Emerging Roles for Natural MicroRNA Sponges

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Minireview

Margaret S. Ebert and Phillip A. Sharp

Recently, a non-coding RNA expressed from a human pseudogene was reported to regulate the corresponding protein-coding mRNA by acting as a decoy for microRNAs (miRNAs) that bind to common sites in the 3′ untranslated regions (UTRs). It was proposed that competing for miRNAs might be a general activity of pseudogenes. This study raises questions about the potential ability of thousands of non-coding transcripts to interact with miRNAs and influence the expression of miRNA target genes. Three years ago, artificial miRNA decoys termed 'miRNA sponges' were introduced as a means to create loss-of-function phenotypes for miRNA families in cell culture and in virally infected tissue and transgenic animals. Given the efficacy of miRNA sponges expressed from stable chromosomal insertions, it seemed plausible that natural non-coding RNAs might have evolved to sequence-specifically sequester miRNAs. The first such endogenous sponge RNA was discovered in plants and found to attenuate a miRNA-mediated response to an environmental stress. More recently, a viral non-coding RNA was observed to sequester and promote the degradation of a cellular miRNA in infected primate cells. In this review we discuss the potential and proven roles for endogenous miRNA sponges and consider some criteria for screening candidate sponge RNAs.

Introduction

microRNAs (miRNAs) are ~21–23 nucleotide RNAs that are derived from hairpin precursors and that associate with Argonaute proteins to post-transcriptionally regulate target genes, typically by binding to partially complementary sequences in the 3′ untranslated region (UTR). Over the past few years miRNAs have been established as important regulators of development and physiology in animals and plants. Inhibition of miRNA activity by antisense oligonucleotides or antagomirs has been used to study their functions but in many cases a more biological approach is preferable. This approach is to block the activity of a specific miRNA of interest using a competitive inhibitor called a miRNA sponge or target mimic [1,2]. Sponge RNAs contain binding sites for the miRNA either in a non-coding transcript or in the 3′ UTR of a reporter gene, and their expression is driven to a high level by strong promoters such as U6 or CMV in mammalian cells (Figure 1). Partial miRNA inhibition is achievable when sponge RNAs are expressed from chromosomal transgene insertions [1], and the use of lentiviral and retroviral sponge vectors has enabled continuous miRNA inhibition in dividing and non-dividing cells over long durations. These stable sponge constructs have been used to probe the roles of miRNAs in a variety of systems: *in vitro* differentiation of neurons [3] and mesenchymal stem cells [4]; xenografts of cancer cell lines [5,6]; and bone marrow reconstitutions from hematopoietic stem/progenitor cells [7–9]. Germline transgenic fruitflies have been shown to generate hypomorphic miRNA phenotypes when sponge expression is induced in a tissue-specific manner via the Gal4–UAS system [10].

Department of Biology, Massachusetts Institute of Technology, Cambridge, MA 02139, USA. Koch Institute for Integrative Cancer Research, Cambridge, MA 02139, USA.
E-mail: sharppa@mit.edu

Hypothetical Roles for Natural miRNA Sponges

Given the ability of stably integrated mRNA-based miRNA sponges to specifically, and in some cases inducibly, inhibit miRNA seed families, it seems reasonable to expect that nature might also have invented this type of miRNA inhibitor. There are further reasons to support this hypothesis. First, miRNAs have been shown to be very stable [11], some with *in vivo* half-lives of more than a week [12]; thus, it should be more effective to induce a sponge RNA to sequence-specifically sequester a miRNA than to sequence-specifically degrade the mature miRNA strand, which is encased in an Argonaute protein complex. Sequestration by a target mimic RNA would likely operate through seed specificity, as appears to be the case for most target mRNAs, so in this case an entire functional class of miRNA seed family members would be inhibited. Finally, effective sponges should be easy to evolve as they require only short stretches of complementarity to miRNA seeds in regions of relatively unstructured RNA. A sponge could contain sites for one miRNA family or for a combination of miRNAs such that it could serve as a specific rescue molecule for one or a few target genes.

One can imagine several scenarios in which the expression of a sponge RNA could add a layer of regulation to post-transcriptional control of miRNA targets. During a developmental transition or in response to a cellular stress, when a miRNA is transcriptionally down-regulated, induction of a sponge RNA could sharpen the loss of that miRNA activity over time (Figure 2A). A miRNA induced to respond to a transient stress could be inhibited shortly thereafter by the accumulation of a stress-induced sponge (Figure 2B). Alternatively, such a stress-induced sponge could act as a quality control mechanism, setting a threshold above which miRNA expression must rise to adequately repress the expression of critical target genes. A viral sponge RNA could inhibit a host miRNA to change the infected cell's gene expression program so as to evade immune response or hijack cellular pathways to promote viral propagation. A sponge RNA expressed in a specific tissue could uncouple the activity of an intron-derived miRNA from the expression of its host gene. A tissue-specific sponge could also neutralize passenger strand miRNA-containing ribonucleoprotein complexes (miRNPs) to enhance the specificity of active miRNA complexes (beyond what is determined by the thermodynamic asymmetry of the miRNA duplex that normally controls strand assembly), as has been done with artificial sponges to prevent passenger strand-mediated off-target effects from short hairpin RNA (shRNA) vectors [13]. A sponge could be constitutively expressed to fine-tune the activity of a miRNA to a slightly lower level. In certain cellular contexts, such as

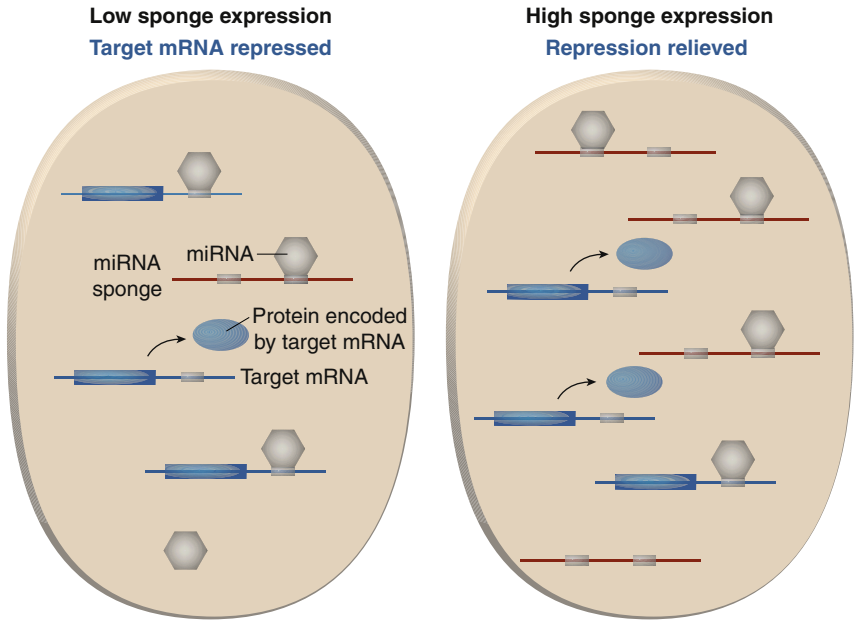

Figure 1. Sponge RNAs compete with target mRNAs for miRNA binding.

Sponge RNAs (in red) contain binding sites (grey rectangles) for a miRNA of interest (grey octagons). Left: in the presence of low sponge expression, target mRNAs (in blue) are post-transcriptionally repressed by the miRNA. Right: in the presence of high sponge expression, target mRNAs are relieved of repression; higher protein output (blue ovals) results.

in neurons, spatially separated zones of translation could experience major consequences from local sequestration of miRNA and the ensuing rescue of expression of a small pool of messages.

Evidence for Natural miRNA Sponges

The first evidence for natural miRNA sponges was discovered in plants [2]. The TPSI family of non-coding RNAs (IPS1 and its paralog At4) are processed as mRNAs but contain very short, poorly conserved open reading frames. They also contain in the 3′ UTR a 23-nucleotide sequence that is highly conserved among different plant species and that can act as a single bulged binding site for miR-399. In fact, the miRNA's nucleotides 1–10 are perfectly paired in more than 80% of *IPS1* genes; there is additional strong, conserved pairing to the miRNA's 3′ end. The mismatches opposite nucleotides 10 and 11 protect the mRNA from endonucleolytic cleavage by miR-399-loaded Argonautes. While the TPSI RNAs are induced upon phosphate starvation, miR-399 expression rises earlier, and the miR-399 target gene *PHO2* is initially down-regulated [14]. Franco-Zorrilla *et al.* [2] found that overexpressing *IPS1* in the presence of miR-399 was able to rescue the level of PHO2 mRNA and thereby lower the shoot phosphate content. (Whether the endogenous TPSI levels are sufficient to derepress *PHO2* to incur the same physiological response remains to be shown.) As miR-399 and its sponge inhibitor are both induced by phosphate stress, they appear to act in an incoherent manner to regulate *PHO2* target expression. Depending on the relative production and turnover rates of the miRNA and the sponge RNA, this type of regulatory architecture could serve to generate a brief pulse of miRNA activity followed by an attenuation period during which target mRNA levels recover [14].

mRNAs that act as competitive inhibitors of regulatory small RNAs (sRNAs) were also recently discovered in prokaryotes [15,16]. In this case a constitutively expressed, long-lived sRNA binds to and is destabilized by a target mimic RNA which is induced by chitobiose, a breakdown product of chitin from the outer membrane [17]. What results is derepression of a chitoporin gene whose message is normally degraded by the sRNA.

Sequence-specific miRNA destabilization was also recently observed in an animal system. Marmoset T cells transformed with the primate virus *Herpesvirus saimiri* (HSV) contain abundant viral non-coding transcripts called HSURs (*H. saimiri* U-rich RNAs). Highly conserved regions of HSURs 1 and 2 have potential to base-pair with host miRNAs miR-16, -27, and -142-3 p [18]. Psoralen cross-linking experiments and knockdown of specific HSURs confirm the interaction of miR-27 with HSUR 1. Both miR-27 family members, miR-27a and -27b, are post-transcriptionally down-regulated in HSV-transformed cells in a manner dependent on the presence of HSUR 1, and a pulse-chase assay shows accelerated turnover of the miR-27a guide strand. Additionally, protein expression of the miR-27 target gene *FOXO1* is up-regulated in the presence of HSUR 1. It is not clear in which cellular compartment the HSUR interacts with the mature miRNA; both miRNPs and HSURs might shuttle between the nucleus and the cytoplasm. The mechanism by which HSUR–miR-27 binding induces destruction of the miRNA is also not yet known, but clearly involves more than simple sequestration. Some users of artificial miRNA sponges have reported substantial reduction in the level of the inhibited miRNA [19–21]. In *Drosophila* and in mammalian cells, target reporter sites with extensive complementarity to the 3′ end of the miRNA also appear to stimulate miRNA turnover, by accelerating exonucleolytic trimming of the miRNA [22]. This trimming phenomenon may be taking place in the case of artificial target mimics and perfectly complementary antisense oligonucleotide inhibitors ('antagomirs') [22] and in the case of HSUR–miR-27 interaction.

There are also hints that a viral miRNA sponge may be produced in cells lytically infected with murine cytomegalovirus [23]. Upon infection, Buck *et al.* [23] observed rapid post-transcriptional down-regulation of miR-27a and -27b, in a manner dependent on RNA polymerase activity; higher multiplicity of infection correlated with lower miR-27 levels. A gain-of-function experiment showed that the miR-27 family suppresses viral replication, supporting the possibility that inhibition of this miRNA family by a viral sponge RNA could facilitate viral replication.

PTENP1 Pseudogene as a Source of Sponge Activity

Recently, a mammalian cellular non-coding RNA was proposed as a miRNA sponge. *PTENP1* is a pseudogene of PTEN derived from retrotransposition and containing a

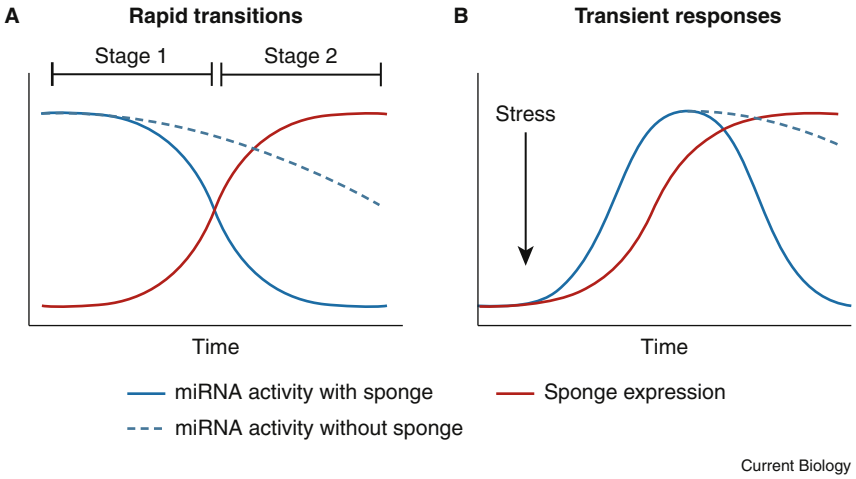

Figure 2. Roles for natural sponges in regulating miRNA activity.

(A) Rapid transitions: transcriptional down-regulation of a miRNA is sharpened by induction of a sponge RNA that sequesters the lingering mature miRNA. (B) Transient responses: a stress-induced miRNA is allowed a pulse of activity before being inhibited by accumulating stress-induced sponge RNAs.

mutated start codon such that its mRNA does not produce protein [24]. The *PTENP1* 3′ UTR is truncated but its proximal region has 95% identity with the 3′ UTR of *PTEN* and contains sites for five of the miRNAs with conserved binding sites in *PTEN*'s 3′ UTR: miR-26, -17-5p/20, -21, -19, and -214. Of these, miR-17-5 p/20 p and -19 (which are both naturally expressed from the sometimes oncogenic 17∼92 cluster) are able to repress both *PTENP1* and *PTEN* RNA levels to a similar degree (even though the *PTEN* 3′ UTR contains two additional conserved miR-19 sites). Retroviral overexpression of the *PTENP1* UTR derepresses *PTEN* in a Dicer-dependent manner. More importantly, knockdown of endogenous PTENP1 in prostate cancer cells results in a decrease in *PTEN* mRNA and protein levels, and those of the miR-17-5 p/20 target p21 and potentially other relevant targets. This is accompanied by accelerated cell proliferation. *PTENP1* and *PTEN* have correlated mRNA expression in prostate tumor and normal prostate samples, and some sporadic colon cancer samples are found to have *PTENP1* copy number losses at the genomic level that correlate with decreased *PTEN* mRNA expression. A similar correlation of expression is found between *KRAS* and its pseudogene *KRAS1P*.

Poliseno *et al.* [24] invoked a decoy activity for the pseudogene RNA, suggesting it regulates *PTEN* expression by competing for the same combination of miRNAs. However, it seems unlikely that in the DU145 cells analyzed in the study, in which the *PTENP1* RNA is expressed at a much lower level than the *PTEN* mRNA (∼1%), the pseudogene RNA could significantly modulate the level of PTEN and other target genes through interaction with the miRNAs. In some prostate cancer samples, the pseudogene is reported to be expressed at approximately 10% the level of the *PTEN* mRNA and in a few cases the two are approximately equal. It is unclear how RNAs from the pseudogene expressed at these lower levels could successfully compete for hundreds or thousands of miRNA molecules in the presence of hundreds of target mRNAs. That said, it is conceivable that an RNA regulator with special properties such as those mentioned above for HSUR RNAs, perhaps working by a catalytic miRNA-turnover mechanism, could influence the expression of a target gene expressed at a higher level. It is also conceivable that an RNA regulator could be effective if the target genes it derepresses are sensitive to subtle changes in protein level. *PTEN* is a haploinsufficient tumor suppressor for which even a 20% decrease in expression can promote cancer growth [25]. In plants, in which target mRNAs are dramatically inhibited by miRNAs through endonucleolytic cleavage, the target expression profile should be more drastically shifted by the introduction of a sponge RNA than in animals, in which fine-tuning of targets by miRNAs may be the norm.

Potential Additional Natural miRNA Sponges

The plant TPSI RNAs, viral HSURs, and pseudogene RNAs implicate classes of non-coding RNAs that could be further investigated for potential miRNA sponges. There are several other classes that should also be considered. Recently genome-wide analysis of chromatin marks has uncovered hundreds of large intergenic non-coding RNAs (lincRNAs) [26]. Some of these transcripts act in the nucleus to regulate gene expression by interaction with chromatin [27], while others localize to the cytoplasm where they could interact with mature miRNAs. It should be noted, however, that having predominantly nuclear localization does not preclude an RNA from being able to inhibit miRNA, as in the case of HSUR 1 [18] or the U6 promoter-driven artificial miRNA sponges [1]. There are also dozens of RNA-polymerase-III- and II-generated mRNA-like non-coding RNAs of undetermined function listed in non-coding RNA databases; some have been detected at high levels in specific cell types or under specific conditions [28]. Such RNAs may be transcribed from intergenic promoters or from promoters within 3′ UTRs. Another mechanism that can generate a 3′ UTR RNA was recently observed in mouse embryonic development: an exon exclusion event causes the entire coding region of the mRNA to be spliced out, leaving the untranslated regions in a non-coding transcript [29]. Gene fusions that are generated by translocation events can also create new expression patterns for 3′ UTRs or UTR fragments. Can target mRNAs be miRNA sponges? It is possible that some miRNA target genes whose repression is functionally inconsequential evolved binding sites to act as sponges, tuning miRNA availability to a precise level for the regulation of a small number of targets whose repression does have important phenotypic consequences [30]. Some observations of 3′ UTR-mediated effects from the literature dating before the discovery of miRNAs might now be appreciated in light of a possible sponge mechanism. For example, physiological levels of the 3′ UTRs of alpha-cardiac actin, tropomyosin, and skeletal muscle troponin 1 were shown to boost the expression of myogenin and promote differentiation of myoblasts [31]; and the 3′ UTRs of prohibitin [32] and MAT1/PEA-15 [33] influenced proliferation in cancer cell lines.

Several criteria may be helpful for screening candidate sponge RNAs. As with target genes, the miRNA binding sites are more likely to be functional when located in regions of little secondary structure (although effective decoys have been reported in which the miRNA binding sites are presented specifically in the unpaired sequences of short stem-loop elements [34]), outside the footprint of ribosomes or RNA binding proteins, and when they show sequence conservation among related species. There must be overlap in the expression and subcellular localization of the sponge RNA and the miRNA(s) whose sites it contains in order for their molecular encounters to occur. The higher the expression of the sponge RNA, the more binding sites it contains, and the more extensive the complementarity at the binding sites, the greater the expected effect of sponge RNA on miRNA. When validating a putative sponge, there must be demonstrable derepression of target genes at physiological sponge RNA (and miRNA) expression levels and the derepressive effect must be attributable to the miRNA binding sites.

Outlook

The discovery of natural transcripts that block miRNA activity has revealed a new layer of post-transcriptional regulation with many potential roles in the biology of animals, plants, and viruses. A growing collection of non-coding RNAs will be under investigation for their potential to interact with miRNAs. Perhaps the search should also consider competitive inhibitors for other classes of small RNAs, such as endogenous small interfering RNAs (siRNAs) and Piwi-interacting small RNAs (piRNAs).

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