



RNA INTERFERENCE: TRENDS IN SMALL REGULATORY RNAS AND GENE SILENCING

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ABSTRACT

RNAi pathway is a biologically conserved phenomenon which regulates the expression of genes through transcriptional or post-transcriptional gene silencing mechanism. The pathway involves the network of different proteins to degrade the target mRNA and triggered by small regulatory RNAs. These small regulatory RNAs are generated via action of dicer enzyme on long double-stranded RNA or hairpin RNA. Herein, we have explained the significant classes of small RNAs, their functions, and mechanism of RNAi as well. Along with, we have highlighted the recent findings in RNAi, glimpse of available small RNAs repositories, and softwares/tools for their efficient designing.

KEYWORDS: *RNAi, Small RNAs, Small RNA databases, Designing tools/softwares.*



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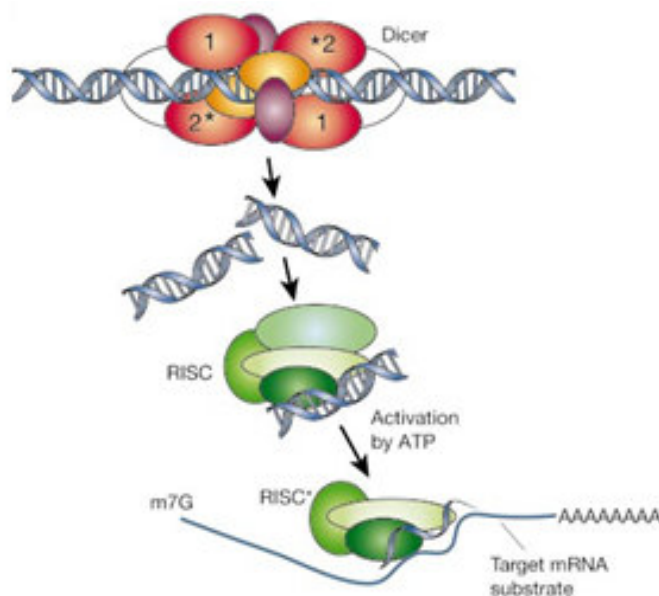
INTRODUCTION

RNA interference (RNAi) is a biologically conserved gene silencing pathway and has founded a new paradigm for getting insight of eukaryotic gene regulation¹. It has unveiled the novel host defenses against transposons and viruses. It has become a powerful knockdown tool for the functional annotation of genome and countenance highly specific and efficient gene silencing in mammalian systems, and other animals as well^{2,3}. In addition to determining the gene functions, it is holding a promise as a therapeutic strategy also. In 1990, the phenomenon was first reported by Jorgensen & Napoli and in 1992 by Macino & Romano in *Neurospora crassa*⁴. Guo and Kemphues reported that the process of gene silencing is heritable in *Caenorhabditis elegans*^{5,6}. The immense potential of RNAi in research and therapeutics has been valued in 2006 by awarding the Nobel Prize in Medicine to Andrew Fire (Stanford University School of Medicine) and Craig Mello (University of Massachusetts Medical School) for uncovering RNAi⁷. It is a highly sequence-specific phenomenon and a single difference in

nucleotides (nts) among the targets results in striking differences in gene silencing. Thus, this attribute of RNAi can be used to target the alleles with a single nucleotide mutation from their wild type⁸. This phenomenon has also been noticed in nematodes, protozoa, animals, viruses, insects, bacteria, plants and fungi⁹⁻¹¹. In plants, post-transcriptional gene silencing (PTGS) and in fungi, quelling is fundamentally related phenomenon as they are also triggered by double-stranded (ds) RNA.

MECHANISM OF RNAI

The RNAi machinery is consisting of three core components: Dicer enzyme, Argonaute protein family and RNA-dependent RNA polymerases (RDRP). To date, the proposed model to explain RNAi machinery is that the pathway is triggered by double-stranded (ds) small RNA (~21-26 nucleotide), which in turn binds to endogenous homologous messenger RNA (mRNA) and mediates its demolition^{12,13}, causing the silencing of gene of interest. RNAi pathway generation has briefly explained in figure 1.

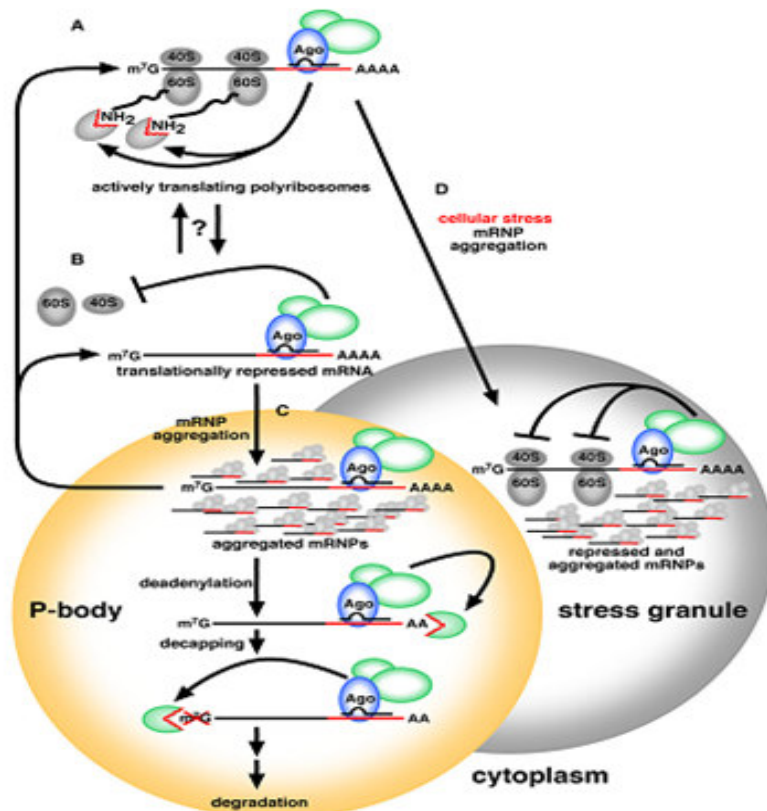


Enzyme dicer initiate the RNAi and processes long double-stranded RNA or hairpin RNA into ~22-nucleotide small double-stranded RNAs¹⁴. These RNAs are then incorporated into RNA Induced Silencing Complex (RISC) (green) and RISC then uses the unwound small RNAs as a guide to substrate selection¹⁵. Reproduced with the permission from⁸.

Figure 1
Diagram representing the Dicer binding and cleavage of dsRNA.

These small RNAs are generated by cleaving of dsRNA by endogenous enzyme Dicer. Dicer is having an RNase III-like activity. It acts in a complex with some other proteins such as Argonaute protein (Ago) family members, and supposed to work as a dimeric enzyme¹⁴⁻¹⁵. Subsequently, small RNAs associate to Ago proteins that together form a nuclease complex, termed as RNA-Induced Silencing Complex (RISC). Unwinding of small RNAs causes activation of RISC. As RISC becomes

activated, small RNAs hybridize the target mRNA through complementary base pairing. The mRNA is then cleaved, causing silencing of corresponding gene. Translational repression of mRNA by Ago proteins has described in figure 2. RNAi has also been generated with synthetic siRNAs¹⁶, hairpin RNAs¹⁷, plasmid vector-based RNAi techniques^{18,19}, and virus-based RNAi systems²⁰.



These proteins stifle translation either during polysome translation (A) or during initiation of translation (B). Aggregation of stified Ago containing mRNPs to P bodies degrade repressed mRNAs via deadenylation and decapping enzymes (C). During cellular stress polysome aggregate to form stress granules and Ago proteins stifle those mRNA translation which are inefficient for stress response (D). Reproduced with the permission from¹².

Figure 2
A model representing translational repression by Ago proteins.

The Argonaute protein family can be split into two subfamilies a) Piwi subfamily and b) Ago subfamily²¹⁻²³. Ago subfamily is cosmically expressed, whereas Piwi subfamily's expression is restricted to germ cells^{24,25}. Members of Piwi subfamily are characterized by amino-

terminal: Piwi-Argonaute-Zwille (PAZ) domain and carboxy-terminal: PIWI domains²⁶ as their structural features (figure 3). The molecular weight of Argonaute protein has been estimated about 100 kDa²⁷.

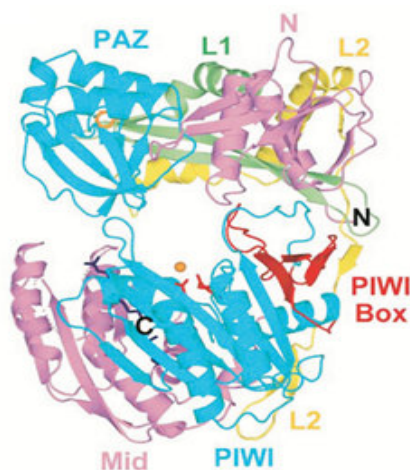


Figure 3
Structure of the Argonaute protein from Aquifex aeolicus representing that PAZ domain (blue) is linked to the amino-terminal domain (N, magenta) by linker 1 (L1, green), and connected with the MID domain (magenta) followed by the PIWI domain (blue) by linker 2 (L2, yellow) at the carboxy-terminal.
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SMALL RNAs

Small RNAs are known to be non-coding molecules. Their size varies from 20 to 40 nts. They play potential roles in repression of translation or RNA demolition, transcriptionally, by affecting chromatin structure or post-transcriptionally, by affecting translation or mRNA stability²⁸⁻³⁰. Thus they are a key player in RNA silencing. They are mobile molecules and guide the RISC to the target mRNA, resulting in gene degradation. They come in many pretexts based on their RNA precursor and biogenesis, such as: stRNAs, siRNAs, tncRNAs, miRNAs, qiRNAs, tRFs, miRNAs, piRNAs³¹⁻³⁴, scnRNAs and heterochromatic siRNAs³⁵. Besides

exogenous siRNAs, some endogenous siRNAs: rasiRNAs, tasiRNAs, casiRNAs, natsiRNAs have also been reported in plants and animals³⁶⁻⁴⁰. Among these some small RNAs are known to be used as RNAi reagent for cell-based High Throughput Screenings (HTSs). A comparative study of various small RNAs found in eukaryotes have been done in table I. Available useful databases of various classes of small RNAs and various tools & softwares to design them have been mentioned in table II and table III, respectively. Table IV is containing the web links of various companies and labs for designing of effective small RNAs.

Table 1
A comparative study of various small RNAs

Small RNAs	Full Name	length (Nt)	Organism	Precursor	Biogenesis	References
tasiRNAs	Trans-acting Acting siRNAs	~21	Plants	miRNA-cleaved TAS RNAs	DCL4	Rana 2007 ¹² , Fire 1998 ¹³
piRNAs	Piwi-interacting RNAs	~23-31	In animal's germ cells	Transposons and repetitive DNA elements	AGO family proteins: PIWIL1, PIWIL2, PIWIL3, PIWIL4, AGO3, AGO4, AGO6	Carmell ¹⁴ , 2002 ¹⁴ , Hammond 2000 ¹⁵ , Elbashir 2001 ¹⁶ , Paul 2002 ¹⁸
tncRNA	Tiny non-coding RNA	~20	—	—	Dicer	Ambros 2007 ³¹
casiRNA	cis-acting siRNAs	24	Plants	Transposons, repeats	DCL3	Hamilton 2002 ³⁸ , Xie 2004 ³⁹
rasiRNA	Repeat-associated siRNA	~24-26 (in plants) ~24-27 (in fruit flies)	Plants, fruit fly	long dsRNAs derived from repetitive sequences	Dicer	Hamilton 2002 ³⁸ , Xie 2004 ³⁹
natsiRNA	Natural antisense transcript-derived siRNAs	~21-24	Plants	Bidirectional transcripts induced by stress	DCL1, DCL2	Katiyar 2006 ⁴⁰
qiRNAs	QDE-2-interacting Small RNAs	~21	Described only in <i>N. crassa</i>	Aberrant RNAs (aRNAs)	QDE-1, QDE-3, Dicer	Lee 2009 ⁴¹ , Li 2010 ⁴²
tRFs	tRNA-derived RNA fragments	~20-22	<i>S. cerevisiae</i> , <i>M. oryzae</i> , <i>fumigatus</i> , Human	tRNA	Dicer, RNaseZ	Kawaji 2008 ⁴³ , Nunes 2011 ⁴⁴
siRNAs	Small-interfering RNAs	~21-24	Eukaryotes	dsRNA	Dicer, AGO	Nunes 2011 ⁴⁴
esRNAs	Endogenous short RNAs	~21	<i>M. circinelloides</i>	Exonic-siRNAs	RdRp1, Dicer-like2 (DCL2)	Nicolas 2010 ⁴⁵
miRNAs	MicroRNA	~21-25	Plants and Animals	Endogenous miRNA genes	DCL1, AGO	Winter 2009 ⁴⁶
scnRNAs	Small-scan RNAs	~28	<i>Tetrahymena thermophila</i>	long dsRNAs by	Dicer	Sui 2002 ¹⁹ , Liu 2004 ⁴⁷
21U-RNA piRNAs	—	~21	<i>Caenorhabditis elegans</i>	Individual transcription of each piRNA	Dicer-independent	Ruby 2006 ⁴⁸
26G RNA	—	~26	<i>Caenorhabditis elegans</i>	Enriched in sperm	RdRP	Ruby 2006 ⁴⁸ , Ambros 2007 ³¹
smRNA	Small modulatory RNA	~20	—	—	—	Kuwabara 2004 ⁴⁹

Table 2
Known small RNAs repositories

Repositories	Attributes	Source links
HIVsirDB a) siRNAmaps b) HIVsirblast c) siRNAalign	A manually curated and freely accessible database of 750 siRNAs which can inhibit HIV.	http://crdd.osdd.net/raghava/hivsir/
HuSida siRNAdb	An open access human siRNA database. The siRNA sequence database gives a view of siRNA experimental data.	http://www.human-siRNA-database.net http://siRNA.cgb.ki.se
GenomeRNAi COLT	Cell-based RNAi phenotypes database. A database of pooled shRNA with a web interface for shRNA querying and visualization.	http://rmai.dkfz.de http://colt.cabr.utoronto.ca/cancer
RNAiAtlas ASRP Rfam	A database of siRNA libraries of human genome. Arabidopsis Small RNA Project Database. An open access database of miRNA and RNA family.	http://rnaiatlas.ethz.ch/ http://asrp.cgrb.oregonstate.edu http://www.sanger.ac.uk/Software/Rfam/mirna/ or http://rfam.sanger.ac.uk/

Table 3
Useful web servers/ softwares for designing of effective shRNA/siRNA

Softwares/Web servers	Attributes	Source links
VIRsiRNAPred	A siRNA prediction web server against essential human virus genes, based on Support Vector Machine (SVM)	http://crdd.osdd.net/servers/virsimapred/ submit.php
siDirect	A web tool for designing efficient and target-specific siRNA for mammalian RNAi.	http://sirect2.rmai.jp/ .
desiRM	A web application to design highly effective complementary and mismatched siRNAs.	http://www.human-siRNA-database.net .
AsiDesigner	Software for designing siRNAs for silencing of multiple genes simultaneously along with the capacity to search for off-targets with FASTA and BLAST algorithms.	http://sysbio.kribb.re.kr/AsiDesigner/
DEQOR	A web tool to design siRNA based on state-of-the-art parameters scoring system.	http://cluster-1.mpi-cbg.de/Deqor/deqor.html
OligoWalk	A web application to design siRNA which utilizes hybridization thermodynamics to predict the free energy changes during the binding of oligonucleotides to a target RNA.	http://rna.umc.rochester.edu/servers/oligowalk
dsCheck	A web-based highly sensitive software for reckoning off-target effects for RNAi studies.	http://dsCheck.RNAi.jp/
E-RNAi	The E-RNAi, a web-based tool to design and evaluate RNAi reagents, siRNAs and esiRNAs as well.	http://e-rnai.dkfz.de/
SiDESIGN Center	An advanced web tool to design siRNA based on the likelihood of identifying functional siRNA.	http://www.dharmacon.com/designcenter/designcenterpage.aspx
Sfold (siRNA)	For rational siRNA designing.	http://sfold.wadsworth.org/cgi-bin/sirna.pl
siVirus	A web-based software for designing of highly effective, off-target minimized antiviral siRNAs and finding of highly conserved target sites for antiviral RNAi.	http://sivirus.rmai.jp/
TROD	T7 RNAi Oligo Designer (TROD) is a web tool to design oligodeoxynucleotide sequences for <i>in vitro</i> synthesis of siRNA duplexes.	http://www.cellbio.unige.ch/RNAi.html
passRNAMiner	A web-based server to predict small RNA phase clusters in plants.	http://bioinfo3.noble.org/pssRNAMiner/
psRNATarget	A server for analyzing small RNA targets in plants.	http://plantgrn.noble.org/psRNATarget/
siDRM	Disjunctive Rule Merging (DRM) algorithm based software for effective selection of siRNAs.	http://siRecords.umn.edu/siDRM/
siPRED	A support vector regression and neural networks based prediction server.	http://predictor.nchu.edu.tw/siPRED
DSIR	Web tool for siRNA (19 or 21 nt) and shRNA prediction against selected target gen.	http://biodev.extra.cea.fr/DSIR/ DSIR.html

Table 4
Web links of various companies and labs for effective designing of small RNAs.

Labs/Company	Web source
Ambion	www.ambion.com/techlib/misc/siRNA_finder.html
Hannon Lab	katahdin.cshl.org:9331/portal/scripts/main2.pl
Integrated DNA Technologies	http://scitools.idtdna.com/site/order/designtool/index/DSIRNA_CUSTOM
Invitrogen	http://rnaidesigner.thermofisher.com/rnaiexpress/
Qiagen	www1.qiagen.com/Products/GeneSilencing/CustomSiRna/SiRnaDesigner.aspx
Whitehead Institute	jura.wi.mit.edu/siRNAext

RNAi IN FUNGI

The fungi kingdom is estimated to have more than 1 million species. In fungi, RNAi pathway performs three major functions: heterochromatin formation, gene regulation and genomic defense. Studies of different fungal system have revealed the occurrence of diverse classes of small RNAs and their biogenesis pathways. Major RNAi studies have been done on *Schizosaccharomyces pombe*, *Neurospora crassa*, *Cryptococcus neoformans*, *Saccharomyces cerevisiae*, *Saccharomyces castellii*, *Ustilago maydis*, *Candida albicans*, *Aspergillus fumigates*, *Aspergillus nidulans*, *Aspergillus oryzae*, *Mucor circinelloides* and *Mucor grisea*⁵⁰. In filamentous fungi, posttranscriptional gene silencing exists, whereas heterochromatin formation at transcriptional level results in gene silencing in fission yeast⁵¹. As discussed above RNAi have three components (Dicer, Argonaute, RDRP) in its core, but gene silencing in *Aspergillus nidulans* is RDRP independent, and requires RsdA, a PAZ PIWI domain (PPD) protein⁵².

RNAi SCREENING

Recently RNAi screening is being used to get insight on a gene function in the organisms which were not accessible for gene manipulation earlier such as planarians⁵³. The screening is helping in getting the insight on health-relevant filed such as: fertility, biocide resistance, viability and innate immunity^{54,55}. Cell-based RNAi screening, RNAi screening *in-vivo*, and *ex-vivo* screening are having a strong impact in understanding cancer biology, RNA biology, general cell biology, signal transduction, interactions between bacterial or viral pathogens and host cells⁵⁶. The accessibility of transcriptomic data for cell-lines, tumors and tissues has made possible the interpretation of cell-based RNAi screening^{57,58}. The RNAi reagents: dsRNAs, shRNAs, endoribonuclease-prepared siRNAs and siRNAs, are used for cell-based HTSs. But, in comparison to cell-based RNAi screening, RNAi screening *in-vivo* provides a straightforward and fast route for phenotype screening. RNAi libraries, so far, have been developed for ticks, Hydra, planarians, Lepidoptera, many plants and trypanosomes^{54,55}. In this regard Retroviral Short

Hairpin RNA (shRNA) and synthetic siRNA libraries are publicly available for high-throughput genetic screening and have been mentioned under table II. In addition, still there is a need for improved gene models, development of algorithms for designing more efficient RNAi reagents, generation of better genome-scale RNAi libraries, and improved methods for delivering RNAi reagents to support HTSs.

CONCLUDING REMARKS

In the past few years, RNAi has revolutionized studies of determining gene functions over other genetic approaches and manipulations. Perhaps, it has become a predominant means of appraising loss of gene function in most organisms and addressing the most fundamental queries in biological processes. Gene silencing via RNAi has led to the generation and screening of genome-wide siRNA libraries. It has opened a new era for designing specific methods to silence the gene of interest by mining the basic cellular mechanism in most of the eukaryotic cells. Treatment of genetic diseases and gene function analysis are now the most important applications of RNAi. This can be achieved by *in-vivo* delivery of shRNAs or synthetic siRNAs via viral particles, transfection of plasmid DNAs, liposome-peptide complex, lipoplexes, dendriplexes, polyplexes, microspheres and microsponges. Advancement in the understanding of mammalian RNAi has made it feasible to use this mechanism as therapeutics against a variety of human diseases. It has shown a demonstrable therapeutic potential against neurodegenerative diseases such as: Alzheimer, Amyotrophic Lateral Sclerosis (ALS), Spinocerebellar Ataxia (SCA), Huntington and Prion in animal models. Thus, RNAi has the admiring potential to cure the undruggable diseases, and its diverse applications in medicines, plants, agriculture and biology is apparently limitless.

CONFLICT OF INTEREST

Conflict of interest declared none.

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