EXPERT OPINION

Decoy activity through microRNAs: the therapeutic implications

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Introduction: microRNAs (miRNAs), small noncoding RNAs, are deregulated in several diseases including cancer. miRNAs regulate gene expression at a post-transcriptional level by binding to 5′UTR, coding regions or 3′UTR of messenger RNAs (mRNA), inhibiting mRNA translation or causing mRNA degradation. The same miRNA can have multiple mRNA targets, and the same mRNA can be regulated by various miRNAs.
Areas covered: Recently, seminal contributions by several groups have implicated miRNAs as components of an RNA–RNA language that involves cross-talk between competing endogenous RNAs through a decoy mechanism. We review the studies that described miRNAs as players in a biological decoy activity. miRNAs can either be trapped by competing endogenous RNAs or interact with proteins that have binding sites for mRNAs.
Expert opinion: The miRNA decoy functions have implications for the design of therapeutic approaches in human diseases, including specific ways to overcome resistance to drug therapy and future miRNA-based clinical trials design.

Keywords: mimic, regulation, sponge, therapeutic potential

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1. Introduction

Contrary to the case with protein-coding genes, there is a proportional increase between the number of noncoding transcripts (ncRNA, RNAs that do not codify for proteins but influence by various mechanisms their expression and function) and the complexity among the species [1,2], with the ratio of noncoding to coding sequences being about 47:1 in humans [3]. The use of massively parallel sequencing platforms of "next-generation sequencing," with use of the RNA sequencing (RNA-seq) application, revealed thousands of transcripts and opened the door to a comprehensive study of the nonprotein-coding transcriptome [4,5]. Up to 97% of the human genome consists of nonprotein-coding DNA [6], and noncoding transcripts comprise a heterogeneous group that includes, among other, microRNAs (miRNAs), small nucleolar RNAs (snoRNAs), infrastructural RNAs (ribosomal RNA [rRNA], transfer RNAs [tRNAs], and small nuclear RNAs [snRNAs]), long intergenic noncoding RNAs (lincRNAs), antisense transcripts, noncoding transcribed ultraconserved regions, and promoter upstream transcripts (PROMPTs) [7]. The ncRNAs can be intergenic or can overlap with the coding regions loci, being antisense or intronic [2]. The paradigm of RNA as a mere intermediary between DNA and protein became obsolete when it became evident that the previously called "junk" DNA [8] could be of major importance for biologic diversity [5] and may have key biological functions [9]. Although much still needs to be studied about the function of ncRNAs, the top of the iceberg is being revealed as we increase our understanding of the importance of ncRNAs in the regulation of gene expression.

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Article highlights.

- RNA transcripts that have the same specific miRNA binding sites can compete for miRNAs binding.
- miRNAs can be trapped by ceRNAs that function as natural miRNA sponges.
- miRNAs function as RNA decoys upon interaction with heterogeneous ribonucleoproteins, as hnRNP (that have binding sites for mRNAs).
- Decoy mechanisms involving miRNAs and the balance between levels of specific ceRNAs should be considered when designing new therapeutic approaches aiming to correct gene expression.
- The hypothesis that miRNA-based decoy mechanisms play a role in the resistance to therapy is worthy of being explored.
- For therapeutic approaches using precursor microRNAs, when two mature forms are processed, their expression, function and mRNA targets should be considered, as some of these could be ceRNAs for both 3p and 5p of that specific miRNA.

This box summarizes key points contained in the article.

In this review, we intend to focus on the studies that identified the miRNA decoy activity, and we propose to emphasize the potential significance of these studies for therapy.

2. Current evidence

miRNAs are small ncRNAs, approximately 20 nucleotides long, that control gene expression at a posttranscriptional level by mRNA degradation or translation inhibition, through the binding to 5′UTR, coding sequences, or 3′UTR of target mRNAs [10]. miRNAs can regulate multiple target genes, and simultaneously, target genes can be regulated by multiple miRNAs. Victor Ambros' and Gary Ruvkun´s groups first discovered miRNAs in 1993 as a new mechanism of gene regulation in *Caenorhabditis elegans* [11,12]. In 2000, Pasquinelli *et al.* proved that let-7 was conserved among species, opening the door for the study of miRNAs in humans [13]. Later, in 2002, it was reported that miRNAs were deregulated in cancer [14]. To date, thousands of studies have analyzed the expression of miRNAs and its role in cancer and in cardiovascular, autoimmune, and neurodegenerative diseases [10].

Recently, another function for miRNAs was found: the decoy activity. In early 2010, Perrotti´s group reported the paradigm shift in miRNA function [15]. hnRNP E2 is a member of the family of RNA-binding proteins whose members are involved in mRNA processing, nucleocytoplasmic export, and translation of mRNAs [16]. In cells in which the BCR/ABL oncogene is expressed, causing an arrest of differentiation in myeloid blast crisis chronic myelogenous leukemia, there is an increase of hnRNP E2 protein [17]. hnRNP E2 interacts with the transcription factor *CEBPA*, inhibiting the translation of mRNA. Using RNA electrophoretic mobility shift assays, UV crosslinking, and RNA immunoprecipitation assays, the authors found that miR-328 (that is downregulated in a BCR/ABL-dependent manner) competes with *CEBPA* mRNA for the hnRNP E2 binding site [15]. They also proved that restoration of miR-328 expression interferes with hnRNP E2 function of translation inhibition by preventing *CEBPA*:: hnRNPE2 binding and consequently restores, *in vivo* and *in vitro*, *CEBPA* mRNA translation [15] (**Figure 1A**). Besides the decoy activity, miR-328 also functions in the canonical way by suppressing translation of mRNA encoding the PIM1 protein kinase through base pairing interaction [15].

Later the same year, Poliseno *et al.* reported in *Nature* the intriguing discovery that pseudogenes could function as a decoy for miRNAs' effects on corresponding protein-coding genes [9]. The authors used as a model the well-known tumor suppressor *PTEN* and its pseudogene *PTENP1*, which has a high sequence homology with part of the *PTEN* 3′UTR [9]. The authors proved that *PTENP1* is targeted by some of the miRNAs that target also *PTEN*, including miR-19b and miR-20a. Through a miRNA-dependent mechanism, overexpression of *PTENP1* 3′UTR resulted in the derepression of *PTEN* (and consequently proved that *PTENP1* has a role as a tumor suppressor), and expression of *PTEN* 3′UTR resulted in the derepression of *PTENP1* [9]. In addition, the authors showed that the same decoy mechanism is present when analyzing other genes and their related pseudogenes, such as the *KRAS* gene and its pseudogene *KRAS1P* [9]. This new concept was further developed 1 year later, when the same group showed that not only noncoding genes can compete for miRNAs binding sites, but also protein-coding transcripts can compete with one another [18]. Transcripts that have the same miRNA binding sites (or miRNA response elements [MREs]) are called "competing endogenous RNAs" (ceRNAs) [19] and may act as natural miRNA sponges. The authors used bioinformatics (MRE enrichment—MuTaME—analysis) and biological approaches to validate ceRNA for *PTEN* [18]. Some of these mRNAs are *SERINC1*, *VAPA*, and *CNOT6L*, whose expression in human prostate cancer and glioblastoma samples was significantly different between PTEN-high and PTEN-low groups [18]. In addition, silencing of these ceRNAs resulted in a decrease in luciferase activity when cells were co-transfected with a luciferase vector containing the *PTEN* 3′ UTR. The authors further proved that this correlation was dependent on the miRNAs, since regulation of *PTEN* expression by *SERINC1*, *VAPA*, and *CNOT6L* ceRNAs vanished in the cells with a defect in the miRNA processing machinery [18] (**Figure 1B**).

In the same issue of *Cell*, three articles reported "out-of-the-box" discoveries about coding transcripts and competing endogenous RNAs. Sumazin *et al.* used a multivariate analysis method, named Hermes, to combine gene expression data with miRNA profiles in glioblastoma and found 7,000 genes whose transcripts were involved in sponge regulatory interactions (modulator and sponges share miRNAs binding sites) and 148 genes that were involved in nonsponge regulatory interactions (modulator and sponges that do not necessarily share miRNA binding sites) [20]. Moreover, the authors focused on the *PTEN* transcript to perform validation

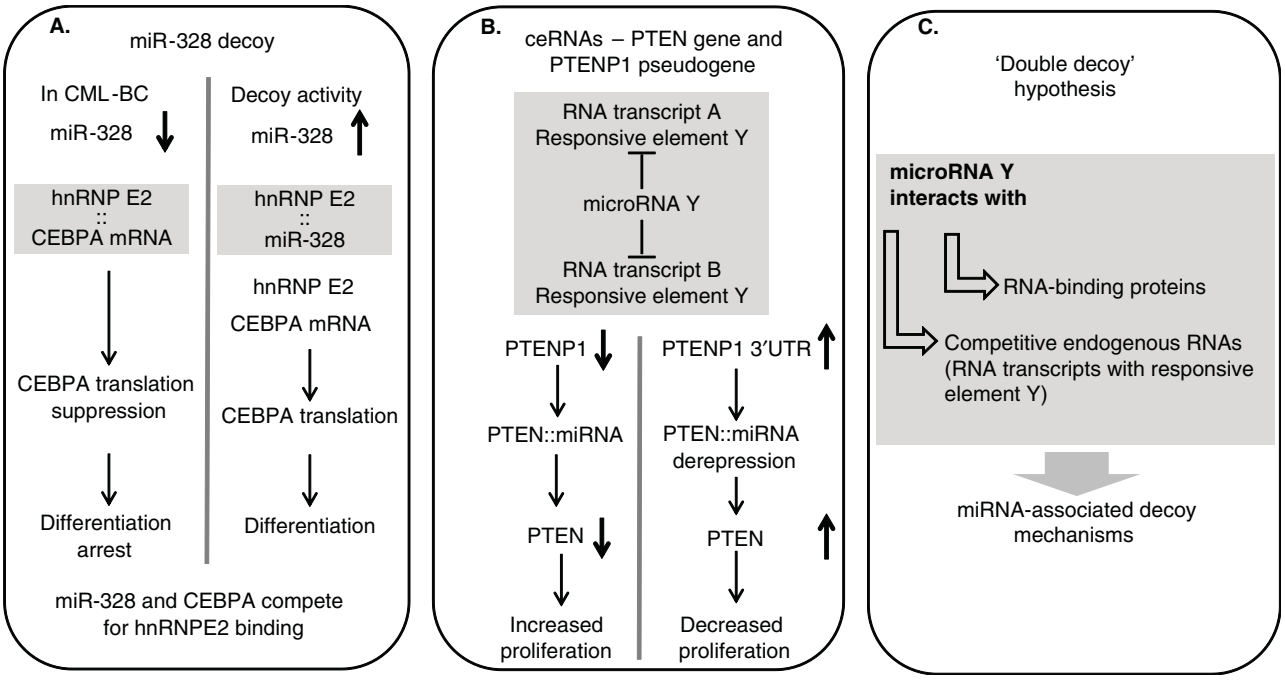

Figure 1. The decoy by microRNAs. A. miR-328 decoy. In blast crisis chronic myelogenous leukemia (CML-BC), miR-328 is downregulated. The RNA binding protein hnRNP E2 interacts with *CEBPA* mRNA, suppressing it translation and causing a differentiation arrest. When miR-328 restoration is induced, miR-328 interacts with hnRNP E2, releasing *CEBPA* from the translation inhibitory effects of hnRNAP E2 and leading to *CEBPA* mRNA translation. **B**. Complementary endogenous RNAs (ceRNAs) – *PTEN* gene and pseudogene. The basic principle of ceRNA is that different RNAs (e.g., RNA transcript A and RNA transcript B) that contain the same microRNA binding sites (e.g., responsive element Y) can compete with each other for those microRNAs (e.g., microRNA Y). The first example of ceRNAs was the *PTEN* gene and it pseudogene *PTENP1*, that share same microRNA responsive elements. When *PTENP1* is silenced, the tumor suppressor PTEN is decreased leading to an increase in cell proliferation. Accordingly, when *PTENP1* 3ÇUTR is overexpressed, PTEN levels increased due to a decoy for the microRNAs, causing a decrease in cell proliferation. **C**. The hypothesis states that the same miRNA can be trapped between binding to proteins and to ceRNAs. This represents a combination of the two experimentally identified instances presented in (**A**) and (**B**). When miRNA Y interacts with an RNA binding protein, the effect of this interaction on the competing mRNA species could be variable. In the case of miR-328 and hnRNP E2, the interaction of miRNA Y with the protein induces mRNA translation. However, one could hypothesize that interaction of miRNA Y with a protein that stabilizes mRNAs and induces translation (e.g. hnRNP A1) could result in reduced expression of mRNA containing the same miRNA binding sites.

studies for *PTEN* miRNA-mediated decoy in glioblastoma cell lines [20].

In another study, Karreth *et al.* identified *PTEN* ceRNAs in a mouse model of melanoma with use of the sleeping beauty transposon system [21]. The authors further validated *ZEB2* as a ceRNA decoy for *PTEN* by demonstrating that ZEB2 depletion downregulates PTEN and that this reduction was dependent on the 3′UTR (ZEB depletion suppressed luciferase activity of *PTEN* 3′UTR reporter) and on miRNAs (ZEB2 depletion does not reduce PTEN expression in cells with a defect in the miRNA processing machinery) [21]. Finally, Cesana *et al.* reported that a long noncoding RNA, *linc-MD1*, acts as a ceRNA for *MAML1* and *MEF2C* mRNAs, two transcription factors that regulate muscle-specific genes, by interacting with miR-133 and miR-135, thereby regulating muscle differentiation [22].

All of the above-mentioned studies were crucial to a new understanding of the importance of miRNAs in mediating mRNA decoys, and their significance has been highlighted in several articles. For example, McCarthy [23] stated that a subtle reduction in few mRNA could cause widespread effects. Rigoutsos and Furnari [24] indicated that the relative amount of mRNAs and corresponding ceRNAs should be considered and they introduced the intriguing hypothesis that the decoy mechanism may also occur in cases in which PTEN levels are reduced without mutation, such as in Cowden disease and Bannayan-Zonana syndrome. In addition, Swami [25] questioned how this decoy applied in noncancer-related genes. In the "Expert Opinion" section, we give our opinion from a therapeutic point of view.

3. Expert opinion: potential therapeutic consequences

The capacity of multiple mRNAs to compete for available miRNAs introduces a new challenge for miRNA-based

cancer therapies because the expression levels of the several transcripts regulated by the miRNAs need to be specifically taken into account. miRNAs have been suggested not only as therapeutic targets in several diseases but also as therapeutic tools because miRNAs can regulate the protein expression of major genes involved in disease and therefore influence patient outcome. There are two major strategies to modulate miRNA expression and consequently influence gene expression: miRNA antagonists (e.g., to knock down miRNA oncogenic activity) and miRNA mimics (e.g., to restore miRNA tumor suppressor activity). The decrease in miRNA levels may be achieved by using chemically modified antisense oligonucleotides such as 2′-O-methyl, 2′-O-methoxyethyl, morpholino oligomers and locked nucleic acid (LNA) [26]. All of these molecules function as miRNA sponges, since they are designed to be complementary to miRNAs and to sequester miRNAs and derepress the mRNA targets. Studies have indicated that research using LNAs (first described in the late 1990s) is quickly progressing. miR-122 reduction using LNA (complementary with miR-122 sequence) was tested in primates [27]. Lanford *et al.* successfully suppressed chronic hepatitis C virus infection in chimpanzees by using LNA against miR-122 and obtained long-lasting but no secondary effects [27]. In contrast to the chemically engineered antagomirs, ceRNAs function as natural biological sponges. In the case of *PTEN* and its pseudogene *PTENP1*, if we could therapeutically overexpress the pseudogene, *PTEN* would be automatically derepressed since miRNAs would bind mainly to the pseudogene. If we could modulate the levels of ceRNAs, we could restore the expression of a full network of mRNA through miRNAs interaction. It is therefore crucial, when deciding on a therapeutic approach involving miRNAs, to consider not only the miRNA levels in that specific cancer, but also the expression balance between the levels of the ceRNAs. In addition, not only the amount of transcripts but also the stability of the miRNA::mRNA binding should be analyzed. Furthermore, in light of the Perrotti group's publication, the same miRNA could bind both ceRNAs and also proteins with binding sites for specific mRNAs (maybe themselves ceRNAs). This introduces another level of complexity since, for example, hnRNPs are members of a large family of proteins that are usually highly overexpressed in human cancers [15] and therefore could easily trap the interactor miRNAs (**Figure 1C**).

It is well known that miRNAs are involved in drug resistance [28]. An unexpected consequence of the ceRNA concept is the fact that resistance to therapy can be caused by an abnormal balance between the ceRNA network components. For example, the cancer-specific overexpression of pseudogene(s) of a gene involved in blocking the activity of a drug can "trap" miRNA molecules that otherwise in normal cells would bind to and downregulate the specific drug-resistance gene. In this case, the gene involved in blocking the activity of a drug would be translated causing drug resistance. Indeed, if a miRNA that targets genes inducing drug resistance is trapped by ceRNAs, it is expected that the mRNAs involved in drug resistance are derepressed, which would contribute to increased resistance to specific drugs. Also, the opposite mechanism can be postulated – the release of miRNAs that target genes involved in the induction of cell death by the drug, due to downregulation of ceRNAs. This will lower the malignant cell sensitivity to drug with consequent increased cell survival and resistance to particular chemotherapeutic regimens. Therefore, in cancer cells, the interactor miRNAs are "used" by the interaction with the multiple ceRNA messengers instead of directly targeting and consequently downregulating the coding genes (**Figure 2**). The mechanisms of ceRNA expression variation are the well-known ones for any coding or noncoding gene: genomic deletions or amplifications, chromosome translocations, loss-of-function or gain-of-function mutations or promoter methylation. Of course, in the absence of experimental proof on the therapeutic consequences of ceRNAs, this is a hypothesis, but one well worth exploring!

Finally, another intriguing possibility is the fact that the two distinct miRNAs generated from the same hairpin precursor can function as a decoy if they share also common targets. This could profoundly influence the design of clinical studies involving miRNA-based therapy. Although studies involving miRNA inhibitors are already being developed in primates, efforts to apply miRNA mimics to therapy are progressing more slowly. One important aspect to consider when designing therapeutic approaches for miRNA mimics is the fact that precursor miRNAs can generate two different mature miRNAs, -5p and -3p, depending on the hairpin stem-loop strand of the precursor miRNA, which may have cooperative or different functions. This is the case of miR-28, which we recently reported to be downregulated in colon cancer [29]. We reported that miR-28-5p, but not miR-28-3p, affects colon cancer cell proliferation by increasing apoptosis and causing G1 arrest. On the other hand, miR-28-5p decreases while miR-28-3p increases colon cancer cells migration and invasion. These differential effects are partially explained by the different mRNA targets. *In vivo*, we observed that miR-28 expression disrupts tumor growth but increases metastasis [29]. Furthermore, we identified several common mRNA targets that are predicted to interact in independent sites for 5p and 3p miR-28, sometimes separated only by few nucleotides. Similarly, Jiang *et al.* reported that miR-125a-5p and miR-125a-3p, which are downregulated in non-small cell lung cancer, have opposite effects *in vitro* on invasion and migration in cells of this tumor type [30]. Consequently, when designing vectors using precursor miRNA, it is essential to consider the function of both -5p and -3p mature forms because one of the strands may cause adverse effects. Furthermore, the main challenge for miRNA mimics will be the *in vivo* delivery strategies. Since the ceRNA mechanism suggests the existence of multiple miRNAs that target simultaneously the same spectrum of coding genes and pseudogenes/other ncRNAs, the 5p/3p pairs of miRNAs could represent examples of such decoy miRNAs.

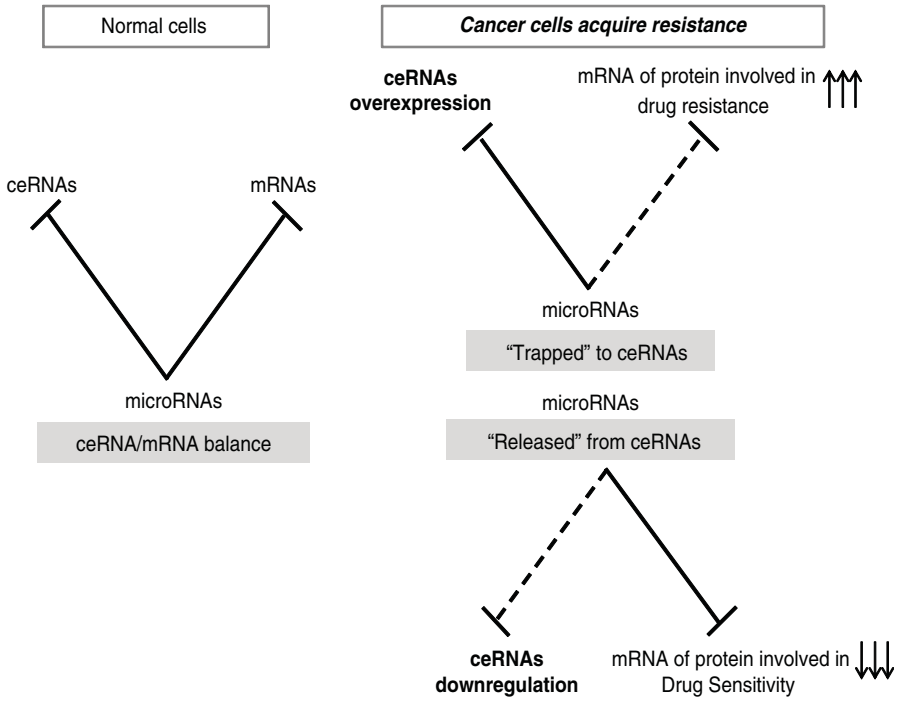

Figure 2. Schematic hypothesis of the involvement of ceRNA/miRNA/mRNA in resistance to therapy. Under normal conditions, there is a balance between the "RNA language" components: ceRNAs, miRNAs, and mRNAs. In cancer, cells may overexpress ceRNAs (with the same miRNA-binding sites as mRNAs involved in drug resistance), which will trap the available miRNAs, causing a derepression of mRNAs involved in drug resistance. This will consequently cause overexpression of drug-resistant mRNA levels and therefore contribute to increased resistance to drug treatment. Other possibility is that in cancer cells specific ceRNAs are downregulated with consequent "release" of one or more miRNAs that could target messengers of genes involved in proper activity of a specific drug. The consequence is that sensitivity to the drug decrease. Note that in this model, the actual levels of the specific miRNA(s) did not change; what change is the ratio between the various targets and consequently the availability of "free" miRNA molecules ready to interact. Of course, the outcome could be further influenced by intrinsic variations in the expression of the specific miRNA (overexpression or downregulation).

4. Conclusion

One of the main challenges for application of miRNA-based therapies is the *in vivo* delivery method. In most cases, the delivery should be tissue-specific (and in some cases, even cell type-specific); in addition, the secondary undesirable effects should be controlled and the effect produced should be stable and long-lasting. Furthermore, the ceRNAs network and the expression levels of ceRNAs should be considered when designing the therapeutic approach for a particular miRNA-target gene.

In conclusion, it seems to be a question of time until miRNA-based therapies can be used to treat cancer as well as cardiovascular, inflammatory, and neurodegenerative diseases. Until then, efforts should be made to solve all of the above-mentioned issues. It is expected that in the next few years, similar to what happened in the miRNAs field, a second boon in the long noncoding RNAs field will emerge and additional functions for these genes will be revealed. This is the case, for example, of regions in the genome that have a high level of homology and conservation between species and whose ultraconserved transcripts have been shown to be deregulated in cancer [31] but whose function has not yet been described. Certainly, the efficiency of ceRNA effects *in vivo* and the simplicity of this powerful concept remind us that the ncRNA El Dorado is a land where both scientists and clinicians should step without dogmas set in stone, but instead should be ready to accommodate new ideas that could benefit the people who need them at most —the patients!

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