REVIEW ARTICLE

RNA Intereference: A powerful laboratory tool and its therapeutic implications

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Abstract
Ever since RNA intereference (RNAi) was discovered in the early 1990s, a number of scientists from the academic and biotechnology world have continued to view it as the revolutionary discovery of the century. Unequivocally, RNAi can be considered as an important regulator of gene expression in many eukaryotic cells. The term RNAi describes a natural process by which a double-stranded RNA molecule, when introduced into the cell is processed into short RNA duplexes and drives gene silencing by specific and distinct mechanisms. Many of the players involved in this cellular defense network have been elucidated but a more complete understanding of the process is essential. Worldwide interest on RNAi in the last decade is mainly attributed to its power as a laboratory tool for the experimental manipulation of gene expression. RNAi assisted already in the dissection of numerous cellular pathways and revealed the role of many proteins in an approach aimed to drug discovery. This new technology has the potential to improve our understanding of physiologic and pathologic processes and lead to the discovery of new drugs. More importantly, there is growing interest among the scientific community for the potential therapeutic applications of RNAi. *Hippokratia 2006; 10 (3): 112-115*

Key words: *RNA interference, gene silencing, siRNA, miRNA*

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Mechanism of RNAi

RNAi was first discovered in plants and worms in 1998 when Andrew Fire described the specific silencing of gene expression by double stranded RNA molecules[1]. However, the first indication that such mechanism existed came much earlier, in 1990, when plant scientists working with petunias found that introducing numerous copies of a gene that encodes for the purple color of the flower, resulted not to an even darker coloration, but rather to plants with white or patchy flowers[2]. It appeared that the transgenes had inactivated both themselves as well as the endogenous gene. The results were understood only after the work performed in *C. elegans,* a few years later when it became clear that the silencing mechanism is mediated by dsRNA through an evolutionarily conserved natural process.

A key in determining the mechanism of RNAi was the discovery that dsRNA, when introduced into cells is cleaved into short RNA entities, 20-25nt long, with a specific secondary structure. The short interfering RNA molecules (siRNA) were double-stranded in nature and consisted of 19 nucleotides of dsRNA with two unpaired nucleotides at the 3' ends[3-5] (Figure 1).

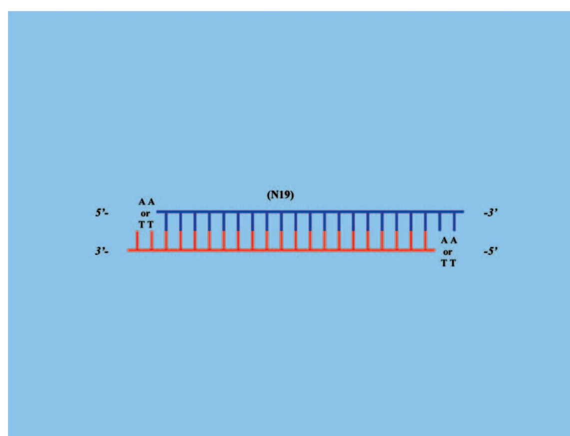

Figure Legends
Figure 1: **Representative structure of an siRNA molecule**. *The duplex consist of 19 base pairs with two nucleotides overhanging on the 3'-ends.*

Initiation Phase

RNA intereference involves two phases. The initiation phase during which short dsRNA molecules are generated and the execution phase in which gene specific inactivation occurs, either by degradation of the target mRNA or by translational inhibition. An enzyme called Dicer[6] cleaves the long dsRNA. Dicer is a multifunctional enzyme that contains numerous domains including, a catalytic RNAse III domain, a dsRNA -binding domain and RNA helicase domain. The processing of dsRNA to siRNA by Dicer is an ATP-dependent process and requires a functional RNA helicase domain.

Dicer recognizes the presence of a dsRNA, associates with it through its RNA binding domain (RBD), unwinds the molecule and cleaves it in a sequence-specific manner[7]. More than one Dicer enzymes have been identified in different organisms. *Drosophila melanogaster* has two paralogues: Dicer-1 (DCR-1) and Dicer-2 (DCR-2)[8] and *Arabidopsis thaliana* has four Dicer-like genes (DCL-1 to DCL-4) [9]. On the other hand, only one Dicer enzyme has been identified in humans. It is believed that each Dicer protein can preferentially recognize RNA duplexes coming from different sources, although this has not been proven yet [8] (Figure 2).

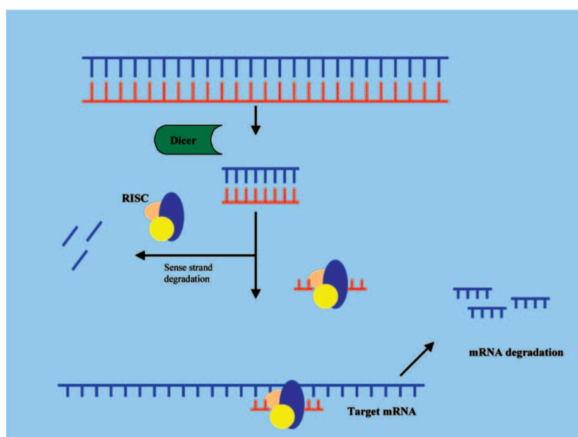

Figure 2: Mechanism of RNA interference.
Long dsRNA is cleaved by Dicer to generate short interfering RNA molecules. The newly formed siRNA associates with a multiprotein complex called RISC. The sense strand of the duplex is degraded and the antisense strand directs the RISC complex to the target mRNA. Association of RISC with the target mRNA results in the degradation of the mRNA.

Execution phase

The newly formed siRNA duplexes are then associated with a multiprotein complex called RISC (RNA-induced silencing complex) which collectively represent the effector complex of the RNAi pathway. Upon association with RISC, the sense strand of the siRNA duplex is degraded. The siRNA antisense strand which remains associated with RISC guides the sequence-specific degradation of complimentary target mRNA, upon hybridization [10]. The RISC complex includes a number of enzymes and structural proteins of which only a fraction has been identified[11]. In addition, RISC complexes with different sizes and compositions are present in different organisms and these presumably differ in activity or even in function. Proteins isolated from RISC complexes include members of the Argonaute (Ago) protein family, which may be required for the incorporation of the siRNA into the RISC complex[12], RNA helicases [13] and exonucleases[14]. Although the RISC complex is known to have endonuclease activity the enzyme responsible has not yet been identified.

Upon binding of the RISC complex to a target mRNA, the endonuclease of RISC cleaves the mRNA at a position approximately middle of the recognized 21 nucleotide sequence. This mRNA degradation takes place in the cytoplasm of the cell and results to silencing the target gene [15] (Figure 2).

Amplification of the RNAi signal in plants and worms

In organisms like plants and worms small amounts of dsRNA are able to silence a vast amount of target mRNA [1]. There are many theories regarding this phenomenon. First, the Dicer enzyme can generate many different siRNAs from a long dsRNA and each one of them can target a homologous mRNA providing a level of amplification. Second, a catalytic mechanism might exist by which siRNA molecules are used multiple times. Finally, a model termed " random degenerative PCR" might occur in which an RNA-dependent RNA polymerase (RdRP) uses the siRNA strands as primers for the target mRNA. This event will generate more dsRNA to be used as a substrate for Dicer and result in amplification of the signal[16]. Human cells do not posses a RdRP and this might explain the absence of RNAi signal amplification in humans.

MicroRNAs

The discovery of siRNAs generated from a long dsRNA, prompted an enquiry for short interfering RNA molecules that are encoded by genes in the genome. Surprisingly, instead of siRNA, a new class of short RNAs was identified, named microRNAs (miRNA). MicroRNAs are single stranded molecules and they are encoded in the host genome, but unlike mRNA they are not translated to a protein. Their function is to regulate the expression of mRNA using a mechanism different from that employed by siRNAs.

MicroRNAs are transcribed from the genome to give rise to a "hairpin" RNA structure called pri-miRNA. This molecule is processed by the enzyme Drosha inside the nucleus and generates a smaller hairpin RNA, about 70 nucleotides long. Pre-miRNAs are exported to the cytoplasm where they are processed by Dicer to generate the mature miRNAs [17,18]. The mature miRNAs are then assembled into a ribonucleoprotein complex (miRNP) that is similar to RISC. Unlike RISC, this complex binds to the 3' untranslated region of particular mRNAs through partial complementarity, and prevents the mRNA from being translated (Figure 3) presumably by interfering with the polyribosomes. MicroRNAs have been recently discovered and their function has not been fully elucidated. However, their ability to regulate mRNA expression by partial complementarity suggests that they can simultaneously regulate a plethora of genes. On the other hand, this same quality makes it very difficult to identify the physiologic mRNA targets for the miRNAs. What complicates the situation even more is that not all miRNAs bind to their targets with imperfect binding. Many of them show perfect complementarity to cellular mRNAs, implying that these

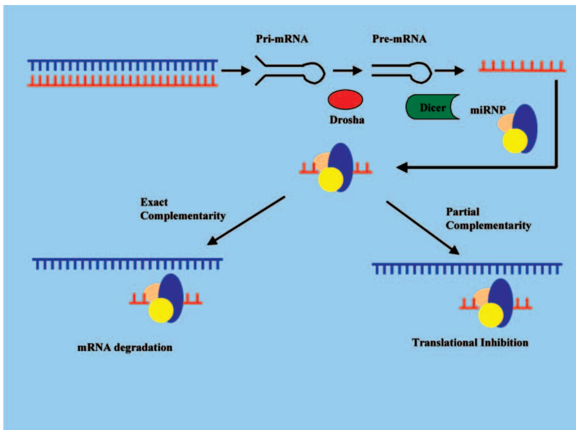

Figure 3: Mechanism of generation of microRNA (miRNA). *MicroRNAs are encoded by the host genome to form a "hairpin" structure called pri-miRNA. Drosha processes the pri-miRNA, to generate pre-miRNA which are exported from the nucleus. Pre-miRNAs are cleaved by Dicer in the cytoplasm to generate the mature miRNA. MicroRNAs then associate with a ribonucleoprotein complex and inactivate gene expression by two mechanisms. If their sequence is partially complimentary to the target mRNA, the miRNP complex binds to the 3'UTR of the mRNA and inhibit its translation. On the other hand, if their sequence is fully complimentary to the target mRNA, the target gene is silenced by degradation of the mRNAs.*

mRNAs will be targeted for degradation [17]. In simple terms it can be assumed that in the cellular context an miRNA can behave as siRNA and vice versa depending mainly on the level of the sequence complementarity.

Therapeutic applications of RNAi

The discovery of the RNAi has rapidly replaced antisense and ribozymes technologies to become the tool of choice for functional genomic analysis, target validation and gene knockdown models. The primary interest of pharmaceutical companies, however, is to develop RNAi for therapeutic purposes. In spite of this, there are many issues that need to be addressed before RNAi can end up in the clinic.

First is the issue of the siRNA stability. Second, the siRNA should be efficiently delivered into the target cells. Finally, non-specific effects and induction of an innate immune response should be reduced or even eliminated completely.

The issue of the stability of the RNA duplexes was immediately addressed. Companies were able to modify specific nucleotide positions on the siRNA molecule with altered nucleotides and augmented the stability of the siRNA from minutes to days.

The key challenge in the usage of RNAi as a therapeutic agent is the delivery to the desired organ and into the target cell. Unless the siRNAs are administered to an animal model in large volumes, something that cannot be performed in humans, they are unable to penetrate the tissue. To enter the target cells, the negatively charged siRNAs need to be encapsulated into cationic lipid vesicles so that they can be taken up by endocytosis. Several biotech companies are developing cationic molecules for *in vivo* delivery of siRNAs. Cationic liposomes composed of polyethylenimine or synthetic cardiolipin analogue can reproducibly and effectively deliver siRNA into cells with low toxicity. One further step to target the encaged siRNA to a specific tissue is to incorporate ligands into the cationic coat that bind to cell surface receptors on the target tissue[19].

Some researchers believe that the delivery obstacle can be bypassed by the use of viral vectors rather than chemically synthesized coating. Viral vectors contain DNA inserts linked to an RNA polymerase III promoter and are designed to express siRNA inside the target cells. Lentilvirus vectors of this type have been successfully used in creating "knock-out" transgenic mice in just three to four weeks (20). Viral vectors have the advantage of introducing siRNA into non-dividing cells such as neurons. Many scientists are currently studying the use of lentilviral or adenoviral siRNAi-expressing vectors as therapeutic agents against HIV, hepatitis C virus (HCV) and muscular dystrophy[19].

Another major obstacle for the in vivo use of RNAi, that is obvious to any RNAi user, is the silencing of genes other than the ones being targeted. The situation becomes even more complicated if someone considers the fact that a specific siRNA can act as miRNA and inhibit gene expression of multiple of genes by partial complementarity in sequence. To minimize these problems the criteria used to design siRNAs must be rigorously examined. Presently, microarray analysis is being used to aid in siRNA specificity studies, especially if this siRNA is being developed for therapeutic purposes. In this way, the effect of the designed siRNA on the whole genome expression pattern can be examined.

The final obstacle in the utilization of RNAi in the clinic is that siRNA administration can result in induction of the innate immune system [21,22]. Two recent reports showed that administration of siRNA containing the sequences 5'-UGUGU-3' or 5'-GUCCUUCAA-3' induced IFN-a and IL-6 in the serum when injected in mice. It is believed that more immunostimulatory sequences will be identified and only careful siRNA design and chemical modifications will prevent this effect.

RNAi technology brought a revolution in the scientific world. Irrespective of how the therapies pan-out, it is clear that RNAi is already a promising powerful tool for identifying disease pathways and targets for therapy.

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