RNA-mediated gene regulation system: Now and the future (Review)

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Abstract. The discovery of RNA-mediated gene regulation system has brought human beings another big surprise on the wisdom and power of nature. Nature can not only create genetic codes with 4 nucleotides and produce proteins with 21 amino acids, but also control their intermediates, mRNAs, with double-stranded RNA molecules composed of ~22 nt. It has been clear that ~22 nt dsRNAs can specifically and efficiently modulate gene expression through sequence-specific targeting thus silencing of RNA. This system promises to be an excellent model for the identification of gene functions and the exploitation of gene expression and phenotype alteration, and a promising way for the prevention and treatment of human diseases.

Contents

1. Introduction

The growth, development, and differentiation of normal cells are involved in the initiation and termination of various genes while the abnormal expression levels and expression forms of genes or invasion of exogenous genetic elements may induce defensive process in cells. It has been discovered recently that cells of different species from *Plasmodium falciparum* (1), T*rypanosome brucei* (2), *Arabidopsis thaliana* (3), *Neurospora crassa* (4), *Caenorhabditis elegans* (5), *zebrafish* (6), *Drosophila melanogaster* (7), *Xenopus laevis* (8), mice and rats (9-11), to human beings (12,13) conserve a powerful ancestral RNA-mediated regulation system through which unwanted mRNAs can be down-regulated, aberrant RNAs can be degraded, epigenetic elements such as virus, transgenes and transposable elements can be suppressed (14). Although this newly discovered system within cells appears to play similar roles in the global immune system in mammals and humans, this defensive system is mediated not by antibodies or immune cells but by RNA molecules. This system seems to be a fascinating approach for cells to modulate normal expression of genes (15,16). Single- or double-stranded ~22 nt RNA with a 5'-monophosphate and a 3'-hydroxyl groups (17-20) can be an effective trigger to initiate sequence-specific suppression on target mRNAs, genomic DNAs or other types of RNAs and DNAs such as aberrant RNAs and transgenes (21). Over past several years, there has been a wide range of investigations into virus-induced gene silencing (VIGS) (22), transcriptional gene silencing (TGS) (23,24), and post-transcriptional gene silencing (PTGS) in plants (25), RNA interference (RNAi) in animals (5) and quelling in fungi (4,26). Abundant evidence suggests that the molecular basis for all these phenomena is an elegant gene regulation system operated by sequence-specific targeting and silencing of target genes (14). The global profiles of these phenomena from their chemical structures, biological functions, genetic resources and metabolic pathways (21,27-30) have been characterized. Pertinent initiators, effectors, complexes, and interactive networks are being identified by genetic screening, biochemical analysis, viral suppressors (31-33) and bioinformatics assistance. In the present review, we attempt to explore how this ancient and complicated network system has been integrated into the normal development of cells and the defense against environmental insults, and to envision the future that this exciting and compelling research can bring us to overcome human diseases such as cancer.

2. Triggers of RNA silencing pathways

What is the structure and feature of RNA trigger for RNAi? As an RNAi trigger, RNA molecule needs to meet at least two requirements. One is to contain a characteristic structural

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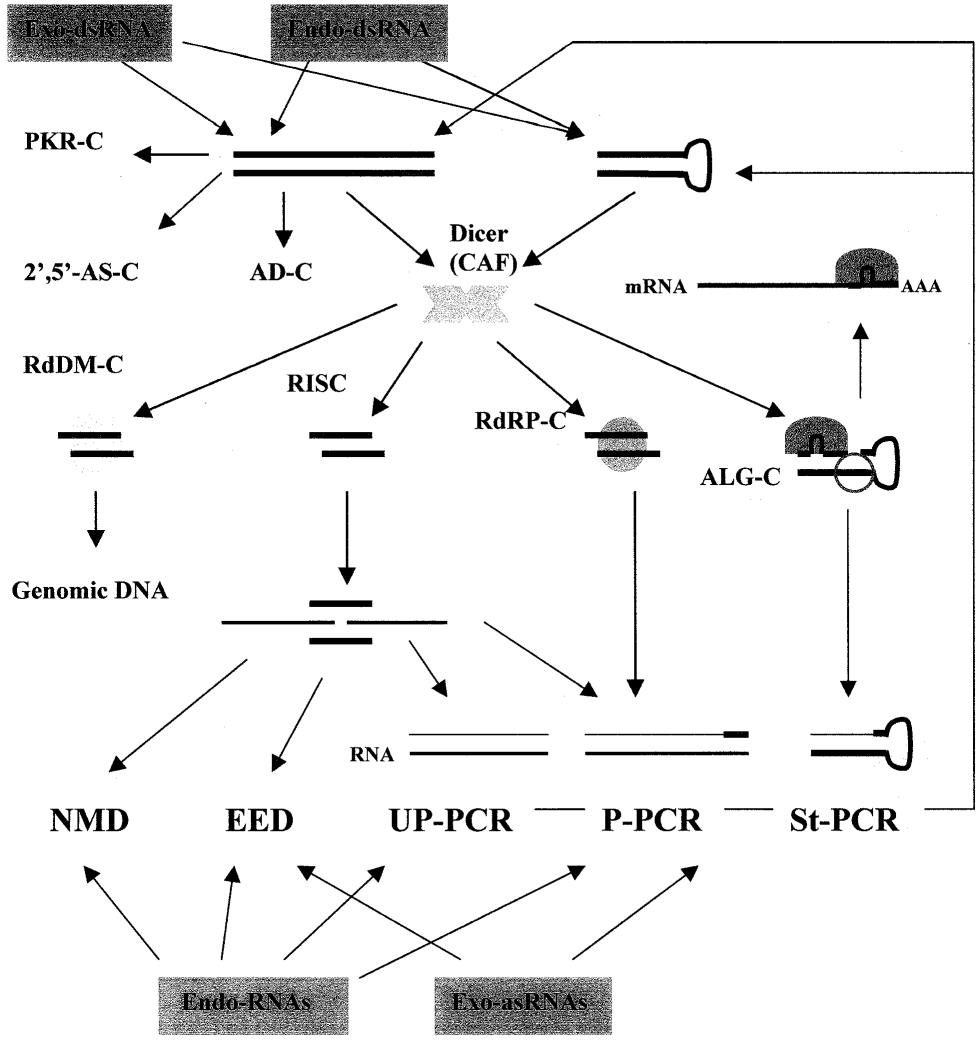

Figure 1. Long or short double-stranded or single-stranded RNAs come from three main aspects including endogenous and exogenous genetic elements and metabolic pathways. When long dsRNA appears in cells, it has at least four different metabolic pathways for choice. Specific complexes for corresponding pathways refer to PKR complex (PKR-C), adinosine deaminase complex (AD-C), 2',5'-oligoadenylate synthetase (2',5'-ASC) and Dicer complex. Through the process of Dicer, dsRNA is chopped into 21-23 nt dsRNA. The resulting siRNA can be transferred into three different complexes such as RNA-dependent DNA methylation complex (RdDM), RNA-inducible silencing complex (RISC) RNA-dependent RNA polymerase complex (RdRP-C), while stRNA can bind to the alg1/alg2 proteins (ALG-C). RISC will make a specific cleavage in the middle of homologous region of a target mRNA. The two products stemmed from the mRNA target will be further processed through NMD, exonucleases, primer PCR, or unprimer PCR pathways. Some siRNA may directly act as a primer to synthesize the strand complementary to a target mRNA molecule. Subsequently, these siRNAs and long dsRNAs may functions as a systemic signal moving from a cell to another cell. Although it is not clear how stRNA can be produced in a local site of cytoplasm, it is likely that a remnant of stRNA precursor functions as both a template and a primer for RdRP reaction.

sequence that is homologous to target gene, and the other is to meet the defined length. Generally, RNA triggers can be divided into two major classes, i.e. natural and artificial. The natural triggers may be single-stranded aberrant RNAs or double-stranded RNAs derived from transposons, viruses or other sources (21). The artificial ones could be introduced transgenes, injected dsRNAs, or transfected single stranded sense or antisense RNAs. These agents fall into five groups according to the length, number and nature of strand. The first group is characterized with long dsRNAs (>38 bp), and dsRNA of less than 38 bp does not suppress target RNA expression (12,29,30). This group includes dsRNA viruses, transposable elements, and transgenes; dsRNA constructs from the inverted DNA repeats of endogenous genes (34,35); and simple dsRNA molecules such as dsRNA fragments designed for research. The second group is related to long single-stranded sense RNAs (ssRNAs). Although no research to date indicates how long single-stranded RNA should be to become effective in RNAi, a recent report showed that short RNA of 22-40 bp can trigger cognate RNA degradation (17). ssRNA virus is an example of this group. Most plant and animal viruses contain a single-stranded RNA genome. A ssRNA virus can use virus RNA-dependent polymerase to synthesize both sense and antisense RNA in the cytoplasm of host cells. Subsequently, these antisense and sense RNAs can form full-length dsRNA and inhibit the expression of endogenous genes by gene-specific regulation mechanisms. This phenomenon is known as VIGS (21). Aberrant ssRNAs or other types of RNAs also belong to this catagory (23,36). The third group is related to ssRNAs that can form a local duplex structure. This group includes derivative RNAs from pre-stRNAs (short temporal RNAs) or miRNA (micro-RNA),

RNAs with a local duplex structure, such as a lariat RNA from intron (37,38), aberrant transcripts derived from the chromatin structure modified by DDM1 and QDE-3 proteins. This type of RNA molecules forms a local duplex structure (39). The fourth group is related to short dsRNAs (about 20-26 nt), consisting of the derivatives cleaved from long dsRNAs by an RNase III-like endonuclease, exogenous short dsRNA such as those synthesized siRNAs (12,29,30). *In vivo* and *in vitro* experiments have revealed that a short dsRNA is incorporated into a complex known as RISC only if their size matches to its dsRNA-binding domain. This dsRNA recognition motif tightly embraces a dsRNA fragment with the length from 20 to 23 nt. The fifth group is related to short antisense RNAs (asRNA) (22 to 40 nt). This group includes asRNA that may be a component of a putative complex containing mut-7 and mut-14 (17). The antisense RNA triggers may be oligonucleotides complementary to different regions or the whole line of an mRNA. The length of antisense RNA is closely related to its function. Recent evidence has demonstrated that 15 to 18 nt asRNAs are ineffective in inhibiting corresponding mRNAs. When the length of asRNAs extends from 22 to 40 nt, those oligos could knock down cognate mRNA molecules. Further evidence comes from natural antisense RNAs occurring in the imprinting. A much longer asRNA is transcribed from the other strand of IGF genomic DNA, which then efficiently blocks the activity of IGF mRNA possibly through degradative PCR pathway.

3. Machinery of RNA silencing pathways

Cellular RNAs usually exist in the combined yet complicated form. They can form different complexes with related proteins or enzymes to function as parts of metabolic pathways or to be degraded as substrates of nucleases. In RNA-mediated gene regulation system, different types of RNAs have been found to interplay with corresponding enzymes and other factors. The resulting complexes are important players for different pathways and essential executives for biological functions shown in Fig. 1.

The complexes related to the metabolism of long dsRNAs. Experimental data to date indicate that four active components in cells are involved in the regulation of long dsRNAs. These four different types of enzymes all contain similar dsRNA-binding domains. When dsRNA molecules occur in cells, they can inevitably initiate competition among these four enzymes. However, the mechanisms by which a cell makes these traffics in order remain to be determined. The four complexes are as follows: i) the PKR complex, in which PKR is a basic protein (pI 8.6) and contains an effective dsRNA binding domain similar to the conserved dsRNA-recognition motif found in the RNase III family. Once PKR is activated through interaction with dsRNA, it can phosphorylate the eukaryotic initiation factor EIF2 to block the initiation pathway of the protein synthesis (40). The effective length and the best size of dsRNA to bind and to activate PKR are 30 and 85 bp, respectively; ii) the 2',5'-oligo(a) polymerase complex, in which 2',5'-oligo(a) polymerase binding dsRNA molecules is an antiviral enzyme. This enzyme catalyzes synthesis of 2',5'-oligoadenylates (2-5A) in a dsRNA-dependent manner. The resulting 2',5'-oligoadenylates trigger non-specific mRNA degradation through the activation of RNase L. The preferred substrate of 2-5A synthetase is a dsRNA containing a sequence longer than 65 to 80 nucleotides (41); iii) the Dicer complex, in which Dicer may contain a red4 protein, a helicase activity (42) and a dsRNase III-like endonuclease (43). Red4 has been found to be capable of enhancing the cleavage rate of Dicer (44). The primary structure of Dicer includes an ATP-dependent RNA helicase domain, a Piwi/Argonaute/Zwille (PAZ) domain (45), two RNase III-like domains and a COOH-terminal dsRNA-binding domain. Recently identified bidentate RNase is responsible for the cleavage of long dsRNA into siRNAs and for the production of mature stRNAs from their precursors (46-49). The Dicer-like enzymes have thus far been identified in humans (helicase MOI), *D. melanogaster* (Dicer), and *C. elegans* (helicase K12H4.8) (50). The effective length of dsRNA should be more than 38 bp, whereas the better inhibitive rate can be achieved by those dsRNAs of more than 150 bp (12,29,30); iv) the adenosine deaminase (AD) complex, in which AD is able to act on both long and short (21-25 nt) dsRNAs with a covalent processing reaction. AD catalyses the hydrolytic deamination of adenone (A) to inosine (I) in dsRNA. The substitution of A by I progressively suppresses the production of siRNA, while the mismatch of antisense siRNA and target mRNA causes the failure of sequence-specific RNA-mediated degradation process (27,51).

The complexes related to the metabolism of short dsRNAs. The siRNAs from different resources can be recognized by specific adaptors, which in turn help those siRNAs into the following complexes: i) the RNA-induced silencing complex (RISC), which may include an endonucleolytic activity, a helicase, an exonuclease, a PAZ/Piwi protein (rde1), a helicase, an adenosine triphosphatase, a homology-researching domain (19,52,53). RISC is a multicomponent nuclease involved in making a specific cleavage in the middle of homologous region of mRNA under the guidance of siRNA. The PAZ/Piwi protein is essential for the initiation of RNAi and stem cell renewal (54,55). The PAZ domain is crucial for the interaction of Dicer with RISC or RdRP. Interestingly, the last three components may be embraced by a member of SMG family known as SMG-2 in animals and Upf1 in humans and yeasts. SMG-2 has been proven related to the maintenance of RNAi (56). However, the question is whether SMG-2 exists in RISC or how it helps RISC function; ii) the RNA-dependent RNA polymerase (RdRP) complex, which may include a PAZ/Piwi protein (RDE1/AGO1/QDE2) (57), a RNA-dependent RNA polymerase, a helicase (i.e. mut-14 and/or SMG-2/SDE3), RNaseD-type 3'-5' exonucleases (Mut-7) and others. The MUT-7 gene of *C. elegans* encodes a protein similar to RNaseD that displays 3'→5' exonuclease activity (58-60). SMG-2 recruits mRNA through the interaction of polysomes. Recent evidence has shown that the RdRP activity is required for amplifying the gene silence process mediated by a small amount of siRNAs (18,61,62) and the activity is closely related to dispersion and maintenance of RNAi signals; iii) the dsRNA-dependent DNA methylation (RdDM) complex, which involves a chromomethylase or a methyltransferase, a putative RecQ DNA helicase (SDE3/QDE3) (35), a SWI2/SNF2 component, a Piwi/Argonaute/

elF2C component (63,64) and other factors such as polycomb proteins (65-68). SWI2/SNF2 appears to be a chromatin-remodeling factor. DNA methylation may be required for the initiation or maintenance of transcriptional suppression of cognate promoters (23,24) as well as the production of aberrant RNA (21).

The complexes related to the metabolism of short ssRNAs. A alg1/2 complex may be composed of an alg1/2, a stRNA and other repressive molecules. Alg1 and alg2 were found to be crucial for small RNA production, targeting, and function (69,70) as well as interaction with other regulators (71). Alg1/2-like protein or other agents may be required for the targeting of a short antisense RNA to the RdRP pathway (17).

Interfaces between different complexes in RNA silencing pathways. It remains to be an interesting issue that different complexes talk to each other and constitute perfect transportable chains to ensure a cascade of reactions to happen in an orderly fashion. Several streams of experimental data have implied that there are special interfaces among different complexes.

RNA silencing was first reported in transgene plants in 1990 (72,73). In the pilot research to obtain a deeper due of the flower, Napoli and coworkers (72) introduced a transgene for chalone synthase, the gene related to pigment synthesis, into petunia. Unexpectedly, the petunia grew up white or variegated. This was termed cosuppression (74) because the expression of both the transgene and the endogenous gene were suppressed by a silencing signal. In 1998, Hamilton and Baulcombe (65) identified a species of 25 nt small antisense RNAs, specific for the transgene in one type of virus-induced PTGS and three types of transgene-mediated PTGS. It is now clear that 25 nt dsRNA is the specific signal for PTGS in plants, while 21 to 23 nt fragments from a dsRNA trigger their target mRNA degradation in flies (75). Three years later, another group of researchers transfected *Drosophila* S2 cells, human embryonic kidney or HeLa cells with synthesized 21 nt siRNA duplex in order to determine if siRNA molecules were able to trigger RNAi reaction in cell culture. Successfully, they made the important discovery that 21 nt siRNA duplexes with the 2-nucleotide 3' overhang could specifically degrade cognate mRNA molecules transcribed from endogenous or exogenous genes in different cell lines (12,29,30). However, genetic research demonstrates that naked 21 nt siRNA could not induce gene silencing without the assistance of a member of PAZ/Piwi protein family (44). Mutations in piwi prohibit the production of siRNA and PTGS, and partially interfere with TGS (64).

Besides the efficient degradation of homology-dependent target mRNAs mediated by dsRNAs, it is interesting to explore whether single-stranded small antisense RNAs could trigger a similar process operated by gene-specific RNA silencing. To test whether short ssRNA can induce RNAi phenomenon under the assistance of some RNAi machinery components, Tijsterman and colleagues (17) injected single-stranded RNAs (ssRNAs) into *C. elegans*. It was surprisingly found that ssRNA oligomers of antisense polarity with the length 22 to 40 nt did suppress cognate mRNA targets. This result suggests that ssRNA trigger the RNAi pathway in a manner different from that of small dsRNAs, because ssRNA could bypass the mutation of rde1. Recently, emerging evidence from bio-informatics analysis indicates that a large number of human antisense RNAs exist in mammalian cells (76,77). Moreover, several lines of experiments demonstrate that endogenous antisense RNAs (stRNAs) are active in regulating gene expression during the period of embryonic development in *Drosophila*, *C. elegans*, and humans (16). Although stRNA is different from antisense RNA in structure, it is likely that a similar carrier may be required for antisense RNA pathway.

Small stRNAs, let-7 and lin-4, were first identified in 1993 (78). They belong to a subset of micro-RNAs (miRNAs) that is a large group of gene regulators. These miRNAs may be stemmed from intergenetic regions or introns of gene encoding regions (37). Owing to their non-encoding characteristics and small size, miRNA can be quickly transcribed. Their precursors usually are about 70 mer and can form a stem-loop structure. Just as double-stranded siRNAs mentioned above, a 21-25 nt single-stranded stRNA is cleaved off from its precursor by an endonuclease known as Dicer. However, the resulting stRNAs take another pathway that is different from siRNAs. Under the assistance of alg1/alg2, members of ade family, they are guided to the 3' non-translation region of target mRNA, for suppressing the translation of corresponding protein.

All evidence listed above implied that the members of Paz and Piwi domain family control small RNAs to enter into different RNA metabolic pathways, though the proteins used by exogenous asRNA are yet to be characterized. The reasons include: i) different members can help recognize and transfer different types of small RNAs; ii) these adapters can interact with both Dicer and its downstream complex; and iii) this conjecture has been supported by several streams of findings as described above.

The Paz and Piwi domain (PPD) family is a group of proteins composed of 1020 amino acid, in which there are 24 (RDE) in *C. elegans*, 7 (AGO) in *Arabidopsis*, 5 in *Drosophila*, and 4 in humans. The diverse members of PPD family have their own properties specific for the recognition of different trigger constructs and the assembly of them into the appropriate downstream complexes. For instance, RDE1/AGO1 can stabilize siRNA and transfer it into a multicomponent nuclease known as RISC (23,24) or a RdRP complex (21) from Dicer through the interaction of their PAZ domains. Genetic analysis indicated that in mutant animals without rde1 and rde4, RNAi cannot be initiated, but animals remain normal. Further cell-free extracts from animals lacking rde1 can effectively chop long dsRNA into siRNAs, but resulting siRNAs would not be taken into related complexes (44,79). This result suggests that rde1/ago1 is necessary for transferring siRNAs into RISC and RdRP complexes. The questions when and how rde1/ago1 delivers siRNAs to RISC or RdRP, respectively remain to be addressed. Similarly, when alg1 and alg2, two other members of rde family, become mutated, mutants can fully go through the process of RNAi, but show developmental defects because longer precursor of *let-7* and *lin-4* cannot be cleaved into the mature forms of stRNAs for the temporal regulation. It implies two possibilities, one is that stRNA cannot trigger endonucleolytic activity and RdRP, and the other is that alg1 and alg2 may help Dicer identify which

strand of stRNA precursor should be cleaved off. However, no research to date indicates whether the other half of precursor can bind to rde1 as a template for the RdRP reaction and whether alg1/alg2 complex has any interactions with RISC or RdRP.

The second interface worth mentioning is a member of the SMG family. Of seven members of SMG family, three members are involved in both RNAi and NMD, including SMG-2, SMG-5 and SMG-6 SMG-2. SMG-2 is composed of an adenosine triphosphatase, an RNA binding domain and a helicase motif. Genomic homology searching has found that *Arabidopsis SDE3*, *C. elegans* SMG-2 and human and yeast Upf1 (80) have similar structure and function, which can recruit mRNA through the contact with polysomes. More recent data have further revealed that the products of genes, *smg-1, smg-3* and *smg-4* can phosphorylate SMG-2, while *smg-5, smg-6* and *smg-7* are required for the dephosphorylation of SMG-2 protein. More importantly, *in vitro* biochemical analysis has shown that ATP is required for RISC process (19). In addition, SMG-2 can also contact the translation termination factors eRF1 and eRF3, and interfere with translation termination. Mutant assay revealed that the mutation of *smg-2, smg-5* or *smg-6* could initially cause temporary RNAi, suggesting that SMG-2 is required for persistence of post-transcriptional silencing (80). For the explanation of this phenomenon, one hypothesis is that SMG-2 could function as a search engine to help RISC or RdRP find corresponding target mRNA. In order to accomplish the searching task and avoid to be trapped with mRNA molecule, the dephorsphorylated SMG-2 is required. This could possibly explain why only three members of SMG family are required for RNAi, but all the SMG family members should be intact for NMD. The further question to be answered may be whether SMG-2 plays any functions with ALG1/ALG2 in suppressing the synthesis of proteins.

The third interface in RNA-mediated reaction may be members of polycomb family that contains more than 30 isoforms. With other associated components, the polycomb protein can form a transcriptional silencing complex responsible for cosuppression in *C. elegans* and *Drosophila* (81,82). Recent investigation has found that polycomb may interplay with siRNA (64), and then play a role in searching the specific chromatin site containing the homogeneous sequence siRNA used (83) and make an entry to the chromatin for the RNA. Similarly, as polycomb proteins do, chromomethylase and histone acetylase MOF also contain chromodomains that have been shown to act as an interface between RNAs and chromatin regulatory factors. However, the properties of this complex remain to be characterized.

4. RNA silencing pathway

RICS branch of RNA silencing pathway. It has been reported that the complexes containing the RNase III-like enzyme and RISC from extracts of *Drosophila* are completely different entities (11), and yet can be separated by high-speed centrifugation (84). Biochemical analysis showed that the activity of RISC is regulated by sense-strand of siRNA. By comparing the prohibitive effects of 5'-phosphorylated and non-phosphorylated siRNA on expression of corresponding target mRNAs, Boutla and coworkers found that both types of siRNAs could suppress the expression of genes effectively (20). This result may be used to explicate that the converse sequence has the same effectiveness as the direct sequence because organisms really contain certain converse sequences within an encoding gene. It also suggests that the correctness of base-pairing of an antisense sequence and its cognate mRNA may be more important than the direction of antisense strand of siRNA. Evidence that 5' phosphate can be headed on siRNA by the enzyme supports the above notion (19).

Further study on siRNA architecture indicates that the inhibitive effect of the siRNA would decline remarkably if either sense or antisense strand of siRNA molecule is substituted by DNA strand (85). Another experiment from biochemical anatomy of siRNA structure (12,29,30) demonstrated that the alternation of the length of sense strand would influence the efficacy of the siRNA in cleaving corresponding mRNA target. The merging of these findings illustrates the overall relationships of siRNA' structure and the activity of RISC, suggesting that a double-stranded RNA molecule is required for the activity of an endonulease in RISC. Why does RISC need a double-stranded RNA? Three possibilities may exist: i) RISC cannot discriminate which strand of double-stranded siRNA molecule is the antisense complementary to a corresponding mRNA target, and therefore, both strands are required for a matching trial, through which the base-pairing can distinguish the antisense strand part from the sense strand of a siRNA held by RISC; ii) active molecules need a double-stranded RNA in searching its homologous region of target mRNA. Thus, when an RISC moves along cognate mRNA once not twice, it can find the sequence of an mRNA complementary to a strand of the siRNA; iii) the strand not for base-pairing may act as a activator or localization marker.

StRNA (antisense RNA) branch of RNA silencing pathway. Clear-cut evidence of RNA-mediated gene regulation has been found in the normal developmental process of cells (86-88). Intriguingly, sequences that meet the requirements for RNAi (inverted repeats in introns that could fold into an RNA hairpin loop which are homologous to sequences in the exons of other genes) are common in the human genome (37). Genes *lin-4* and *let-7* do not contain obvious protein-coding sequences, and the surrounding genomic sequences suggest that both are derived from non-coding genes for regulation or functional introns surrounded by vestigial exons (37,38). The expression of the heterochronic genes *lin-14* and *lin-41*, which adjust *C. elegans* temporal development, are regulated by *lin-4* and *let-7* stRNAs (89). The regulators can interact with repeated elements in the 3' non-translated region of target mRNAs, resulting in the termination of translation, but not the damage of the structure of mRNA.

Why does the RICS require double-stranded RNA, while alg1 and alg2 just need single-stranded RNA? The possible explanation may be that the specific structure of stRNA precursors force Dicer to cut off an antisense stRNA from one strand but not the other strand of its precursor. Alg1 and alg2 are not associated with RNAi. They may not have contact with RISC. This is one of the reasons why stRNA cannot degrade target mRNA. Another explanation may be that a

special bulge structure of stRNA blocks the RISC activity. If the phenomenon is really due to partial base pairing with its target, one artifact precursor with an antisense stRNA complementary to target mRNA perfectly should cause the degradation of target mRNA, and could be targeted to regions other than only 3' UTR of mRNA target. Direct experimental evidence is still lacking. If it is not so, why should miRNAs bypass RNAi and take other modes of action? The biggest difference between siRNA and miRNA is the resulting forms of mRNAs they induce, one is irreversible degradation and the other for reversible blockage. Obviously, cells want to intermittently use those mRNAs and/or get large amounts of corresponding proteins in a special time. These characteristic requirements could be found in the cases of nervous system and the differentiation of stem cells.

Because alg1/alg2 or alg1/alg2-like complex contains only one antisense strand or a mature stRNA molecule and may fail to have an endonucleolytic activity, it would become much safer for the asRNA or stRNA to interact with its target mRNA. Although several lines of laboratory results have shown that stRNA are not involved in RNAi or a RdRP reaction, the question of what mechanisms by which RdRP reaction can not be primed by this type of small RNA molecule remains. It is likely that the 3' terminal of stRNA is completely coated by alg1/alg2 proteins. Support for this notion comes from the analysis on the incomplete base-paring of stRNA with regulatory sequences in the 3'-untranslated regions of the mRNA and G/U mismatches. On one hand, the appearance of bulged nucleotides might cause the withdrawal of stRNA strand in alg1/alg2 proteins so that the free 3'-hydroxyl group could be embedded in the alg1/alg2 complex. Thus, stRNA does not act as primer for the RdRP reaction. On the other hand, alg1 and alg2 family may provide cells another device, which is to act as a carrier for the delivery of an antisense RNA completely complementary to target mRNA for RdRP reaction. This model awaits further experiments to examine whether alg1/alg2 genes are responsible for the delivery of both endogenous and exogenous single-strand antisense polarity or to see whether new members of PPD family serve as the carrier of asRNA for the PCR degradation pathway.

RdRP branch of RNA silencing pathway. The effects of RNAi can be maintained and propagated in plants and certain animals. The molecular mechanisms underlying these phenomena have been recently investigated by several groups of scientists. Strong evidence from several lines of experiments has indicated that the RNA-directed RNA polymerase (RdRP) is required for these RNAi features. About 3, 6, 4 or one RdRP genes have been identified such as rrpA in *Dictyostelium discoideum* (90), sgs2 in *Arabidopsis* (68), ego1 in *Caenorhabditis elegans* (91) and qde1 in *Neurospora* (92), respectively. The first direct evidence showed that RdRP can catalyse a cellular PCR on mRNA as a template and antisense siRNA as a primer (61). The genetic screen demonstrated that the production of *in vivo* 23 nt siRNA is dependent on both mRNA and RdRP. In the RdRP mutant strains, 23 nt siRNAs are not detectable (90). The genetic analysis and biochemical assay suggested that there exist three different PCR metabolic pathways for RNA-mediated signals, including unspecific degradation PCR (UD-PCR), specific degradation PCR (S-PCR), and specific interference PCR (SI-PCR). The UD-PCR refers to the PCR process by which many unspecific siRNAs that can trigger more than two family mRNA silencing are produced, while S-PCR just makes a population of siRNAs specific for a single gene or a set of family genes. These two types of PCR pathways may be initiated by three different modes. The first one is to copy a single-stranded RNA into dsRNA without the needs of any primers. The second is the PCR mediated by the direct interaction of an siRNA acting as a primer with an intact mRNA. The last one is composed of a cascade of reactions (Fig. 1). Interestingly, this model hypothesizes that the RISC pathway may be upstream from the degradative PCP pathway that may be necessary and important for the initiation of S-PCR.

Why are normal mRNAs not attacked by a degradative PCR pathway in cells? By what mechanisms do cells protect those normal RNAs, and degrade aberrant RNAs? In another words, how does an RdRP know to take an aberrant RNA but not a normal RNA as the template to synthesize a complementary strand? Although current *in vitro* experiments have shown that siRNA can act as a primer to initiate RdRP reaction, the key point is that the templates employed in experiments may be not the intact mRNA molecules while *in vivo* experiment cannot rule out the cooperation presented by RISC. In cell culture experiments, the mRNAs used as templates were processed by biochemical reagents, and might have been damaged. This result suggests that the initiation of RdRP reaction needs an injury signal of mRNAs. The signal flows to the specific degradation RdRP from the beginning of endonucleolytic cleavage at one or more sites, or to other degradation such as nonsense-mediated decay (NMD) pathway (93), un-primer degradative PCR reaction, or exonucleolytic decay. After the endonucleolytic cleavage of an mRNA was made, two unstable RNA fragments would be produced. One is 5' capped fragment and the other is 3' adenylated portion of target mRNA. It has been shown that the former may enter the pathway mediated by NMD, a highly conserved pathway in eukaryotes that targets and degrades RNAs containing a premature stop codon. Alternatively, 5' RNA fragment also can function as a template for the primer degradative PCR reaction, while 3' RNA fragment may prefer to be copied in an un-primer synthesis or a 5' to 3' degradation mediated by NMD pathway (Fig. 1).

The specific sequence, or the small fragment in target mRNA attacked by 21 nt siRNA can extend to a generalized sequence, or the whole sequence of an mRNA, even to other upstream sequences through the amplification mediated by RdRP reaction (61). Indeed, both the content and amount of trigger siRNA become enlarged and increased. The second generation of siRNAs becomes different from the first generation of 21 nt siRNAs (62). In content, the second generation of siRNAs contains not only the first generation of 21 nt siRNAs but also the siRNAs produced from the upstream sequence. In total, all the next generation of siRNAs upstream from the sequence as a primer is amplified because the primer will be used out as a primer at every RdRP cycle. Thus, the degradative PCR is not unlimited in amplification. Its limiting value is dependent on the length of sequence that is amplified. Interestingly, recent experiments have shown that the primed RdRP pathway can induce the

production of the secondary siRNAs (61), which in turn trigger a phenomenon known as 'transitive RNAi' (62). The secondary siRNAs may cause degradation of additional mRNAs. The emerging problem, however, may be the decrease in specificity. The data from multiple DNA sequence alignments and biochemical analysis have demonstrated that different regions in the same mRNA molecule can contain different amounts of homologous sequences from the same gene family and other gene families. The heading 21 nt sequence, middle 21 nt sequence and ending 21 nt sequence of an mRNA may be homologous to 2, 20, and 200 other corresponding mRNA molecules, respectively. Obviously, 21 nt dsRNA stemmed from different regions of RNAs can produce different specificity and sensitivity in silencing pertinent mRNAs. One may specifically suppress only one corresponding mRNA while others may degrade much more mRNAs containing the sequence identical to that of the siRNA used. In the efficacy, different sequences may have a wide range of changes in silencing their target mRNAs, because their competitive mRNA molecules are different in the number and expression levels. A particular siRNA produced from a degraded mRNA may also silence another mRNA's activity. And the short homologous sequence of either end in different genes may be targeted by siRNA molecule at the same time. It may be one of the molecular mechanisms by which a cluster of genes composed of many isoforms can be regulated in a naturally occurring manner (94). The same stream of genes may play similar or related roles in the growth, development and differentiation of cells.

PCR branch for stRNA precursor regulation. There is a lack of knowledge on the regulation of miRNA metabolic pathways although about 100 miRNAs have been found in different organisms (16,71). The question how to maintain sufficient amount of stRNA specific for its target mRNA remains to be answered. An attractive hypothesis is that the miRNAs may be amplified from their precursors (15). Although there is no direct evidence to support this notion, some indirect data from observation on miRNA precursors in *C. elegans* indicated that ~70 nt precursor can be detected as the major form of miRNAs from embryonic to adult worms. The localization studies revealed that processing of stRNA precursors occurred in the cytoplasm, suggesting that there is a life cycle for miRNA in cytoplasm in three steps including: i) synthesized precursor miRNAs are transferred into cytoplasm from nucleus; ii) precursor miRNAs are processed into a mature single-stranded RNA, and the other part of precursor by Dicer; and iii) RdRP reaction is initiated by using the cleaved 3' end of the lariat stRNA as a primer and the 5' portion of the lariat as a template to synthesize a new strand. However, this powerful generator to product regulators in the local area of cells is still a conjecture for experimental proof.

RdDM branch of RNA silencing pathway. Twenty nt double-stranded RNAs have been shown as the guide for an endonuclease complex that specifically cleaves mRNA, for an RdRP complex that initiates the degradative PCR reaction, or for a methyltransferase complex that methylates the genomic DNA containing its homologous sequence. DNA methylation can both directly suppress the expression of genes and increase the probability that affects genes to undergo a mutational event.

RNA-directed DNA methylation was first discovered by Wassenegger and coworkers in 1994 (95). In their investigation, replication of the potato spindle tuber viroid was shown to cause the hypermethylation of both the viroid transgene DNA and the tobacco genomic DNA. The above study, along with the observation from subsequent studies on plant transgene systems (96,97) demonstrated that DNA sequence as short as 30 basepairs could be targeted for RdDM at both symmetric (CpG or CpNpG) and non-symmetric sites. Research in animals to date have indicated that both transcriptional and post-transcriptional transgene silencing in *Drosophila* (64) and *C. elegans* (44) could be induced by the tranfection of a transgene construction or injection of dsRNAs. It has also been found that polycomb and a PAZ/Piwi protein are required for TGS. Their mutants can block PTGS and one portion of TGS. Biochemical analysis revealed that both polycomb and heterochromatin responsible for repression of gene expression contain a common structure known as the chromodomain. Interestingly, *in vitro* assays of the chromo-domain of the histone acetylase MOF in *Drosophila* have indicated that these special domains may act as RNA inter-action modules. The binding of specific RNA and the domain may guide regulators to cognate genomic DNA site (98). Although there is no evidence to support this notion that DNA methylation in mammalian cells could be mediated by siRNAs derived from viral genomic sequence, the possibility does not seem unreasonable. Increasing evidence indicates that distinct and abnormal patterns of methylation exist in cancers. In particular, methylation of the promoter regions of several genes, including known tumor suppressor genes, has been shown to induce the suppression of related gene expression. Just as the results caused by RNA-dependent DNA methylation, methylation within the promoter regions of tumor suppressor genes results in gene silencing, and methylation within the gene itself can induce mutational events. More importantly, tumor-causing viruses have, in fact, the capacity of inducing RdDM, because these viruses have their own RNA-dependent polymerases that are not found in human cells. When a viral sequence is inserted downstream of a host promoter, its high expression may cause siRNA generation and RNA degradation. Subsequently, resulting siRNA population should lead sequence-specific DNA modification and/or other inhibitive effects. However, this idea remains to be proved by future experiments.

5. Practical implications

With the accomplishment of human genome sequencing, scientists are eager to obtain a rapid, efficient and powerful tool for identifying gene function, regulating gene expression, and understanding relations among genes. Recently, more and more researchers and labs are becoming increasingly interested in investigating genomic functions and regulation of gene expression by using the sequence-directed technology. It is predicted that great theoretical values and huge application benefits exist for better understanding the sequence-mediated gene immunity and homology-directed gene regulation in cells.

Basic research. RNA-mediated gene regulation is superior to other methods in studying functional genomics (99). Its merits include but are not limited to: i) easily available compared to antibodies; ii) accessible to a scientist in a small lab (40); iii) short experimental period compared to gene knockout; iv) flexible for many different experimental conditions (100); v) high specificity and efficacy compared to antisense technology (12,29,30,40); and vi) high-throughput gene function identification (101). It has been recently shown that miRNA play very important roles in regulating the differentiation of stem cells. It is reasonable to hypothesize that many RNAs with their corresponding miRNAs may co-exist in a balancing status before the mitosis of stem cells. With many times of mitosis of stem cells, the original balancing has been altered: some mRNAs become more active in some cells than others owing to different assignment of regulating miRNAs, and thereby the differentiation of cells starts with a developmental cascade of cytoplasm and nucleus differentiation. An intriguing study has shown that lin-14 or lin-28 mRNAs remain associated with polyribosomes (102) when they are suppressed by their regulating lin-4 stRNA, implying that the protein synthesis may stop temporally, and will continue when the suppression is removed. Obviously, RNAi technology provides a new approach for the studying of how stem cells develop and differentiate.

Although there are high levels of adinosine deaminases in the nervous system, several successful experiments have been performed with RNAi technology (103). The formation of the pioneer pathway of axons and synaptogenesis were investigated by combinatorial RNAi (100). Indeed, RNA-mediated gene silencing system can be an excellent molecular microscopy by which the interaction of miRNAs with other repressive molecules can be observed in dendrites of neurons. If there is no synaptic transmission, synthesis of some proteins can be inhibited in post-synaptic sites. Once a synaptic transmission is coming, the miRNA complex will be removed away by other activated inhibitors. Thus, the peptides can immediately continue synthesizing. The initial and terminal signals for peptide synthesis may be a way to build up the basis for long-term memory.

RNAi technology also provides a useful tool to silence genes of interest in order to explore their characteristic phenotypes. In the current mammalian cell cultures, about 13 genes essential for the growth, survival and development of cells were investigated (10). The introduction of synthesized 21 nt siRNAs with 2 nt 3' overhang into human and other mammalian cells have been repeatedly proved to be effective in prohibiting different genes including onco-genes and other essential genes (104-106). More excitingly, recent advances in a large-scale suppression of gene expression have revealed that about 10,000 genes could be analyzed by a high-throughput RNAi-by-soaking technology, of which 600 displayed their own phenotypes (107). The combination of this method with genechip microarrays will undoubtedly promote the gene identification and phenotypic analysis.

Clinical perspective. Just as RNAi can be employed to silence endogenous genes, the introduction of siRNA or dsRNA corresponding to a viral genome would be possible to suppress the virus. This idea has been confirmed by the observation that dsRNA generated by a recombinant double-subgenomic Sindbis virus was able to prevent yellow fever virus and DEN-1, 2, 3 and 4 viruses (108-110). It has been shown that the transgene containing a hybrid sequence made from two different DEN viruses could protect cells of *A. aegypti* from the infection mediated by both DEN viruses containing the homologous sequence the transgene used. Similarly, using RNA interference technology, McRobert and McConkey have studied the human malaria parasite, *P. falciparum* (1). They first concluded that dsRNA could be used as a powerful tool for investigating gene functions of intracellular organisms, and potential approaches for developing related anti-malarial agents and vaccine. Taken together, we cannot imagine that cells could survive without this immune arm mediated by RNAs. It is no exaggeration to say that RNA-induced arm is essential for cells to counter the invasion of parasitic genetic elements, and sufficient for forming an intact immune system with both humoral and cellular arms in mammals.

A number of experimental studies have made it clear that different types of mRNAs can be efficiently, specifically and rapidly inactivated by powerful RNAi technology. Using stable production of siRNAs in numerous cell lines, Paddison and his colleagues (13) have demonstrated that continuous expression of dsRNA can induce stable homology-dependent suppression of the target gene. Phenotypic changes induced by complete knock-down of specific sequences can be used as reliable indications for genomic functional study and new gene identification. Thus, along with possible RdRP-mediated amplification of effectors in a cell (62) and inconceivable signal propagation among mammalian and human cells and tissues (111), long-term and complete silencing of oncogenes in tumor cells may be a new and powerful way for the prevention and treatment of cancer.

If over-expression of oncogenes is involved in uncontrolled growth and proliferation of cells, the specific suppression of oncogene activities can reverse the phenotype of tumor cells and control their growth and proliferation. This notion has been tested by a number of experiments. It has been discovered that tomato and *Arabidopsis* could be prevented from the crown gall tumorigenesis by RNAi-induced oncogene suppression (112). In those plant models, the expression of two oncogenes such as iaaM and ipt could be specifically degraded by the transformation with a transgene expressing two self-complementary iaaM and ipt constructions. Recently, we used human skin cancer cells as a model to test if small RNAs can silence their cognate mRNAs. Preliminary experimental results indicated that human melanoma cells could be prevented from abnormal proliferation following the transfection with a group of oncogene siRNA molecules (unpublished data). More recently, a new method for stable expression of siRNAs with a new pSuper vector in mammalian cells has been developed (113). The model will allow researchers to better analyze phenotypic changes caused by longer-time and complete suppression of gene functions. It can be envisioned that this exciting yet challenging technology will be widely used to dissect signaling pathways of cancer cells, to reverse functional phenotypes of tumor cells, to determine oncogene functions, to explore interactions among different genes, and eventually to provide clinical management of cancer.

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