FEATURE ARTICLE

# RNA Interference: An Instant Update on the Mechanism, Functions, & Therapeutic Applications

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**Abstract**

*This article reviews the discovery of RNA interference, its mechanism of action and its functions, and the principles, challenges, and strategies for RNA therapeutics.*

**Key Words:** *RNA interference; small interfering RNA; microRNA; RNA-interference-based therapies.*

## Introduction to RNA Interference

RNA is known to be a molecule of multiple functions. It plays a key role in the central dogma of molecular biology. The genetic information stored in DNA is first transcribed onto an mRNA molecule, and ribosomes interact with mRNA and tRNA molecules to translate the information in the mRNA into a polypeptide. RNA is also the genetic material of some viruses. RNA has also been shown to have catalytic capacity and can function as an enzyme (Cech, 2002). The objective of this paper is to inform biology teachers about a recently discovered function of RNA – that RNA plays a role in gene regulation.

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RNA interference (or RNAi) is a natural process that cells use to silence the activity of specific genes, discovered by Andrew Fire and Craig Mello in 1998. These two American scientists were awarded the 2006 Nobel Prize in Physiology or Medicine for uncovering this fundamental mechanism of gene regulation. They have shown that double-stranded RNA molecules can silence a gene by elimination of the mRNA corresponding to that gene (Fire et al., 1998). RNA interference has been shown to occur in essentially all organisms whose cells contain a nucleus. RNA interference has also been developed into a highly useful research tool that is primarily used to study the functions of specific genes. The hope is that, in the future, RNA interference may be used to treat diseases.

## Discovery of RNA Interference

RNA interference was first observed in petunias around 1990, when plant biologists attempted to intensify the hue of red petunias by introducing a gene that controls the formation of red pigment. Instead of intensified color, the resulting flowers had white patches or were completely white (Napoli et al., 1990). It turned out that the mRNA for the red pigment had disappeared. The cause of these unexpected effects remained puzzling until the discoveries of Fire and Mello (Fire et al., 1998).

The important experiments that led to the discovery of RNA interference were carried out by Fire and Mello in the 1990s using the nematode *Caenorhabditis elegans* – a millimeter-long roundworm commonly used to study cellular processes. Fire and Mello were studying how gene expression was regulated in *C. elegans*. Among several things, they were studying mRNA for the *unc-22* gene, which encodes a component of myosin protein. When they injected the roundworm gonads with sense RNA (a molecule identical to the mRNA), there was no effect on the roundworms or their offspring. Similarly, injecting the roundworm gonads with antisense RNA (a molecule complementary to the mRNA) that could bind to the corresponding mRNA for the muscle protein also had no effect on the roundworms. However, when a mixture of sense RNA and antisense RNA was injected into the roundworms, their progeny showed severe twitching movements. Decrease in *unc-22* activity was known to produce such movement patterns. Fire and Mello established that annealed sense–antisense RNA, but neither antisense nor sense RNA alone, caused the twitching phenotype in the progeny (Fire et al., 1998). One would have expected that only single-stranded antisense RNA would bind to the corresponding mRNA and silence that gene. What could be the explanation for the unexpected results?

Fire and Mello persevered with their investigation by injecting single-stranded or double-stranded *mex-3* RNA into the gonads of *C. elegans*. Using a special staining technique, they were able to show

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the presence of mRNA for genes that were active in the roundworm embryos. When the gene was active, the embryo could be stained because its cells contained the mRNA. Injecting antisense RNA only decreased the amount of mRNA to some extent. However, injection of double-stranded RNA (sense RNA and antisense RNA) resulted in no staining at all, implying that the mRNA was absent and the gene *mex-3* had been silenced.

Fire and Mello continued with their investigation by injecting different roundworm embryos with double-stranded RNA corresponding to the genetic code of several other *C. elegans* genes. For all the experiments, double-stranded RNA molecules silenced their corresponding genes. They drew a number of conclusions from their research with *C. elegans* genes, which can be summarized as follows (Fire et al., 1998). (1) Gene silencing was elicited efficiently by injecting double-stranded RNA, but was weak or nonexistent when single-stranded RNA (sense RNA or antisense RNA) was injected. (2) The mRNA affected by the double-stranded RNA disappeared, which suggests that it was degraded. (3) The double-stranded RNA injected must correspond to the mature mRNA for the gene. Neither intron nor promoter sequences elicited gene silencing. This suggested that the interference occurred after post-transcriptional modification, presumably in the cytoplasm rather than in the nucleus. (4) Only the mRNA that was identical to the sense RNA strand of the double-stranded RNA was silenced; other mRNAs were unaffected. This suggested that gene silencing was specific for the gene with a code corresponding to that of the mRNA. (5) Only several double-stranded RNA molecules per cell were needed to silence a gene completely. This suggested that the double-stranded RNA played a catalytic role rather than acting as a reactant in the process. (6) The effect of the double-stranded RNA could spread from cell to cell and from tissue to tissue and could even be passed on to the progeny.

## Mechanism of RNA Interference

RNA interference is an intricate process that involves several proteins (Wilson & Doudna, 2013). The mechanism differs considerably from the simple antisense mechanism discovered in bacteria in the early 1980s (Wagner & Simons, 1994).

RNA interference works by destroying mRNA molecules (Wilson & Doudna, 2013). Upon entering a cell, the long double-stranded RNA molecules that trigger RNA interference are cut into small fragments known as "small interfering RNAs" (siRNAs) by an enzyme called "Dicer" (Figure 1). Dicer is an endonuclease of the RNase III family; it acts as a "molecular ruler" and precisely produces RNA duplexes approximately 21–25 nucleotides in length, with characteristic termini. The 3′ end carries a dinucleotide overhang, and the 5′ end terminates in a monophosphate group. Once generated, an siRNA duplex is loaded by Dicer, with the help of the Trans-activation response RNA-binding protein (TRBP), onto the RNA-induced silencing complex (RISC). The length and distinctive ends of the siRNA are necessary features for efficient recognition by, and loading onto, the RISC.

At the center of the RISC is an Argonaute protein, which is the principal executer of RNA interference–mediated gene silencing. There are four types of Argonaute proteins in humans (AGO 1-4), but silencing by siRNAs is accomplished via AGO2. Upon loading of siRNA onto the RISC, AGO2 selects the siRNA guide strand, then cleaves and ejects the passenger strand (Figure 1). While attached to AGO2, the guide strand serves as a search probe and subsequently pairs with its complementary target mRNA long enough for AGO2 to slice the target. After slicing, the cleaved target mRNA is released and RISC is recycled, using the same loaded guide strand for slicing more mRNA molecules (Wilson & Doudna, 2013). The gene for which the mRNA is a messenger is silenced, and therefore no protein is formed.

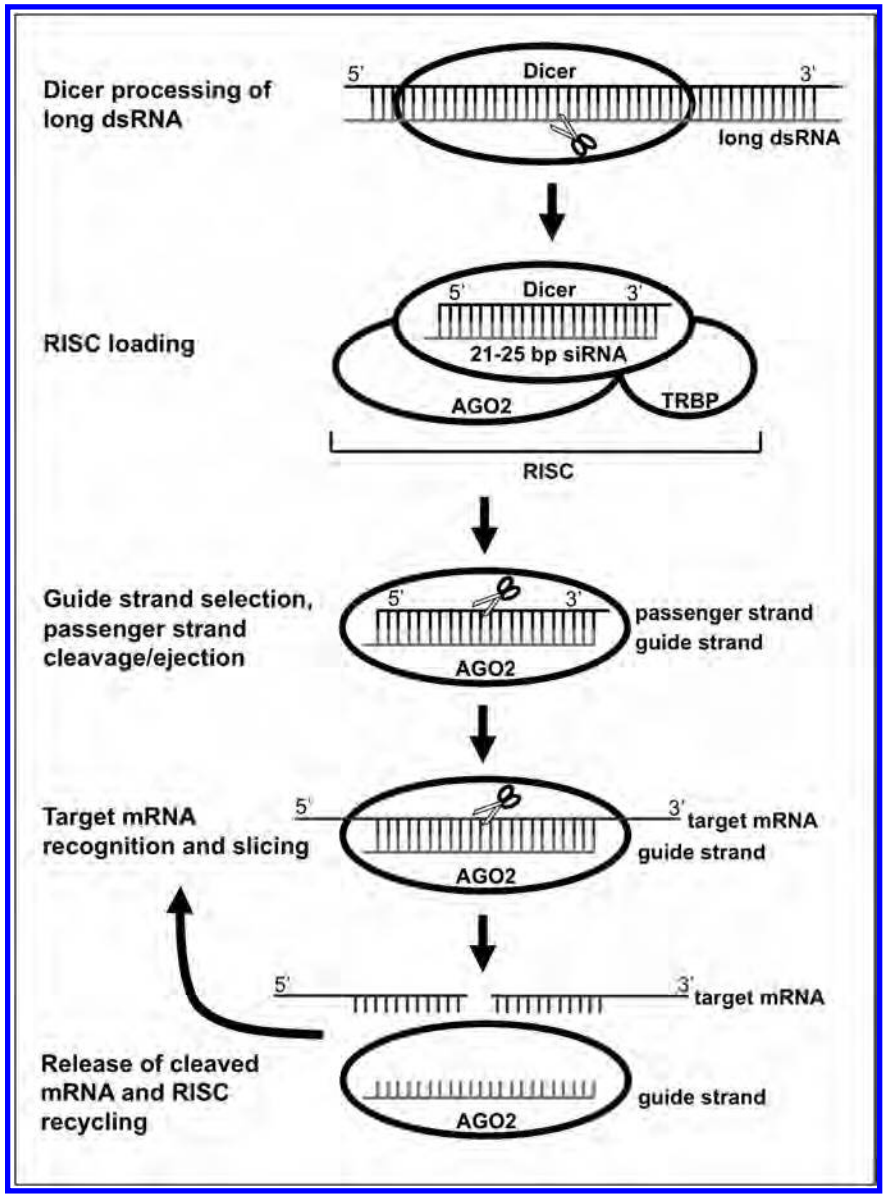


**Figure 1.** The mechanism of RNA interference.

## ○ Functions of RNA Interference

Humans and other vertebrates have efficient immune systems as defense against invading microorganisms; for instance, interferons and various types of T cells protect us against viral infections. But among organisms that lack an efficient immune system, RNA interference has proved to be an important mechanism for protection against viral infections (Obbard et al., 2009). Some viruses have genetic material that consists of double-stranded RNA, and many viruses with single-stranded RNA have their RNA in double-stranded form at some point during the reproductive life cycle within the host cell. A viral infection starts when viral RNA enters the cell. The double-stranded RNA molecule would trigger the RNA interference machinery and silence the virus's genes, preventing a viral infection.

RNA interference may also have evolved to fight the spread of transposable genetic elements within the genome of a cell (Obbard et al., 2009). Transposable elements can wreak havoc by moving from one location to another within the genome, sometimes causing mutations that can lead to cancer and other diseases. Like RNA viruses, transposable elements can take on the form of a double-stranded RNA that would trigger RNA interference to prevent the potentially harmful transposition of genetic elements. It has been shown that when components of the RNA interference machinery are mutated in *C. elegans*, transposable elements are activated that cause disturbances in the function of the genome.

## ○ Other Cellular Pathways Similar to RNA Interference

Soon after RNA interference was discovered, researchers uncovered a related process, the microRNA (miRNA) pathway (Ghildiyal & Zamore, 2009). MicroRNAs have been identified in many eukaryotes from nematode to humans to plants. Both RNA interference and the microRNA pathway involve double-stranded RNA, but the source of the RNAs is different. The double-stranded RNA that triggers RNA interference is from an exogenous source such as viruses, whereas microRNAs are encoded in the genome. In addition, microRNAs are not completely double-stranded but form hairpin-like structures that contain double-stranded regions. Unlike RNA interference, the microRNA pathway focuses on regulating the cell's own genes. Researchers estimated that humans have >200 microRNAs that regulate >30% of our genes.

The microRNA pathway is distinct from RNA interference (Ghildiyal & Zamore, 2009). Primary microRNAs are transcribed from microRNA genes by RNA polymerases. Primary microRNAs are then trimmed in the nucleus to precursor microRNAs by the ribonuclease Drosha (Figure 2). Precursor microRNAs contain a loop and usually have interspersed mismatches along the duplex. Precursor microRNAs are then exported from the nucleus to the cytoplasm by Exportin-5. The cytoplasmic ribonuclease Dicer excises microRNA–microRNA duplexes from precursor microRNAs. The duplex associates with an Argonaute protein within the RISC. One strand of the duplex (called the "passenger strand") is removed. The remaining strand ("guide strand") can regulate gene expression by base-pairing to mRNA, which results in either degradation of the mRNA or suppression of translation.

Shortly after the discovery of RNA interference, it was shown that gene silencing in plants occurs at the transcriptional level and operates via RNA-interference-like mechanisms (Covey et al., 1997). Now it is known that transcriptional gene silencing is the plant equivalent to RNA interference. RNA-interference-like mechanisms keep chromatin condensed and suppress transcription.

## ○ Applications of RNA Interference in Medicine & Biomedical Research

Using RNA interference to treat diseases is theoretically viable because RNA interference is a natural process and double-stranded RNA is an endogenous substance. RNA interference offers the ability to shut down any gene in the body. It is possible to design small RNA that matches any gene, or any part of that gene, and silence it. Although no drug based on RNA interference has been approved so far, successful animal experiments have been performed and ~20 substances are being tested in clinical trials in humans (Davidson & McCray, 2011).

Diseases that can be blocked by knocking down the activity of one or several genes are the most promising targets for RNA-interference-based therapies. Cancer is often caused by overactive genes, and suppressing their activities could halt the disease. Several pharmaceutical companies are currently testing RNA-interference-based therapies for various forms of cancer such as familial adenomatous polyposis, liver tumor, chronic myeloid leukemia, metastatic melanoma, and solid tumors (Gomes-da-Silva et al., 2012).

Viral infections are also potential targets for RNA-interference-based therapies. Decreasing the activity of key viral genes would disable the virus, and numerous studies have pointed to the promise of RNA interference for treating viral infections. Researchers have stopped the growth of HIV, polio, hepatitis B, hepatitis C, and other

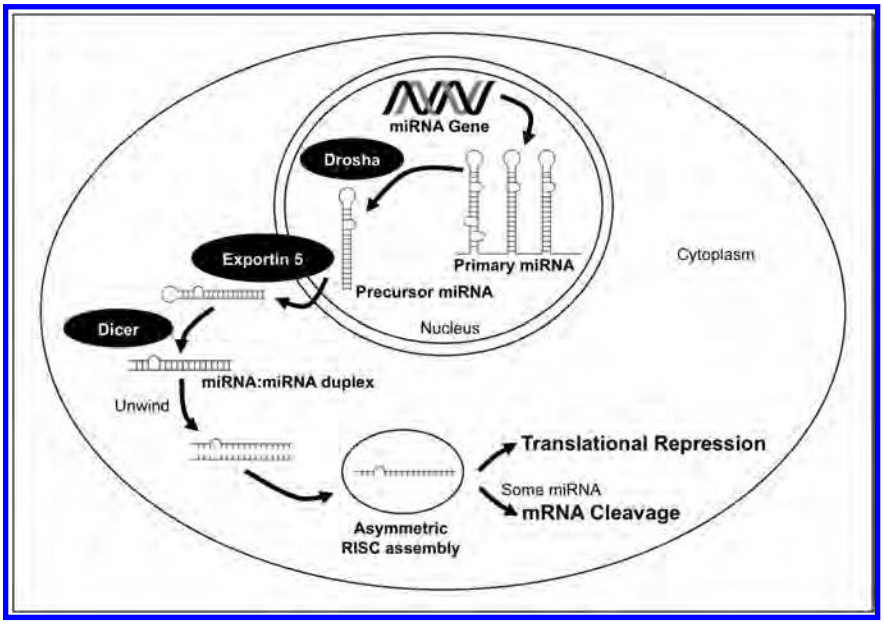


**Figure 2.** The microRNA pathway.

viruses in laboratory-grown human cells. RNA-interference-based therapies against HIV, hepatitis B, hepatitis C, and RS viruses are also being tested in clinical trials (Davidson & McCray, 2011).

Double-stranded RNA is also being tested as a way to treat age-related degeneration of the fovea. Blood vessels grow into and damage the fovea – the part of the retina that has the highest concentration of photoreceptors. The growth of blood vessels is due to a substance known as "vascular endothelial growth factor" (VEGF). If double-stranded RNA corresponding to the mRNA for VEGF is injected into the eye, it can decrease the amount of VEGF produced and prevent new blood vessels from forming and invading the fovea (Garba & Mousa, 2010). Various other genes are also involved in ocular diseases and are potential targets for RNA interference-based-therapies. These include TGFβ and its receptors (fibrotic eye diseases), c-Jun (apoptosis in glaucoma), and p66Shc (oxidative damage) (Campochiaro, 2007).

RNA-interference-based therapies also show potential in targeting neurodegenerative disorders. Important examples are polyQ repeat disorders that are currently untreatable with conventional drugs, including spinocerebellar ataxias and Huntington's disease. Further relevant targets are Parkinson's disease and Alzheimer's disease, as well as amyotrophic lateral sclerosis. Using appropriate small-animal models, the results were encouraging and ranged from improvements in motor coordination and muscle function to delayed disease onsets and substantial extensions of life spans (Davidson & Boudreau, 2007).

RNA interference can be used as a research tool to study the functions of genes (Shan, 2010). Knocking down the activity of a gene yields a lot of information about its functions in cellular pathways. Prior to the discovery of RNA interference, the process was a laborious task and could take months. Currently, by making use of RNA interference, researchers can silence selected genes quickly and easily and can answer questions that were once beyond their reach. This technique can be applied to cells from almost all organisms.

RNA interference can also be used as a research tool to study gene regulation. For many years, researchers have been studying how proteins known as "transcription factors" regulate gene activity. Now RNA, through RNA interference and related processes, is recognized as a key partner in the cell's multilayered approach to gene regulation (Castel & Martienssen, 2013).

## ○ Challenges of RNA Therapeutics

The therapeutic application of RNA interference is promising because of its efficient and specific gene silencing. However, for this treatment to work optimally, a number of problems must be solved to realize the full potential of this technology (Gavrilov & Saltzman, 2012).

One major challenge is the accumulation of siRNAs at the target sites to therapeutically appropriate levels (Guo et al., 2010); siRNAs are highly susceptible to degradation by enzymes found in the serum, the half-life of siRNAs in serum ranging from several minutes to an hour. When siRNAs reach their target cells, the large sizes and negative charges of siRNA molecules hinder their diffusion across the plasma membranes and, thus, their accumulation in the cytoplasm. Within the cytoplasm, the siRNAs remain susceptible to degradation by intracellular RNases. The molecules must also be recognized by and loaded onto the RISC before they can exert their gene silencing effects.

Another challenge of RNA therapeutics is off-target gene silencing, which refers to the inhibition of genes other than the desired gene targets (Aagaard & Rossi, 2007). Studies have shown that treatment with siRNAs can sometimes cause off-target gene silencing, which can lead to unanticipated cell transformation. Off-target silencing usually results from base pairing between a nontarget and six or seven nucleotides in the siRNA sequence. This problem is of great concern in the context of miRNA gene silencing, because miRNAs exert their gene-silencing effects through having only partial complementarity (6 to 8 nucleotides) with the mRNA targets. Currently, all potential therapeutic siRNA sequences are rigorously tested for disruption of normal gene expression. As our understanding of the mechanism of action of siRNAs deepens, the use of predictive bioinformatics approaches at the stage of siRNA design will eventually eradicate off-target silencing.

Another problem is that siRNAs can sometimes trigger an immune response. It has been shown that double-stranded siRNA can activate interferon responses and cause cell death in culture (Reynolds et al., 2006). Another study showed that certain siRNAs can bind to and activate Toll-like receptor 7 (TLR7) if they contain the 5′-GUCCUUCAA-3′ or similar GU-rich sequences that can also be recognized by TLR7. Immunogenicity is thus an area of concern that must be worked on in developing RNA therapeutics.

## ○ Strategies for RNA Therapeutics

Before siRNA therapies can become useful, researchers must figure out how to effectively deliver siRNA molecules into target cells. Although virus-based delivery systems are efficient, they are unsafe to use because they can induce mutations and trigger immunogenic responses. Hence, extensive research has been carried out to develop efficacious nonviral delivery systems such as chemical modifications of siRNA, liposome formulations, and nanoparticles.

Chemical modifications can significantly enhance the stability and uptake of naked siRNAs (Choung et al., 2006). It is important to be able to modify siRNAs directly without diminishing their ability to silence their targets. Chemical modifications have been extensively explored for every part of siRNA molecules, from the bases to the backbone and termini, with the aim of designing siRNA with prolonged half-life and increased cellular uptake. The sugar moiety is most commonly modified. For example, the incorporation of 2′-halogen, 2′-amine, or 2′-deoxy can significantly increase the stability of siRNA in serum. Modifications of the backbones of siRNA duplexes can decrease susceptibility to nucleases in both the serum and cytoplasm. For example, replacing the internucleotide phosphate linkage (P=O) with boranophosphate (P=B) increases resistance to nucleases by more than tenfold compared with unmodified constructs, without causing cytotoxicity or damage to the siRNA silencing function.

A liposome is an artificially prepared vesicle that consists of a lipid bilayer that encloses an inner aqueous environment. Using liposomes is an important strategy for delivering siRNAs to target cells. Liposomes facilitate efficient internalization of siRNA via membrane fusion with the target cell (Gao et al., 2011). Recently, researchers are working on a delivery method that packages siRNA within a layer of lipid-like molecules called "lipidoids" (Whitehead et al., 2011). It was found that ineffective single lipidoids could be formulated together in a single delivery vehicle to facilitate intracellular delivery of siRNAs.

Polymeric nanoparticles are promising siRNA delivery systems because they offer stability and controlled release, have the capacity to encapsulate large amounts of siRNA, and can be surface modified to enhance transport properties, targeting, or uptake. Polymers that are biodegradable, biocompatible, and nontoxic make good candidates for constructing in vivo delivery vehicles. Chitosan, cyclodextrin, polyethyleneimine, dendrimers, and metallic core nanoparticles have become popular for use in delivery systems, although none of these materials possess all the desirable properties (Gao et al., 2011).

## ○ Conclusion

The discovery of RNA interference has caused much excitement. Most exciting is the potential therapeutic application of this technology. Although multiple challenges stand in the way of realizing this potential, the biggest hurdle being siRNA delivery, more than a decade of innovative engineering has resulted in solutions to a number of these challenges, laying the foundation for progress toward making widespread RNA therapeutics a reality in the near future.

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