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Review

Small RNAs: An expanding world with therapeutic promises

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ABSTRACT

Small non-coding RNAs (sncRNAs), such as microRNAs (miRNAs), small interfering RNAs (siRNAs), PIWI-interacting RNAs (piRNAs), and transfer RNA (tRNA)-derived small RNAs (tsRNAs), play essential roles in regulating various cellular and developmental processes. Over the past three decades, researchers have identified novel sncRNA species from various organisms. These molecules demonstrate dynamic expression and diverse functions, and they are subject to intricate regulation through RNA modifications in both healthy and diseased states. Notably, certain sncRNAs in gametes, particularly sperm, respond to environmental stimuli and facilitate epigenetic inheritance. Collectively, the in-depth understanding of sncRNA functions and mechanisms has accelerated the development of small RNA-based therapeutics. In this review, we present the recent advances in the field, including new sncRNA species and the regulatory influences of RNA modifications. We also discuss the current limitations and challenges associated with using small RNAs as either biomarkers or therapeutic drugs.

1. Introduction

Small non-coding RNAs (sncRNAs) refer to non-coding RNAs (ncRNAs) less than 200-nucleotide (nt) and were first discovered in *C. elegans* nearly three decades ago [1,2]. Since then, the kingdom of sncRNAs has rapidly expanded with increasing numbers of novel species identified in various organisms. SncRNAs are broadly classified into two categories: the housekeeping ones, including small nuclear RNAs (snRNAs) and small nucleolar RNAs (snoRNAs) [3], and the regulatory ones, including microRNAs (miRNAs), small interfering RNAs (siRNAs) and PIWI-interacting RNAs (piRNAs). Such regulatory sncRNAs are known to play critical roles in gene expression at post-transcriptional, translational, and epigenetic levels. Recent advances in next-generation sequencing technologies have identified new sncRNA species derived from other RNA molecules, such as transfer RNAs (tRNAs) [4,5] and ribosomal RNAs (rRNAs) [6]. While the functions of such sncRNAs require further elucidation, their involvement in specific biological processes has been suggested. Additionally, studies have demonstrated that RNA modifications play an important role in the biogenesis and functionality of sncRNAs, thus shaping their regulatory roles. These findings increase the potential of sncRNAs in the diagnosis, prognosis, and therapeutics for human diseases. In this review, we summarize the recent advances and breakthroughs within the field, including novel sncRNA species and the regulatory roles of RNA modifications to the biogenesis and functionality of sncRNAs. Moreover, we discuss the potential applications of sncRNAs in clinical practice (Fig. 1).

2. Sequential and functional diversity of sncRNAs

The most intensely researched classes of endogenous sncRNAs are miRNAs, endogenous small interfering RNAs (endo-siRNAs), and piRNAs. The mechanisms that distinguish these three classes from one another are primarily based on the differences in their biogenesis and protein partners. To create miRNAs, RNase III enzymes Drosha and Dicer cleave hairpin-shaped precursors whereas endo-siRNAs are generated from double-stranded RNA (dsRNA) through Dicer [7,8]. On the other hand, the precursors of piRNAs are single-stranded. Their production is independent of Drosha and Dicer [9,10]. miRNAs (~22-nt) and endo-siRNAs (~21-nt) form complexes with AGO-family proteins such as AGO2, which mediate post-transcriptional regulation by inducing RNA cleavage [11] and translation repression [8,12]. piRNAs (24–32-nt), as their name indicates, are mainly associated with PIWI proteins, a germline-specific AGO subfamily. piRNAs primarily repress transposable elements (TE) at both post-transcriptional and epigenetic levels [13]. Furthermore, piRNAs also repress protein-coding genes in animal germlines via siRNA and miRNA-like mechanisms [14–16]. Newly emerging roles and functional mechanisms of these well-researched sncRNAs are still continually discovered. For instance, recent studies

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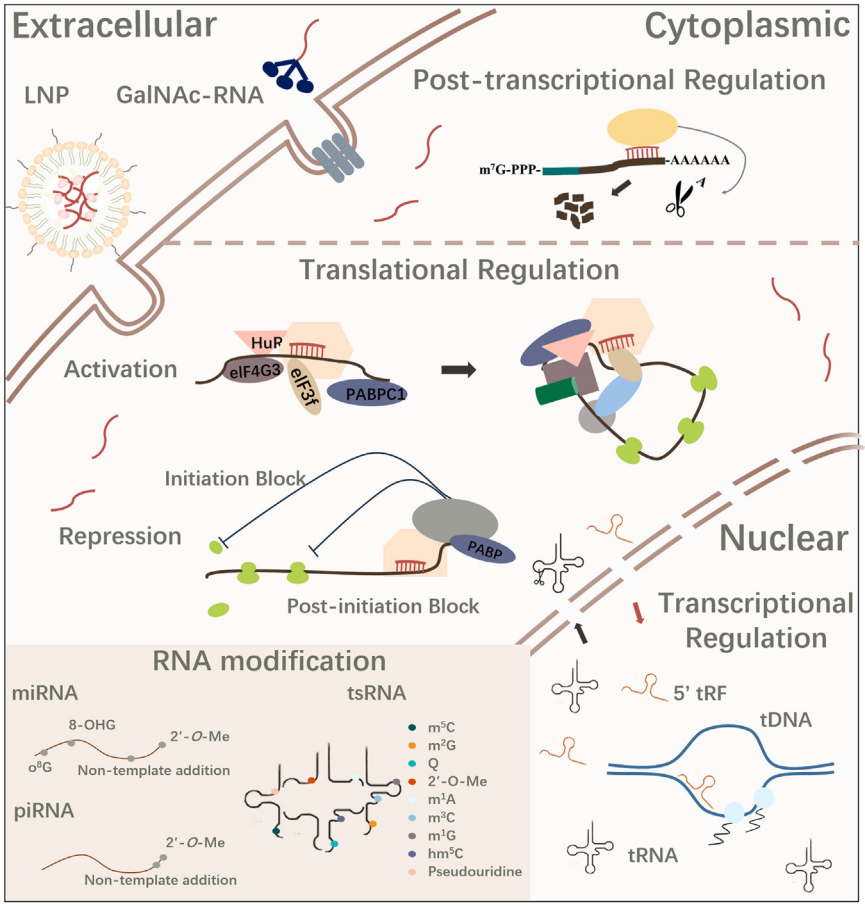

Fig. 1. Schematic diagram summarizing the regulatory roles of sncRNAs, modifications and delivery approaches for RNA therapeutics. sncRNAs are key regulators for various biological processes, which function to regulate gene expression at transcriptional, post-transcriptional, and translational levels. Multiple sncRNA species have been identified with advanced detection and annotation methods, among which many are carried with various types of chemical modifications, adding another layer of complexity to the sncRNA kingdom. sncRNAs have strong potentials in therapeutics, and a few sncRNA-based therapeutics have been approved by FDA since 2018.

highlight that piRNAs and PIWI proteins promote the activation of translation in mice and flies, and the underlying molecular mechanisms are remarkably conserved between the two species. In mouse spermatids and fly embryos, PIWI proteins interact with translation initiation factor 3 (eIF3) subunits and other RNA-binding proteins, forming a translation-activating complex [17,18]. In mouse spermatids, the selectivity of this complex depends on a specific base pairing between piRNAs and the 3′ UTR of their target mRNAs [17]. Additionally, a new class of piRNAs was recently identified in human, monkey, and hamster oocytes, which are associated with rodent-lacking PIWIL3 and termed oocyte short piRNAs (os-piRNAs) [19–21].

In addition to the increasing understanding of well-known sncRNA species, the sncRNA realm is expanding owing to advanced detection and annotation methods. NGS (Next-Generation Sequencing) is probably the most widely used method for the detection and annotation of sncRNAs. Many sncRNA species that were initially overlooked during earlier NGS data analysis are mappable to longer and often structured RNA species [22]. Apart from tsRNAs and rsRNAs, fragments originating from yRNAs (a class of soluble ribonucleoproteins (Ro RNPS)-associated non-coding RNAs. The “y” prefix is used to emphasize their primarily cytoplasmic localization and to distinguish them from nuclear-localized snRNAs such as U6) [23–25], vault RNAs (vtRNAs) [26], snRNAs [27], snoRNAs [28,29], long non-coding RNAs (lncRNAs) [30], and mRNAs [31,32] have also been recently reported. Although most small RNA sequencing approaches aimed to profile well-studied sncRNAs (such as miRNAs), improvements in library preparation, in particular removal of RNA modifications interfering with adaptor ligation and/or reverse transcription (RT), have paved the way for new advancements in the sncRNA landscape [33]. One example is the recently developed panoramic RNA display by overcoming RNA modification aborted sequencing (PANDORA-seq) [34] which utilizes a combination of enzymes to remove RNA methylation and 3′-phosphate (3′-P) of sncRNAs. Bioinformatics analysis is an integral part of small RNA sequencing. It is no surprise that newly built specialized computer tools and pipelines have improved the speed and achieved better identification and annotation of different types of sncRNAs, in particular the non-canonical ones [35,36]. Nevertheless, accurately quantifying and comparing those novel sncRNAs between various conditions, as well as identifying them in low-input scenarios like early embryos or extracellular biofluids, remain significant challenges.

Comprehending the functions of sncRNAs necessitates understanding their biogenesis, tissue abundance, and biological importance. A portion of sncRNAs is derived from tRNAs and rRNAs, demonstrating that tsRNAs and rsRNAs could be functional sncRNAs [37–40]. Recent studies have likewise shown tsRNAs and rsRNAs are abundant in multiple tissues [34], implying their biological significance. The cleavage of mature tRNAs or tRNA precursors can occur on several sites, contributing to the generation of diverse tsRNAs. Both the tRNA structure and the RNA modifications on them, such as DNMT2 and NSUN2-mediated 5-methylcytosine (m5C) modification [41–46], contribute to tRNA cleavage site preference. Similar to tsRNAs, the biogenesis of rsRNAs is also under strict regulation. Various preferred cleavage sites in rRNAs are

often observed, depending on the rRNA types. Hence, different rRNAs can generate rsRNAs with favored lengths, leading to highly context-specific profiles of rsRNAs [47–49]. In particular, the tsRNA and rsRNA population in mammalian sperm has become a leading topic in the field [50,51]. In 2016, two separate groups found that the 30–40 nt small RNA population in mature sperm, primarily composed of tsRNAs and rsRNAs, was sensitive to environmental stimuli. More importantly, these sncRNAs could be carried into oocytes during fertilization and presumably play a part in the intergenerational inheritance of acquired traits [34,52,53]. The majority of tsRNAs in mammalian sperm is derived from 5′ half of mature tRNAs, termed 5′-tRNA halves (5′ tRNAF or 5′ tRFl) [54]. On the other hand, the majority of rsRNAs are derived from 28S rRNA [51]. Subsequent studies using different lifestyle models, as well as environmental stress and toxicants, have further validated the involvement of sperm-borne tsRNAs in epigenetic inheritance [53,55-59]. Despite the proposal that epididymis and epididymosomes might be potential sources [60], the biogenesis of sperm-borne sncRNAs remains a myth. tsRNAs have a varied range of functions, including Argonaute (Ago) family-dependent gene silencing [61–63], translational inhibition [64–66], and retrotransposon repression [67]. A recent study further indicates some 5′-tRNA halves are vital for early embryonic development in zebrafish, where they may enhance tRNA transcription by forming stable, transcriptionally inhibitory hybrids with the template strand of DNA, competing with existing tRNA [68]. This suggests that RNA-derived fragments may function by mimicking their precursors based on the sequence and structure similarity [67,69]. Although tsRNAs [70] and rsRNAs [47] are now thoroughly profiled, the molecular mechanisms underlying their roles in different biological processes remain largely unknown, including epigenetic inheritance. Moreover, knowledge about additional types of RNA-derived fragments, such as snRNA- and snoRNA-derived sncRNAs, is limited. Some of these RNA-derived fragments were mistakenly identified as miRNAs [71] or piRNAs [72], but their unique biogenesis, cellular abundance, and functionality remain unknown. Although intriguing, it will be a challenge to comprehensively capture those sncRNAs in various biological situations and decipher their complex information in future studies [33].

3. RNA modifications and structures: the additional layers of complexity in the sncRNA kingdom

The complexity of the sncRNA field isn't solely due to diversity in sequence and genomic sources. Various factors have been discovered over the past decade, influencing sncRNA functionality. Among these factors, several well-characterized RNA modifications can regulate sncRNA function (Table S1), particularly in mammalian germ cell development and epigenetic inheritance.

To date, more than 150 chemical modifications, termed 'epitranscriptomics', have been identified in different RNA species including sncRNAs [73]. Advances in NGS methods for detecting RNA modifications demonstrate that RNA modifications play key roles in RNA metabolism and functions. Alterations in RNA modifications may lead to abnormalities in biological processes and cause diseases such as cancers [74,75]. In particular, sncRNAs and their precursors have multiple modifications, such as N6-methyladenosine (m6A), 5-methylcytosine (m5C), ADAR-mediated adenosine-to-inosine (A-to-I) editing, non-templated uridine addition and pseudouridylation (Ψ). The m6A may enhance the recognition and processing of primary miRNAs (pri-miRNAs) by DGCR8, promoting the maturation of miRNAs [76,77]. Conversely, miRNAs may affect m6A abundance on mRNAs by modulating the activity of METTL3, an m6A writer [78]. A-to-I editing, the most studied RNA editing form, produces an RNA sequence different from its template DNA. A-to-I editing is catalyzed by the Adenosine deaminase (AD) domain-containing proteins ADAR1 and ADAR2 in humans (ADAR and ADARB1 in mice) [79]. ADAR-mediated A-to-I editing is quite prevalent in miRNAs and their precursors [80] and is known to affect miRNA processing [81,82] as well as miRNA-mRNA interaction [83,84]. However, A-to-I editing, although essential for multiple biological events including embryonic development [82], appears to be dispensable for mammalian male fertility [85]. Non-templated addition of nucleotides (NTA), mainly consisting of 3′ mono- and poly-adenylation or uridylation, is generated by terminal nucleotidyltransferases (TENTs). NTA occurs on multiple RNA species, including miRNAs and snoRNAs [86], and is involved in the regulation of miRNA processing [87–89], interaction with target mRNAs [90], and stability [91,92]. Importantly, NTA is essential for miRNA functionality during various processes such as embryonic development [93,94]. In mouse male germ cells, the loss of TUT4 and TUT7, which are the primary uridylyl transferases, resulted in a significant loss of 3′ uridylation of piRNAs [95]. During spermatogenesis, 3′ uridylation, with mono-uridylation as a major form, enhances the targeting efficacy of piRNAs bound to MIWI [96]. Intriguingly, 3′ uridylation appears to compete with the signature 2′-*O*-methylation modification at the 3′ end of piRNAs. Hen1 (Henmt1 in mammals) deposits 2′-*O*-methylation on the 3′ terminal of piRNAs [97–101]. Loss of 2′-*O*-methylation is often linked with increased 3′ uridylation and enhanced degradation [102–105]. However, mono-uridylation of MIWI-bound piRNAs can co-exist with 2′-*O*-methylation [96], which suggests that HENMT1 may compete with TUT4/7, leading to most mono-uridylated piRNAs immediately methylated by HENMT1 to prevent further extension of the 3′ tailing. tRNAs and tsRNAs might be the sncRNA species that are most heavily decorated with RNA modifications. Up to 39 types of modifications have been identified in human tRNAs and 50 tRNA modification enzymes have been deposited in MODOMICS [73,106,107]. These modifications provide a regulatory mechanism for tRNA functions and stability, including tRNA cleavage or generation of tsRNAs. For example, m5C modification, catalyzed by DNMT2 or NSUN2, promotes tRNA stability by inhibiting the binding of endonuclease angiogenin (ANG), thereby protecting the tRNAs from being cleaved into tsRNAs [44,45]. Loss of DNMT2 leads to dysregulation of tsRNAs and rsRNAs in spermatozoa, and in turn, impairs the transmission of diet-induced metabolic disorders [44]. N1-methyladenine (m1A), N3-methylcytidine (m3C), and N1-methylguanine (m1G) modifications on tRNAs have similar ANG-inhibiting roles and thus repress the generation of tsRNA [108–111]. TET2, which converts the m5C on tRNAs to 5-hydroxymethylcytosine (hm5C), enhances the generation of 5′ tRFs (i.e. tRFs containing the 5′ sequence of tRNAs) but surprisingly also represses the generation of some 3′ tRFs (i.e. tRFs containing the 3′ sequence of tRNAs). tsRNAs generated from modified tRNAs can inherit those chemical modifications, although it remains elusive whether certain tsRNAs might also be targets of some tRNA modifiers. Multiple RNA modifications, such as m5C, N2-methylguanosine (m2G), m6A, and pseudouridylation, have been detected in tsRNAs in different biological samples, including mature sperm in mammals [44]. Pseudouridylation at position 8 (Ψ8) of tRFs, potentially mediated by Pseudouridine synthase 7 (PUS7) and additional factors [112], was shown to repress translation initiation by sequestering polyadenylate-binding protein 1 (PABPC1), a key translation factor [66]. This suggests that the chemical modifications in tsRNAs may have unique biological relevance beyond being inherited remnants from tRNAs.

4. The clinical application of small RNAs in human diseases

4.1. sncRNAs as biomarkers in diagnosis and prognosis

sncRNAs have great potential to be novel biomarkers for various diseases, including cancers [113], neurological [114,115], metabolic [116], cardiovascular [117], reproductive [118,119], and infectious diseases [120,121]. sncRNAs in biological fluids, which are often encapsulated in exosomes or bound with carrier proteins [122–124], can be stable and abundant enough for use as clinical biomarkers. Clinical usage of these extracellular sncRNAs is an especially appealing concept in liquid biopsy. Studies have profiled the sncRNA population in tissues and biological fluids (such as blood and seminal plasma) and identified

particular sets of sncRNAs of which the abundance is strongly correlated with diagnosis, prognosis, and treatment in human diseases. While current studies primarily focus on miRNAs in liquid biopsy as biomarkers, as technology improves, other sncRNA species may become applicable as biomarkers in clinics. For example, multiple piRNAs have been suggested as diagnostic or prognosis biomarkers in cancers [125]. The quality of in vitro cultured embryos may also be significantly associated with a panel of miRNAs, tsRNAs, and rsRNAs in sperm. Thus, this specific set of sncRNAs in sperm may be considered biomarkers for successful in vitro fertilization (IVF) [126]. Furthermore, sncRNAs, including miRNAs and piRNAs, have also been detected in the used culture media of IVF embryos, indicating that in vitro cultured embryos secret sncRNAs-containing extracellular vesicles [127]. As a result, the panels of sncRNAs from the spent culture media may have an indicative potential for embryo quality, such as ploidy and pregnancy outcomes [128–134].

The major challenge in clinical usage is that technical differences in sample handling procedures (e.g. contaminations and degradation) and detection methods (e.g. qRT-PCR, microarray, or NGS) [135] can readily affect the sncRNA profile. Therefore, the sncRNA signatures reported by different studies tend to be quite different and even contradictory. Cross-validation is crucial to determine the clinical utility of sncRNA signatures, and standardization of handling and detection procedures is much needed. Once cross-validated, these sncRNA signatures can be effective molecular targets for early diagnosis and effective treatment. In addition, the sncRNA profiles of spermatozoa, which are sensitive to environmental disturbances such as cigarettes [136,137], alcohol [138,139], endocrine disruptors [140,141], and air pollution [142], can reflect parental exposures. Furthermore, the sncRNA profiles of seminal plasma and follicular fluid may respond to parental exposures. Although not affecting sperm morphology, the sperm RNA code may still transmit adverse information to the next generations in response to environmental exposures. Therefore, the sncRNA profiles of sperm, seminal plasma, and follicular fluid may have the potential to serve as indicators for certain exposures in parents, which would be highly valuable in understanding disease predisposition in the Developmental Origins of Health and Disease (DOHaD) field.

4.2. Small RNA-based therapeutics

There have been significant advancements in the field indicating that sncRNAs have strong therapeutic potential. RNA-based therapy is an active and promising area of pharmaceutical development, which expands the range of druggable targets from disease-causing proteins to RNAs. Currently, there are 11 RNA-based therapeutics that have been approved by the FDA and/or the European Medicines Agency (EMA), and a few more are in late-stage clinical development (beyond phase II trial) [143–145]. Among the approved RNA-based drugs, 4 are double-stranded siRNA that exploit the endogenous RNAi pathway to silence target genes. The first RNAi drug that was approved by the FDA was Patisiran (Onpattro) [146,147], which was followed by Givosiran in 2019 [148], Lumasiran in 2020 [149] and Inclisiran in 2021 [150] (Table S2). These approvals marked the beginning of a new generation of RNA therapeutics.

Compared to small molecule drugs, The hydrophilic nature and larger size of unmodified RNAs make it difficult for them to efficiently move across cell membranes. In addition, naked RNAs are prone to degradation by RNases and unlikely delivered into specific tissues. Therefore, designing RNA-based drugs must consider key properties like Absorption, Distribution, Metabolism, Excretion, and Toxicity (ADMET), similar to other fields of pharmaceutical development. To improve the ADMET properties, a variety of delivery approaches have been developed for RNA-based drugs [143,151,152]. Patisiran, which is administrated intravenously, is encapsulated in a lipid nanoparticle (LNP) that protects the siRNA during plasma circulation. The LNP absorbs apolipoprotein E (ApoE), the ligand of low-density lipoprotein receptor (LDLR), which leads to a selective accumulation of patisiran in hepatocytes that express a high level of LDLR [153,154]. The other three RNAi drugs adopt alternative delivery approaches. Instead of being incorporated into nanoparticulate carriers, the therapeutic siRNA is directly conjugated to trimeric N-acetyl galactosamine (GalNAc) which is a tissue-targeting ligand that binds to asialoglycoprotein receptor (ASGPR) primarily expressed on hepatocytes, allowing effective and specific transport of the drug into the liver [148-150,155].

Aside from siRNAs, microRNA-based drugs, including microRNA mimics and antagomiR (i.e. microRNA inhibitors), show promise as therapeutic agents [144,156,157]. While no miRNA-based candidate drug has been approved by the FDA, a handful have reached phase II clinical trials (Table S2). As one miRNA can target multiple mRNAs often involved in one pathway, miRNA-based therapeutics, compared to siRNA-based therapeutics, may elicit a broader therapeutic effect. In contrast to gene silencers such as siRNAs and microRNAs, small activating RNAs (saRNAs) are double-stranded RNAs that activate target genes, making them a valuable addition to RNA therapeutics [158,159]. The first and currently the only saRNA drug entering clinical trials is MTL-CEBPA which upregulates C/EBP-α, a master transcriptional factor that regulates hepatic and myeloid functions. MTL-CEBPA is being developed to treat advanced hepatocellular carcinoma (HCC). [160,161].

5. Conclusion and future perspective

Over the past decades, studies have largely expanded our knowledge of the sncRNA kingdom, revealing the diversity of sncRNA classes, the complexity of their regulation, and the abundance of their functions. Yet, it is very likely that our current knowledge represents just the tip of the iceberg. Among the newer classes of sncRNAs, only tsRNAs appear to be actively being studied, and our understanding of these novel sncRNAs is still in its early stages, with little known about their biogenesis, cellular abundance, or context-dependent biological relevance. More importantly, while the characterization of identified sncRNA species may begin soon, another pressing question remains: whether there are more sncRNA species to be discovered. Multiple NGS methods and bioinformatic tools have been developed for the discovery of sncRNAs, yet profiling and quantification remain particularly challenging for sncRNA species that are structured, heavily modified, and/or highly conserved in sequences, such as tRNAs and tsRNAs. As the profiling of sncRNAs using NGS methods can be enormously interfered with by RNA modifications, mapping of RNA modifications and uncovering new sncRNA classes are also challenges. Some base-resolution NGS methods, as well as accompanied bioinformatics analyses, have been developed for a few prevalent RNA modifications, such as m6A, m5C, and pseudouridylation [33,162], but most appear to only profile one modification at one time. Multiple modifications may co-exist on one sncRNA molecule. Therefore, it is tempting to speculate that crosstalk exists between different modifications, and combinations of modifications may elicit a more profound effect compared to individual ones. This awaits the development of new approaches that can simultaneously profile multiple RNA modifications. To explore the sncRNA kingdom further, combining NGS methods with mass spectrometry, such as MS ladder complementation sequencing (MLC-seq) [163], as well as direct sequencing, may provide alternative solutions [33,164]. Additionally, profiling of novel sncRNAs and their modifications in rare samples, such as preimplantation embryos, remains mostly unachievable, presenting obstacles to further understanding in areas such as epigenetic inheritance.

Despite the tremendous breakthrough achieved in the clinical applications of small RNAs in the past decade, the field still faces major challenges when it comes to specificity, delivery, and tolerability, all of which need to be seriously considered during the design of RNA-based therapeutics [144]. Though challenging, with continued advancements in technology and our understanding of the sncRNA kingdom, small RNA-based therapeutics will no doubt have the potential to revolutionize the treatment modality for a wide spectrum of untreatable diseases.

It is important to note that this review only covers a few aspects of the vast and thriving sncRNA field. There are many other active directions in sncRNA research, such as sncRNA-protein interaction [165,166], counteracting or synergistic effects among sncRNAs and other RNA species [167], transport of sncRNAs within or across cells, i.e., the subcellular compartmentalization [168] and extracellular existence of sncRNAs [123,124,169], all of which are integral to our current exploration of this intriguing field. Collectively, we strongly believe that research in the sncRNA field will continue to be active and future studies will increase our understanding of sncRNAs, as well as their pioneering applications.

Declaration of competing interest

The authors declare that they have no conflicts of interest in this work.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fmre.2023.03.003.

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