Chapter 12

RNA Interference

RNA Aid for Future Therapeutics?

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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.
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 (Drosophila 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.)
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. 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. 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.

Another working model proposes a monomeric action of Dicer cleaving the dsRNA during translocation of the enzyme along its substrate. 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. This mode of action might occur in Drosophila and other invertebrates but not in mammals, where Dicer is acting in an ATP-independent way.

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. 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. 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.

The recently discovered protein R2D2 harbors a tandem dsRNA-binding domain (R2) and a Drosophila Dicer-2-binding domain (D2), both required for the transfer of siRNAs. It assurses 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. 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.

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. So far, Drosophila RISC was first proposed to be an approx 500-kDa complex bound to ribosomes in cell-free extracts. 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. 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 liposome-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 versatility, 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 autoimmunity, 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 siRNAs 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 possibility of systematically testing a gene function in the context of the entire organism. Further animal models can be generated in a straightforward 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, acquired 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 apoptosis 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                       | HeLa cells, lung adenocarcinoma cells, hepatoma cells, ovarian carcinoma cells, melanoma cells, etc. | shRNA/lentivirus     | Proliferation, cell division                                                    | 219,238,239    |
| Bcl-2    | Colorectal carcinoma cells, cervix carcinoma, leukemia |                                                                                   | siRNAs               | Apoptosis inhibitor, chemotherapy resistance                                    | 204,210,214,218,219 |
| VEGF     | Most cancer types                 | HeLa cells, lung adenocarcinoma cells, hepatoma cells, ovarian carcinoma cells, 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/vector        | Apoptosis inhibitor                                                              | 173,263        |
| CXCR4    | Breast cancer, tumors             | Breast cancer cells                                                          | siRNAs               | Cell migration                                                                   | 264            |
| Wee1     |                                   | HeLa cells, lung adenocarcinoma cells, hepatoma cells, ovarian carcinoma cells, melanoma cells, etc. | siRNAs               | Apoptosis inhibitor, cell cycle control                                          | 203,219        |
| Chk1     |                                   | PANC1, MIAPaCa2, BxPC3,Capan2                                                 | siRNAs               | Growth factor, tumor invasiveness, metastasis                                    | 202            |
| Myt1     |                                   | HeLa                                                                        | siRNAs               | Proliferation, apoptosis inhibitor                                               | 265            |
| Cdc2     |                                   | Endothelial cells                                                            | siRNAs               | Angiogenesis                                                                     | 187,225        |
| Eph2     | Pancreatic adenocarcinoma         |                                                                            |                      |                                                                                  |                |
| receptor |                                   |                                                                            |                      |                                                                                  |                |
| FASE     | Prostate cancer                   |                                                                            |                      |                                                                                  |                |
| Tie-2    | Tumors                            |                                                                            |                      |                                                                                  |                |
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.

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