

Minireview

Small RNAs: Classification, Biogenesis, and Function

V. Narry Kim*

Department of Biological Sciences and Institute of Molecular Biology and Genetics, Seoul National University, Seoul 151-742, Korea.

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Eukaryotes produce various types of small RNAs of 19-28 nt in length. With rapidly increasing numbers of small RNAs listed in recent years, we have come to realize how widespread their functions are and how diverse the biogenesis pathways have evolved. At the same time, we are beginning to grasp the common features and rules governing the key steps in small RNA pathways. In this review, I will summarize the current classification, biogenesis, action mechanism and function of these fascinating molecules.

Keywords: Argonaute; MicroRNA; RNA Interference; RNA Silencing; RNase III; siRNA; Small RNA.

Introduction

Small RNAs constitute a family of regulatory non-coding RNAs of 19-28 nt in length, which are derived from double-stranded RNAs (dsRNAs). Small RNAs can induce gene silencing through specific base-pairing with the target molecules. Small RNA-mediated gene silencing has been observed in a number of eukaryotes for almost two decades but the mechanism underlying the silencing began to be unraveled only recently. Because these phenomena were seemingly unrelated at the time of discovery, they were referred to as several different terms such as RNA interference (RNAi), co-suppression, gene silencing (post-transcriptional or transcriptional depending on the affected process), or quelling. The RNAi pathway was originally recognized in *Caenorhabditis elegans* as a response to dsRNA leading to sequence-specific mRNA cleavage (Fire *et al.*, 1998). It soon turned out that RNAi is not restricted to nematode and can be induced in *Drosophila melanogaster* (Kennerdell and Carthew, 1998), *Trypanosoma* (Ngo *et al.*, 1998), and vertebrates (Elbashir *et al.*, 2002; Wianny and Zernicka-Goetz, 2000; Yang *et*

al., 2001). This discovery had been preceded by the observation of similar phenomena in plants and fungi although the involvement of dsRNA was uncertain at the time. For instance, in petunia, introduction of exogenous transgenes silenced expression of the homologous endogenous loci (Napoli *et al.*, 1990; van der Krol *et al.*, 1990). These phenomena were called 'co-suppression' (also termed post-transcriptional gene silencing, PTGS) in plants and 'quelling' in fungi. This wide range of silencing pathways is now collectively known as 'RNA silencing'. Although the term 'RNAi' is also often used to indicate 'small RNA-mediate silencing phenomena' in general, it usually means only the mRNA cleavage event induced by the administration of dsRNA. 'RNAi' can also refer to the technology in which small RNA is used as an experimental tool to shut off gene expression.

Classification of small RNAs

The common key players in RNA silencing are small RNAs (also referred to as 'sRNA' in plants) of 19-28 nucleotides (nt) in length. Small RNAs are derived from dsRNAs through the processing mediated by RNase III type enzymes. Two relatively well-defined classes of small RNAs are involved in RNA silencing: microRNAs (miRNAs) and small interfering RNAs (siRNAs) (Table 1, shaded in yellow). MiRNA is often pronounced in full (*ma-i-kro-RNA*), while siRNA is usually pronounced letter by letter (*es-aa-i-RNA*). Because the active forms of miRNA and siRNA are sometimes biochemically or functionally indistinguishable, they are classified based on their origins. MiRNAs are generated from the dsRNA region of the hairpin-shaped precursors while siRNAs are derived from long dsRNAs. The first endogenous small RNAs to be discovered were miRNAs (Lagos-Quintana *et al.*, 2001; Lau *et al.*, 2001; Lee and Ambros, 2001; Lee *et al.*, 1993; Mourelatos *et al.*, 2002). Endogenous siRNAs have since been identified in *Schizosaccharomyces pombe* (Reinhart and Bartel, 2002), *Trypanosoma brucei* (Djikeng *et al.*, 2001), *C. elegans* (Ambros *et al.*, 2003b), *D.*

* To whom correspondence should be addressed.

Tel: 82-2-880-9120; Fax: 82-2-887-0244

E-mail: narrykim@snu.ac.kr

Table 1. Classification of endogenous small RNAs.

Classes	Sub-classes	Length (nt)	Biogenesis	Action mechanism	Biological function	References
MicroRNA (miRNA)	N.A.	~22 (19-25)	Two-step cleavage of hairpin precursors by Drosha and Dicer ^a	Translational repression, mRNA cleavage	Diverse (often function in development and cell differentiation)	(Bartel, 2004)
Short interfering RNA (siRNAs)	Endogenous trans-acting siRNA	21-22 (in nematode) 21 (in plants)	Cleavage of long endogenous dsRNAs by Dicer	mRNA cleavage	Unknown	(Peragine <i>et al.</i> , 2004; Xie <i>et al.</i> , 2004)
	Repeat-associated siRNA (rasiRNA) ^c	24-26 (in plants) 24-27 (in fruit flies)	Cleavage of long dsRNAs derived from repetitive sequences by Dicer ^b	Modification of histone and/or DNA	Silencing of transposons, repetitive genes, and viruses	(Djikeng <i>et al.</i> , 2001; Hamilton <i>et al.</i> , 2002; Ketting <i>et al.</i> , 1999; Mette <i>et al.</i> , 2002; Pal-Bhadra <i>et al.</i> , 2004; Schramke and Allshire, 2003; Tabara <i>et al.</i> , 1999; Xie <i>et al.</i> , 2004)
	Small scan RNA (scnRNA)	~28	Cleavage of long dsRNAs by Dicer	Histone methylation leading to DNA elimination	Genome Rearrangement during conjugation	(Liu <i>et al.</i> , 2004b; Mochizuki <i>et al.</i> , 2002; Mochizuki and Gorovsky, 2004a)
Tiny non-coding RNA (tncRNA) ^d	N.A.	~20	Produced by Dicer from unidentified precursor	Unknown	Unknown	(Ambros <i>et al.</i> , 2003b)
Small modulatory RNA (smRNA) ^d	N.A.	~20	Unknown	Transcriptional transactivation	Neuronal differentiation	(Kuwabara <i>et al.</i> , 2004)

^a In animals, a two-step processing model by two RNase III (Drosha and Dicer) is well established whereas in plants, processing is achieved by sequential cleavage by the nuclear Dicer-like protein DCL1. The detailed biogenesis mechanism in plants is not clear yet.

^b RasiRNAs are longer than other siRNAs. In *Arabidopsis*, DCL3 is responsible for rasiRNA biogenesis. The rasiRNAs in *Drosophila* are also thought to be processed by an RNase III type protein although the identity of the enzyme has not been determined.

^c RasiRNAs have not been identified in mammalian systems to date.

^d TncRNA and smRNA are shown in grey. These RNA classes should be considered still provisional. They may be re-grouped into other classes with more information available in the future.

melanogaster (Aravin *et al.*, 2001; 2003; 2004; Pal-Bhadra *et al.*, 2002), and *A. thaliana* (Llave *et al.*, 2002a; Xie *et al.*, 2004). There are apparently several different subclasses of siRNAs: endogenous trans-acting siRNA (tasiRNA), repeat-associated siRNA (rasiRNA), and small scan RNA (scnRNA) (Table 1). There are at least two provisional classes of small RNAs (Table 1, shaded in grey) whose biogenesis pathway is not yet apparent enough to be used as the basis for classification: tiny noncoding RNA (tncRNA) and small modulatory RNA (smRNA). Some of the small RNAs are fragments of mRNAs, whereas others

are from intergenic regions of the genome. Exogenous dsRNAs can also induce the production of small RNAs (not shown in Table 1). Naturally occurring exogenous siRNAs include virus-induced siRNAs. Both naturally occurring and experimentally introduced small RNAs will be described.

Useful databases of small RNAs have been constructed. MiRNA sequences are available in the Rfam miRNA registry (<http://www.sanger.ac.uk/Software/Rfam/mirna/>) (Griffiths-Jones, 2004) and plant small RNAs are now listed in the Small RNA Database (

state. edu) (Xie *et al.*, 2004).

Action mechanisms of small RNAs

Small RNAs mediate gene silencing through at least four different mechanisms (Fig. 1): (1) endonucleolytic cleavage of the cognate mRNAs, (2) translational repression, (3) transcriptional repression through the modification of DNA and/or histone, and (4) DNA elimination through the modification of histone.

When small RNA targets mRNA, silencing occurs at the post-transcriptional level, which is accordingly known as post-transcriptional gene silencing (PTGS) in plants [reviewed in (Bartel, 2004; Meister and Tuschl, 2004)]. The fate of the target mRNA when bound to small RNA depends on the degree of complementarity between the small RNA and its target mRNA. If the basepairing between small RNA and target is almost perfect, the target mRNA is cleaved between positions 10 and 11 nt of the paired bases relative to the 5' end of the guide small RNA. If the complementarity is lower, the interaction results in translational repression. In either case, the highest level of complementarity (near perfect match) is found at positions 2-7 nt relative to the 5' end of small RNA. Basepairing at this region appears to be important for target recognition. For translational inhibition, multiple binding of miRNAs on a single mRNA induces a synergistic effect. The mechanism of translational repression remains elusive because the polysome profiling on the target mRNA indicates that ribosomes still proceed on the mRNA as if they are normally translated.

Repeat-associated siRNAs (rasiRNAs) target the cognate DNA and induce modification of DNA and histone presumably by recruiting DNA-cytosine methyltransferases and histone-modifying enzymes [reviewed in (Lippman and Martienssen, 2004; Matzke and Birchler, 2005)]. Because repetitive elements with methylatoins on DNA and methylation on histone H3 at Lys9 are silenced at the transcription level, this process is known as transcriptional gene silencing (TGS).

Small scanRNA (scnRNA) can also induce histone methylation that in turn mediates gene silencing through the elimination of DNA in ciliate protozoa. In this case, methylated histone is thought to recruit proteins required for DNA elimination.

Key protein factors in small RNA pathways

Essential factors in small RNA pathways are often encoded by multigene families conserved among eukaryotes [reviewed in (Meister and Tuschl (2004))] (Fig. 2).

RNase III type enzymes are essential components of small RNA pathways. There are two RNase III subfamilies

involved in small RNA pathways: Dicer (class III) and Drosha (class II). Both are large proteins with tandem catalytic domains and a dsRNA-binding domain (dsRBD) at the C-termini. Dicer is a highly conserved protein that has a long N-terminus that contains a DEXH RNA helicase/ATPase domain, as well as the DUF283 domain and the PAZ domain. Dicer cleaves dsRNA precursors into 21-22 nt RNA duplexes. There is one Dicer homologue in fission yeast (*Dcr*), one in human (*Dicer*, also known as *Helicase-MOI*), one in nematode worm (*DCR-1*), two in *Drosophila* (*DCR-1* and *DCR-2*), and four in *Arabidopsis* (*DCL1*, *DCL2*, *DCL3*, *DCL4*). Drosha, on the other hand, is conserved only among metazoans. Drosha initiates miRNA maturation by cleaving the primary transcript of miRNA, releasing short hairpin-like precursor (pre-miRNA). Only one Drosha homologue is found in each animal species.

Argonaute (*Ago*) proteins play a central role in various aspects of small RNA pathways, by directly interacting with small RNAs and by forming effector complexes. These effector complexes are known as RNA-induced silencing complex (RISC), miRNP, or RNA-induced initiation of transcriptional silencing complex (RITS). Argonaute family proteins are highly basic proteins of ~100 kDa that contain two common domains; PAZ and PIWI domains. The PAZ domain consisting of ~130 amino acids is usually located at the center of the protein and interacts with the 3' overhang of dsRNA (Lingel *et al.*, 2004; Ma *et al.*, 2004; Song *et al.*, 2003; Yan *et al.*, 2003). The C-terminal PIWI domain containing ~300 amino acids exhibits structural homology to RNase H (Song *et al.*, 2004). Human *Ago2* was recently shown to act as the 'slicer' enzyme that cleaves target mRNA (Liu *et al.*, 2004a; Meister *et al.*, 2004; Song *et al.*, 2004). The biochemical functions of other *Ago* proteins are still blurred. There is one *Ago* family member in *S. pombe* (*Ago1*), more than 20 in *C. elegans*, five in *Drosophila* and eight in human (*hAgo1/eIF2C1*, *hAgo2/eIF2C2*, *hAgo3/eIF2C3*, *hAgo4/eIF2C4*, *PIWIL1*, *PIWIL2*, *PIWIL3*, *PIWIL4*), and ten in *Arabidopsis*.

Several dsRBD-containing proteins have also been isolated in genetic screening as well as in biochemical purifications. *Drosophila* R2D2, for example, forms a tight complex with *DCR-2* and functions in strand selection and RISC assembly (Liu *et al.*, 2003; Tomari *et al.*, 2004b). Another dsRBD-containing protein, *DGCR8*, and its *Drosophila* homologue *Pasha* interact with Drosha and functions as an essential cofactor in the initiation of miRNA processing (Denli *et al.*, 2004; Gregory *et al.*, 2004; Han *et al.*, 2004a; Landthaler *et al.*, 2004).

Putative RNA helicases have been genetically or biochemically recognized to be required for RNA silencing pathways. Some of the RNA helicases such as Armitage and spindle E are thought to function in the assembly of effector complexes (Aravin *et al.*, 2001; Kennerdell *et al.*,

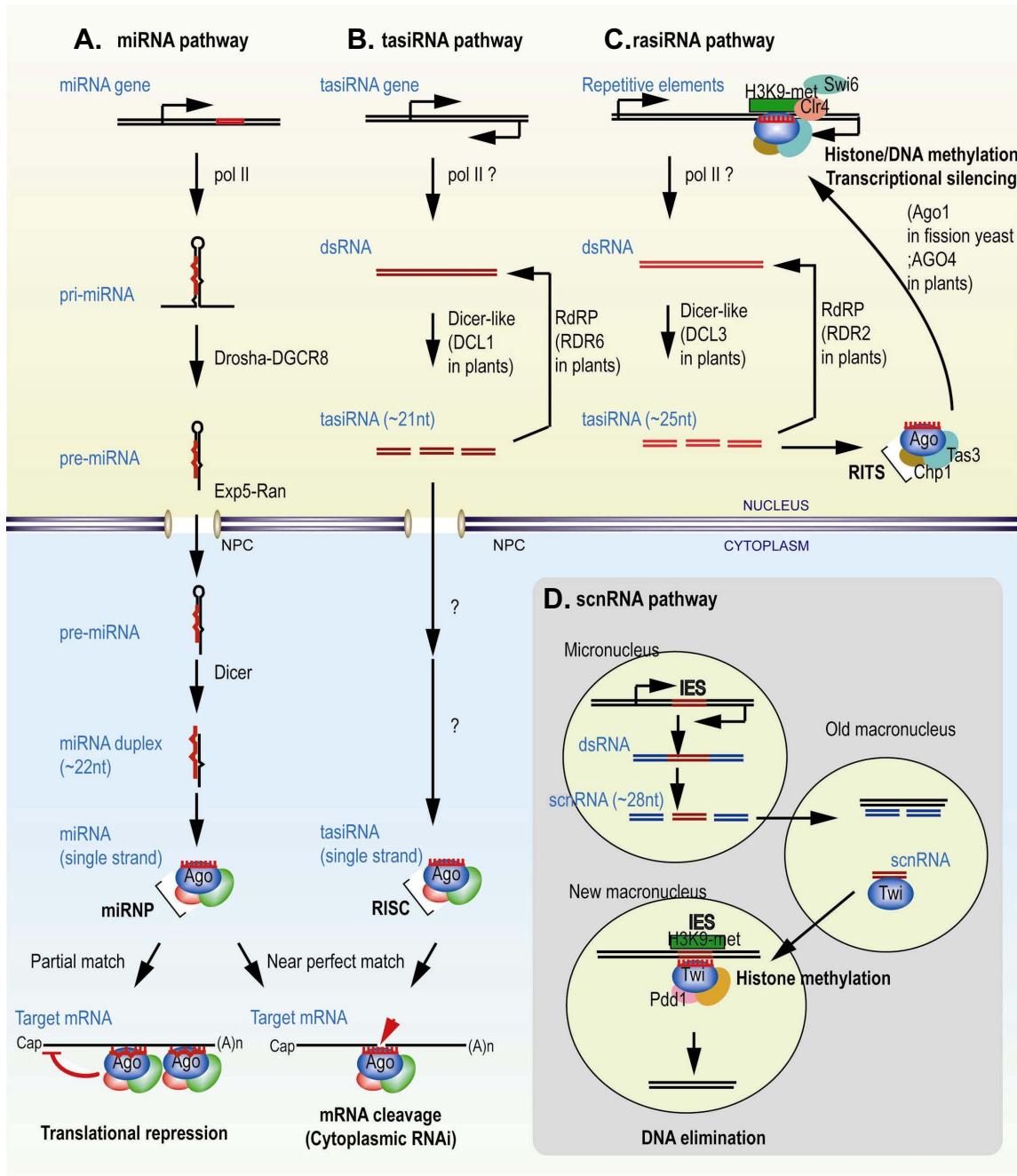


Fig. 1. Model for RNA silencing pathways. MiRNAs are generated from stem-loop precursors whereas siRNAs are processed from long dsRNAs. **A.** MiRNA genes are transcribed by RNA polymerase II to generate the primary transcripts (pri-miRNAs). The initiation step (cropping) by the Drosha-DGCR8 complex results in pre-miRNAs of ~70-nt, which are exported by the Exp5-Ran complex. Upon export, Dicer participates in the second step of processing (dicing) to produce miRNA duplexes. The duplex is separated and usually one strand is selected as mature miRNAs, whereas the other strand is degraded. The final products act as guide molecules in translational control or cleavage of certain mRNAs. **B.** TasiRNA genes are synthesized in both orientations to generate dsRNA molecules. The dsRNA gets cleaved by Dicer-like proteins. TasiRNA is incorporated into RISC and induces target mRNA cleavage. **C.** RasiRNA genes are transcribed in both orientations to generate dsRNA molecules. In organisms expressing RdRP, dsRNAs are amplified by RdRP and cleaved by Dicer-like proteins. RasiRNA is incorporated into RITS complex that associates with chromatin and induce histone/DNA modification. **D.** The micronuclear genome is transcribed in both orientations to generate dsRNA molecules, which is cleaved by Dicer-like protein. ScnRNA diffuses to old macronucleus and subsequently to new macronucleus to scan for the region to be eliminated. ScnRNA is incorporated into RITS-related complex that associates with chromatin and induces histone modification. Methylated histone is thought to recruit proteins required for DNA elimination.

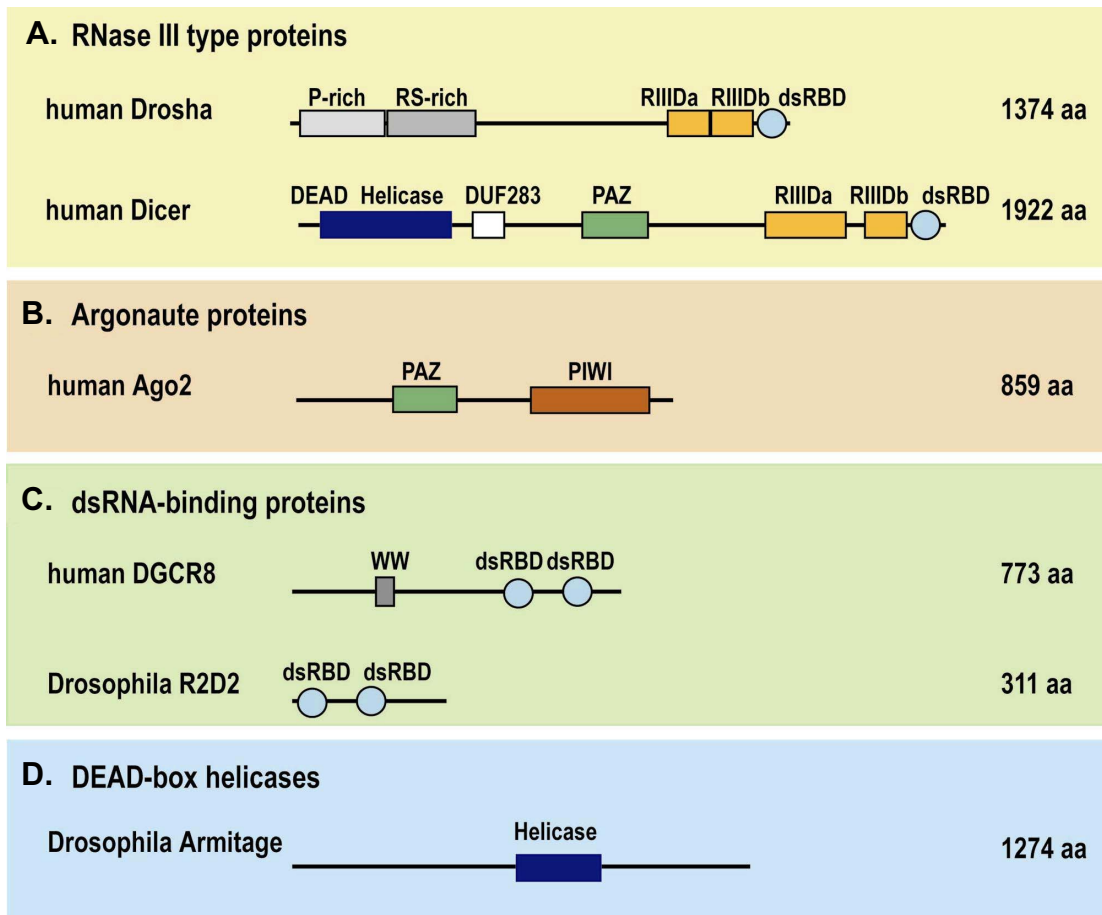


Fig. 2. Domain organization of representative RNA silencing factors. **A.** RNase III proteins. The RIIID, RNase III domain, is the catalytic domain that executes endonucleolytic reaction. The dsRBD, double-stranded RNA binding domain, is also a well conserved motif in many dsRNA binding proteins of diverse functions. The “P-rich” indicates a proline-region, whose biochemical significance remains unknown. The “RS-rich” is a region that is abundant in arginine and serine. Dicer homologues contain additional domains such as Helicase domain and PAZ domain. **B.** Argonaute (Ago) family proteins contain PAZ domain and PIWI domain. **C.** dsRBD-containing protein family. Two dsRBDs are found in R2D2 and DGCR8/Pasha. **D.** RNA helicase family. Shown here is Armitage from *Drosophila* which contain DEAD box helicase motif.

2002; Tomari *et al.*, 2004a).

RNA-dependent RNA polymerases (RdRPs) synthesize dsRNA from single stranded RNA templates to initiate or amplify the RNA silencing process. There are four RdRP in nematode worm, six in *Arabidopsis*, but none in mammals or fire fly.

Different classes of small RNAs

MiRNAs According to the current convention, a microRNA is defined as a single-stranded RNA of 18-24-nt in length (average 21-22 nt), which is generated by the RNase-III-type enzyme Dicer from an endogenous transcript that contains a local hairpin structure (Ambros *et al.*, 2003a) (Fig. 1A). At the time of writing, the miRNA database (<http://www.sanger.ac.uk/Software/Rfam/mirna/>) contains 116 *C. elegans*

miRNAs, 78 *D. melanogaster* miRNAs, 30 *Danio rerio* miRNAs, 121 *Gallus gallus* (chicken) miRNAs, 222 *H. sapiens* miRNAs, 112 *A. thaliana* miRNAs and 5 Epstein Barr virus miRNAs. The list is still expanding as a result of both intensive cloning and computational prediction approaches.

The biogenesis of miRNA is more deeply understood in animals. MiRNA genes are transcribed by RNA polymerase II to generate long primary transcripts (pri-miRNAs) (Cai *et al.*, 2004; Lee *et al.*, 2004a) (Fig. 1A). Pri-miRNAs are first trimmed to release the hairpin intermediates (pre-miRNAs) (Lee *et al.*, 2002). This cleavage is executed by RNase III type enzyme Drosha in the nucleus (Lee *et al.*, 2003). Drosha forms a large complex (500–650 kDa, known as the microprocessor complex) together with its essential cofactor DGCR8/Pasha, a protein containing two dsRNA-binding domains) (Denli *et al.*, 2004; Gregory *et al.*

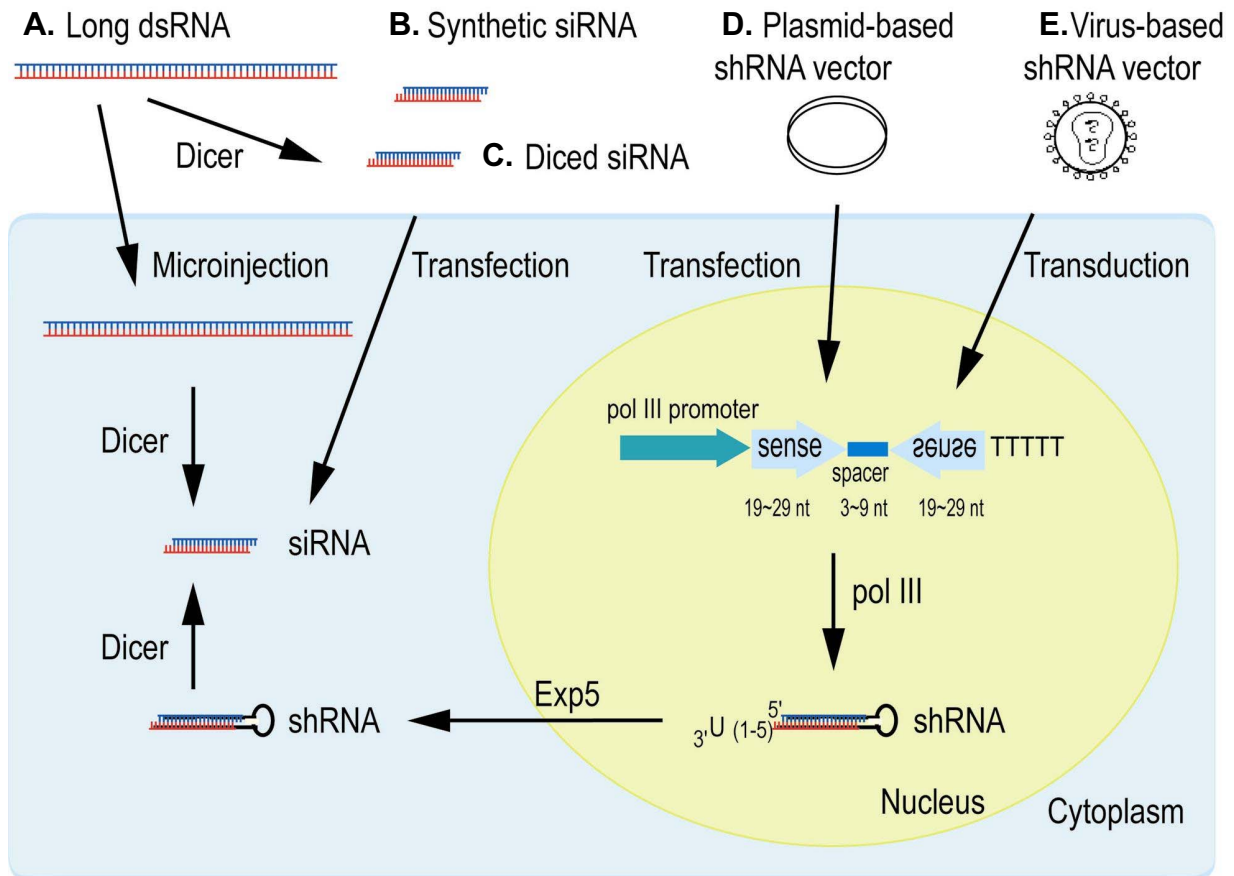


Fig. 3. Various strategies for RNAi in mammalian cells. **A.** long dsRNAs can induce specific RNAi in oocytes, early embryos, and undifferentiated embryonic stem cells. **B.** Chemically synthesized siRNA duplex can be efficiently transfected into cultured cells. **C.** siRNA can be prepared in vitro from dsRNAs by incubating with recombinant Dicer protein. The diced products are purified based on their size (~21 nt) and transfected into cells. **D.** Short hairpin RNAs (shRNAs) are expressed in the nucleus from expression plasmids. The pol III-derived expression system is shown here as an example. Upon export by Exp5, shRNAs are processed by Dicer releasing siRNAs. **E.** ShRNA expression cassette can be delivered by viral vectors such as retroviral vector, lentiviral vector, and adenoviral vector.

al., 2004; Han *et al.*, 2004a; Landthaler *et al.*, 2004). Pre-miRNA then gets exported to the cytoplasm by Exportin-5 (Exp5), which is a member of the Ran-dependent nuclear transport receptor family (Bohnsack *et al.*, 2004; Lund *et al.*, 2004; Yi *et al.*, 2003). Upon arrival in the cytoplasm, pre-miRNAs are subjected to the second processing by Dicer, the cytoplasmic RNase III type protein (Bernstein *et al.*, 2001; Grishok *et al.*, 2001; Hutvagner *et al.*, 2001; Ketting *et al.*, 2001; Knight and Bass, 2001). Pre-miRNA is cleaved into the short-lived miRNA duplex, whose one strand is degraded by an unknown nuclease while the other strand remains as a mature miRNA (Khvorova *et al.*, 2003; Schwarz *et al.*, 2003).

Homologues of Drosha and DGCR8/Pasha are not found outside the animal kingdom, indicating that the Drosha-dependent stepwise processing model applies only to animal cells. In fact, precursors of plant miRNAs are quite diverse in structure. Plant miRNA biogenesis is mediated by the nuclear protein DCL1, one of the four

Dicer-like proteins in *Arabidopsis* (Kurihara and Watanabe, 2004; Papp *et al.*, 2003; Park *et al.*, 2002; Reinhart *et al.*, 2002). Additional biogenesis factors include HYL1, a two dsRBD-containing nuclear protein of unknown biochemical function (Han *et al.*, 2004b; Vazquez *et al.*, 2004a) and HEN1, a protein with a dsRBD and a methyltransferase domain (Boutet *et al.*, 2003; Park *et al.*, 2002; Yu *et al.*, 2005). It was recently shown that HEN1 methylates miRNA duplex at the 2' hydroxyl groups on the 3' most nucleotides (Yu *et al.*, 2005). The biochemical role of this 2'-O-methyl group on miRNA and the presence of similar mechanisms in other organisms await further investigation. HASTY is a putative homologue of Exp5 based on the amino acid sequences and the mutant showed pleiotropic phenotypes, suggesting that this protein too may function in miRNA biogenesis (Bollman *et al.*, 2003; Telfer and Poethig, 1998).

Out of hundreds of miRNAs, only a handful of miRNAs are known for their biological functions (Table

2). The paradigm for the function of miRNAs has been originally provided by *lin-4* and *let-7*, which were identified by genetic analysis of *C. elegans* developmental timing (Lee *et al.*, 1993; Reinhart *et al.*, 2000). They were initially called small temporal RNAs (stRNAs) because of their temporal expression pattern and their roles in temporal regulation. *Lin-4* and *let-7* act as post-transcriptional repressors of their target genes when bound to their specific sites in the 3' untranslated region of the target mRNA (Lee *et al.*, 1993; Moss *et al.*, 1997; Olsen and Ambros, 1999; Slack *et al.*, 2000; Wightman *et al.*, 1993). The level of target mRNA does not change, suggesting that the inhibition occurs at the level of translation. Other animal miRNAs act similarly in various pathways (Ambros, 2004; Bartel, 2004). Another nematode miRNA, *lxy-6* RNA, was identified in a gene screening process for left/right asymmetry of neuronal chemoreceptor expression (Chang *et al.*, 2004). *Lxy-6* RNA targets the *cog-1* transcription factor (Chang *et al.*, 2004). The *bantam* RNA from *Drosophila* suppresses apoptosis and stimulates cell proliferation by inhibiting translation of *hid* mRNA during development (Brennecke *et al.*, 2003). In mammals, miR-181 is involved in the control of hematopoiesis through as yet unknown target(s) (Chen *et al.*, 2004). Mouse miR-196 miRNAs represses the expression of the *HOXB8* gene that is a transcription factor important in developmental regulation (Yekta *et al.*, 2004). MiR-196 RNAs are the first examples of animal miRNAs that cause target mRNA cleavage rather than translational repression (Yekta *et al.*, 2004). Plant miRNAs generally display a higher degree of complementarity to the target mRNAs, resulting in target cleavage (Llave *et al.*, 2002b), although some plant miRNAs appear to repress protein synthesis (Aukerman and Sakai, 2003; Chen, 2003). Interestingly, most of the known targets of plant miRNAs are transcription factors, particularly those involved in developmental regulation or cell differentiation. Functions of the targets of animal miRNAs appear to be more diverse than plant miRNAs.

A single miRNA species can bind to many different mRNA targets and, conversely, several different miRNAs can cooperatively control a single mRNA target. Thus, miRNAs and their targets seem to constitute remarkably complex regulatory networks.

TasiRNAs Endogenous trans-acting siRNAs direct cleavage of endogenous cognate mRNAs *in trans* (the target genes are different from the gene that the siRNA originates) (Fig. 1B). A recently identified set of tasiRNAs was shown to be generated from an intron of a non-coding gene in *Arabidopsis* (Vazquez *et al.*, 2004b). For other tasiRNAs, the hosting gene structures remain to be determined. Interestingly, biogenesis of these RNAs is dependent on genes that belong to two distinct pathways: AGO1, DCL1, HEN1, HYL1 (required for miRNA pathways) and RDR6

and SGS3 (required for virus-induced cis-acting siRNA pathways) (Peragine *et al.*, 2004; Vazquez *et al.*, 2004b; Xie *et al.*, 2004). Further experimentation is likely to reveal a link between these pathways.

Target genes of some of tasiRNAs can be predicted based on their extensive complementarity (Park *et al.*, 2002; Sunkar and Zhu, 2004). Recently, some of the tasiRNA target genes were verified experimentally (Vazquez *et al.*, 2004b). Although tasiRNAs may be of fundamental importance in regulating endogenous cellular function, the endogenous role of tasiRNAs remains unclear. This is because the function of the target genes regulated by tasiRNAs remains unknown and the mutants in *rdr6* and *sgs3* genes do not exhibit severe phenotypes compared to miRNA pathway gene mutants. TasiRNA genes are not conserved among other plant species, suggesting that they may have been recently evolved. So far tasiRNAs have been found only in plants and nematode worms, which possess RNA-dependent RNA polymerases (RdRPs). TasiRNAs may be confined in organisms with RdRP-dependent dsRNA production system but not in organisms such as mammals that lack this system.

RasiRNAs RasiRNAs are presumably derived from long dsRNAs and therefore belong to the siRNA family (Fig. 1C). RasiRNAs match repetitive sequence elements in sense and antisense orientation (Djikeng *et al.*, 2001; Llave *et al.*, 2002a; Reinhart and Bartel, 2002) and function in the establishment of heterochromatin in repetitive elements leading to transcriptional silencing. RasiRNAs are found in plants (Hamilton *et al.*, 2002; Llave *et al.*, 2002a; Mette *et al.*, 2002), *Trypanosoma brucei* (Djikeng *et al.*, 2001), *Drosophila melanogaster* (Aravin *et al.*, 2001; 2003; 2004; Pal-Bhadra *et al.*, 2002), and fission yeast (Hall *et al.*, 2002; Reinhart and Bartel, 2002; Volpe *et al.*, 2002).

In fission yeast, rasiRNAs can suppress the transcription of repetitive transposable elements at the level of transcription (Schramke and Allshire, 2003). The biochemical basis of rasiRNA-induced heterochromatin formation is most comprehensively studied in fission yeast (Lippman and Martienssen, 2004; Matzke and Birchler, 2005). The heterochromatin DNA in fission yeast consists of the simple transposon-derived tandem array that surround the central core centromeric region of each chromosome. In mutants deficient in Ago1, Dicer, and RdRP genes, the centromeric outer-transposon repeats are de-repressed (Volpe *et al.*, 2002). Hallmarks of heterochromatin are also reduced: both histone H3 Lys9 (H3K9) methylation and Swi6 (heterochromatin-associated bromodomain protein) association are decreased in this region (Volpe *et al.*, 2002). In these mutants, transcripts from both strands of this DNA region can be detected (Volpe *et al.*, 2002) and, importantly, small RNAs corresponding to this region have been identified (Reinhart and Bartel, 2002). The transcripts from opposite strands are thought to generate dsRNA molecules that may

be amplified by RdRP, which was found to be tightly associated with repeat DNA. Dicer is believed to cleave dsRNA into small RNAs. Ago1 binds to small RNAs and also interacts with two more proteins, Chp1 and Tas3, to form an effector complex, known as 'RITS' (RNA-induced initiation of transcriptional silencing) (Noma *et al.*, 2004; Verdel *et al.*, 2004). Chp1 is a centromere-associated chromodomain protein and Tas3 is a serine-rich protein that is specific to fission yeast (Verdel *et al.*, 2004). The association of RITS with the silenced loci is required for transcriptional gene silencing and siRNA production in the associated region (Noma *et al.*, 2004).

In plants, rasiRNAs are known to mediate histone H3 (Lys9) methylation and asymmetric DNA methylation to repress mobile genetic elements (Xie *et al.*, 2004; Zilberman *et al.*, 2003). They are also known to induce systemic silencing in plants (Hamilton *et al.*, 2002). Biogenesis of long siRNAs (mostly associated with repeat sequences) requires DCL3, RDR2, AGO4, and SDE4 but not DCL1, DCL2, or RDR1 (Xie *et al.*, 2004). The current model depicts that RDR2 (a RNA-dependent RNA polymerase) and SDE4 (a DNA-dependent RNA polymerase IV) transcribe and amplify precursor dsRNA (Herr *et al.*, 2005), which is then cleaved by DCL3 to produce rasiRNA (Chan *et al.*, 2004; Vazquez *et al.*, 2004b; Xie *et al.*, 2004). The biochemical role of AGO4 is unclear although it has been genetically proved to act downstream of rasiRNA generation and to be required for the methylation of DNA and histone (Zilberman *et al.*, 2003; 2004). The biochemical mechanism of RNA-dependent DNA methylation (RdDM) has not been elucidated. However, genetic data suggest that the rasiRNA-AGO4 complex may interact with DRD1 and DDM1 (chromatin-remodeling protein SNF2-like proteins), HDAC6 (histone deacetylase 6), and MET1 (DNA methyltransferase 1) to trigger methylation of tandem repeats (Lippman *et al.*, 2004).

Nematode worms defective in RNAi pathways are unable to silence transposons in germline tissues (Ketting *et al.*, 1999; Tabara *et al.*, 1999; Vastenhouw and Plasterk, 2004). Fruit flies with mutations in RNA silencing machinery such as the Argonaute family (*piwi* and *aubergine*) and the RNA helicase *homelss/spindle E* are defective in H3K9 methylation and HP1 (the Swi6 homologue) association in heterochromatic regions (Pal-Bhadra *et al.*, 2004). It has been reported that experimentally introduced siRNA can induce DNA methylation in human cells (Kawasaki and Taira, 2004; Morris *et al.*, 2004), although the generality of this phenomenon requires further examination. Despite the seeming ubiquity of small RNA-guided mechanisms, however, it should be noted that there are RNAi-independent pathways for heterochromatin formation and DNA methylation, which are not discussed in this review.

ScnRNAs The programmed excision of excess DNA observed in *Tetrahymena thermophila* can be viewed as 'the

ultimate form of gene silencing' (Mochizuki and Gorovsky, 2004b). Ciliated protozoans contain two distinct types of nuclei. The diploid micronucleus contains the complete genomic contents and is transcriptionally silent during vegetative growth. The polyploidy macronucleus that lacks the germline sequences serves as the transcribed, somatic nucleus. During conjugation, two haploid micronuclei fuse to form a zygotic nucleus, which is later divided into two nuclei that differentiate into a micronucleus and a macronucleus. Differentiation into a new macronucleus necessitates elimination of large parts of the genome. In *Tetrahymena*, ~6000 segments called internal eliminated segment (IES) sequences (each ranging from 0.5 kb to > 20 kb) are deleted, which all together account for ~15% of the genome.

For years, the mechanism for DNA elimination remained enigmatic. The critical role of RNA silencing in DNA elimination was recently revealed by the following findings. Firstly, the micronuclear genome is transcribed at both directions during conjugation (Chalker and Yao, 2001). Secondly, an experimentally introduced sequence in the old macronucleus could affect the elimination of the homologous sequence in the new macronucleus (Meyer and Garnier, 2002). Thus, it was proposed that the double stranded transcripts may serve as the sequence-specific signals that diffuse between the nuclei. Thirdly, small scan RNAs (scnRNAs) of ~28 nt in length appear before elimination (Mochizuki *et al.*, 2002). Lastly, in *Tetrahymena* cells with mutated Twi1 (an Argonaute family protein), scnRNAs do not accumulate and DNA elimination fails to occur (Mochizuki *et al.*, 2002). Recently, Twi1 was revealed to be required for H3K9 methylation of IES sequences, which in turn is required for IES excision (Liu *et al.*, 2004b). Methylated H3K9 recruits two chromodomain-containing proteins, Pdd1p and Pdd3p, which are required for the deletion of IES sequences (Taverna *et al.*, 2002). Taking these observations together, it was proposed that scnRNAs are produced by Dicer from micronuclear dsRNAs and translocate to the old macronucleus to scan for the macronuclear genome (Fig. 1D). Any small RNAs that are complementary to IES sequences cannot basepair with the macronuclear genome because the old macronuclear genome lacks these sequences. So these 'subtracted-out' scnRNAs are free to diffuse into the developing macronuclei, where they guide the H3K9 methylation of IES sequences, leading to IES elimination. It is currently unclear if scnRNAs can also induce cytosine methylation of DNA apart from histone methylation. It is believed that IES sequences have derived from transposons and that DNA elimination is a way of suppressing these selfish DNA elements.

Provisional classes of small RNAs Intensive cloning efforts in nematode worm have revealed a number of new small RNAs (Ambros *et al.*, 2003b). Apart from miRNAs, three groups of small RNAs were found in this study: tiny

non-coding RNAs (tncRNAs), endogenous siRNAs, and X cluster small RNAs.

Unlike miRNAs, tncRNAs are not processed from hairpin precursors and they are not well conserved outside *C. elegans*. The average length of tncRNAs found in this cloning is ~20 nt, which is slightly shorter than the 22 nt average length of miRNAs. On the other hand, tncRNAs are similar to miRNAs in that they are encoded in intergenic regions, exhibit developmentally regulated expression pattern, and do not perfectly match to known messenger RNAs. Most tncRNAs were dependent on Dicer for accumulation. So if their origin from the long dsRNA precursor can be verified, they should be classified as members of endogenous tasiRNAs.

The second group of small RNAs found in this study were grouped into 'endogenous siRNAs' because they are mostly antisense to protein-coding genes. Transposon sequences were also found in this group so some of the RNAs corresponding to transposon sequences may have to be regrouped into rasiRNAs. Endogenous siRNAs are similar to tncRNAs in that they are also ~20 nt in length and that both tncRNAs and siRNAs show similar sequence bias towards G at the 5' end.

Over forty cDNA sequences cloned in this study are found in a locus on chromosome X, all oriented in the same direction. Some of these X cluster RNAs are contained within predicted hairpin structures but they are not yet classified as miRNAs because the majority of small RNAs from this dense locus do not belong to the miRNA class. None of these small RNAs have been characterized to the extent that is sufficient to provide the grounds for proper classification at this point. If they are shown to be produced from long dsRNAs, they too should be classified into the 'siRNA' class.

Kuwabara and colleagues identified a new small RNA through the cloning of 20-40 nt RNAs from adult hippocampal neural stem cells (Kuwabara *et al.*, 2004). This small RNA of ~20 nt is potentially part of dsRNA because a probe to the antisense orientation could also detect ~20 nt RNA. SmRNA is expressed at the early stage of neural differentiation and experimental introduction of smRNA increased the expression of neuronal markers in neuronal progenitor cultures. Localized in the nucleus, smRNA functions as a transcriptional modulator. SmRNA is complementary to a promoter element known as NRSE/RE1 which is usually found within promoter regions of neuron-specific genes. The NRSE/RE1 is known as a binding site for the NRSF/REST protein that functions as a transcriptional repressor. Presumably through interaction with NRSF/REST, smRNA converts NRSF/REST from a repressor to an activator. As such the expression of NRSE/RE1-containing genes can be restricted to neural lineages. It is currently unclear how smRNA is made in cells. In fact, one of the key questions is whether any of the RNA silencing-related machinery (such as RNase III or Argonaute family proteins) is involved in smRNA biogenesis and function.

naute family proteins) is involved in smRNA biogenesis and function.

Virus-induced or virus-encoded small RNAs In plants, RNA silencing serves as an effective antiviral defense system (Baulcombe, 2004; Lecellier and Voinnet, 2004). Mutations in some RNAi factor genes such as Dicer-like protein 2 (DCL2), a RNA-dependent RNA polymerase (RDR6/SGS2), and a plant-specific protein of unknown function (SGS3) exhibit delayed accumulation of viral siRNA and increased susceptibility and sensitivity towards virus (Mourrain *et al.*, 2000; Xie *et al.*, 2004). Apparently these proteins have specificity for different viral RNAs and additional RNAi components are needed in anti-viral defense. For instance, RDR6 mutants are susceptible to cucumber mosaic virus but not to tobacco mosaic virus (Xie *et al.*, 2004). Upon infection of viruses, viral DNA or RNA is used as the template for RDR6 to generate dsRNA molecules. It is ambiguous yet how RdRP and DCL differentiate among distinct RNA types. DCL2 is thought to cleave these dsRNAs to generate primary siRNAs, which may serve as primers for RdRP resulting in amplification of secondary siRNAs. These siRNAs presumably interact with AGO1 and mediate cytoplasmic degradation of viral RNAs (Fagard *et al.*, 2000). There is no clear evidence that animal viruses also induce siRNAs and it seems unlikely that RNA silencing is a general anti-viral defense mechanism at least in mammals.

Viruses have evolved various strategies to suppress the host's antiviral RNA silencing (Baulcombe, 2004). Some viruses express viral suppressor proteins that interfere with the host's RNAi machinery. For instance, p19 encoded by tomato bushy stunt virus binds tightly to siRNAs duplexes and inhibits incorporation of siRNA into RISC (Baulcombe and Molnar, 2004; Chapman *et al.*, 2004; Lakatos *et al.*, 2004; Silhavy *et al.*, 2002; Vargason *et al.*, 2003; Ye *et al.*, 2003). Other viruses counteract silencing by making themselves inaccessible to RNAi machinery through protective secondary structure in their RNAs or through compartmentalization in certain subcellular locations.

Some animal viruses such as Epstein-Barr virus encode miRNAs in their genome (Pfeffer *et al.*, 2004). These viruses seem to have evolved to express their own miRNA genes to regulate viral and host gene expression to optimize infectivity in the host cells.

Small RNA as an experimental tool Because of its exquisite specificity and efficiency, RNAi has drawn much attention as a powerful gene knockdown technique. The long dsRNA can be experimentally introduced by microinjection or ingestion, as is in RNAi-mediated gene knockdown experiments (Hannon and Rossi, 2004). While this technique revolutionized the genetic studies of *C. elegans*, development of RNAi techniques in mammalian cells was belated

because long dsRNA nonspecifically suppressed gene expression in differentiated cells (Gil and Esteban, 2000; Kumar and Carmichael, 1998). This limitation was soon circumvented by using synthetic siRNA duplexes (21-nt) that are too short to induce non-specific inhibition (Caplen *et al.*, 2001; Elbashir *et al.*, 2001a; 2001b) (Fig. 3). This method involves transfection of synthetic siRNA into cultured cells. Because of its straightforward protocol, siRNA transfection is the most widely used RNAi technique so far. Despite of its potent knockdown capabilities, however, the siRNA transfection method has its weak points such as transient effect and difficulties in transfection of certain cell types. Stable gene silencing was later achieved by developing a method based on the expression of siRNAs from DNA templates (Fig. 3). When short hairpin RNA (shRNA) that resembles pre-miRNA is transcribed from the pol III promoter, shRNA gets processed by Dicer to generate siRNAs. To construct a shRNA expression cassette, the gene-specific targeting sequence (~19-nt sequences from the target transcript separated by a short spacer from the reverse complement sequences) is inserted between the pol III promoter and the terminator. Similarly, hairpin RNA can be embedded downstream of pol II promoters so that the resulting transcript can be processed like a primary miRNA (pri-miRNA) (Zeng *et al.*, 2002). Viral vectors such as retroviral vectors and lentiviral vectors are often employed to efficiently deliver shRNA expression cassettes.

Divergence and convergence of pathways in different organisms

The RNA silencing machinery of fission yeast is perhaps the simplest, which possesses only one homologue each for Dicer, Argonaute, and RdRP. *Arabidopsis* contains much more elaborate RNA silencing machinery: four Dicer homologues, at least ten Argonaute homologues, and six RdRP homologues.

Different homologues are often assigned to take on distinct roles. For instance, *Arabidopsis* Dicer-like genes DCL1, DCL2, and DCL3 appear to function in the biogenesis of miRNA, virus-induced siRNA, and rasiRNA, respectively (Xie *et al.*, 2004). The function of DCL4 is not known. The RNA silencing machinery in *Drosophila* shows another neat example of functional specialization. DCR-1 is required for miRNA processing whereas DCR-2 is needed for siRNA processing (Lee *et al.*, 2004b). Likewise, *Drosophila* AGO1 functions in the miRNA pathway while *Drosophila* AGO2 is critical in siRNA-mediated mRNA cleavage (Okamura *et al.*, 2004).

In contrast, mammals and nematode worm have more converged pathways for small RNA biogenesis: a single Dicer homologue functions in all small RNA pathways in these organisms. Human Argonaute proteins bind to both miRNA and siRNA to constitute miRNP and the RNA-

induced silencing complex (RISC) (Martinez *et al.*, 2002; Mourelatos *et al.*, 2002). In human, the miRNA-containing RISC and the siRNA-containing RISC cannot be distinguished in terms of composition and function.

Some proteins, such as *Arabidopsis* AGO1 and AGO10 (ZWILLE), have partially overlapping functions. Other proteins function in multiple pathways. For instance, AGO1 in *Arabidopsis* are involved in both miRNA and siRNA pathways (Xie *et al.*, 2004).

Conclusions

The natural role of RNA silencing is thought to be in fine tuning gene regulation (Bartel, 2004) as well as in defense against invasive nucleic acids such as transposable elements and viruses (Ketting and Plasterk, 2004).

A recent computational study has suggested that over one third of human genes are possibly targeted by miRNAs (Lewis *et al.*, 2005). If this is correct, the unique combination of miRNAs that are present in each cell type may affect the utilization of thousands of mRNAs in the cell (Bartel and Chen, 2004). Endogenous tasiRNAs may also participate in such gene regulation. With more refined methods for small RNA identification and target prediction, we may be able to dissect the complex gene network directed by small RNAs.

The role of RNA silencing as a self-defense system is also very intriguing. Although RNA-mediated defense may not be apparent in somatic cells of mammals, it may play a critical role in germline cells and early embryos (Houbaviy *et al.*, 2003; Suh *et al.*, 2004). It would be important to identify small RNAs expressed in such cells and the protein factors involved in RNA silencing-mediated self-defense.

Although the link between small RNA and human disease has not been firmly established, some miRNAs have been implicated in tumorigenesis (Calin *et al.*, 2002; 2004; Metzler *et al.*, 2004). Identification of the molecular targets for these miRNAs will be necessary to provide a direct link between miRNA genes and cancer. Another clinically relevant finding is that FMR1 (also named FMRP) is associated with miRNAs and Argonaute proteins in *Drosophila* and humans (Caudy *et al.*, 2002; Ishizuka *et al.*, 2002) [reviewed in (Murchison and Hannon, 2004)]. The loss of function of FMR1/FMRP causes fragile X mental retardation syndrome in humans. The biochemical role of FMR1 in RNA silencing is not known yet. Understanding the mechanism of RNA silencing will shed light on the molecular basis of human disease.

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