RNA Interference – Towards RNA becoming a Medicine

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The central dogma in molecular biology stipulates that the flow of genetic information is from DNA (i.e. the gene) through RNA to proteins, which is expressed as the phenotype of the gene. Any change in the gene by mutation would normally lead to a change in the phenotype. The approach where the mutant phenotype is used to determine the function of the gene is called *Forward genetics*. With the advent of genome sequencing projects the situation has changed; thousands of genes are identified without knowing their function. *Reverse genetics*, where genetic analysis proceeds from the genotype to the phenotype is now the most effective way to assess the function of a gene. Among the various reverse genetics approaches that are available, like homologous recombination and antisense technology, the most effective method is a recently discovered phenomenon called RNA interference. RNA interference or RNAi is sequence specific gene silencing mediated by double stranded RNA (dsRNA) 21-25 nt long called short interfering RNA or siRNA. The ability of siRNA to knock out any gene has revolutionized reverse genetics approaches. This phenomenon is found only among eukaryotic organisms and was initially independently discovered and studied in different organisms before it was recognized that the underlying mechanism was, at some level the same. (*Box* 1 lists various gene silencing phenomena related to RNAi). Recent findings that genes could be effectively suppressed *in vivo* in mammals by RNAi have potential implications in disease and research. A brief history of the development of RNAi is shown in *Box* 2.

Keywords
RNA interference, siRNA, gene silencing,Dicer.

Mechanism of RNA Interference

The RNAi pathway is initiated by a process where dsRNA is cleaved into 21-25 nt fragments of siRNA. dsRNA which can be

Box 1. Lexicon of Gene Silencing Effects Related to RNAi

Co-suppression: Ability of some transgenes to suppress themselves and homologous chromosomal loci simultaneously. Observed in plants.

Post transcriptional gene silencing: Ability of some virus, transgenes or RNAs to trigger the post transcriptional degradation of homologous cellular RNA. Observed in plants.

Quelling: A co-suppression phenomenon in *Neurospora crassa*

RNAi: Ability to block activity of cellular genes by homologous RNA of 21-25 nucleotides. First observed in *Caenorhabditis elegans*.

exogenous (injected or fed) or viral dsRNA, can enter RNAi pathway directly. In several organisms RNAi can also be induced by dsRNA formed from inverted repeat sequences that are transcribed into hairpin dsRNA. Dicer, an enzyme of the RNase III ribonuclease family displays specificity for dsRNA and can generate siRNA. It is a dimeric enzyme having dual catalytic domains and an additional helicase and PAZ (*piwi-argonaute-zwille*) domain, which is also found in several other proteins involved in the RNAi pathway (*Box* 3). Subtle alterations in the Dicers active site can alter the spacing of the catalytic center and hence explain the species specific variation in the siRNA length. The double stranded siRNA is then incorporated into a 250-500 kDa multiprotein complex known as RNA induced silencing

Box 2. Brief History of RNAi

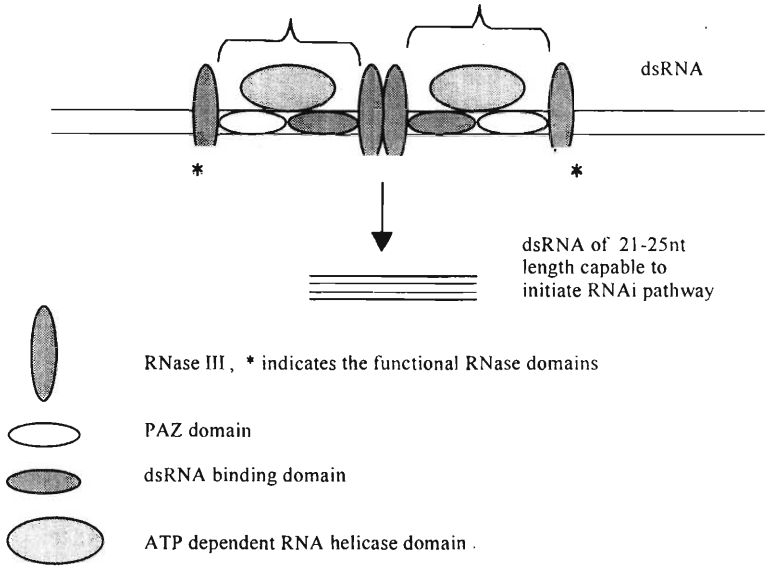
The phenomenon of post translational gene silencing was identified in 1990 when genes to enhance petal colors of petunia surprisingly inhibited the expression of any color in the petal. This was the first report of RNA mediated gene silencing.

The term 'RNA interference' was coined by Fire and Mello when they discovered the gene silencing phenomenon in *C.elegans*. They were attempting to use antisense RNA as an approach to inhibit gene expression. They found that sense and antisense RNA forming a double stranded RNA was a better silencing trigger than antisense RNA.

After the discovery of RNAi in *C.elegans*, identification of the RNAi pathway was extended to almost all eukaryotic model systems like *Neuro-spora, Arabdopsis, Drosophila,* mouse and humans.

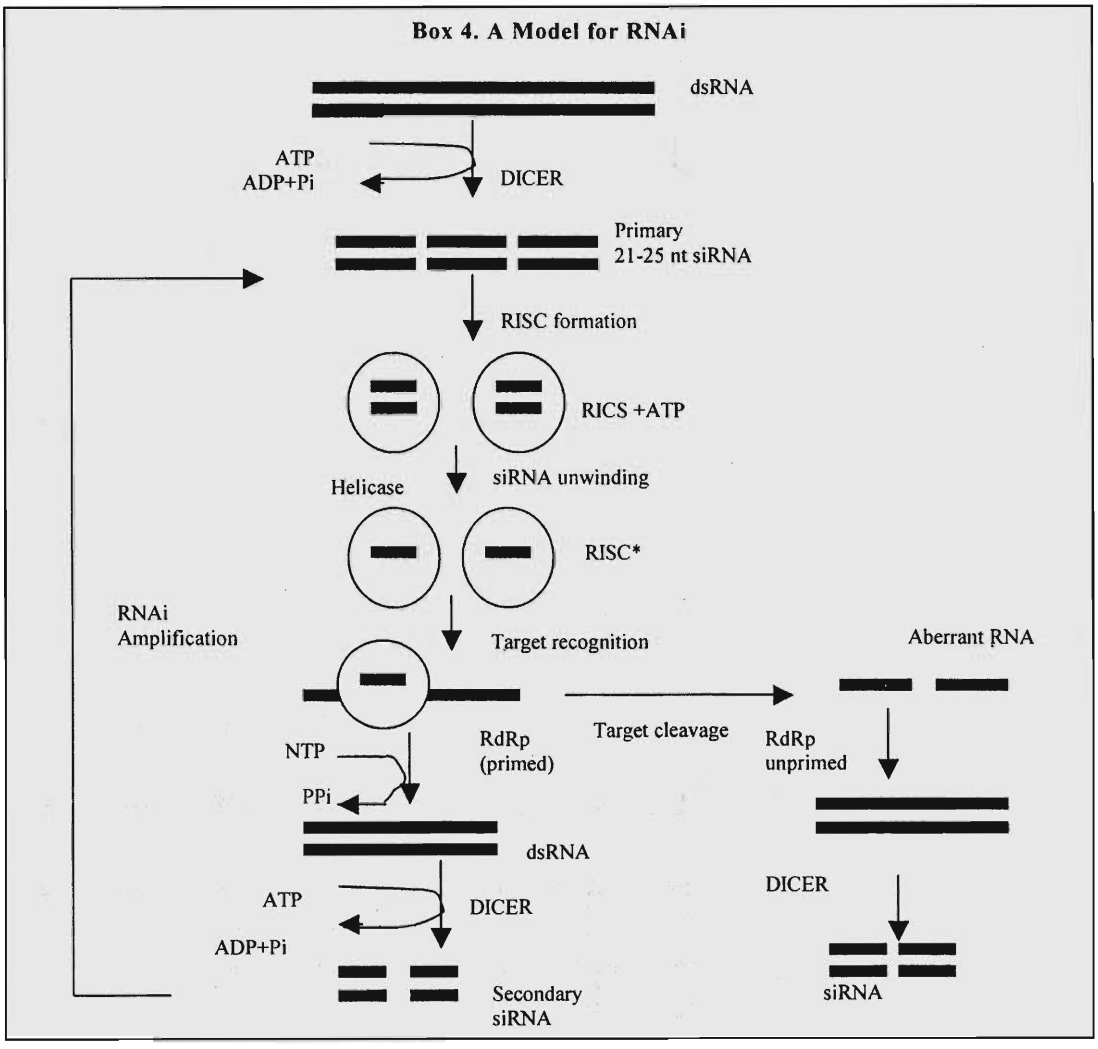
Box 3. Dicer and its Mechanism

The Dicer enzyme with its different domains as shown, mediates the cleavage of double stranded RNA to 21- 25 nt double stranded siRNA characteristic of RNAi



complex (RISC). RISC then undergoes an ATP dependent activation step that results in the unwinding of the double stranded RNA to single stranded RNA. Activated RISC* uses the single stranded siRNA as a guide to identify complementary mRNA. An endoribonuclease is supposed to cleave the mRNA-siRNA hybrid almost at the middle leading to the formation of an aberrant RNA, which is susceptible to other RNA degradation pathways (*Box* 4).

Various members of the RISC complex and other proteins involved in the RNAi pathway are identified using genetic and biochemical approaches like yeast two-hybrid screening. Diverse lines of evidences suggest that some aspect of the RNA silencing might involve a cellular RNA dependent RNA polymerase (RdRP). A 'Random degradative PCR' model proposes that the RdRP uses the guide siRNA as a primer to synthesize a new RNA strand using target RNA as the template and thereby converting it into a dsRNA that can act as a substrate and be destroyed by Dicer. This in turn would release a new crop of siRNA (secondary siRNA) to prime additional rounds of synthesis and target destruction. Thus RdRP plays a central role in the



The double stranded(ds) siRNA created after Dicer activity gets incorporated into RISC and is converted to single stranded(ss) siRNA. The activated RISC* can cleave target mRNA, or act as a primer for synthesis of ds RNA mediated by RdRp. The dsRNA generated can be further cleaved by Dicer.

amplification of RNAi. But the mammalian system seems to lack the RdRP activity. It has also been found that aberrant RNA can be converted to a dsRNA by a primer independent activity of RdRP.

Minute quantities of dsRNA can cause cell-to-cell spread of the silencing effect. This systemic silencing may occur over short

range in plants by transmission through plasmodesmata, but silencing signal may also pass over longer range through the plant vasculature. A gene *sid1* from *C.elegans* has been found to encode a transmembrane protein that may act as a channel for import of silencing signal and influence systemic signaling. There has been evidence of *sid1* homologues in mice and humans that raises the interesting possibility of systemic spread of RNAi in mammals.

Biological Functions of RNA Interference

Micro RNA and Short temporal RNA in Developmental Regulation

Micro RNA (miRNA) constitute a class of noncoding small RNAs that are phylogenetically conserved among eukaryotes. The short temporal RNA (stRNA) are a subclass of miRNA that are temporally expressed. stRNA like *lin4* and *let7* in *C. elegans* are involved in regulating developmental timings. Dicer is thought to excise precursor stRNA from their ~70 nt stem loop precursor to single stranded stRNA unlike the double stranded siRNA that is formed in the RNAi pathway. The stRNA precursors contain bulges and mismatches in the stem region that encodes the stRNA, unlike siRNA which have a perfect match. The pairing of stRNA with complementary mRNA regulates expression by blocking translation, whereas in siRNA there is cleavage of the target mRNA.

Chromatin Remodeling

The Polycomb group of proteins are involved in organizing the chromatin structure and normally involved in chromatin repression. It is found that mutation of certain genes in RNAi in *C. elegans* derepress genes that are silenced by polycomb dependent mechanism. Another evidence of chromatin remodeling is histone methylation and heterochromatin formation. dsRNA can induce H3 methylation and heterochromatin formation; H3 methylation may also lead to DNA methylation and subsequently gene silencing.

Viral Immunity and Control of Parasitic Nucleic Acids

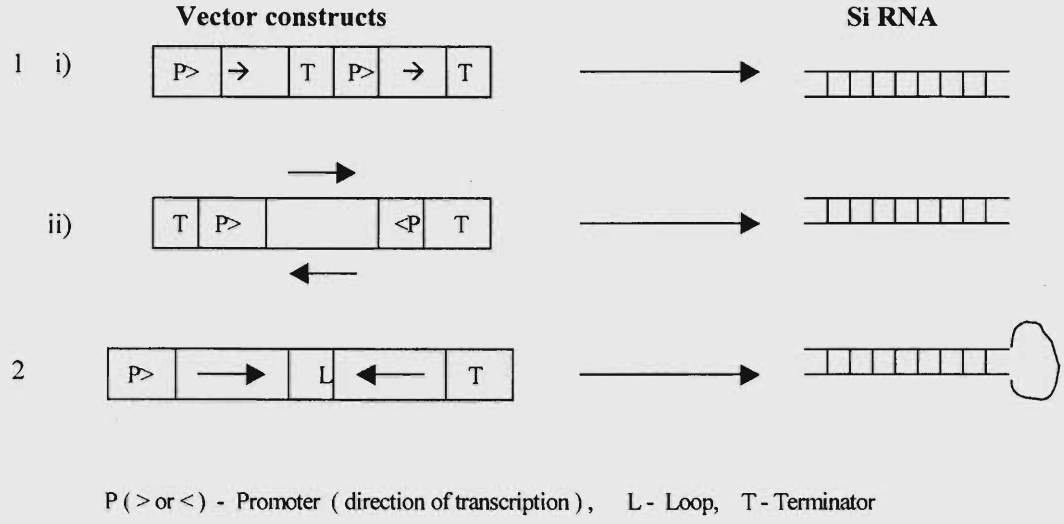
RNAi forms the basis of virus induced gene silencing in plants. It has been observed that mutations in RNA-dependent RNA polymerase (RdRp) homologs in *Arabdopsis* increases the accumulation of virus; conversely, virus infection can stimulate expression of RdRp genes.

It has been observed that loss of function of certain genes involved in RNAi can activate transposons. From this it has been proposed that RNAi may be involved in stabilizing the genome by sequestering transposons and making them unavailable for recombinational events that may lead to chromosomal translocation.

Stable Expression of siRNA

From the above discussion we see that there exists a mechanism that can effectively silence a particular gene. But there are certain problems that have to be considered. Firstly, in humans the amplification of RNAi is lacking due to absence to RdRp. Secondly, large dsRNA (30-85 nt) can stimulate an antiviral state that can dramatically change the cellular metabolism that eventually lead to induction of apoptosis, so this pathway should not be induced. These problems can be avoided by using a vector mediated delivery of siRNA. Intracellular transcription of small RNA molecules can be achieved by cloning the siRNA templates to RNA polymerase III transcription units. Two approaches have been developed for expressing siRNA: First, sense and antisense strands constituting the siRNA duplex are transcribed by individual promoters, that can hybridize to form siRNA. In the second, siRNA are expressed as fold back stem loop structures that give rise to siRNA after intracellular processing (*Box* 5). At present the transfection ability of cells especially mammalian cells is the rate-limiting step in using siRNA as a therapeutic agent. Transfection using cationic lipid carriers like Oligofectamine and Lipofectamine are being tried.

Box 5. Endogenous Synthesis of siRNA



P (> or <) - Promoter (direction of transcription), L - Loop, T - Terminator

1. i) Sense and antisense RNA strands of required sequence length synthesized from individual promoters which hybridize to form siRNA.
   ii) Sense and antisense RNA synthesizes as transcript and countertranscript from individual promoters.
2. siRNA expressed as fold back stem-loop structures.

RNAi in Therapeutics and Research

RNAi possesses great potential as a therapeutic agent by its virtue to silence genes. Many diseases are being targeted, like AIDS, tumors, Hepatitis C, Malaria, Polio to name a few. Inactivation of critical proteins involved in pathogenesis is the principle use of siRNA. In case of AIDS, CD4 is the receptor for the entry of HIV1 virus. Targeting CD4 stops viral entry, but this approach has limitations as CD4 has an important role in the normal immune functions. This can be overcome by identifying other targets. One such target is CCR5, a co-receptor of CD4. A group of individuals in the Scandinavian region who are homozygous recessive for a mutation in CCR5 are immune to AIDS. Among other targets that are being tried out are p24 protein which is involved integration of the viral genome into the host genome and the Rev protein which is involved in transport of nucleic acid from cytoplasm to the nucleus. Certain

forms of tumors develop as a result of a mutant form of the Ras protein. Ras is a switch protein involved in Receptor Tyrosine Kinase signaling cascade. Function of normal Ras protein is necessary for the cell. The mutant form of the Ras protein can bind to GTP but GTP is not hydrolysed, thereby is always in an active form. This physiological change can cause neoplastic transformations. Targeting the mutant form of Ras protein, leaving the normal form unaltered can inhibit the formation of tumors.

RNAi approach can also be used to study biochemical processes and pathways. Biochemical approaches that can identify protein-protein interactions involved in a certain process like yeast two-hybrid screening pick up large numbers of false positives. Knockout using RNAi based approaches provide a better system to determine the functional relevance of the protein in that particular process.

Conclusion

Elucidation of the RNA interference pathway has proved to be a boon to biologists. With the development and realization of the importance of this phenomenon, the *Science* magazine has named it as the 'Breakthrough of the Year-2002'. It is likely that vectors that encode siRNA or miRNA will become widely accepted as a gene knockout tools. It is certain that the RNA interference technology will transform future studies of cellular systems and introduce a new wave of therapeutic RNA molecules in the years to come.

Suggested Reading

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