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RNA Interference in *C. elegans*: History, Application, and Perspectives

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Abstract: RNA interference (RNA*i*) is the phenomenon of gene silencing by double-stranded RNA (dsRNA) at transcriptional and post-transcriptional levels in a sequence-specific manner. Reverse genetic approaches using RNA interference (RNA*i*) have become a major tool for biological researches since its discovery in the nematode *Caenorhabditis elegans*. In this review, we overview how the RNA*i* phenomenon was discovered and how the underlying mechanism has been elucidated. We also describe and discuss how RNA*i* experiments can be performed and how RNA*i* can be used for genetic studies.

Key words: *C. elegans*, RNA interference

Reverse genetics using RNA interference (RNA*i*) has become a major trend in biological research after its first application for the nematode Caenorhabditis elegans (*C. elegans*) (Fire et al., 1998). RNA*i* refers to silencing genes by the use of double-stranded RNA (dsRNA) at transcriptional and post-transcriptional levels in a sequence-specific manner.

Discovery of RNA interference in *C. elegans* and mammals

There were several reports that an introduced transgene for overexpression silenced itself as well as the endogenous gene(s) with high sequence similarity in plants and fungus (Jorgensen, 1990; Romano and Macino, 1992; Vaucheret et al., 1998). This transgene silencing is defined as occurring at the post-transcriptional level when RNA does not accumulate even though transcription occurs. This phenomenon was named post-transcriptional gene silencing (PTGS) while its mechanism was unclear. Guo and Kemphues reported that injection of antisense RNA into the *C. elegans* germ line results in downregulation of gene expression (Guo and Kemphues, 1995). Interestingly, injection of sense RNA also downregulated gene expression as efficient as antisense RNA (Guo and Kemphues, 1995). Andy Fire, Craig Mello and their colleagues had studied on this phenomenon, and found that delivering dsRNA into worms downregulated gene expression dramatically (Fire et al., 1998). They found that it was different from antisense gene silencing, and named it RNA interference (RNA*i*). This discovery led to the possible explanation for the mechanism of PTGS: introduction of transgenes in the genome may generate dsRNA by cryptic promoters and/or inverted repeats (Montgomery and Fire, 1998).

In the nematode *C. elegans*, introduction of dsRNA covering full-length genes efficiently and specifically downregulated target gene expression. However, introduction of long dsRNA into vertebrate organisms met an obstacle. In mammalian cells, detection of dsRNA longer than 30 nucleotides triggers type-1 interferon response in neighbor cells, and leads to global shutdown of translation resulting in cell lethality (Gil and Esteban, 2000; Kaufman, 1999). This problem was solved by Elbashir et al., when they introduced chemically synthesized 23-nucleotide dsRNAs into mammalian cells, which led to sequence-specific degradation of target mRNA without activating non-specific lethality (Elbashir et al., 2001). This finding have led to the widespread use of RNA*i* technique for reverse genetic approach in various organisms from worm to humans (Hannon, 2002).

Mechanisms of RNA interference

The works for revealing the mechanism responsible for RNA*i* have been performed by numerous researchers with

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various organisms. Biochemical studies indicate that RNA*i* is mediated by small-interfering RNAs (siRNA), which are cleaved from long dsRNAs by RNase III type endonuclease called "dicer". Dicer was first identified from the fruit fly (*Drosophila Melanogaster*) extract, and its putative homologs have been identified in fungi, *C. elegans*, plants, and mammals (Bernstein et al., 2001). The siRNAs processed by dicer are about 21-25 nucleotides in length, and are incorporated into a large nuclease complex, the RNA*i* inducing silencing complex (RISC). RISC performs endonucleoytic cleavage of mRNA targeted by siRNAs, resulting in further degradation of target mRNA molecules by the mRNA surveillance mechanism (Zamore et al., 2000).

Interestingly, Tijsterman et al. reported that only antisense siRNAs are accumulated in vivo, dependent on the presence of target mRNA (Tijsterman et al., 2002). This finding raised the possibility of a mechanism for amplification of RNA*i* silencing signal. RNA-dependent-RNA polymerases (RdRPs) have been found to be essential in RNA*i* mechanism. Indeed, primary siRNAs cleaved from introduced dsRNA acts as a primer for RdRP to generate secondary dsRNAs using cleaved mRNA as a template (Sijen et al., 2001; Smardon et al., 2000). Secondary dsRNAs produced by RdRP are likely to be processed by dicer and yield more secondary siRNAs. Secondary rather than primary siRNAs seem to play a major role in gene silencing mechanism during RNA*i* (Aoki et al., 2007; Pak and Fire, 2007). Most gene silencing events by RNA*i* in *C. elegans* seem to occur at the post-transcriptional level. There are a few reports concerning RNA*i*-mediated gene silencing at the transcriptional level in yeast (Noma et al., 2004) and *C. elegans* (Grishok et al., 2005; Robert et al., 2005), which is named as RNA*i*-induced Transcriptional Gene Silencing (RNA*i*-TGS) to be distinguished from posttranscriptional control in most RNA*i* cases. According to these reports, RNA*i* pathway downregulates transcription by modifying histones and producing heterochromatin structures.

RNA*i* techniques and their applications

While some scientist had been making efforts to uncover its mechanism, others started to use RNA*i* as a tool for genetic studies to knock down genes of their interest. Micro-injection is the first method used to deliver dsRNA into organisms (Fire et al., 1998). Injecting dsRNA that had been synthesized in vitro generated knock-down phenotypes from thousands of genes in *C. elegans* and this method is used for delivering dsRNA into eggs of fly (Kennerdell and Carthew, 1998), frog (Nakano et al., 2000), and mice (Wianny and Zernicka-Goetz, 2000). *C. elegans* has an unusual ability to transport dsRNA across cell boundaries, and researchers found alternative methods for RNA*i*, including soaking the worms in a dsRNA solution (Tabara et al., 1998) and feeding the worms dsRNA-expressing bacteria (Timmons and Fire, 1998). These techniques of RNA*i*, coupled with the availability of the complete genomic sequence of *C. elegans* (The *C. elegans* Sequencing Consortium, 1998), has made possible the rapid study of gene function, both on a single gene and at the genomewide scale. The following are more detailed descriptions of the RNA*i* methods mentioned above.

Injection RNA*i*

Remarkably, RNA*i* is heritable in *C. elegans* and this phenomenon had not been observed before (Fire et al., 1998; Grishok et al., 2000). Early RNA*i* experiments in *C.elegans* were performed by injecting dsRNA into the germline of hermaprodites, similarly to the DNA transformation method. However, injection of dsRNA into body cavity and intestine also reduced gene expression with great efficiency. Spreading of the injected dsRNA into most cells of the worm, including the germline, allows the inheritance of the injected material and the establishment of the RNA*i* effect in the progeny of the injected worms. Thus, injection RNA*i* is performed by injecting dsRNA produced *in vitro* into young adult hermaphrotides and examine the phenotypes in the next generations (Fig. 1A). RNA*i* by injection gives rise to very reliable gene inhibition from worm to worm. However, injection RNA*i* requires relatively expensive equipments and is labor intensive. This method is not preferred for large scale RNA*i* experiments.

Soaking RNA*i*

Tabara et al. showed that simply soaking worms into the solution containing dsRNA was also effective in producing knock-down phenotypes (Tabara et al., 1998). The procedure of soaking RNA*i* is quite simple: worms are soaked in a high concentration dsRNA solution produced by in vitro transcription and then their progeny scored for phenotypes (Fig. 1B). Soaking RNA*i* is useful for treating a moderately large number of animals (e.g., tens to hundreds). Worms of any stage can be soaked and larger amount of dsRNA is required for the soaking RNA*i* method than for the injection method. If automation is feasible, then soaking RNA*i* procedure can be a method of choice for performing genomewide RNA*i* experiments.

Feeding RNA*i*

Timmons and Fire developed a technique for feeding worms dsRNA-expressing bacteria (Timmons and Fire, 1998). This was an exciting and insightful finding, because the researchers had been feeding the nematodes *E. coli* as

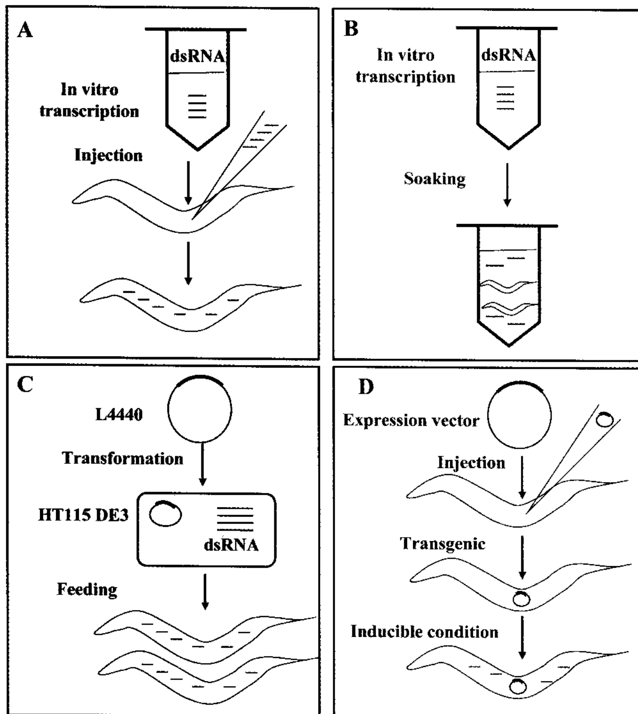

Fig. 1. Four typical methods of RNA*i*. (A) The classical injection RNA*i*. Unlike DNA injection, dsRNA can be injected anywhere inside the body. The progeny form the injected animals are analyzed for their phenotypes. (B) Soaking RNA*i*. Animals are soaked with in vitro transcribed dsRNA. (C) Feeding RNA*i*. Animals are fed with bacteria that are engineered to produce specific dsRNA. (D) Transgenic RNA*i*. A DNA construct that can produce dsRNA in specific conditions is introduced into the nematode. RNA*i* can be induced by certain conditions such as heat shock and hypoxia (see the text for more detail).

food since *C. elegans* was adopted as a model organism in the lab and *E. coli* had long been the organism of choice for overexpressing genes of other species; they had transformed the food to the dsRNA delivery vehicle. The procedure is now simple: one can feed the worms with *E. coli* that had been engineered to produce dsRNA of interest (Fig. 1C). Feeding RNA*i* is the least laborious and most inexpensive method but produces slightly more variable results than soaking or injection RNA*i*. Worms of any stage can be subjected to RNA*i* by feeding. Different from other dsRNA delivery methods, L1s can be used instead of L4s in the feeding RNA*i* procedure. An advantage of using L1s over using adults is that some phenotypes can be scored in the fed worms instead of their progeny, allowing an easily scored synchronized population to be used. However for some genes, inherited maternal product will be sufficient for gene activity, preventing induction of a phenotype in the fed worms. Also, since some genes are needed at several stages in development, different phenotypes can be seen when using L1s compared to L4s. For example, RNA*i* of some genes induces sterility of the fed L1s whereas L4 feeding induces embryonic lethality of the progeny. This will preclude scoring of progeny for these genes if L1s are fed. However, using L1s is beneficial if the assay is for any form of lethality such as sterility, larval lethality, or embryonic lethality. Feeding RNA*i* can be used to treat a large number of animals at once or for high throughput screening, both on agar plates and in liquid culture. It is fortunate that a research group at MRC cloned about 18,000 genes covering over 85% of the *C. elegans* genome into a dsRNA producing vector, and generated a feeding RNA*i* library consisting of bacterial strains each capable of producing distinct dsRNA. This feeding RNA*i* library is now commercially available. Production of genome-wide collections of feeding RNA*i* constructs and bacteria made possible the genomewide RNA*i*-based screening in *C. elegans* (Ashrafi et al., 2003; Fraser et al., 2000; Kamath and Ahringer, 2003).

Transgenic RNA*i*

Another alternative method for dsRNA delivery is the use of transgenes to make dsRNA *in vivo*. Tavernarakis et al. succeeded to establish transgene worms that are subject to inducible RNA*i* by introducing transgenes designed for producing dsRNA of interest under the control of a heat shock promoter (Tavernarakis et al., 2000). RNA*i* can be induced simply by putting the worms to a higher temperature (30°C) at the desired time point (Figure 1D). This approach has been used successfully in various organisms, including protozoan (Bastin et al., 2000; Ngo et al., 1998), plants (Chuang and Meyerowitz, 2000; Waterhouse et al., 1998) and fly (Kennerdell and Carthew, 2000). The advantage of the transgenic RNA*i* is that the transgenic lines can be maintained over multiple generations and so are the RNA effects, whereas other methods result in transient and reversible knock-downs. Furthermore, the stage of dsRNA expression can be regulated due to the heat shock inducible promoter, which is not available in other methods. However, there are limitations to this method. For example, it has been observed that dsRNA expressed form a transgene in one tissue is often not efficient in gene silencing in other tissues, while exogenous dsRNAs spread very efficiently. This is because exogenously provided dsRNA is packaged in the endocytic vesicles while endogenous dsRNA is not (Timmons et al., 2003). Another potential problem is that high temperature might cause deleterious effects on the transgenic worms, which may make it difficult to analyze the phenotypes. One way to avoid this problem, or a compensatory method, is to use a *hsp-16* promoter and to induce RNA*i* by hypoxic condition (Hong et al., 2004). The procedure is as simple as heat shock: a large number of worms are put in an eppendorf tube in M9 buffer with the lid closed. As the animals consume oxygen, the environment becomes hypoxic, and RNA*i* turns on.

Table 1. A list of genes on chromosome I that show different phenotypes in the mutations and different RNA*i* experiments

Gene name	ORF	Mutant phenotype	RNA*i* phenotype in N2 (Kamath and Ahringer, 2003)	RNA*i* phenotype in the *rrf-3* mutant strain (Simmer et al., 2003)
aph-2	ZC434.6	Emb	Normal	Normal
apr-1	K04G2.8	Emb	Unc, Bmd, Lvl	Emb
cdc-25	K06A5.7	Emb	Normal	Emb
ceh-6	K02B12.1	Emb, Bmd	Unc, Mlt	Clr, Lvl, Unc
cye-1	C37A2.4	Emb/Ste, Clr	Emb (10%), Clr	Emb
gpc-2	F08B6.2	Emb	Normal	Emb (40%)
hmr-1	W02B9.1	Emb	Emb (10%), Unc, Bmd, Dpy	Emb
mom-4	F52F12.3	Emb	Normal	Normal
mom-5	T23D8.1	Emb	Unc, Bmd	Emb (50%)
spe-11	F48C1.7	Emb	Normal	Normal
sup-17	DY3.7	Emb	Normal	Normal

Using RNA*i* resistant and sensitive strains

The RNA*i* process in *C. elegans* is modulated by several genes limiting its efficiency. Therefore, strains containing mutations in such genes show better RNA*i* efficiency. The *rrf-3* mutation was the first to be demonstrated to have enhanced RNA*i* responses (Simmer et al., 2002). *rrf-3* encodes a putative RdRP that is thought to compete with other RdRPs, such as *rrf-1* and *ego-1*, the components of the RNA*i* mechanism and plays a role in an RNA*i*-opposing pathway differently from other RdRPs. A genome-wide RNA*i* screens were also performed with a *rrf-3* mutant strain by Simmer et al., and more phenotypes associated with gene knock-down were reported (Simmer et al., 2003).

Genetic screens for mutants with enhanced RNA*i* sensitivity (*eri*) were carried out by Kennedy et al., and an RNA*i* sensitive mutant, *eri-1,* has been isolated (Kennedy et al., 2004). *eri-1* encodes an RNase that contains a SAP/SAF box domain and a DEDDh-like 3'-5' exonuclease domain. ERI-1 degrades siRNAs, but not single strand RNA, in vitro, therefore antisense strands of siRNAs are accumulated in the *eri-1* mutants, making them more sensitive to RNA*i*. *eri-1* is expressed preferentially in the neurons and the somatic gonad, which may explain why the nervous system is resistant to RNA*i* in *C. elegans*. The *eri-1* mutant strain is therefore a favored strain for RNA*i* experiments for genes expressed in the nervous system.

Recently, another class of *eri* mutants was identified by Wang et al., that is, the retinoblastoma (RB) pathway mutants (Wang et al., 2005) including *lin-15b* mutant. This genes of this class are components of histone deacetylase complex (HDAC) and are involved in the chromatin repressive pathway through histone modification. This pathway functions independently of *rrf-3* or *eri-1* and is directly involved in the germline specific gene repression in the somatic tissue of *C. elegans*. The *eri-1;lin-15b* double mutant strain has become one of most widely used supersensitive strains in RNA*i* application.

Wild type worms may give good results in analyzing most phenotypes and assays by RNA*i* experiments, However, phenotypes are often stronger in RNA*i* supersensitive strains, so it is a good idea to try these as well. *rrf-3* and *eri-1* mutants have smaller brood sizes than the wild type strain and are sterile at 25°C, so using these strains in RNA*i* need more attention than using wild type animals .

Promises and limitations

RNA*i* has become one of the most widely used tools to study loss-of-function phenotypes of genes of interest in *C. elegans*. Of four methods described above, feeding RNA*i* is less labor-intensive and less expensive than any other method. Development of feeding RNA*i* method enabled researchers to perform genome-wide RNA*i* screening. With this, many researchers could find genes involved in lifespan (Dillin et al., 2002; Lee et al., 2003), synaptic function (Gottschalk et al., 2005), fat regulation (Ashrafi et al., 2003), and development (Zipperlen et al., 2001) in *C. elegans*. However, there are several important factors that must be considered when interpreting RNA*i* results. First, knock-down of gene activities often resulted in different phenotypes depending on RNA*i* methods (Kamath et al., 2001). This implies that feeding RNA*i* and injection RNA*i* may downregulate gene expression in different manners, which remains unclear. Second, knock-down phenotypes by RNA*i* are often different from that of a genetic mutant phenotype. For example, Table 1 represents genes in Chromosome I of *C. elegans* that showed embryonic lethal phenotype in their mutants but resulted in normal or mild phenotypes when they were knocked down by RNA*i*. This problem seems to be partially overcome by using the *rrf-3*

mutant strain, an RNA*i* supersensitive strain. But knocking down some essential genes such as *aph-2*, *mom-4*, *spe-11*, and *sup-17* did not cause any phenotype even in the *rrf-3* mutant background. This result could represent the different sensitivities of each gene to RNA*i*. It is possible that genes that are highly expressed in some tissues can be difficult to silence, or that genes encoding proteins with long half lives may have little chance to show its knock down phenotypes by RNA*i* since mRNA degradation does not reduce the quantity of the protein. Despite all these limitations, RNA*i* still is a favored way of gene silencing for genes with no mutations identified, because gene targeting by homologous recombination is not available in *C. elegans* and it will take a long time before researchers will get genetic mutations in the gene of interest. Furthermore, RNA*i* is a way of choice for genome-wide screening such as searching for interacting genes or for chemical target identification.

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