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ceRNA crosstalk in cancer: when ce-bling rivalries go awry

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Abstract

The cancer transcriptome is characterized by aberrant expression of both protein-coding and non-coding transcripts. Similar to mRNAs, a significant portion of the non-coding transcriptome including long non-coding RNAs and pseudogenes harbors microRNA-response elements. The recent discovery of competitive endogenous RNAs (ceRNA), natural decoys that compete for a common pool of microRNAs, provides a framework to systematically functionalize MRE-harboring non-coding RNAs and integrate them with the protein-coding RNA dimension in complex ceRNA networks. Functional interactions in ceRNA networks aid coordinating a number of biological processes and, when perturbed, contribute to disease pathogenesis. In this review, we discuss recent discoveries that implicate natural miRNA decoys in the development of cancer.

Keywords

ceRNA; ceRNET; miRNA; decoy; cancer

Introduction

Diseases such as cancer are often associated with aberrant transcriptomes. This is not limited to the production of abnormal levels of protein-coding mRNAs, but also includes deregulated expression of the non-coding dimension of the human genome. Intriguingly, recent analyses of the human transcriptome have revealed that a mere 2% of the genome encode protein-coding transcripts even though over three-quarters of the genome are transcribed (1, 2). The function of several classes of non-coding transcripts, such as tRNAs, small nucleolar RNAs (snoRNAs), and micro RNAs (miRNAs) is relatively well understood. However, the functions of a huge portion of the genomic “dark matter” remain to be unraveled. This undertaking is, at least in part, hindered by the lack of systematic means to functionally annotate the non-coding transcriptome. As the non-coding space likely plays critical roles in promoting cancer, a better understanding of its functions in physiology and disease is imperative for the development of novel therapeutic strategies.

The ceRNA hypothesis

miRNAs - gene-regulatory non-coding RNAs that are expressed in plants, fungi, animals, and viruses (3) – direct the RNA-induced silencing complex (RISC) to miRNA-response elements (MREs), thereby repressing protein production by either inhibiting translation or destabilizing the mRNA (4, 5). MREs are localized on the 3 untranslated region (UTR), coding sequence (CDS) and the 5 UTR, and, besides on mRNAs, can also be found on non-

protein coding transcripts such as pseudogenes and long non-coding RNAs (lncRNAs) (6). Importantly, each miRNA has numerous RNA targets and the vast majority of RNA molecules harbors several MREs and are thus repressed by different miRNAs. This target multiplicity has led to the hypothesis that different RNAs (either pseudo-targets or legitimate targets) compete for limiting pools of miRNAs (7, 8), thus acting as competitive endogenous RNAs (ceRNAs) (Figure 1A). The ceRNA hypothesis postulates that any RNA transcript that harbors MREs can sequester miRNAs from other targets sharing the same MREs, thereby regulating their expression (Figure 1B, C). Importantly, the ability of two transcripts to cross-regulate each other can be bioinformatically predicted based on the MRE overlap, i.e. the MREs that they have in common. The ceRNA hypothesis thus proposes two very intriguing possibilities. Firstly, a major function of non-coding RNAs such as lncRNAs and pseudogenes that harbor MREs may be to act as endogenous miRNA decoys. As over two thirds of the human genome is transcribed (1), the ability to predict putative ceRNA interactions may allow functionalization of a sizeable portion of the human transcriptome. Secondly, besides serving as a blueprint for protein production, mRNAs may be involved in the post-transcriptional regulation of other transcripts.

ceRNA activity is evident in different biological contexts

Artificial sponges have been utilized for several years to investigate the function of miRNAs. Such sponges harbor one or more complementary binding sites to a miRNA family and are delivered to cells or transgenic animals, where they sequester the miRNAs of interest and thereby abolish their function (9). Experimental evidence for natural sponges (ceRNAs) was found in several species and contexts and includes a variety of RNA species including mRNAs, pseudogenes, and lncRNAs. The *A. thaliana* non-coding RNA IPS1 sequesters miR-399 from its target mRNA PHO2 and thereby regulates shoot phosphate content (10). This process, termed ‘target mimicry’, relies on imperfect complementarity of miR-399 and IPS1 at the miRNA cleavage site, which results in sequestration of the miRNA (10). In zebrafish, miR-430 targets both the Nodal agonist *squint* and the Nodal antagonist *lefty* (11) and, thus, both agonist and antagonist can be considered to be natural sponges for one another that ensure balanced Nodal signaling. Similarly, the ligand *Sdf1a* and its sequestration receptor *Cxcr7b* compete for miR-430 to control germ cell migration in the zebrafish gonad (12). The first evidence for ceRNA activity in mammalian cells came from the analysis of the PTEN pseudogene in regulating its parental gene (13) and is discussed in detail below. In addition, Bozzoni and co-workers ascribed a ceRNA function to a lncRNAs, *linc-MD1*, which liberates the differentiation factors MEF2C and MAML1 from repression by decoying miR-135 and miR-133, respectively, thus controlling muscle differentiation (14). Duchenne muscle cells exhibit reduced levels of *linc-MD1* suggesting that this axis plays a role in the pathogenesis of Duchenne muscle dystrophy (14). Another lncRNAs, *linc-RoR*, controls self-renewal of human embryonic stem cells by sequestering miR-145 from the stemness factors OCT4, NANOG, and SOX2 (15). Based on these experimental findings, it was hypothesized that a myriad of natural sponges may exist and play critical roles in biological processes (16). Thus, competition for shared miRNAs may lend robustness to signaling pathways during development and physiology, and it is likely that a multitude of such functional interactions remain to be discovered.

Interestingly, ceRNAs are not only found in higher organisms. Viruses have evolved to express ceRNAs to ablate antiviral host miRNAs as discovered by Steitz and colleagues who identified miR-27 binding sites in the Herpesvirus saimiri non-coding RNA HSUR1 (17). Indeed, virally transformed T cells expressed lower levels of miR-27 and increased levels of the miR-27 target FOXO1 (17). Moreover, the virally expressed and highly abundant m169 transcript binds to and promotes the degradation of host miR-27, a process that is important for the replication of murine cytomegalovirus (18, 19). The examples described above

indicate that endogenous miRNA decoys have important functions in various biological processes and cell types, and it is likely that ceRNAs can be found in all organisms that utilize miRNAs to regulate gene expression. Given the prominent roles of ceRNAs in physiology, it is conceivable that their deregulation is a common occurrence in, and promotes progression of, various diseases such as cancer.

Pseudogenes as ceRNAs in cancer

Pseudogenes were widely considered “junk” DNA and genomic relicts of evolution because the vast majority of pseudogenes do not encode for functional proteins. Pseudogenes can develop through several mechanisms: gene duplication gives rise to unprocessed pseudogenes that contain introns, 5 UTRs, and 3 UTRs, and are under the control of similar promoter elements as their parental genes. Processed pseudogenes form through retrotranscription of spliced mRNA into DNA, which is then re-integrated into the genome. Processed pseudogenes may contain 5 UTRs and 3 UTRs but their expression is regulated by promoter and enhancer elements that differ from their parental genes. Finally, unitary pseudogenes develop *de novo* and have no protein-coding counterparts. The three classes of pseudogenes harbor mutations that disrupt the start codon and prevent the production of a functional protein (20). Processed and unprocessed pseudogenes typically maintain a high sequence homology with their parental genes, and thus display also a high degree of MRE overlap (i.e. the number and identity of MREs) with their parental genes. Given this notable MRE overlap, pseudogenes may, at least theoretically, constitute perfect endogenous miRNA sponges.

This theory was first interrogated by examining the PTEN pseudogene, PTENP1. PTEN is a haploinsufficient tumor suppressor that is commonly lost or mutated in a variety of human malignancies such as cancers of the prostate, breast, colon, glioblastoma and melanoma (21). Importantly, even a 20% reduction of PTEN levels can elicit a cancer phenotype in mice (22, 23). The proximal 3 UTR region of PTENP1 is highly homologous to the proximal PTEN 3 UTR and contains numerous seed matches for miRNAs that were previously validated to repress PTEN expression (13), suggesting that PTENP1 could act as a ceRNA for PTEN. Indeed, PTENP1 is repressed by several validated PTEN-targeting miRNAs and overexpression and RNAi silencing experiments confirmed that PTENP1 post-transcriptionally regulates expression of PTEN (13). Notably, PTENP1 expression levels positively correlate with those of PTEN in human tissues and prostate cancer and genomic losses of the locus containing *PTENP1* occur in human colon cancer and are accompanied by reduced PTEN expression (13). Genomic losses of *PTENP1* were also reported in melanoma (24), a cancer known to be promoted by reduction of PTEN expression (25), further emphasizing the tumor suppressive function of PTENP1. Thus, the ceRNA activity of PTENP1 contributes to post-transcriptional regulation of PTEN, and alterations of PTENP1 expression levels or miRNA decoy activity may lead to modest variation in PTEN levels to promote cancer development.

A recent study added a further layer of complexity to the regulation of PTEN by PTENP1. Johnsson et al. reported that the *PTENP1* locus encodes two antisense transcripts, PTENpg1 asRNA and PTENpg2 asRNA, that differ in their transcriptional start sites but overlap with the PTENP1 transcript (26). Intriguingly, these two PTENpg1 asRNA transcripts have opposing functions. PTENpg1 asRNA recruits the chromatin remodeling factors EZH2 and DNMT3a to the PTEN promoter where they catalyze the addition of a repressive H3K27me3 chromatin mark (26). The PTENpg2 asRNA isoform thus negatively regulates PTEN expression independent of the PTENP1 sense transcript. Conversely, PTENpg1 asRNA associates with PTENP1 sense to increase the stability of the PTEN pseudogene transcript. The 3 UTR of PTENP1 lacks a robust polyA tail and this RNA element is likely provided by

PTENpg1 asRNA in the RNA-RNA complex to increase RNA stability (26). Altering the levels of PTENpg1 asRNA changed expression of PTENP1 as well as PTEN, indicating that PTENpg1 asRNA promotes the miRNA decoy function of PTENP1 (26). The fact that the PTENP1 locus is lost in cancer (13, 24) may suggest that the stimulatory ceRNA function of the PTENP1–PTENpg1 asRNA RNA complex is more critically involved in regulating PTEN expression than the inhibitory function of PTENpg1 asRNA. These findings suggest that the PTEN pseudogene is not just a mere artifact of evolution but rather a regulated modulator of PTEN expression that, when perturbed, may contribute to cancer formation.

It is currently unknown whether PTENP1 is an exception among pseudogenes, and whether other pseudogenes have similar miRNA-dependent regulatory functions towards their parental genes and possibly other genes with which they share miRNAs remains to be investigated. Notably, however, overexpression of the 3' UTR of the KRAS pseudogene KRAS1P increases KRAS levels and promotes cells growth (13). Moreover, the KRAS and KRAS1P transcripts show a positive correlation of expression and the KRAS1P locus is amplified in human cancers (13). In addition, several other pseudogenes such as the OCT4, FOXO, NPM and CDK4 pseudogenes were shown to share binding sites for common miRNAs with their parental genes (13), suggesting that gene regulation by miRNA-sequestering pseudogenes could be a frequently occurring phenomenon. Interestingly, RNA-seq analyses revealed that a myriad of pseudogenes is expressed in human cells and cancers. Some of these pseudogenes are expressed almost ubiquitously, while others are found only in a subset of the analyzed cell types or are specific to a single cell or cancer type (27). While many of these pseudogenes may have no function or miRNA-independent functions, a large fraction of these likely represents ceRNAs. Further work is required to identify pseudogenes with the ability to decoy miRNAs from their parental genes, determine whether the abundance of such pseudogenes relative to their parental genes permits ceRNA crosstalk, and analyze whether perturbed expression of these pseudogenes contributes to cancer development using *in vitro* and, where appropriate, mouse modeling approaches.

mRNAs as ceRNAs in cancer

Similar to pseudogenes, mRNAs may promote cancer by regulating the expression of tumor suppressors and oncogenes in a miRNA-dependent manner provided that they share MREs that permit ceRNA crosstalk. Intriguingly, miRNA sequestration is independent of the protein-coding function of mRNAs, thus allowing genes to participate in diverse and potentially even opposing biological processes through the mRNA transcript and the protein that they encode. Our laboratory employed a computational approach to identify mRNAs that contain at least 7 out of 10 MREs that are complementary to validated PTEN-targeting miRNAs in their 3' UTRs, an approach termed Mutually Targeted MRE Enrichment (MuTaME) (28). This analysis identified numerous putative PTEN ceRNAs, two of which, CNOT6L and VAPA, were validated to regulate PTEN expression in a miRNA-dependent and protein coding-independent manner (28). Moreover, silencing CNOT6L and VAPA induced AKT signaling and promoted cell proliferation (28). Using a different bioinformatic approach, Califano and colleagues identified multiple mRNAs with putative ceRNA activity toward PTEN and experimental validation confirmed their 3' UTR-dependent regulation of PTEN (29). Interestingly, both studies found reduced expression levels of PTEN ceRNAs in human cancers (28, 29). In addition, a forward genetic screen in a B-Raf^{V600E}-driven mouse model of melanoma revealed that a remarkable 10% of the identified hits in protein-coding genes were putative ceRNAs for PTEN (30). CNOT6L was also identified in this melanoma screen, and silencing of AFF1, JARID2, MBNL1, RBM9, TNRC6a, TNRC6b, and ZEB2 decreased PTEN levels (30), suggesting that these transcripts could function as PTEN ceRNAs and, thus, tumor suppressors *in vivo*. One putative PTEN ceRNA, the ZEB2

mRNA, was validated to regulate PTEN levels through a miRNA-dependent and protein coding-independent mechanism (30). As the ZEB2 protein possesses oncogenic properties by promoting epithelial-to-mesenchymal transition (31), this represents an intriguing example of a gene encoding a mRNA and a protein with unrelated functions. The potency of concurrent genetic lesions in B-Raf and PTEN has been demonstrated in mice and even heterozygous loss of PTEN significantly accelerated B-Raf^{V600E}-induced melanoma development (32). Therefore, moderate changes of PTEN levels elicited by loss of PTEN ceRNA expression are likely to contribute to melanomagenesis in the context of a B-Raf^{V600E} initiating mutation. Whether aberrant ceRNA-mediated regulation of PTEN promotes cancer in other cell types or cancer-sensitizing genetic backgrounds remains to be determined in mouse models. Moreover, whether haplosufficient tumor suppressors are similarly sensitive to aberrant ceRNA regulation will be an interesting topic for future research. Computational analyses suggest that aberrant expression of numerous mRNAs with putative ceRNA activity correlates with neoplastic phenotypes (29, 33). However, both their ability to decoy miRNAs and potential to promote cancer remain to be experimentally validated.

3'UTRs as ceRNAs in cancer

Another important implication of the ceRNA theory pertains to the role of 3 UTRs in cancer and other diseases. The 3 UTRs of protein-coding transcripts are critical for mRNA stability and posttranscriptional regulation, and typically contain MREs for several different miRNAs. In light of the ceRNA hypothesis, 3 UTRs may not only regulate the stability of their own transcripts *in cis*, but likely decoy miRNAs from transcripts with shared MREs, thereby regulating such transcripts *in trans*. This, in turn, suggests that mutations or changes in abundance, structure or length of 3 UTRs may affect their ability to sponge miRNAs. Curiously, alternative poly-adenylation has been observed, leading to 3 UTR lengthening during embryogenesis (34), and 3 UTR shortening in proliferating cells (35) and in cancer (36). Changes in 3 UTR length alter the interaction of miRNAs with such transcripts and affect protein output (35, 36). In addition, due to a reduced number of MREs, 3 UTR shortening will also modify the ability of these mRNAs to compete for miRNAs and function as ceRNAs. Hence, by alternative poly-adenylation, oncogenes may circumvent miRNA-mediated reduction in their abundance and, as a side effect, no longer sequester tumor suppressor-targeting miRNAs. The analysis of such alternative poly-adenylation holds promise to reveal additional facets of ceRNA activity and their contribution to cancer biology.

Intriguingly, Mattick and colleagues recently reported that numerous 3 UTRs may be expressed independently of their normally associated CDSs, a process that is developmentally regulated in a cell type-, and stage-specific fashion (37). Only a minority of independently expressed 3 UTRs appears to be generated by *de novo* transcription and a mechanism involving posttranscriptional cleavage followed by CDS degradation is more likely (37) (Figure 2A). Future work will reveal how such 3 UTR transcripts are produced. Nevertheless, their occurrence indicates that 3 UTRs possess genetic information in addition to regulating stability and translation of their normally associated CDSs. Independently expressed 3 UTRs may have two trans-acting ceRNA functions (Figure 2B). Firstly, they could modulate the expression of the CDS with which they are normally associated. As the sequence and, hence, the number and identity of MREs is identical between the CDS-associated and independently expressed 3 UTR isoforms, such ceRNA regulation could be extremely potent because the independently expressed 3 UTR would compete for every single miRNA species targeting the CDS-associated 3 UTR. Secondly, independently expressed 3 UTRs may act as miRNA sponges for and affect expression of numerous other transcripts with which they compete for common miRNAs.

Several reports have described that expression of 3 UTRs independent of their associated protein-coding sequences modulates differentiation and proliferation of mammalian cells (38-42). However, as these studies were carried out before the advent of the ceRNA hypothesis, it is unclear whether these 3 UTRs act as miRNA decoys to regulate the expression of their CDS-containing counterparts or other transcripts. More recently, Yang and colleagues generated transgenic mice overexpressing the 3 UTR of versican. In these mice, the versican 3 UTR potentially sequesters miR-199a* to increase protein expression of versican and another miR-199a* target, fibronectin, resulting in abnormal adhesion of the liver to its surrounding organs or the inner body wall (43). In an additional study, the versican 3 UTR was shown to decoy four miRNAs from versican and three each from fibronectin and CD34, and versican 3 UTR transgenic mice developed hepatocellular carcinoma (44). Moreover, ectopic expression of the versican 3 UTR in HepG2 cells enhanced transformation *in vitro* and tumor growth in xenografts *in vivo* (44). Aberrant expression of versican, fibronectin, and CD34 is associated with cancer; however, other genes are likely affected by versican 3 UTR overexpression and these studies do not provide functional evidence that the versican 3 UTR promotes hepatocellular carcinoma by deregulating expression of these three genes. Indeed, overexpression of the versican 3 UTR in the mouse breast carcinoma cell line 4T1 enhances expression of the tumor suppressors PTEN and Rb1, leading to reduced proliferation and tumor growth (45). Thus, the effect of deregulated expression of the versican 3 UTR appears to be context-dependent and is likely modulated by the abundance of mRNAs with shared MREs as well as the pool of miRNAs for which they are in competition.

Other examples of 3 UTRs functioning as miRNA decoys include the nephronectin 3 UTR, which affects the abundance of β -catenin and GSK3 to promote differentiation of the osteoblast progenitor cell line MC3T3-E1 (46). In addition, ectopic expression of the CD44 3 UTR in the human breast cancer cell line MT-1 relieved repression of CD44 and CDC42 by sequestering at least three shared miRNAs (47). This resulted in reduced proliferation but elevated tube formation *in vitro*, and the formation of smaller, more vascularized tumors in xenograft experiments (47). In a different human breast cancer cell line, MDA-MB-231, overexpression of the CD44 3 UTR enhanced cell motility and invasion and promoted metastatic spread *in vivo* (48). Collagen type 1 $\alpha 1$ and fibronectin were also among the mRNAs affected by the CD44 3 UTR, suggesting that, similar to the versican 3 UTR, the CD44 3 UTR regulates expression of several protein-coding transcripts (48). Which of these transcripts promote the observed phenotypes in what context awaits further investigation.

Finally, the PTEN 3 UTR, which is among those expressed independently of their CDS (37), affects expression of PTEN (13); however, the incidence and abundance of independently expressed PTEN 3 UTR remains to be determined. Taken together, these studies provided evidence that 3 UTRs have the ability to act as ceRNAs in certain contexts and that their deregulation can facilitate cancer development and progression.

Aberrant 3'UTR expression by genomic translocation, alternative splicing and read-through

An alternative means to express 3 UTRs independently of their CDS is by genomic translocations that fuse two unrelated genes. This may place a low abundance 3 UTR under the control of a strong promoter or *vice versa* (Figure 2A). Genomic translocations are common in hematologic malignancies and also occur in solid tumors, thus warranting investigations into the capability of their transcripts to serve as cancer promoting ceRNAs. Other processes leading to aberrant expression of 3 UTRs include alternative trans-splicing as well as read-through transcription of conjoined genes. Such events result in chimeric transcripts that may be generated independently of their parent genes' transcription. Notably,

expression of hundreds of chimeric transcripts has been detected in prostate cancer samples and their abundance is increased compared to benign control tissue (49). A key role of chimeric transcripts may be to serve as miRNA decoys and future work will assess their functionality and potency as ceRNAs.

lncRNAs and circRNAs as ceRNAs with potential roles in tumorigenesis

3' UTRs, but also the CDSs and 5' UTRs of mRNAs are peppered with MREs and hence many, if not most, mRNAs have the potential to engage in ceRNA crosstalk. Intriguingly, other RNA species such as lncRNAs have recently begun to emerge as natural miRNA decoys. Indeed, lncRNAs are extensively targeted by miRNAs (3, 6), suggesting that they may serve as ceRNAs. The long non-coding RNA highly upregulated in liver cancer (HULC) promotes liver cancer (50) and, interestingly, regulates its own expression in a ceRNA-dependent feed forward loop (51). HULC expression is induced by PKA-mediated phosphorylation of CREB followed by phospho-CREB recruitment to the HULC promoter. The HULC transcript decoys miR-372 from PRKACB, the catalytic subunit of PKA, thus completing this regulatory circuit (51).

A novel class of RNA molecules, termed circular RNAs (circRNAs), was demonstrated to act as endogenous ceRNAs by two recent studies. CircRNAs form through head-to-tail splicing of exons within the same transcript and thus include intronic and exonic sequences. Such transcripts are very stable and there may be thousands of circRNAs encoded in the human genome that are expressed in context-dependent manners and are found in normal and malignant cells (52-54). The groups of Rajewski and Kjems demonstrated that the circRNA encoded by the cerebellar degeneration-related protein 1 (CDR1) gene, which they termed CD1Ras and ciRS-7, respectively, contains dozens of miR-7 seed matches and is loaded with miRNA effector complexes (52, 55). The circRNA is insensitive to miR-7-mediated destabilization and serves as an endogenous miRNA sponge that regulates expression levels of miR-7 target genes (52, 55). Moreover, the testis-specific circRNA sex-determining region Y (Sry) circRNA acts as a ceRNA by decoying miR-138 (55). Given the sheer abundance of circRNAs, their stability, and the potential number of MREs that they may contain, circRNAs are bound to become a potent class of ceRNAs with important roles in both normal physiology and diseases.

ceRNETS

Most studies that reported ceRNA crosstalk between RNA molecules focused on binary and sometimes reciprocal interactions that occur between two transcripts and are mediated by one or more miRNAs. Most transcripts, however, contain numerous MREs for different miRNAs. In addition, miRNAs typically repress multiple target transcripts. This complexity suggests that miRNAs and their targets are connected in complex ceRNA networks, or ceRNETS. ceRNETS likely contain smaller sub-networks where the nodes (i.e. the ceRNAs) are linked by a higher number of connections (i.e. miRNAs), and ceRNA crosstalk may be more efficient in such sub-networks (Figure 3A). Using a computational approach, Califano and colleagues described an extensive ceRNA network in glioblastoma containing approximately 7,000 putative ceRNAs and near a quarter of a million miRNA mediated interactions (29). This ceRNET consists of many highly interconnected sub-networks that show a strong positive correlation of expression of their individual ceRNA components and contain numerous transcripts of cancer-associated genes (29). In addition, our group bioinformatically predicted the optimal ceRNA network based on MRE overlap and gene expression correlation. Interestingly, gene ontology analysis of this network revealed that numerous nodes are involved in regulation of gene expression (33). Furthermore, we predicted putative ceRNAs of the oncogenic transcription factor translocation PAX/FKHR

and some of its transcriptional targets in Rhabdomyosarcoma, and found that the presence of the PAX/FKHR chimeric transcription factor resulted not only in increases in its transcriptional targets but also in higher abundance of some of its predicted ceRNAs and ceRNAs of its transcriptional targets (33). This indicates that transcription factor networks and ceRNETs are tightly coupled as transcription factor expression controls both its transcriptional targets as well as intertwined ceRNA networks, thus expanding the regulatory potency of transcription factors (Figure 3B). This may have critical implications in cancers driven by genomic translocation that result in aberrant expression of (chimeric) transcription factors such as PAX/FKHR, ERG, and c-myc.

Given the complexity of ceRNETs, future work will focus on identifying the critical nodes that have potent effects on the network or sub-networks when their expression levels are perturbed. Recently developed mathematical models will help to understand the complexity and behavior of ceRNETs (33, 56). These models were used to perform relatively simple simulations involving only a handful of ceRNAs and miRNAs but they are scalable to replicate larger networks. Moreover, while these networks currently only allow for qualitative predictions, a detailed knowledge of the identity and abundance of all components of a ceRNET will enable us to quantitatively simulate the behavior of a ceRNET in response to perturbation of a single factor. We combined the mathematical ceRNA model with cell biological approaches in an attempt to further define the molecular conditions for optimal ceRNA crosstalk and made several interesting observations (33). First, *in silico* simulations showed that ceRNAs communicate best at near-equimolarity of all components involved in the crosstalk (i.e. ceRNAs and miRNAs). Second, the relative abundance of ceRNAs and miRNAs influences crosstalk as does the number of MREs (i.e. the number of miRNAs) that two ceRNAs have in common. Third, and critically, indirect interactions where two linked ceRNAs are also connected through a third ceRNA greatly amplify ceRNA crosstalk (Figure 3C). These results shed some light on the molecular environment that permits RNAs to serve as efficient miRNA decoys, while more work is required to fully elucidate the mechanisms of ceRNA crosstalk.

Conclusion and future challenges

The last few years have heralded the ceRNA hypothesis as an exciting novel aspect of RNA biology. Studies by several groups illustrate that competitive endogenous RNAs serve as posttranscriptional regulators of gene expression by decoying miRNAs from other target transcripts. Such sponge activity has been demonstrated for various examples of mRNAs, pseudogenes, lncRNAs, and circRNAs but virtually any transcript containing miRNA seed matches may participate in ceRNA crosstalk. Indeed, a recent study reported canonical and non-canonical miRNA-binding sites on numerous ribosomal RNAs and tRNAs (57). The ever-expanding variety of ceRNAs promises an exciting future where we will uncover the importance of newly discovered transcripts such as pseudogenes, lncRNAs, and circRNAs, and ascribe novel, protein coding-independent functions to well-studied mRNAs. The ceRNA field is certainly still in its infancy and several open questions remain to be addressed. For instance, what is the dynamic range of ceRNAs in tumorigenesis and normal physiology? A low abundance ceRNA may have a potent effect when overexpressed several-fold, while its silencing may cause very little harm. But how low abundant is too low to have an effect? PTENP1 appears to be expressed at lower levels than PTEN in some tissues and yet its silencing has a significant effect on PTEN (13). Conversely, VAPA is more abundant than PTEN and has a much more profound effect on PTEN than vice versa (28, 33). Future work will need to address this issue. Why does the silencing of a single ceRNA have profound effects on another, even though the entire ceRNET should lend robustness and “dilute” the effect? Threshold levels, relative ceRNA ratios, miRNA affinity to seed matches, and crosstalk-amplifying indirect interactions may all contribute to the

ceRNA effect (33), but this awaits experimental confirmation. How do RNA-binding proteins that could mask MREs, secondary structures of transcripts, RNA turnover, and subcellular compartmentalization influence ceRNA crosstalk? Many answers to these questions will only be applicable to a certain ceRNA or ceRNET and possibly only in a specific tumor type – when the abundance or localization of ceRNET components changes, the efficiency of ceRNA crosstalk will likely change as well. Thus, each and every ceRNA will have to be evaluated individually and in the context and cell type of interest. Finally, the tumor promoting capability of under- or over-expressed ceRNAs remains to be investigated in mouse models and a relevance to human cancers needs to be firmly established. Even though numerous open questions remain, the discovery of endogenous miRNA decoys and ceRNETS could guide the functionalization of a sizeable portion of the cancer transcriptome, and may stimulate the development of novel and exciting cancer treatment strategies to target key nodes in ceRNETs that will add to existing approaches of miRNA therapy (58).

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Abbreviations

ceRNA	competitive endogenous RNA
ceRNET	ceRNA network
miRNA	microRNA
MRE	microRNA-response element
lncRNAs	long non-coding RNA
circRNA	circular RNA

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Significance

Crosstalk between competitive endogenous RNAs through shared microRNAs represents a novel layer of gene regulation that plays important roles in physiology and development of diseases such as cancer. As crosstalk can be predicted based on the overlap of microRNA-binding sites, we are now one step closer to a complete functionalization of the human transcriptome, especially the non-coding space.

Key concepts

- Competitive endogenous RNAs (ceRNAs) share MREs and sequester common miRNAs, thereby regulating each other's expression.
- mRNAs, pseudogenes, lncRNAs and circRNAs may all serve as ceRNAs.
- ceRNAs and miRNAs form complex regulatory networks termed ceRNETs.
- Perturbations of critical nodes in ceRNETs may disrupt the balance of the network and thereby contribute to disease development.

Figure 1.

The ceRNA concept. (A) The schema outlines the concept of ceRNAs. Two transcripts, ceRNA1 in orange and ceRNA2 in green, have two different MREs (blue and red boxes, complementary to miRNAs miR1 and miR2, respectively) in common. Thus, miR1 and miR2 repress both ceRNAs. When miR1 and miR2 target ceRNA1 and are therefore eliminated from the pool of available miRNA, ceRNA1 effectively inhibits the repressive activity of miR1 and miR2 towards ceRNA2 (dashed inhibition arrow). As ceRNA crosstalk is, in theory, reciprocal, binding of miR1 and miR2 to ceRNA2 inhibits their activity towards ceRNA1. (B) Changing the abundance of one ceRNA will lead to a similar effect in the levels of the other ceRNA, i.e. upregulation of ceRNA1 will lead to increased levels of ceRNA2. (C) Deregulation of one ceRNA results in changes in the pool of available, unbound ceRNAs and miRNAs (adapted from reference 33). Increased expression of ceRNA1 will first lead to a decrease in unbound miRNA molecules. Once the majority of miRNA molecules are bound, the levels of unbound, unrepressed ceRNA1 and ceRNA2 can increase.

Figure 2.

3 UTRs as ceRNAs. (A) Independently expressed 3 UTRs can arise through various mechanisms. (i) 3 UTRs are typically associated with the CDSs of their genes, but they nevertheless could serve as ceRNAs for other transcripts. (ii) Alternative promoters may drive independent expression of 3 UTRs. (iii) Posttranscriptional cleavage of transcripts may dissociate the CDS from its 3 UTR. (iv) Genomic translocations resulting in fusion genes may place 3 UTRs under the control of foreign promoters. (v) Transcriptional read-through of two adjacent genes could result in chimeric transcripts and abnormal levels of the downstream transcript. (vi) Aberrant trans-splicing could join two unlinked transcripts, also resulting in chimeric transcripts. (B) Independently expressed 3 UTRs can either serve as

miRNA sponges to de-repress expression of their CDS-associated counterparts, or other transcripts with which they share MREs.

Figure 3. ceRNA networks. (A) Example of a ceRNETS. The schematic depicts a ceRNET with a moderate degree of complexity that contains several indirect interactions (outlined by 'glowing' connections). The nodes highlighted in red could represent critical nodes that are connected to numerous other ceRNAs and thus may have the most impact on the network when deregulated. (B) Intertwined transcriptional and ceRNA networks. A transcription factor may act as ceRNA via its mRNA transcript, while its protein stimulates transcription of targets that in turn also decoy miRNAs. (C) Indirect interactions amplify ceRNA crosstalk. These two networks consisting of three ceRNAs differ only by a miRNA connection between ceRNA2 and ceRNA3. When this connection is lacking, ceRNA1 (shown in green) has only direct effects on ceRNAs 2 and 3 (top panel). If the connection between ceRNAs 2 and 3 is present, then increasing the abundance of ceRNA1 will have direct effects as well as indirect effects (bottom panel). In the latter case, elevated levels of ceRNA1 will increase ceRNA2, which in turn will increase ceRNA3. Moreover, ceRNA1 will affect ceRNA3, which will then impact ceRNA2 and thus ceRNAs 2 and 3 are doubly regulated through ceRNA crosstalk.