

Mechanism of RNA interference (RNAi): Current concept

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Abstract. RNAi is an evolutionary conserved silencing pathway in which the double stranded RNA is broken down into small interfering RNA (siRNA) with the help of dicer and RNA-induced silencing complex (RISC) complex by a series of steps. The two component of DICER, dcr-2/r2d2 an ATP dependent binds to siRNA and helps it to load into RISC by forming RISC loading complex (RLC). RLC recruits their associates to form the effective machinery for gene silencing wherein it may remain binds to the complementary mRNA or may degrade the target. In this way, the activated RISC could potentially target multiple mRNAs, and thus function catalytically

Key word: Gene silencing, RNAi, DICER, RISC.

1. Introduction

RNA interference (RNAi) is a phenomenon primarily can be demonstrated as interference by RNA in the expression of genetic information. It is a phenomenon primarily for the regulation of gene expression; self or non self depending upon the surrounding factors or conditions, with the help of RNA molecules that are non coding in nature to control cellular metabolism and help in maintaining genomic integrity by preventing the invasion of viruses and mobile genetic elements.

RNA molecules were long believed to serve only as messengers, bearing genetic information from DNA. But in the early 1980s it was revealed in *Escherichia coli* that small RNA molecules (about 100 nucleotides in length) can bind to a complementary sequence in mRNA and inhibit translation (21). This inactivates mRNA and therefore prevents it from passing on the genetic information. The phenomena of RNAi first came into notice in the year 1990, when plant scientists were trying to intensify the hue of red petunias for commercial benefits. Rich Jorgensen introduced a gene that controls the formation of red pigments in petunia flowers but in some cases it was observed that the colour disappeared altogether. The reason behind such an observation was not clear at that time. The phenomenon was named as co-suppression. Co-suppression is a classical form of eukaryotic post-transcriptional gene silencing. It was first reported in transgenic petunia, where a sense transgene meant to overexpress the host Chalcone Synthase-A (CHS-A) gene caused the degradation of the homologous transcripts and the loss of flower pigmentation. Subsequently such phenomenon was also observed in fungi, which was termed as quelling (6, 8, 26)

In animals, RNA silencing was first reported when Guo and Kemphues (28) were attempting to use antisense RNA to shut down expression of the par-1 gene in order to assess its function. As expected, injection of the antisense RNA disrupted expression of par-1, but quizzically, injection of the sense-strand control did too used antisense RNA to block par-1 mRNA expression in *Caenorhabditis elegans* but found that the par-1 mRNA itself also repressed par-1. Their paradoxical observation— subsequently dubbed RNA interference (RNAi)—inspired the experiments of Fire, Mellow, and colleagues (4)

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Fire et al. (4) studied on the gene function especially on the gene responsible for movement in *C.elegans*. When this nematodes were injected with the mixture of sense and antisense RNA, nematodes were observed with the impaired movement suggesting a defective muscle gene protein. They concluded that the dsRNA injected must match the mature “trimmed” mRNA sequence for the gene and the interference could not be elicited by intron sequences. This implies that interference takes place after transcription, probably in the cytoplasm rather than in the cell nucleus (4)

The mRNA was revealed to be targeted with large complex proteins to form RNA induced silencing complex, commonly called RISC; in association with si RNA (25). Subsequently, it has been shown that RISC contains at least one member of the argonaute protein family, which is likely to act as an endonuclease and cut the mRNA (often referred to as the Slicer function). It was also demonstrated that a ribonuclease III-like nuclease, called Dicer, is responsible for the processing of dsRNA to short RNA. In certain systems, in particular plants, worms and fungi, an RNA dependent RNA polymerase (RdRP) plays an important role in generating and/or amplifying siRNA (14).

2. Mechanism of RNA interference

Mechanism of RNA interferences as understood is that it comes into play when a double stranded RNA is introduced either naturally or artificially in a cell. An endoribonuclease enzyme cleaves the long dsRNA into small pieces of RNA. The small pieces could be mi RNA or si RNA depending upon the origin of long dsRNA i.e. endogenous or exogenous respectively. A double stranded RNA may be generated by either RNA dependent RNA polymerase or bidirectional transcription of transposable elements or physically introduced. The mechanism of RNA interference can be divided into 2 stages: 1st Initiation, 2nd Effector (Fig 1).

2.1. Initiation

This stage is characterised by generation of siRNA mediated by type III endonuclease Dicer. In *Drosophila*, Dicer, which is a large multidomain RNase III enzyme has been identified in existence into two forms: Dcr-1/Loquacious (Loqs)-PB (also known as R3D1-L[long]): generate miRNA and Dcr-2/R2D2 generates siRNA (15,22,20). Dcr-1 share a structural homology with Dcr-2, despite that they display different sets of properties such as ATP requirements and substrate specifications (15).

Dcr-1 is an enzyme that show ATP independent functions and affinity towards stem-loop form of RNA (precursor of miRNA) (15). It has been identified that Dcr-1 requires a double stranded RNA binding protein partner. In *Drosophila* Dicer-1 is seen to interact with the dsRBD protein Loquacious (Loqs). Immuno-affinity purification experiments reveal that Loqs resides in a functional pre-miRNA processing complex, and stimulates and directs specific pre-miRNA processing activity. These results support a model in which Loqs mediates miRNA biogenesis and, thereby, the expression of genes regulated by miRNAs (20). Loquacious protein is supposed to be composed of three dsRNA binding domains, which is encoded by *loqs* gene into two types of proteins PA and PB; isoform to each other, by alternate splicing, among which PB is known to enhance the affinity of Dcr-1 towards pre-miRNA (15, 20, 22).

Dcr-2 shows ATP dependent activity with substrate specificity to double stranded RNA (15). Structurally homologous to Dcr-1, it also requires a double stranded RNA binding protein, namely R2D2, which function in association with specific RNase enzyme Dcr-2 forming a heterodimeric complex. R2D2, unlike Loq is supposed to be composed of two dsRNA binding domains (15,22,20) that interact with long double stranded RNA but does not regulate si RNA generating activity of Dcr-2 (15). Studies of the physiological functions of R2D2 by the design of mutant strain have shown that R2D2 protein plays an important in development. It has also been shown to be responsible for the stability of Dcr-2 and decrease in the concentration of R2D2 in cells reduces the concentration of Dcr2 and vice versa. These results indicate that both dsRNA binding domains of Dcr-2 and R2D2 are critical for Dcr-2/R2D2 complex to bind and load siRNA into siRISC complex (24). Dcr-2 contains an RNA helicase domain, a DUF283 domain, and a PAZ domain at the N terminus as well as tandem RNase III motifs and a dsRBD motif at the C terminus.

It is unclear which of these domains physically contact siRNA. Since neither Dcr-2 nor R2D2 bind siRNA alone, it is possible that siRNA is bound at the interface between Dcr-2 and R2D2, which triggers a conformational change in either or both proteins, allowing them to bind siRNA co-operatively (24).

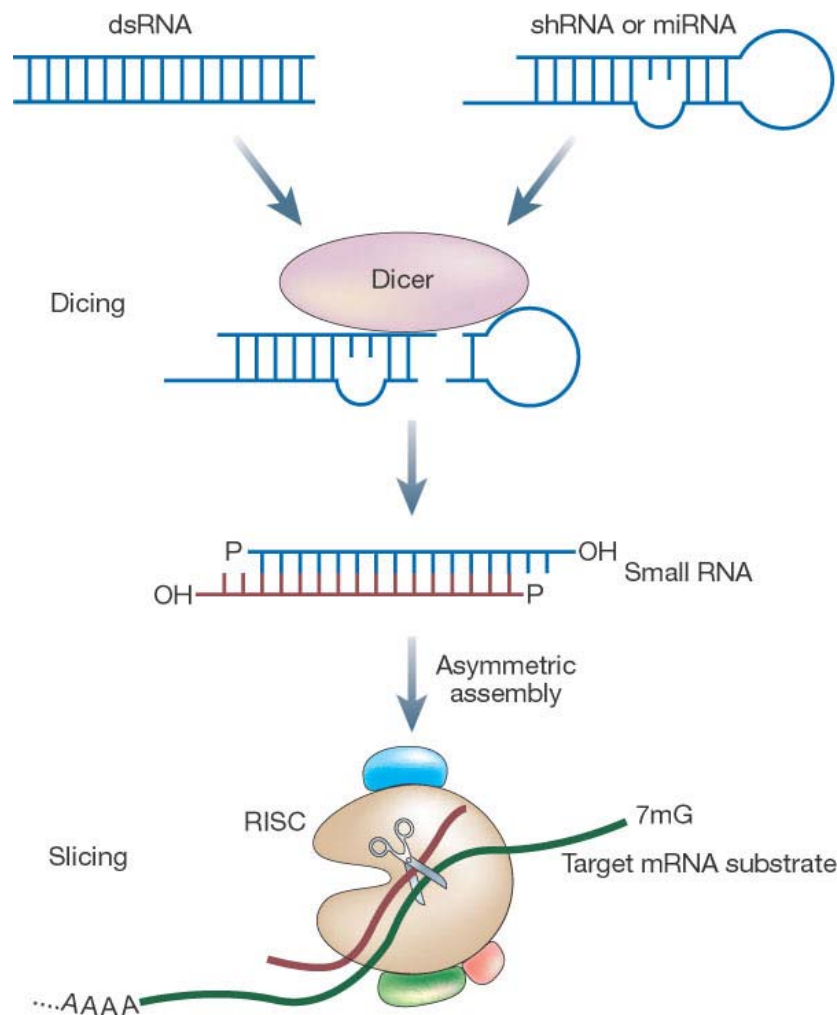


Fig. 1: Mechanism of RNA silencing in different systems. Long dsRNA and miRNA precursors are processed to siRNA/miRNA duplexes by the RNase-III-like enzyme Dicer. These short dsRNAs are subsequently unwound and assembled into effector complexes, RISCs, which can direct RNA cleavage, mediate translational repression or induce chromatin modification. *S. pombe*, *C. elegans* and mammals carry only one Dicer gene. 7mG, 7-methyl guanine; AAAA, poly-adenosine tail; Me, methyl group; P, 5' phosphate. (Courtesy: Gregory J. Hannon and John J. Rossi, 2004)

2.2. Effector

The second step of RNA interference mechanism is referred as effectors step; the incorporation of guide strand. This is characterized by the formation of silencing complex.

Single strands of siRNA and miRNA duplexes, referred to as guide strands, are incorporated into RNA silencing effector complexes such as the RNA-induced silencing complex (RISC), or the RNA-induced initiation of transcriptional gene silencing (RITS) complex.

1. RNA Induced Silencing Complex (RISC) cleaves m RNA or represses their translation by homology dependent m-RNA degradation. 2. RNA-induced Initiation of Transcriptional gene Silencing complex (RITS) regulates heterochromatin assembly.

The core of these RNA silencing effector complexes, RISC was found to be composed of PPD Proteins (PAZ PIWI Domain proteins) which are highly conserved super-family. Members of PPD proteins contains centrally located PAZ (100 amino acids) and C-Terminal located PIWI (300 amino acids).

Subsequently it was revealed that the PPD protein in RISC was Ago proteins. Ago proteins are members of PPD protein super-family. Not all PPD proteins are known to perform the same task in all systems (Table

1), but when studies were performed and compared the number of PPD in different systems; Nematodes were found to have more specific function of PPD than others.

Table 1. Function of PPD Proteins (PAZ PIWI Domain proteins) in variable living system

Type of system	No of PPD proteins exist	Type of PPD Protein involved in siRNA mediated silencing	Function performed
Nemetode (<i>C. elegans</i>)	3	PPD, RDE-1, PPW-1	For efficient siRNA mediated m-RNA cleavage
Arthropoda (<i>D. melanogaster</i>)	1	Ago-2	For incorporation of siRNA into RISC
Chordata (<i>Homo sapiens</i>)	1	hAgo-2	Catalytic activity required for m-RNA cleavage

The purification of complete eukaryotic Ago protein was difficult so experiments were performed with their prokaryotic homolog which revealed that it has a structure similar to RNase H ribonuclease (19). The ago protein was found to be comprised of 4 major domains: N- terminal, PAZ domain, Middle and PIWI domain. The purification and study of RISC revealed that it consist of Ago proteins in a major concentration which was suspected to be the catalytic subunit part of this protein (27). Structure directed mutagenesis experiments implicate Ago protein as the catalytic subunit of RISC (17). A crystal structure of a full-length eubacterial Ago protein from Aquifex aeolicus (AaAgo) was determined and further confirmed the relationship to RNase H. Among the four domains of Ago proteins, Eukaryotic Ago proteins are found to be characterised by two functional domains PAZ and PIWI.

The RNA interference model has taken a shape as; Dcr-2/R2D2 binds with siRNA, it forms RDI complex (R2D2 Dcr Initiator complex). RDI orients siRNA duplex so that the 5 prime end with lower melting temperature preferentially interacts with Dcr-2 the less stable end, R2D2 binds the more stable end of siRNA (23). This leads to thermodynamic asymmetry which helps the separation of guide strand from the passenger strand (other strand). Studies have shown that small change in the structure of siRNA effects on determination of the guided strand of the RISC complex which concludes, enzyme that governs the RISC assembly selects the siRNA strand incorporated into RISC on the basis of structure. (3, 11). Also the orientation of Dicer2/R2D2 on siRNA determines the guide strand. It suggests that structure siRNA determines the orientation of Dcr2/R2D2 which in turn determines the strand that will be incorporated into RISC as guide strand.

Ago2-dependent complex, pre-RISC, was identified and Ago2 was found to be responsible for conversion of pre-RISC to holo-RISC i.e. the effective removal of passenger strand. The pre-RISC complex contains duplex siRNA whereas holo-RISC contains just the guide strand of the siRNA, the passenger strand having dissociated from holo-RISC. 5 prime end of siRNA acts as a target recognition guide which is required for the efficient RNA interference mechanism (1, 11). Here the PIWI domain of the catalytic subunit of RISC (holo-RISC), Ago-2, acts as RNase H (highly conserved in Eukaryotes) and cleaves the target m-RNA backbone.

It has been observed that, a small induction was enough for silencing a particular gene in the whole organism. This indicates that RNA interference silencing mechanism could amplify its signal intensity. This amplification mechanism was termed as Transitivity; siRNA bearing new homology sequences (upstream/downstream) of the target zone are synthesized. These newly synthesized siRNA were used to destroy the target. A role of RNA dependent RNA polymerase (RdRP) was assumed in this mechanism (12,2). The siRNA derived in RNA interference pathway may have two different fates, either it may be converted into holo-RISC for the destruction of the target m-RNA, or it may contribute to the amplification step by the generation of secondary siRNA. Two models were proposed as the mode of its action for the

generation of secondary siRNA. 1. RdRP generates the newly synthesized siRNA directly. 2. RdRP use siRNA as a template for the production of long dsRNA, which are then cleaved into siRNA (13). It was found that the newly synthesized RNA was of 24-26 nucleotides long instead of 20-22 nucleotides (5). The reason behind this is unexplained. The newly synthesized siRNA again joins the cycle for the production of long double stranded RNA and sustains the amplification process.

3. Conclusion and future prospects

During the studies of silencing mechanism it was noticed that in Plants as well as *C. elegans*, local silencing could be spread to distant sites. This observation suggested evidence for the existence of mobility of the silencing signal. SYSTEMY is the mobility of the silencing signal, which is based on the fact that RNAi is not cell autonomous. Later it was suggested that mobile signals are able to spread through specific proteins where it can activate RNAi (10). In plants 2 types of systemy known to exist: 1. cell to cell movement 2. Long range spreading. The movement of the former is uncertain but the later is supposed to move through phloem and activate silencing through plasmodesmata. The mechanism of RNAi shows that it is a powerful tool for biochemical studies and developing technologies and a ray of hope for various challenging diseases.

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