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RNA interference – significance and applications

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Summary

RNA interference (RNAi) is a post-transcriptional, highly conserved process in eukaryotes that leads to specific gene silencing through degradation of the target mRNA. This mechanism is mediated by double-stranded RNA (dsRNA) that is homologous in sequence to the silenced gene. The dsRNA is processed into small interfering RNA (siRNA) by an enzyme called Dicer, and the siRNAs are then incorporated into a multi-component RNA-induced silencing complex, which finds and cleaves the target mRNA. In plants and worms, amplification of the silencing signal and cell-to-cell RNAi spreading is observed. The proposed biological roles of RNAi include resistance to viruses, transposons (mainly in plants), and the silencing and regulation of gene expression, particularly during development. In developmental gene control, specific small RNAs (micro RNA and small temporal RNA) are involved, which are processed in the same way as dsRNAs but act at the level of translation. RNAi technology has become a powerful tool in functional genomic analyses and may prove to be a useful method to develop highly specific gene-silencing therapeutics against viral infections and cancer in the future.

Key words:

RNA interference • gene silencing • dsRNA • siRNA

Abbreviations:

dsRNA – double-stranded RNA, **miRNA** – micro RNA, **PKR** – protein kinase R, **RdRp** – RNA-dependent RNA polymerase, **RISC** – RNA-induced silencing complex, **RNAi** – RNA interference, **siRNA** – small interfering RNA, **stRNA** – small temporal RNA

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INTRODUCTION

In recent years many RNA-based silencing mechanisms, e.g. post-transcriptional gene silencing, co-suppression, quelling, and RNA interference (RNAi), have been discovered among species of different kingdoms (fungi, plants, and animals)^{9,13, 20, 51, 53, 78}. One of the most interesting discoveries was RNAi, a post-transcriptional sequence-specific gene-silencing mechanism initiated by the introduction of double-stranded RNA (dsRNA) homologous in sequence to the silenced gene^{35, 44, 54, 76, 78}. The phenomenon of RNAi was first discovered in plants during experiments connected with changes in pigmentation in the petunia. Introducing supernumerary copies of a pigment gene designed to increase the color intensity in the flower did not deepen flower color as expected: the flowers became even less colorful than the wild flowers^{8, 14, 22, 28}. The term RNAi was first coined after the discovery that injection of dsRNA into the nematode *Caenorhabditis elegans* leads to the specific silencing of genes highly homologous in sequence to the delivered dsRNA^{17, 21}. RNAi has also been observed in a wide range of other species, including plants, fungi, nematodes, protozoa, insects (*Drosophila melanogaster*), and vertebrates. This suggests an ancient evolutionary origin of this phenomenon^{10, 45, 53}. The conservation of RNAi by eukaryotes clearly suggests that this adaptive response means a lot to these organisms: RNAi protects the most sensitive part of a species, its genetic code⁵³. RNAi has not been found in prokaryotes, so it is probably a eukaryotic innovation^{28, 43}.

MECHANISM OF RNAI

A combination of genetic and biochemical studies suggests that RNAi has a very similar mechanism in many organisms and that the enzymes involved in this process exhibit high homology across species⁵¹. The initial step of RNAi is connected with the appearance of dsRNA in the cell which is perfectly homologous in sequence to the silenced gene^{3, 19, 27, 29, 56, 58, 60}. dsRNA can either be intracellularly synthesized or exogenously introduced directly into cells⁵⁸. The minimal length of dsRNA to induce RNAi is 26 nucleotides, although long dsRNAs are more effective. That is why nucleotides about a few hundred nucleotides long are often used^{17, 57}. On the other hand, transfection of long dsRNA (hundreds of nucleotides) into cultured mammalian cells induces the interferon response, whereas short dsRNAs (about 20 nucleotides) only induce RNAi^{3, 8, 29, 65}. The interferon response protects neighboring cells from infection and blocks viral replication⁸. Long dsRNAs activate a dsRNA-dependent kinase, protein kinase

R (PKR)^{8, 58}, which phosphorylates and inactivates the translation factor eIF2 α ^{23, 51, 58}. This activation leads to a generalized inhibition of translation and can dramatically alter cellular metabolism and often activates apoptotic and nonapoptotic pathways^{10, 58, 64}. PKR may be one of several kinases in mammalian cells that can mediate this response^{10, 23}. A second dsRNA-response pathway activates 2'-5' oligoadenylate polymerase, the product of which is an essential cofactor for a sequence-non-specific ribonuclease, RnaseL^{10, 51, 58}. To overcome these difficulties, short dsRNAs are used in many cases to trigger RNAi in mammalian cells (dsRNAs less than 30 nucleotides long are unable to activate PKR, and full activation requires ~80 nucleotides)^{10, 42, 64}.

The dsRNAs are recognized by the Dicer enzyme, which is an Rnase III ribonuclease family member^{8, 16, 27, 33, 56, 67} (Fig. 1). Dicer enzymes are evolutionarily conserved and these proteins have been found in *D. melanogaster*, *C. elegans*, tobacco, and mammals²⁸. Humans and *C. elegans* have only one Dicer, but *Drosophila* has two (Dicer-2 is the major small interferin RNA – siRNA – producer in RNAi, and Dicer-1 probably binds micro RNA – miRNA) and *Arabidopsis* four Dicer enzymes⁶⁶. Dicer is ATP dependent and contains several characteristic domains: an N-terminal helicase domain, a PAZ (a conservative domain found in proteins in *Drosophila* and *Arabidopsis*, involved in developmental control), dual Rnase III domains, and a dsRNA-binding domain^{4, 64}. Dicer acts as a dimeric enzyme,

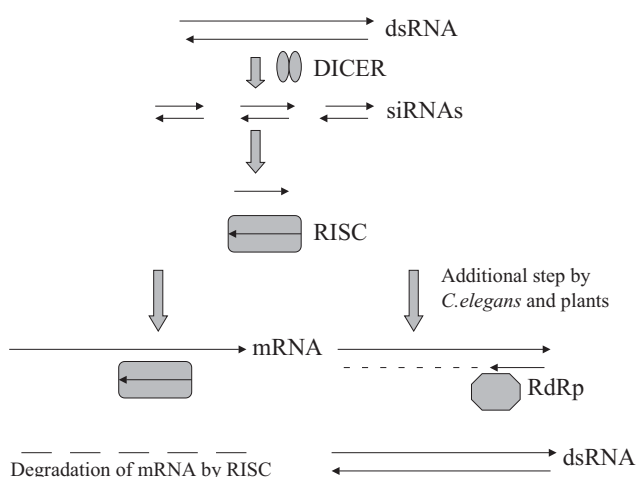


Figure 1. Schematic model of the RNAi mechanism. The cellular enzyme Dicer cleaves intracellularly synthesized or exogenously administered dsRNA into 21-25 nucleotide siRNAs. The siRNAs are incorporated into the RNA-induced silencing complex (RISC), which uses the antisense strand of the siRNA to find and destroy the target mRNA. The siRNAs can also be used as primers for the generation of new dsRNA by RNA-dependent RNA polymerase (RdRp). This step has only been found in plants and *C. elegans*.

although one of the active sites in each Dicer enzyme is defective²⁸. Dicer processes dsRNA into double-stranded siRNA 21-25 nucleotides in length, depending on the species^{4, 16, 17, 19, 26, 28, 57, 60} (Fig. 1), although plants produce two distinct classes of siRNAs: short (21-23 nucleotides) and long (24-25 nucleotides)⁷⁴. These molecules contain 3' hydroxyl termini and additional characteristic 2-nucleotide 3' overhangs and 5' phosphorylated termini^{12, 49}. It was shown that the described structural features of siRNAs are crucial for the next stages of the RNAi pathway^{12, 42, 59, 64}. For example, modification of the 5' end of the antisense strand inhibits siRNA activity, and blunt-ended siRNAs become inefficient intermediates in the further steps^{42, 59, 64}. The siRNAs produced by Dicer are incorporated into a multicomponent nuclease complex, RNA-induced silencing complex (RISC)^{1, 8, 17, 19, 27, 28, 49, 67} (Fig. 1), which must be converted from a latent form, containing a double-stranded siRNA, to an active form by unwinding the siRNA by a helicase in an ATP-dependent process (an ATP-dependent RNA helicase)^{28, 49, 53}. The RISC also contains an endoribonuclease which, using the sequence encoded by the antisense siRNA strand, finds and destroys the complementary sequence of mRNA^{8, 49, 64} (Fig. 1). During studies on the biochemistry of RNAi, some other proteins in the RISC have been identified and characterized. For example, in human RISC two proteins were found: eIF2C1 (Argonaute1, a translation factor)³⁶ and eIF2C2/GERp95 (a translation factor, component of the Golgi apparatus or endoplasmic reticulum, and a component of the survival of motor neurons complex)^{8, 43, 64}. The argonaute protein has two characteristic domains: PAZ, also found in Dicer, and PIWI. The PAZ domain probably acts as an RNA binding module for Argonautes, recognizing and binding the characteristic 2-nucleotide 3' overhang of siRNAs³⁶. The 3' end recognition by PAZ may be important for only two protein families which contain these domains, Dicers and Argonautes, to distinguish between siRNAs and other RNA molecules³⁶. In *Drosophila* there is also the RISC loading complex that contains Dicer-2 and R2D2 (Dicer-2-associated protein)^{36, 66}. The exact role of these proteins in RISC is still unclear, and the identification of other RISC components is still being awaited. The target mRNA is cleaved for fragments of about 22 nucleotides (21-23 nucleotide intervals) long^{17, 68}. When the cleavage is completed, the RISC departs and the siRNA can be used in a new cycle of mRNA recognition and cleavage, which protects this molecule from rapid degradation, the normal fate of small single-stranded RNA in cells⁷⁵.

The described processes probably take place in the cytoplasm^{6, 78}. There are two indications of this: first,

RISC has been found to copurify with ribosomes and, secondly, it was also reported that dsRNAs targeted against intronic and promoter sequences are not effective as inducers of RNAi^{27, 78}. In plants and worms, amplification of the silencing signal and cell-to-cell RNAi spreading have been observed^{8, 58}. As yet this "systemic" spreading has not been noticed in mammalian cells. In both plants and *C. elegans*, enzyme RNA-dependent RNA polymerase (RdRp) has been found^{8, 57}. It is responsible for the generation and amplification of siRNA into dsRNA^{1, 18, 28} (Fig. 1). These siRNAs are used as primers for the generation of new dsRNAs by RdRp, which can subsequently serve as targets for the Dicer enzyme and be processed into new siRNAs^{1, 43} (Fig. 1). Thanks to this system in plant and worm cells, specific numbers of dsRNA molecules are able to inactivate more copies of endogenous transcripts^{1, 6}. RdRps are also encoded by a wide variety of RNA viruses for genome replication, mRNA synthesis, RNA recombination, and other processes. Many if not most eukaryotes also encode RdRps whose roles are still being uncovered^{18, 28}.

Besides the signal amplification system, there is also a system of cell-to-cell signal transmission throughout most tissues of the organism. This effect is termed systemic suppression²⁸. In plants, the movement of dsRNA or siRNA between cells can take place through cytoplasmic bridges (plasmodesmata) and/or via the vascular system^{28, 64}. It is still unclear how this process proceeds in worms, but recent experiments show that products of the *sid-1*, *sid-2*, and *sid-3* genes are required for systemic spreading in the worm body⁷². It is possible that the SID-1 membrane protein can act as a channel for importing the silencing signal. Such transfer could involve a sequence-specific component, probably nucleic acid^{58, 72}. SID-1 has homologues in the mouse as well as in humans, and these proteins share significant amino-acid identity and have strikingly similar transmembrane profiles⁵⁸.

SIGNIFICANCE OF RNAI

In plants and invertebrates, RNAi is a system of defense against mobile genetic elements such as transposons and viruses^{8, 17, 27, 58, 69, 70}. It is clear that RNAi protects and controls viral infections in plants^{69, 70}. For a lot of viruses, a mutation in the RNAi system can cause an increase in the severity of viral pathogenesis. In addition, many plant viruses encode proteins which are able to inactivate essential genes in the RNAi machinery^{17, 40}. These viruses promote in this way their replication in the host genome²⁸. For example, the tombusvirus p19 protein

blocks RNAi in plants by binding RNA duplexes, thereby blocking their assembly into the RISC⁷⁴. There is no evidence that RNAi is an antiviral system in vertebrates⁵, and its specific function in these organisms is unclear⁴³. In plants, dsRNA induces genomic methylation at sites of sequences homology. This methylation is asymmetric and is not restricted to CpG sequences^{11, 18, 28}. If methylation occurs in promoter sequences, it can cause transcriptional gene silencing before the proper mRNA transcript arises⁴⁷. In worms and vertebrates, RNAi is probably involved in developmental gene control^{8, 27, 57}. For this process, specific small RNAs, miRNAs or small temporal RNAs (stRNAs such as lin4 and let7), are probably very important^{28, 50}. In plants, miRNAs can be the targets for a viral silencing suppressor such as p19 tombusvirus protein. This protein binds miRNAs in a double-stranded state, preventing maturation of miRNAs and their function as developmental regulators⁷⁴. The family of endogenously encoded small RNAs is very large, and over 100 of these RNAs have been identified in *D. melanogaster*, *C. elegans*, and mammals^{38, 78}. It is still not known how many functions these small RNAs perform in eukaryotic cells besides their functions in gene silencing and developmental timing^{28, 71}. New classes of small RNAs are still being discovered. Tiny non-coding RNAs (tncRNAs), for instance, were found in *C. elegans* last year⁴⁸. They are not evolutionarily conserved, but some are developmentally regulated. The function of tncRNAs is still unknown⁴⁸.

The small RNAs are transcribed from the genome as hairpin precursors 21–22 nucleotides long⁵⁷. Similar to dsRNAs, stRNAs and miRNAs are cleaved by Dicer and only one strand is then used in the further stages^{15, 25, 32}. The small RNAs do not have to show perfect complementarity to their target, in contrast to dsRNAs. These molecules bind mRNA, but degradation of the transcript is not initiated because miRNAs and stRNAs act mostly at the level of translation and inhibit protein chain elongation (plants and animals)^{27, 47, 50, 66}. In plants, silencing via miRNAs can also occur via destruction of the target mRNA⁶⁶. It is possible that there are two analogous RISC complexes, containing either siRNAs or stRNAs and miRNAs^{28, 66}. In the first case, post-transcriptional mRNA degradation is induced and in the second case, ribosomal elongation is blocked⁶⁶. It is suggested that the RISC may be a flexible enzyme complex involved in different regulatory strategies²⁸. One of the many biological functions of the RNAi machinery may be to form heterochromatic domains (transcriptional inactive chromatin) in the nucleus, which is a very important process for genome organization and stability¹¹. RNAi can affect gene expression at

the level of chromatin structure in *D. melanogaster*, *C. elegans*, and fungi¹⁶. There are interconnections between RNAi and other metabolic pathways. In *C. elegans*, seven genes, *smg1-7*, are responsible for non-sense-mediated mRNA decay^{41, 64}. Three of these genes are also required for persistence of RNAi. It is clear that gene silencing is connected with other cellular processes, but it is not known how these metabolic pathways interplay and influence each other⁶⁴.

RNAi – PERSPECTIVES FOR THE FUTURE

RNAi is a potentially powerful research tool for a wide variety of gene-silencing applications^{2, 30, 46, 56}. Possible repercussions of RNAi in mammals are its use in the fight against certain diseases, such as cancer or virus and parasite infections², as well as in the analysis of problems in cell and developmental biology¹⁹: there are, for example, many efficient human and murine siRNA sequences against members of apoptotic pathways, such as caspase-1, -2, -3, -8, and Fas⁷⁷. RNAi can also be used to study the functions and interactions of genes⁶. siRNAs are easily synthesized and used to silence genes in cell cultures, and it is possible that silencing cell lines will be obtained^{52, 63}. One of the earliest uses of RNAi technology in drug development has been its application in functional genomic analyses. During these studies many components of complex pathways have been identified and isolated and their relevance to various drug discovery applications has been assessed⁵⁸. RNAi can be used as a tool to identify possible novel targets in drug discovery. This approach has several advantages: it permits rapid target identification and processing and does not depend on preexisting knowledge of target biology. Using bioinformatics, libraries of designed siRNAs (several different siRNAs oligos per gene) can be used to elucidate novel targets for any biological pathway. This method allows for the functional analysis of thousands of genes simultaneously, is highly reproducible, and requires small amounts of siRNA oligos. This procedure allows for high-throughput testing of potential targets without compromising high specificity and sensitivity⁷³. siRNAs could also represent the next generation of antiviral therapeutics, and DNAs encoding siRNAs should be useful in various forms of gene therapy⁷⁵. The activation of siRNAs appears to be short-lived in mammals. They are sequence-specific natural cellular products, do not produce toxic metabolites, have a long life-span in cell culture and calf serum, and are efficient even in low concentrations^{75, 77}.

Introducing siRNAs reduces the immune response against newly introduced agents, which may be a problem in gene therapy. Delivering of siRNAs to

the appropriate cells is a major challenge. Effective strategies to deliver siRNAs to target cells in a cell culture include physical (electroporation, injection) or chemical (lipid-mediated gene delivery or a new designed method: the peptide-based gene delivery system MPG) transfection^{54, 61} (Fig. 2). Chemical synthesis of siRNA is the most commonly used method to generate RNAi⁵⁸. T7-transcribed siRNAs as well as siRNAs isolated from *D. melanogaster* embryo protein extracts were also shown to be effective⁵⁸ (Fig. 2). It may be difficult to introduce short dsRNAs directly into cells. An alternative strategy uses the endogenous expression of siRNAs by various RNA polymerase III promoter systems (mouse U6, human H1, tRNA promoters) that allow transcription of functional siRNAs or their precursors^{39, 56, 65} (Fig. 2). This

structured an arrayed siRNA expression cassette library that targets about 8000 genes with two sequences per gene⁸⁰. Injection of plasmid DNA expressing long cytoplasmic dsRNA induces efficient RNAi in non-embryonic mammalian cells without stress response pathways (Fig. 2). This approach may prove to be the best method of inducing RNAi in mammals. In this case there will be simultaneous expression of a large number of siRNAs from a single precursor dsRNA, and longer dsRNA could include more than one message in a single construct. Liver cells seem to be particularly receptive to exogenous RNA⁷⁵. Neurons seem to be more resistant to RNAi than other cell types, perhaps because of differences related to RNA transport across the cell membrane or the RNAi pathway in these cells³⁷. It has been claimed that very high concentrations of dsRNAs (15 µg/ml) can induce inhibition of target gene expression in proliferating and differentiating cells in a nematode neuronal culture³⁷. Better delivery methods are required before siRNAs can be used as therapeutics, especially to suppress gene expression in tissues other than liver. Recently, vectors have been investigated which contain a cytomegalovirus (CMV) promoter and express long (about 500 nucleotides) dsRNAs, but these dsRNAs are not transported into cytoplasm and do not induce the interferon response²². These dsRNAs are cleaved into siRNAs in the nucleus and are then transported to the cytosol, where they silence the target mRNA. This system is based on the polymerase II promoter and, although the CMV promoter is active in most cell types, these findings are a first step toward the use of tissue-specific polymerase II promoters. The potential advantage of this method is that there are numerous tissue-specific polymerase II promoters available²².

Physical methods		Chemical methods	
1. Electroporation 2. Injection		1. Lipid-mediated gene delivery 2. Peptide-mediated gene delivery	

siRNA		
Isolated from <i>Drosophila</i> embryo	Synthetic RNAs 1. siRNAs, 2. dsRNA	Plasmid generated RNAs 1. siRNAs pol III promoters-mouseU6, humanH1, tRNA promoter 2. dsRNAs pol II and T7promoters

Figure 2. siRNA delivery strategies.

way the produced siRNAs could be expressed for longer periods than exogenously introduced siRNAs, particularly in cells where the expression unit will integrate with the host genome^{7, 58}. Zheng et al.⁸⁰ have developed a dual-promoter siRNA expression system (pDual) in which a synthetic DNA encoding a gene-specific siRNA sequence is inserted between two different opposing polymerase III promoters, the mouse U6 and human H1 promoters. Upon transfection into mammalian cells, the sense and antisense strands of the duplex are transcribed by these two promoters from the same template, resulting in an siRNA duplex with a uridine overhang on each 3' terminus, similar to the siRNA generated by Dicer. These siRNAs can be incorporated into the RISC without any further modifications and specifically and efficiently suppress gene functions. Furthermore, they have developed a single-step PCR protocol that allows the production of siRNA expression cassettes in a high-throughput manner and they have con-

RNAi is a good candidate for an antiviral drug⁸. Recently some studies have shown the capacity of chemically synthesized siRNAs to specifically inhibit some viruses^{31, 35}. Pre-treatment of human and mouse cells with siRNAs to a virus genome reduced the titer of virus progeny and promoted clearance of the virus from most infected cells²⁴. For example, RNAi can inhibit HIV-1 replication in mammalian cells at multiple steps of the HIV life cycle^{8, 34, 39, 55}. SiRNAs may induce the cleavage of pre-integrated RNA or interfere with post-integration HIV-1 RNA transcripts and block progeny virus production^{39, 43}. SiRNAs targeting CD4, CXCR4, or CCR5 RNA transcripts inhibit virus attachment to the CD4 receptor (the main receptor for HIV on the cell surface) or chemokine receptors (coreceptors for HIV) mediating HIV-1 fusion and entry⁴³. Effective blockade of receptors or coreceptors which are expressed on the cell surface represents a new strategy in therapy.

Good examples are chemokine receptors involved in inflammatory, allergic, and immunoregulatory disorders. Overexpression of CXCR4 has been associated with a number of malignant disorders, such as metastasis of breast carcinoma cells, angiogenesis of normal and tumor tissue, or B cell chronic lymphocytic leukemia⁴³. Thus, CXCR4 may be an important therapeutic target. The involvement of CCR5 in inflammatory processes and rheumatoid arthritis has been documented⁴³. Selective viral vectors containing siRNAs directed to CXCR4 and CCR5 in specifically targeted cells or after *ex vivo* manipulation of stem cells could be used to alter the role of chemokine receptors in various diseases.

Another receptor involved in the development of some disorders is the Fas receptor. Fas-mediated apoptosis is implicated in a broad spectrum of liver diseases, where inhibition of hepatocyte death would be a life-saving measure⁷⁵. Song et al.⁶² investigated the *in vivo* silencing effect of siRNAs targeting the Fas gene encoding the Fas receptor to protect mice from liver failure and fibrosis in two models of autoimmune hepatitis. Intravenous injection of Fas siRNA specifically reduced Fas mRNA levels in mouse hepatocytes. Hepatocytes isolated from mice treated with Fas siRNAs were resistant to apoptosis, which is a sign that silencing expression with RNAi could be used in therapy to prevent liver injury by protecting hepatocytes from cytotoxicity⁶². In another investigation siRNA was used against caspase-8 to prevent not only Fas-specific liver injury, but also acute liver failure induced by the wild-type adenovirus⁷⁷.

The RNAi methodology has also been used as a tool against severe acute respiratory syndrome-associated

coronavirus (SARS-CoV), which is responsible for SARS⁷⁹. The siRNA used specifically and effectively inhibited Spike protein from SARS-CoV. This protein is probably important in the interaction with the host cell surface receptors and the fusion event between the viral envelope and cellular membrane. Therefore, thanks to RNAi technology it is possible to block viron entrance into host cells and inhibit SARS-CoV infection⁷⁹.

RNAi also blocks poliovirus^{15, 24}, respiratory syncytial virus, and human papilloma virus infections and could be easily extended to other human viruses^{31, 35, 43}.

CONCLUSIONS

The use of genetic modeling systems such as RNAi has been the key to understanding gene structure and function, the biology of cells and organisms and, ultimately, the molecular aspects of human disease. Libraries of short RNAs or of DNA vectors encoding short RNAs have been generated to target a few thousand human genes to determine their functions. Unlike classical antisense techniques, dsRNAs act as powerful gene silencers, which influences their therapeutic potential. As an ideal therapeutic, RNAi should also act selectively, long-term, and be able to systemically modulate gene targets distal from the inoculation area. A major problem in therapeutic gene silencing is still the delivery problem. The improvement of *in vivo* nucleic acid delivery technologies is the most important obstacle to overcome for scientists in the coming years. These small RNA molecules will probably have several applications in biology and in medicine in the future.

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