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RNA Interference and Cancer

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Abstract
RNA interference (RNAi) is an evolutionally conserved gene silencing mechanism present in a variety of eukaryotic species. RNAi uses short double-stranded RNA (dsRNA) to trigger degradation or translation repression of homologous RNA targets in a sequence-specific manner. Over the past decade RNA interference (RNAi) has emerged as a natural mechanism for silencing gene expression. This system can be induced effectively in vitro and in vivo by direct application of small interfering RNAs (siRNAs), or by expression of short hairpin RNA (shRNA) with non-viral and viral vectors. This review briefly describes the molecular principles underlying of RNAi phenomenon and discuss the main technical issues regarding optimization of RNAi experimental design.
Keywords: RNA interference (RNAi), double strand RNA (dsRNA), short hairpin RNA (shRNA)

Introduction

RNAi is a sequence specific process, operating at the mRNA level, which is able to regulate gene transcripts negatively. This posttranscriptional gene silencing phenomenon is triggered by dsRNA, homologous to a coding region in the target gene (1). It has first been reported by Izant et al. (*1984*) that RNA introduction into cells is able to interfere with gene functions. They observed that thymidine kinase (TK) activity could be reduced after introduction of TK anti-sense RNA (2). In 1998 Fire and coll. coined the term RNA interference (RNAi) referring to the phenomenon of post-translational silencing of gene expression that occurs in response to the introduction of double-stranded RNA (dsRNA) into a cell. RNAi promises to revolutionize key areas of medical research, as demonstrated by the preliminary findings obtained in the fields of cancer, infectious diseases and neurodegenerative disorders (3). This system was examined in a broad variety of species including plants, fungi, yeasts, nematodes, flies and mammals. In fact, RNAi serves as a safeguard for the preservation of genomic integrit. It protects the host from viral infections and invasion by mobile genetic elements by degrading the exogenous genomic material (e.g. viral RNAs). RNAi is triggered by small double-stranded RNA (dsRNA) and functions at all levels, including transcription (4), post-transcription (5) and translation (6). The mechanism of action remained enigmatic until 1998, when Fire and Mello discovered that dsRNA, instead of the single-stranded sense or antisense RNA, mediated gene silencing by degrading endogenous mRNAs in a sequence specific manner (7). RNAi promises to revolutionize key areas of medical research, as demonstrated by the preliminary findings obtained in the fields of cancer, infectious diseases and neurodegenerative disorders. In the present review, we discuss the mechanism of RNAi, and its role in the cancer biology. RNAi technology is rapidly spreading in research laboratories worldwide, as it is associated with a number of practical and theoretic advantages
over preexisting methods of suppressing gene expression (Table 1).

Table 1: Comparison between different methods for gene silencing.

Method	Advantages	Drawbacks
RNA interference	*Specific* *Relatively easy*	*Knock-down (not knock-out)* *Needs transfection*
Anti-sense DNA	*Easy* *Inexpensive*	*Variable efficiency* *Variable specificity* *Needs transfection*
Knock-out animal	*Complete gene silencing*	*Labor intensive, expensive* *Lethal mutants may prevent embryonic development*
Small molecule inhibitors	*Easy delivery*	*Variable specificity* *Labor intensive development*

Mechanisms of the RNAi and key protein factors in small RNA pathways

Extensive research has been conducted during the past decade in order to reveal the mechanism behind RNAi (1).

dsRNAs that cause selective gene silencing make RNAi the gold standard for studying loss of function phenotype. Two types of small RNAs, siRNAs and microRNAs (miRNAs), are known to mediate RNAi. siRNAs are double-stranded RNAs of 21 to 23 nucleotides in length and contain 3′ overhangs of 2 nucleotides on each strand. siRNAs are generated from long dsRNAs of exogenous or endogenous origin in the cytoplasm by the enzyme Dicer (8, 9).

Biochemical and genetic studies have revealed the detailed mechanism by which dsRNA-mediated gene silencing takes place. In general, the mechanism includes two major steps: the initiator step and the effector step (Figure 1) (10).

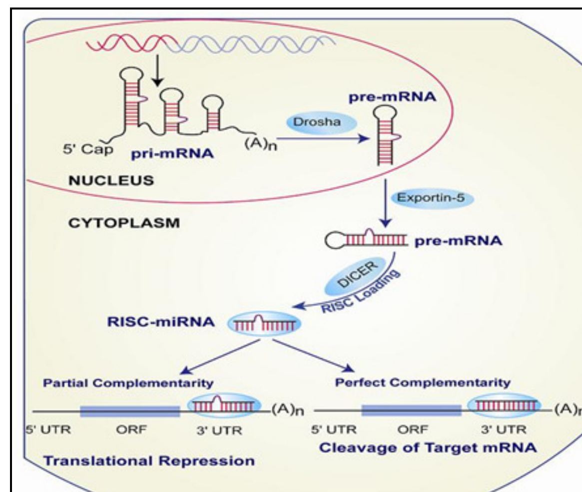

Figure 1 The RNA interference pathways

In the initiator step, long dsRNAs, which are produced by endogenous genes, invading viruses, transposons or experimental transgenes, are initially recognized by a dsRNA-binding protein, RDE-4/R2D2 (11,12). They are then submitted to and cleaved by the RNase III-like nuclease Dicer (13). RNase III type enzymes are essential components of small RNA pathways. There are two RNase III subfamilies involved in small RNA pathways: Dicer (class III) and Drosha (class II). Both are large proteins with tandem catalytic domains and a dsRNA-binding domain (dsRBD) at the C-termini. Dicer is a highly conserved protein that has a long N-terminus that contains a DExH RNA helicase/ATPase domain, as well as the DUF283 domain and the PAZ domain. Dicer cleaves dsRNA precursors into 21-22 nt RNA duplexes with overhanging 3' ends, called small interfering RNAs (siRNA). There is one Dicer homologue in fission yeast (Dcr), one in human (Dicer, also known as Helicase- MOI), one in nematode worm (DCR-1), two in Drosophila (DCR-1 and DCR-2), and four in Arabidopsis (DCL1, DCL2, DCL3, DCL4). Drosha, on the other hand, is conserved only among metazoans. Drosha initiates miRNA maturation by cleaving the primary transcript of miRNA, releasing short hairpin-like precursor (premiRNA). Only one Drosha homologue is found in each animal species (14).

In the effector step, siRNAs are incorporated into a multicomponent nuclease complex, the RNA-induced silencing complex (RISC) (15). The antisense strand of the duplex directs RISC to recognize and to cleave cognate target RNAs, which undergoes specific base pairing and endonucleolytic cleavage. This leads to the degradation of the unprotected and single-stranded target RNA. To date several components of the RISC have been identified, including some conserved argonaute proteins that share the PAZ domain with Dicer family proteins (16).

Argonaute (Ago) proteins play a central role in various aspects of small RNA pathways, by directly interacting with small RNAs and by forming effector complexes. These effector complexes are known as RNA-induced silencing complex (RISC), miRNP, or RNA-induced initiation of transcriptional silencing complex (RITS). Argonaute family proteins are highly basic proteins of ~100 kDa that contain two common domains; PAZ and PIWI domains. The PAZ domain consisting of ~130 amino acids is usually located at the center of the protein and interacts with the 3′ overhang of dsRNA (17). In some organisms (*C. elegans*, *Arabidopsis thaliana*) an additional step in the RNAi pathway has been described involving a population of secondary siRNAs derived from the action of a cellular RNA-dependent RNA polymerase (RdRp). They are most likely generated during cyclic amplification in which RdRp is primed on the target mRNA template by existing siRNAs (18).

Strategies for RNAi and cancer

There are two general abnormalities in cancer cells - they exhibit dysregulation of the cell cycle resulting in uncontrolled growth and they are resistant to death as a result of abnormalities in one or more proteins that mediate apoptosis (19). Over the past decades, investigations of the molecular mechanisms of cancer have revealed a number of oncogenes. With the tremendous efforts in identification, cloning, sequencing and functional analysis of oncogenes, novel preventive and therapeutic strategies are being developed to conquer cancers. Of them, RNA-mediated gene regulation system is a powerful gene-silencing arm within cells. This approach has been used to identify genes critical for the growth, differentiation and death of cells, to protect cells from invasion of viruses, to inhibit cancer genesis, and to investigate molecular mechanisms of other diseases (20). The goals for RNAi approaches for cancer therapy are therefore to knock out the expression of a cell cycle gene and/or an anti-apoptotic gene in the cancer cells thereby stopping tumor growth and killing the cancer cells. To selectively eliminate cancer cells without damaging normal cells, the siRNAs would be selectively delivered into the cancer cells (21). In order to study the functional genomics and biology of RNA interference, much effort has gone into the study of artifi cial RNAi-inducing gene silencing. Strategies for delivery of RNAi reagents into mammalian cells can be divided into two types, the transient RNAi and the stable/inducible RNAi. The methods commonly used in producing siRNA extraneously include chemical synthesis, in vitro transcription, and recombinant human Dicer/E. coli RNase III digestion of long dsRNAs. These siRNAs can be transiently transfected into target cells.Alternatively, the short hairpin RNAs (shRNAs) are expressed endogenously from plasmids and viral vectors. The shRNA expression cassettes can be stably integrated into the genome of target cells, transcribed intranuclearly and processed into siRNAs by Dicer in the cytosol. In general, RNA Pol III promoters (i.e. U6, H1 and tRNA promoters) are commonly used to drive shRNA expression in the RNAi studies (22).

There are some general and some specific rules to design an efficient siRNA (23). The overall efficiency of the artificial siRNA or shRNA will depend upon additional characteristics: low G/C content (30–60%); beginning with a G or C residue after an AA dimer in the 50-flanking sequence; sense strand base preferences at positions 3 (A), 10 (U), 13 (A) and 19 (A); lack of four or more consecutive T or Ato avoid premature pol III transcription termination signals (figure 2) (24).

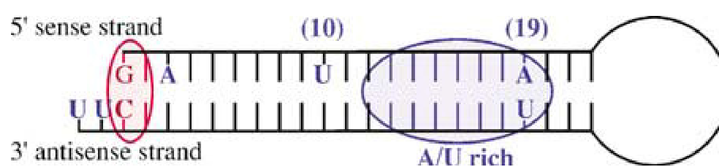

Figure 2. Schematic view of an shRNA transcript, showing stability rules for RISC preferences.

Although these vectors provide certain advantages over chemically synthesized siRNAs, numerous disadvantages remain, including low and variable transfection efficiency and difficulties of integrating into the host DNA to become stable (25).

RNAi technology can help to discover genes essential for viability in cancer cells that can be then used as targets for suicide. Inhibiting overexpressed oncogenes, such as *ras* or *myc,* should block pathways that cancer cells depend on. In most cancers, however, it may be necessary to block pathways at several points, or even to target several pathways. Identifying the genes that are altered in the stepwise progression to malignancy has become one of the central goals of cancer research; automation of data generation (robotics) and computer analysis (bioinformatics) have significantly accelerated the process of discovering cancer-linked genes. Once an oncogene that is highly represented in a particular type of cancer (melanoma or glioblastoma, for example) is identified, there is the hope that this will lead to clinically useful targeted therapies. One of the most important signaling pathways to control growth and proliferation of our cells is the mitogen-activated protein kinase (MAPK) pathway. Ras is mutated to an oncogenic form in about 15% of human cancer; stable suppression of tumorigenicity was achieved by virus-mediated RNAi to inhibit specifically the oncogenic allele of K-ras (K-rasV12) in human tumor cells (26).

Three more papers have recently (27-29) reported the use of siRNA to block further the Ras-Raf-Mek-Erk-Map kinase cascade, one at the Raf level, and the other one through NADPH oxidase1 (Nox1). The three Raf genes (A-raf, B-raf and C-raf) code for cytoplasmic serine/threonine kinases that are regulated by binding Ras. Raf proteins phosphorylate Mek 1/2, which in turn phosphorylate Erk 1/2 and these act upon MAPK. Melanoma, on the other hand, is an aggressive skin cancer derived from melanocytes resistant to most current treatments once cells have spread to other parts of the body. The oncogene B-raf was found to be mutated in 59% of melanoma cell lines, in 80% of short-term melanoma cell cultures and 66% of uncultured melanomas, making it a putative excellent target

against this type of tumor. In this context siRNA have been designed to knock down the activating mutation B-raf^{V599E} present in more than 60% of malignant melanomas. Suppression of B-raf V599E expression caused growth arrest and apoptosis in melanoma cell lines and in vivo (30). RNAi can be directed against cell-cycle control genes to block cell division and promote apoptosis. Cyclin E is required for progression through the G1 phase, and its depletion would lead to G1/S transition arrest. siRNAs have been successfully directed against the messengers of this protein (31).

RNAi can be used to increase the apoptotic susceptibility of cancer cells by inhibiting antiapoptotic genes such as bcl-2, livin or survivin. In esophageal adenocarcinoma, the levels of the apoptosis-resistant determinant BCL-XL are dangerously high. 50% decrease in protein expression was reported using RNAi against BCL-XL mRNA (32). In Hela cells, silencing of livin strongly increased the apoptotic rate in response to different proapoptotic stimuli (33). Also, siRNAs are small in size and it is possible to include more than one antiangiogenic siRNA into a single retroviral vector, which could inhibit multiple pathways simultaneously (34). RNAi can help in the identification of ''synthetic lethals'', a combination of two nonlethal mutations that, together, result in cell death. In general, functional mammalian cancer genetic screens have expanded markedly, allowing the discovery of new combinations of activating mutations in oncogenes, and loss-of-function mutations in oncosuppressors. These are important for tumor development, angiogenesis, progression, or metastasis (35).

Conclusions

In recent years, RNAi has become a powerful tool to probe gene functions and to rationalize drug design. It has been employed as a prophylactic and therapeutic agent for combating a wide range of disorders, including infectious diseases, tumors and metabolic disorders. Many studies have proliferated that describe notable success in provoking cancer cell death in culture by siRNA treatments, but very few yet are complemented with the eradication of well-developed tumors in experimental animals, a necessary step before using this technology in human trials. Also, the ability to deliver efficiently sufficient amounts of siRNA to a particular tumor requires refinement before this new technology can be tried clinically. Initial in vivo studies reported effective tumor suppression in nude mice by chemically synthesized siRNAs. More recently, many researchers have used plasmid and viral vectors for transcription of shRNAs, both in vitro and in vivo. With these systems, gene expression was more stably inhibited, but the efficiency of selective delivery will continue to be a limiting factor. Despite all this, there is no doubt that RNAi represents a new front in the war on cancer.Human trials exploiting this powerful technology are likely to follow soon.

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