12 RNA Interference

RNAid for Future Therapeutics?

Mustapha Diallo, Dipl Biol,
Katja Schmitz, PhD, and Ute Schepers, PhD

Contents

Summary

RNA interference (RNAi) is an evolutionarily conserved phenomenon of double-stranded (ds)RNA-mediated mRNA degradation that leads to the posttranscriptional silencing of the corresponding gene. First reports on RNAi emerged in 1998, and since then, it has become one of the most fascinating fields of molecular biology. RNAi has provided important insights about the diversity of RNA molecules and their implication in many biological processes such as the regulation of developmental genes in eukaryotic organisms. Furthermore, RNAi has rapidly developed into a powerful instrument with a great potential for functional genomics and therapeutic applications by silencing normal and disease-related gene functions. To date, the use of RNAi for genetic-based therapies is widely studied, especially in viral infections, cancers, and inherited genetic disorders. Despite the many unanswered questions on how this technology can be efficiently applied to humans, the development of novel approaches, such as vaccines or novel delivery agents, is certainly one of the major goals in RNAi research.

Key Words: RNA interference; RNAi; cancer; gene therapy; siRNA; knockout; viral infections; functional genomics.

From: *Cancer Drug Discovery and Development: The Oncogenomics Handbook*
Edited by: W. J. LaRochelle and R. A. Shimkets © Humana Press Inc., Totowa, NJ

1. INTRODUCTION

Discovered only a few years ago, RNA interference (RNAi) has already gained access to almost all biomedical and even some chemical laboratories as a technique that allows the suppression of gene function in a very effective way. RNAi is a specific form of RNA silencing that is induced by double-stranded (ds) RNA and results in sequence-specific cleavage of the homologous mRNA *(1)*. RNAi has been first observed in the nematode *Caenorhabditis elegans* by Guo and Kemphues *(2)* during antisense experiments.

However, the decisive discovery was made by Fire and Mello, who tried to explain the silencing activity of sense control RNA found in the Guo and Kemphues experiment *(1,2)*. By simultaneous injection of sense and antisense RNA strands in *C. elegans*, they obtained a 10-fold stronger effect on the silencing of homologous mRNA than with antisense RNA alone. This led to the conclusion that dsRNA triggers an efficient silencing mechanism in which exogenous dsRNA significantly reduces the overall level of target mRNA *(1,3)*.

During further investigations of the phenomenon, a number of features were discovered that made RNAi an exiting new tool for molecular biology *(4)*. RNAi is highly selective in mRNA degradation if the exogenously added dsRNA shares sequences of perfect homology with the target. Because the translation of the protein is inhibited by specific degradation of its encoding mRNA, the transcription of the gene remains unaffected. Because dsRNA homologous to intron sequences or promoters comprised by the DNA sequence showed no effect at all, the silencing appears to take place at the posttranscriptional level *(1,5,6)*. Substoichiometric amounts of dsRNA are sufficient to decrease mRNA levels within 2–3 h, and the RNAi phenotype can progress systemically (distribution between different organs) in a variety of organisms *(7,8)*. Moreover, cultured cells transfected with dsRNA maintain the loss-of-function phenotype for up to nine cell divisions *(9)*.

We know today that RNAi is related to a well-known form of posttranscriptional gene silencing (PTGS), which, until recently, was believed to occur exclusively in plants, *Drosophila* (cosuppression), and in *Neurospora crassa* (quelling) as a response to retroviral and transposable element invasion *(10–13)*. The trigger for this cellular defense mechanism is dsRNA, which occurs during replication of those elements but never from tightly regulated endogenous genes. Intermediate dsRNA will be recognized and degraded. Moreover, the RNAi machinery is presumed to carry out numerous additional functions. There is evidence that it eliminates defective mRNAs by degradation *(14)*, as there are genes that function simultaneously in RNAi and nonsense-mediated mRNA decay (NMD) *(15)*. RNAi is further assumed to tightly regulate protein levels in response to various environmental stimuli, although the extent to which this mechanism is employed by specific cell types remains to be estimated *(16)*. This suggests that RNAi is evolutionarily conserved among all eukaryotes occurring in response to the presence of dsRNA.

In the last few years, intensive molecular and biochemical studies have been undertaken to identify and characterize the participating players of the RNAi pathway. Furthermore, researchers have developed RNAi into a standard tool for in vivo reverse genetic studies in many eukaryotic systems.

This chapter will give an overview about the discovery and the current state of RNAi research and what is known about the mechanism and its implication in functional genomic studies and in designing therapeutic approaches against diseases.

2. MECHANISM OF RNAi

Soon after the discovery of RNAi, questions on the mechanism arose. Many laboratories directed their research toward the identification of the proteins implicated in the RNAi pathway and their molecular and biochemical characterization. Even though the RNAi pathway is still not fully understood, many of the main players are already well characterized and many more will follow for sure in the near future.

A first success was the correlation of RNAi with long known RNA silencing phenomena such as cosuppression and quelling. All these phenomena are now referred to as RNA silencing. Indeed, genetic studies in RNA-silencing-deficient mutants of *Arabidopsis (17)*, *N. crassa (18,19)*, and *C. elegans (20,21)* revealed several genes that are conserved throughout all three phenomena, including members of the helicase family, RNase III-related nucleases, members of the Argonaute family, and RNA-dependent RNA polymerases (RdRp).

3. DICER

Among the first proteins to be identified and thoroughly investigated was Dicer, a member of the RNase III family. Its discovery was based on the observation that dsRNA is processed to smaller fragments comprising a length of 21–25 nucleotides (nt), depending on the organism in which they are generated *(22,23)*. Such small dsRNAs, complementary to both strands of the silenced gene, were initially observed in plants undergoing transgene- or virus-induced posttranscriptional gene silencing or cosuppression *(22)*. Later, Zamore and co-workers identified the same small RNA species after the incubation of *Drosophila* S2-cell extracts with long dsRNA *(23,24)*. To date, they are known as the active dsRNA species in the RNAi pathway and are referred to as small interfering RNAs (siRNAs). They exhibit the characteristic features of dsRNA fragments originating from the cleavage of long exogenous dsRNA by a dsRNA-specific RNase III.

In addition to the already characterized members of the RNase III family such as the regular canonical RNase III and Drosha, localized to the nucleus *(25)*, homology screens of genomic data from *Drosophila* revealed new candidate genes with RNase III-like domains *(26)*. Among those candidates was a nuclease of 2249 amino acids predicted from *Drosophila* sequence data *(27)*. It contains two RNase III domains *(28,29)*, a dsRNA-binding motif (DSRM) *(30)*, an amino-terminal DexH/DEAH RNA helicase/ATPase domain, and a so-called "PAZ domain" *(31)*. All of these properties characterize members of the ribonucleases (RNase) III family. With reference to its ability to generate equally sized fragments from long dsRNA, this enzyme was called Dicer *(27,32)*.

Catalysis of the "initiator" step in the RNAi pathway by recognition and degradation of dsRNA into siRNAs has been confirmed *(27)* (Fig. 1). Although some organisms only encode one (human, mouse, *C. elegans*, and *Schizosaccharomyces pombe*) or two (*Drosophilia melanogaster*) homologs, some plants like *Arabidopsis* encode at least four Dicer-like enzymes (DCL1–4) *(33)*. The evolutionarily conserved PAZ domain *(27)* is assumed to function as a nucleic-acid-binding motif *(27,34)*. By this means, the multiplication of parasitic elements is prevented and their integration into the host genome inhibited *(23)*.

Furthermore, Dicer plays a dominant role in the processing of another small RNA species, the micro-RNAs (Fig. 1). miRNAs that also resemble a length of 21–22 nt have been recently identified in eukaryotic organisms, including plants, *C. elegans*, and humans

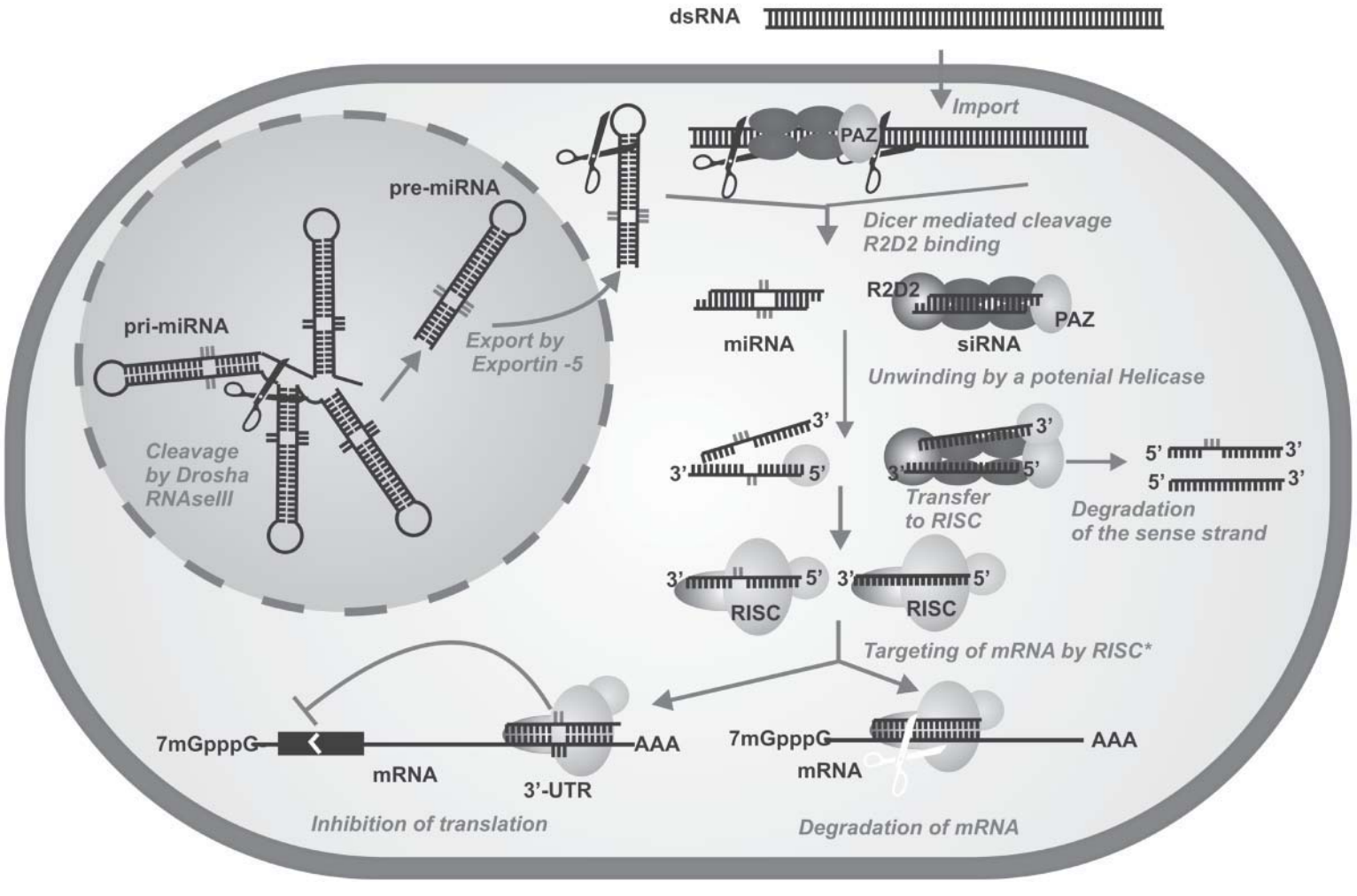

Fig. 1. Proposed mechanism of RNAi. (Modified from ref. *260*.)

(35–40). miRNAs are processed from long stem loop precursors encoded by evolutionarily conserved and nonprotein coding genes and are very heterogeneous in their structure. They have been shown to be *bona fide* regulators of gene expression by repressing transcription. Like all RNase III cleavage products, siRNAs and miRNAs bear a 5'-phosphate and a 3'-hydroxyl-group and a two-nucleotide overhang at both 3' ends. These structural features are indispensable for their capacity to exhibit their silencing activity *(41)*. In accordance with the structure of bacterial RNaseIII, in which two active centers embrace a cleft that can accommodate a dsRNA substrate, Dicer has been proposed to function as an antiparallel dimer *(42)*. Dicer contains two catalytic domains and it is suggested that in the dimer, only two of the four centers remain active to process the dsRNA into siRNAs of appropriate size *(27,43)*.

Another working model proposes a monomeric action of Dicer cleaving the dsRNA during translocation of the enzyme along its substrate *(27)*. This process would require the partial unwinding of the dsRNA, probably by the helicase domain, and an energy-consuming step to drive the translocation of the enzyme along the dsRNA in an ATP-dependent manner *(27,32,44–47)*. This mode of action might occur in *Drosophila* and other invertebrates but not in mammals, where Dicer is acting in an ATP-independent way *(45)*.

In vivo, Dicer is part of a protein complex. Even though the molecular mechanism of Dicer-mediated dsRNA cleavage is partially unraveled, it is still not fully clarified how the "initiator step" is connected to the "effector" step, because Dicer is not directly involved in the cleavage of the target mRNA *(48)*. During the last 2 yr, several proteins were identified that seem to play a role as interaction partners or even RNAi signal transporters.

The siRNAs are not moving freely throughout the cytoplasm *(49)*. To ensure a specific target recognition by siRNAs, they are bound by Dicer-associated proteins and, thus, transferred to the "effector" complex, also known as the RNA-induced silencing complex (RISC). The interaction might occur between the two PAZ domains of Argonaute-2 and Dicer, facilitating the transfer of siRNAs *(50)*.

The recently discovered protein R2D2 *(51)* harbors a tandem dsRNA-binding domain (R2) and a *Drosophila* Dicer-2-binding domain (D2), both required for the transfer of siRNAs. It assures the stabilization of the Dicer cleavage products by forming a stable complex with the nascent siRNAs and serves as the transfer shuttle for siRNA from Dicer to RISC *(51)*. Although Dicer alone is sufficient to cleave dsRNA, R2D2 is required to bind not only the nascent siRNAs but also synthetic siRNAs. It appears that newly generated symmetric siRNAs are not released from the complex but, rather, retained by DCR-2/R2D2 in a fixed orientation, which might determine the guiding strand for target cleavage *(51)*.

4. RNA-INDUCED SILENCING COMPLEX

The siRNAs serve as templates for the sequence-specific cleavage of the endogenous target mRNA. This was proven by the identification of siRNAs associated to RISC *(52)*. So far, *Drosophila* RISC was first proposed to be an approx 500-kDa complex bound to ribosomes in cell-free extracts *(49)*. Closer studies identified it as a multifunctional ribonucleotide protein complex (RNP) containing a DEAD-box helicase and a nuclease, which both seem to be conserved in *Drosophila*, *C. elegans*, and mammals, although the overlap is not complete *(53)*. Unwinding of the siRNA mediated by an ATP-dependent helicase converts RISC into its active form RISC*. The antisense siRNA strand remains bound to RISC* and serves as a guiding strand for the recognition of the target mRNA.

Target binding occurs via conventional Watson–Crick base-pairing. Perfect homology promotes the cleavage by the nuclease activity of the RISC* *(52)*.

Recent studies have shown that antisense RNA ranging from 19 to 29 nt can also enter the RNAi pathway, albeit less efficiently *(48,54)*. Because of the lack of information about the correct strand orientation, siRNA sense strands may enter the RNAi pathway. Even if the siRNAs are bound by Dicer/R2D2, they are not transferred to RISC in a predetermined orientation. On the other hand, separated antisense and sense strands of a distinct siRNA can reveal similar intrinsic efficacy in targeting their specific mRNA but show different activities when hybridized to a duplex siRNA. The stability of the 5' end determines which strand enters into RISC and which one is released for degradation *(55)*. Because 5' ends starting with a less stable A-U base-pairing are preferred over those beginning with the more stable G-C, it is suggested that the RISC helicase preferably acts from the less stable 5' end, leading to a preferential incorporation of the respective strand by RISC *(55)*. This preference is even enhanced by G:U wobble basepairs at the respective 5' end. Statistical analysis of the internal thermodynamic stability of hundreds of synthetic siRNAs has recently confirmed that a decreased stability at the 5' ends of the functional duplexes referring to the antisense strand *(55,56)* facilitates the incorporation of the antisense strand into RISC. Thus, sense strands with instable 5' ends might be incorporated by RISC, presumably leading to off-target effects *(57)*.

The siRNA single strand eventually resides in the RISC together with homologs of Argonaute proteins Ago-2, eIF2C1, and/or eIF2C2 *(48)*, which all contain a PIWI and a PAZ domain. Screening of Argonaute mutants in plants, *C. elegans*, and *N. Crassa*, established a link between this gene family and RNAi *(31)*. Even though the binding affinity for nucleic acids usually is low, PAZ domains exhibit enhanced affinity for siRNA binding *(58,59)*, recently underlined by the molecular structure of the PAZ domain *(58–60)*.

Across the examined species, mutants for genes of the Argonaute family and their homologs not only exhibit severe defects in RNA silencing but also in developmental timing.

The current research on RISC is now focusing on the antisense RNA guided degradation of the target mRNA. Target cleavage occurs between the ninth and tenth basepair from the 5 end of the guiding antisense RNA strand. Recently, a novel protein comprising five repeats of a nuclease domain known from staphylococcus bacteria could be purified in association with RISC *(61–63)*. One of the nuclease domains is fused to a Tudor domain, leading to the name Tudor–staphylococcal nuclease (Tudor-SN) *(63)*. Despite its conservation in many species, this nuclease is unlikely to be responsible for the siRNA-mediated mRNA cleavage *(63)*. Other nucleases remain to be identified.

5. RNAi IN REVERSE GENETIC STUDIES

Traditionally, the function of a gene was determined by forward genetic experiments that start with a mutant phenotype and the analysis of the protein defect and end with the conversion of the protein sequence into genetic information. Mutant phenotypes were found in patients with inherited diseases or knockout animals *(64)*. Today, most gene functions are determined by reverse genetics, which work in the opposite direction. This approach was made possible by huge progress in recombinant DNA technology and the sequencing of a variety of genomes *(65,66)*. Reverse genetic studies, sometimes also referred to as functional genomics, meet the challenge of deciphering the steadily accumulating genetic data into functional information. Meanwhile, the genetic information

of several organisms has been deciphered, including *C. elegans*, *D. melanogaster*, and humans *(65,66)*. So far, many methods have been developed to manipulate the expression of genetic information at different levels.

Gene silencing at the genomic level mainly proceeds by subjecting the isolated wild-type gene to in vitro mutagenesis by nucleotide substitution, deletion, or insertion. The mutated gene is then placed back into cultured cells or into the organism of interest, where it replaces the functional gene after homologous recombination. This method has been especially exploited to silence genes in animals such as mice and flies. Many practical approaches have been published, including inducible systems that enable the spatial, tissue-specific, and temporal inhibition of gene expression *(67–69)*. Many of these methods are very time-consuming and laborious.

Other common methods act at the posttranslational level, such as the depletion of proteins by antibodies masking the protein, inhibitors that block protein function by imitating substrates, or docking sites for other interaction partners, as well as RNA-based aptamers and intramers *(70,71)*. With the discovery of RNAi, posttranscriptional silencing is receiving more and more interest.

In antisense technology the target mRNA is bound by homologous strands of antisense DNA, modified DNA, or PNA (peptide nucleic acids) to prevent the binding of the ribosome and, thus, translation. Many modifications have been introduced to stabilize the antisense binding partner and a few systems have made their way to clinical trials. Yet, antisense technology has never quite met its high expectations, whereas RNAi with its catalytic nature seems to create a real hype.

In the generation of loss-of-function phenotypes, RNAi procedures are much faster and straightforward than traditional genetic approaches *(64)*, certainly displaying the method for initial and high-throughput experiments.

In addition, it offers many advantages over other methods comprising specificity and efficiency. Only mRNA sharing perfect homology with catalytic amounts of exogenously applied dsRNA is cleaved, whereas other mRNAs, even those with point mutations, remain unaffected *(1,72,73)*. It further offers a very simple handling. In *C. elegans*, RNAi can be induced by simply injecting adult worms with dsRNA, by soaking the animals in the dsRNA, by electroporation of dsRNA, or by engineering *Escherichia coli* to produce the appropriate dsRNA and feeding the bacteria to the worms *(3,9,74–76)*. Soaking also functions in *Drosophila* S2 cell culture *(77)*. Moreover, in plants and worms, the RNAi effect can diffuse across tissue boundaries and can be transmitted to the progeny *(78,79)*. Moreover, RNAi can be used to simultaneously silence several genes.

Many techniques have already been established by a variety of laboratories, as described earlier. In *C. elegans*, RNAi has already yielded impressive results in investigating the functions of the whole genome *(80)*, including genes implicated in cell division *(81,82)*—fat regulatory genes *(83)*.

Genomewide RNAi screens became feasible with the generation of a library of bacterial strains that each produce dsRNA for an individual nematode gene. The current library contains 16,757 bacterial strains targeting approx 86% of the 19,427 currently predicted genes of the *C. elegans* genome. The loss-of-function phenotype when performing systemic RNAi on a genomewide scale is estimated to be approx 65% *(75,84)* (For review, see refs. *83* and *85–87*). These investigations revealed not only a detailed knowledge about the function of the targeted genes, but also allowed to estimate its relationship to conserved homologs in other species.

Like *C. elegans*, *Drosophila* is a prominent organism for genomewide functional RNAi studies. A huge number of *Drosophila* genes have been silenced since the early days of RNAi research, starting with frizzled-2 and wingless, which are involved in wing development *(88)*. Recently, a genomewide RNAi screen in *Drosophila* Schneider cells (S2 cells) has been reported for the study of phenotypes affecting cell morphology *(89,90)*. Moreover, RNAi has been used to successfully dissect mitosis and cytokinesis and to unravel cell signaling pathways in *Drosophila* tissue culture and cell lines *(91,92)*. All *Drosophila* kinesins and cytoplasmic dynein have been targeted for mitotic phenotypes in S2 cells. For the analysis of functional redundancy and coordinated activity, RNAi was subsequently performed to simultaneously target multiple kinesin genes, an approach that was made feasible only by the RNAi technique *(93)*.

6. RNAi IN MAMMALS

Although RNAi constitutes a very powerful tool for studying gene functions in plants and invertebrates, its application in mammalian systems turned out to be a major problem because mammals have evolved a different and more elaborate response to dsRNA. In mammals, dsRNA longer than 30 bp mediates an interferon response, which leads to the simultaneous activation of RNase H, which unspecifically degrades all mRNA transcripts, and protein kinase R (PKR). The latter phosphorylates and, thus, inactivates transcription factor eIF2α leading to a global shutdown of protein biosynthesis and as a result to apoptosis *(94,95)*.

Despite the first impression that RNAi would not work in mammalian cells, several independent groups proved the existence of mammalian RNAi pathways by the introduction of dsRNA or vectors producing dsRNA into cell lines lacking the interferon machinery, like mice oocytes or mice embryonic cancer cell lines *(96,97)*. The most important experiment that established RNAi as the same powerful tool in mammals has been performed by Tuschl and co-workers *(98)*, who used synthetic 21-nt duplexes (siRNAs) to trigger the RNAi pathway. They achieved the knocking down of the activity of transfected and endogenous genes without induction of the interferon response. Obviously, the siRNAs, which act as the active intermediate of RNAi, are too short for the activation of PKR. dsRNAs shorter than 21 bp and longer than 25 bp are inefficient in initiating RNAi as well as siRNAs with blunt ends *(41)*. Only short dsRNAs with a two-nucleotide 3-overhang resembling the naturally active products of Dicer are efficient mediators of RNAi. With this technology, even somatic primary neurons have been successfully treated to produce knock-down RNAi phenotypes *(99)*.

Investigation of the minimal chemical requirements for siRNAs and a detailed mutation scan suggest that the 3'-end modification of the antisense strand usually reduces activity, and mispairing is more crucial for the first 10 nucleotides from the 5'-end of the sense strand. The 10th nucleotide seems to be important for RISC-mediated cleavage of the target mRNA *(41,48,54,100,101)*. Further, the 5'-phosphate residue is essential for siRNAs to direct target–RNA cleavage, but nonphosphorylated siRNAs have been shown to be phosphorylated in vivo by a cytosolic kinase prior to their entrance into the RNAi pathway *(54)*. Shortly after the discovery of siRNA-mediated RNAi in mammals, empirical rules were put up for the design of efficient synthetic siRNAs that are now often referred to as the Tuschl rules *(56)*.

The recent finding that only one strand of the siRNAs enters the RISC depending on thermodynamic properties will help to design siRNAs displaying an even higher efficacy *(55)*.

Novel rules for the design of siRNAs and algorithms, which are based on those rules and consider the accessibility of siRNA binding sites on the secondary structure of the target mRNA, are currently being developed and refined *(55,56,102)*. They also circumvent off-target effects by evaluating the antisense sequence in a Smith–Waterman or BLAST search for possible targets.

To date, the use of siRNAs has become a state-of-the-art tool for the study of gene function in mammals and in cultured mammalian cells. Depending on the type of cells, different chemical and physical methods are used to deliver siRNAs, such as liposom-mediated transfection *(98,103–105)*, electroporation *(106)*, and microinjection. The most common delivery method so far is the regular transfection as described for DNA. Even though electroporation has come into focus lately, transfection is still the most reliable technique, especially for the delivery into adherent cells, such as HeLa, NIH3T3, or 293T cells. It should be mentioned that transfection by calcium phosphate is not as efficient for siRNA as it is for DNA *(107,108)*. Following the use of calcium phosphate and polybrene, various cationic liposomal formulations were developed to increase transfection efficiency to up to 90%, depending on the cell type.

Electroporation is an alternative, which should be considered for nondividing cells or cells resistant to chemical transfection reagents *(109–112)*. Other approaches are exploiting peptides such as the short MPG that forms stable noncovalent complexes with nucleic acids. MPG is a chimeric protein composed of gp41 (the human immunodeficiency virus [HIV-1] fusion peptide domain) and the nuclear localization sequence (NLS) of SV40 large T antigen. Using the MPG bearing a mutation in its NLS prevents nuclear entry and distributes the siRNAs throughout the cytosol *(113)*. Other approaches use the great versatility of cell-penetrating peptides (CPPs) *(114–118)* with respect to cargo and cell type as a valuable tool for the introduction of siRNAs into mammalian cells and even fully grown organisms. The covalent coupling of CPPs with siRNAs yields the so-called pepsiRNAs (peptide-coupled siRNAs) *(119,120)*.

The use of siRNAs has already helped to screen a large number of mammalian genes for their function and to unravel the molecular basis of several important biochemical processes like signal transduction, cell cycle regulation, development, cell motility, cell death, and many more.

The number of targeted mRNAs is constantly increasing together with the validation of a vast number of siRNAs. The field is constantly growing and the first genomewide studies have already been carried out but have just been published in part *(121)*.

Until now, many gene functions responsible for embryogenesis or stem cell differentiation remain to be determined. Although these systems have hardly been accessible by traditional methods, RNAi appears as a practical approach that has already contributed to the characterization of many developmental genes and, thus, constitutes a promising tool for the elucidation of mechanisms involved in development and disease.

The siRNA-mediated silencing of a single isoform of shcA in HeLa cells predicted a crucial role of this protein in regulating cell proliferation *(122)*. Likewise, siRNA-mediated silencing of the phosphatidylinositol 3-kinase causes a drastic decrease in growth and tissue invasiveness of tumor cells *(123)*. By RNAi, the IP3 receptor-1 in germinal vesicle-intact oocytes was found to be responsible for intracellular calcium oscillations, the first steps of development after insemination *(124)*. RNA interference was also used to silence several molecular players of the cell cycle and DNA replication *(125–128)*. The identification and characterization of novel proteins intervening in embryonic *(129,130)*

and neuronal development *(99)* are further examples of insights made possible by this new technique.

Its versatily, its high specificity, and the minute amounts required to inhibit gene function render RNAi a highly promising technique to combat diseases. Because RNA silencing serves as a defense against retroviruses in plants and invertebrates it appears only challenging to reintroduce RNAi to fight viruses in mammals and humans. Many studies have been performed to treat HIV-1, herpes simplex virus, and hepatitis B and C by dsRNA-based approaches. Moreover, the high sensitivity toward point mutations qualifies RNAi as a potential tool for the cure of cancers and inherited diseases. In a study of the Ras oncogene, it was possible to target mutated Ras without affecting unmutated Ras, demonstrating the exquisite specificity of RNAi as a therapeutic tool. However, the same specificity could pose problems for therapeutics against viral infection. Viral escape from RNAi selection in poliovirus by mutation of the target sequence has already been demonstrated. The high specificity is already put into doubt by recent findings that the RNAi mechanism can tolerate some sequence mismatches, particularly away from the middle cleavage site. In fact, a recent in vitro study showed that some genes with incomplete homology could be partially silenced, an effect that was more pronounced at higher concentrations of siRNA.

Site-specific delivery of siRNAs broadens the list of genes that can be silenced without inducing toxicity. To target mutated sequences in inherited diseases, the individual gene mutation needs to be determined in order to synthesize appropriate siRNAs. This patient-specific approach would be much more costly and difficult to execute. Therefore, the in vitro studies of RNAi carried out to date focus mainly on viral infection and cancer, which are likely to be the areas in which siRNAs make their way to clinical studies.

The siRNAs are further being studied as therapeutic treatment against genes involved in autoimmuninty, neurodegenerative diseases like Alzheimer's disease, infectious diseases, and inflammatory response and many more.

To date, many prerequisites still need to be fulfilled and obstacles overcome before RNAi can make its way into clinical studies. First of all, silencing is often incomplete—a knockdown rather than a knockout—and residual gene expression might be sufficient to maintain the present phenotype, especially if only low amounts of protein are required to fulfill their cellular function.

Some residual gene expression might be attributed to untransfected cells or to a low affinity of the selected siRNAs toward the target mRNA. In rapidly dividing transformed cells, the silencing effect is rather short-lived, as the transfected siRNAs are rapidly diluted.

Further obstacles of the clinical application of RNAi often are insufficient transfection efficiency and the limited persistence of the transient RNAi phenotype. To obtain persistent RNA silencing in cells and organisms, different plasmid and viral vectors were developed to express short hairpin RNAs under the control of RNA polymerase III (Pol III) and RNAse polymerase II (Pol II) promoters. Upon transfection of these vectors, siRNAs can be constitutively and endogenously expressed.

The currently used vectors employ short hairpin RNA (shRNA) expression cassettes that resemble pre-miRNAs and undergo processing by Dicer *(131–136)*. They are designed according to the same rules as synthetic siRNAas to match perfectly with the target mRNA and, thus, trigger its degradation. To achieve a constitutive silencing effect, the shRNA transcript must translocate from the nucleus to the cytoplasm, where it has to be recognized by Dicer and transferred to RISC. siRNA hairpin-expressing plasmids can specifically

suppress gene expression in a transient or persistent manner depending on their design. Thus, loss-of-function phenotypes can be generated if longer periods of time are required to fully deplete the cytosol of residual target protein. The potency of shRNAs to trigger RNAi in mammalian cells is comparable to synthetic siRNA duplexes.

In many cases, strong RNA polymerase III promoters, such as the human H1 and the murine U6, are employed to control the expression of the shRNAs. *(131–136)*. In vivo, RNA polymerase III is responsible for the transcription of a limited number of genes, including 5S RNA, tRNA, 7SL RNA, U6 snRNA, and a number of other small stable RNAs that are involved in RNA processing *(137)*.

It is assumed that long dsRNAs are a more effective trigger because they are more efficiently processed into siRNAs *(49)*. This can be the result of a highly cooperative binding of long dsRNA strands or additional features of cleavage by Dicer, such as incorporation of the nascent siRNAs into transfer protein complexes *(138)*.

Despite some concerns, which are discussed within the field of RNAi, it was recently shown that endogenously expressed long hairpin dsRNAs (lhRNAs) are capable of inducing RNAi in mammalian somatic cells including human primary fibroblasts, melanocytes, HeLa cells *(139,140)*, and even whole mice *(141)*.

To be taken up by cells or tissues, those expression vectors require classical methods like electroporation, microinjection, or liposomal transfer of the DNA precursor vector. Most cell lines are easy to transfect and recombinant cell clones permanently expressing RNAi phenotypes can be selected. However, without cell division, the shRNA (DNA) construct cannot enter the nucleus as required for DNA transcription and it passively resides in cytosol. Therefore, most nondividing cells are not susceptible to transfection, as are primary cells and stem cells. A number of important cell types have been resistant to the introduction of both siRNAs and shRNAs because of the lack of an appropriate delivery system.

Even more problematic is the delivery of siRNAs directly to entire vertebrate animals. High toxicity of most cationic transfection reagents prohibits whole-body application of siRNAs aided by liposomal or chemical approaches, whereas physical techniques like the hydrodynamic transfection method was shown to be successful in mice. Naked siRNAs applied to mice via tail-vein injection caused the knock down of a reporter gene by 80–90% in the liver, kidney, spleen, lung, and pancreas *(142,143)*. Yet, the effect is rather short-lived, lasting only a few days, and not all organs and cell types are accessible.

Another method to circumvent many of those difficulties makes use of viral vectors to infect cells with the dsRNA expression construct. Retroviral vectors *(144)*, like adenoviral vectors, and predominantly lentiviral vectors *(145,146)* are currently being used as viral delivery systems. By this approach, almost every cell or tissue, including stem cells and neurons, can be treated with shRNA-producing vectors.

The advantages of the lentiviral approach lies in the possiblity of systematically testing a gene function in the context of the entire organism. Further animal models can be generated in a straigthforward manner to determine which genes are important to the function of different tissues and organs and which might be effective therapeutic targets in diseases.

Even though Tuschl and co-workers *(98)* observed that siRNAs shorter than 30 nt do not trigger an interferon response, recent reports are concerned about nonspecific effects induced by endogenously expressed shRNAs. Both transfection of siRNAs and transcription

of shRNAs leads to an interferon (IFN)-mediated activation of the Jak–Stat pathway and global upregulation of interferon-stimulated genes within the nucleus *(147–149)*. It was found that the dsRNA-dependent protein kinase (PKR) is activated by the intracellular presence of 21-bp siRNAs and triggers the upregulation of IFN and possibly other, cellular signaling molecules. Comparative studies on interferon induction by siRNAs and their shRNA counterparts showed a significantly stronger interferon response after the application of shRNA *(149)*, so that these side effects need to be studied more closely to settle the current controversy. It has to be kept in mind that almost all plasmid vectors can induce interferon response upon transfection independent of the type of insert they are bearing *(150)*.

7. RNAi AND GENE THERAPEUTIC SETTINGS

Therapeutic strategies for siRNA are based on silencing of disease-related genes without altering the expression of other essential genes. At the beginning of RNAi research, it was assumed that targeting mRNAs with homologous siRNAs or dsRNA does not influence the expression of other mRNAs. To date, we know that a single knockdown of a gene can induce a variety of changes in the overall gene expression. As long as we cannot distinguish the changes resulting from the silencing of a single gene from those caused by unspecific effects, the approach resembles all of the benefits and drawbacks that were already described for gene therapy.

7.1. Infectious Diseases

The first approach aiming at the use of RNAi in therapeutic applications was the silencing of viral genes. With regard to the number of outbreaks and people infected worldwide, several groups of viruses are currently recognized as the most important human pathogens. Among those, aquired immunodeficiency syndrome (AIDS), hepatitis, and influenza are viral diseases of global dimension, bringing along high morbidity and mortality. Influenza even occurs in annual epidemics and in pandemics of infrequent occurrence but very high attack rates *(151)*. Although RNAi is an ancient antiviral defense in plants and invertebrates, it does not play an important role in mammals. Nevertheless, the RNAi machinery is present in mammals and might be exploited to inhibit viral infections upon triggering by siRNAs.

So far, many groups have been successful in activating the pathway against viral targets with diverse replication strategies, including HIV *(136,152–162)*, hepatitis B and C (HBV/HCV) *(163–170)*, human papilloma virus (HPV) *(171–173)*, polio virus *(174,175)*, herpes virus *(176)*, human T-cell leukemia virus-1 *(177)*, respiratory syncytial virus *(178)*, influenza *(179)*, Epstein–Barr virus *(180)*, and, finally, corona viruses such as SARS *(181–185)*.

At the beginning, mostly genes of the respective virus were targeted. Bitko and Barik were the first to observe specific antiviral effects of siRNA against the respiratory syncytial virus. Shortly after that, the same effects were reported for poliovirus in infected human cells *(174)* and HIV-1 using siRNAs directed against different regions of the HIV-1 genome, including its long terminal repeat and the genes encoding for the highly conserved genes *vif* and *nef* *(152)*. siRNA-treated cells were particularly immune to subsequent HIV-1 infection in short-term experiments. Novina and co-workers used a contrary approach that notably reduced the ability of the HIV-1 to enter cells by targeting the CD-4 receptor, which is responsible for HIV-1 entry into the host cells. This indicates that the viral infectivity can also be stopped if host genes involved in the viral life cycle are targeted *(162)*.

These studies clearly show that RNAi will hold its promise to become an efficient tool in the treatment of viral and other infectious diseases in human. However, before RNAi can make its way to therapeutic applications, an appropriate siRNA delivery system is required to guide the active species to the target organs. Interestingly, siRNA delivery to mice by hydrodynamic injection into the tail vein has led to a gene downregulation of 90% in the majority of liver cells. However, this technique requires high pressure and large volumes of siRNA solutions, making it inappropriate for application in humans *(142,143)*. On the other hand, after the coinjection of luciferase-targeting siRNA and plasmid-luciferase into the tail vein of mice, gene silencing was observed in several organs, including the liver, spleen, kidney and lung. Mice have been also healed for 10 d from Fas-induced fulminant hepatitis by the injection of the appropriate siRNAs *(168)*.

More striking results were obtained on HBV. RNAi could inhibit the production of HBV replicative intermediates in cell culture and in immunocompetent and immunodeficient mice transfected with an HBV plasmid *(186)*. Upon siRNA treatment, substantially reduced levels of HBV RNAs and replicated HBV genomes were found in mouse liver. In these studies, most of the targets are genes that are relatively conserved between different viral strains and exhibit a low mutation activity, as found for the genes essential for replication. Likewise, host genes required for viral entry or playing an essential role in the viral life cycle are also potential targets *(187)*.

In spite of the great excitement created by those studies, it has to be kept in mind that most of the experiments were performed in cell culture or model animals using laboratory strains of viruses that are well characterized.

Some recent studies indicated that a therapeutic treatment of viral infection does not stand up to its expectations *(188,189)*. In the long term, siRNA directed against the highly conserved *Nef* gene (siRNA-Nef) confers resistance to HIV-1 replication. However, the inhibition of replication is not complete. After several weeks of culture, HIV-1 escape variants appeared that were no longer inhibited by siRNA-Nef because of nucleotide substitutions or deletions in the *Nef* gene that altered the homology to the siRNA-Nef sequence *(188,189)*. To minimize the risk of viral escape, several viral and host genes must be targeted simultaneously.

Other infectious diseases such as those spread by parasites are also the subject to therapeutical RNAi approaches *(190–195)*. Protozoan parasites and pathogenic fungi often resist manipulation by standard molecular genetic approaches. The discovery of RNAi in *Trypanosoma brucei* provides a convenient method for generating knockout phenotypes of the parasite *(196)*. Further, erythrocyte-infecting stages of the malaria parasite *Plasmodium falciparum* were successfully treated with dsRNA encoding the dihydroorotate dehydrogenase (DHODH) *(192)*.

The application of RNAi to fight diseases reaches its limit when it comes to bacterial infections, because prokaryotes are not amenable to silencing by siRNA. Yet, the manipulation of the host genes involved in bacterial invasion or in immune response might help to immunize cells and tissues against bacterial infections *(197)*. The recent discovery of the DNA or RNA editing roles of prokaryotic PIN domains, which are strikingly numerous in thermophiles and in organisms such as *Mycobacterium tuberculosis* now supports the idea that, similar to their eukaryotic counterparts, bacterial PIN domains participate in a related RNA silencing mechanism and nonsense-mediated RNA degradation *(198)*. After an initial flood of mainly descriptive reports about the effects of bacterial infection, transcriptome and proteome studies are now becoming more refined in their approach

and are shedding light on the role of pathogen–host interactions, which eventually can be targeted by RNAi *(199)*. First, RNAi experiments targeting pro-inflammatory cytokines (interleukin-1 [IL-1], or tumor necrosis factor α [TNF-α]) successfully reduced the sepsis triggered by lipopolysaccharide (LPS) in mice *(200)*. The intervention with the peroxisome proliferation-activated receptor (PPAR)-γ-dependent anti-inflammatory mechanism for the treatment of chronic inflammation is only one of many further possibilities *(201)*.

7.2. Cancer

Several in vitro studies have already demonstrated the potential use of RNAi in cancer treatment. Cancer cells usually differ from normal cells by their uncontrolled growth and the ability to escape programmed cell death (apoptosis). For nutrition supply, they assemble a network of blood vessels around tumors, and to evade chemotherapy, they might change surface composition. Therefore, targets for a possible RNAi treatment are genes that are either involved in cell division and proliferation such as growth factors, tumor suppressor genes, transcription factors, and apoptosis inhibitors *(106,171,202–217)* or proto-oncogenes that take part in many regulatory events and signaling cascades such as Ras and Bcl-2 *(208)*, as well as viral oncogenes, such as from human papilloma virus (HPV). Impaired apoptosis signaling is associated with tumor development and confers resistance to chemotherapy and apoptosis triggered by the death receptor pathway *(204)*. In many tumors, antiapoptotic proto-oncogenes are overexpressed and inhibition of their expression has potential to facilitate tumor necrosis factor-related apoptosis inducing ligand (TRAIL)-induced apoptosis *(218–220)*. Multidrug resistance proteins that pump chemotherapeutic drugs out of tumors and factors that stimulate angiogenesis to connect the tumor to nutrition resources are also potential RNAi targets *(221–235)*.

A selection of targets is listed in Table 1. Well-known targets are Bcr-Abl, mutated Ras, human papilloma virus (HPV) E6 and E7, and proto-oncogenes like bcl-2 and c-myb.

Bcl-2 (B-cell lymphoma protein 2) targeting by RNAi is a promising example of how apoptosis can be triggered in tumor cells *(106,210,214,218,236)*. Bcl-2 is an important regulator of programmed cell death, and its overexpression has been implicated in the pathogenesis of some lymphomas. Resistance to chemotherapy, at least in vitro, might also be related to *Bcl-2* overexpression. In a comparative study, single siRNAs or combinations of siRNAs were successfully transfected into HeLa cells, lung adenocarcinoma cells, hepatoma cells, ovarian carcinoma cells, and melanoma cells with cationic lipid complexes. Downregulation of other proto-oncogenes and apopotosis inhibitors, such as cdk-2, mdm-2, pkc-alpha, tgf-beta1, h-Ras, and vegf, effectively suppressed the proliferation of cancer cells to different extents, leading to the conclusion that chemically synthesized and vector-driven siRNAs can inhibit the growth and proliferation of cancer cells *(219)*.

Ras is a powerful regulator of several interconnected receptor-signaling pathways. In its mutated form, it is constitutively active and acts as an oncogene. This process is thought to contribute to malignant transformation in many cell types, which makes elements of this signaling pathway attractive targets for inhibition by RNAi *(237)*. Previous attempts to silence the Ras oncogene with phosphorothioate oligonucleotides succeeded in stabilizing the disease. Silencing of crucial effectors in Ras signaling like the Raf-c kinase by oligonucleotides shows low to moderate effects in vitro and in an in vivo tumor–xenograft model, whereas treatment with siRNAs can specifically silence expression of oncogenic K-Ras in tumor cells *(238,239)*. A tumor suppressor that is often genetically altered is Bcr-Abl. However, targeting the Abl sequence in Bcr-Abl by RNAi might be a therapy for

Table 1
Selection of Cancer-Related Genes Subjected to si/shRNA Treatment

Gene	*Cancer Types*	*Cell Types*	*RNA Species*	*Mode of Action*	*Ref.*
k-Ras	Most tumors		shRNA/lentivirus	Proliferation, cell division	*219,238,239*
Bcl-2	Colorectal carcinoma cells, cervix carcinoma, leukemia	HeLa cells, lung adenocarcinoma cells, hepatoma cells, ovarian carcinoma cells, melanoma cells, etc.	siRNAs	Apoptosis inhibitor, chemotherapy resistance	*204,210,214, 218,219*
VEGF	Most cancer types	HeLa cells, lung adenocarcinoma cells, hepatoma cells, ovarian carcinoma cells, and melanoma cells, K562	siRNAs, shRNas/vector	Angiogenesis	*219,223*
Bcr-Abl	Chronic myeloid leukemia	Leukemic cells	siRNAs, shRNAs / lentivirus	Chemotherapy resistance	*211,261*
MDR1	Human pancreatic carcinoma, gastric carcinoma	EPP85-181RDB EPG85-257RDB	siRNAs	Chemotherapy resistance	*262*
HPV-E6/E7	HPV-positive tumors	HeLa	siRNAs shRNA/vector	Apoptosis inhibitor	*173,263*
CXCR4	Breast cancer,	Breast cancer cells	siRNAs	Cell migration	*264*
Wee1 Chk1 Myt1 Cdc2	tumors	HeLa cells, lung adenocarcinoma cells, hepatoma cells, ovarian carcinoma cells, melanoma cells, etc.	siRNAs	Apoptosis inhibitor, cell cycle control	*203,219*
Eph2 receptor	Pancreatic adenocarcinoma	PANC1, MIAPaCa2, BxPC3,Capan2	siRNAs	Growth factor, tumor invasiveness, metastasis	*202*
FASE	Prostate cancer	HeLa	siRNas	Proliferation, apoptosis inhibitor	*265*
Tie-2	Tumors	Endothelial cells	siRNAs	Angiogenesis	*187,225*

chronic myeloid leukemia. This is only a selection of commonly studied target genes that is supplemented by Table 1. However, the major drawback for the application in therapy so far remains the issue of delivery. Despite the specific targeting of proto-oncogenes that leaves the expression of the wild-type gene unaltered, many of the other target proteins also play their role in nonproliferating cells and healthy organs. Downregulation of those targets requires methods that allow direct delivery to the tumor without affecting other cells. Those delivery agents need to be tailor-made to each tumor and the different organs from which the tumor is derived. So far, many approaches have been made with either modified dendrimers and liposomes or modified lentiviruses and adenoviruses. To date, no reliable results have been obtained.

7.3. Cardiovascular and Neurodegenerative Diseases

Apart from cancer and infectious diseases, cardiovascular diseases cause the highest mortality in the Western world. The development of strategies for the prevention of those diseases is, therefore, of high priority. RNAi is currently used to elucidate the underlying mechanisms of cardiovascular diseases *(230,240,241)*; however, therapy is still way out of reach. Likewise, the number of patients with neurodegenerative diseases such as Alzheimer's disease, Parkinson's disease, or amyotrophic lateral sclerosis is growing with the increasing life-span of humans.

Many studies support the therapeutic potential of RNAi-based methods for the treatment of diseases like myotrophic lateral sclerosis *(242–244)*. The search for possible candidate genes responsible for Alzheimer's disease *(245–251)* and Parkinson's disease *(252–254)* is proceeding at a rapid pace. However, delivery is even more a problem when the trespassing of the blood–brain barrier is involved.

Neurones in culture are very sensitive, and transfection efficiencies by conventional methods are poor. In vivo, the blood–brain barrier prevents the uptake of substances from the remaining vascular system with the exception of small (less than 500 Da) and lipid-soluble molecules and, consequently, reduces the bioavailability of intravenously applied oligonucleotides in the brain to virtually zero *(255)*.

Even though it is already possible to treat primary neurons and other neuronal cell lines with siRNAs *(99,256,257)*, in vivo studies in mice are just at their beginning, and many of methods published so far bear their drawbacks. Nonetheless, it could be shown that adenoviral vectors successfully passed the blood–brain barrier after intravenous application and were expressed in the brain *(258)*. Others injected siRNA expressing adenoviral vectors directly into the brain and observed a decent RNAi phenotype *(259)*.

8. PERSPECTIVES ON THE FUTURE

In a time when biologists are facing the enormous challenge of decrypting genetic messages encoded by whole genomes, the RNAi technique represents a precious tool. The use of either synthetic or stably expressed siRNAs will greatly facilitate and accelerate genomewide functional screening in model organisms and navigation within the decrypted genetic landscape. Also, siRNA-mediated gene silencing will be one standard method in the studying of specific roles of proteins in integrated cellular pathways. However, although RNAi has turned out to be an efficient approach to downregulate viral activity in cultured mammalian cells, its application as a therapeutic approach in humans will

only be feasible if all risks of unspecific responses and other side effects have been eliminated. This demands a better understanding of the RNAi-related processes, particularly the correlation between structural features of the natural miRNAs and their strictly specific mode of action. This, in turn, will provide a basic reference design of siRNAs to be used for therapeutic purposes. At last, an appropriate system of siRNA delivery in vivo still needs to be developed.

RNAi is now one of the largest growing fields in biology, pharmacology, medicine, and even chemistry. Although the mechanism by which dsRNA modulates gene expression is not at all clarified, the development of novel approaches to cure diseases related to gene expression has proceeded to the point at which in vivo experiments in animal models are afoot. The recent development of leukemia cells and other cancer cells resistant to the well-known small-molecule inhibitors is calling for novel treatments. Even though a cell might be able to evolve mutated proteins that evade a small-molecule protein inhibitors, this does not happen if the encoding mRNA is degraded. RNAi might have held many promises, as yet, there is no experience in patients. Without doubt, the time to celebrate significant achievements in the clinical trials will be forthcoming soon.

REFERENCES

1. Fire A, Xu SQ, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. Nature 1998; 391(6669):806–811.
2. Guo S, Kemphues KJ. Par-1, a gene required for establishing polarity in *C. elegans* embryos, encodes a putative Ser/Thr kinase that is asymmetrically distributed. Cell 1995; 81(4):611–620.
3. Timmons L, Fire A. Specific interference by ingested dsRNA. Nature 1998; 395(6705):854.
4. Schmitz K, Schepers U. Silencio: RNA interference, the tool of the new millennium. Biol Chem 2004; in press.
5. Montgomery MK, Xu SQ, Fire A. RNA as a target of double-stranded RNA-mediated genetic interference in *Caenorhabditis elegans*. Proc Natl Acad Sci USA 1998; 95(26):15,502–15,507.
6. Montgomery MK, Fire A. Double-stranded RNA as a mediator in sequence specific genetic silencing and co-suppression. Trends Genet 1998; 14(7):255–258.
7. Timmons L, Tabara H, Mello CC, Fire AZ. Inducible systemic RNA silencing in *Caenorhabditis elegans*. Mol Biol Cell 2003; 14(7):2972–2983.
8. Winston WM, Molodowitch C, Hunter CP. Systemic RNAi in *C. elegans* requires the putative transmembrane protein SID-1. Science 2002; 295(5564):2456–2459.
9. Tabara H, Grishok A, Mello CC. RNAi in *C. elegans*: soaking in the genome sequence. Science 1998; 282(5388):430–431.
10. Voinnet O. RNA silencing as a plant immune system against viruses. Trends Genet 2001; 17(8):449–459.
11. Ruiz MT, Voinnet O, Baulcombe DC. Initiation and maintenance of virus-induced gene silencing. Plant Cell 1998; 10(6):937–946.
12. Ketting RF, Haverkamp TH, van Luenen HG, Plasterk RH. Mut-7 of *C. elegans*, required for transposon silencing and RNA interference, is a homolog of Werner syndrome helicase and RNaseD. Cell 1999; 99(2):133–141.
13. Tabara H, Sarkissian M, Kelly WG, et al. The rde-1 gene, RNA interference, and transposon silencing in *C. elegans*. Cell 1999; 99(2):123–132.
14. Plasterk RHA. RNA silencing: the genome's immune system. Science 2002; 296(5571):1263–1265.
15. Domeier ME, Morse DP, Knight SW, Portereiko M, Bass BL, Mango SE. A link between RNA interference and nonsense-mediated decay in *Caenorhabditis elegans*. Science 2000; 289(5486):1928–1930.
16. McManus MT, Petersen CP, Haines BB, Chen J, Sharp PA. Gene silencing using micro-RNA designed hairpins. RNA 2002; 8(6):842–850.
17. Mourrain P, Beclin C, Elmayan T, et al. Arabidopsis SGS2 and SGS3 genes are required for posttranscriptional gene silencing and natural virus resistance. Cell 2000; 101(5):533–542.
18. Cogoni C, Macino G. Gene silencing in *Neurospora crassa* requires a protein homologous to RNA-dependent RNA polymerase. Nature 1999; 399(6732):166–169.

19. Cogoni C, Macino G. Isolation of quelling defective (QDE) mutants impaired in posttranscriptional transgene-induced gene silencing in neurospora crassa. Proc Natl Acad Sci USA 1997; 94(19):10,233–10,238.
20. Smardon A, Spoerke JM, Stacey SC, Klein ME, Mackin N, Maine EM. EGO-1 is related to RNA-directed RNA polymerase and functions in germ-line development and RNA interference in *C. elegans*. Curr Biol 2000; 10(4):169–178.
21. Qiao L, Lissemore JL, Shu P, Smardon A, Gelber MB, Maine EM. Enhancers of glp-1, a gene required for cell-signaling in *Caenorhabditis elegans*, define a set of genes required for germline development. Genetics 1995; 141(2):551–569.
22. Hamilton AJ, Baulcombe DC. A species of small antisense RNA in posttranscriptional gene silencing in plants. Science 1999; 286(5441):950–952.
23. Zamore PD, Tuschl T, Sharp PA, Bartel DP. RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. Cell 2000; 101(1):25–33.
24. Yang D, Lu H, Erickson JW. Evidence that processed small dsRNAs may mediate sequence-specific mRNA degradation during RNAi in *Drosophila* embryos. Curr Biol 2000; 10(19):1191–1200.
25. Wu H, Xu H, Miraglia LJ, Crooke ST. Human RNase III is a 160-kDa protein involved in preribosomal RNA processing. J Biol Chem 2000; 275(47):36,957–36,965.
26. Filippov V, Solovyev V, Filippova M, Gill SS. A novel type of RNase III family proteins in eukaryotes. Gene 2000; 245(1):213–221.
27. Bernstein E, Caudy AA, Hammond SM, Hannon GJ. Role for a bidentate ribonuclease in the initiation step of RNA interference. Nature 2001; 409(6818):363–366.
28. Rotondo G, Frendewey D. Purification and characterization of the Pac1 ribonuclease of *schizosaccharomyces pombe*. Nucleic Acids Res 1996; 24(12):2377–2386.
29. Mian IS. Comparative sequence analysis of ribonucleases Hii, Iii, Ii Ph and D. Nucleic Acids Res 1997; 25(16):3187–3195.
30. Aravind L, Koonin EV. A natural classification of ribonucleases. Methods Enzymol 2001; 341:3–28.
31. Cerutti L, Mian N, Bateman A. Domains in gene silencing and cell differentiation proteins: the novel PAZ domain and redefinition of the Piwi domain. Trends Biochem Sci 2000; 25(10):481–482.
32. Ketting RF, Fischer SEJ, Bernstein E, Sijen T, Hannon GJ, Plasterk RHA. Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in *C. elegans*. Genes Dev 2001; 15(20):2654–2659.
33. Schauer SE, Jacobsen SE, Meinke DW, Ray A. DICER-LIKE1: blind men and elephants in Arabidopsis development. Trends Plant Sci 2002; 7(11):487–491.
34. Ketting RF, Plasterk RHA. A genetic link between co-suppression and RNA interference in *C. elegans*. Nature 2000; 404(6775):296–298.
35. Lau NC, Lim LP, Weinstein EG, Bartel DP. An abundant class of tiny RNAs with probable regulatory roles in *Caenorhabditis elegans*. Science 2001; 294(5543):858–862.
36. Lee RC, Ambros V. An extensive class of small RNAs in *Caenorhabditis elegans*. Science 2001; 294 (5543):862–864.
37. Lagos-Quintana M, Rauhut R, Yalcin A, Meyer J, Lendeckel W, Tuschl T. Identification of tissue-specific microRNAs from mouse. Curr Biol 2002; 12(9):735–739.
38. Lee Y, Jeon K, Lee JT, Kim S, Kim VN. MicroRNA maturation: stepwise processing and subcellular localization. EMBO J 2002; 21(17):4663–4670.
39. Reinhart BJ, Weinstein EG, Rhoades MW, Bartel B, Bartel DP. MicroRNAs in plants. Genes Dev 2002; 16(13):1616–1626.
40. Lagos-Quintana M, Rauhut R, Meyer J, Borkhardt A, Tuschl T. New microRNAs from mouse and human. RNA 2003; 9(2):175–179.
41. Elbashir SM, Martinez J, Patkaniowska A, Lendeckel W, Tuschl T. Functional anatomy of siRNAs for mediating efficient RNAi in *Drosophila melanogaster* embryo lysate. EMBO J 2001; 20(23):6877–6888.
42. Blaszczyk J, Tropea JE, Bubunenko M, et al. Crystallographic and modeling studies of RNase III suggest a mechanism for double-stranded RNA cleavage. Structure 2001; 9(12):1225–1236.
43. Hannon GJ. RNA interference. Nature 2002; 418(6894):244–251.
44. Hutvagner G, McLachlan J, Pasquinelli AE, Balint E, Tuschl T, Zamore PD. A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. Science 2001; 293(5531):834–838.

45. Myers JW, Jones JT, Meyer T, Ferrell JE Jr. Recombinant Dicer efficiently converts large dsRNAs into siRNAs suitable for gene silencing. Nature Biotechnol 2003; 21(3):324–328.
46. Provost P, Dishart D, Doucet J, Frendewey D, Samuelsson B, Radmark O. Ribonuclease activity and RNA binding of recombinant human Dicer. EMBO J 2002; 21(21):5864–5874.
47. Zhang H, Kolb FA, Brondani V, Billy E, Filipowicz W. Human Dicer preferentially cleaves dsRNAs at their termini without a requirement for ATP. EMBO J 2002; 21(21):5875–5885.
48. Martinez J, Patkaniowska A, Urlaub H, Luhrmann R, Tuschl T. Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. Cell 2002; 110(5):563–574.
49. Nykänen A, Haley B, Zamore PD. ATP requirements and small interfering RNA structure in the RNA interference pathway. Cell 2001; 107(3):309–321.
50. Hammond SM, Boettcher S, Caudy AA, Kobayashi R, Hannon GJ. Argonaute2, a link between genetic and biochemical analyses of RNAi. Science 2001; 293(5532):1146–1150.
51. Liu Q, Rand TA, Kalidas S, et al. R2D2, a bridge between the initiation and effector steps of the *Drosophila* RNAi pathway. Science 2003; 301(5641):1921–1925.
52. Hammond SM, Bernstein E, Beach D, Hannon GJ. An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. Nature 2000; 404(6775):293–296.
53. Carmell MA, Xuan Z, Zhang MQ, Hannon GJ. The Argonaute family: tentacles that reach into RNAi, developmental control, stem cell maintenance, and tumorigenesis. Genes Dev 2002; 16(21):2733–2742.
54. Schwarz DS, Hutvagner G, Haley B, Zamore PD. Evidence that siRNAs function as guides, not primers, in the *Drosophila* and human RNAi pathways. Mol Cell 2002; 10(3):537–548.
55. Schwarz DS, Hutvagner G, Du T, Xu Z, Aronin N, Zamore PD. Asymmetry in the assembly of the RNAi enzyme complex. Cell 2003; 115(2):199–208.
56. Khvorova A, Reynolds A, Jayasena SD. Functional siRNAs and miRNAs exhibit strand bias. Cell 2003; 115(2):209–216.
57. Jackson AL, Bartz SR, Schelter J, et al. Expression profiling reveals off-target gene regulation by RNAi. Nature Biotechnol 2003; 21(6):635–637.
58. Song JJ, Liu J, Tolia NH, et al. The crystal structure of the Argonaute2 PAZ domain reveals an RNA binding motif in RNAi effector complexes. Nature Struct Biol 2003; 10(12):1026–1032.
59. Yan KS, Yan S, Farooq A, Han A, Zeng L, Zhou MM. Structure and conserved RNA binding of the PAZ domain. Nature 2003; 26:468–474.
60. Lingel A, Simon B, Izaurralde E, Sattler M. Structure and nucleic-acid binding of the *Drosophila* Argonaute 2 PAZ domain. Nature 2003; 426:465–469.
61. Ponting CP. Evidence for PDZ domains in bacteria, yeast, and plants. Protein Sci 1997; 6(2):464–468.
62. Callebaut I, Mornon JP. The human EBNA-2 coactivator p100: multidomain organization and relationship to the staphylococcal nuclease fold and to the tudor protein involved in *Drosophila melanogaster* development. Biochem J 1997; 321(Pt 1):125–132.
63. Caudy AA, Ketting RF, Hammond SM, et al. A micrococcal nuclease homologue in RNAi effector complexes. Nature 2003; 425(6956):411–414.
64. Arenz C, Schepers U. RNA interference: from an ancient mechanism to a state of the art therapeutic application? Naturwissenschaften 2003; 90(8):345–359.
65. Venter JC, Adams MD, Myers EW, et al. The sequence of the human genome. Science 2001; 291 (5507):1304–1351.
66. Lander ES, Linton LM, Birren B, et al. Initial sequencing and analysis of the human genome. Nature 2001; 409(6822):860–921.
67. Nagy A, Perrimon N, Sandmeyer S, Plasterk R. Tailoring the genome: the power of genetic approaches. Nature Genet 2003; 33(Suppl):276–284.
68. Rajewsky K, Gu H, Kuhn R, et al. Conditional gene targeting. J Clin Invest 1996; 98(3):600–603.
69. Nagy A. Cre recombinase: the universal reagent for genome tailoring [review]. Genesis 2000; 26(2): 99–109.
70. Famulok M. Oligonucleotide aptamers that recognize small molecules. Curr Opin Struct Biol 1999; 9(3):324–329.
71. Sullenger BA, Gallardo HF, Ungers GE, Gilboa E. Overexpression of TAR sequences renders cells resistant to human immunodeficiency virus replication. Cell 1990; 63(3):601–608.
72. Bosher JM, Labouesse M. RNA interference: genetic wand and genetic watchdog. Nature Cell Biol 2000; 2(2):E31–E36.
73. Hunter CP. Genetics: a touch of elegance with RNAi. Curr Biol 1999; 9(12):R440–R442.

74. Feinberg EH, Hunter CP. Transport of dsRNA into cells by the transmembrane protein SID-1. Science 2003; 301(5639):1545–1547.
75. Kamath RS, Ahringer J. Genome-wide RNAi screening in *Caenorhabditis elegans*. Methods 2003; 30(4):313–321.
76. Montgomery MK. The use of double-stranded RNA to knock down specific gene activity. Methods Mol Biol 2004; 260:129–144.
77. Clemens JC, Worby CA, Simonson-Leff N, et al. Use of double-stranded RNA interference in *Drosophila* cell lines to dissect signal transduction pathways. Proc Natl Acad Sci USA 2000; 97(12):6499–6503.
78. Grishok A, Mello CC. RNAi (Nematodes: *Caenorhabditis elegans*). Adv Genet 2002; 46:339–360.
79. Grishok A, Tabara H, Mello CC. Genetic requirements for inheritance of RNAi in *C. elegans*. Science 2000; 287(5462):2494–2497.
80. Maeda I, Kohara Y, Yamamoto M, Sugimoto A. Large-scale analysis of gene function in *Caenorhabditis elegans* by high-throughput RNAi. Curr Biol 2001; 11(3):171–176.
81. Schumacher JM, Golden A, Donovan PJ. AIR-2: an Aurora/Ip11-related protein kinase associated with chromosomes and midbody microtubules is required for polar body extrusion and cytokinesis in *Caenorhabditis elegans* embryos. J Cell Biol 1998; 143(6):1635–1646.
82. Chase D, Serafinas C, Ashcroft N, et al. The polo-like kinase PLK-1 is required for nuclear envelope breakdown and the completion of meiosis in *Caenorhabditis elegans*. Genesis 2000; 26(1):26–41.
83. Ashrafi K, Chang FY, Watts JL, et al. Genome-wide RNAi analysis of *Caenorhabditis elegans* fat regulatory genes. Nature 2003; 421(6920):268–272.
84. Fraser AG, Kamath RS, Zipperlen P, Martinez-Campos M, Sohrmann M, Ahringer J. Functional genomic analysis of *C. elegans* chromosome I by systematic RNA interference. Nature 2000; 408(6810): 325–330.
85. Castillo-Davis CI, Hartl DL. Conservation, relocation and duplication in genome evolution. Trends Genet 2003; 19(11):593–597.
86. Gonczy P, Echeverri C, Oegema K, et al. Functional genomic analysis of cell division in *C. elegans* using RNAi of genes on chromosome III. Nature 2000; 408(6810):331–336.
87. Lee SS, Lee RY, Fraser AG, Kamath RS, Ahringer J, Ruvkun G. A systematic RNAi screen identifies a critical role for mitochondria in *C. elegans* longevity. Nature Genet 2003; 33(1):40–48.
88. Kennerdell JR, Carthew RW. Use of dsRNA-mediated genetic interference to demonstrate that frizzled and frizzled 2 act in the wingless pathway. Cell 1998; 95(7):1017–1026.
89. Kiger A, Baum B, Jones S, et al. A functional genomic analysis of cell morphology using RNA interference. J Biol 2003; 2(4):27.
90. Boutros M, Kiger AA, Armknecht S, et al. Genome-wide RNAi analysis of growth and viability in *Drosophila* cells. Science 2004; 303(5659):832–835.
91. Somma MP, Fasulo B, Cenci G, Cundari E, Gatti M. Molecular dissection of cytokinesis by RNA interference in *Drosophila* cultured cells. Mol Biol Cell 2002; 13(7):2448–2460.
92. Maiato H, Sunkel CE, Earnshaw WC. Dissecting mitosis by RNAi in *Drosophila* tissue culture cells. Biol Proc Online 2003; 5:153–161.
93. Goshima G, Vale RD. The roles of microtubule-based motor proteins in mitosis: comprehensive RNAi analysis in the *Drosophila* S2 cell line. J Cell Biol 2003; 162(6):1003–1016.
94. Clemens MJ, Elia A. The double-stranded RNA-dependent protein kinase PKR-structure and function [review]. J Interferon Cytokine Res 1997; 17(9):503–524.
95. Clemens MJ. Pkr—a protein kinase regulated by double-stranded RNA. Int J Biochem Cell Biol 1997; 29(7):945–949.
96. Wianny F, Zernicka-Goetz M. Specific interference with gene function by double-stranded RNA in early mouse development. Nature Cell Biol 2000; 2(2):70–75.
97. Billy E, Brondani V, Zhang H, Muller U, Filipowicz W. Specific interference with gene expression induced by long, double- stranded RNA in mouse embryonal teratocarcinoma cell lines. Proc Natl Acad Sci USA 2001; 98(25):14,428–14,433.
98. Elbashir SM, Harborth J, Lendeckel W, Yalcin A, Weber K, Tuschl T. Duplexes of 21-nucleotide RNAs mediate RNA interference in cultured mammalian cells. Nature 2001; 411(6836):494–498.
99. Krichevsky AM, Kosik KS. RNAi functions in cultured mammalian neurons. Proc Natl Acad Sci USA 2002; 99(18):11,926–11,929.
100. Chiu YL, Rana TM. siRNA function in RNAi: a chemical modification analysis. RNA 2003; 9(9):1034–1048.

101. Chiu YL, Rana TM. RNAi in human cells: basic structural and functional features of small interfering RNA. Mol Cell 2002; 10(3):549–561.
102. Reynolds A, Leake D, Boese Q, Scaringe S, Marshall WS, Khvorova A. Rational siRNA design for RNA interference. Nat Biotechnol 2004; 22:326–330.
103. Caplen NJ, Parrish S, Imani F, Fire A, Morgan RA. Specific inhibition of gene expression by small double-stranded RNAs in invertebrate and vertebrate systems. Proc Natl Acad Sci USA 2001; 98(17): 9742–9747.
104. Gitlin L, Andino R. Nucleic acid-based immune system: the antiviral potential of mammalian RNA silencing. J Virol 2003; 77(13):7159–7165.
105. Martins LM, Iaccarino I, Tenev T, et al. The serine protease Omi/HtrA2 regulates apoptosis by binding XIAP through a reaper-like motif. J Biol Chem 2002; 277(1):439–444.
106. Cioca DP, Aoki Y, Kiyosawa K. RNA interference is a functional pathway with therapeutic potential in human myeloid leukemia cell lines. Cancer Gene Therapy 2003; 10(2):125–133.
107. Donze O, Picard D. RNA interference in mammalian cells using siRNAs synthesized with T7 RNA polymerase. Nucleic Acids Res 2002; 30(10):e46.
108. Weil A, Garcon L, Harper M, Dumenil D, Dautry R, Kress M. Targeting the kinesin Eg5 to monitor siRNA transfection in mammalian cells. Biotechniques 2002; 33(6):1244–1248.
109. Dunne J, Drescher B, Riehle H, et al. The apparent uptake of fluorescently labeled siRNAs by electroporated cells depends on the fluorochrome. Oligonucleotides 2003; 13(5):375–380.
110. Walters DK, Jelinek DF. The effectiveness of double-stranded short inhibitory RNAs (siRNAs) may depend on the method of transfection. Antisense Nucleic Acid Drug Dev 2002; 12(6):411–418.
111. Scherr M, Morgan MA, Eder M. Gene silencing mediated by small interfering RNAs in mammalian cells. Curr Med Chem 2003; 10(3):245–256.
112. Heidenreich O, Krauter J, Riehle H, et al. AML1/MTG8 oncogene suppression by small interfering RNAs supports myeloid differentiation of t(8; 21)-positive leukemic cells. Blood 2003; 101(8):3157–3163.
113. Simeoni F, Morris MC, Heitz F, Divita G. Insight into the mechanism of the peptide-based gene delivery system MPG: implications for delivery of siRNA into mammalian cells. Nucleic Acids Res 2003; 31(11):2717–2724.
114. Prochiantz A. Getting hydrophilic compounds into cells—lessons from homeopeptides (Commentary). Curr Opin Neurobiol 1996; 6(5):629–634.
115. Schwarze SR, Ho A, Vocero-Akbani A, Dowdy SF. In vivo protein transduction: delivery of a biologically active protein into the mouse [comment]. Science 1999; 285(5433):1569–1572.
116. Schwarze SR, Dowdy SF. In vivo protein transduction: intracellular delivery of biologically active proteins, compounds and DNA. Trends Pharmacol Sci 2000; 21(2):45–48.
117. Derossi D, Joliot AH, Chassaing G, Prochiantz A. The third helix of the Antennapedia homeodomain translocates through biological membranes. J Biol Chem 1994; 269(14):10,444–10,450.
118. Derossi D, Chassaing G, Prochiantz A. Trojan peptides—the penetratin system for intracellular delivery. Trends Cell Biol 1998; 8(2):84–87.
119. Schmitz K, Schepers U. Cell penetrating peptides in siRNA delivery. Expert Opin Biol Ther 2004; in press.
120. Schmitz K, Diallo M, Mundegar R, Schepers U. pepsiRNAs for RNAi in mammals. Submitted.
121. Berns K, Hijmans EM, Mullenders J, et al. A large-scale RNAi screen in human cells identifies new components of the p53 pathway. Nature 2004; 428(6981):431–437.
122. Kisielow M, Kleiner S, Nagasawa M, Faisal A, Nagamine Y. Isoform-specific knockdown and expression of adaptor protein ShcA using small interfering RNA. Biochem J 2002; 363(1):1–5.
123. Czauderna F, Fechtner M, Aygun H, et al. Functional studies of the PI(3)-kinase signalling pathway employing synthetic and expressed siRNA. Nucleic Acids Res 2003; 31(2):670–682.
124. Xu Z, Williams CJ, Kopf GS, Schultz RM. Maturation-associated increase in IP3 receptor type 1: role in conferring increased IP3 sensitivity and Ca^{2+} oscillatory behavior in mouse eggs. Dev Biol 2003; 254(2):163–171.
125. Stucke VM, Sillje HH, Arnaud L, Nigg EA. Human Mps1 kinase is required for the spindle assembly checkpoint but not for centrosome duplication. EMBO J 2002; 21(7):1723–1732.
126. Ohta T, Essner R, Ryu JH, Palazzo RE, Uetake Y, Kuriyama R. Characterization of Cep135, a novel coiled-coil centrosomal protein involved in microtubule organization in mammalian cells. J Cell Biol 2002; 156(1):87–99.
127. Salisbury JL, Suino KM, Busby R, Springett M. Centrin-2 is required for centriole duplication in mammalian cells. Curr Biol 2002; 12(15):1287–1292.

128. Prasanth KV, Sacco-Bubulya PA, Prasanth SG, Spector DL. Sequential entry of components of the gene expression machinery into daughter nuclei. Mol Biol Cell 2003; 14(3):1043–1057.
129. Cabot RA, Prather RS. Cleavage stage porcine embryos may have differing developmental requirements for karyopherins alpha2 and alpha3. Mol Reprod Dev 2003; 64(3):292–301.
130. Kim VN. RNA interference in functional genomics and medicine [review]. J Korean Med Sci 2003; 18(3):309–318.
131. Paddison PJ, Caudy AA, Bernstein E, Hannon GJ, Conklin DS. Short hairpin RNAs (shRNAs) induce sequence-specific silencing in mammalian cells. Genes Dev 2002; 16(8):948–958.
132. Brummelkamp TR, Bernards R, Agami R. A system for stable expression of short interfering RNAs in mammalian cells. Science 2002; 296(5567):550–553.
133. Sui G, Soohoo C, El Affar B, Gay F, Shi Y, Forrester WC. A DNA vector-based RNAi technology to suppress gene expression in mammalian cells. Proc Natl Acad Sci USA 2002; 99(8):5515–5520.
134. Miyagishi M, Taira K. U6 promoter driven siRNAs with four uridine 3' overhangs efficiently suppress targeted gene expression in mammalian cells. Nature Biotechnol 2002; 20(5):497–500.
135. Paul CP, Good PD, Winer I, Engelke DR. Effective expression of small interfering RNA in human cells. Nature Biotechnol 2002; 20(5):505–508.
136. Lee NS, Dohjima T, Bauer G, et al. Expression of small interfering RNAs targeted against HIV-1 rev transcripts in human cells. Nature Biotechnol 2002; 20(5):500–505.
137. Paule MR, White RJ. Survey and summary: transcription by RNA polymerases I and III. Nucleic Acids Res 2000; 28(6):1283–1298.
138. Zamore PD. RNA interference: listening to the sound of silence. Nature Struct Biol 2001; 8(9):746–750.
139. Diallo M, Arenz C, Schmitz K, Sandhoff K, Schepers U. RNA Interference: a new method to analyze the function of glycoproteins and glycosylating proteins in mammalian cells. Knockout experiments with UDP-glucose/ceramide-glucosyltransferase. Methods Enzymol 2003; 363:173–190.
140. Diallo M, Arenz C, Schmitz K, Sandhoff K, Schepers U. Long endogenous dsRNAs can induce a complete gene silencing in mammalian cells and primary cultures. Oligonucleotides 2003; 13(5):381–392.
141. Shinagawa T, Ishii S. Generation of Ski-knockdown mice by expressing a long double-strand RNA from an RNA polymerase II promoter. Genes Dev 2003; 17(11):1340–1345.
142. Lewis DL, Hagstrom JE, Loomis AG, Wolff JA, Herweijer H. Efficient delivery of siRNA for inhibition of gene expression in postnatal mice. Nature Genet 2002; 32(1):107–108.
143. McCaffrey AP, Meuse L, Pham TTT, Conklin DS, Hannon GJ, Kay MA. Gene expression—RNA interference in adult mice. Nature 2002; 418(6893):38–39.
144. Hemann MT, Fridman JS, Zilfou JT, et al. An epi-allelic series of p53 hypomorphs created by stable RNAi produces distinct tumor phenotypes in vivo. Nature Genet 2003; 33(3):396–400.
145. Tiscornia G, Singer O, Ikawa M, Verma IM. A general method for gene knockdown in mice by using lentiviral vectors expressing small interfering RNA. Proc Natl Acad Sci USA 2003; 100(4):1844–1848.
146. Rubinson DA, Dillon CP, Kwiatkowski AV, et al. A lentivirus-based system to functionally silence genes in primary mammalian cells, stem cells and transgenic mice by RNA interference. Nature Genet 2003; 33(3):401–406.
147. Sledz CA, Holko M, de Veer MJ, Silverman RH, Williams BR. Activation of the interferon system by short-interfering RNAs. Nature Cell Biol 2003; 5(9):834–839.
148. Stark GR, Kerr IM, Williams BR, Silverman RH, Schreiber RD. How cells respond to interferons. Annu Rev Biochem 1998; 67:227–264.
149. Bridge AJ, Pebernard S, Ducraux A, Nicoulaz AL, Iggo R. Induction of an interferon response by RNAi vectors in mammalian cells. Nature Genet 2003; 34(3):263–264.
150. Akusjarvi G, Svensson C, Nygard O. A mechanism by which adenovirus virus-associated RNAI controls translation in a transient expression assay. Mol Cell Biol 1987; 7(1):549–551.
151. Koopmans M, Duizer E. Foodborne viruses: an emerging problem. Int J Food Microbiol 2004; 90(1): 23–41.
152. Jacque JM, Triques K, Stevenson M. Modulation of HIV-1 replication by RNA interference. Nature 2002; 418(6896):435–438.
153. Martinez MA, Clotet B, Este JA. RNA interference of HIV replication. Trends Immunol 2002; 23(12): 559–561.
154. Yamamoto T, Omoto S, Mizuguchi M, et al. Double-stranded nef RNA interferes with human immunodeficiency virus type 1 replication. Microbiol Immunol 2002; 46(11):809–817.

155. Anderson J, Banerjea A, Akkina R. Bispecific short hairpin siRNA constructs targeted to CD4, CXCR4, and CCR5 confer HIV-1 resistance. Oligonucleotides 2003; 13(5):303–312.
156. Anderson J, Banerjea A, Planelles V, Akkina R. Potent suppression of HIV type 1 infection by a short hairpin anti-CXCR4 siRNA. AIDS Res Hum Retroviruses 2003; 19(8):699–706.
157. Boden D, Pusch O, Lee F, Tucker L, Shank PR, Ramratnam B. Promoter choice affects the potency of HIV-1 specific RNA interference. Nucleic Acids Res 2003; 31(17):5033–5038.
158. Pusch O, Boden D, Silbermann R, Lee F, Tucker L, Ramratnam B. Nucleotide sequence homology requirements of HIV-1-specific short hairpin RNA. Nucleic Acids Res 2003; 31(22):6444–6449.
159. Michienzi A, Castanotto D, Lee N, Li S, Zaia JA, Rossi JJ. RNA-mediated inhibition of HIV in a gene therapy setting. Ann NY Acad Sci 2003; 1002:63–71.
160. Boden D, Pusch O, Silbermann R, Lee F, Tucker L, Ramratnam B. Enhanced gene silencing of HIV-1 specific siRNA using microRNA designed hairpins. Nucleic Acids Res 2004; 32(3):1154–1158.
161. Dunn SJ, Khan IH, Chan UA, et al. Identification of cell surface targets for HIV-1 therapeutics using genetic screens. Virology 2004; 321(2):260–273.
162. Novina CD, Murray MF, Dykxhoorn DM, et al. siRNA-directed inhibition of HIV-1 infection. Nature Med 2002; 8(7):681–686.
163. Andino R. RNAi puts a lid on virus replication. Nature Biotechnol 2003; 21(6):629–630.
164. Hamasaki K, Nakao K, Matsumoto K, Ichikawa T, Ishikawa H, Eguchi K. Short interfering RNA-directed inhibition of hepatitis B virus replication. FEBS Lett 2003; 543(1-3):51–54.
165. McCaffrey AP, Meuse L, Karimi M, Contag CH, Kay MA. A potent and specific morpholino antisense inhibitor of hepatitis C translation in mice. Hepatology 2003; 38(2):503–508.
166. Sen A, Steele R, Ghosh AK, Basu A, Ray R, Ray RB. Inhibition of hepatitis C virus protein expression by RNA interference. Virus Res 2003; 96(1-2):27–35.
167. Shlomai A, Shaul Y. Inhibition of hepatitis B virus expression and replication by RNA interference. Hepatology 2003; 37(4):764–770.
168. Song E, Lee SK, Wang J, et al. RNA interference targeting Fas protects mice from fulminant hepatitis. Nature Med 2003; 9(3):347–351.
169. Wilson JA, Jayasena S, Khvorova A, et al. RNA interference blocks gene expression and RNA synthesis from hepatitis C replicons propagated in human liver cells. Proc Natl Acad Sci USA 2003; 100(5): 2783–2788.
170. Yokota T, Sakamoto N, Enomoto N, et al. Inhibition of intracellular hepatitis C virus replication by synthetic and vector-derived small interfering RNAs. EMBO Rep 2003; 4(6):602–608.
171. Milner J. RNA interference for treating cancers caused by viral infection. Expert Opin Biol Ther 2003; 3(3):459–467.
172. Butz K, Ristriani T, Hengstermann A, Denk C, Scheffner M, Hoppe-Seyler F. siRNA targeting of the viral E6 oncogene efficiently kills human papillomavirus-positive cancer cells. Oncogene 2003; 22(38): 5938–5945.
173. Hall AH, Alexander KA. RNA interference of human papillomavirus type 18 E6 and E7 induces senescence in HeLa cells. J Virol 2003; 77(10):6066–6069.
174. Gitlin L, Karelsky S, Andino R. Short interfering RNA confers intracellular antiviral immunity in human cells. Nature 2002; 418(6896):430–434.
175. Wang QC, Nie QH, Feng ZH. RNA interference: antiviral weapon and beyond. World J Gastroenterol 2003; 9(8):1657–1661.
176. Godfrey A, Laman H, Boshoff C. RNA interference: a potential tool against Kaposi's sarcoma-associated herpesvirus. Curr Opin Infect Dis 2003; 16(6):593–600.
177. Stewart SA, Dykxhoorn DM, Palliser D, et al. Lentivirus-delivered stable gene silencing by RNAi in primary cells. RNA 2003; 9(4):493–501.
178. Bitko V, Barik S. Phenotypic silencing of cytoplasmic genes using sequence-specific double-stranded short interfering RNA and its application in the reverse genetics of wild type negative-strand RNA viruses. BMC Microbiol 2001; 1(1):34.
179. Ge Q, McManus MT, Nguyen T, et al. RNA interference of influenza virus production by directly targeting mRNA for degradation and indirectly inhibiting all viral RNA transcription. Proc Natl Acad Sci USA 2003; 100(5):2718–2723.
180. Li XP, Li G, Peng Y, Kung HF, Lin MC. Suppression of Epstein–Barr virus-encoded latent membrane protein-1 by RNA interference inhibits the metastatic potential of nasopharyngeal carcinoma cells. Biochem Biophys Res Commun 2004; 315(1):212–218.

181. Zhang J, Hua ZC. Targeted gene silencing by small interfering RNA-based knock-down technology. Curr Pharm Biotechnol 2004; 5(1):1–7.
182. Zhang Y, Li T, Fu L, et al. Silencing SARS-CoV Spike protein expression in cultured cells by RNA interference. FEBS Lett 2004; 560(1-3):141–146.
183. He ML, Zheng B, Peng Y, et al. Inhibition of SARS-associated coronavirus infection and replication by RNA interference. JAMA 2003; 290(20):2665–2666.
184. Qin L, Xiong B, Luo C, et al. Identification of probable genomic packaging signal sequence from SARS-CoV genome by bioinformatics analysis. Acta Pharmacol Sin 2003; 24(6):489–496.
185. Wu HY, Guy JS, Yoo D, Vlasak R, Urbach E, Brian DA. Common RNA replication signals exist among group 2 coronaviruses: evidence for in vivo recombination between animal and human coronavirus molecules. Virology 2003; 315(1):174–183.
186. McCaffrey AP, Nakai H, Pandey K, et al. Inhibition of hepatitis B virus in mice by RNA interference. Nature Biotechnol 2003; 21(6):639–644.
187. Lieberman J, Song E, Lee SK, Shankar P. Interfering with disease: opportunities and roadblocks to harnessing RNA interference. Trends Mol Med 2003; 9(9):397–403.
188. Das AT, Brummelkamp TR, Westerhout EM, et al. Human immunodeficiency virus type 1 escapes from RNA interference-mediated inhibition. J Virol 2004; 78(5):2601–2605.
189. Boden D, Pusch O, Lee F, Tucker L, Ramratnam B. Human immunodeficiency virus type 1 escape from RNA interference. J Virol 2003; 77(21):11,531–11,535.
190. Bastin P, Ellis K, Kohl L, Gull K. Flagellum ontogeny in trypanosomes studied via an inherited and regulated RNA interference system. J Cell Sci 2000; 113(18):3321–3328.
191. LaCount DJ, Bruse S, Hill KL, Donelson JE. Double-stranded RNA interference in *Trypanosoma brucei* using head-to-head promoters. Mol Biochem Parasitol 2000; 111(1):67–76.
192. McRobert L, McConkey GA. RNA interference (RNAi) inhibits growth of *Plasmodium falciparum*. Mol Biochem Parasitol 2002; 119(2):273–278.
193. Lillico S, Field MC, Blundell P, Coombs GH, Mottram JC. Essential roles for GPI-anchored proteins in African trypanosomes revealed using mutants deficient in GP18. Mol Biol Cell 2003; 14(3): 1182–1194.
194. Mohmmed A, Dasaradhi PV, Bhatnagar RK, Chauhan VS, Malhotra P. In vivo gene silencing in *Plasmodium berghei*—a mouse malaria model. Biochem Biophys Res Commun 2003; 309(3):506–511.
195. Blackman MJ. RNAi in protozoan parasites: what hope for the Apicomplexa? Protist 2003; 154(2): 177–180.
196. Ngo H, Tschudi C, Gull K, Ullu E. Double-stranded RNA induces mRNA degradation in *Trypanosoma brucei*. PNAS 1998; 95(25):14,687–14,692.
197. Aballay A, Drenkard E, Hilbun LR, Ausubel FM. *Caenorhabditis elegans* innate immune response triggered by *Salmonella enterica* requires intact LPS and is mediated by a MAPK signaling pathway. Curr Biol 2003; 13(1):47–52.
198. Arcus VL, Backbro K, Roos A, Daniel EL, Baker EN. Distant structural homology leads to the functional characterisation of an archaeal PIN-domain as an exonuclease. J Biol Chem 2004; 279:16,471–16,478.
199. Walduck A, Rudel T, Meyer TF. Proteomic and gene profiling approaches to study host responses to bacterial infection. Curr Opin Microbiol 2004; 7(1):33–38.
200. Sorensen DR, Leirdal M, Sioud M. Gene silencing by systemic delivery of synthetic siRNAs in adult mice. J Mol Biol 2003; 327(4):761–766.
201. Kelly D, Campbell JI, King TP, et al. Commensal anaerobic gut bacteria attenuate inflammation by regulating nuclear-cytoplasmic shuttling of PPAR-gamma and RelA. Nature Immunol 2004; 5(1): 104–112.
202. Duxbury MS, Ito H, Benoit E, Zinner MJ, Ashley SW, Whang EE. RNA interference targeting focal adhesion kinase enhances pancreatic adenocarcinoma gemcitabine chemosensitivity. Biochem Biophys Res Commun 2003; 311(3):786–792.
203. Wang Y, Decker SJ, Sebolt-Leopold J. Knockdown of Chk1, Wee1 and Myt1 by RNA interference abrogates G(2) checkpoint and induces apoptosis. Cancer Biol Ther 2004; 3:305–313.
204. Zangemeister-Wittke U. Antisense to apoptosis inhibitors facilitates chemotherapy and TRAIL-induced death signaling. Ann NY Acad Sci 2003; 1002:90–94.
205. Chatterjee B. The role of the androgen receptor in the development of prostatic hyperplasia and prostate cancer. Mol Cell Biochem 2003; 253(1-2):89–101.

206. Wall NR, Shi Y. Small RNA: can RNA interference be exploited for therapy? Lancet 2003; 362(9393): 1401–1403.
207. Brummelkamp TR, Nijman SM, Dirac AM, Bernards R. Loss of the cylindromatosis tumour suppressor inhibits apoptosis by activating NF-kappaB. Nature 2003; 424(6950):797–801.
208. Tanaka Y, Gavrielides MV, Mitsuuchi Y, Fujii T, Kazanietz MG. Protein kinase C promotes apoptosis in LNCaP prostate cancer cells through activation of p38 MAPK and inhibition of the Akt survival pathway. J Biol Chem 2003; 278(36):33,753–33,762.
209. Milner J. RNA interference for treating cancers caused by viral infection [review]. Expert Opin Biol Ther 2003; 3(3):459–467.
210. Jiang M, Milner J. Bcl-2 constitutively suppresses p53-dependent apoptosis in colorectal cancer cells. Genes Dev 2003; 17(7):832–837.
211. Wilda M, Fuchs U, Wossmann W, Borkhardt A. Killing of leukemic cells with a BCR/ABL fusion gene by RNA interference (RNAi). Oncogene 2002; 21(37):5716–5724.
212. Lin SL, Chuong CM, Ying SY. A novel mRNA–cDNA interference phenomenon for silencing bcl-2 expression in human LNCaP cells. Biochem Biophys Res Commun 2001; 281(3):639–644.
213. Chen YG, Lui HM, Lin SL, Lee JM, Ying SY. Regulation of cell proliferation, apoptosis, and carcinogenesis by activin. Exp Biol Med (Maywood) 2002; 227(2):75–87.
214. Subramanian T, Chinnadurai G. Pro-apoptotic activity of transiently expressed BCL-2 occurs independent of BAX and BAK. J Cell Biochem 2003; 89(6):1102–1114.
215. Grzmil M, Thelen P, Hemmerlein B, et al. Bax inhibitor-1 is overexpressed in prostate cancer and its specific down-regulation by RNA interference leads to cell death in human prostate carcinoma cells. Am J Pathol 2003; 163(2):543–552.
216. Dzitoyeva S, Dimitrijevic N, Manev H. Gamma-aminobutyric acid B receptor 1 mediates behavior-impairing actions of alcohol in *Drosophila*: adult RNA interference and pharmacological evidence. Proc Natl Acad Sci USA 2003; 100(9):5485–5490.
217. Aza-Blanc P, Cooper CL, Wagner K, Batalov S, Deveraux QL, Cooke MP. Identification of modulators of TRAIL-induced apoptosis via RNAi-based phenotypic screening. Mol Cell 2003; 12(3):627–637.
218. Lima RT, Martins LM, Guimaraes JE, Sambade C, Vasconcelos MH. Specific downregulation of bcl-2 and xIAP by RNAi enhances the effects of chemotherapeutic agents in MCF-7 human breast cancer cells. Cancer Gene Ther 2004; 11:309–316.
219. Yin JQ, Gao J, Shao R, Tian WN, Wang J, Wan Y. siRNA agents inhibit oncogene expression and attenuate human tumor cell growth. J Exp Ther Oncol 2003; 3(4):194–204.
220. Konnikova L, Kotecki M, Kruger MM, Cochran BH. Knockdown of STAT3 expression by RNAi induces apoptosis in astrocytoma cells. BMC Cancer 2003; 3(1):23.
221. Sun B, Nishihira J, Suzuki M, et al. Induction of macrophage migration inhibitory factor by lysophosphatidic acid: relevance to tumor growth and angiogenesis. Int J Mol Med 2003; 12(4):633–641.
222. Liu LT, Chang HC, Chiang LC, Hung WC. Histone deacetylase inhibitor up-regulates RECK to inhibit MMP-2 activation and cancer cell invasion. Cancer Res 2003; 63(12):3069–3072.
223. Zhang L, Yang N, Mohamed-Hadley A, Rubin SC, Coukos G. Vector-based RNAi, a novel tool for isoform-specific knock-down of VEGF and anti-angiogenesis gene therapy of cancer. Biochem Biophys Res Commun 2003; 303(4):1169–1178.
224. Ameri K, Lewis CE, Raida M, Sowter H, Hai T, Harris AL. Anoxic induction of ATF-4 through HIF-1-independent pathways of protein stabilization in human cancer cells. Blood 2004; 103(5):1876–1882.
225. Niu Q, Perruzzi C, Voskas D, Lawler J, Dumont DJ, Benjamin LE. Inhibition of Tie-2 signaling induces endothelial cell apoptosis, decreases akt signaling, and induces endothelial cell expression of the endogenous anti-angiogenic molecule, thrombospondin-1. Cancer Biol Ther 2004; 3:402–405.
226. Tien ES, Davis JW, Vanden Heuvel JP. Identification of the CBP/p300 interacting protein CITED2 as a PPARalpha coregulator. J Biol Chem 2004; 279:24,053–24,063.
227. Zippo A, De Robertis A, Bardelli M, Galvagni F, Oliviero S. Identification of Flk-1-target genes in vasculogenesis: Pim-1 is required for endothelial and mural cell differentiation in vitro. Blood 2004; 103:4536–4544.
228. Bachelder RE, Lipscomb EA, Lin X, et al. Competing autocrine pathways involving alternative neuropilin-1 ligands regulate chemotaxis of carcinoma cells. Cancer Res 2003; 63(17):5230–5233.
229. Sun Y, Mochizuki Y, Majerus PW. Inositol 1,3,4-trisphosphate 5/6-kinase inhibits tumor necrosis factor-induced apoptosis. J Biol Chem 2003; 278(44):43,645–43,653.

230. Schafer R, Abraham D, Paulus P, et al. Impaired VE–cadherin/beta–catenin expression mediates endothelial cell degeneration in dilated cardiomyopathy. Circulation 2003; 108(13):1585–1591.
231. Liu Y, Lashuel HA, Choi S, et al. Discovery of inhibitors that elucidate the role of UCH-L1 activity in the H1299 lung cancer cell line. Chem Biol 2003; 10(9):837–846.
232. Riss D, Jin L, Qian X, et al. Differential expression of galectin-3 in pituitary tumors. Cancer Res 2003; 63(9):2251–2255.
233. Deroanne C, Vouret-Craviari V, Wang B, Pouyssegur J. EphrinA1 inactivates integrin-mediated vascular smooth muscle cell spreading via the Rac/PAK pathway. J Cell Sci 2003; 116(Pt 7):1367–1376.
234. Nagashima K, Endo A, Ogita H, et al. Adaptor protein Crk is required for ephrin-B1-induced membrane ruffling and focal complex assembly of human aortic endothelial cells. Mol Biol Cell 2002; 13 (12):4231–4242.
235. Jarad G, Wang B, Khan S, et al. Fas activation induces renal tubular epithelial cell beta 8 integrin expression and function in the absence of apoptosis. J Biol Chem 2002; 277(49):47,826–47,833.
236. Futami T, Miyagishi M, Seki M, Taira K. Induction of apoptosis in HeLa cells with siRNA expression vector targeted against bcl-2. Nucleic Acids Res Supplement 2002; (Suppl 2):251–252.
237. Cunningham JM, Weinberg RA. Ras oncogenes in human tumours: identification, mechanism of activation and cooperative role in transformation. IARC Sci Publ 1985(60):359–364.
238. Duursma AM, Agami R. Ras interference as cancer therapy. Sem Cancer Biol 2003; 13(4):267–273.
239. Brummelkamp TR, Bernards R, Agami R. Stable suppression of tumorigenicity by virus-mediated RNA interference. Cancer Cell 2002; 2(3):243–247.
240. Wolff JA, Herweijer H. Nonviral vectors for cardiovascular gene delivery. Ernst Schering Res Found Workshop 2003(43):41–59.
241. Lorenz K, Lohse MJ, Quitterer U. Protein kinase C switches the Raf kinase inhibitor from Raf-1 to GRK-2. Nature 2003; 426(6966):574–579.
242. Maxwell MM, Pasinelli P, Kazantsev AG, Brown RH Jr. RNA interference-mediated silencing of mutant superoxide dismutase rescues cyclosporin A-induced death in cultured neuroblastoma cells. Proc Natl Acad Sci USA 2004; 101(9):3178–3183.
243. Ding H, Schwarz DS, Keene A, et al. Selective silencing by RNAi of a dominant allele that causes amyotrophic lateral sclerosis. Aging Cell 2003; 2(4):209–217.
244. Xia XG, Zhou H, Ding H, Affar el B, Shi Y, Xu Z. An enhanced U6 promoter for synthesis of short hairpin RNA. Nucleic Acids Res 2003; 31(17):e100.
245. Miller VM, Xia H, Marrs GL, et al. Allele-specific silencing of dominant disease genes. Proc Natl Acad Sci USA 2003; 100(12):7195–7200.
246. Francis R, McGrath G, Zhang J, et al. aph-1 and pen-2 are required for Notch pathway signaling, gamma-secretase cleavage of betaAPP, and presenilin protein accumulation. Dev Cell 2002; 3(1): 85–97.
247. Asai M, Hattori C, Szabo B, et al. Putative function of ADAM9, ADAM10, and ADAM17 as APP alpha-secretase. Biochem Biophys Res Commun 2003; 301(1):231–235.
248. Mattson MP. Neurobiology: ballads of a protein quartet. Nature 2003; 422(6930):385–387.
249. Takasugi N, Tomita T, Hayashi I, et al. The role of presenilin cofactors in the gamma-secretase complex. Nature 2003; 422(6930):438–441.
250. Marlow L, Canet RM, Haugabook SJ, Hardy JA, Lahiri DK, Sambamurti K. APH1, PEN2, and Nicastrin increase Abeta levels and gamma-secretase activity [erratum Biochem Biophys Res Commun 2003 Aug 1; 307(3):756]. Biochem Biophys Res Commun 2003; 305(3):502–509.
251. Kao SC, Krichevsky AM, Kosik KS, Tsai LH. BACE1 suppression by RNA interference in primary cortical neurons. J Biol Chem 2004; 279(3):1942–1949.
252. Kiehl TR, Shibata H, Pulst SM. The ortholog of human ataxin-2 is essential for early embryonic patterning in *C. elegans*. J Mol Neurosci 2000; 15(3):231–241.
253. Yang Y, Nishimura I, Imai Y, Takahashi R, Lu B. Parkin suppresses dopaminergic neuron-selective neurotoxicity induced by Pael-R in *Drosophila*. Neuron 2003; 37(6):911–924.
254. Love R. First disease model created by RNA interference. Lancet Neurol 2004; 3(1):7.
255. Trulzsch B, Wood M. Applications of nucleic acid technology in the CNS. J Neurochem 2004; 88(2): 257–265.
256. Omi K, Tokunaga K, Hohjoh H. Long-lasting RNAi activity in mammalian neurons. FEBS Lett 2004; 558(1-3):89–95.
257. Trulzsch B, Davies K, Wood M. Survival of motor neuron gene downregulation by RNAi: towards a cell culture model of spinal muscular atrophy. Brain Res Mol Brain Res 2004; 120(2):145–150.

258. Moon C, Kang WS, Jeong DC, Jin JY. Distribution of adenoviral vector in brain after intravenous administration. J Korean Med Sci 2003; 18(1):108–111.
259. Xia H, Mao Q, Paulson HL, Davidson BL. siRNA-mediated gene silencing in vitro and in vivo. Nature Biotechnol 2002; 20(10):1006–1010.
260. Schepers U. Die Wiederentdeckung der RNA. Nachr Chem 2004; 52:302–305.
261. Wohlbold L, van der Kuip H, Miething C, et al. Inhibition of bcr-abl gene expression by small interfering RNA sensitizes for imatinib mesylate (STI571). Blood 2003; 102(6):2236–2239.
262. Nieth C, Priebsch A, Stege A, Lage H. Modulation of the classical multidrug resistance (MDR) phenotype by RNA interference (RNAi). FEBS Lett 2003; 545(2-3):144–150.
263. Crnkovic-Mertens I, Hoppe-Seyler F, Butz K. Induction of apoptosis in tumor cells by siRNA-mediated silencing of the livin/ML-IAP/KIAP gene. Oncogene 2003; 22(51):8330–8336.
264. Chen Y, Stamatoyannopoulos G, Song CZ. Down-regulation of CXCR4 by inducible small interfering RNA inhibits breast cancer cell invasion in vitro. Cancer Res 2003; 63(16):4801–4804.
265. Zha S, Ferdinandusse S, Denis S, et al. Alpha-methylacyl-CoA racemase as an androgen-independent growth modifier in prostate cancer. Cancer Res 2003; 63(21):7365–7376.