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Hypothesis

RNA silencing: A remarkable parallel to protein-based immune systems in vertebrates?

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Abstract Sequence-specific gene silencing by double-strand RNA has been observed in many eukaryotes. Accumulating data suggest that it is the major antiviral defense mechanism in plants and invertebrates. The discovery that this cellular mechanism is also highly conserved though somewhat impaired in mammals has stimulated debate about the evolution of antiviral systems. Here we suggest that the existence of the interferon response as an evolutionary intermediate could account for both the relative decline of RNA silencing and the development of protein-based immune systems in vertebrates. In addition, we emphasize the opportunities presented by RNA silencing and the deeper understanding of vertebrate antiviral systems that is needed.

Keywords: RNA silencing; RNA interference; Interferon; Protein-based immune system; Vertebrates; Evolution

1. Introduction

RNA silencing or post-transcriptional gene silencing (PTGS) is a process of degradation of cognate mRNA in response to the introduction of a double-strand RNA (dsRNA). It is commonly accepted that RNA silencing is the major antiviral defense system in plants and invertebrates. The important role of short dsRNA molecules in initiating sequence-specific gene silencing was first discovered in the nematode *Caenorhabditis elegans* [1]. This process, also termed RNA interference (RNAi), is mediated by small interfering RNAs (siRNAs) (19–27 bp long), which are generated by cleavage of dsRNA by an RNaseIII-like enzyme, Dicer [2]. There is recent evidence that this form of gene silencing is conserved in mammals [3–5]. These remarkable observations have led to demonstrations of a protective effect of siRNAs against mammalian viruses, although it has been reported that siRNAs also activate the protein kinase R (PKR)-RNase L pathway [6,7], an important innate antiviral mechanism regulated by interferon (IFN) [8].

These findings raise a number of questions. Does RNAi occur naturally in virally infected cells of vertebrates thus representing a nucleic acid-based immune system, by analogy with the protein-based immune system? If so, what is the relationship between the two, are they quite separate or linked in some way? Why is this antiviral system in mammalian cells not powerful enough to combat viral infection on its own without the artificial introduction of siRNAs? How can we achieve a better understanding of the capacity of vertebrates to defend against viruses? What new approaches might lead to more effective procedures for treating vertebrate viral diseases?

2. RNAi as a natural antiviral mechanism

Li et al. [9] postulated that RNA silencing is a natural antiviral response in mammals. Their hypothesis is supported by three lines of evidence: first, the RNA silencing machinery is conserved in mammals and can inhibit viral infection when the formation of siRNAs is experimentally induced. Second, mammalian viruses encode suppressors (e.g., the E3L and NS1 proteins by the influenza and vaccinia viruses) of RNAi as an essential feature, as has been established for viruses of plants and invertebrates. Third, suppressors of RNAi also act as inhibitors of the innate mammalian antiviral response regulated by the IFN system.

These discoveries not only suggest a strategy for treating viral diseases in mammals but also promise a deeper understanding of the evolution of mammalian antiviral potential. Biology students are taught that the classical protein-based immune response is the major antiviral mechanism in vertebrates. Nevertheless there is increasing evidence that RNA silencing is an evolutionarily conserved mechanism that protects genomes from exogenous (viral) and endogenous (transposon) invasion, and impacts on cellular programs of gene expression and development [10–12]. Both protein-based and siRNA-based mechanisms at least in part share the same function, namely combating invaders. These facts prompted us to consider the evolutionary relationship between them. Table 1 compares the features of RNAi and of vertebrate protein-based immune systems. They are comparable in at least seven crucial aspects, some of which had been described by Ding et al. [13]. In addition, dsRNA (and even siRNA), induces a non-specific antiviral response involving the PKR-RNase L pathway, similar to that mediated by cytokines in protein-based immune systems. Furthermore, RNA silencing leads to non-cytopathic viral clearance [14,15], whereas the protein-based immune response brings about widespread microphagocytosis or apoptosis of virally infected cells [16].

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Table 1
Comparison of protein-based immune systems and RNA silencing

Features	Protein-based immune system	RNA silencing
1	Antigen	Viral genome/transposon/ aberrant RNAs/mRNA expressed from plasmid backbones
2	Antibody	siRNA
3	Lymphocytes (B- and T-cells)	Dicer/RISC[a]
4	Cytokines (IL, IFN, etc.)	PKR/RNase L/IFN pathways
5	Microphagocytosis/apoptosis	Non-cytopathic viral clearance
6	Immunological memory	A rudimentary form of memory
7	Immune response (several days to a few months)	RNAi effect (several hours to a few days)

[a]In animal RNAi pathways, target RNA destruction is catalyzed by the siRNA-guided, RNA-induced silencing complex (RISC).

It seems likely that a nucleic acid-based immune system evolved prior to the appearance of the protein-based immune system of vertebrates [10]. Many scientists have suggested that life originated as an RNA world and have proposed models of the evolution of life from the late stages of the RNA world to the emergence of eukaryotes and prokaryotes [17–19]. In the hypothetical RNA world, RNA had both genotypic and phenotypic capabilities. RNAi is a mechanism in which gene-specific dsRNA triggers the degradation of corresponding transcripts. The observations that *Entamoeba histolytica* and *Giardia intestinalis* have RNAi pathways and that RNAi genes are present in *Giardia* support the view that gene silencing by dsRNA appeared very early in the evolution of the eukaryotic lineage [20]. Interestingly, Billy et al. [3] have demonstrated that extracts of embryonal carcinoma (EC) P19 and F9 cells contain much higher levels of Dicer than extracts of differentiated cells such as rat REF52, mouse NIH 3T3 or human Hela cells. Moreover, these researchers showed that specific RNAi could be induced by long dsRNA in mouse embryonal teratocarcinoma cell lines, whereas induction of RNAi in response to long dsRNA has generally been unsuccessful in differentiated mammalian cells, most likely due to non-specific effects of dsRNA-dependent PKR and RNase L, both of which are effectors of the IFN response [21]. It is well known that protein-based immune systems cannot function as strong defenses against invaders early in mammalian embryogenesis. These phenomena also imply that RNA silencing, which acts as the major natural antiviral mechanism in plants and invertebrates, could have become somewhat defective and have eventually been functionally replaced by a superior protein-based immune system during the evolution of vertebrates.

At present there is no evidence that RNAi is employed in bacterial and archeal cells; hence one needs to be cautious in suggesting a very early origin of RNAi. However, the existence of ribozymes (or catalytic RNAs) discovered a little more than 20 years ago is suggestive [22]. Ribozymes catalyze sequence-specific reactions (cleavage or ligation of the RNA phosphodiester backbone) controlled by RNA–RNA interactions between the ribozyme and its substrate molecules [23]. They occur widely in viruses, bacteria, plants, and lower eukaryotes, but are rare in vertebrates. The structure and functional similarity, and cell type distribution of ribozymes and RNA silencing machinery inevitably suggest the idea that RNAi is a mechanism that evolved from a primordial molecular information-processing system such as ribozymes. To test this hypothesis, it will be necessary to look for possible intermediates in lower species.

3. RNAi could be a co-agent of protein-based immune systems in vertebrates

Gitlin and Andino [10] have discussed the possibility that RNA silencing, as a versatile antiviral system, may have been conserved during evolution, since viruses (as well as other molecular parasites like transposons) probably maintain an unrelenting selective pressure on their hosts, despite the fact that vertebrates have evolved a sophisticated immune mechanism based on protein recognition. Since an impressive number of RNAi effects involving viral infection of mammals have been demonstrated [14,24–28], we wish to emphasize the possibility that RNA silencing acts synergistically with conventional mammalian protein-based immune systems in defense against viruses.

As described in Table 1, RNA silencing based on recognition at the level of RNA, and sequence-specific and rapid inhibition of viral infection, could complement and strengthen protein-based immunity in controlling important vertebrate pathogens. First, RNAi has been shown to occur in the cytoplasm [29,30], where protein-based recognition of virus by lymphocytes, antibodies and cytokines cannot take place. RNA silencing can be triggered by viruses or transposons that generate dsRNA during their replication [31], and aberrant RNAs that have not been well characterized are thought to be capable of initiating RNAi responses in plants. Second, as mentioned, RNAi as a natural antiviral mechanism involves non-cytopathic viral clearance [14,15], whereas the protein-based immune response induces broad range microphagocytosis, or apoptosis, of virally infected cells [16]. Moreover, viral infections, acting on the protein-based immune system, can ablate self-tolerance, mimic immune responses to self-antigens, and induce autoimmune disease [32–34]. Third, RNAi leads to rapid and efficient resistance against viral infection in response to siRNA [35] and can achieve relatively long lasting viral suppression in mammalian cells [36], whereas it takes traditional vaccines based on inactivated virus or subunit peptides a few days to induce an immune response strong enough to prevent disease; even so-called emergency vaccines require 4–5 days to elicit sufficient levels of interfering factors to achieve protection [37].

Is it possible that siRNAs generated by viral infection, chemical synthesis or direct transcription from vectors (plasmid or virus), could help to develop protein-based immunity? Previous work by Suzuki et al. [38] showed that the introduction of fragments of dsRNA as short as 25 bp in length into the cytoplasm of non-immune cells could cause abnormal expression of the major histocompatibility complex (MHC), as well as the expression or activation of other genes or gene products essential for antigen presentation. This effect was sequence-independent, not duplicated by single-stranded polynucleotides, and control experiments eliminated the involvement of CpG motifs, which act directly on cells of the immune system.

It suggests that dsRNA introduced into the cytoplasm may cause normal cells such as rat FRTL-5 thyroid cells to become ''non-professional'' antigen-presenting cells (APCs) and trigger

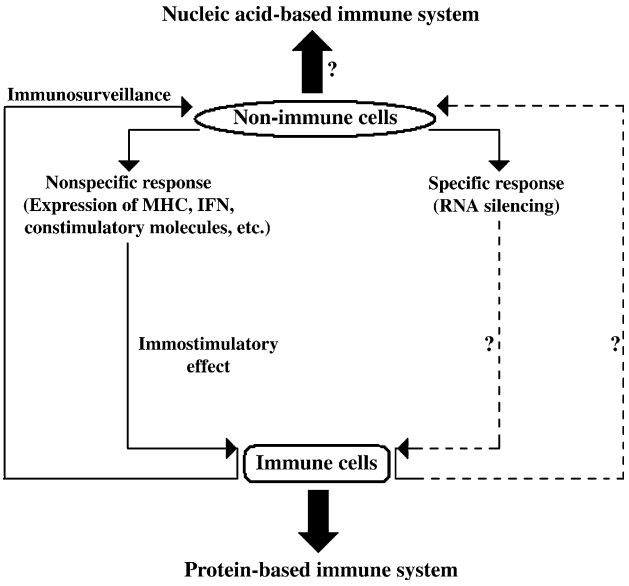

Fig. 1. A brief schematic representation of the antiviral responses in vertebrates. In addition to the lymphocytes, the non-immune cells have been employed to elicit antiviral response in specific or non-specific manner, due to activation by dsDNA and dsRNA generated by viral infection or artificial methods. The non-specific response has shown an interaction between the non-immune cells and the protein-based immune system. A role of RNA silencing in inducing the immunositmulatory effect to or immunosurveillance by the traditional system should be further determined.

immunosurveillance of these cells by activating cells of the immune system [38,39]. In the case of RNAi, the initial dsRNA is cleaved to siRNAs, 19–27 nucleotides long, by a protein complex containing Dicer [2], and induction of an IFN response by siRNAs or RNAi vectors has been reported in mammalian cells [6,7,40,41]. IFNs are involved in numerous immune interactions during viral infection, and contribute to both the induction and regulation of innate and adaptive antiviral mechanisms [8]. Thus, it will be important to seek evidence for the involvement of siRNAs in protein-based immune systems (Fig. 1).

4. The IFN response: A bridge between RNA silencing and protein-based immune systems?

There is evidence of antiviral synergy between the RNAi machinery and the IFN response during embryogenesis (Fig. 2). In embryonic cells of mammals, RNA silencing can be efficiently and specifically induced by even long dsRNA, presumably due to the high level of Dicer expression, and induction of IFN genes by dsRNA or viral infection is defective because of a lack of dsRNA- and IFN-activated enzymes [42–46]. In differentiated cells, however, the specific RNAi effect seems to be impaired due to downregulation of Dicer expression [3], while IFN-α/β is produced rapidly in the early phase of a viral infection when viral factors interact with cellular pattern-recognition receptors (PRRs) [8]. Both RNA silencing and the IFN response confer rapid resistance during the early phase of viral infection. These results suggest that these two systems synergize in their antiviral actions. Future work will be necessary to determine if virus infection indeed elicits an RNA silencing

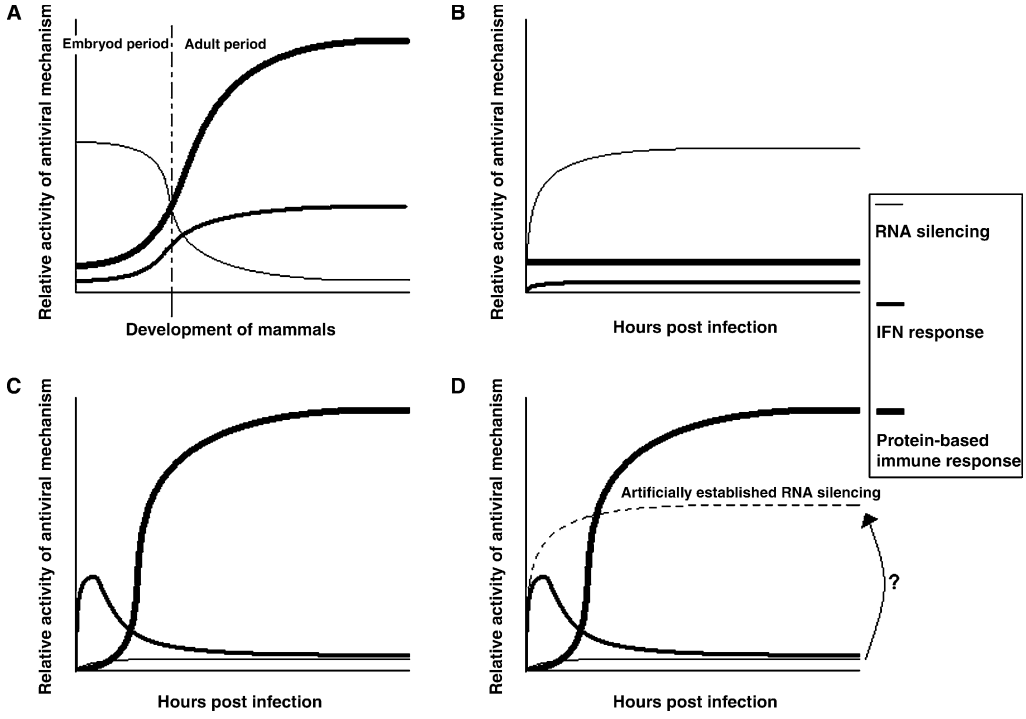

Fig. 2. Representation of the relative activity of antiviral mechanisms in vertebrates. Based on the hypothesis that RNA silencing would be conserved in mammals and naturally induced by viral infection, the potentialities of three antiviral mechanisms employed is simply described during (A) development of mammals as a whole, (B) embryo period when challenged with virus, (C) adult period when challenged with virus, and (D) adult period with an artificially established RNA silencing.

response in mammalian embryonic cells that is much stronger than the IFN response or protein-based immunity (Fig. 2B).

Furthermore, we suggest that the IFN response may have played a key role in the evolutionary development of protein-based immune systems, and that it served as a bridge between this adaptive system and RNA silencing during the evolution of antiviral mechanisms. The following is a summary of the published studies and theoretical ideas supporting this hypothesis.

(a) As an apparently more primitive RNA-based surveillance system, the RNA silencing pathway is not only operational in mammalian cells, but there is evidence that it is part of the mammalian innate antiviral immunity. For example, the NS1 and E3L proteins encoded by mammalian viruses (influenza and vaccinia viruses) function in mammalian hosts as inhibitors of the innate antiviral response regulated by the IFN system, which represents one of the first lines of defense against viral infections [47]. These proteins were shown recently by Li et al. [9] to act as suppressors of RNA silencing.

(b) However, it is well known that the protein-based immune system is the major antiviral mechanism in vertebrates and that the RNA silencing machinery seems to be somehow defective since no direct evidence has been found to support a strong natural antiviral activity of RNAi. Thus, it is conceivable that RNA silencing provided the impetus for the evolution of more effective antiviral systems. The induction of an IFN response by siRNAs has been reported in differentiated mammalian cells [6,7]. This suggests that siRNAs may have widespread and complex effects beyond the selective silencing of target genes. Because of the very restricted specificity of siRNAs, the RNA silencing system could have been selected against in evolution but have laid the foundation for the evolution of a more efficient rapidly acting immunity mechanism, such as the IFN system.

(c) From a theoretical point of view it is understandable that there should have evolved a mechanism that responded to a range of foreign entities to replace. RNA silencing is triggered by siRNAs, since these latter are severely restricted in structure. By contrast there is a wide range of effectors of the IFN response including envelope glycoproteins, CpG motifs, random dsDNAs or dsRNAs, etc. Moreover, RNA silencing occurs in the cytoplasm, whereas rapid and direct recognition of effectors by cellular receptors on the cell membrane leading to the expression of IFN genes, is a key property of protein-based immune systems.

(d) IFNs have been identified in non-mammalian vertebrates such as chicken and fish, suggesting that the IFN response as an antiviral defense appeared prior to the evolution of the more sophisticated protein-based immune systems. Gobel et al. [48] reported that release of IFN-γ by $CD4^+$ T cells of the chicken can be stimulated by interleukin 18 (IL-18). They therefore suggested that a fully functional IL-18-IFN-γ system arose before divergence of birds and mammals from a common ancestor ~300–350 million years ago [49]. Interestingly, a recent study revealed that high titers of a type I IFN were produced in fish in response to infection with UV-activated grass carp hemorrhagic virus (GCHV) [50].

(e) Later, in vertebrates, IFNs became involved in numerous immune interactions as inducers, regulators, and effectors of both innate and adaptive antiviral mechanisms. IFN-α/β and IFN-γ influence the activities of macrophages, NK cells, dendritic cells (DC), and T cells by enhancing antigen presentation, cell trafficking, cell differentiation and expression profiles, ultimately enhancing and prolonging antiviral effector functions [8].

(f) Importantly, Sledz et al. [6] have shown, using cell lines deficient in specific components of IFN action, that the RNAi mechanism itself is independent of the IFN system. Similarly the IFN system as an innate antiviral mechanism can act independently of protein-based immune systems in vertebrates. It is therefore quite possible for the IFN system to have served as an intermediate antiviral mechanism during evolution.

5. Outlook: A challenging opportunity for RNA silencing

The discovery of RNA silencing machinery promises to open up two major avenues of investigation. First, it raises the opportunity of developing novel therapeutic approaches. This conclusion derives in essence from the natural antiviral potential of RNA silencing. Second, RNAi is now routinely used in reverse genetic approaches to study gene functions. However, there remain many challenges, as described elsewhere [10]. Several critical problems will need to be addressed before a fully successful outcome can be achieved; these include: stabilization and enhancement of RNA silencing, improved siRNA delivery, systemic effects and target mutation. Fortunately, recent studies suggest that these hurdles can be overcome [35,36,51–56].

In addition, RNA silencing may provide a key to understanding the evolution of modern cells (bacterial, archeal, and eukaryotic cells). Several theories have already been proposed in this area [57]. Typically, primitive attempts to model cellular evolution focus on the fundamentally different types of genetic machinery involved in information processing, namely translation, transcription, and genome structure and replication. After biology entered the genomic era in the 1990s, horizontal gene transfer (HGT) was recognized as an evolutionary force comparable in power to classical vertical evolutionary mechanisms [58]. RNA silencing and related cellular defenses against alien genetic material could also have played a part in evolutionary strategies.

6. Conclusions

We have summarized accumulating evidence that RNA silencing acted as a natural antiviral mechanism during the evolution of life and that a legacy of it remains in vertebrates. Furthermore, we have argued that the existence of the IFN response could account for both the decline in importance of RNA silencing machinery in the vertebrate kingdom and the development of protein-based immune systems. Although more decisive evidence for a natural antiviral role of RNA silencing and its synergistic interaction with the protein-based immune system may be anticipated from studies of a variety of viruses in vertebrates, an overall picture of the interaction of

antiviral mechanisms is beginning to emerge. As mentioned above, we believe that antiviral strategies based on a combination of nucleic acid-based and protein-based immune systems would be more effective than either alone.

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References

[1] Fire, A. (1999) RNA-triggered gene silencing. Trends Genet. 15, 358–363.
[2] Alisky, J.M. and Davidson, B.L. (2004) Towards therapy using RNA interference. Am. J. Pharmacogenomics 4, 45–51.
[3] Billy, E., Brondani, V., Zhang, H., Muller, U. and Filipowicz, W. (2001) Specific interference with gene expression induced by long, double-stranded RNA in mouse embryonal teratocarcinoma cell lines. Proc. Natl. Acad. Sci. USA 98, 14428–14433.
[4] Caplen, N.J., Parrish, S., Imani, F., Fire, A. and Morgan, R.A. (2001) Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. Proc. Natl. Acad. Sci. USA 98, 9742–9747.
[5] Tuschl, T. (2002) Expanding small RNA interference. Nat. Biotechnol. 20, 446–448.
[6] Sledz, C.A., Holko, M., de Veer, M.J., Silverman, R.H. and Williams, B.R. (2003) Activation of the interferon system by short-interfering RNAs. Nat. Cell Biol. 5, 834–839.
[7] Kariko, K., Bhuyan, P., Capodici, J., Ni, H., Lubinski, J., Friedman, H. and Weissman, D. (2004) Exogenous siRNA mediates sequence-independent gene suppression by signaling through toll-like receptor 3. Cells Tissues Organs 177, 132–138.
[8] Malmgaard, L. (2004) Induction and regulation of IFNs during viral infections. J. Interferon Cytokine Res. 24, 439–454.
[9] Li, W.X., Li, H., Lu, R., Li, F., Dus, M., Atkinson, P., Brydon, E.W., Johnson, K.L., Garcia-Sastre, A., Ball, L.A., Palese, P. and Ding, S.W. (2004) Interferon antagonist proteins of influenza and vaccinia viruses are suppressors of RNA silencing. Proc. Natl. Acad. Sci. USA 101, 1350–1355.
[10] Gitlin, L. and Andino, R. (2003) Nucleic acid-based immune system: the antiviral potential of mammalian RNA silencing. J. Virol. 77, 7159–7165.
[11] Bernstein, E., Denli, A.M. and Hannon, G.J. (2001) The rest is silence. RNA 7, 1509–1521.
[12] Vastenhouw, N.L. and Plasterk, R.H. (2004) RNAi protects the *Caenorhabditis elegans* germline against transposition. Trends Genet. 20, 314–319.
[13] Ding, S.W., Li, H., Lu, R., Li, F. and Li, W.X. (2004) RNA silencing: a conserved antiviral immunity of plants and animals. Virus Res. 102, 109–115.
[14] Gitlin, L., Karelsky, S. and Andino, R. (2002) Short interfering RNA confers intracellular antiviral immunity in human cells. Nature 418, 430–434.
[15] Randall, G., Grakoui, A. and Rice, C.M. (2003) Clearance of replicating hepatitis C virus replicon RNAs in cell culture by small interfering RNAs. Proc. Natl. Acad. Sci. USA 100, 235–240.
[16] Guidotti, L.G. and Chisari, F.V. (2001) Noncytolytic control of viral infections by the innate and adaptive immune response. Annu. Rev. Immunol. 19, 65–91.
[17] Poole, A.M., Jeffares, D.C. and Penny, D. (1998) The path from the RNA world. J. Mol. Evol. 46, 1–17.
[18] Joyce, G.F. (2002) The antiquity of RNA-based evolution. Nature 418, 214–221.
[19] Brosius, J. (2003) Gene duplication and other evolutionary strategies: from the RNA world to the future. J. Struct. Funct. Genomics 3, 1–17.
[20] Ullu, E., Tschudi, C. and Chakraborty, T. (2004) RNA interference in protozoan parasites. Cell Microbiol. 6, 509–519.
[21] Cullen, B.R. (2002) RNA interference: antiviral defense and genetic tool. Nat. Immunol. 3, 597–599.
[22] Puerta-Fernandez, E., Romero-Lopez, C., Barroso-delJesus, A. and Berzal-Herranz, A. (2003) Ribozymes: recent advances in the development of RNA tools. FEMS Microbiol. Rev. 27, 75–97.
[23] Tanner, N.K. (1999) Ribozymes: the characteristics and properties of catalytic RNAs. FEMS Microbiol. Rev. 23, 257–275.
[24] Jiang, M. and Milner, J. (2002) Selective silencing of viral gene expression in HPV-positive human cervical carcinoma cells treated with siRNA, a primer of RNA interference. Oncogene 21, 6041–6048.
[25] Shlomai, A. and Shaul, Y. (2003) Inhibition of hepatitis B virus expression and replication by RNA interference. Hepatology 37, 764–770.
[26] Song, E., Lee, S.K., Wang, J., Ince, N., Ouyang, N., Min, J., Chen, J., Shankar, P. and Lieberman, J. (2003) RNA interference targeting Fas protects mice from fulminant hepatitis. Nat. Med. 9, 347–351.
[27] Lee, N.S., Dohjima, T., Bauer, G., Li, H., Li, M.J., Ehsani, A., Salvaterra, P. and Rossi, J. (2002) Expression of small interfering RNAs targeted against HIV-1 rev transcripts in human cells. Nat. Biotechnol. 20, 500–505.
[28] Novina, C.D., Murray, M.F., Dykxhoorn, D.M., Beresford, P.J., Riess, J., Lee, S.K., Collman, R.G., Lieberman, J., Shankar, P. and Sharp, P.A. (2002) siRNA-directed inhibition of HIV-1 infection. Nat. Med. 8, 681–686.
[29] Hammond, S.M., Caudy, A.A. and Hannon, G.J. (2001) Post-transcriptional gene silencing by double-stranded RNA. Nat. Rev. Genet. 2, 110–119.
[30] Sharp, P.A. (2001) RNA interference-2001. Genes Dev. 15, 485–490.
[31] Sijen, T. and Plasterk, R.H. (2003) Transposon silencing in the *Caenorhabditis elegans* germ line by natural RNAi. Nature 426, 310–314.
[32] Guardiola, J. and Maffei, A. (1993) Control of MHC class II gene expression in autoimmune, infectious, and neoplastic diseases. Crit. Rev. Immunol. 13, 247–268.
[33] Gianani, R. and Sarvetnick, N. (1996) Viruses, cytokines, antigens, and autoimmunity. Proc. Natl. Acad. Sci. USA 93, 2257–2259.
[34] Horwitz, M.S., Bradley, L.M., Harbertson, J., Krahl, T., Lee, J. and Sarvetnick, N. (1998) Diabetes induced by Coxsackie virus: initiation by bystander damage and not molecular mimicry. Nat. Med. 4, 781–785.
[35] Chen, W., Yan, W., Du, Q., Fei, L., Liu, M., Ni, Z., Sheng, Z. and Zheng, Z. (2004) RNA interference targeting VP1 inhibits foot-and-mouth disease virus replication in BHK-21 cells and suckling mice. J. Virol. 78, 6900–6907.
[36] Song, E., Lee, S.K., Dykxhoorn, D.M., Novina, C., Zhang, D., Crawford, K., Cerny, J., Sharp, P.A., Lieberman, J., Manjunath, N. and Shankar, P. (2003) Sustained small interfering RNA-mediated human immunodeficiency virus type 1 inhibition in primary macrophages. J. Virol. 77, 7174–7181.
[37] Barnett, P.V. and Carabin, H. (2002) A review of emergency foot-and-mouth disease (FMD) vaccines. Vaccine 20, 1505–1514.
[38] Suzuki, K., Mori, A., Ishii, K.J., Saito, J., Singer, D.S., Klinman, D.M., Krause, P.R. and Kohn, L.D. (1999) Activation of target-tissue immune-recognition molecules by double-stranded polynucleotides. Proc. Natl. Acad. Sci. USA 96, 2285–2290.
[39] Rott, O., Tontsch, U. and Fleischer, B. (1993) Dissociation of antigen-presenting capacity of astrocytes for peptide-antigens versus superantigens. J. Immunol. 150, 87–95.
[40] Bridge, A.J., Pebernard, S., Ducraux, A., Nicoulaz, A.L. and Iggo, R. (2003) Induction of an interferon response by RNAi vectors in mammalian cells. Nat. Genet. 34, 263–264.
[41] Pebernard, S. and Iggo, R.D. (2004) Determinants of interferon-stimulated gene induction by RNAi vectors. Differentiation 72, 103–111.
[42] Burke, D.C., Graham, C.F. and Lehman, J.M. (1978) Appearance of interferon inducibility and sensitivity during differentiation of murine teratocarcinoma cells in vitro. Cell 13, 243–248.
[43] Barlow, D.P., Randle, B.J. and Burke, D.C. (1984) Interferon synthesis in the early post-implantation mouse embryo. Differentiation 27, 229–235.
[44] Krause, D., Silverman, R.H., Jacobsen, H., Leisy, S.A., Dieffenbach, C.W. and Friedman, R.M. (1985) Regulation of ppp(A2′p)-

nA-dependent RNase levels during interferon treatment and cell differentiation. Eur. J. Biochem. 146, 611–618.
[45] Francis, M.K. and Lehman, J.M. (1989) Control of beta-interferon expression in murine embryonal carcinoma F9 cells. Mol. Cell. Biol. 9, 3553–3556.
[46] Harada, H., Willison, K., Sakakibara, J., Miyamoto, M., Fujita, T. and Taniguchi, T. (1990) Absence of the type I IFN system in EC cells: transcriptional activator (IRF-1) and repressor (IRF-2) genes are developmentally regulated. Cell 63, 303–312.
[47] Garcia-Sastre, A. (2002) Mechanisms of inhibition of the host interferon alpha/beta-mediated antiviral responses by viruses. Microbes Infect. 4, 647–655.
[48] Gobel, T.W., Schneider, K., Schaerer, B., Mejri, I., Puehler, F., Weigend, S., Staeheli, P. and Kaspers, B. (2003) IL-18 stimulates the proliferation and IFN-γ release of CD4+ T cells in the chicken: conservation of a Th1-like system in a nonmammalian species. J. Immunol. 171, 1809–1815.
[49] Hedges, S.B., Parker, P.H., Sibley, C.G. and Kumar, S. (1996) Continental breakup and the ordinal diversification of birds and mammals. Nature 381, 226–229.
[50] Zhang, Y. and Gui, J. (2004) Molecular characterization and IFN signal pathway analysis of *Carassius auratus* CaSTAT1 identified from the cultured cells in response to virus infection. Dev. Comp. Immunol. 28, 211–227.
[51] Brummelkamp, T.R., Bernards, R. and Agami, R. (2002) Stable suppression of tumorigenicity by virus-mediated RNA interference. Cancer Cell 2, 243–247.
[52] Stewart, S.A., Dykxhoorn, D.M., Palliser, D., Mizuno, H., Yu, E.Y., An, D.S., Sabatini, D.M., Chen, I.S., Hahn, W.C., Sharp, P.A., Weinberg, R.A. and Novina, C.D. (2003) Lentivirus-delivered stable gene silencing by RNAi in primary cells. RNA 9, 493–501.
[53] Heggestad, A.D., Notterpek, L. and Fletcher, B.S. (2004) Transposon-based RNAi delivery system for generating knock-down cell lines. Biochem. Biophys. Res. Commun. 316, 643–650.
[54] Lewis, D.L., Hagstrom, J.E., Loomis, A.G., Wolff, J.A. and Herweijer, H. (2002) Efficient delivery of siRNA for inhibition of gene expression in postnatal mice. Nat. Genet. 32, 107–108.
[55] Ge, Q., Filip, L., Bai, A., Nguyen, T., Eisen, H.N. and Chen, J. (2004) Inhibition of influenza virus production in virus-infected mice by RNA interference. Proc. Natl. Acad. Sci. USA 101, 8676–8681.
[56] Tompkins, S.M., Lo, C.Y., Tumpey, T.M. and Epstein, S.L. (2004) Protection against lethal influenza virus challenge by RNA interference in vivo. Proc. Natl. Acad. Sci. USA 101, 8682–8686.
[57] Woese, C.R. (2002) On the evolution of cells. Proc. Natl. Acad. Sci. USA 99, 8742–8747.
[58] Woese, C.R. (2000) Interpreting the universal phylogenetic tree. Proc. Natl. Acad. Sci. USA 97, 8392–8396.