

RNA Interference: A revolution in drug development

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RNA interference (RNAi) or gene silencing technology is a phenomenon by which double stranded RNAs elicit degradation of a target mRNA containing homologous sequence. It is essentially a new incarnation of well-established antisense principle. This technology enables the researchers to trigger post-transcriptional gene silencing *in vivo*. It is a robust method for lowering specific protein levels, compared to traditional techniques such as antisense, ribozymes or microinjection of function-blocking antibodies. RNAi offers a powerful tool for ascribing functions to genes while its application to *in vivo* models of disease opens up tremendous opportunities to develop a novel generation of oligonucleotide-based drugs, thus offering an enormous potential of being developed as a therapeutic modality.

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Introduction

More than a decade ago, Rich Jorgensen and colleagues made a surprising observation in petunias, while trying to deepen its purple colour by introducing a pigment-producing gene under the control of a powerful promoter. Instead of expected deep purple colour, many of the flowers appeared variegated or even white. They named this phenomenon, as 'cosuppression' as expression of both the induced gene and the homologous endogenous gene was suppressed¹. This phenomenon of gene silencing of an endogenous gene by the introduction of a transgene or infection by a virus is called as cosuppression. Transgene-induced silencing in some plants appears to involve gene-specific methylation (transcriptional gene silencing, or TGS) but in others, silencing of endogenous gene is caused by the introduction of homologous double stranded RNA. (dsRNA), transgene or virus and is called post transcriptional gene silencing (PTGS)². In PTGS, the transcript of the silenced gene is synthesized but does not accumulate because it is rapidly degraded. This phenomenon of cosuppression can occur at post-transcriptional or transcriptional level and has been found to occur in fungi like *Neurospora crassa* where it is known as quelling³.

In the year 1998, the first evidence that dsRNA could lead to gene silencing and could be used as a tool to understand gene function came from A Fire and C Mello in the nematode, *Caenorhabditis elegans*⁴. dsRNA-a mixture of both sense and antisense strands was injected into *C. elegans*, which resulted in more efficient silencing than injection of either sense or antisense strands alone. Furthermore, injection of dsRNA into gut of the worm, caused gene silencing not only throughout the worm, but also in its first generation offspring. The researchers studying *C. elegans* first introduced the term RNA interference (RNAi) to describe post-transcriptional gene silencing induced by direct introduction of dsRNA. RNAi has also been observed in *Drosophila*⁵ and over the last few years these RNAi strategies have been used as reverse genetic tools in *Drosophila* organism, embryo lysates and cells to characterize various loss-of-function phenotypes⁶.

While the natural presence of RNAi has been observed in a variety of organisms (plants, protozoa, insects and nematodes), evidence for the existence of RNAi in mammalian cells took longer to establish. Elbashir and coworkers have demonstrated that duplexes of 21- nucleotide (nt).

RNAs mediate RNA interference in cultured mammalian cells⁷. The RNA interference (RNAi) describes the phenomenon in which introduction of double stranded RNAs (dsRNA) into the diverse

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range of organisms and cell types elicit degradation of a complimentary or target mRNA containing homologous sequence. Thus, this phenomenon of gene silencing through RNAi is essentially a new incarnation of the well-established antisense principle—the inactivating agent interacts with the target through complementary interaction and results in inability of the latter to produce corresponding protein⁸

Antisense Technology: RNA Mediated Translational Inhibition

Protein molecules are the expressions of a gene. However, to get to a protein the cell must undergo two complex processes, transcription and translation. Transcription is the process in which an RNA copy is made of the DNA. In order to get the copy, many enzymes such as polymerase, helicase, exonuclease, ligase and single stranded binding proteins work together to unwind the double helix and match the base pairs of RNA to the DNA. Once the copy is made, the RNA molecule, which is now in the heterogenous nuclear RNA (hnRNA) mode, is still not ready to express the gene by making a protein. The hnRNA must be spliced to remove non-coding sequences and protected from the cellular environment with a 5' cap and poly-A tail. Finally, the hnRNA is transported out of the nuclear membrane into the cytoplasm, where it achieves the status of mRNA. In the cytoplasm, the mRNA hooks up with ribosomes where the protein production can start. Every three nucleotides in the mRNA molecule code for a specific amino acid and are appropriately called a codon. The codon pairs with an anticodon of tRNA that has attached to an amino acid. In this manner a polypeptide chain is formed. It will eventually twist and contort itself into a unique configuration, which aids in the function of the protein. Occasionally, a bad mRNA molecule is synthesized so that the resulting protein cannot function properly. Abnormalities of proteins cause many diseases that afflict humans. Therefore, it seems logical to conclude that if the expression of these malfunctioning proteins could be stopped, the sources of disease would be obliterated and the disease will be treated, if not cured. This idea is the basis for antisense technology.

A sense strand is a 5' to 3' mRNA molecule or DNA molecule. The complementary strand or mirror strand to the sense is called an antisense. Antisense technology is the process in which the antisense

strand forms hydrogen bonds with the targeted sense strand. When an antisense strand binds to mRNA sense strand, a cell will recognize the double helix as foreign to the cell and proceed to degrade the faulty mRNA molecule, thus preventing the production of undesired protein. Although DNA is already a double stranded molecule, antisense technology can be applied to it, building a triplex formation.

A DNA antisense molecule must be approximately seventeen bases in order to function and approximately thirteen bases for an RNA molecule. RNA antisense strands can be either catalytic or noncatalytic. The catalytic antisense strands, also called ribozymes, cleave the RNA molecule at specific sequences. A non-catalytic RNA antisense strand blocks further RNA processing, i.e. modifying the mRNA strand or transcription.

The exact mechanism of antisense-based translational inhibition has not been determined. The current hypotheses include blocking RNA splicing, accelerating degradation of the RNA molecule, preventing introns from being spliced out of the hnRNA, impeding the exportation of mRNA into cytoplasm hindering translation and the triplex formation in DNA (Fig: 1 a & b).

Applications of Antisense Technology

Antisense technology has been used for several years to “knock down” the expression of specific genes in living tissues to study the functions of their protein products¹⁰. Specifically designed antisense oligonucleotides (ODNs) are short lengths of man-made genetic material i.e. single stranded nucleic acids, which function by selectively hybridizing to their target mRNA, thereby blocking translation. Translation is inhibited by either RNase H nuclease activity at the DNA: RNA duplex or by inhibiting ribosome progression, thereby inhibiting protein synthesis¹¹. This can shed light on the function of the protein; alternatively, if a disease process results from an excess of certain protein, the method can in theory correct the problem. Faster than transgenic techniques, more similarly problematic in practice, antisense has been seen as something of a “black art”. There are many technical problems to understand and overcome.

Applications of antisense technology are diverse, like selective protein knockdown, gene function analysis, drug target validation and therapeutics. It was first successfully used in plants. In *Flavr Savr* it was used to block the enzyme that is involved in its

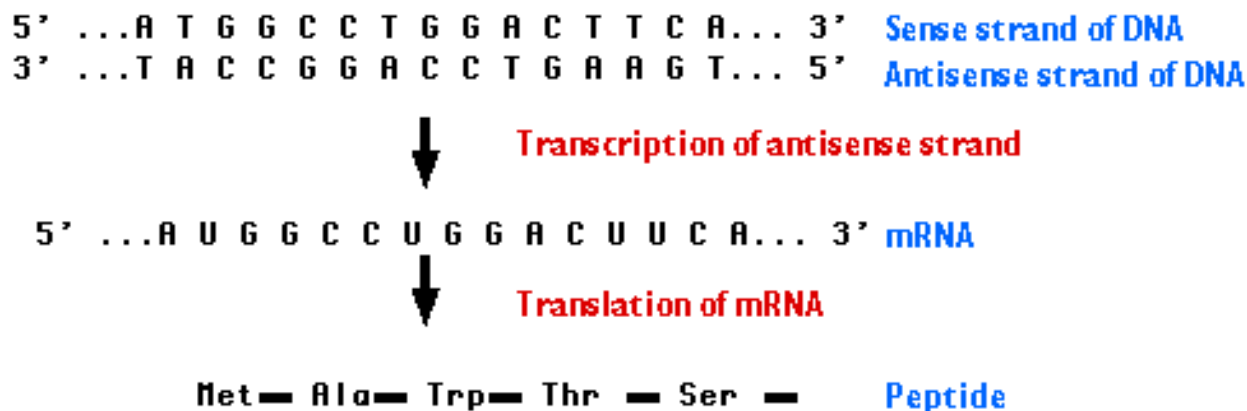


Fig.1—(a): Cartoon sequence of transcription of antisense DNA strand into the sense mRNA strand, which is then translated into a polypeptide.

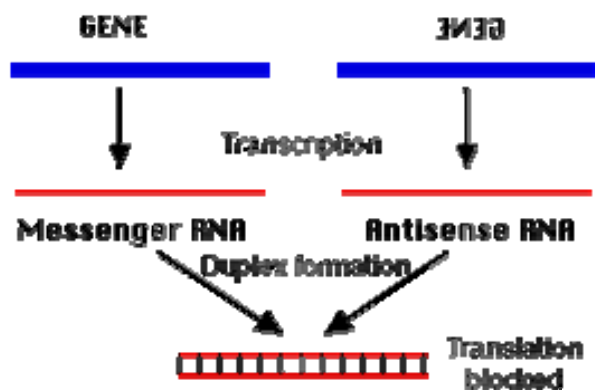


Fig.1—(b): Cartoon of how sense mRNA and antisense RNA are transcribed and then anneal to form double-stranded RNA, which blocks translation of the protein coded for by the sense mRNA.

spoilage, thereby increasing the length of time a tomato could be sold¹². Antisense technology is now used in mammalian cells. The promising fields of study in humans include functionalization of genes and their validation as potential targets for cancer gene therapy and AIDS. Antisense oligonucleotides have great potential as therapeutic agents e.g. against proto-oncogenes and viral genes.

The Mechanism of RNA Interference

Long double-stranded RNAs (dsRNAs typically >200 nt) can be used to silence the expression of target genes in a variety of organisms and cell types (e.g., worms, fruit flies, plants). Upon introduction into a cell, the long dsRNAs enter a cellular pathway called RNA interference pathway¹³. First the dsRNAs are cleaved into short 20-25 nucleotide (nt) small interfering RNAs (siRNA) by a ribonuclease III – like enzyme called Dicer (initiation step). Then, the

siRNAs assemble with protein components into endoribonuclease-containing complexes known as RNA-induced silencing complexes (RISCs), unwinding in the process. The siRNA strands subsequently guide the activated RISCs to complementary RNA molecules, where they bind to complementary transcript by base pairing interactions between antisense strand and the mRNA. The bound mRNA is cleaved and sequence specific degradation of mRNA results in gene silencing (effector step). Cleavage of cognate RNA takes place near the middle of the region bound by the strand (Fig. 2).

Short Interfering RNA (siRNA)

Endogenous short interfering RNAs or siRNAs are small RNA duplexes approximately 21 nucleotides (nts) long. They have a characteristic structure that includes 2-nucleotide (nt) 3' overhangs and 5' phosphate group. They were first identified in plants and *Drosophila*, where their presence was associated with sequence-specific inhibition of gene expression observed as a result of PGTs in plants and RNAi in *Drosophila*.

In mammalian cells, introduction of exogenous long dsRNA (>30 nt) initiates a potent antiviral response, exemplified by non-specific inhibition of protein synthesis, decrease in gene expression, RNA degradation and often cell death. To circumvent this, RNA duplexes based on the structure of siRNAs are used to induce RNAi in mammalian cells and organisms without non-specific responses. The principal natural triggers of these responses are the dsRNAs intermediates formed during viral replication and requires RNA duplexes of ~60 to 80 nts in length for full activation¹⁴. siRNA methodology results in the

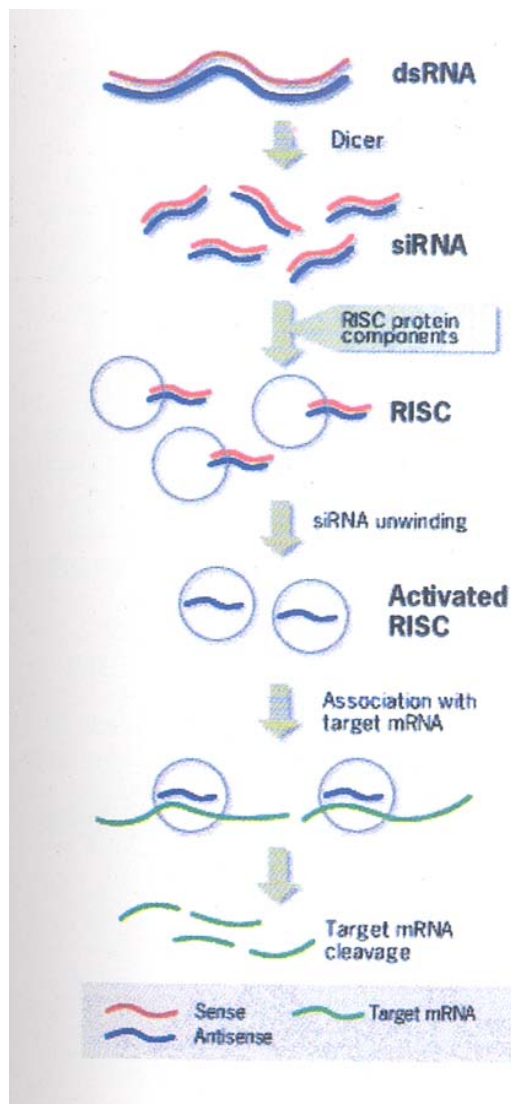


Fig .2— RNAi mechanism

silencing of gene expression by mechanism similar to antisense technology. However, in contrast to antisense methodology, siRNA generally requires much lower concentrations to show similar gene expression inhibition effects. The design of functionally active siRNA is a crucial step in RNAi experiments. Optimally designed siRNA is both potent and specific. Potent duplexes provide high levels of silencing at low concentrations.

In the last few years, siRNAs have been used in number of different experimental settings to silence gene expression. In some, chemically synthesized or *in vitro* transcribed siRNAs have been transfected into cells, injected into mice or introduced into plants by a particle gun. In others, siRNAs have been expressed

endogenously from expression vectors or PCR products in cells or in transgenic animals¹⁵.

In addition to their role in gene silencing, siRNAs have been determined to play diverse biological functions *in vivo*. The various roles include antiviral defense, transposon silencing, gene regulation, centromeric silencing, and genomic rearrangements¹⁶. siRNA introduced by transient transfection were found to effectively induce RNAi in mammalian cultured cells in a sequence-specific manner. The effectiveness of siRNA varies—the most potent siRNAs result in >90% reduction in target RNA and protein levels¹⁷. The most effective siRNA turns out to be 21nt dsRNA with 2nt 3' overhangs and 5' phosphate group. Sequence specificity of siRNA is very stringent, as single base pair mismatches between siRNA and its target mRNA dramatically reduce silencing⁵.

Naturally occurring siRNAs in lower eukaryotes have characteristic structural elements, however, little is known about what features are critical for an exogenous siRNA to mediate RNAi in mammals. Caplen and Mousses have recently determined some of the critical parameters that influence the efficiency of siRNA mediated RNAi in mammalian cells. These features can aid in designing an effective siRNA.

Characteristics for siRNA that influence its ability to mediate response is as follows¹⁴:

1. Length of siRNA should be 20-22 nts.
2. It should target coding sequences only.
3. As RNAi acts in cytoplasm, intronic sequences present in pre-mRNA should not be targeted.
4. It is recommended that regions at least 70 to 100 nucleotides from translational start site should be targeted to avoid regions likely to be associated with the binding of the regulatory proteins.
5. AU: GC (or GC: AU) ratios should be within the range of 40-60% and ideally as close to 50:50 as possible.
6. Specificity of siRNA sequence against a particular target should be ensured and extensive bioinformatic analysis should be performed to ensure that the siRNA targets only the transcript under study.
7. siRNA should have 2 nt 3' overhangs on both the strands however it has been found that at least some of the mammalian cell lines are more tolerant of variation in the length of overhang (0-5nts) than has been reported in *Drosophila* cells.

8. Unphosphorylated siRNA has been found to be equally effective as those carrying a 5'-phosphate group. Addition of fluorescence tags to the 3' end of these sense strands has minimum effect on their ability to mediate silencing. It can be used to track the uptake of siRNA and assess transfection efficiency.
9. siRNA which are the basis of this technology, must be highly specific for genes in question to avoid unintentional silencing of non-target genes and potent to provide high level of silencing at low concentration.

Advances are continuously being made in siRNA design, effectiveness, delivery and specificity. As pharmaceutical and biotechnology companies race to take advantage of the abundance of sequence data available for the drug discovery research, synthetic siRNA will become a vital technology for high throughput screening and target validation.

Advantages of RNAi Over Other Similar Technologies

After going through all the hype about RNAi, one is bound to ask what is wrong with the original antisense approaches and how RNAi is different from them? The most logical answer that one might hear is that 'RNAi simply works'. It has been demonstrated quite clearly that various antisense approaches including ribozymes, traditional cleaving and blocking oligonucleotides can inactivate target genes. But use of RNAi in mammalian cells has following advantages over the other antisense type approaches.

- 1) The hit rate and efficacy of RNAi is much better than those of ribozymes.
- 2) RNAi is less toxic than phosphorothioate -based cleaving oligonucleotides.
- 3) RNAi has overcome the obstacle of delivery to mammalian cell that has hindered the use of neutral charges blocking type antisense oligonucleotides¹⁵.

Applications of RNAi

RNAi is generating quite a buzz in the biotechnology field and is referred to as 'Billion Dollar Breakthrough of The Year' because it offers the sequence specific knock-down of the target genes followed by *in vitro* and *in vivo* phenotypic analysis thus providing a straight forward method for ascribing function to genes. The researchers are most excited about RNAi's potential use as functional genomics tool⁸. Already, RNAi has been used to ascertain both

broad and specific functions to many genes for which no known function had been discovered previously in *Drosophila*, *C. elegans* and several species of plants. With the knowledge that RNAi can be induced in mammalian cells by transfection of siRNA, many more researches are beginning to use RNAi as a tool in human, mouse and other mammalian cell culture systems. Since the reports of induction of RNAi by siRNAs in mammalian cells, over 200 different transcripts have been targeted and down regulated by RNAi in mammalian cells¹⁸.

The inhibition of gene expression by RNAi has been rapidly exploited as a reverse genetic tool to facilitate the elucidation of gene function in a number of species and has been an important breakthrough for scientists involved in both basic and applied drug discovery research. In addition, the application of RNAi technology to *in vivo* models of disease opens up tremendous opportunities to develop a novel generation of oligonucleotide-based drugs. The development of such drugs would be accelerated as they would bypass the need for the time and money-consuming steps of high through put screening, lead identification and validation as development of new drugs would require simply the knowledge of sequence of genes causing a corresponding disease⁸. Although, the interest in the application of RNAi *in vivo* is increasing, the vast majority of RNAi based research is focused in the cell culture systems for gene function analysis and drug target identification and validation. The use of RNAi in cell culture-based models of disease has yielded important data that provides insight into the function of target genes on the cellular level. But, true validation of the target as a potential point of therapeutic intervention requires evaluation in animal models of disease.

Recently, advances have been made in the administration of RNAi in animal models to evaluate the effect of target knockdown on various phenotypes. Further advances in delivery methodology will pave the way for widespread use of RNAi *in vivo*, which will accelerate the drug discovery process.

With the help of gene silencing, geneticists can inactivate a gene that may cause disease or be defective. It has been used to treat several viral diseases including AIDS, herpes, chicken pox and hepatitis¹⁹. RNAi has been shown to block a pathophysiological pain response and provides relief from neuropathic pain in rat disease model by down regulating an endogenous, neuronally expressed gene.

These observations open a path towards the use of siRNA as a genetic tool for drug target validation in the mammalian central nervous system as well as for proof of concept studies and therapeutic agents in man²⁰.

A new gene silencing technology has been developed at the North Carolina State University, which may offer potential benefits to crops with increased resistance to drought and disease, soybeans with more proteins and vegetables with more vitamins¹³. Researchers at the University of California, Davis, have also developed a gene silencing technology that would offer resistance to bacterial disease in crop plants¹³.

Researchers at the Fred Hutchinson Cancer Research Center report that they have discovered inhibitors of gene silencing, (a compound called splitomicin) that reverses the process of gene silencing in which genes or chromosomal regions are shut off. The inhibitors could have significant applications in the treatment of a variety of cancers, including certain forms of breast cancers that result from genetic abnormalities. These compounds might be useful against sickle-cell anemia too²¹.

siRNA Against Potential Therapeutic Targets

Recently, several studies have been suggested that could be used directly as therapeutic approach in that both siRNA and shRNAs (short hair pin RNAs) have been shown to knockdown gene expression *in vivo* in mice transiently and stably through generation of transgenic mice²². There are many disorders in which down-regulation of proteins could modulate the disease initiation or progression, including cancer, infectious disease and dominant genetic disorders. Several studies have shown the feasibility of using RNAi to down-regulate the cancer-related genes, including potentially therapeutic targets like tyrosine kinase receptor ErbB2. High level expressions of ErbB2 is a common feature of human breast cancer due to increased transcription and gene amplification. Transtuzumab is a humanized antibody that has shown to have a significant clinical benefit in patients with metastatic breast cancer, both as monotherapy and in combination with taxanes and anthracyclins by down regulation of ErbB2. So in future, RNAi may even hold promise for development of gene-specific therapeutics as functional genomics²³.

Conclusion

RNAi or gene silencing is proving to be a very important functional genomics tool. It is a

technique of choice for successful *in vitro* drug target validation and has enormous potential as a therapeutic agent. Important issues that remain to be addressed are the identification of most effective gene sequences that can be targeted by the siRNAs and the development of high throughput systems for validation and analysis of RNAi against specific targets in mammalian cells.

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