REVIEW

RNA Networks that Regulate mRNA Expression and their Potential as Drug Targets

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Natural antisense transcripts (asRNAs) transcribed from eukaryotic genes are primarily long transcripts that do not code for proteins. Transcriptome analyses have revealed that asRNAs exhibit diverse functional roles in the regulation of gene expression. In the case of inducible genes, asRNAs epigenetically affect their expression or post-transcriptionally affect stability and translatability of their mRNAs. Many low-copy-number asRNAs regulate the expression levels of mRNAs through *cis*-controlling elements in the mRNA in concert with *trans*-acting factors, such as RNA-binding proteins and microRNAs. Recently, a competitive endogenous RNA (ceRNA) hypothesis was postulated as the basis of a functional network, comprising mRNAs, asRNAs, and microRNAs. This network finely tunes mRNA expression by common microRNA-responsive elements being present among mRNAs and asRNAs, permitting the redirection of microRNAs between the two. Examples of the ceRNA-mediated cross-regulation of mRNA expression are observed in the phosphatase and tensin homolog mRNA network and the interferon-alpha1 mRNA network. In such regulatory RNA networks, an mRNA, its corresponding asRNAs (high specificity), microRNAs (low specificity), and RNA-binding proteins mutually interact. Both asRNAs and microRNAs are involved in the pathogenesis or pathophysiology of various diseases, such as cancer, inflammation, and infection. Simple disruption of an asRNA or a microRNA can often show off-target effects due to complicated interactions inside and outside the regulatory RNA networks. Therefore, drugs that target asRNAs should be developed to minimize off-target effects and to target interactions that are dysregulated in disease.

Keywords: regulatory RNA network; antisense transcript; microRNA; RNA-binding protein; competitive endogenous RNA; drug

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Natural antisense RNA transcripts as a therapeutic target

Natural antisense transcripts (asRNAs) are transcribed from many eukaryotic genes, as well as from pseudogenes [1]. An asRNA has the same sequence as that of the complementary (antisense) strand of a gene, whereas the mRNA has the same sequence as that of the sense strand. Therefore, most asRNAs do not encode proteins, are more than 200 nucleotides in length, and are distinct from small non-coding RNAs such as microRNAs. Most asRNAs are thus classified as long non-coding RNAs (lncRNAs) [2]. Transcriptome analyses of mammalian genomes have demonstrated that asRNAs are frequently transcribed, indicating a role for asRNAs in gene regulation [3, 4].

However, to date, only a few asRNAs have been functionally validated.

Accumulated data have revealed that asRNAs have a broad array of functions in the regulation of gene expression by a variety of mechanisms. Both asRNAs and lncRNAs can alter gene expression at almost all steps of gene expression, from transcription to translation [1, 5]. In the expression of inducible genes in response to various stimuli, asRNAs epigenetically enhance the expression of these genes or post-transcriptionally affect stability and translatability of mRNA. asRNAs are assumed to regulate the expression of mRNAs through their *cis*-controlling elements (usually single-stranded regions) in concert with *trans*-acting factors, such as RNA-binding proteins and microRNAs [1]. Indeed, the interferon (IFN)-alpha1 asRNA modulates stability and thereby translatability of IFN-alpha1 mRNA at a post-transcriptional level [6].

The expression levels of asRNAs are generally much lower than those of mRNAs [1]. Despite this, asRNAs have distinct and diverse functions, and the mechanism of asRNA-mediated gene regulation differs for each gene [1]. Genome-wide expression analyses of nine different tissues from humans, mice, and rats show that asRNA expression is conserved [7]. Additionally, despite low levels of asRNAs expression, conservation of asRNA nucleotide sequences and secondary structures is also observed among species [1].

In various diseases, such as inflammatory or infectious diseases, neurodegenerative disorders, and cancer, many asRNAs are over-expressed, implying that asRNAs may be involved in the pathogenesis or pathophysiology of these diseases [8]. Initial approaches to target and 'knockdown' asRNAs involved the use of short interfering RNAs (siRNAs), which resulted in destabilization of cognate mRNAs, and a decrease in their levels. This silencing method successfully decreased levels of the mRNAs encoding endothelial nitric oxide synthase (eNOS) [9] and beta-site amyloid precursor protein-cleaving enzyme 1 (BACE1), which is involved in the development of Alzheimer's disease [10].

Unexpectedly, disruption of asRNAs also led to increases in the levels of some mRNAs, including those for brain-derived neurotrophic factor (BDNF), glial-derived neurotrophic factor (GDNF), and ephrin receptor B2 [11]. Therefore, the asRNA knockdown approach results in both concordant mRNA regulation (mRNA decrease) and discordant mRNA regulation (mRNA increase) [12]. These findings suggest that the asRNA-mediated mechanism of mRNA regulation is much more complicated than previously predicted.

Here, we focus on the networks among regulatory RNAs (asRNAs and microRNAs) that regulate mRNA expression, and we provide an overview of interactions in the networks that can potentially be exploited as drug targets.

Involvement of asRNAs in disease

asRNAs function to regulate mRNA levels as part of the overall mechanism that controls gene expression. However, dysregulated gene expression frequently results in disease and altered asRNA expression has been reported in many diseases, such as cancer, Alzheimer's disease, Parkinson's disease, spinocerebellar ataxia, Huntington's disease, and autoimmune diseases [8, 13, 14]. Here, we describe several examples of asRNA-mediated post-transcriptional mechanisms involved with disease.

Cancer

Tumor suppressor genes, such as *P53* and the phosphatase and tensin homolog (*PTEN*), are post-transcriptionally regulated by asRNAs [15, 16]. PTEN, phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase, negatively regulates the phosphatidylinositol-4,5-bisphosphate 3-kinase (PI3K)-Akt pathway, and its gene is mutated at a high frequency in a large number of cancers [17]. Furthermore, a subtle decrease in *Pten* gene expression is sufficient to promote cancer susceptibility in mice that express 80% normal levels of *Pten* mRNA [18]. *PTEN* expression is regulated by the action of a *PTEN* pseudogene (*PTENpg1*) at a post-transcriptional level [16]. The sense transcript transcribed from this pseudogene is an lncRNA (PTENpg1 lncRNA), which shares common microRNA-responsive elements (MREs, to which microRNAs may bind) with *PTEN* mRNA. Therefore, PTENpg1 lncRNA sequesters numerous PTEN-targeting microRNAs by acting as an endogenous microRNA 'sponge'. This competition with *PTEN* mRNA for microRNA binding, results in upregulation of *PTEN* mRNA. This type of transcript (PTENpg1 lncRNA) is designated as a competitive endogenous RNA (ceRNA) [19].

Two asRNAs isoforms (alpha and beta) are transcribed from the *PTENpg1* locus [16]. PTENpg1 asRNA-alpha epigenetically modulates *PTEN* transcription. In contrast, PTENpg1 asRNA-beta stabilizes PTENpg1 lncRNA through an RNA–RNA interaction, thereby increasing stability of PTENpg1 lncRNA and maintaining its microRNA sponge activity [16]. When PTENpg1 asRNA-beta is depleted, microRNAs are released from the PTENpg1 lncRNA and bind to *PTEN* mRNA, leading to a decrease in PTEN protein levels. These data show that the network mediated by the sense and antisense transcripts from the *PTENpg1*

pseudogene finely regulates PTEN mRNA degradation and translation.

Inflammation and infection

During infection-provoked inflammation, mRNAs are rapidly transcribed from inducible genes. Among them, early response genes [20], such as inducible nitric oxide synthase (iNOS), proinflammatory cytokines [*e.g.*, tumor necrosis factor-alpha (TNF-alpha)], and chemokines, are intimately involved in the pathophysiology of inflammation and infection. In the presence of interleukin 1beta, iNOS asRNA interacts with and stabilizes iNOS mRNA [21]. Single-stranded sense oligodeoxyribonucleotides corresponding to the iNOS mRNA sequence decrease iNOS mRNA levels by interfering with mRNA-asRNA interactions [21]. This method is referred to as natural antisense transcript-targeted regulation (NATRE) technology [22]. In contrast, when sense oligonucleotides corresponding to other mRNAs that are involved in inflammation (*e.g.*, mRNAs encoding TNF-alpha, nuclear factor-kappaB p65 subunit, and several chemokines) were introduced into hepatocytes, their mRNA levels increased (discordant regulation) [23, 24].

Following viral infection, IFN-alpha proteins induce the expression of IFN-stimulated genes, leading to antiviral activity. IFN-alpha proteins, which are classified as type I IFNs, consist of 13 functional subtypes that show high homology to IFN-alpha1 (*i.e.*, the IFN-alpha multigene family) [25]. Infection of human Namalwa lymphocytes with Sendai virus or infection of guinea pig fibroblasts with influenza virus elevated the expression of IFN-alpha1 asRNA [6]. IFN-alpha1 asRNA stabilizes IFN-alpha1 mRNA by interacting with a bulged stem-loop (BSL) region formed by the IFN-alpha1 mRNA [6]. The IFN-alpha1 asRNA may mask the microRNA-1270 (miR-1270)-binding site in the BSL region of the IFN-alpha1 mRNA and prevent microRNA-induced destabilization of the target mRNA, leading to IFN-alpha1 mRNA stabilization. Furthermore, antisense oligoribonucleotides (*i.e.*, asRNA mimics of IFN-alpha1 asRNA) elevated IFN-alpha1 mRNA expression [6].

Regulatory RNA networks

mRNA–asRNA interactions

asRNAs involved in post-transcriptional mechanisms are hypothesized to be potential drug targets, with disruption of the interaction between asRNAs and mRNAs a promising strategy. Both sense oligodeoxyribonucleotides [11, 21–24] and antisense oligoribonucleotides (asRNA mimics) [6] have been successfully used to interfere with this interaction. The copy number of an asRNA is generally lower than that of an mRNA, although asRNAs have high specificity to their cognate mRNAs. A recycling model for mRNA–asRNA interactions has been postulated [26]. When an asRNA interacts with an mRNA by hybridizing to loops in secondary structures, it recruits RNA-binding protein(s) and then detaches itself from the mRNA to interact with another mRNA. This model may explain how a low-copy number asRNA affects the functions of its mRNA by recycling of the asRNA molecule.

The lncRNA and mRNAs in the PTEN gene-centered ceRNA network

A high-copy number microRNA targets an mRNA through its seed sequence at relatively low specificity and may be another pivotal player in the regulation of mRNA expression. As mentioned above, PTENpg1 asRNA-beta stabilizes PTENpg1 lncRNA, which indirectly regulates the levels of PTEN mRNA as a ceRNA. Any transcript that is non-coding and that shares common MREs (*i.e.*, a lncRNA or asRNA) may be a ceRNA [19]. ceRNA crosstalk has been discovered in many species and is assumed to be involved in mRNA degradation [19].

Another type of ceRNA is represented by several protein-coding transcripts (*e.g.*, VCAN, VAPA, and ZEB2 mRNAs) that share microRNAs with the PTEN mRNA [27–29]. These mRNAs up-regulate the PTEN mRNA through MREs to modulate PTEN protein levels in a microRNA-dependent, protein-coding-independent fashion. Because the expression levels of these mRNAs correlate with various types of human cancer, the *PTEN* gene-centered ceRNA network may contribute to PTEN function and tumorigenesis [29].

asRNAs and mRNAs in the IFN-alpha1 gene-centered ceRNA network

Recent studies have reported asRNAs that share several MREs with their corresponding mRNAs. Kimura *et al.* investigated a possibility that IFN-alpha1 asRNA may also function as a ceRNA, in addition to competing with miR-1270 for IFN-alpha1 mRNA [25]. The authors found that IFN-alpha1 asRNA harbors multiple MREs for miR-1270 and that IFN-alpha1 asRNA forms a ceRNA network specifically with both mRNAs and asRNAs of the IFN-alpha family genes to antagonize miR-1270, thereby positively affecting IFN-alpha1 mRNA levels. These results suggest that IFN-alpha1 asRNA, together with asRNAs (alpha7, 8, 10, and 14) and mRNAs (alpha8, 10, 14, and 17) of specific IFN-alpha subtypes, act as competing molecules in the ceRNA network [25]. The *IFN-alpha1* gene-centered ceRNA

network is newly identified and forms a large regulatory network among many IFN-alpha family genes.

Furthermore, introduction of antimiR-1270 (an oligoribonucleotide complementary to the seed sequence of miR-1270) resulted in specific de-repression of other five mRNAs, indicating that these mRNAs also function in the ceRNA network [25]. Among the five mRNAs, the cell cycle-associated protein 1 (*CAPRIN1*) mRNA is included, and *CAPRIN1* can modulate signaling events that are necessary for translation of IFN-stimulated genes [30–32]. The use of antimiR led to the discovery that *CAPRIN1* mRNA modulates antiviral immunity by finely tuning the IFN-alpha response in a protein-coding-independent fashion [25].

The coordinated regulatory networks for IFN-alpha1 mRNA expression suggest a vital function for the innate immune system to precisely maintain physiological expression levels of IFN-alpha proteins [25]. Indeed, an antisense oligoribonucleotide that stabilizes IFN-alpha1 mRNA caused a reduction in human influenza A virus titers in the respiratory tract of infected guinea pigs (Kimura *et al*., unpublished results). The *IFN-alpha1* gene-centered ceRNA network may be critical for the innate immune responses against viral infection.

RNA–RNA interactions in a regulatory RNA network

Several parameters may affect the interactions among mRNAs, asRNAs, and microRNAs in a regulatory RNA network. First, the stoichiometry of RNA molecules and MREs should be considered. Examining the stoichiometry of RNA molecules in a ceRNA network raises several questions, such as how a low-copy-number asRNA with high specificity interacts with high-copy-number microRNAs with low specificity [29, 33]. These questions will be solved as the details of ceRNA networks are clarified.

Second, structures of mRNAs and asRNAs, as well as conformation of their hybrids, should be considered. Single-stranded regions (loops or bulges) in the secondary structures of mRNAs are assumed to be sites of interaction with asRNAs and microRNAs [6, 21]. Exposure of the single-stranded regions in an mRNA may trigger binding to its partner RNA. This may induce changes in duplex conformation and to local structures, which may enhance or suppress binding of RNA-binding proteins [1, 34]. Additionally, various drugs may alter the stem-loop structures and duplex conformation of RNA, for example by intercalating in RNA duplexes or binding to RNA [1]. Because new approaches to analyze *in vivo* RNA structures have recently been developed [35, 36], clarification of detailed RNA structures will be possible in the future.

Third, accessibility and affinity of an RNA molecule for its partners should be considered [1]. A recent study clarified the function of N^6-methyladenosine (m^6A), which is the prevalent internal modification present in mRNAs of all higher eukaryotes [37]. The m^6A modification regulates degradation of mRNA and lncRNA through RNA-binding proteins (YTHDF2 proteins) that recognize and bind to m^6A [37]. Because asRNAs are classified as lncRNAs, asRNAs might be m^6A modified, and the modification might regulate stability of the asRNA. Interestingly, m^6A modification also controls the RNA structure-dependent accessibility of RNA-binding proteins to affect mRNA–protein interactions [38]. When m^6A-modified asRNAs interact with mRNAs, the asRNAs may indirectly regulate mRNA stability and its accessibility to RNA-binding proteins.

Finally, small polypeptides encoded by lncRNAs might also be functional [39]. Recent reports suggest a possibility that small polypeptides (20–100 amino acids) translated from short open reading frames (ORFs) of lncRNAs have biological activities. Although such ORFs in asRNAs and lncRNAs are generally ignored in gene annotation algorithms, the small polypeptides translated from these ORFs might affect the interactions in the regulatory RNA network, leading to changes of gene expression.

Detailed knowledge of the various interactions within these regulatory networks remains limited. Future studies will help pave the way for the development of new drugs that target critical interactions in these networks.

Drugs that target asRNAs in regulatory RNA networks

RNA has long been a drug target, although drug–RNA interactions have not been well studied. A drug that targets an asRNA is expected to affect multiple interactions in a regulatory RNA network, including RNA–RNA interactions. The mechanism of asRNA-mediated gene regulation differs among genes, and sometimes involves a ceRNA network. Therefore, the drug should be specific in its targeting of asRNA with minimal off-target effects. Several drugs have been designed to regulate the functions of asRNAs based on their mechanisms of action, in a similar manner to drugs that target lncRNAs [13].

(1) *Degradation of asRNAs*. In this strategy, siRNA is used as a potential drug to disrupt a single asRNA molecule, resulting in impairment of its functions [9, 10]. However, if the asRNA functions as a ceRNA and its MREs are shared by other asRNAs or mRNAs, off-target effects may occur. Therefore, silencing of an asRNA alone is sometimes insufficient to normalize dysregulated gene expression in disease.

(2) *Functional block of asRNAs*. Interaction sites for binding partners (mRNAs, microRNAs, or RNA-binding proteins) are blocked by sense oligonucleotides, asRNA mimics (oligoribonucleotides), or antimiRs (oligonucleotides complementary to microRNA seed regions) [6, 11, 21, 22, 25]. These oligonucleotides have the potential to be used for a therapeutic purpose. Some of these oligonucleotides may also possess activity to degrade asRNA to affect stability and translatability of mRNA. Alternatively, when an asRNA harbors multiple MREs, asRNA mimics may sequester microRNAs. Therefore, these oligonucleotides have potential to be nucleic acid drugs that can modulate RNA interactions in disease.

(3) *Structure disruption*. A drug may bind to a specific structure in an asRNA to inhibit binding with partners. This type of drug is usually associated with the loss of asRNA function [1]. Previous studies have reported classical low-molecular-weight drugs that specifically bind to RNA to disrupt structure and block function. A famous example is aminoglycoside antibiotics, which bind to the aminoacyl-transfer RNA decoding site (A site) on the 16S ribosomal RNA to interfere with translation in bacteria [40]. However, only a few other drugs that specifically recognize RNA structures are known. For example, acetylpromazine, a psychotropic drug, recognizes and binds to the specific structure of transactivation-responsive (TAR) RNA, to which the transactivating regulatory (Tat) protein of human immunodeficiency virus type I (HIV-1) binds [41]. RNA aptamers, which bind to asRNA (or RNA-binding proteins) in a structure-specific, but sequence-independent manner, may also function as drugs to disrupt structures.

Many constituents of herbal medicines, which have low molecular weights (less than 1,000), destabilize iNOS mRNA, indicating that they might interfere with the iNOS mRNA–asRNA interaction [1]. Indeed, epigallocatechin gallate and apigenin, both of which are flavonoids in herbal medicines, bind to RNA with high affinity [42, 43], suggesting the possibility that flavonoids interfere with mRNA–asRNA interactions. Detailed studies on such drugs are required to elucidate how the drug–RNA interactions affect various interactions in a regulatory RNA network.

Perspectives

Here, we provide an overview of regulatory RNA networks. Within a network, regulatory RNAs (asRNAs, microRNAs, and other lncRNAs), as well as RNA-binding proteins, mutually interact to finely tune mRNA expression. In this sense, a regulatory RNA network resembles the solar system, which harbors the sun (mRNA), planets (asRNAs), and asteroids (microRNAs and RNA-binding proteins) (Figure 1). MicroRNAs and RNA-binding proteins cross-interact with other networks, and an asRNA or mRNA may act as a ceRNA to sequester microRNAs from inside and outside the network. Therefore, the transcriptome in the cell, which consists of many networks, can appear as a 'microcosmos'. Understanding this '*RNA microcosmos*' will lead to the development of new drugs.

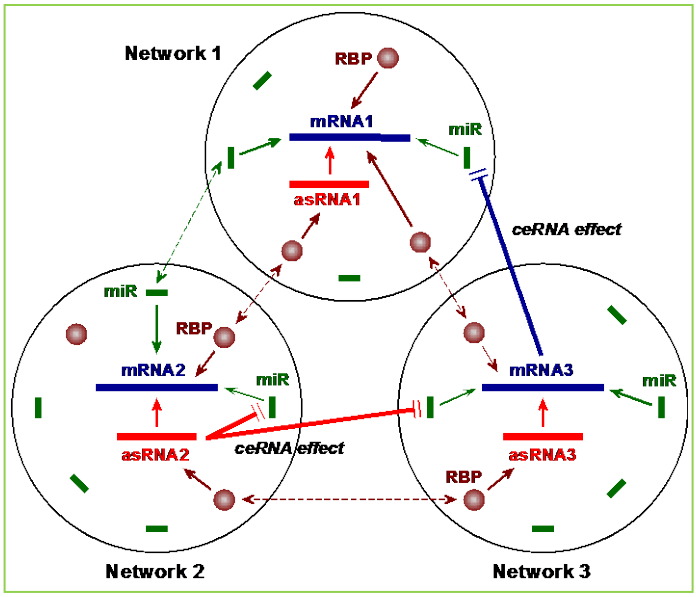

Figure 1. The RNA microcosmos in the cell. A model of regulatory RNA networks in the cell is schematically depicted. Each network (shown by a circle) consists of an mRNA, an antisense transcript (asRNA), many microRNAs (miRs), and various RNA-binding proteins (RBPs). The asRNAs, miRs, and RBPs affect the expression of mRNAs (arrows). miRs and RBPs may enter another regulatory RNA network and regulate the expression of another mRNA (broken double-headed arrows). As ceRNAs, an asRNA (red) or mRNA (blue) may sequester miRs inside and outside the network (bold stopped lines), resulting in the up-regulation of mRNA (*i.e.*, asRNA2 *versus* mRNA2, and asRNA2 *versus* mRNA3). An mRNA may also show the ceRNA effect on another mRNA (*i.e.*, mRNA3 *versus* mRNA1), such as in the *PTEN* gene- and *IFN-alpha1* gene-centered networks. Therefore, a regulatory RNA network cross-interacts with other networks to affect mRNA expression via miRs, RBPs, and ceRNAs. All the regulatory RNA networks in the cell form a transcriptome, the '*RNA microcosmos*'.

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Conflict of Interest

M.N. and T.K. declare that there are no potential conflicts of interest.

Abbreviations

asRNA: antisense transcript; lncRNA: long non-coding RNA; IFN: interferon; siRNA: short interfering RNA; PTEN: phosphatase and tensin homolog; *PTENpg1*: phosphatase and tensin homolog pseudogene 1; iNOS: inducible nitric oxide synthase; TNF-alpha: tumor necrosis factor-alpha; ceRNA: competitive endogenous RNA; MRE: microRNA-responsive element; miR-1270: microRNA-1270.

Each author's contribution

M.N. and T.K. wrote the manuscript together. Final editing and manuscript preparation were coordinated by M.N. and T.K.

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