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
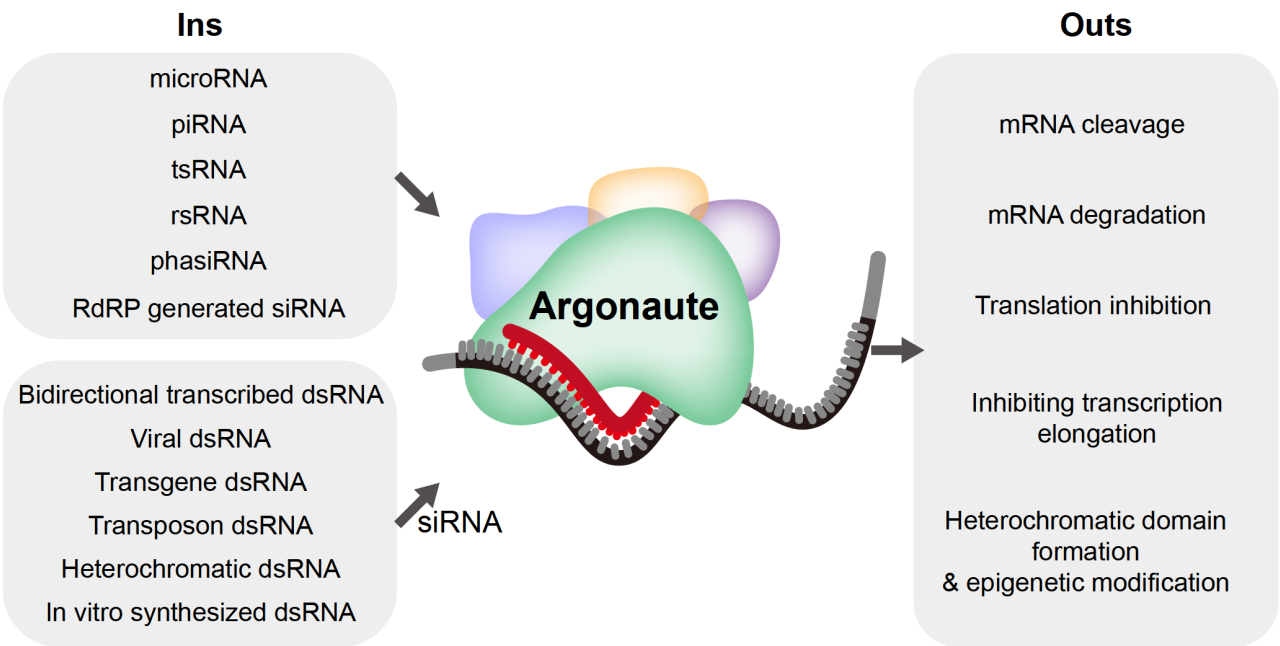
Figure 1. The inputs and outputs of RNAi Small regulatory RNAs can be derived from multiple inputs, which subsequently associate with conserved Argonaute family proteins. Small regulatory RNA/Argonaute complexes then recognize their targeted nucleic acids harboring complementary sequences and conduct 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 *Drosophil*a, 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.

Conflict of Interest

The authors declare that they have no conflict of interest.

References

1. Fire A, Xu SQ, Montgomery MK, Kostas SA, Driver SE, Mello CC. Potent and specific genetic interference by double-stranded RNA in *Caenorhabditis elegans*. *Nature* 1998, 391: 806–811

2. Hammond SM, Bernstein E, Beach D, Hannon GJ. An RNA-directed nuclease mediates post-transcriptional gene silencing in *Drosophila* cells. *Nature* 2000, 404: 293–296

3. Nykänen A, Haley B, Zamore PD. ATP requirements and small interfering RNA structure in the RNA interference pathway. *Cell* 2001, 107: 309–321

4. Hammond SM, Boettcher S, Caudy AA, Kobayashi R, Hannon GJ. Argonaute2, a link between genetic and biochemical analyses of RNAi. *Science* 2001, 293: 1146–1150

5. Zamore PD, Tuschl T, Sharp PA, Bartel DP. RNAi: double-stranded RNA directs the ATP-dependent cleavage of mRNA at 21 to 23 nucleotide intervals. *Cell* 2000, 101: 25–33

6. Tabara H, Yigit E, Siomi H, Mello CC. The dsRNA binding protein RDE-4 interacts with RDE-1, DCR-1, and a DExH-Box helicase to direct RNAi in *C. elegans*. *Cell* 2002, 109: 861–871

7. Ketting RF, Fischer SEJ, Bernstein E, Sijen T, Hannon GJ, Plasterk RHA. Dicer functions in RNA interference and in synthesis of small RNA involved in developmental timing in *C. elegans*. *Genes Dev* 2001, 15: 2654–2659

8. Bernstein E, Allis CD. RNA meets chromatin. *Genes Dev* 2005, 19: 1635–1655

9. Matzke MA, Birchler JA. RNAi-mediated pathways in the nucleus. *Nat Rev Genet* 2005, 6: 24–35

10. Carthew RW, Sontheimer EJ. Origins and mechanisms of miRNAs and siRNAs. *Cell* 2009, 136: 642–655

11. Guang S, Bochner AF, Burkhart KB, Burton N, Pavelec DM, Kennedy S. Small regulatory RNAs inhibit RNA polymerase II during the elongation phase of transcription. *Nature* 2010, 465: 1097–1101

12. Ketting RF. The many faces of RNAi. *Dev Cell* 2011, 20: 148–161

13. Castel SE, Martienssen RA. RNA interference in the nucleus: roles for small RNAs in transcription, epigenetics and beyond. *Nat Rev Genet* 2013, 14: 100–112

14. Liao S, Chen X, Xu T, Jin Q, Xu Z, Xu D, Zhou X, *et al*. Antisense ribosomal siRNAs inhibit RNA polymerase I-directed transcription in *C. elegans*. *Nucleic Acids Res* 2021, 49: 9194–9210

15. Bartel DP. MicroRNAs: genomics, biogenesis, mechanism, and function. *Cell* 2004, 116: 281–297

16. Ghildiyal M, Zamore PD. Small silencing RNAs: an expanding universe. *Nat Rev Genet* 2009, 10: 94–108

17. Bagijn MP, Goldstein LD, Sapetschnig A, Weick EM, Bouasker S, Lehrbach NJ, Simard MJ, *et al*. Function, targets, and evolution of *Caenorhabditis elegans* piRNAs. *Science* 2012, 337: 574–578

18. Okamura K, Lai EC. Endogenous small interfering RNAs in animals. *Nat Rev Mol Cell Biol* 2008, 9: 673–678

19. Hang R, Xu Y, Wang X, Hu H, Flynn N, You C, Chen X. *Arabidopsis* HOT3/eIF5B1 constrains rRNA RNAi by facilitating 18S rRNA maturation. *Proc Natl Acad Sci USA* 2023, 120: e2301081120

20. Zhou X, Feng X, Mao H, Li M, Xu F, Hu K, Guang S. RdRP-synthesized antisense ribosomal siRNAs silence pre-rRNA via the nuclear RNAi pathway. *Nat Struct Mol Biol* 2017, 24: 258–269

21. Wahba L, Hansen L, Fire AZ. An essential role for the piRNA pathway in regulating the ribosomal RNA pool in *C. elegans*. *Dev Cell* 2021, 56: 2295–2312.e6

22. Xie Y, Yao L, Yu X, Ruan Y, Li Z, Guo J. Action mechanisms and research methods of tRNA-derived small RNAs. *Sig Transduct Target Ther* 2020, 5: 109

23. Kim HK, Fuchs G, Wang S, Wei W, Zhang Y, Park H, Roy-Chaudhuri B, *et al*. A transfer-RNA-derived small RNA regulates ribosome biogenesis. *Nature* 2017, 552: 57–62

24. Sobala A, Hutvagner G. Transfer RNA-derived fragments: origins, processing, and functions. *Wiley Interdiscip Rev RNA* 2011, 2: 853–862

25. Liu Y, Teng C, Xia R, Meyers BC. PhasiRNAs in plants: their biogenesis, genic sources, and roles in stress responses, development, and reproduction. *Plant Cell* 2020, 32: 3059–3080

26. Shi J, Zhang Y, Tan D, Zhang X, Yan M, Zhang Y, Franklin R, *et al*. PANDORA-seq expands the repertoire of regulatory small RNAs by overcoming RNA modifications. *Nat Cell Biol* 2021, 23: 424–436

27. Wang H, Huang R, Li L, Zhu J, Li Z, Peng C, Zhuang X, *et al*. CPA-seq reveals small ncRNAs with methylated nucleosides and diverse termini. *Cell Discov* 2021, 7: 25

28. Lai H, Feng N, Zhai Q. Discovery of the major 15-30 nt mammalian small RNAs, their biogenesis and function. *Nat Commun* 2023, 14: 5796

29. Guo H, Ingolia NT, Weissman JS, Bartel DP. Mammalian microRNAs predominantly act to decrease target mRNA levels. *Nature* 2010, 466: 835–840

30. Andersen PR, Tirian L, Vunjak M, Brennecke J. A heterochromatin-dependent transcription machinery drives piRNA expression. *Nature* 2017, 549: 54–59

31. Wang X, Ramat A, Simonelig M, Liu MF. Emerging roles and functional mechanisms of PIWI-interacting RNAs. *Nat Rev Mol Cell Biol* 2023, 24: 123–141

32. Brennecke J, Aravin AA, Stark A, Dus M, Kellis M, Sachidanandam R, Hannon GJ. Discrete small RNA-generating loci as master regulators of transposon activity in *Drosophila*. *Cell* 2007, 128: 1089–1103

33. Lau NC, Seto AG, Kim J, Kuramochi-Miyagawa S, Nakano T, Bartel DP, Kingston RE. Characterization of the piRNA complex from rat testes. *Science* 2006, 313: 363–367

34. Rouget C, Papin C, Boureux A, Meunier AC, Franco B, Robine N, Lai EC, *et al*. Maternal mRNA deadenylation and decay by the piRNA pathway in the early *Drosophila* embryo. *Nature* 2010, 467: 1128–1132

35. Goh WSS, Falciatori I, Tam OH, Burgess R, Meikar O, Kotaja N, Hammell M, *et al*. piRNA-directed cleavage of meiotic transcripts regulates spermatogenesis. *Genes Dev* 2015, 29: 1032–1044

36. Chen Q, Zhou T. Emerging functional principles of tRNA-derived small RNAs and other regulatory small RNAs. *J Biol Chem* 2023, 299: 105225

37. Kuhle B, Chen Q, Schimmel P. tRNA renovatio: rebirth through fragmentation. *Mol Cell* 2023, 83: 3953–3971

38. Yang D, Wan X, Dennis AT, Bektik E, Wang Z, Costa MGS, Fagnen C, *et al*. MicroRNA biophysically modulates cardiac action potential by direct binding to ion channel. *Circulation* 2021, 143: 1597–1613

39. Lyons SM, Kharel P, Akiyama Y, Ojha S, Dave D, Tsvetkov V, Merrick W, *et al*. eIF4G has intrinsic G-quadruplex binding activity that is required for tiRNA function. *Nucleic Acids Res* 2020, 48: 6223–6233

40. Plasterk RHA. RNA silencing: the genome's immune system. *Science* 2002, 296: 1263–1265

41. Almeida R, Allshire RC. RNA silencing and genome regulation. *Trends*

Cell Biol 2005, 15: 251–258

42. Ketting RF, Haverkamp THA, van Luenen HGAM, Plasterk RHA. Mut-7 of *C. elegans*, required for transposon silencing and RNA interference, is a homolog of Werner syndrome helicase and RNaseD. *Cell* 1999, 99: 133–141

43. Tabara H, Sarkissian M, Kelly WG, Fleenor J, Grishok A, Timmons L, Fire A, *et al.* The rde-1 gene, RNA interference, and transposon silencing in *C. elegans*. *Cell* 1999, 99: 123–132

44. Sijen T, Plasterk RHA. Transposon silencing in the *Caenorhabditis elegans* germ line by natural RNAi. *Nature* 2003, 426: 310–314

45. Ruby JG, Jan C, Player C, Axtell MJ, Lee W, Nusbaum C, Ge H, *et al.* Large-scale sequencing reveals 21U-RNAs and additional microRNAs and endogenous siRNAs in *C. elegans*. *Cell* 2006, 127: 1193–1207

46. Das PP, Bagijn MP, Goldstein LD, Woolford JR, Lehrbach NJ, Sapetschnig A, Buhecha HR, *et al.* Piwi and piRNAs act upstream of an endogenous siRNA pathway to suppress Tc3 transposon mobility in the *Caenorhabditis elegans* germline. *Mol Cell* 2008, 31: 79–90

47. Burton NO, Burkhart KB, Kennedy S. Nuclear RNAi maintains heritable gene silencing in *Caenorhabditis elegans*. *Proc Natl Acad Sci USA* 2011, 108: 19683–19688

48. Batista PJ, Ruby JG, Claycomb JM, Chiang R, Fahlgren N, Kasschau KD, Chaves DA, *et al.* PRG-1 and 21U-RNAs interact to form the piRNA complex required for fertility in *C. elegans*. *Mol Cell* 2008, 31: 67–78

49. Shen EZ, Chen H, Ozturk AR, Tu S, Shirayama M, Tang W, Ding YH, *et al.* Identification of piRNA binding sites reveals the argonaute regulatory landscape of the *C. elegans* germline. *Cell* 2018, 172: 937–951

50. Tsai HY, Chen CCG, Conte Jr. D, Moresco JJ, Chaves DA, Mitani S, Yates Iii JR, *et al.* A ribonuclease coordinates siRNA amplification and mRNA cleavage during RNAi. *Cell* 2015, 160: 407–419

51. Shukla A, Yan J, Pagano DJ, Dodson AE, Fei Y, Gorham J, Seidman JG, *et al.* poly(UG)-tailed RNAs in genome protection and epigenetic inheritance. *Nature* 2020, 582: 283–288

52. Pak J, Fire A. Distinct populations of primary and secondary effectors during RNAi in *C. elegans*. *Science* 2007, 315: 241–244

53. Buckley BA, Burkhart KB, Gu SG, Spracklin G, Kershner A, Fritz H, Kimble J, *et al.* A nuclear Argonaute promotes multigenerational epigenetic inheritance and germline immortality. *Nature* 2012, 489: 447–451

54. Mao H, Zhu C, Zong D, Weng C, Yang X, Huang H, Liu D, *et al.* The nrde pathway mediates small-RNA-directed histone H3 lysine 27 trimethylation in *Caenorhabditis elegans*. *Curr Biol* 2015, 25: 2398–2403

55. Schwartz-Orbach L, Zhang C, Sidoli S, Amin R, Kaur D, Zhebrun A, Ni J, *et al. Caenorhabditis elegans* nuclear RNAi factor SET-32 deposits the transgenerational histone modification, H3K23me3. *eLife* 2020, 9: e54309

56. Robert VJP, Vastenhouw NL, Plasterk RHA. RNA interference, transposon silencing, and cosuppression in the *caenorhabditis elegans* germ line: similarities and differences. *Cold Spring Harb Symposia Quantitative Biol* 2004, 69: 397–402

57. Zaratiegui M, Irvine DV, Martienssen RA. Noncoding RNAs and gene silencing. *Cell* 2007, 128: 763–776

58. Aravin AA, Hannon GJ, Brennecke J. The Piwi-piRNA pathway provides an adaptive defense in the transposon arms race. *Science* 2007, 318: 761–764

59. Carmell MA, Girard A, van de Kant HJG, Bourc'his D, Bestor TH, de Rooij DG, Hannon GJ. MIWI2 is essential for spermatogenesis and repression of transposons in the mouse male germline. *Dev Cell* 2007, 12: 503–514

60. Megosh HB, Cox DN, Campbell C, Lin H. The role of PIWI and the miRNA machinery in *Drosophila* germline determination. *Curr Biol* 2006, 16: 1884–1894

61. Gonzalez LE, Tang X, Lin H, Sundaram MV. Maternal Piwi regulates primordial germ cell development to ensure the fertility of female progeny in *Drosophila*. *Genetics* 2021, 219: iyab091

62. Ku HY, Lin H. PIWI proteins and their interactors in piRNA biogenesis, germline development and gene expression. *Natl Sci Rev* 2014, 1: 205–218

63. Vagin VV, Sigova A, Li C, Seitz H, Gvozdev V, Zamore PD. A distinct small RNA pathway silences selfish genetic elements in the germline. *Science* 2006, 313: 320–324

64. Aravin AA, Klenov MS, Vagin VV, Bantignies F, Cavalli G, Gvozdev VA. Dissection of a natural RNA silencing process in the *Drosophila melanogaster* germ line. *Mol Cell Biol* 2004, 24: 6742–6750

65. van Lopik J, Alizada A, Trapotsi MA, Hannon GJ, Bornelöv S, Czech Nicholson B. Unistrand piRNA clusters are an evolutionarily conserved mechanism to suppress endogenous retroviruses across the *Drosophila* genus. *Nat Commun* 2023, 14: 7337

66. Song SU, Kurkulos M, Boeke JD, Corces VG. Infection of the germ line by retroviral particles produced in the follicle cells: a possible mechanism for the mobilization of the gypsy retroelement of *Drosophila*. *Development* 1997, 124: 2789–2798

67. Wang G, Reinke V. A *C. elegans* Piwi, PRG-1, regulates 21U-RNAs during spermatogenesis. *Curr Biol* 2008, 18: 861–867

68. Gu W, Lee HC, Chaves D, Youngman EM, Pazour GJ, Conte Jr. D, Mello CC. CapSeq and CIP-TAP identify Pol II start sites and reveal capped small RNAs as *C. elegans* piRNA precursors. *Cell* 2012, 151: 1488–1500

69. Montgomery BE, Vijayasarathy T, Marks TN, Cialek CA, Reed KJ, Montgomery TA. Dual roles for piRNAs in promoting and preventing gene silencing in *C. elegans*. *Cell Rep* 2021, 37: 110101

70. Weng C, Kosalka J, Berkyurek AC, Stempor P, Feng X, Mao H, Zeng C, *et al.* The USTC co-opts an ancient machinery to drive piRNA transcription in *C. elegans*. *Genes Dev* 2019, 33: 90–102

71. Huang X, Cheng P, Weng C, Xu Z, Zeng C, Xu Z, Chen X, *et al.* A chromodomain protein mediates heterochromatin-directed piRNA expression. *Proc Natl Acad Sci USA* 2021, 118: e2103723118

72. Weick EM, Miska EA. piRNAs: from biogenesis to function. *Development* 2014, 141: 3458–3471

73. Thomson T, Lin H. The biogenesis and function of PIWI proteins and piRNAs: progress and prospect. *Annu Rev Cell Dev Biol* 2009, 25: 355–376

74. Hasuwa H, Iwasaki YW, Au Yeung WK, Ishino K, Masuda H, Sasaki H, Siomi H. Production of functional oocytes requires maternally expressed PIWI genes and piRNAs in golden hamsters. *Nat Cell Biol* 2021, 23: 1002–1012

75. Roovers EF, Rosenkranz D, Mahdipour M, Han CT, He N, Chuva de Sousa Lopes SM, van der Westerlaken LAJ, *et al.* Piwi proteins and piRNAs in mammalian oocytes and early embryos. *Cell Rep* 2015, 10: 2069–2082

76. Loubalova Z, Fulka H, Horvat F, Pasulka J, Malik R, Hirose M, Ogura A, *et al.* Formation of spermatogonia and fertile oocytes in golden hamsters requires piRNAs. *Nat Cell Biol* 2021, 23: 992–1001

77. Zhang H, Zhang F, Chen Q, Li M, Lv X, Xiao Y, Zhang Z, *et al.* The piRNA pathway is essential for generating functional oocytes in golden hamsters. *Nat Cell Biol* 2021, 23: 1013–1022

78. Gou LT, Kang JY, Dai P, Wang X, Li F, Zhao S, Zhang M, *et al.* Ubiquitination-deficient mutations in human piwi cause male infertility by impairing histone-to-protamine exchange during spermiogenesis. *Cell* 2017, 169: 1090–1104

79. Wang X, Tan YQ, Liu MF, Defective piRNA processing and azoospermia. *N Engl J Med* 2022, 386: 1674–1676

80. Nagirnaja L, Mørup N, Nielsen JE, Stakaitis R, Golubickaite I, Oud MS, Winge SB, *et al.* Variant PNLDC1, defective piRNA processing, and azoospermia. *N Engl J Med* 2021, 385: 707–719

81. Zhang Y, Guo R, Cui Y, Zhu Z, Zhang Y, Wu H, Zheng B, *et al.* An essential role for PNLDC1 in piRNA 3′ end trimming and male fertility in mice. *Cell Res* 2017, 27: 1392–1396

82. Nishimura T, Nagamori I, Nakatani T, Izumi N, Tomari Y, Kuramochi-Miyagawa S, Nakano T. PNLDC 1, mouse pre-piRNA Trimmer, is required for meiotic and post-meiotic male germ cell development. *EMBO Rep* 2018, 19: e44957

83. Pasquinelli AE. MicroRNAs and their targets: recognition, regulation and an emerging reciprocal relationship. *Nat Rev Genet* 2012, 13: 271–282

84. Bushati N, Cohen SM. microRNA functions. *Annu Rev Cell Dev Biol* 2007, 23: 175–205

85. Bernstein E, Kim SY, Carmell MA, Murchison EP, Alcorn H, Li MZ, Mills AA, *et al.* Dicer is essential for mouse development. *Nat Genet* 2003, 35: 215–217

86. Förstemann K, Tomari Y, Du T, Vagin VV, Denli AM, Bratu DP, Klattenhoff C, *et al.* Normal microRNA maturation and germ-line stem cell maintenance requires loquacious, a double-stranded RNA-binding domain protein. *PLoS Biol* 2005, 3: e236

87. Lee RC, Feinbaum RL, Ambros V. The *C. elegans* heterochronic gene lin-4 encodes small RNAs with antisense complementarity to lin-14. *Cell* 1993, 75: 843–854

88. Reinhart BJ, Slack FJ, Basson M, Pasquinelli AE, Bettinger JC, Rougvie AE, Horvitz HR, *et al.* The 21-nucleotide let-7 RNA regulates developmental timing in *Caenorhabditis elegans*. *Nature* 2000, 403: 901–906

89. Bagga S, Bracht J, Hunter S, Massirer K, Holtz J, Eachus R, Pasquinelli AE. Regulation by let-7 and lin-4 miRNAs results in target mRNA degradation. *Cell* 2005, 122: 553–563

90. Meyer WJ, Schreiber S, Guo Y, Volkmann T, Welte MA, Müller HAJ, Frankel WN. Overlapping functions of argonaute proteins in patterning and morphogenesis of *Drosophila* embryos. *PLoS Genet* 2006, 2: e134

91. Hutvágner G, McLachlan J, Pasquinelli AE, Bálint E, Tuschl T, Zamore PD. A cellular function for the RNA-interference enzyme Dicer in the maturation of the let-7 small temporal RNA. *Science* 2001, 293: 834–838

92. DeVeale B, Swindlehurst-Chan J, Blelloch R. The roles of microRNAs in mouse development. *Nat Rev Genet* 2021, 22: 307–323

93. Liu W, Bi P, Shan T, Yang X, Yin H, Wang YX, Liu N, *et al.* MiR-133a regulates adipocyte browning *in vivo*. *PLoS Genet* 2013, 9: e1003626

94. Wang D, Zhang Z, O'Loughlin E, Wang L, Fan X, Lai EC, Yi R. MicroRNA-205 controls neonatal expansion of skin stem cells by modulating the PI(3)K pathway. *Nat Cell Biol* 2013, 15: 1153–1163

95. Andl T, Murchison EP, Liu F, Zhang Y, Yunta-Gonzalez M, Tobias JW, Andl CD, *et al.* The miRNA-processing enzyme dicer is essential for the morphogenesis and maintenance of hair follicles. *Curr Biol* 2006, 16: 1041–1049

96. Harris KS, Zhang Z, McManus MT, Harfe BD, Sun X. *Dicer* function is essential for lung epithelium morphogenesis. *Proc Natl Acad Sci USA* 2006, 103: 2208–2213

97. Zhu C, Yan Q, Weng C, Hou X, Mao H, Liu D, Feng X, *et al.* Erroneous ribosomal RNAs promote the generation of antisense ribosomal siRNA. *Proc Natl Acad Sci USA* 2018, 115: 10082–10087

98. You C, He W, Hang R, Zhang C, Cao X, Guo H, Chen X, *et al.* FIERY1 promotes microRNA accumulation by suppressing rRNA-derived small interfering RNAs in *Arabidopsis*. *Nat Commun* 2019, 10: 4424

99. Zinta R, Tiwari JK, Buckseth T, Thakur K, Goutam U, Kumar D, Challam C, *et al.* Root system architecture for abiotic stress tolerance in potato: lessons from plants. *Front Plant Sci* 2022, 13: 926214

100. Wang HL, Chekanova JA. SmallRNAs: essential regulators of gene expression and defenses against environmental stresses in plants. *Wiley Interdiscip Rev RNA* 2016, 7: 356–381

101. Sunkar R, Li YF, Jagadeeswaran G. Functions of microRNAs in plant stress responses. *Trends Plant Sci* 2012, 17: 196–203

102. Mallory AC, Vaucheret H. Functions of microRNAs and related small RNAs in plants. *Nat Genet* 2006, 38: S31–S36

103. Wang S, Sun X, Hoshino Y, Yu Y, Jia B, Sun Z, Sun M, *et al.* MicroRNA319 positively regulates cold tolerance by targeting OsPCF6 and OsTCP21 in rice (*Oryza sativa L.*). *PLoS One* 2014, 9: e91357

104. Yang C, Li D, Mao D, Liu X, Ji C, Li X, Zhao X, *et al.* Overexpression of microRNA 319 impacts leaf morphogenesis and leads to enhanced cold tolerance in rice (*Oryza sativa L.*). *Plant Cell Environ* 2013, 36: 2207–2218

105. Islam W, Naveed H, Idress A, Ishaq DU, Kurfi BG, Zeng F. Plant responses to metals stress: microRNAs in focus. *Environ Sci Pollut Res* 2022, 29: 69197–69212

106. Srivastava S, Suprasanna P. MicroRNAs: tiny, powerful players of metal stress responses in plants. *Plant Physiol Biochem* 2021, 166: 928–938

107. Vaucheret H, Mallory AC, Bartel DP. AGO1 homeostasis entails coexpression of MIR168 and AGO1 and preferential stabilization of miR168 by AGO1. *Mol Cell* 2006, 22: 129–136

108. Wang Y, Sun F, Cao H, Peng H, Ni Z, Sun Q, Yao Y, *et al.* TamiR159 directed wheat TaGAMYB cleavage and its involvement in anther development and heat response. *PLoS One* 2012, 7: e48445

109. Reyes JL, Chua N. ABA induction of miR159 controls transcript levels of two MYB factors during *Arabidopsis* seed germination. *Plant J* 2007, 49: 592–606

110. Sun M, Yang Z, Liu L, Duan L. DNA methylation in plant responses and adaption to abiotic stresses. *Int J Mol Sci* 2022, 23: 6910

111. Gutzat R, Mittelsten Scheid O. Epigenetic responses to stress: triple defense? *Curr Opin Plant Biol* 2012, 15: 568–573

112. Matzke MA, Mosher RA. RNA-directed DNA methylation: an epigenetic pathway of increasing complexity. *Nat Rev Genet* 2014, 15: 394–408

113. Singh RK, Prasad A, Maurya J, Prasad M. Regulation of small RNA-mediated high temperature stress responses in crop plants. *Plant Cell Rep* 2022, 41: 765–773

114. Bronkhorst AW, van Rij RP. The long and short of antiviral defense: small RNA-based immunity in insects. *Curr Opin Virol* 2014, 7: 19–28

115. Ding SW. RNA-based antiviral immunity. *Nat Rev Immunol* 2010, 10: 632–644

116. Haasnoot J, Westerhout EM, Berkhout B. RNA interference against viruses: strike and counterstrike. *Nat Biotechnol* 2007, 25: 1435–1443

117. Sidahmed A, Abdalla S, Mahmud S, Wilkie B. Antiviral innate immune response of RNA interference. *J Infect Dev Ctries* 2014, 8: 804–810

118. Buchon N, Vaury C. RNAi: a defensive RNA-silencing against viruses and transposable elements. *Heredity* 2006, 96: 195–202

119. Jose AM, Hunter CP. Transport of sequence-specific RNA interference information between cells. *Annu Rev Genet* 2007, 41: 305–330

120. Hubel P, Urban C, Bergant V, Schneider WM, Knauer B, Stukalov A, Scaturro P, *et al.* A protein-interaction network of interferon-stimulated genes extends the innate immune system landscape. *Nat Immunol* 2019, 20: 493–502

121. Matskevich AA, Moelling K. Dicer is involved in protection against influenza A virus infection. *J Gen Virol* 2007, 88: 2627–2635

122. Haussecker D, Cao D, Huang Y, Parameswaran P, Fire AZ, Kay MA. Capped small RNAs and MOV10 in human hepatitis delta virus replication. *Nat Struct Mol Biol* 2008, 15: 714–721

123. Parameswaran P, Sklan E, Wilkins C, Burgon T, Samuel MA, Lu R, Ansel

KM, *et al.* Six RNA viruses and forty-one hosts: viral small RNAs and modulation of small RNA repertoires in vertebrate and invertebrate systems. *PLoS Pathog* 2010, 6: e1000764

124. Pumplin N, Voinnet O. RNA silencing suppression by plant pathogens: defence, counter-defence and counter-counter-defence. *Nat Rev Microbiol* 2013, 11: 745–760

125. Zhang X, Zhao H, Gao S, Wang WC, Katiyar-Agarwal S, Huang HD, Raikhel N, *et al. Arabidopsis* argonaute 2 regulates innate immunity via miRNA393(*)-mediated silencing of a golgi-localized SNARE gene, MEMB12. *Mol Cell* 2011, 42: 356–366

126. Katiyar-Agarwal S, Morgan R, Dahlbeck D, Borsani O, Villegas Jr. A, Zhu JK, Staskawicz BJ, *et al.* A pathogen-inducible endogenous siRNA in plant immunity. *Proc Natl Acad Sci USA* 2006, 103: 18002–18007

127. Katiyar-Agarwal S, Gao S, Vivian-Smith A, Jin H. A novel class of bacteria-induced small RNAs in *Arabidopsis. Genes Dev* 2007, 21: 3123–3134

128. Grewal SIS, Klar AJS. Chromosomal inheritance of epigenetic states in fission yeast during mitosis and meiosis. *Cell* 1996, 86: 95–101

129. Cavalli G, Paro R. The *Drosophila* Fab-7 chromosomal element conveys epigenetic inheritance during mitosis and meiosis. *Cell* 1998, 93: 505–518

130. Morgan HD, Sutherland HGE, Martin DIK, Whitelaw E. Epigenetic inheritance at the agouti locus in the mouse. *Nat Genet* 1999, 23: 314–318

131. Miska EA, Ferguson-Smith AC. Transgenerational inheritance: models and mechanisms of non-DNA sequence–based inheritance. *Science* 2016, 354: 59–63

132. Fitz-James MH, Cavalli G. Molecular mechanisms of transgenerational epigenetic inheritance. *Nat Rev Genet* 2022, 23: 325–341

133. Rechavi O, Lev I. Principles of transgenerational small RNA inheritance in caenorhabditis elegans. *Curr Biol* 2017, 27: R720–R730

134. Rechavi O, Minevich G, Hobert O. Transgenerational inheritance of an acquired small RNA-based antiviral response in *C. elegans. Cell* 2011, 147: 1248–1256

135. Shirayama M, Seth M, Lee HC, Gu W, Ishidate T, Conte Jr. D, Mello CC. piRNAs initiate an epigenetic memory of nonself RNA in the *C. elegans* germline. *Cell* 2012, 150: 65–77

136. Wan G, Fields BD, Spracklin G, Shukla A, Phillips CM, Kennedy S. Spatiotemporal regulation of liquid-like condensates in epigenetic inheritance. *Nature* 2018, 557: 679–683

137. Xu F, Feng X, Chen X, Weng C, Yan Q, Xu T, Hong M, *et al.* A cytoplasmic argonaute protein promotes the inheritance of RNAi. *Cell Rep* 2018, 23: 2482–2494

138. Grishok A, Tabara H, Mello CC. Genetic Requirements for Inheritance of RNAi in *C. elegans. Science* 2000, 287: 2494–2497

139. Rechavi O, Houri-Ze'evi L, Anava S, Goh WSS, Kerk SY, Hannon GJ, Hobert O. Starvation-induced transgenerational inheritance of small RNAs in *C. elegans. Cell* 2014, 158: 277–287

140. Schott D, Yanai I, Hunter CP. Natural RNA interference directs a heritable response to the environment. *Sci Rep* 2014, 4: 7387

141. Morgan HD, Santos F, Green K, Dean W, Reik W. Epigenetic reprogramming in mammals. *Hum Mol Genet* 2005, 14: R47–R58

142. Chen Q, Yan M, Cao Z, Li X, Zhang Y, Shi J, Feng G, *et al.* Sperm tsRNAs contribute to intergenerational inheritance of an acquired metabolic disorder. *Science* 2016, 351: 397–400

143. Skvortsova K, Iovino N, Bogdanović O. Functions and mechanisms of epigenetic inheritance in animals. *Nat Rev Mol Cell Biol* 2018, 19: 774–790

144. Chen Q, Yan W, Duan E. Epigenetic inheritance of acquired traits through sperm RNAs and sperm RNA modifications. *Nat Rev Genet* 2016, 17: 733–743

145. Rodgers AB, Morgan CP, Bronson SL, Revello S, Bale TL. Paternal stress exposure alters sperm microRNA content and reprograms offspring HPA stress axis regulation. *J Neurosci* 2013, 33: 9003–9012

146. Sarker G, Sun W, Rosenkranz D, Pelczar P, Opitz L, Efthymiou V, Wolfrum C, *et al.* Maternal overnutrition programs hedonic and metabolic phenotypes across generations through sperm tsRNAs. *Proc Natl Acad Sci USA* 2019, 116: 10547–10556

147. Raad G, Serra F, Martin L, Derieppe MA, Gilleron J, Costa VL, Pisani DF, *et al.* Paternal multigenerational exposure to an obesogenic diet drives epigenetic predisposition to metabolic diseases in mice. *eLife* 2021, 10: e61736

148. Guo Q, Liu Q, A. Smith N, Liang G, Wang MB. RNA silencing in plants: mechanisms, technologies and applications in horticultural crops. *Curr Genomics* 2016, 17: 476–489

149. Boettcher M, McManus MT. Choosing the right tool for the job: RNAi, TALEN, or CRISPR. *Mol Cell* 2015, 58: 575–585

150. Boutros M, Ahringer J. The art and design of genetic screens: RNA interference. *Nat Rev Genet* 2008, 9: 554–566

151. Sharma S, Rao A. RNAi screening: tips and techniques. *Nat Immunol* 2009, 10: 799–804

152. Fraser AG, Kamath RS, Zipperlen P, Martinez-Campos M, Sohrmann M, Ahringer J. Functional genomic analysis of *C. elegans* chromosome I by systematic RNA interference. *Nature* 2000, 408: 325–330

153. Kamath RS, Fraser AG, Dong Y, Poulin G, Durbin R, Gotta M, Kanapin A, *et al.* Systematic functional analysis of the Caenorhabditis elegans genome using RNAi. *Nature* 2003, 421: 231–237

154. Cronin SJF, Nehme NT, Limmer S, Liegeois S, Pospisilik JA, Schramek D, Leibbrandt A, *et al.* Genome-wide RNAi screen identifies genes involved in intestinal pathogenic bacterial infection. *Science* 2009, 325: 340–343

155. Dietzl G, Chen D, Schnorrer F, Su KC, Barinova Y, Fellner M, Gasser B, *et al.* A genome-wide transgenic RNAi library for conditional gene inactivation in *Drosophila. Nature* 2007, 448: 151–156

156. Mohr SE, Smith JA, Shamu CE, Neumüller RA, Perrimon N. RNAi screening comes of age: improved techniques and complementary approaches. *Nat Rev Mol Cell Biol* 2014, 15: 591–600

157. Collinet C, Stöter M, Bradshaw CR, Samusik N, Rink JC, Kenski D, Habermann B, *et al.* Systems survey of endocytosis by multiparametric image analysis. *Nature* 2010, 464: 243–249

158. Sessions OM, Barrows NJ, Souza-Neto JA, Robinson TJ, Hershey CL, Rodgers MA, Ramirez JL, *et al.* Discovery of insect and human dengue virus host factors. *Nature* 2009, 458: 1047–1050

159. Kaelin Jr. WG. Use and abuse of RNAi to study mammalian gene function. *Science* 2012, 337: 421–422

160. Wagner DE, Ho JJ, Reddien PW. Genetic regulators of a pluripotent adult stem cell system in planarians identified by RNAi and clonal analysis. *Cell Stem Cell* 2012, 10: 299–311

161. Molnar A, Melnyk C, Baulcombe DC. Silencing signals in plants: a long journey for small RNAs. *Genome Biol* 2011, 12: 215

162. Ashfaq MA, Dinesh Kumar V, Soma Sekhar Reddy P, Anil Kumar C, Sai Kumar K, Narasimha Rao N, Tarakeswari M, *et al.* Post-transcriptional gene silencing: basic concepts and applications. *J Biosci* 2020, 45: 128

163. Baulcombe D. RNA silencing in plants. *Nature* 2004, 431: 356–363

164. Feldmann KA. Steroid regulation improves crop yield. *Nat Biotechnol* 2006, 24: 46–47

165. Chen S, Hajirezaei MR, Zanor M, Hornyik C, Debast S, Lacomme C, Fernie AR, *et al.* RNA interference-mediated repression of sucrose-phosphatase in transgenic potato tubers (*Solanum tuberosum*) strongly

affects the hexose-to-sucrose ratio upon cold storage with only minor effects on total soluble carbohydrate accumulation. *Plant Cell Environ* 2008, 31: 165–176

166. Cheng W, Song XS, Li HP, Cao LH, Sun K, Qiu XL, Xu YB, *et al.* Host-induced gene silencing of an essential chitin synthase gene confers durable resistance toF usarium head blight and seedling blight in wheat. *Plant Biotechnol J* 2015, 13: 1335–1345

167. Kamthan A, Chaudhuri A, Kamthan M, Datta A. Small RNAs in plants: recent development and application for crop improvement. *Front Plant Sci* 2015, 06: 208

168. Niu QW, Lin SS, Reyes JL, Chen KC, Wu HW, Yeh SD, Chua NH. Expression of artificial microRNAs in transgenic *Arabidopsis thaliana* confers virus resistance. *Nat Biotechnol* 2006, 24: 1420–1428

169. De Francesco A, Simeone M, Gómez C, Costa N, García ML. Transgenic Sweet Orange expressing hairpin CP-mRNA in the interstock confers tolerance to citrus psorosis virus in the non-transgenic scion. *Transgenic Res* 2020, 29: 215–228

170. Yu N, Christiaens O, Liu J, Niu J, Cappelle K, Caccia S, Huvenne H, *et al.* Delivery of dsRNA for RNAi in insects: an overview and future directions. *Insect Sci* 2013, 20: 4–14

171. Koch A, Biedenkopf D, Furch A, Weber L, Rossbach O, Abdellatef E, Linicus L, *et al.* An RNAi-based control of fusarium graminearum infections through spraying of long dsRNAs involves a plant passage and is controlled by the fungal silencing machinery. *PLoS Pathog* 2016, 12: e1005901

172. Dalakouras A, Jarausch W, Buchholz G, Bassler A, Braun M, Manthey T, Krczal G, *et al.* Delivery of hairpin RNAs and small RNAs into woody and herbaceous plants by trunk injection and petiole absorption. *Front Plant Sci* 2018, 9: 1253

173. Vadlamudi T, Patil BL, Kaldis A, Sai Gopal DVR, Mishra R, Berbati M, Voloudakis A. DsRNA-mediated protection against two isolates of Papaya ringspot virus through topical application of dsRNA in papaya. *J Virol Methods* 2020, 275: 113750

174. Anastasiadou E, Jacob LS, Slack FJ. Non-coding RNA networks in cancer. *Nat Rev Cancer* 2018, 18: 5–18

175. Loganathan T, Doss C GP. Non-coding RNAs in human health and disease: potential function as biomarkers and therapeutic targets. *Funct Integr Genomics* 2023, 23: 33

176. Hogg DR, Harries LW. Human genetic variation and its effect on miRNA biogenesis, activity and function. *Biochem Soc Trans* 2014, 42: 1184–1189

177. Esteller M. Cancer epigenomics: DNA methylomes and histone-modification maps. *Nat Rev Genet* 2007, 8: 286–298

178. Poller W, Dimmeler S, Heymans S, Zeller T, Haas J, Karakas M, Leistner DM, *et al.* Non-coding RNAs in cardiovascular diseases: diagnostic and therapeutic perspectives. *Eur Heart J* 2018, 39: 2704–2716

179. Santulli G. Non-coding RNAs in clinical practice: from biomarkers to therapeutic tools. *J Pharmacol Exp Ther* 2023, 384: 225–226

180. Backes C, Leidinger P, Altmann G, Wuerstle M, Meder B, Galata V, Mueller SC, *et al.* Influence of next-generation sequencing and storage conditions on miRNA patterns generated from PAXgene blood. *Anal Chem* 2015, 87: 8910–8916

181. Nemeth K, Bayraktar R, Ferracin M, Calin GA. Non-coding RNAs in disease: from mechanisms to therapeutics. *Nat Rev Genet* 2024, 25: 211–232

182. Si ML, Zhu S, Wu H, Lu Z, Wu F, Mo YY. miR-21-mediated tumor growth. *Oncogene* 2007, 26: 2799–2803

183. Gong C, Tan W, Chen K, You N, Zhu S, Liang G, Xie X, *et al.* Prognostic value of a BCSC-associated microRNA signature in hormone receptor-positive HER2-negative breast cancer. *EBioMedicine* 2016, 11: 199–209

184. Lin XJ, Chong Y, Guo ZW, Xie C, Yang XJ, Zhang Q, Li SP, *et al.* A serum microRNA classifier for early detection of hepatocellular carcinoma: a multicentre, retrospective, longitudinal biomarker identification study with a nested case-control study. *Lancet Oncol* 2015, 16: 804–815

185. Gupta RA, Shah N, Wang KC, Kim J, Horlings HM, Wong DJ, Tsai MC, *et al.* Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. *Nature* 2010, 464: 1071–1076

186. Li X, Wu Z, Mei Q, Li X, Guo M, Fu X, Han W. Long non-coding RNA HOTAIR, a driver of malignancy, predicts negative prognosis and exhibits oncogenic activity in oesophageal squamous cell carcinoma. *Br J Cancer* 2013, 109: 2266–2278

187. Suwardjo S, Gilang Permana K, Aryandono T, Heriyanto D, Anwar S. Long-noncoding-RNA HOTAIR upregulation is associated with poor breast cancer outcome: a systematic review and meta analysis. *Asian Pac J Cancer Prev* 2024, 25: 1169–1182

188. Chen X, Ba Y, Ma L, Cai X, Yin Y, Wang K, Guo J, *et al.* Characterization of microRNAs in serum: a novel class of biomarkers for diagnosis of cancer and other diseases. *Cell Res* 2008, 18: 997–1006

189. Schwarzenbach H, da Silva AM, Calin G, Pantel K. Data normalization strategies for microRNA quantification. *Clin Chem* 2015, 61: 1333–1342

190. Setten RL, Rossi JJ, Han S. The current state and future directions of RNAi-based therapeutics. *Nat Rev Drug Discov* 2019, 18: 421–446

191. Hu B, Zhong L, Weng Y, Peng L, Huang Y, Zhao Y, Liang XJ. Therapeutic siRNA: state of the art. *Sig Transduct Target Ther* 2020, 5: 101

192. Li C, Ni YQ, Xu H, Xiang QY, Zhao Y, Zhan JK, He JY, *et al.* Roles and mechanisms of exosomal non-coding RNAs in human health and diseases. *Sig Transduct Target Ther* 2021, 6: 383

193. Mahajan S, Choudhary S, Kumar P, Tomar S. Antiviral strategies targeting host factors and mechanisms obliging +ssRNA viral pathogens. *Bioorg Med Chem* 2021, 46: 116356

194. Jopling CL, Yi MK, Lancaster AM, Lemon SM, Sarnow P. Modulation of hepatitis C virus RNA abundance by a liver-specific microRNA. *Science* 2005, 309: 1577–1581

195. Yan L, Zhang Y, Wang M, Wang L, Zhang W, Ge ZR. Circulating LIPCAR is a potential biomarker of heart failure in patients post-acute myocardial infarction. *Exp Biol Med (Maywood)* 2021, 246: 2589–2594

196. Kamerkar S, LeBleu VS, Sugimoto H, Yang S, Ruivo CF, Melo SA, Lee JJ, *et al.* Exosomes facilitate therapeutic targeting of oncogenic KRAS in pancreatic cancer. *Nature* 2017, 546: 498–503

197. Tang Q, Khvorova A. RNAi-based drug design: considerations and future directions. *Nat Rev Drug Discov* 2024, 23: 341–364

198. Santel A, Aleku M, Keil O, Endruschat J, Esche V, Durieux B, Löffler K, *et al.* RNA interference in the mouse vascular endothelium by systemic administration of siRNA-lipoplexes for cancer therapy. *Gene Ther* 2006, 13: 1360–1370

199. Guo S, Zhang M, Huang Y. Three 'E' challenges for siRNA drug development. *Trends Mol Med* 2024, 30: 13–24

200. Dykxhoorn DM, Lieberman J. Running interference: prospects and obstacles to using small interfering RNAs as small molecule drugs. *Annu Rev Biomed Eng* 2006, 8: 377–402

201. Paroo Z, Corey DR. Challenges for RNAi *in vivo*. *Trends Biotechnol* 2004, 22: 390–394

202. Bramsen JB, Laursen MB, Nielsen AF, Hansen TB, Bus C, Langkjær N, Babu BR, *et al.* A large-scale chemical modification screen identifies design rules to generate siRNAs with high activity, high stability and low toxicity. *Nucleic Acids Res* 2009, 37: 2867–2881

203. Suzuki Y, Hyodo K, Suzuki T, Tanaka Y, Kikuchi H, Ishihara H. Biodegradable lipid nanoparticles induce a prolonged RNA interference-

mediated protein knockdown and show rapid hepatic clearance in mice and nonhuman primates. *Int J Pharm* 2017, 519: 34–43

204. Jadhav V, Vaishnaw A, Fitzgerald K, Maier MA. RNA interference in the era of nucleic acid therapeutics. *Nat Biotechnol* 2024, 42: 394–405

205. Fu Z, Zhang X, Zhou X, Ur-Rehman U, Yu M, Liang H, Guo H, *et al.* *In vivo* self-assembled small RNAs as a new generation of RNAi therapeutics. *Cell Res* 2021, 31: 631–648

206. Li K, Lu M, Xia X, Huang Y. Recent advances in photothermal and RNA interfering synergistic therapy. *Chin Chem Lett* 2021, 32: 1010–1016

207. Hoy SM. Patisiran: first global approval. *Drugs* 2018, 78: 1625–1631

208. Lin KP, Yang CC, Lee YC, Lee MJ, Vest J, Sweetser MT, White MT, *et al.* Patisiran, an RNAi therapeutic for hereditary transthyretin-mediated amyloidosis: sub-analysis in Taiwanese patients from the APOLLO study. *J Formos Med Assoc* 2024, 123: 975–984

209. Kristen AV, Ajroud-Driss S, Conceição I, Gorevic P, Kyriakides T, Obici L. Patisiran, an RNAi therapeutic for the treatment of hereditary transthyretin-mediated amyloidosis. *Neurodegener Dis Manag* 2019, 9: 5–23

210. Raal FJ, Kallend D, Ray KK, Turner T, Koenig W, Wright RS, Wijngaard PLJ, *et al.* Inclisiran for the treatment of heterozygous familial hypercholesterolemia. *N Engl J Med* 2020, 382: 1520–1530

211. Desai AS, Webb DJ, Taubel J, Casey S, Cheng Y, Robbie GJ, Foster D, *et al.* Zilebesiran, an RNA interference therapeutic agent for hypertension. *N Engl J Med* 2023, 389: 228–238

212. Alterman JF, Godinho BMDC, Hassler MR, Ferguson CM, Echeverria D, Sapp E, Haraszti RA, *et al.* A divalent siRNA chemical scaffold for potent and sustained modulation of gene expression throughout the central nervous system. *Nat Biotechnol* 2019, 37: 884–894

213. Cheng Q, Wei T, Farbiak L, Johnson LT, Dilliard SA, Siegwart DJ. Selective organ targeting (SORT) nanoparticles for tissue-specific mRNA delivery and CRISPR–Cas gene editing. *Nat Nanotechnol* 2020, 15: 313–320

214. Hariharan VN, Shin M, Chang CW, O'Reilly D, Biscans A, Yamada K, Guo Z, *et al.* Divalent siRNAs are bioavailable in the lung and efficiently block SARS-CoV-2 infection. *Proc Natl Acad Sci USA* 2023, 120: e2219523120

215. Mi S, Cai T, Hu Y, Chen Y, Hodges E, Ni F, Wu L, *et al.* Sorting of small RNAs into *Arabidopsis* argonaute complexes is directed by the 5′ terminal nucleotide. *Cell* 2008, 133: 116–127

216. Andre M, Caobi A, Miles JS, Vashist A, Ruiz MA, Raymond AD. Diagnostic potential of exosomal extracellular vesicles in oncology. *BMC Cancer* 2024, 24: 322

217. Zhang X, Zuo X, Yang B, Li Z, Xue Y, Zhou Y, Huang J, *et al.* MicroRNA directly enhances mitochondrial translation during muscle differentiation. *Cell* 2014, 158: 607–619

218. Gao K, Cheng M, Zuo X, Lin J, Hoogewijs K, Murphy MP, Fu XD, *et al.* Active RNA interference in mitochondria. *Cell Res* 2021, 31: 219–228

219. Won Lee J, Kyu Shim M, Kim H, Jang H, Lee Y, Hwa Kim S. RNAi therapies: expanding applications for extrahepatic diseases and overcoming delivery challenges. *Adv Drug Deliver Rev* 2023, 201: 115073