Research in Biotechnology, 2(3): 75-80, 2011 **ISSN: 2229-791X**
www.researchinbiotechnology.com

Review Article

RNAi: An innate gene knockdown mechanism

Thangavelu Srinivasan and Dorairaj Sudarsanam

Department of Advanced Zoology and Biotechnology,
School of Genomics and Proteomics, Loyola College, Chennai, Tamil Nadu, India

RNA interference (RNAi) is an evolutionary conserved mechanism in all eukaryotic cells whose role is to down-regulate the gene expression in the nucleus known as Transcriptional Gene silencing (TGS) and in the cytoplasm known as Post Transcriptional Gene Silencing (PTGS). It can occur at different stages of cell cycles during cell proliferation, developmental stage and cell death. An artificially induced Double Stranded RNA (dsRNA) in a eukaryotic organism like *C. elegans* can also cause RNAi by sequence specific gene silencing. The Double Stranded RNA (dsRNA) derived small RNAs (19-28nt in length) along with Argonaute protein, Dicer (RNase III like enzyme) and other cofactors act as molecular scissors which degrade the homologous mRNA. This effector-protein complex is termed as RNA-induced silencing complex (RISC) which searches for the homologous transcripts of mRNA to degrade them. The Small RNA which might be either a Small Interference RNA (siRNA) or a microRNA (miRNA) along with the effector complex directs the endonuclease cleavage to occur on the target mRNA thereby preventing the expression of transcripts. This overall process is termed as RNAi (RNA interference).

Keywords: RNA interference (RNAi); Gene Silencing; Methylation; Transcriptional Gene Silencing (TGS); Post Transcriptional Gene Silencing (PTGS)

Discovery of RNAi

Before the discovery of RNAi, a homology dependent post transcriptional gene silencing was first observed in1990 in petunia, a widely-cultivated genus of flowering plants of South American origin. When the Chalcone synthase gene was introduced in petunia, the expression of both the Transgene and the homologous gene were suppressed (Marjori et al., 2001; Wang et al., 2001). Initially, this phenomenon was referred to as Co-suppression. A similar mechanism was observed in Neurospora crassa, but it was termed as quelling. This phenomenon suggests that an increased copy of expressed gene leads to silencing by dsRNA either by mRNA degradation (PTGS) or by DNA methylation (TGS) (Vaucheret et al., 2001). The transgenes involved in gene silencing mechanism were first observed in certain plant species. Subsequently, many eukaryotes such as nematodes, fungi, insects and protozoans were also found to exhibit such gene silencing (Fire, 1999).

It was Andrew Fire and Craig Mello who first reported the dsRNA mediated gene silencing in the worm, *Caenorhabditis elegans*. Mello coined the term 'RNA interference'. They injected the sense, the anti-sense Single Stranded RNAs and the dsRNA into *C. elegans*. By injecting the sense and the antisense RNAs, they found

no obvious change in the worm. But some phenotypic effects were observed when dsRNA was injected. From their experiments, Fire and Mello concluded that gene silencing was triggered efficiently by injecting dsRNA, but only weakly by injecting the sense or the anti-sense Single Stranded RNAs (ssRNA). They also concluded that the injected dsRNA will affect only its homologous mRNA while the other mRNA will be unaffected. They observed that the injected dsRNA will bind only with its complimentary mature mRNA while it will not affect the introns or the promoter sequences. The target mRNA disappeared suggesting that it was degraded and only a few dsRNA molecules per cell were sufficient to accomplish full silencing. This indicated that the dsRNA was amplified and it could spread between tissues even to the progeny (The Nobel Prize in Physiology or Medicine, 2006).

Classes of small RNA

Small non-coding RNAs are 19-28ntd long that can be found in diverse organisms. On the basis of its origin and biogenesis it has been broadly classified into two types, namely, siRNA and miRNA. The siRNA are 22nt long, which are derived from a long double stranded RNA whereas miRNAs are single stranded RNA (ssRNA) of 19-25nt length, which are derived from the hairpin shaped dsRNA. The reaction is catalyzed by the Dicer enzyme. TasiRNA is a 21nt long sequence, which is generated from an intronic region of the gene in Arabidopsis (plant).

Table: 1 Classes and sub classes of small RNAs and functions

S.No	Classes	Sub classes	Organism	Function
1	miRNA (19-25nt)	Not yet identified	*C. elegans, D. melanogaster*	Diverse functions, degradation of mRNA repression of translation
2	siRNA (22nt)	TasiRNA (21nt)	*Arabidopsis*	Regulatory endogenous cellular function.
		RasiRNA (24-26nt)	*T. brucei*	Heterochromatin in repetitive element of the sense, anti-sense orientation of genome.
		ScnRNA (28nt)	*Tetrahymena thermophila*	Methylation of H3K9
3	tncRNA (~22nt)	Not yet identified	*C. elegans*	Unknown
4	smRNA (~20nt)	Not yet identified	Hippocampus	Neural differentiation and its function as transcriptional modulator.
5	piRNA (26-31nt)	Not yet identified	Mouse testes	Spermatogenesis.

TasiRNA: Trans-acting siRNA, RasiRNA: Repeat assosciated siRNA, ScnRNA: Small scan RNA, tncRNA: Tiny non-coding RNA, smRNA: Small modulatory RNA, piRNA: Piwi-interacting RNA.

Recently the TasiRNA has been identified in nematode worms also. RasiRNAs are found in plants, *Trypanosoma brucei, Drosophila melanogater* and *Saccharomyces cerevisiae*. The function of rasiRNA is to form heterochromatin by arrangement of repetitive elements of the sense and anti-sense strands in the genome. During conjugation of ciliated protozoans (*Tetrahymena thermophila*) 30ntd long small

scan RNA (scnRNA) has been identified which originates as an internally eliminated segmented sequence (ranges from 0.5kb to 20kb) (Kim, 2005; Kim, 2006). Tiny non-coding RNA (tncRNA) and small modulatory RNA (smRNA) have also been identified in the class of small RNA. These were identified through various cloning experiments. The tncRNAs are shorter than the miRNA that is found in C.elegans. The smRNA was identified from the adult hippocampal neural stem cells. The smRNA is expressed at the early stages of neural differentiation and it functions as a transcriptional modulator. However, its biogenesis is still unclear. The piwi-interacting RNA (piRNA) was identified in mouse testes and is found abundant in chromosomes 2, 4, 5 and 17 but lesser in the intergenic regions. The piRNA sequences are frequently found in retrotransposons, majority of which are clustered in short genomic loci (below 1kb to above 100kb). Its function is believed to be in spermatogenesis by possibly regulating meiosis and/or suppressing tetrotransposons (Kim, 2006).

Mechanism of RNAi

Dicer is an enzyme highly conserved in all eukaryotic organisms. Human dicer homologues are multi domain proteins of ~200kDa, 1,922 amino acids in length. It consists of two RNase III domains (RIIIDa and RIIIDb) and double stranded RNA binding domain (dsRBD). In addition, it has a long N terminal segment that consists of a PAZ domain, DEAD-BOX RNA HELICASE DOMAIN and DUF283 domain. The PAZ domain binds to the 3′ ends of small RNAs and the DEAD-box RNA helicase domain hydrolyzes ATP resulting in unwinding of an RNA duplex. In total, there is one Ago family protein in S.pombe, more than 20 in C.elegans, 5 in Drosophila, 8 in human and about 10 in Arabidopsis. Ago protein is about ~100kDa in size that contains two domains namely PAZ and PIWI [8]. PAZ domain is ~130 amino acids which located at the center of the protein. It interacts with the 3′ overhang of dsRNA. The PIWI domain has ~300 amino acids and is structurally homologous to RNase H.

Human drosha enzyme is classified under RNase III family protein. It is a large protein of ~160kDa containing 1,374 amino acids, two RIIIDs domains, dsRBD, proline rich region (P-rich) , arginine and serine rich residues (RS-rich). Drosha binds with its cofactor, the DGCR8 protein for processing of pri-miRNA those results in the formation of a mature miRNA. The human homologues DGCR8 (DiGeorge syndrome critical region gene8) protein is also known as Pasha in D.melanogaster and C.elegans. It is a ~120kDa protein of 773 amino acids that contains two dsRBD (dsRNA binding protein). The biochemical pathway of these proteins is still unclear (Kim, 2005a).

In mammals and plants, formation of heterochromatin (transcriptional inactive form) occurs by hypermethylation of cytosine in DNA and methlylation in histone H3 of lysine K9 (H3K9) that is directed by small non-coding RNA. This methylation process is carried out by DNA methyltrasferase (DNMT) and Histone methyltrasferase enzyme (HMT) (Tariq et al., 2004). The functions of RNA dependent DNA and/or histone methylation are to control gene expression and to act as an epigenetic marker. RNA directed DNA methylation (RdDM) process was first discovered in viral infected plants (Tobacco virus, cytoplasmic RNA viruses). Plants produce 24nt siRNA to form RdDM complex and methylation of cytosine residues at symmetrical and asymmetrical sites (CpGp and CpHpHpG H= A, C, or T) that depend upon HEN1 protein and domain rearrangement methyltransferase2

(DRD2) (Wassenegger et al., 2005). In nucleus, dsRNA triggered gene silencing is initiated by an aberrant transgene, inverted repeat sequence of dsRNA, or secondary siRNA produced by RNA dependent RNA polymerase (RdRP). Normally, mammals lack RdRP based production of dsRNA (Tariq et al., 2004; Voinnet, 2008). RNA dependent DNA polymerase will synthesis secondary siRNA from an aberrant primary siRNA which acts as a template. This Secondary siRNA will be processed by Dicer to form secondary 24nt siRNA which will be linked to sequence specific cytosine methylation that potentially triggers transcriptional gene silencing (TGS) (Voinnet, 2008).

Table: 2 Key proteins involved in RNA silencing pathway

S. No	Key Proteins	*Schizosaccharo -myces pombe*	*Arabidopsis thaliana*	*Caenorhabditis elegans*	*Drosophila melanogaster*	Human
1	Class 3 RNAase III enzymes	Dicer 1	Dicer 1,2,3 and 4	Dicer 1	Dicer 1 and 2	Dicer 1
2	Class 2 RNAase III enzymes	Not yet identified	Not yet identified	Not yet identified	Drosha	Drosha
3	Argonaute Protein	Ago 1	Ago 1 to 10	Ago 1 and 2	Ago 1 and 2	Ago 1 to 4
4	RdRP	RdRP 1	RdRP1 to 6	RdRP 1 to 3	Not yet identified	Not yet identified

RNAi pathways are slightly varied in eukaryotes due to miRNA, siRNA biogenesis and exist of key proteins and enzymes which involved in transcriptional and post transcriptional pathways. RdRP (RNA dependent RNA polymerase), Ago (Argonaute protein).

The miRNA and siRNA posttranscriptional gene silencing (PTGS) processes are slightly different from one another on the basis of biogenesis and assembly of RISC complex. These differences have been identified in some eukaryotes. For example, humans and *Caenorhabditis elegans* have only one dicer enzyme while *Drosophila* has two Dicer enzymes (Dicer-1, and Dicer-2), in which short interference RNA production is associated with Dicer-2, but not Dicer-1. In *Drosophila* at embryo stage, maturation of miRNAs and siRNAs are required respectively by Ago1 and Ago2 for the assembly of the RNA-induced Silencing complex (RICS). *Arabidopsis thaliana* contains four Dicer enzymes, in which Dicer-2, 3 and Dicer-4 are involved in the production of different sizes of siRNAs (21, 22, 24 nucleotide in length), wheres Dicer-1 produces variable sizes of miRNA (Katsutomo et al 2004).

MicroRNAs belong to family of large endogenous RNA. A short single strandard miRNAs are formed in two phases. In nucleus, miRNA transcripts (~60-70nt) are synthesized by RNA polymerase II, which is recognized by Drosha-DGCR8 complex (microprocessor). The primary RNA (pri-miRNA) contains a hairpin shaped structure that indicates the stem loop, a cap structure and a poly A tail structure (Richard et al 2005). Exportin5, a nuclear membrane protein, functions in the export of priRNA into the cytoplasm (Hutvagner, 2005). The Dicer (RNase III) cleaves the pri-miRNA to form short 22nt miRNA with 2nt 3' overhang. The mature

miRNA is recognized by Argonaute protein, Dicer and it finally forms RNA induced silencing complex (RISC) which results from the cleavage of a complementary mRNA known as PTGS (Kim, 2003). In plants, miRNA target interaction is more complementary and involves the coding regions. But animal miRNA targets are interrupted by gaps, mismatches and 3'UTR of mRNA. Some miRNA are responsible for translational repression (David et al 2005).

Long dsRNA can be synthesized from various sources such as transgenes, transposons and promoters of bidirectional transcription (Christophe et al., 2002). Double standard RNA can be formed by pairing of the sense RNAs and anti-sense RNAs that result from aberrant transcription of their genes (Meins, 2000). RNA-dependent RNA polymerase (RdRP) possibly involves in production of dsRNA, which triggers PTGS. RdRP is present in a large variety of organisms like plants, worms, fungi and fission yeast. This enzyme helps in the conversion of primary and aberrant transcripts into dsRNA. Short interfering RNAs are generated by Dicer from exogenous or endogenous long dsRNA and the resulting short siRNA along with a ribonucleoprotein (argonaute protein), DICER and other co-factors form the RICS complex. The initial RICS containing siRNA is inactive and transforms into the active form by loss of one of the strands (Annaleen et al., 2005). Thus the final RICS complex which contains a single-stranded RNA molecule causes endonucleolytic cleavage of the target mRNA. In the siRNA the 3' overhang is mainly involved in the endonucleolytic cleavage of the target mRNA (Philipp et al., 2006). The 3' overhangs are more efficient in degradation of target mRNA compared to the blunt ended siRNA molecules (Siomi et al 2009).

Conclusion

The various classes of RNase enzymes cleave the ssRNA or dsRNA and act as a defense mechanism against RNA viruses. They also regulate the gene expression. The key proteins which are involved in RNAi mechanism such as the Dicer, Argonaute protein, Drosha, Pasha all fall under the RNase family which are involved in the RNAi pathway. This mechanism is a very complex process in plants when compared to other eukaryotes since it has more isoenzymes (argonaute and dicer enzymes). The classes of small RNA and sub classes are found in many eukaryotes, but their biogenesis and pathways are quite different from one another. For example, in plants the major role of RNAi is to act as an anti-viral agent while in mammals they are found in large numbers in the germ cells. The RNAi is believed to control the gene expression at all stages i.e., transcriptional, post transcriptional, and translational level. Its function is more advanced than the ribozyme and the anti-sense RNA. These results suggest that, the eukaryotic genome has gene down regulatory mechanisms during cell proliferation and developmental stages. Recent studies mention that plants and human cells contain a large numbers of small RNAs with the potential to down regulate the gene expression. Still this mechanism is unclear in many aspects like the number and type of small RNAs that are present, their unique biological functions, generation and pathway regulations that exist in the different eukaryotic organisms. Artificially Induced RNAi based down regulation of gene expression can be used to disrupt the gene function and study the gene which serves a potential tool in the upcoming field of reverse genetics.

References

Annaleen V, et al., 2005. The contributions of dsRNA structure to dicer specificity and efficiency. Cold Spring Harbor Laboratory Press. 11: 674-682.

Christophe B, et al., 2002. A branched pathway for transgene-induced RNA silencing in plants. Current Biology., 12: 684-688.

David T.H, et al., 2005. MicroRNAs control translation initiation by inhibiting eukaryotic initiation factor 4E/cap and poly (A) tail function. PNAS., 102: 16961-16966.

Fire A. 1999. RNA-triggered gene silencing, Bayesian statistical methods., 15: 358-363.

Hutvagner G. 2005. Small RNA asymmetry in RNAi: Function in RISC assembly and gene regulation. Federation of European Biochemical Societies., 579: 5850-5857.

Katsutomo O, et al., 2004. Distinct roles for argonaute proteins in small RNA-directed RNA cleavage pathways. Genes and Development.,18:1655-1666.

Kim V. N, 2003. RNA interference in functional genomics and medicine. J Korean Med Sci., 18:309-318.

Kim V. N, 2005. Small RNAs: Classification, biogenesis, and function. Mol. Cells., 19: 1-15.

Kim V. N, 2005a. MicroRNA Biogenesis Coordinated cropping and dicing. Nature., 6: 376-385.

Kim V. N, 2006. Small RNAs just got bigger, Piwi-interacting RNAs (piRNAs) in mammalian testes, Gene and Development., 20:1993-1997.

Marjori A M, et al. 2001. RNA-based silencing strategies in plants. Current Opinion in Genetics & Development., 11: 221-227.

Meins F. Jr, 2000. RNA degradation and models for post-transcriptional gene silencing. Plant Molecular Biology., 43: 261-273.

Philipp J.F, et al., 2006. Cleavage of the si RNA passenger strand during RISC assembly in human cells. EMPO Reports., 7: 314-320.

Richard I.G, et al., 2005. Human RISC couples microRNA biogenesis and posttranscriptional gene silencing. Cell, 8: 631-640.

Siomi H and Mikiko C. Siomi, 2009. On the road to reading the RNA – interference code. Nature., 457: 396-494.

Tariq M and Paszkowski J, 2004. DNA and histone methylation in plants. Trends in Genetics., 20: 244-251.

The Nobel Prize in Physiology or Medicine. 2006; 1-10.

Vaucheret H and Fagard H, 2001. Transcriptional gene silencing in plants: targets, inducers and regulators. Trends in Genetics., 17:29-35.

Voinnet O, 2008. Use, tolerance and avoidance of amplified RNA silencing by plants. Trends in Plant Science., 13: 317-328.

Wang M B and Waterhouse P M, 2001. Application of gene silencing in plants. Current opinion in Plant Biology., 5:146-150.

Wassenegger M, 2005. The role of the RNAi machinery in Herterochromatin formation, 122: 13-16.