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Review

Review

Functions and applications of RNA interference and small regulatory RNAs

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Abstract

Small regulatory RNAs play a variety of crucial roles in eukaryotes, influencing gene regulation, developmental timing, antiviral defense, and genome integrity via a process termed RNA interference (RNAi). This process involves Argonaute/small RNA (AGO/sRNA) complexes that target transcripts via sequence complementarity and modulate gene expression and epigenetic modifications. RNAi is a highly conserved gene regulatory phenomenon that recognizes self- and non-self nucleic acids, thereby defending against invasive sequences. Since its discovery, RNAi has been widely applied in functional genomic studies and a range of practical applications. In this review, we focus on the current understanding of the biological roles of the RNAi pathway in transposon silencing, fertility, developmental regulation, immunity, stress responses, and acquired transgenerational inheritance. Additionally, we provide an overview of the applications of RNAi technology in biomedical research, agriculture, and therapeutics.

Key words RNAi, siRNA, microRNA, piRNA

Introduction

Small regulatory RNAs direct sequence-specific regulation of gene expression via a mechanism termed RNA interference (RNAi), which was first described in nematodes in 1998 [1]. In a range of eukaryotic organisms, small regulatory RNAs and their associated Argonaute proteins play essential roles in RNAi-mediated gene silencing, whereby small regulatory RNAs guide the Argonaute-containing protein complexes to targeted nucleic acids with sequence complementarity [2–7]. Small RNA/Argonaute complexes modulate gene expression via several mechanisms, including degrading targeted RNAs, inhibiting translation, inducing epigenetic modifications and heterochromatin formation, and inhibiting transcription elongation or triggering alternative splicing [8–14].

There are three main classes of small regulatory RNAs: microRNAs (miRNAs), small interfering RNAs (siRNAs) and PIWI-interacting RNAs (piRNAs) [15–18]. Additional classes of small regulatory RNAs, such as antisense ribosomal siRNAs (risiRNAs) [19–21], tRNA-derived small RNAs (tsRNAs), and phased secondary small interfering RNAs (phasiRNAs), have also been shown to act through the RNAi pathway (Table 1) [22–25]. Notably, recent developments in novel RNA-sequencing techniques have significantly expanded our knowledge of small regulatory RNAs by overcoming sequencing obstacles, which are caused either by specific modifications or by terminus multiplicities of small RNAs. More importantly, many of these newly detected small RNAs have important functions in different biological processes, such as reprogramming, lineage specification and apoptosis [26–28].

Small regulatory RNAs are produced and function through a variety of mechanisms (Figure 1). In eukaryotes, most miRNAs are generated through the cleavage of a stem-loop precursor by the conserved RNase III-like ribonucleases Drosha, followed by export to the cytoplasm and processing by Dicer into 21–23 nucleotide mature miRNAs. Usually, miRNAs silence gene expression in the cytoplasm by degrading mRNAs or inhibiting translation [10,15,18,29]. siRNAs are generated by the cleavage of long double-stranded RNAs into mature 21–23 nt segments, which guide the RNA-induced silencing complex (RISC) to target mRNAs for degradation in the cytoplasm or induce epigenetic modifications

Table 1. The characteristics of different types of small regulatory RNAs

Classification	Length (nt)	Precursor	Production and processing	Function
microRNA (miRNA)	~20–25	hairpin loop-like precursor	transcribed by RNAP II, cleaved by Drosha and Dicer	transcriptional gene silencing, inhibition of translation and inducing mRNA decay
PIWI-interacting RNA (piRNA) (*Drosophila*, mammals etc)	~18–30	long single-stranded precursor	transcribed by RNAP II, followed by processing pathway, amplified by ping-pong amplification involving Zuc, Aub and AGO3 in *Drosophila*	silence gene expression and regulate genome stability
21U RNA (nematode)	21	pre-piRNA precursor	start with U, transcribed by RNAP II, followed by processing pathway	silence gene expression and regulate genome stability, recognize self and non-self nucleic acids
phasiRNA (plant)	21 or 24	long dsRNA	iteratively cleaved by Dicer	mRNA degradation and development regulation
Small interfering RNA (siRNA)	~20–25	long dsRNA	transcribed by RNAP II, virus-derived, cleaved by Dicer	mRNA decay and epigenetic modification, transgenerational inheritance
Secondary siRNA (nematode)	~22		starting with G, using mRNA as templates, synthesized by RdRP	mRNA decay, inhibit RNAP II transcription and epigenetic modification
Antisense ribosomal siRNA (risiRNA)	~22		starting with G, using rRNA as templates, synthesized by RdRP	rRNA decay, inhibit RNAP I transcription
tRNA-derived small RNA (tsRNA)	~15–40	tRNA	cleaved by Angiogenin, Dicer, RNase T2, RNase Z, ELAC2 and Rnylp, etc	mimicry or displacement of tRNA, or acting like microRNA
rRNA-derived small RNA (rsRNA)	~21–44	rRNA	unclear	unclear, may involve in transgenerational inheritance

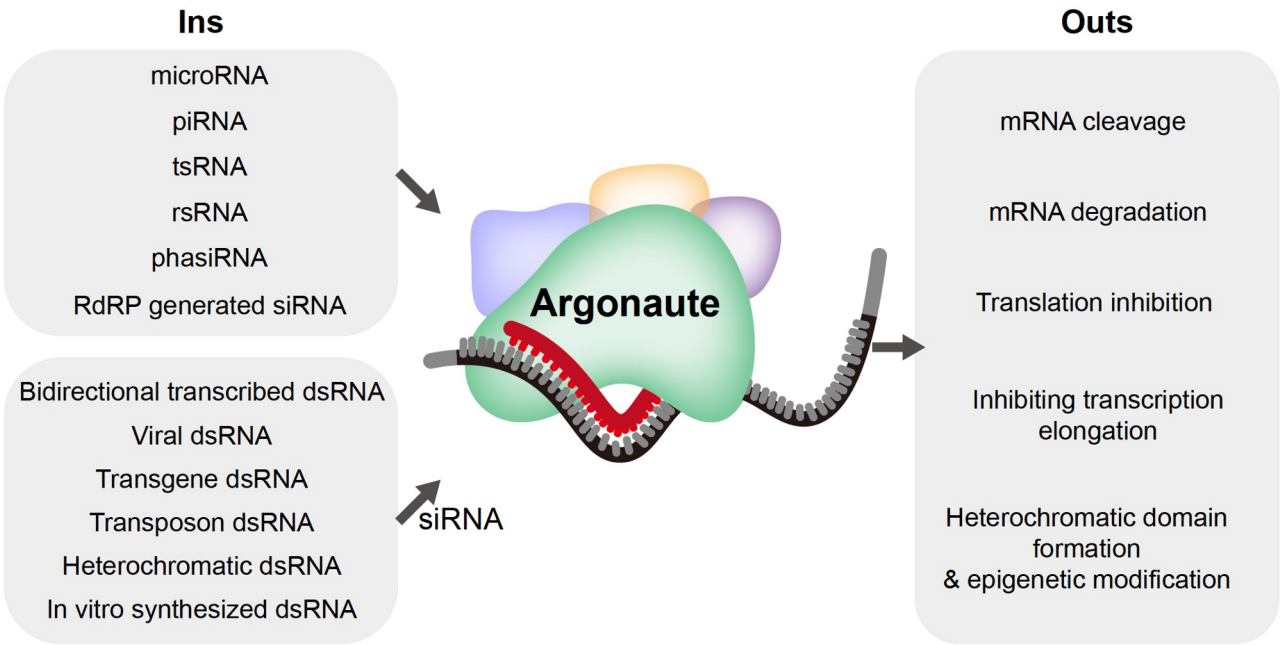

Figure 1. The inputs and outputs of RNAi Small regulatory RNAs can be derived from multiple inputs, which subsequently associate with conserved Argonaute family proteins. Small regulatory RNA/Argonaute complexes then recognize their targeted nucleic acids harboring complementary sequences and conduct downstream gene silencing via a variety of transcriptional and post-transcriptional mechanisms.

and inhibit transcription elongation in the nucleus [11,13,18]. In animal gonads, piRNAs are generated via distinct mechanisms in different organisms [30,31]. The piRNA pathway is thought to recognize nascent transcripts of selfish genetic parasites, such as transposable elements (TEs), and guide sequence-specific silencing at both the cotranscriptional and posttranscriptional levels [32,33]. Recent evidence indicates that piRNAs are involved in regulating the expression of protein-coding genes [34,35]. The production of risiRNAs is elicited by erroneous ribosomal RNA sequences, which subsequently silence rRNA expression in the nucleolus to maintain the balance of ribosome biogenesis [14,20]. Depending on the splicing site, tsRNAs can be divided into two main classes: tRNA halves (tiRNAs) and tRNA-derived fragments (tRFs) of 18–20 nt. They have been suggested to exert regulatory functions similar to

those of siRNAs or miRNAs rather than performing the roles of tRNAs in translation [22,24].

In addition to their roles in RNAi-like mechanisms, some small regulatory RNAs also exhibit AGO-independent base pairing or 3D structure-based aptamer-like functions [36,37]. For example, in mouse cardiomyocytes, miR-1 interacts with the potassium channel protein Kir2.1 and regulates cardiac electrophysiology through a core sequence located outside its RNAi seed region [38]. Additionally, certain 5′ tiRNAs can assemble into a tetrameric G-quadruplex (G4) structure, which interacts with the translation initiation complex eIF4F and disrupts its stability. This G4 structure formation depends on a stretch of oligoguanine at the 5′ end of the tiRNAs [39]. Overall, the diverse types and working mechanisms of small regulatory RNAs underscore their crucial roles in regulating gene expression and maintaining cellular homeostasis.

Since its discovery, RNAi has been used as a powerful experimental technique for studying gene functions in a wide range of organisms. Small regulatory RNAs of different structures and origins have become increasingly recognized for their crucial roles in regulating gene expression across a wide range of biological processes in eukaryotes, such as fertility, development, immunity and maintenance of genome stability. Progress in understanding the mechanism of RNAi and the biogenesis and functions of small regulatory RNAs has led to important practical applications in therapeutics, agricultural biotechnology and many other areas.

Functions of RNAi

RNAi and transposon suppression

TEs are common in all living organisms. These repetitive elements are interspersed throughout the genome and can move from one region to another. By inserting themselves into various locations within the host genome, TEs can cause sequence alterations, abnormal chromosomal recombination and consequently genome instability. To protect the genome, hosts have developed a number of defense mechanisms, among which small regulatory RNAs and RNAi play crucial roles [40,41].

RNAi-based TE silencing was first reported in *C. elegans* in 1999. Forward genetic screening was conducted to isolate RNAi-defective (Rde) mutants, and the results revealed that these mutants also presented increased rates of transposition [42–44]. *Mutator* (*Mut*) genes, which are known to suppress transposons in *C. elegans*, were cloned from the collected Rde mutants. A subsequent deep sequencing study revealed numerous TE-derived siRNAs from the nematode genome, further supporting the role of RNAi in TE suppression [45].

In most animals, piRNAs, in conjunction with Piwi proteins, are responsible for silencing TEs in the germline. In *C. elegans*, the majority of piRNA genes are located within two clusters on chromosome IV [46]. Following transcription and processing, the mature piRNAs bind with PRG-1, the sole functionally characterized Piwi ortholog in *C. elegans* [47]. The piRNA-PRG-1 complex targets transposon RNAs, which are subsequently cleaved by the endonuclease RDE-8 and poly-tailed by the poly (UG)-polymerase RDE-3 [48–52]. Although the piRNA-PRG-1 complex can reduce the abundance of TE RNAs, its effect is significantly enhanced by the generation of secondary siRNAs. The poly (UG)-tailed transposon RNAs serve as templates and further recruit RNA-dependent RNA polymerases (RdRPs) to generate secondary siRNAs. These abundant secondary siRNAs either bind with an expanded group of worm-specific AGOs (WAGOs) to mediate posttranscriptional gene silencing (PTGS) or associate with the nuclear Argonaute protein HRDE-1 to inhibit transposon transcription through the nuclear RNAi pathway [53–55].

Although RNAi-based TE suppression is particularly important for both plants and invertebrates, it also plays critical roles in repressing the activities of TEs in many other eukaryotes [56,57]. For example, in mammals, mutations in two of the three mouse *Piwi* homologs lead to increased expression of some TEs, which leads to male infertility [58,59].

piRNAs and fertility maintenance

The roles of PIWI proteins and their associated piRNAs in fertility have been widely studied. In *Drosophila*, PIWI proteins are essential for primordial germ cell specification and differentiation [60–62]. More recently, it was reported that deletion of the piRNA clusters *Su* (*Ste*) and *flam* caused infertility in male and female *Drosophila*, respectively, providing more direct evidence of the importance of piRNAs in reproduction [63–66].

In *C. elegans*, piRNAs are depleted in *prg-1* mutants. The *prg-1* mutant or its downstream nuclear RNAi-defective (Nrde) mutants displayed progressive sterility, which is termed the mortality (Mrt) phenotype [67,68]. Interestingly, *prg-1* mutation or piRNA depletion shifted the cellular RNAi machinery toward the progressive accumulation of risiRNAs and ultimately led to Mrt [21,69]. Moreover, recent progress in the study of the piRNA transcription machinery revealed that the chromodomain protein UAD-2 directs clustered piRNA expression in a temperature-sensitive manner. At elevated temperatures, *uad-2* mutants expressed much lower levels of piRNAs and presented a temperature-sensitive fertility defect [70,71].

PIWI proteins are conserved across various organisms. In mice, three PIWI homologs, MIWI, MILI and MIWI2, function in different stages of spermatogenesis [72,73]. Most species, however, encode an additional PIWI gene, PIWIL3, which is highly expressed throughout oogenesis in humans, bovines and hamsters [74,75]. In golden hamster, PIWIL3 mutants present a decreased 19 nt piRNA population in oocytes and reduced female fertility [74,76,77].

Humans have all four PIWI proteins. Mutations in PIWIL1 have been identified in patients with azoospermia, and modeling such mutations in mice suggests that one role of PIWI proteins is to regulate histone-to-protamine exchange during spermiogenesis [78–80]. Additionally, defects in piRNA processing may also lead to male infertility. For example, poly(A)-specific RNase-like domain containing 1 (PNLDC1), which participates in the maturation of piRNAs by trimming the 3′ end of pre-piRNAs, is essential for male fertility in mice [81]. Patients with azoospermia who carry PNLDC1 mutations exhibit diminished expressions of piRNA-processing proteins, lower levels of pachytene piRNAs and spermatogenic arrest [80,82].

RNAi and developmental regulation

Small regulatory RNAs and the RNAi pathway are also well known for their roles in developmental regulation by directing the precise spatiotemporal expression of specific genes. Genes involved in developmental processes are often enriched for miRNA binding sites [83,84], and animals that are unable to produce mature miRNAs usually do not grow or reproduce [85,86]. The first two

identified miRNAs, *lin-4* and *let-7*, were both found to regulate developmental processes in *C. elegans*. *Lin-4* suppresses the expression of the heterochronic gene *lin-14* to allow the transition from larval stage L1 to L2 [87], whereas *let-7* modulates the expression of multiple genes to promote progression to adulthood [88,89]. In *Drosophila*, both Ago1 and Dicer1 are required for miRNA-induced gene silencing, and *ago1*; *dcr*-1 double mutant embryos present reduced Wg protein (Wingless) expression and strong segmentation defects [90]. Similarly, in *Arabidopsis*, mutations in the Dicer homolog CARPEL FACTORY lead to developmentally defective leaves and excessive proliferation of floral meristems [91].

In mammals, although some miRNA-depleted tissues are able to differentiate and pattern properly, the morphogenesis of many tissues or organs is often disrupted [29,92]. In mice, miRNA-133 promotes the differentiation of adult satellite cells into brown adipose tissue by targeting the transcription factor Prdm16 [93], and depletion of miR-133 leads to reduced brown adipose tissue formation and potentially affects energy metabolism and thermoregulation. During epidermal development, miR-205 is crucial for the proliferation and maintenance of follicular progenitors because it targets genes involved in cell cycle regulation and differentiation, such as *ZEB1*, *Foxo1* and *PTEN*. Ablation of miR-205 impairs the proliferation of follicular progenitors and causes severe defects in epidermal growth [94,95]. During lung development, more than 100 miRNAs are differentially expressed. Some of these miRNA clusters were found to regulate the balance between the proliferation and differentiation of the lung epithelium or affect the size of the lumen during a specific developmental window [96]. Thus, RNAi pathways, particularly those regulated by miRNAs, are required for the proper timing and direction of development [92].

RNAi and anti-stress reaction

Organisms are challenged by enormous amounts of endogenous and environmental stimuli, which can affect the homeostasis of a variety of RNAs. In *C. elegans*, lower temperatures, UV irradiation, and deficiencies in rRNA modification or splicing can result in the accumulation of risiRNAs [97]. In turn, risiRNAs silence pre-rRNA expression via the nucleolar RNAi pathway [14]. A similar phenomenon has also been observed in *Arabidopsis*: aberrant rRNA processing events in fiery1 (fry1) mutants or xrn2 xrn3 double mutants are accompanied by risiRNA production [98]. Therefore, the risiRNA/RNAi-directed feedback loop is speculated to act as a mechanism to prohibit the accumulation and spread of erroneous rRNAs in the population.

In plants, various abiotic stresses, such as salinity, drought, heat and cold, also elicit differential expression of different types of small regulatory RNAs [99,100]. For example, under high-salinity stress, many miRNAs are up- or downregulated in maize [101]. Some of these miRNAs target transcription factors involved in plant development and organ formation, such as MYBs, NAC1, and HD-ZIP. In *Arabidopsis*, miR319 is upregulated in response to salt stress [102]. Interestingly, overexpressing one member of the rice miR319 gene family, Osa-miR319b, led to increased tolerance to cold in rice [103,104]. Another abiotic stress that threatens the health of plants is heavy metal pollution, which is often resulted from the large-scale use of chemical fertilizers and pesticides. Recent studies have shown that following metal exposure, a group of miRNAs exhibit differential expression, which may coordinate with plant responses through regulating antioxidant functions, root growth, hormone signals, and the expressions of metal transporters [105,106].

Like normal physiological conditions, some of these abiotic stress-regulated small regulatory RNAs induce RNAi responses via transcript cleavage, translation inhibition, or changes in epigenetic modifications [107–109]. In plants, RNAi responses are often mediated via the hypermethylation of DNA sequences, which is commonly known as the RNA-directed DNA methylation (RdDM) pathway [110,111]. Plants employ RNA polymerase IV, RNA-dependent RNA polymerase 2, and Dicer-like protein 3 for the generation of 24-nt siRNAs. These 24-nt siRNAs initiate the formation of a silencing effector complex, which directs the methylation of homologous DNA loci [112]. In *Arabidopsis*, this canonical RdDM pathway accounts for at least one-third of the methylated loci [113]. Additionally, these DNA methylations could serve as transgenerational stress memories, allowing subsequent generations to adapt better to future exposures [110,113].

RNAi and pathogen defense

RNAi is a strong antiviral defense mechanism in plants, worms, and insects [114,115]. Upon infection, RNA viruses generate dsRNAs, which are further processed by Dicer into 19–25 siRNAs and loaded into Argonaute-containing complexes for the cleavage and degradation of viral RNA [116]. In addition to these virus-induced primary siRNAs, which are usually expressed at low levels, *C. elegans* and plants can generate more antiviral siRNAs through RdRP-mediated amplification [117,118]. These secondary siRNAs also serve as important prerequisites for non-cell-autonomous RNAi, by which the silencing signal is transported from one cell to another or even to distant tissues [119].

Mammalian cells lack RdRPs. Thus, the level of virus-specific siRNAs is relatively low [117]. Upon viral infection, mammalian cells activate the interferon response by inducing the expressions of interferon-stimulated genes (ISGs), which are the first line of antiviral defense [120]. Nevertheless, recent evidence has also suggested a direct role of RNAi in controlling viral infection in mammalian cells. After viral infection, multiple virus-derived siRNAs are detected via deep sequencing in mammalian host cells. In addition, viral replication is enhanced in cells with mutations in the components of the RNAi machinery [121–123]. It is likely that the RNAi and interferon pathways are both critical components of antiviral innate immunity.

In addition to functioning in viral infection, RNAi is also a common defense mechanism against other pathogens. In *Arabidopsis*, *Ago2* mutants are more susceptible to *Pseudomonas syringae* pv. *tomato* (*Pst*) infection, the most well-characterized bacterial disease in plants [124]. Leaves treated with *Pst*-derived peptide express relatively high levels of miR-393, which confers antibacterial defense through the suppression of auxin signaling [125]. In addition, RNA-dependent RNA polymerase 6 (RDR6) is a key RNAi factor against bacterial infection in plants. RDR6 is involved in the biogenesis of the bacterium-induced long siRNA AtlsiRNA-1 and natural antisense transcript (NAT)-associated siRNAs. Consequently, *rdr6*-knockout mutants are highly susceptible to various bacterial infections [126,127].

RNAi and transgenerational inheritance

Organisms have developed a variety of strategies to remember their exposure to invaders and transmit experiences to their descendants.

Some of the information is transmitted in the form of non-DNA sequence-based signals, such as DNA methylation, histone modification and noncoding RNAs [128–131]. It is proposed that the inherited signals are either preserved during gametogenesis and fertilization and thus directly transmitted from the parental generation to the progeny or that the primary epigenetic signals are erased but later reconstructed from various secondary signals [132].

The ability of small regulatory RNAs to induce gene silencing over generations has been known since the discovery of the RNAi phenomenon in *C. elegans* [133]. Upon stimulation or infection by nonself nucleic acids, small regulatory RNAs direct the RNAi machinery to deposit suppressive epigenetic modifications on the targeted locus, which can persist for at least tens of generations [53,134–136]. These transgenerational suppression effects depend on the RdRP-mediated production of secondary 22G siRNAs, which ensures the maintenance of these signals across many generations without dilution [137,138]. Certain environmental conditions, such as starvation or heat, can also induce the expression of a set of endogenous, transgenerationally transmitted siRNAs for at least three to four generations. Importantly, these starvation-induced siRNAs target genes involved in metabolism, and the descendants of the starved parents consistently exhibit increased lifespans compared with those of control worms [139,140].

During the early development of mammals, reprogramming occurs in primordial germ cells and early embryos, which potentially resets chromatin modifications and limits transgenerational information flow [141]. Despite these barriers, recent studies in mice suggest that sperm small regulatory RNAs, particularly miRNAs, tsRNAs, and certain RNA modifications, may serve as vectors for epigenetic inheritance [142–144]. For example, stress exposure can increase the expression of a group of miRNAs in sperm, which contributes to reduced hypothalamic–pituitary–adrenal (HPA) axis responsivity in offspring [145]. Additionally, injecting small regulatory RNAs derived from the sperm of male mice fed with a high-fat, high-sugar diet into normal zygotes produced offspring with traits reflecting paternal metabolic disorders [142,146,147].

Despite these intriguing observations in mice, several issues remain to be addressed in future studies. One critical issue is to carefully track how many generations of these inherited epigenetic signals can persist. Additionally, since mammals lack RdRP or its orthologs, elucidating the mechanism underlying the maintenance of small regulatory RNAs in each generation will also be critical for fully understanding the mechanism of transgenerational inheritance in mammals.

The Applications of Small Regulatory RNAs and RNAi

RNAi as a research tool for reverse genetics

For more than 25 years after its discovery, RNAi has been widely used as a pivotal reverse genetic tool in functional genomic studies. By harnessing the specificity of small regulatory RNAs, RNAi enables the targeting of any specific genes and uncovering their roles in development, metabolism, aging, stress response and other critical processes [148–151]. A variety of powerful RNAi-based research products have been developed for high-throughput screening. In *C. elegans*, for example, Ahringer and colleagues produced an RNAi library, which represents approximately 86% of the genes of *C. elegans* [152, 153]. In *Drosophila*, a genome-wide transgenic RNAi library for conditional gene inactivation was also generated, which covers 88% of the predicted protein-coding genes in the *Drosophila* genome [154,155]. A similar strategy has also been successfully applied in multiple other model organisms as well as in humans [57,156–160].

Applications of RNAi in agricultural biotechnology

In agricultural biotechnology, RNAi has been exploited to improve plant agronomic traits by modifying their biochemical and physiological characteristics. For example, plant biologists use this tool to manage pest and pathogen infection, increase abiotic stress tolerance and improve yield and nutritional quality [161–163].

RNAi can be used to increase crop yield by regulating meristem activities and growth patterns. Knocking down *OsDWARF4*, a gene encoding a C-22 hydroxylase in rice, results in dwarf plants with erect leaves. This characteristic potentially enhances photosynthesis in the lower leaves, thereby improving yields in densely growing environments [164]. In potato, downregulating the expression of sucrose-phosphatase SPP in potato tubers via siRNA reduces SPP activity, which greatly affects the hexose-to-sucrose ratio of the tubers [165]. In wheat, the expression of hairpin RNA fragments derived from *Fusarium graminearum* chitin synthase-3b (*FgChs3b*) enhances plant resistance to Fusarium head blight, a serious wheat disease caused by pathogenic fungi [166].

Currently, the applications of RNAi technology in agriculture are largely based on the generation of transgenic plants that express dsRNAs [167–169]. Alternatively, exogenous dsRNAs or siRNAs can be introduced through microinjections, soaking or direct spraying on leaves [170–172]. For example, topical application of *in vitro*-produced dsRNA molecules derived from either the coat protein or the proteinase of Papaya ringspot virus-Tirupati (PRSV) isolate can efficiently protect papaya against PRSV infection [173].

Although the application of RNAi technology for plant protection and improvement appears promising, several issues, such as optimizing the concentration, length and stability of the applied regulatory RNAs, improving the efficiency of production and delivery techniques, and minimizing off-target effects, need to be resolved before practical applications can be fully realized.

Application of small regulatory RNAs in diagnosis

Aberrant expression of small regulatory RNAs has been linked to a wide range of human diseases, such as cancers, neurological disorders, infectious diseases, and cardiovascular diseases [174,175]. In diseased tissues, the expression of small regulatory RNAs is usually dysregulated as a consequence of perturbed genomic structure, epigenetic modifications or transcription activities [176,177]. Understanding the physiological meaning and underlying mechanism of these changes could pave the way for the development of innovative diagnostic and intervention tools. This is particularly important for the diagnosis of certain cancers, for which late detection leads to poor prognosis, and ideally, for the discovery of accurate biomarkers to achieve personalized treatment.

Although many small regulatory RNAs have shown potential for use as biomarkers, only a small fraction of them have reached clinical trials [178–180]. One of the most broadly tested small regulator RNA markers in cancer is miR-21-5p, which has been validated as a diagnostic and prognostic biomarker in some highly prevalent cancers, such as lung cancer, breast cancer and colorectal

cancer [181,182]. It has also been reported that the use of a miRNA classifier, which profiles the expression of multiple correlated miRNAs, could yield more reliable results for the diagnosis of certain diseases [183,184]. Notably, in addition to small regulatory RNAs, long non-coding RNAs (lncRNAs) are also potential diagnostic markers for many clinical diseases. For example, HOTAIR, a lncRNA involved in chromatin reprogramming, has been tested as a diagnostic biomarker in ovarian, colorectal, breast, and pancreatic cancers [185–187].

Despite recent technological advancements, there are also some technical limitations to be resolved before small regulatory RNAs, and more broadly, non-coding RNAs, can be widely utilized in diagnostics. Some of the current difficulties include understanding their precise functions, elucidating their disease specificity and accurate quantification [180,188,189].

RNAi and siRNAs in therapy

The concept of using nucleic acids as drugs emerged in the 1970s with the rapid progress of oligonucleotide synthesis techniques, and the discovery of RNAi as a fundamental mechanism for silencing gene expression further suggested that small regulatory RNAs hold great potential in the development of new drugs and therapeutic approaches [190–192]. The prospect of controlling any disease-associated gene by simply synthesizing and applying sequence-specific siRNAs appears compelling and promising. For example, viral and human genes that are needed for viral replication can be targeted to generate virus-resistant host cells [193,194], and tumor growth can be inhibited by targeting oncogenes essential for malignant cell replication or molecules important for neovascularization [195–198].

Although many proof-of-concept studies have revealed the potential effectiveness of RNAi-based therapies, early siRNA-derived drugs exhibit a variety of dose-related toxicities or insufficient therapeutic activity. In addition, there are a number of side effects, such as undesired immunostimulatory activity, competition for endogenous RNAi pathway components, and off-target effects [199–201]. In the past few years, with major technical breakthroughs being made in sequence selection, chemical modification, compound delivery, and even synergistic therapeutic approaches, safer and more effective siRNA-based drugs have been developed [202–206]. For example, the conjugation of siRNAs to GalNAc (N-acetylgalactosamine) represents a breakthrough in the tissue-specific targeting of RNA therapeutics, which specifically enhances the uptake of RNA molecules by hepatocytes. Twenty years after the discovery of the RNAi phenomenon, the FDA approved the first siRNA drug, patisiran, for the treatment of hereditary transthyretin amyloidosis [207–209]. By 2024, five additional siRNA therapeutics have been approved, and all six drugs target disease-causing genes in the liver [197,210,211]. Efforts to achieve robust gene silencing in other tissues, such as the central nervous system, eye, lung, and muscle, are rapidly increasing, which will remarkably increase the number of diseases that can be treated by RNAi therapeutics. Currently, multiple candidates for treating kidney injury and eye diseases are in phase II and III clinical trials [197,212–214].

Perspective

Soon after the discovery of RNAi in *C. elegans*, Tuschl and Elbashir demonstrated that RNAi could also function through synthetic 21-nucleotide RNA duplexes in human cells in 2001 [91]. These dsRNAs can be readily synthesized, transfected into cultured cells, and used to control gene expression, suggesting their enormous potential for future applications.

Although the natural mechanisms of small regulatory RNA biogenesis and RNAi have largely been understood after twenty years of extensive study and RNAi and synthetic RNAs have already been widely used as laboratory tools for controlling gene expression, a number of critical questions remain unresolved, both in basic research and in clinical applications.

One critical question is how cellular RNAs are selected for targeting by particular small regulatory RNA pathways. The molecular characteristics of certain genes or transcripts, including their sequence context, strength of regulatory elements, and transcript localization, are among the many features that may contribute to this selection [51]. In plants, Argonaute proteins may determine their bound siRNA partners by recognizing the 5′-end nucleotides of the siRNAs [215]. However, the mechanism by which certain small regulatory RNAs are selected for inclusion in exosomes and subsequently translocated to neighboring or distant cells remains largely unknown [216].

Although RNAi can take place in the cytoplasm as well as in the nucleus via a range of distinct mechanisms, it is unclear how RNAi can be conducted in particular subcellular organelles. Both risiRNAs and mitochondrial miRNAs can silence gene expression at specific cellular locations [217,218]. However, whether and how other organelle-specialized RNAi processes occur remain intriguing.

Third, although the use of dsRNAs and synthetic siRNAs is relatively straightforward, off-target effects are frequently identified. Small regulatory RNAs could have partial complementarity to many other RNA targets, since the complementarity of as few as 6-8 base sequences (seed sequences) between siRNAs and their targets is sometimes sufficient for gene silencing. Additionally, a high dose of introduced small regulatory RNAs can overwhelm the cellular RNAi machinery, leading to off-target silencing and disrupted cellular processes. More critically, dsRNAs can also bind with certain proteins in a sequence-independent manner, and these nonspecific interactions may induce cellular stress, such as interferon responses, which may lead to additional off-target effects.

Fourth, although the chemical synthesis of small regulatory RNAs is quite easy, these nucleic acids frequently require chemical modifications to improve their stability, efficient delivery, and potent gene knockdown [219]. Whether and how these chemical modifications can increase the stability and transportability of siRNA drugs is intriguing. Advances in understanding the chemical and biological characteristics of small RNAs will facilitate their clinical design and continue to increase the likelihood of their successful application.

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Conflict of Interest

The authors declare that they have no conflict of interest.

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