In ovo electroporation involves the injection of nucleic acids into the central canal of the spinal cord, followed by electroporation with five short (25-ms) square wave pulses at low voltages (25 V). Nucleic acid enters the side of the embryo near the positive electrode. The other half of the embryo is not electroporated and serves as a control. In previous studies, electroporation of dominant-negative gene constructs into chick embryos has allowed analysis of vertebrate gene function in the developing spinal cord (Thaler et al., 2002). We have found that RNA interference can be readily induced in the chick embryo spinal cord by in ovo electroporation, which allowed the demonstration in vivo of inhibition of retroviral replication by RNAi. Pekarik et al. (2003) have also demonstrated the use of RNAi by in ovo electroporation.

Initially, to investigate whether RNAi took place in the chick embryo, we used GFP as a target. A *gfp* encoding plasmid was electroporated into the chicken neural tube together with either specific (siGFP) or nonspecific (siLUC against firefly luciferase) siRNAs. Two days after electroporation, in the absence of siRNA or in the presence of the control siRNA bright green cells were detected in the half of the spinal cord proximal to the positive electrode. In embryos electroporated with the gfp plasmid and siGFP, a greater than 90% reduction in GFP expression was observed. Electroporation of siRNAs up to 3 μg/μl showed no interruption of embryo development (Hu et al., 2002). These studies and those of Pekarik's group establish that RNAi can operate in the developing chick spinal cord.

4.2. Inhibiting ALV infection by in ovo electroporation

To investigate whether RNAi could inhibit replication of RCAS in embryos, the two siRNAs against *gag* that had been shown above to be active in cell culture were used. siLUC was used as a nonspecific control. Two days after fertilization, embryos were electroporated with a plasmid encoding the RCAS genome together with siRNAs. The embryos were analyzed at day 4 by sectioning and staining with an antibody against ASLV Gag protein p19.

Electroporation of RCAS DNA with no siRNA or control siRNA resulted in virus spread from the electroporated half of the spinal cord into the surrounding mesenchyme (due to secondary infection). At 2 days post-electroporation, ASLV replication was inhibited efficiently (>90%) by either of the siRNAs targeting *gag*: infection was evident in only a few cells by anti-Gag staining. These results indicate that RNAi can inhibit ASLV replication in chick embryos.

4.3. Inhibiting pathogenesis by RSV

We next asked whether RNAi could inhibit transformation by RSV in the chick embryo model. RSV encodes the oncogenic *src* gene, which disrupts signal transduction and causes sarcomas in chickens. We electroporated embryos with DNA encoding RSV and different siRNAs. Three days after electroporation, none of chick embryos treated with RSV plus control siRNA survived. In contrast, 7 of 12 embryos treated with the siGAG against p12 survived.

In order to examine the effect of RNAi on RSV pathogenesis, chick embryo sections were stained with different antibodies at 36 h post-electroporation. The mpm2 monoclonal antibody detected M-phase phosphoproteins, thereby identifying mitotic cells, and kip1 (cyclin-dependent kinase inhibitor p27) staining detected inhibition of cell cycle progression, showing cells arrested in G1. The Gag (p19) antibody was used to monitor the spread of RSV. In RSV-infected chick embryos, the neural tube was disorganized and normal cell cycle progression disrupted in the electroporated half. In contrast, normal development and properly controlled cell cycle progression were seen when RSV replication was inhibited by RNAi, thereby documenting inhibition of pathogenesis.

5. Applications of RNAi against animal retroviruses

Today, avian retroviruses are a serious problem in the poultry industry. Crowded conditions in poultry farms are ideal for spread of epidemics of infectious disease, and retroviruses remain a prominent threat. Vaccines are largely ineffective, so new measures against disease caused by avian retroviruses are needed.

The three main disease-causing avian retrovirus of agricultural significance are the ASLV, reticuloendotheliosis virus (REV), and lymphoproliferative disease virus (LPDV) of turkeys (for review, see Payne, 1998). The ASLVs are categorized into six subgroups based on their receptor tropism, A–E and J. Of these, A, B, and J occur commonly. Although genetic resistance (due to recessive resistance genes) to subgroups A–E have been observed in some chickens, all chicken strains are susceptible to subgroup J (ALV-J). ALV-J induces myelocytic leukosis, a tumor-inducing infection of white blood cells from the bone

marrow. Control of ALV-J has become a major concern to the poultry industry as losses of breeder birds to ALV-J can be as high as 20–40% in severe outbreaks. The REV family of retroviruses causes a variety of diseases including chronic lymphatomas in chickens, ducks, turkeys, geese, pheasants, and quail. In turkeys, LPDV infection causes a lymphoproliferative disease.

Furthermore, some poxviruses responsible for fowlpox outbreaks in the United States have been found to harbor an intact REV provirus in their genomes (Kim and Tripathy, 2001; Singh et al., 2003). Evidently the REV virus used the poxvirus genome as an integration target rather than the host cell chromosome, thereby incorporating the retroviral genome in the poxvirus genome. The addition of REV to the fowlpox genome has been implicated in increased poxvirus replication in host chickens, and may facilitate infection of previously vaccinated poultry.

An attractive potential technology to suppress retroviral diseases of poultry would be germ line insertion of RNAi constructs targeting either retroviral sequences or dispensable host proteins involved in the infectious process. For example, DNA hairpins initiating RNAi against the *gag* targets described above could be installed in the chicken genome, blocking replication of pathogenic ASLVs. Such an approach, if successful, might be applied to inhibiting viral infection and pathogenesis in a wide range of domestic animals. RNAi is also being investigated as a means of treating HIV infection in humans, but development of RNAi as a drug seems much more difficult—germ line modification of farm animals, in contrast, seems potentially feasible in the near term.

In summary, RNAi appears to be a promising new technology for preventing and treating retroviral diseases, well warranting a review that begins and ends with a bang!

References

Al-Kaff, N.S., Covey, S.N., Kreike, M.M., Page, A.M., Pinder, R., Dale, P.J., 1998. Transcriptional and posttranscriptional plant gene silencing in response to a pathogen. Science 279, 2113–2115.

Banerjea, A., Li, M.J., Bauer, G., Remling, L., Lee, N.S., Rossi, J., Akkina, R., 2003. Inhibition of HIV-1 by lentiviral vector-transduced siRNAs in T lymphocytes differentiated in SCID-hu mice and CD34+ progenitor cell-derived macrophages. Mol. Ther. 8, 62–71.

Bishop, J.M., 1991. Molecular themes in oncogenesis. Cell 64, 235–248.

Bushman, F.D., 2003. RNA interference: applications in vertebrates. Mol. Ther. 7, 9–10.

Caplen, N.J., Fleenor, J., Fire, A., Morgan, R.A., 2000. dsRNA-mediated gene silencing in cultured Drosophila cells: a tissue culture model for the analysis of RNA interference. Gene 252, 95–105.

Caplen, N.J., Parrish, S., Imani, F., Fire, A., Morgan, R.A., 2001. Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. Proc. Natl. Acad. Sci. U.S.A. 98, 9742–9747.

Capodici, J., Kariko, K., Weissman, D., 2002. Inhibition of HIV-1 infection by small interfering RNA-mediated RNA interference. J. Immunol. 169, 5196–5201.

Carmell, M.A., Xuan, Z., Zhang, M.Q., Hannon, G.J., 2002. The Argonaute family: tentacles that reach into RNAi, developmental control, stem cell maintenance, and tumorigenesis. Genes Dev. 16, 2733–2742.

Caudy, A.A., Ketting, R.F., Hammond, S.M., Denli, A.M., Bathoorn, A.M., Tops, B.B.J., Silva, J.M., Myers, M.M., Hannon, G.J., Plasterk, R.H.A., 2003. A micrococcal nuclease homologue in RNAi effector complexes. Nature 425, 411–415.

Coburn, G.A., Cullen, B.R., 2002. Potent and specific inhibition of human immunodeficiency virus type 1 replication by RNA interference. J. Virol. 76, 9225–9231.

Cogoni, C., Macino, G., 1999. Gene silencing in Neurospora crassa requires a protein homologous to RNA-dependent RNA polymerase. Nature 399, 166–169.

Doench, J.G., Petersen, C.P., Sharp, P.A., 2003. siRNAs can function as miRNAs. Genes Dev. 17, 438–442.

Doi, N., Zenno, S., Ueda, R., Ohki-Hamazaki, H., Ui-Tei, K., Saigo, K., 2003. Short-interfering-RNA-mediated gene silencing in mammalian cells requires Dicer and eIF2C translation initiation factors. Curr. Biol. 13, 41–46.

Dykxhoorn, D.M., Novina, C.D., Sharp, P.A., 2003. Killing the messenger: short RNAs that silence gene expression. Nat. Rev. Mol. Cell. Biol. 4, 457–467.

Elbashir, S.M., Harborth, J., Lendeckel, W., Yalcin, A., Weber, K., Tuschl, T., 2001a. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 411, 494–498.

Elbashir, S.M., Lendeckel, W., Tuschl, T., 2001b. RNA interference is mediated by 21 and 22-nucleotide RNAs. Genes Dev. 15, 188–200.

Ellerman, V., Bang, O., 1908. Experimentelle Leukamie bei Huhnern. Zentralbl. Bakteriol. Parasitenkd. Infectionskr. Hyg. Abt. Orig. 46, 595–609.

Federspiel, M.J., Hughes, S.H., 1997. Retroviral gene delivery. Methods Cell. Biol. 52, 179–214.

Fire, A., Xu, S., Montgomery, M.K., Kostas, S.A., Driver, S.E., Mello, C.C., 1998. Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans. Nature 391, 806–811.

Garrus, J.E., von Schwedler, U.K., Pornillos, O.W., Morham, S.G., Zavitz, K.H., Wang, H.E., Wettstein, D.A., Stray, K.M., Cote, M., Rich, R.L., Myszka, D.G., Sundquist, W.I., 2001. Tsg101 and the vacuolar protein sorting pathway are essential for hiv-1 budding. Cell 107, 55–65.

Hamilton, A.J., Baulcombe, D.C., 1999. A species of small antisense RNA in posttranscriptional gene silencing in plants. Science 286, 950–952.

Hammond, S.M., Bernstein, E., Beach, D., Hannon, G.J., 2000. An RNA-directed nuclease mediates post-transcriptional gene silencing in Drosophila cells. Nature 404, 293–296.

Hu, W.Y., Meyers, C.P., Kilzer, J.M., Pfaff, S.L., Bushman, F.D., 2002. Inhibition of retroviral pathogenesis by RNA interference. Curr. Biol. 12, 1301–1311.

Hughes, S.H., Greenhouse, J.J., Petropoulos, C.J., Sutrave, P., 1987. Adaptor plasmids simplify the insertion of foreign DNA into helper-independent retroviral vectors. J. Virol. 61, 3004–3012.

Jacque, J.M., Triques, K., Stevenson, M., 2002. Modulation of HIV-1 replication by RNA interference. Nature 418, 435–438.

Jorgensen, R., 1990. Altered gene expression in plants due to trans interactions between homologous genes. Trends Biotechnol. 8, 340–344.

Keogh, E.V., 1938. Ectodermal lesions produced by the virus of Rous sarcoma. Br. J. Exp. Pathol. 19, 1–9.

Kim, T.J., Tripathy, D.N., 2001. Reticuloendotheliosis virus integration in the fowl poxvirus genome: not a recent event. Avian Dis. 45, 639–663.

Lee, N.S., Dohjima, T., Bauer, G., Li, H., Li, M.J., Ehsani, A., Salvaterra, P., Rossi, J., 2002. Expression of small interfering RNAs targeted against HIV-1 rev transcripts in human cells. Nat. Biotechnol. 20, 500–505.

Li, H., Li, W.X., Ding, S.W., 2002. Induction and suppression of RNA silencing by an animal virus. Science 296, 1319–1321.

Li, M.J., Bauer, G., Michienzi, A., Yee, J.K., Lee, N.S., Kim, J., Li, S., Castanotto, D., Zaia, J., Rossi, J.J., 2003. Inhibition of HIV-1

infection by lentiviral vectors expressing Pol III-promoted anti-HIV RNAs. Mol. Ther. 8, 196–206.
Lipardi, C., Wei, Q., Paterson, B.M., 2001. RNAi as random degradative PCR: siRNA primers convert mRNA into dsRNAs that are degraded to generate new siRNAs. Cell 107, 297–307.
Marathe, R., Anandalakshmi, R., Smith, T.H., Pruss, G.J., Vance, V.B., 2000. RNA viruses as inducers. Plant Mol. Biol. 43, 295–306.
Martinez, J., Patkaniowska, A., Urlaub, H., Luhrmann, R., Tuschl, T., 2002a. Single-Stranded Antisense siRNAs Guide Target RNA Cleavage in RNAi. Cell 110, 563–574.
Martinez, M.A., Gutierrez, A., Armand-Ugon, M., Blanco, J., Parera, M., Gomez, J., Clotet, B., Este, J.A., 2002b. Suppression of chemokine receptor expression by RNA interference allows for inhibition of HIV-1 replication. AIDS 16, 2385–2390.
Nakamura, H., Funahashi, J., 2001. Introduction of DNA into chick embryos by in ovo electroporation. Methods 24, 43–48.
Ngo, H., Tschudi, C., Gull, K., Ullu, E., 1998. Double-stranded RNA induces mRNA degradation in Trypanosoma brucei. Proc. Natl. Acad. Sci. U.S.A. 95, 14687–14692.
Novina, C.D., Murray, M.F., Dykxhoorn, D.M., Beresford, P.J., Riess, J., Lee, S.K., Collman, R.G., Lieberman, J., Shankar, P., Sharp, P.A., 2002. siRNA-directed inhibition of HIV-1 infection. Nat. Med. 8, 681–686.
Palatnik, J.F., Allen, E., Wu, X., Schommer, C., Schwab, R., Carrington, J.C., Weigel, D., 2003. Control of leaf morphogenesis by microRNAs. Nature 425, 257–263.
Park, W.S., Miyano-Kurosaki, N., Hayafune, M., Nakajima, E., Matsuzaki, T., Shimada, F., Takaku, H., 2002. Prevention of HIV-1 infection in human peripheral blood mononuclear cells by specific RNA interference. Nucleic Acids Res. 30, 4830–4835.
Payne, L.N., 1998. Retrovirus-induced disease in poultry. Poult. Sci. 77, 1204–1212.
Pekarik, V., Bourikas, D., Miglino, N., Joset, P., Preiswerk, S., Stoeckli, E.T., 2003. Screening for gene function in chicken embryo using RNAi and electroporation. Nat. Biotechnol. 21, 93–96.
Pickford, A.S., Cogoni, C., 2003. RNA-mediated gene silencing. Cell Mol. Life Sci. 60, 871–882.
Plasterk, R.H., 2002. RNA silencing: the genome's immune system. Science 296, 1263–1265.
Qin, X.F., An, D.S., Chen, I.S., Baltimore, D., 2003. Inhibiting HIV-1 infection in human T cells by lentiviral-mediated delivery of small interfering RNA against CCR5. Proc. Natl. Acad. Sci. U.S.A. 100, 183–188.
Ratcliff, F.G., MacFarlane, S.A., Baulcombe, D.C., 1999. Gene silencing without DNA. RNA-mediated cross-protection between viruses. Plant Cell 11, 1207–1216.
Romano, N., Macino, G., 1992. Quelling: transient inactivation of gene expression in Neurospora crassa by transformation with homologous sequences. Mol. Microbiol. 6, 3343–3353.
Rous, P., 1911. A sarcoma of the fowl transmissible by an agent separable from the tumor cells. J. Exp. Med. 13, 397–411.
Sasaki, T., Shiohama, A., Minoshima, S., Shimizu, N., 2003. Identification of eight members of the Argonaute family in the human genome small star, filled. Genomics 82, 323–330.
Schwarz, D.S., Hutvagner, G., Haley, B., Zamore, P.D., 2002. Evidence that siRNAs function as guides, not primers, in the Drosophila and human RNAi pathways. Mol. Cell. 10, 537–548.
Sijen, T., Fleenor, J., Simmer, F., Thijssen, K.L., Parrish, S., Timmons, L., Plasterk, R., Fire, A., 2001. On the role of RNA amplification in dsRNA-triggered gene silencing. Cell 107, 465–476.
Singh, P., Schnitzlein, W.M., Tripathy, D.N., 2003. Reticuloendotheliosis virus sequences within the genomes of field strains of fowlpox virus display variability. J. Virol. 77, 5855–5862.
Surabhi, R.M., Gaynor, R.B., 2002. RNA interference directed against viral and cellular targets inhibits human immunodeficiency virus type 1 replication. J. Virol. 76, 12963–12973.
Tabara, H., Sarkissian, M., Kelly, W.G., Fleenor, J., Grishok, A., Timmons, L., Fire, A., Mello, C.C., 1999. The rde-1 gene, RNA interference, and transposon silencing in *C. elegans*. Cell 99, 123–132.
Temin, H., Mizutani, S., 1970. RNA-dependent DNA polymerase in virions of Rous sarcoma virus. Nature 226, 1211–1213.
Temin, H.M., Rubin, H., 1958. Characteristics of an assay for Rous sarcoma virus and Rous sarcoma cells in tissue culture. Virology 6, 669–688.
Thaler, J.P., Lee, S.K., Jurata, L.W., Gill, G.N., Pfaff, S.L., 2002. LIM factor Lhx3 contributes to the specification of motor neuron and interneuron identity through cell-type-specific protein-protein interactions. Cell 110, 237–249.
Zeng, Y., Wagner, E.J., Cullen, B.R., 2000. Both natural and designed micro RNAs can inhibit the expression of cognate mRNAs when expressed in human cells. Mol. Cell. 9, 1327–1333.
Zeng, Y., Yi, R., Cullen, B.R., 2003. MicroRNAs and small interfering RNAs can inhibit mRNA expression by similar mechanisms. Proc. Natl. Acad. Sci. U.S.A. 100, 9779–9784.