

RNAi – History, Mechanism and Application

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The History of RNAi

The phenomenon of RNA interference (RNAi) was first discovered in *c.elegans*, in which double-stranded RNA (dsRNA) could specifically silence expression from homologue genes[1]. The first observation of gene silencing induced by RNA was back in early 1990s in plants. In the efforts to make the color of flowers more vivid, researchers created transgenic plants with extra copies of pigment-making gene. To their surprise, instead to be more colorful, some of the transgenic flowers turned out to be white. Production from both the transgenic gene and plants' endogenous gene had been switched off or "co-suppressed"[2-4]. The transgenic copy, intended to make more corresponding gene products, surprisingly made less. This phenomenon, namely post transcriptional gene silencing (PTGS), was not limited to plants. Parallel work in *c.elegans* was carried on in 1995. Guo and Kemphues injected the antisense strand RNAs of certain endogenous gene into worms, with intention that those antisense strands may hybridized to corresponding gene and in turn block translation. Sense strand RNAs, which in fact the same as endogenous target mRNA itself, were used as negative control. Shockingly, sense strand by itself was found to be as effective as antisense to suppress gene expression [5]. This mystery was solved in 1998, when Fire and Mello tested the synergy effect of sense and antisense strand together. They found that double-stranded RNA was at least ten-fold or perhaps a hundred fold more potent as a silence trigger than was sense or antisense alone. Furthermore, they pointed out the repression effect observed by Guo and Kemphues, was in fact caused by trace amount of double-stranded RNA contamination in those samples [1].

Why does it take so long for people to propose dsRNA as a trigger for the silencing effects observed? First, at the time, dsRNA was thought to be a nonspecific silencing agent that triggers a global destruction of mRNA and suppression the protein expression in mammalian cells. Second, dsRNA is rather stable and thought to be unavailable for further base pairing to target mRNAs. Any model including dsRNA as a trigger for RNAi is obligated to involve a whole set of cellular factors to unwind dsRNA and somehow searching the target mRNA sequence in the cellular pool by using a single strand as guide. Although now we know that it is actually how RNAi functions, back then, hypothesis comprising of so many unknown activities are rarely appealing. It takes more than brilliant designs of experiments and unarguable scientific evidences for Fire and Mello to propose dsRNA as the silencing trigger and created the new term –RNAi. And because of that, they were awarded the Noble Prize in 2006.

With the discovery of dsRNA as an extremely potent trigger, it became feasible to explore the mechanism of RNAi in various biological systems. Results from genetic screen in *C.elegans* identified cellular proteins required for RNAi[6]. Comparing of the genes required for RNAi in *C.elegans* to genes required for gene silencing in *Drosophila*, plants and fungi, substantiates that previous observed silencing phenomena –PTGS, co-suppression,

quelling and RNAi were in fact share a common fundamental mechanism. People became to realize that RNAi is a pathway of ancient origin conserved from fungi to human.

A flurry of exciting results was exploding out in relatively short amount of time with the help from genetic and biochemical studies. Baulcombe group [7] reported that during investigation of PTGS in plants, formation of ~25nt small RNAs derivatives was observed only when cells were pre-treated with dsRNA. In 2001, Zamore and his colleagues recapitulated RNAi in *Drosophila* extracts [8]. They found small RNAs in length of 21-23-nt, produced from dsRNA in cell extracts, could serve as a *de novo* silencing trigger for RNAi in cell extracts free of dsRNA treatments. In addition, Hannon group found that these small RNAs were products of ribonuclease and tightly in association with the effector complex of RNAi: RNA-induced silencing complex (RISC) [9]. Unique structure of small interfering RNA (siRNA) is a classic cleavage product pattern of RNase III-like enzyme, which led to the identification of the highly conserved Dicer family of RNase III enzymes as the mediators of dsRNA cleavage. Dicer was later cloned by Bernstein et al. in *Drosophila* [10]. Engineered Dicer-like synthetic RNAs were shown to induce sequence-specific gene silencing in human cells without initiating the nonspecific gene silencing pathways, which made it feasible to use siRNA as a tool to knock-down specific genes in mammalian cells [11]. This application draws a broad attention to this amazing phenomenon of RNAi.

More exciting news came from the observations that naturally expressed small hairpin RNAs [12, 13], now known as microRNAs (miRNAs), were also processed by Dicer and function through a similar pathway as RNAi [14, 15]. In plants, miRNAs mainly function as siRNAs, resulting cleavage of the target mRNA; while in animal, miRNAs appear predominantly to inhibit translations by targeting partially complementary sequences located within the 3' untranslated region (3'UTR) of mRNAs. Finally, the RNAi machinery was linked to chromatin regulation in yeast, plants and mammalian systems (reviewed in [16]). Those breakthroughs expanded the role of RNAi from anti-viral system to key machinery in endogenous gene regulations.

Mechanism of RNAi

Initiation - Processing dsRNA precursors

RNAi can be triggered by various sources of RNA molecules. MicroRNA precursor, termed primary microRNA or pri-miRNA, will fold back and form a long stem-loop structure, severing as an endogenous source

of dsRNA. Certain microRNA precursors are located in the intron regions of host gene and therefore transcribed by the host gene promoter. Other microRNA sequences are clustered in polycistronic transcripts and have their own promoters [17]. Polymerase II are believed to be responsible for the transcription of microRNA precursors [18]. However, recent findings indicate Polymerase III is also involved in transcribing certain microRNAs [19].

Despite the rather uniform structure of microRNA (miRNA), pri-miRNAs are heterogeneously in length and loop sequences. Maturation of microRNA involved two step-wise events. First, the nascent miRNA transcripts (pri-miRNA) are processed into ~70nt precursor (pre-miRNA); in the second event, this pre-miRNA is further cleaved to generate ~21 – 25 nucleotide mature miRNAs [20]. Those two sequential cleavages are executed by two dsRNA specific RNase III-type endonuclease, termed Drosha and Dicer. Both enzymes cut

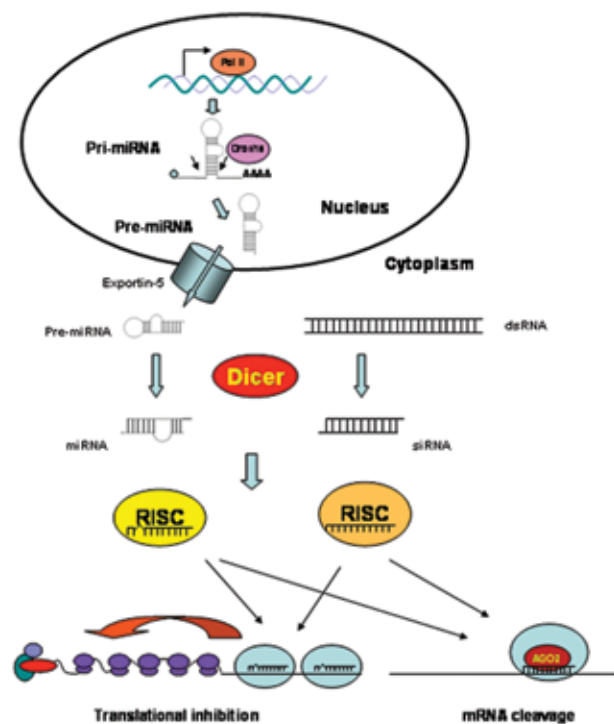


Figure 1. The current model for RNA interference in mammalian cells. The pri-miRNA transcripts are first processed into pre-miRNAs by Drosha inside the nucleus. After transported into cytoplasm through exportin-5, pre-miRNA and dsRNA are both processed by Dicer. Only one strand of the miRNA duplex or siRNA duplex will be loaded into RISC, which subsequently acts on its target by translational repression or mRNA cleavage, depending on the level of complementarity between the small RNA and its target.

dsRNA, leaving two 3' overhang nucleotides and 5' phosphate group at the cleavage site [21]. Droscha, under help of a partner protein Pasha (DGCR8), recognizes various form of pri-miRNA and excises the stem-loop from bottom in nucleus, defining one end of mature microRNA. Pre-miRNAs are transported into cytoplasm through a Ran-GTP dependent transporter, exportin-5 [22]. Once in the cytoplasm, Dicer will trim off the loop from pre-miRNA, defining the other ends of mature microRNAs [15, 23].

Processing of exogenous dsRNA introduced into cells only involves Dicer but not Droscha. Dicer contains two RNase III domains, one helicase domain, one dsRNA binding domain and one Piwi/Argonaute/Zwille domain [24]. The last domain is also present in Argonaute family proteins, already known to be essential for RNAi. The current model believes the binding of Dicer to the end of dsRNA is far more effective than internal binding. Dicer will associate with an existing terminus of dsRNA and cuts ~21 nucleotides away from the end, forming a new end with two 3' overhangs. As a result of this stepwise cutting, a pool of 21-nt long small RNA with two 3' overhangs nucleotides will be generated from long dsRNAs.

Several organisms contain more than one Dicer genes, with each Dicer preferentially processing dsRNAs from different sources. In *Drosophila*, among the two paralogues, Dicer-1 specifically process pre-miRNA while Dicer-2 is required for dsRNA processing [25]. *Arabidopsis thaliana* has four Dicer-like proteins. DLC-1 is involved in microRNA maturation; DLC-2 preferentially process dsRNA from plant virus; DLC-3 is required for generating small RNAs from endogenous repeated-sequences [26]. Interestingly, *S.pombe*, *C.elegans* and mammals carry only one Dicer gene.

Assemble the RNA induced silencing complex (RISC)

Dicer generated siRNA or miRNA will join functional ribonucleoprotein particles RISC. The size and composition of RISC varies, indicating the possible existence of multiple types of RISC, which perhaps suggests functional differences in various cellular processes [27-29]. The most conserved members of RISC are Argonaute proteins, which are essential for RISC functions. Argonaute proteins are highly basic proteins that have been linked to RNAi through mutant screens in plants, yeast and *C.elegans* [6, 30]. Argonaute proteins are characterized by the presence of two homology regions, the PAZ domain and the PIWI domain. Structure analysis revealed that PIWI domain contains putative RNase H – like activates, and therefore a possible functional domain of Argonaute proteins. PAZ domain also appears in Dicer proteins, specifically recognize the unique structure of two 3' nucleotides overhangs of siRNAs [31]. 5' phosphate group is recognize by the PIWI domain in Argonaute proteins and therefore required

for siRNA to assembly into RISC [32, 33]. SiRNA lacking this phosphate group in 5' end will be rapidly phosphorylated by an endogenous kinas [33].

Handover siRNA from Dicer to RISC is rather a protein-associated process than simple diffusion *in vivo*. An ATP-dependent process is needed to activate RISC, which could be explained as a step of unwinding siRNA duplex, leaving only single strand RNA joining the active form of RISC. The strand remained within the RISC function as a guild to locate targets mRNA sequence through Watson-Crick base-pairing while the other stand of duplex siRNA is either cleaved or discarded during the loading process. Two strands of siRNA duplex are not equally eligible for assembly into RISC. RISC prefers the strand have relatively lower thermal stabilities of the base pairs at the 5' ends [34, 35].

Different organisms have different numbers of Ago proteins, ranging from one in *S.pombe* to over 20 in *C.elegans* [36]. There are eight Ago proteins in human and evidences suggest their functions are not redundant [37]. For organism have more than one Dicer proteins, loading small RNA to different Ago containing RISC may be regulated by the specific interaction between individual Dicer and Ago proteins. For organism has only one Dicer but multiple Ago proteins, such as human, it would be of great interests to know how this process is controlled. Besides the Ago proteins, various cellular factors are also reported as component of RISC, including the Vasa intronic gene product (VIG), fragile-X related protein (dFXR), and the tudor staphylococcal nuclease domain containing protein (Tudor-SN) in *Drosophila* [38] and fragile X mental retardation protein (FMRP), Gemin3/4 in human [39]. The detail functions of those proteins in RISC remain unclear.

The effector step – function of various RISC

Single strand of siRNA duplex incorporated in RISC will serve as a guide to find complementary sequence in the target mRNAs. Perfect or near-perfect Watson-Crick base pairing between the mRNA transcript and the guide strand is sufficient and required to lead an endonucleolytic cut in the middle of target complementary region, ten nucleotides upstream of the nucleotide paired with the 5' end of the guide siRNA [40]. The endonuclease Argonaute 2, the only member of the Argonaute subfamily of proteins with observed catalytic activity in mammalian cells, is responsible for this slicing activity [30]. Cleaved transcripts will undergo subsequent degradation by cellular exonucleases. The guiding strand of siRNA duplex inside RISC will be intact during this process and therefore permit RISC function catalytically [41]. This robust cleavage pathway makes it a very attractive method of choice for potential therapeutic applications of RNAi.

In plants, the majority of miRNAs hybridize to target mRNA with a near-perfect complementarity, and mediate an endonucleolytic cleavage through a similar, if not identical, mechanism used by the siRNA pathway [42]. In animals, miRNAs interact with the 3'UTR of target mRNAs. The first identified miRNA, *lin-4*, was found to specifically bind to multiply target sites in the 3'UTR region of *lin-14* and negatively regulate its protein expression [13]. By far, most investigated miRNAs form an imperfect hybridization with sequences solely located in the 3'UTR region of target mRNA. The central mismatch between miRNA-mRNA hybridization is believed to be responsible for the lack of RNAi-mediated mRNA cleavage events. Instead, the association between miRNA programmed RISC and target mRNA induces translational repression through a poorly understood mechanism. Evidences supporting models in which translation repression occurs at the initiation stage or later steps including elongation have been provided [43, 44]. Repressed mRNA and associated Ago proteins are enriched in processing body (P-body) where endogenous cellular mRNA are kept for storage and degradation [44, 45]. It is still not clear whether this localization to P-body is essential for MicroRNA mediated translational repression. Recent evidences suggested it may be a secondary event of the initial translational inhibition [46, 47]. Nonetheless, P-body association may partially explain the observation that miRNA mediated translational inhibition is often coupled with some amount of RISC-independent target mRNA degradation [48].

RNAi induced gene silencing is not limited in posttranscriptional level. In plants, it has been shown that siRNA can also trigger *de novo* DNA methylation and transcriptional silencing [49-51]. This activity in plants may be directed by a separate class of siRNAs [52]. Existing evidence suggests that siRNAs can inactivate transcription through direct DNA methylation and other types of covalent modification in the genomes of certain species other than plants. Several studies also demonstrated that RNAi machinery in the fission yeast *S.pombe* plays a critical role in formation and maintenance of higher-order chromatin structure and function [53, 54]. Deletions in critical RNAi pathway genes lead to loss of epigenetic silencing of centromeric DNA and other types of heterochromatin; these changes are accompanied by changes in the methylation state of these regions, and subsequent loss of centromere cohesion, resulting in chromosome mis-segregation during nuclear division. It is hypothesized that in wild-type cells expression of centromeric repeats results in the formation of a dsRNA that is cleaved by Dicer into siRNAs that direct DNA methylation of heterochromatic sites. Recent researches further extended this phenomenon beyond plants and yeasts. However, conflict results from different reports indicate that the scenario of TGS in mammalian system is much more complicated than that in plants and yeasts [55].

Utilizing RNAi as Gene Knock-down Tools

Application of dsRNA in mammalian cells was limited, because any dsRNA longer than 30-bp will induce interferon pathway, resulting off target effects. This restriction was overcome when Tuschl and his colleagues introduced chemically synthesized siRNA into mammalian cells [11]. Those siRNA was shown to be capable to induce similar inhibition effect as dsRNA, without causing any unspecific degradation. A flood of papers were reported to successfully exploit either synthetic or expressed siRNA in various types of cells, targeting exogenous targets as well as endogenous genes [30]. Chemically synthesized siRNA are generally 21-nt in length, with two 3' nt overhang in both ends and a full complementary of sequence to any part of target mRNA (coding or non-coding region). SiRNAs are not only available for large-scale synthesis and uniform production, but also open to chemical modification that can increase their stabilities. Several companies involved in manufacturing siRNAs provide on-line software to aid design efficient siRNAs based on parameters as target site accessibility, thermodynamic stability, strand biases and minimal off-target effect.

Unlike fungi, plants and worms, which can amplify siRNAs, there is no indication of siRNA replication in mammals. Therefore, RNAi induced by transfection synthetic siRNA is limited in *Drosophila* and mammals by its transient nature. Several groups have developed expressed short hairpin RNA (shRNA), with a loop sequence linking the sense and anti-sense strand of siRNA together. This miRNA structure-like small hairpin RNA could be processed by Dicer in cytoplasm and function in siRNA pathway [56, 57]. Most plasmid-based expression system use RNA polymerase II or polymerase III as promoter to produce short RNA transcripts. Pol III promoters (such as U6 or H1) produce high level of transcripts with well defined initiation points and termination signals. Pol II promoters express shRNA in longer transcripts which are more close to the structures of native miRNA precursors. This artificial structure renders Pol II driven shRNA a better efficiency to assemble into RISC.

Several things need to be taken into consideration when design siRNAs. First, guiding strand of siRNA might function as a miRNA when it contain partial complementary sequence to 3'UTR of certain gene and cause unwanted off-target effect [58]. Second, certain sequence motifs of siRNAs which may induce interferon pathway through toll like receptor (TLR) should be avoided [59]. Third, over-expression of siRNA/shRNA may saturate the endogenous microRNA pathway and therefore cause toxic effect to the host cells [60].

Outlooks

RNAi is a newly discovered pathway with ancient origin. Understanding the mechanism of RNAi was not only providing us a powerful tool to manipulate the expression from any gene of interest, but also shedding light on human disease. Recent evidences link miRNA processing with various diseases including cancer and viral infections [61, 62]. The day that small RNA becomes a practical drug or target of drugs may come sooner than anyone could imagine.

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