Endogenous microRNA sponges: evidence and controversy

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Abstract | The competitive endogenous RNA (ceRNA) hypothesis proposes that transcripts with shared microRNA (miRNA) binding sites compete for post-transcriptional control. This hypothesis has gained substantial attention as a unifying function for long non-coding RNAs, pseudogene transcripts and circular RNAs, as well as an alternative function for messenger RNAs. Empirical evidence supporting the hypothesis is accumulating but not without attracting scepticism. Recent studies that model transcriptome-wide binding-site abundance suggest that physiological changes in expression of most individual transcripts will not compromise miRNA activity. In this Review, we critically evaluate the evidence for and against the ceRNA hypothesis to assess the impact of endogenous miRNA-sponge interactions.

Long non-coding RNAs
(lncRNAs). Transcripts with little or no protein-coding potential that are greater than 200 nucleotides in length.

Competitive endogenous RNA
(ceRNA). RNA that leads to upregulation of expression of a target gene by competing for microRNA binding sites.

MicroRNA
(miRNA). A small (20–22 nucleotide long) non-coding RNA that inhibits gene expression by guiding the RNA-induced silencing complex (RISC) to target genes.

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Despite considerable development in our understanding of long non-coding RNAs (lncRNAs) over the past decade, only a fraction of annotated lncRNAs has been examined for biological function[1]. These several hundred lncRNAs have yielded a growing evidence base from which hypotheses for generalized mechanisms of lncRNA function have been derived. One such hypothesis for assigning lncRNA function that is gaining notable attention is the competitive endogenous RNA (ceRNA) hypothesis. The ceRNA hypothesis posits that specific RNAs can impair microRNA (miRNA) activity through sequestration, thereby upregulating miRNA target gene expression (FIG. 1). Two classes of lncRNAs in particular are reported in an increasing number of studies as acting as functional ceRNAs; pseudogene-derived transcripts and circular RNAs (circRNAs) (BOX 1).

The attraction of the ceRNA hypothesis is its potential to account for the function of a substantial proportion of the thousands of as yet uncharacterized lncRNAs. On this basis, it has been touted as a new paradigm to explain the complexities of pervasive transcription, being described as the 'Rosetta stone of a hidden RNA language' (REF. 2). But the optimism behind this analogy has been met with growing scepticism[3].

The potential for antisense RNA to sequester miRNA activity is not the basis of the dispute; artificial antisense miRNA inhibitors sufficiently demonstrate the efficacy of miRNA competition. The essence of the underlying argument against the ceRNA hypothesis is that any change in expression of an individual miRNA target could constitute only a tiny fraction of the target site abundance[4]. Therefore, physiological changes in expression of an individual lncRNA would be insufficient to suppress miRNA activity. Evidence supporting this counter-argument is growing following a number of recent analyses using transcriptome-wide approaches[5–10]. The controversy over the ceRNA hypothesis has been further compounded by assertions that ceRNA activity is a general phenomenon[11], in which ceRNAs are defined as a functional class to describe the mechanism underlying regulatory functions of circRNAs, lncRNAs and pseudogenes.

Despite the controversy surrounding the ceRNA hypothesis, its fairly widespread and early acknowledgement as a plausible generic mechanism for regulating gene expression has sparked new areas of research. Epitomising this interest is the proliferation of databases of bioinformatically-predicted ceRNA networks[12–17]. These databases acknowledge the capacity for individual miRNAs to simultaneously silence hundreds of targets, as well as to collectively function in synergistic networks in which a single target may have multiple miRNA recognition elements (MREs). ceRNA network predictions rely on the same algorithms commonly used for miRNA target prediction and produce an *in silico* list of putative targets. An intended utility of these databases is to discover putative miRNA binding sites as candidates for experimental validation[18]. However, the practical limitations in creating a database that takes into account the physiological constraints and stoichiometry of ceRNA interactions obfuscates a basis upon which to discriminate signal from noise. Consequently, queries to such databases typically yield a bewildering picture of putative competitive RNA interactions that cannot be meaningfully interpreted.

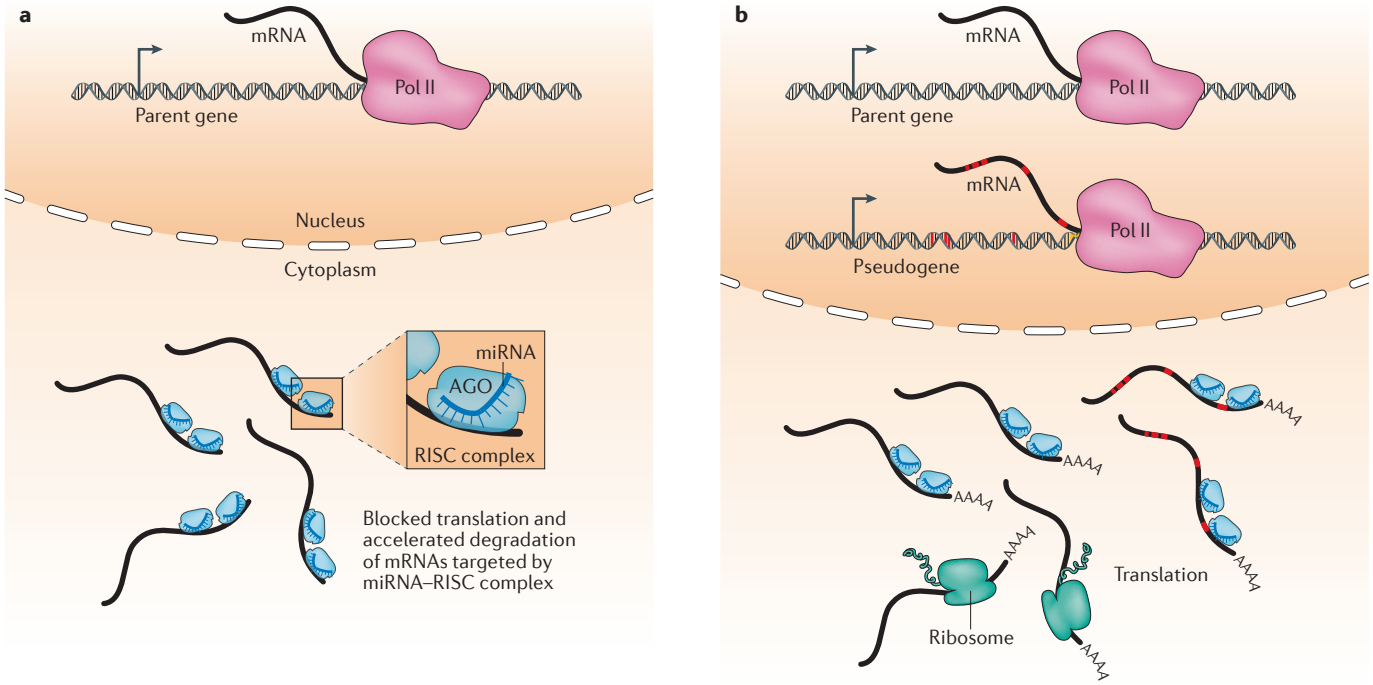

Figure 1 | **The competitive endogenous RNA mechanism.** **a** | While the competitive endogenous RNA (ceRNA), such as a pseudogene, remains transcriptionally silent, the parent mRNA is transcribed and exported to the cytoplasm where it is targeted by the microRNA–guided RNA-induced silencing complex (miRNA–RISC), leading to accelerated degradation, blocked translation and decreased expression. **b** | When the pseudogene with competing target sites (red) becomes transcriptionally active, it competes for miRNA targeting and binding of RISC complexes. This sequesters miRNA–RISC complexes away from the parent gene and results in increased parent gene expression. AGO, Argonaute; Pol II, RNA polymerase II.

Pseudogene
A duplicated gene, identified through DNA homology to its parent gene but with evolutionarily acquired mutations.

Circular RNAs
(circRNAs). A class of RNAs derived mostly from non-canonical splicing in which the exon ends are joined to form a loop.

Antisense RNA
The reverse complement of a specified RNA. This differs from an antisense transcript, which refers to endogenous antisense transcription of an annotated gene.

ceRNA networks
(competitive endogenous RNA networks). Networks of interactions that arise from the concept that a collective pool of transcripts can synergistically compete for microRNA (miRNA) binding and that a single miRNA can silence several targets.

Applying these bioinformatics predictions, the ceRNA hypothesis has found immediate pragmatic application in uncovering non-coding RNA function of poorly annotated RNAs or a dual function for coding transcripts. However, the greatest utility of the ceRNA hypothesis may be to understand how collective ceRNA networks — not individual transcripts — influence post-transcriptional regulation, particularly with the use of transcriptome-wide approaches coupled with bioinformatic prediction.

This Review collects and critically evaluates the accumulating evidence describing functional ceRNA interactions in the context of recent counter-evidence from work modelling transcriptome-wide ceRNA interactions[7,8,16,19–21] and experimentally quantifying the effect of a ceRNA[4,5,22]. The limitations of both experimental and mathematical modelling data are reviewed in an attempt to explain the controversy on the subject and to deliver an objective assessment of the potential significance of the ceRNA hypothesis.

Artificial miRNA inhibitors

The experimental evidence underlying the biochemical principle behind miRNA sponges (specifically, artificial antisense miRNA inhibitors) predates reports of ceRNAs by several years. miRNAs can be inhibited either by the introduction of antisense oligonucleotides[23–25] or by overexpressing transgenic reporters that contain miRNA binding sites[26–28]. The use of antisense RNAs as miRNA inhibitors is now routine in molecular research, and progress has been made towards their application as a new class of drug[29]. A range of commercially available reagents with varying chemistries is available for miRNA inhibition, as reviewed previously[30].

Oligonucleotide miRNA inhibitors (also referred to as antimiRs[31], antagomiRs[24] or other names with commercial propriety) typically comprise small single-stranded RNA oligonucleotides with near perfect complementarity against a miRNA. The oligonucleotides are modified to improve their stability thereby increasing their efficacy. Modifications include 2′-*O*-methylation, cholesterol modification[24], locked nucleic acid (LNA) modification[25] or a hairpin structure such as that found in miRIDIAN inhibitors (Dharmacon) and tough decoy (TuD) constructs[32]. These are introduced into cells by transfection or *in vivo* by viral transduction. In addition, nanoparticle vehicles have been developed for tissue-specific delivery for use as therapeutics[33].

Constructs have also been developed for artificial miRNA sponges that express 3′ untranslated regions (3′ UTRs) containing multiple miRNA-binding sites[28]. The advantage of this type of miRNA sponge compared to antisense oligonucleotides is the potential for inducible, stable expression that is driven by promoters such as U6 or cytomegalovirus (CMV), which are among the strongest drivers of expression in mammalian systems[34].

Box 1 | **Non-coding RNAs**

MicroRNAs
MicroRNAs (miRNAs) are small (20–22 nucleotides long) non-coding RNAs that have become recognized over the past decade as important regulators of gene expression. Functional miRNAs are bound to Argonaute (AGO), the core protein of the RNA-induced silencing complex (RISC). miRNAs control gene expression by guiding RISC to target mRNAs, causing RNA degradation or translational repression. miRNAs are capable of targeting hundreds of genes, as supported by computational, proteomic, transcriptomic and AGO immunoprecipitation studies, which enables their potential to regulate at least two-thirds of a eukaryotic transcriptome.

Competitive endogenous RNA
A competitive endogenous RNA (ceRNA) is a transcript targeted by a miRNA that, in doing so, sequesters the activity of the bound miRNA, effectively de-repressing other targets of that miRNA. It is comparable in action to an artificially introduced miRNA sponge but distinguished by its endogenous origin.

ceRNA network
A collective pool of transcripts that synergistically sequester miRNA activity through competition for the same individual miRNA or a collection of miRNAs.

Long non-coding RNAs
Long non-coding RNAs (lncRNAs) are pragmatically described as transcripts with little or no protein-coding potential that are greater than 200 nucleotides long, distinguishing them from mRNAs and classes of small non-coding RNA, such as miRNAs, small nucleolar RNAs (snoRNAs) or tRNAs. Diverse roles for lncRNAs have been described, including functions in chromosome modification, transcriptional regulation and post-transcriptional processing. There are several classes of lncRNAs described by their origin (such as pseudogene, intergenic- or intronic-derived lncRNAs) or their mechanism of biosynthesis (such as circular RNAs).

Pseudogene transcripts
Pseudogenes are degenerate copies of genes that are synthesized mostly through DNA duplication (duplicated pseudogenes) and retrotransposition of cellular RNAs (processed pseudogenes). In humans, there are 14,467 annotated pseudogenes (Gencode v21), of which ~9% have been recently estimated to be actively transcribed. Although the potential for pseudogene transcripts to code for proteins is acknowledged, the majority of transcribed pseudogenes are thought to be lncRNAs owing to accumulated mutations causing frame shift mutations or premature stop codons. As transcribed pseudogenes commonly share miRNA-binding sites with their parent genes, they are considered attractive candidates as ceRNAs.

Circular RNAs
Circular RNAs (circRNAs) mostly originate from a non-canonical form of alternative splicing, whereby the splice donor site of one exon is ligated to the splice acceptor site of an upstream exon. circRNAs are more resistant to exonucleases than linear transcripts and are therefore more stable, which is thought to enable more efficient suppression of miRNA activity than linear transcripts.

The widespread use of artificial antisense RNAs as miRNA inhibitors is proof of the basic biochemical principle for RNA competition. However, their application is not confined to physiological expression levels, as concentrations that are typically used in miRNA knockdown experiments (10–100 nM transfection of oligonucleotides)[35] far exceed the total cellular concentration of the most highly expressed miRNAs[36]. Nevertheless, studies using antisense miRNA inhibitors illustrate the necessity of high inhibitor concentrations to effectively silence miRNAs. The efficacy of introduced artificial miRNA inhibitors by any method depends on their concentration in the cell, which in turn is also directly influenced by the stability of the inhibitor[35,37,38]. However, the functional quantity is difficult to measure as transfected oligonucleotides accumulate in non-functional vesicles, such as lysosomes, thereby providing an artificially high quantitation following whole-cell lysis[36]. Despite reaching unphysiologically high levels, artificial miRNA sponges are only capable of partial inhibition[34], often no more than 50% against highly expressed miRNAs, which are typically less susceptible to knockdown[35,38].

miRNA recognition elements
(MREs). MicroRNA (miRNA) binding sites that are canonically found in the 3′ untranslated region of a target mRNA and have sequence similarity to the 5′ seed region of the miRNA.

miRNA sponges
Antisense RNA transcripts that inhibit microRNA (miRNA) activity. These molecules can be artificially introduced or, if endogenous, are equivalent to competitive endogenous RNAs.

Experimental evidence for ceRNAs

An appealing aspect of the ceRNA hypothesis is that it provides a pathway for predicting a non-coding function of any uncharacterized RNA transcript by the identification of putative miRNA binding sites. The hypothesis is now supported by experimental evidence for an accumulating number of lncRNAs, particularly circRNAs[39,40] and pseudogene-derived lncRNAs[41–46], but also other lncRNAs[41,44,47–52], expressed 3′ UTRs[53,54], viral non-coding RNAs [55,56] and genomic viral RNAs[4,7,9,34,57–59] (FIG. 2). Furthermore, and consistent with a developing understanding of RNA with multiple functions[60], many protein-coding transcripts (that is, mRNAs) also elicit non-coding function by the ceRNA mechanism[53,61–70].

Despite the relative ease in identification of putative ceRNA interactions using sequence searching algorithms[13,14,71–74], experimentation is essential to identify bona fide miRNA targets and ceRNA interactions. At the most basic level, the experimental framework for identification of a ceRNA interaction involves showing that overexpression of the putative ceRNA leads to increased expression of the competing transcripts or observing the reciprocal relationship when the ceRNA is inhibited. This is often interrogated on a gene-specific level using well-established methods of measuring gene expression, such as quantitative reverse transcription-PCR (qRT-PCR) or western blotting. To demonstrate that changes in expression are the direct result of miRNA competition, experiments can be carried out that involve simultaneous knockdown of specific miRNAs and/or the cellular components required for small RNA biosynthesis, such as by the use of Dicer-deficient cells[75]. The benchmark methodology in demonstrating direct miRNA targeting also incorporates the use of reporter assays containing miRNA binding sites, showing activity specific to the miRNA target sequence[76].

Additional experimental strategies for identifying and validating ceRNA interactions largely mimic those that have been employed for identifying miRNA targets[77]. These involve perturbation of miRNA levels *in vitro* or *in vivo*, followed by gene expression analysis to evaluate coordinated downregulation of the competing target genes. Analyses using microarray or sequencing technologies[78–80] can then provide a transcriptome-wide view of miRNA–target interactions. RNA sequencing, following biochemical enrichment of components of the RNA-induced silencing complex (RISC) components, particularly the Argonaute (AGO) family, has been applied previously for the identification of miRNA target gene networks[81,82]. The same approaches — particularly HITS–CLIP (high-throughput sequencing of RNA isolated by crosslinking immunoprecipitation; also known as CLIP–seq) — are now also being applied and analysed to identify ceRNA networks[5,57].

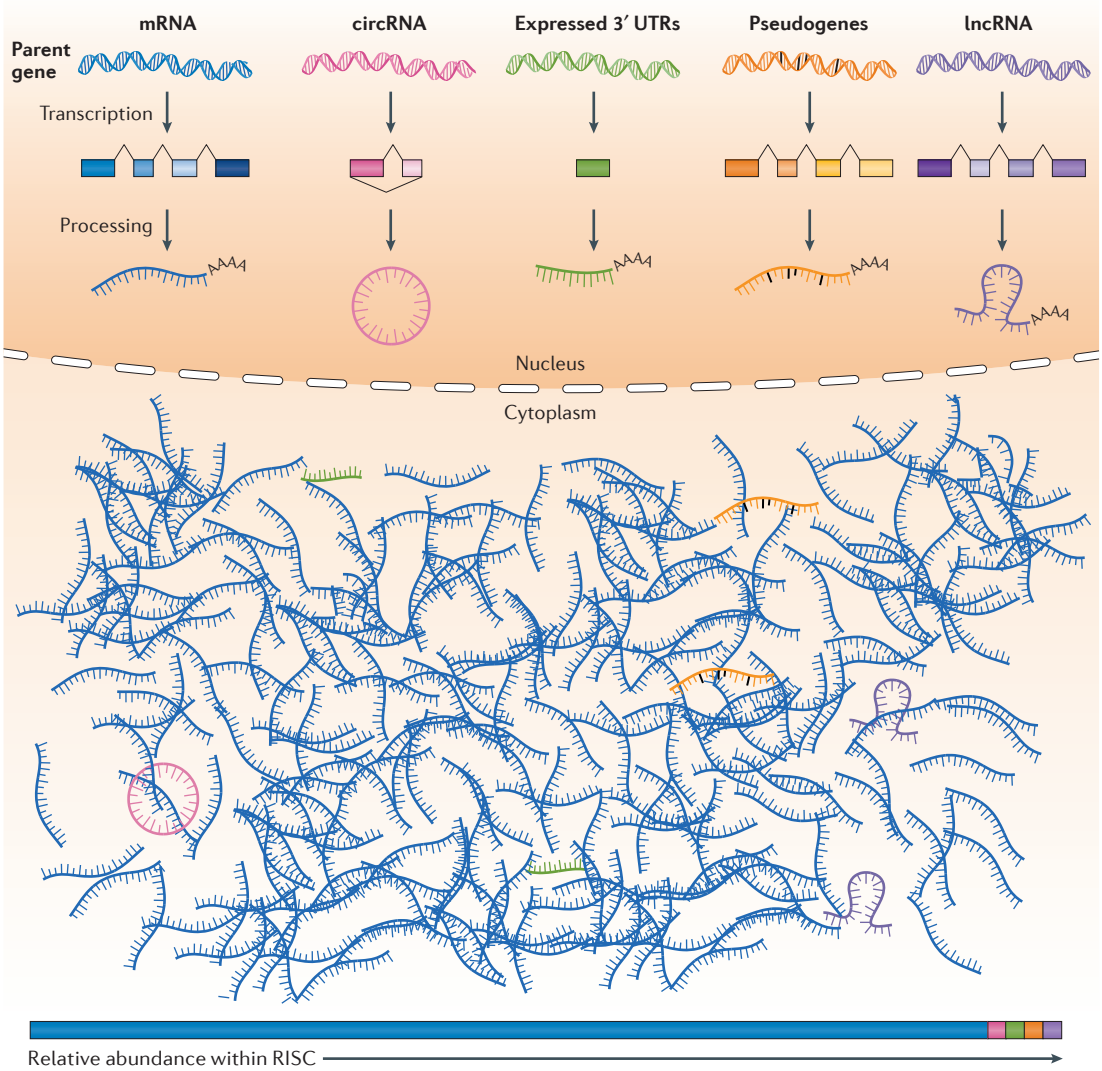

Figure 2 | **The active transcriptome available for microRNA binding competition.** The genome encodes diverse RNA classes including mRNAs, circular RNAs (circRNAs), expressed 3′ untranslated regions (3′ UTRs), pseudogenes and long non-coding RNAs (lncRNAs). Transcription of all RNA classes constitutes the transcriptome, which collectively can compete for miRNA targeting. The majority of active transcripts (those associated with the RNA-induced silencing complex (RISC)) available for microRNA targeting consist of coding transcripts (that is, mRNAs).

3′ untranslated regions
(3′ UTRs). The ends of messenger RNAs that follow the stop codon.

Dicer
A key protein in the microRNA (miRNA) and small interfering RNA (siRNA) biosynthesis pathways. Dicer is an endonuclease that cleaves double-stranded RNA and miRNA precursor transcripts.

RNA-induced silencing complex
(RISC). A protein complex that uses a single stranded guide RNA (for example, a microRNA (miRNA) or small interfering RNA (siRNA)) to elicit post-transcriptional gene silencing.

Argonaute
(AGO). A family of proteins that bind a guide RNA as part of the microRNA (miRNA) or small interfering RNA (siRNA) pathway.

HITS–CLIP
(High-throughput sequencing of RNA isolated by crosslinking immunoprecipitation). A sequencing method to identify microRNA (miRNA) targets and functional miRNAs.

Pseudogenes as ceRNAs. A large number of eukaryotic pseudogenes are actively transcribed, often in a tissue-specific manner, which suggests a coordinated regulation and function[83]. Several modes of action have been proposed for pseudogene transcripts that mostly overlap with the same mechanisms described for lncRNAs[84] (BOX 1), notwithstanding the evidence that many pseudogenes are translated[85].

Owing to their origin as gene copies, pseudogenes typically exhibit high sequence homology to their parent gene. Consequentially, an expressed pseudogene will share miRNA target sites that may compete for miRNA binding.

The capability of pseudogenes to act as ceRNAs is supported experimentally for a number of transcribed pseudogenes including *PTENP1* (REFS 43–45), *OCT4P4* (also known as POU5F1P4)[41], *BCAS4* unitary pseudogene[42], *CYP4Z2P* pseudogene[46] and *BRAFP1* (REF. 75). *PTENP1* was the first reported pseudogene-derived ceRNA to have a role in cancer. *PTENP1* is a sense transcribed pseudogene that is homologous to the tumour suppressor *PTEN* but with a mutation that prevents translation[43]. *PTENP1* shares conserved miRNA seed target sites with *PTEN* for the miR-17, miR-21, miR-214, miR-19 and miR-26 miRNA families[43]. In DU145 prostate cancer cells, miR-19a and miR-19b miRNA mimics were found to downregulate both *PTEN* and *PTENP1* at the mRNA level (by 50–75%), and treatment with an inhibitor cocktail of all predicted targeting miRNAs increased the expression of both *PTEN* (by ~50%) and *PTENP1* (by ~25%)[43]. A luciferase reporter with a 3′ UTR for the miRNAs targeting both *PTEN* and *PTENP1* showed that inhibition of the 3′ UTR was directly due to these shared miRNA

binding sites[43]. Expression of the *PTENP1* 3′ UTR alone was shown to increase expression of PTEN (by 50%) in a Dicer-dependent manner and to act as a tumour suppressor, which was demonstrated with colony-forming assays[43]. However, the generalization of these observations to all other transcribed pseudogenes has little basis. Pseudogene transcripts are frequently localized to the nucleus, and other functions including their translation into proteins or peptides have been reported[85].

The steady-state expression levels of pseudogenes seldom reach that of their parent genes[42,83]. Assuming that a ceRNA needs to be at near equimolar concentration to the collective pool of competing transcripts, as suggested by mathematical models[7–10], most individual pseudogenes are too lowly expressed to function as viable competitive targets. However, characteristics unique to pseudogenes could allow for greater activity. Pseudogenes share several MREs with their parent gene, thereby drastically increasing target abundance. Also, many processed pseudogenes are present as families, by virtue of their derivation from retrotransposition of the same mRNA. As a result, although not yet evaluated experimentally, groups of pseudogenes that have very similar sequences could in principle be accumulatively expressed to reach target site abundance sufficient to act as ceRNAs.

circRNAs as ceRNAs. circRNAs are much more stable than linear transcripts as they do not have free ends that are susceptible to exonuclease digestion, which is a characteristic that is thought to facilitate their effectiveness as miRNA sponges[39,40]. circRNAs are typically synthesized through a non-canonical form of alternative splicing in which the splice donor site of one exon is ligated to the splice acceptor site of an upstream exon[40,86,87]. The first circRNA was discovered over two decades ago, and it is derived from the testis-determining gene *Sry* (sex-determining region Y)[88]. Recently, circRNAs have gained interest following reports describing their widespread and abundant expression in eukaryotes[39,40,87,89–93].

Although the function of most circRNAs remains elusive, at least three circRNAs have been experimentally shown to act as ceRNAs: *cIRS-7* (also known as *CDR1-AS*) regulates miR-7 activity in the central nervous system[40]; an *Sry*-derived circRNA acts as a sponge for miR-138 (REF. 39); and *cir-ITCH* controls the level of itchy E3 ubiquitin protein ligase (*ITCH*) by sponging miR-7, miR-17 and miR-214 in oesophageal squamous cell carcinoma (ESCC)[94].

Following the initial observations of circRNAs acting as ceRNAs, it was asserted that this mechanism may be a common function of all circRNAs[39]. However, there is now considerable counter-evidence to this assumption. With the exception of *cIRS-7*, which contains 73 selectively conserved miR-7 target sites, and *Sry*-derived circRNA, which has sites for miR-138, the vast majority of circRNAs do not contain such distinct miRNA-binding sites[87,91,92]. Depletion of polymorphisms of putative miRNA-binding sites in circRNAs has been suggested to indicate a conserved role involving miRNAs[95]. However, assessment of circRNA conservation is confounded by the majority of annotated circRNAs overlapping coding exon loci[73]. Furthermore, not all miRNAs that target circRNAs are suppressed (for example, miR-671, which downregulates expression of *CDR1-AS* with a concomitant decrease in *CDR1* mRNA levels[96]). In this case, the targeted circRNA is cleaved in an AGO2 (also known as slicer)-dependent manner, limiting its potential to act as a miRNA sponge. In addition, AGO co-immunoprecipitation experiments do not indicate an appreciable enrichment of circRNA-derived exons among AGO family-bound transcripts, which would be expected if circRNAs were acting as ceRNAs[5].

Viral miRNA sponges. Viruses are known to exploit various mechanisms to manipulate host gene expression[97]. Among the most efficient of these, and thought to be preferred over protein-based factors, is a mechanism using non-coding RNAs[98]. There is now growing evidence that viruses produce non-coding RNAs that serve as miRNA sponges, providing interesting adaptions to the ceRNA mechanism that could account for increased ceRNA activity.

A report of two viral small nuclear RNAs (snRNAs) that each downregulate a host miRNA[55] offered the first insight into the potential for viral control of host phenotype through a miRNA-sponge mechanism. The intriguing herpesvirus saimiri (HVS) produces more non-coding RNAs than any other class of mammalian virus[55], of which the HVS uracil-rich RNAs (HSURs) are the most abundantly expressed gene products in HVS-transformed T cells[99,100]. Two of the HSUR snRNAs contain predicted binding sites for the abundant primate (host) miRNAs (miR-16, miR-27a and miR-142-3p). Co-immunoprecipitation with epitope-tagged HSUR constructs validated miR-16 and miR-27a binding to HSUR2 and HSUR1, respectively. Levels of miR-27a were further shown to be downregulated in HVS-transformed cells with concurrent upregulated expression of its target genes[55].

As well as representing the first described case of viral RNA competing with host miRNA expression, the HVS snRNAs represent the first example of a natural small RNA sponge. The high abundance and structure of snRNAs, similar to the short hairpin inhibitors used for miRNA knockdown (for example, the miRIDIAN miRNA hairpin inhibitors manufactured by Dharmacon), bestow these non-coding RNAs with favourable characteristics to act as effective miRNA inhibitors. However, the predominantly nuclear localization of HSURs defies the canonical view that miRNAs act in the cytoplasm, although the authors do note that the HSURs are shuttled between the nucleus and the cytoplasm[55,101].

As well as small RNAs, other genomic RNAs from retroviruses can act as miRNA sponges. For example, the two miRNA binding sites in the 5′ UTR of hepatitis C virus (HCV) bind and sequester host miR-122, reducing the binding of miR-122 to other targets[57]. Importantly, this example delivers a slightly different mode of action of competition for miRNAs. Whereas cellular miRNA targets are degraded following binding, it was shown that HCV RNA is stabilized by miR-122 binding and is required for viral replication[102,103]. This constitutes a

Small nuclear RNAs (snRNAs). A class of nuclear localized RNAs with roles in splicing and RNA modification.

positive feedback loop in which the HCV genomic RNA is its own substrate for replication[57], providing a mechanism by which the miRNA sponging effect is amplified.

mRNAs as ceRNAs. Coding transcripts are, in general, more abundant than lncRNAs[58,59], they show the highest conservation of miRNA binding sites, particularly in the 3′ UTR[104], and make up the majority of miRNA targets[82,104]. A reasonable deduction to make from this is that mRNAs should exert the majority of influence in RNA competition. Focus on lncRNAs may be driven by the attraction of finding function for poorly annotated transcripts; nonetheless, an accumulating number of reports indicate roles of protein-coding transcripts in sequestering miRNAs through the ceRNA mechanism, including *CD44* (REF. 62), *FN1* (REF. 63), *VCAN*[53,64], *FOXO1* (REF. 65), *OCT4B* (also known as *POU5F1B*)[66], *AEG1* (also known as *MTDH*)[67] and *PTEN*[67–70].

The tumour suppressor gene *PTEN* has been studied in depth for its capacity to act as a ceRNA[105]. Because of this, representative suppositions have been postulated towards roles of ceRNAs in general[106]. *PTEN* has been implicated in ceRNA networks in a number of cancers including glioblastoma[68,70], melanoma[69] and prostate cancer[68]. *PTEN* has also been shown to compete for miRNA binding with *ZEB2* (REF. 69), *CNOT6L*[68], *VAPA*[69], *VCAN*[64] and many more coding transcripts that have been identified through analysis of gene expression networks[70]. Not restricted to coding transcripts, *PTEN* competes with miRNA binding with the *PTENP1* pseudogene[43].

An important outcome of these studies on *PTEN,* as well as other studies on mRNAs, is the observation that expression of the 3′ UTR alone is capable of eliciting ceRNA effects[62–65,67], indicating a non-coding function of coding transcripts. These data provide further evidence that transcripts from the same loci can exert multiple functions through independent mechanisms[60], in this case both protein-coding and non-coding functions.

Taken together, studies focusing on *PTEN* have helped elucidate a ceRNA network that puts competition for miRNA binding at the forefront of many cancers[105]. Despite attracting marked interest, these studies highlight the futility of focusing exclusively on individual transcripts (in this case *PTEN*) as ceRNAs, when in fact competition networks may be the influence underlying disease states.

ceRNA networks

Evidence for transcriptome-wide competition for miRNA binding. The majority of evidence experimentally validating ceRNA interactions assesses one or a few miRNAs against a ceRNA–target pair. Owing to experimental limitations, demonstration of direct ceRNA interaction is often restricted to individual genes. However, individual miRNAs are capable of targeting hundreds of genes, as shown by computational, proteomic, transcriptomic and AGO immunoprecipitation studies[104]. Using the highly studied miR-124 as an example, miRNA mimic overexpression downregulates hundreds of mRNAs enriched in the miR-124 seed sequence: at the transcript level measured by microarray[107], at the protein level measured using SILAC (stable isotope labelling by amino acids in cell culture)[108], actively translated transcripts measured using polysome profiling[109] and transcripts associated with the RISC complex demonstrated using AGO HITS–CLIP[82]. Indeed, hundreds of thousands of miRNA–target interactions are estimated, a proportion of which are supported by some experimental evidence ranging from altered expression following miRNA perturbation, to validation at the sequence level with engineered reporter assays[110]. On this basis, the potential for a vast ceRNA network that broadly influences the transcriptome is avidly speculated[111]. The potential for ceRNA networks to influence miRNA activity has been largely investigated by predicting ceRNA interactions using bioinformatics tools developed previously for the prediction of miRNA targets. Indeed, any study of ceRNA interaction relies to some extent on target prediction, whether it is to identify candidate ceRNAs or to model target abundance.

Bioinformatic prediction of ceRNA interactions. The most widely used miRNA target discovery algorithms search for evolutionary conserved, 6-nucleotide interactions between the 5′ end of the miRNA known as the seed region and the 3′ UTR of the target mRNA. These 'rules' of targeting are based on numerous computational and biochemical studies showing the importance of the seed region. Nonetheless, miRNA targeting is also influenced by the 3′ region of the miRNA and the molecular context of the target site. Indeed, several alternative modes for miRNA targeting have been described including centred pairing[112] and G-bulge sites[113]. Therefore, the validity of target prediction tools is hindered by the simplified rules used to represent targeting interactions, with different algorithms producing vastly different results[77,114,115].

Using miRNA target discovery algorithms, such as TargetScan[71] and miRanda[72], several studies have developed databases of ceRNA interactions. These databases include the Competing Endogenous mRNA DataBase (ceRDB)[13], the Database of human long non-coding RNAs acting as competing endogenous RNAs (lnCeDB (Human))[14], Linc2GO[116], miRcode[73], DIANA-LncBase Predicted v.2 (REF. 74) and the LncRNA-associated Competing Triplets DataBase (LncACTdb). Bioinformatic algorithms typically allow specification of various user-defined parameters to maximize true positives and minimize false positives and negatives. In this way, different algorithms limit choices to restrict observations to mRNAs[13] or to include lncRNAs[14,20,73,74,116], circRNAs[74] or viral miRNA targets[117].

Although many predictions rely completely on sequence information, others also incorporate gene expression data to better predict the presence of miRNA–target pairs[118–120] and AGO HITS–CLIP data has been included to enrich for active AGO-bound RNA targets[12,74]. Although incorporation of these analyses improves the capability to predict physiologically relevant ceRNA interactions[16], in practice it is difficult to compile the optimal combination of data sets effectively[77], which is illustrated by the lack of consensus between different databases such as miRSponge[17] compared to starBase, DIANA-LncBase and LncACTdb[17].

SILAC
(Stable isotope labelling by amino acids in cell culture). A proteomic approach using quantitative mass spectrometry.

Polysome profiling
The analysis of mRNAs associated with elongating ribosomes to identify translationally active mRNAs.

Centred pairing
A non-canonical mechanism of microRNA (miRNA) targeting in which six or more nucleotides in the centre of the miRNA bind target genes.

G-bulge
MicroRNA (miRNA) targeting of complementary nucleotides where a guanosine (G) nucleotide is skipped resulting in a 'bulge'.

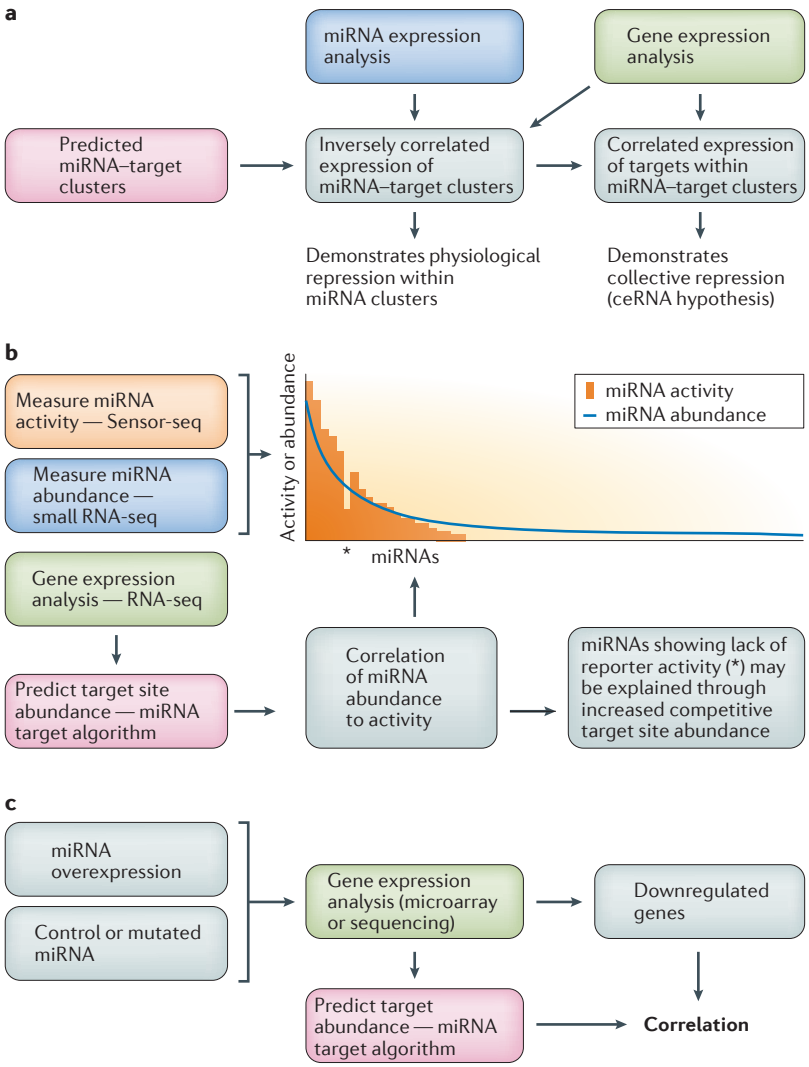

Figure 3 | **Strategies used to assess transcriptome-wide competition for microRNA binding. a** | MicroRNA (miRNA)–target clusters are predicted using target prediction algorithms with no preconceptions of gene expression. miRNA–target cluster predictions are overlayed to databases of gene expression (mRNAs) and miRNA expression. Within predicted miRNA–target clusters, physiological miRNA repression is demonstrated by miRNAs and their targets being inversely correlated in expression. To demonstrate that mRNA targets within the same biocluster are competing for miRNA targeting, common miRNA targets show correlated expression, supporting the competitive endogenous RNA (ceRNA) hypothesis. **b** | miRNA activity (for example, that measured using reporter assays) compared to miRNA abundance (for example, that measured using small RNA sequencing (RNA-seq)) are weakly correlated. Discordance between miRNA activity and abundance (denoted as *) can be investigated by measuring and comparing target abundance of each miRNA. **c** | miRNA target genes are identified by miRNA overexpression followed by gene expression analysis. Comparing downregulated genes to predicted miRNA target site abundance is capable of showing that overexpressed miRNAs with a greater target pool have diluted activity.

Modelling of ceRNA networks from gene expression data. In principle, the extent to which genes are regulated by ceRNAs can be assessed by listing all miRNA–target pairs and evaluating whether mRNAs targeted by the same miRNAs are significantly more correlated in expression (FIG. 3a). Comparison of miRNA target predictions with gene expression databases shows that miRNAs and predicted targets are more likely to have inversely correlated gene expression, as expected for miRNA–target pairs. However, this approach does not consider the effect of common transcription factors or other regulatory mechanisms and is dependent on *in silico* predictions. Nonetheless, these results offer an opportunity to identify 'networks' of ceRNA interaction[7,9,16,70,121].

Using this approach, *in silico* ceRNA networks, also referred to as miRNA–target bioclusters, were compared to Encyclopedia of DNA Elements (ENCODE) gene expression data looking at both mRNA and miRNA expression[16]. Each miRNA–target biocluster represented a manageable number of miRNAs and mRNAs connected by target prediction, and these were shown to be inversely correlated in expression as expected for miRNA–target pairs. In support of the ceRNA hypothesis, mRNAs within miRNA–target bioclusters showed correlation in expression suggestive of expression buffering within commonly targeted mRNAs[16].

Transcriptome-wide RNA competition

A large endogenous target pool can decrease miRNA activity. The suppressive effect that a large pool of endogenous targets can have on miRNA activity has been observed by comparing transcriptome-wide target abundance predictions to a high-throughput assay of miRNA activity[22]. This approach, termed Sensor-seq, uses RNA sequencing (RNA-seq) to quantify the degradation of a reporter construct pool, each with target sites for one of 291 miRNAs. By comparing Sensor-seq results to cellular miRNA abundance, it was noted that some highly expressed miRNAs exhibited relatively weak activity in comparison to similarly highly expressed miRNAs that correlated with a high number of predicted target sites[22] (FIG. 3b). This gives an experimental framework by which the entire pool of target transcripts can detectably decrease miRNA activity.

An important consideration from this work is that only the most abundant miRNAs have detectable activity, as over 60% of miRNAs detected by sequencing have no discernable activity when measured by Sensor-seq[22]. This finding is supported by earlier work in which lowly expressed miRNAs (<100 copies per cell) were found to have little regulatory capacity[26]; the same conclusion was reached from an experiment in which 20 individual reporters were assayed with target sites against miRNAs of varying endogenous expression levels[122].

Overexpressed miRNAs with a larger target pool have diluted activity. An alternative approach to measuring the suppressive effect of a large pool of endogenous targets is to compare predicted target abundance with miRNA target repression following miRNA overexpression (FIG. 3c). Using this approach, predicted miRNA target abundance has been compared to microarray data sets of genes that are downregulated following artificial miRNA overexpression[78–80]. This has revealed that miRNA efficacy can be diluted when a greater number of target transcripts are predicted.

However, as these experiments compare different miRNAs with different binding affinities, the observed differences may be due to the binding strength rather

Encyclopedia of DNA Elements
(ENCODE). An international consortium with the goal to build a comprehensive list of functional elements in the human genome.

than target site frequency. Subsequent experiments to distinguish between these possibilities, by substituting the two adenines in the *Caenorhabditis elegans* miRNA *lsy-6* for the adenine analogue 2,6-diaminopurine (DAP) to increase the binding strength without changing target abundance, found that although either property was associated with the targeting efficiency of 3′ UTR sites, the number of predicted target sites had an independent, statistically significant effect[80].

Physiological ceRNA expression changes do not affect highly expressed miRNAs. An important factor in assessing the potential impact of the ceRNA hypothesis is the capacity of a single transcript to contribute to the greater pool of potential binding sites thereby affecting miRNA activity. To address this question, Denzler *et al.*[4] introduced a transcript *in vivo* at controlled levels to compete for binding to miR-122, a miRNA highly expressed in the liver. The team found that at physiological expression levels of 5.1×10^3 miR-122 target sites per cell, the introduced target was insufficient to affect miRNA levels, target gene expression or downstream physiological responses[4]. This was concluded to be the result of the relatively high abundance of other miR-122 targets, which was calculated to be ~1.5×10^5 binding sites. Furthermore, it was noted that neither the individual endogenous target nor the entire pool of predicted miR-122 target sites were capable of reaching this abundance in a physiological setting or even at levels predicted in pathological states. The model reinforced by Denzler *et al.* is that changes in ceRNA abundance must approach the target abundance of the miRNA to de-repress miRNA targets.

Mathematical models predict that ceRNA inhibition most optimally occurs when the miRNA and targets are at near equimolar concentrations[7–9]. ceRNA expression must approach that of the target abundance before it can exert de-repression of the miRNA target[10]. In the case of miR-122, the most abundant miRNA in hepatocytes and comparable to one of the most highly expressed miRNAs in any mammalian system[4], physiological changes in target expression were unable to reach these levels[4]. Although this work does not exclude the possibility that lowly expressed miRNAs may be susceptible to ceRNA interaction, it provides an indication of the limited potential for individual ceRNAs to influence post-transcriptional regulation. As discussed previously, only the most highly expressed miRNAs are capable of exerting target repression as observed by functional assays[22,122].

The abundance of most individual targets is insufficient to alter active miRNAs. The assessment of miRNA target abundance typically relies on whole-cell RNA-seq quantification. With the understanding that only transcripts associated with RISC and AGO engage in competition, this approach potentially overestimates the number of actively competing transcripts. This limitation can be overcome using AGO HITS–CLIP, which specifically enriches active AGO-bound RNAs[81,82,123], and therefore allows more accurate quantification of the active miRNA target pool[5,57,124].

AGO individual-nucleotide resolution CLIP (iCLIP), a variation of HITS–CLIP, combined with absolute miRNA and mRNA quantitation, in human embryonic and mesenchymal stem cells, showed that with the exception of the most highly expressed miRNA families, the total target pool (defined as all 6-, 7-, and 8-mer 3′ UTR targets in iCLIP libraries) exceeded the miRNA concentration. Moreover, mRNA-seq with or without AGO induction, revealed miRNA-mediated repression. Consistent with previous observations of miRNA functionality[22], only 8–12 miRNA seed families in embryonic stem cells and mesenchymal stem cells exhibited detectable binding activity[5]. Of the eight most active embryonic stem cell miRNA families, only six were expressed at concentrations greater than their 3′ UTR 8-mer target pool. Single-cell miRNA reporter assays further support that the most highly expressed miRNA families, such as miR-294 and let-7, are unsusceptible to ceRNAs. One exception was the active miR-25 miRNA family, which has exceptionally low target abundance relative to its expression, where high-affinity ceRNA induction far below that of the entire target pool (~3,000 copies of 8-mer reporter sites, ~15% of total 6-, 7- and 8-mer pool) led to a modest (~30%) reduction in miRNA activity.

In summary, these experiments support previous evidence[7,9,16] that miRNA–target ratios determine the susceptibility of target repression to target competition[5]. With the exception of extreme scenarios in which the target pool is exceptionally small and the ceRNA has high affinity, the high abundance of the majority of active miRNAs are unlikely to be susceptible to ceRNA competition (FIG. 4). Furthermore, despite the differences in experimental systems, and much lower total miRNA target pool estimates, assessments of ceRNA susceptibility using AGO iCLIP support previous calculations showing the inability of highly abundant miRNAs to be sequestered by ceRNAs within physiological expression levels[4]. The study also highlights that AGO-bound miRNA targets are predominantly composed of 3′ UTR sites of mRNAs with proportionally little binding in non-protein-coding transcripts[5]. As the pseudogene and lncRNA classes constitute only 1.7% of the average 7- and 8-mer miRNA binding pool in embryonic stem cells, coding transcripts are best positioned to exert the major competitive influence to miRNA activity (FIG. 2).

Models of enhanced ceRNA activity

There is a discernable disconnect between the stoichiometric models of ceRNA interaction and the number of individual ceRNAs experimentally reported. Although models assessing transcriptome-wide target abundance indicate that individual transcripts do not (except under exceptional circumstances)[4–6] physiologically reach the required abundance to elicit competition[6], reports supported by experimental evidence are growing. The field would benefit from molecular models to better describe how ceRNA activity can be enhanced beyond solely a function of abundance.

Modelling of transcriptome-wide stoichiometry usually relies on the false assumption that a cell is akin to an aqueous solution. A pertinent argument to explain

Sensor-seq
A high-throughput assay of microRNA (miRNA) activity as measured by high-throughput sequencing of a pool of reporter constructs each with binding sites for individual miRNAs.

Individual-nucleotide resolution CLIP
(iCLIP). A crosslinking immunoprecipitation (CLIP) technique for identifying protein–RNA interactions, in which the direct crosslinking site can be identified where the reverse transcribed cDNA is truncated.

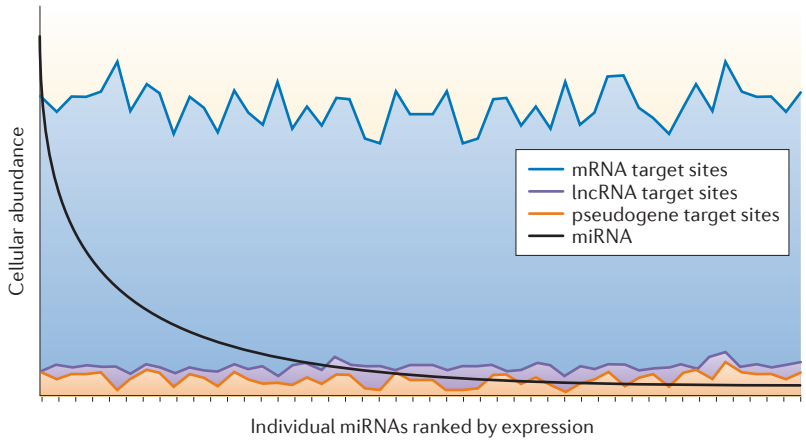

Figure 4 | **Schematic of relative cellular abundance of microRNAs and predicted target sites.** The abundance (copies per cell) of individual microRNAs (miRNAs; black line) is shown whereby the top ~10 most abundant miRNAs make up the majority of all cellular miRNAs. The total number of predicted miRNA target sites for each miRNA typically outnumbers the abundance of the miRNA. Predicted miRNA target sites consist of mRNAs (blue line), long non-coding RNAs (lncRNAs; purple line) and pseudogenes (orange line). The majority of predicted target sites are within mRNAs.

discordance between lowly abundant transcripts and function is that compartmentalization or subcellular localization will increase RNA concentration at the site of activity. This argument is reiterated in recent articles, speculating that trapping miRNAs in P-bodies or other sites of miRNA-mediated RNA decay would amplify ceRNA regulation[6,75]. This concept adheres to the understanding that lncRNAs can show highly specific localization and expression patterns[125]. However, this raises the question: what are the subcellular compartments where ceRNA activity occurs? The premise is that ceRNAs must be in close association with active RISC-associated miRNAs[82,126]. With this assumption, supported by some evidence[126], the application of AGO immunoprecipitation techniques is ideally suited for discerning the specific subcellular localization of active ceRNAs. Thus, investigating ceRNAs at their subcellular localized level should be as straightforward as analysis of the AGO HITS–CLIP data. Nonetheless, the potential for enriched expression through subcellular localization remains as an argument to enhanced ceRNA function. Justifiably, there remains the possibility that compartmentalization of ceRNA activity may occur independently of the RISC complex, although this argument remains largely speculative.

It is also frequently observed that lncRNA expression can be highly temporally regulated, which results in coordinated spikes in expression[127], prompting speculation that coordination of lncRNA expression may enhance ceRNA activity through highly regulated co-expression of a miRNA and its competing targets[128].

Another factor that influences ceRNA activity is the stability of the ceRNA as it is understood to be an important consideration when designing artificial oligonucleotide miRNA inhibitors. Stability of endogenous transcripts is highly variable[129] as there are identified structural components of endogenous transcripts that allow greater stability, such as hairpin secondary structures or in the case of circRNAs, which are far less susceptible to exonucleases[39,40]. Similarly, the structural context of the miRNA binding site, such as that offered by alternative 3′ UTR isoforms, is recognized as influencing miRNA binding[130]. This is supported by the observation that when the target site is unstable, as in the case for perfectly complementary target sites that are cleaved by AGO2, the miRNA is less likely to be sequestered. Imperfect base pairing has been shown to increase miRNA binding for up to 20 times longer than a perfectly complementary target[124]. However, in mammals (as opposed to plants) most miRNA target sites are imperfectly complementary to the miRNA[131], meaning this scenario does not provide an exceptional argument to specifically enhanced ceRNA activity of just a few lncRNAs.

Several molecular pathways have recently been described that explain how subtle regulation of the ceRNA mechanism may be amplified through downstream processes[57,128]. For example, the modest upregulation of transcription factors by miRNA competition with lowly expressed lncRNAs may have magnified downstream consequences through signalling to multiple effector targets, a concept known as pathway divergence. This claim is validated to some extent by the observation that 88% of lncRNAs share miRNA binding sites with transcription factor mRNAs that are also downregulated upon lncRNA knockdown[128]. Another promising mechanism for enhanced ceRNA activity is that of the HCV RNA. In addition to having binding sites to effectively sequester the highly abundant miR-122, the HCV RNA is also stabilized by miR-122, constituting a positive feedback loop, which effectively enhances its ceRNA activity[57].

Limitations of ceRNA validation

The possibility cannot be excluded that the discrepancy between transcriptome-wide modelling data and experimental validation is a consequence of limitations specific to each approach. Here, we summarize notable limitations to both experimental and bioinformatics approaches used to investigate the ceRNA mechanism.

Many of the limitations of experimental evidence towards ceRNA interactions encompass the lack of physiological relevance of overexpression systems. It is difficult to replicate physiological miRNA expression with the introduction of miRNA mimics, particularly owing to the difficulties of directly measuring the activity of introduced miRNAs if transfected oligonucleotides collect in lysosomes where they do not elicit miRNA activity[36]. This is also the case for exogenous expression of putative ceRNAs, whereby absence of a complementary inhibition experiment inherently limits interpretation. miRNA activity at endogenous expression levels can more appropriately be investigated using target reporter constructs or miRNA-sensor reporters (including Sensor-seq). However, expression of a reporter at higher than physiological levels may itself contribute to saturating miRNA activity. This effect is observed when titration of a miRNA sensor reporter reveals increased miRNA activity at lower reporter concentrations[122].

Pathway divergence
A signalling pathway that is amplified when signals from the same ligand activate a variety of different effectors leading to diverse cellular responses.

Small interfering RNA
(siRNA). siRNAs, which are also called silencing RNAs, act within the RNA-induced silencing complex (RISC) to guide gene silencing. The term can refer to a synthetic RNA duplex or an endogenously derived RNA from a double-stranded precursor.

CRISPR
A specific gene-editing technique using guide RNAs and CRISPR-associated protein 9 (Cas9).

AGO immunoprecipitation techniques are capable of quantifying endogenous expression of both miRNAs and targets; however, many AGO HITS–CLIP protocols rely on an epitope-tagged AGO overexpression[132]. Exogenous AGO overexpression has been observed to have differing effects on cellular localization to P-bodies which could potentially affect endogenous RISC activity[133].

In addition, *Dicer*-knockout cell lines have been used extensively to demonstrate the dependence of a molecular or cellular mechanism on miRNAs[75]. The role of Dicer in miRNA biogenesis is widely understood[104]; however, pleiotropic effects of Dicer have also been observed[75,134]. It is also difficult to distinguish the coding from non-coding function of an mRNA through inhibition alone, given that gene knockout or small interfering RNA (siRNA)-induced silencing cannot be designed specifically to either function. Although several studies have used overexpression of the 3′ UTR alone to rectify this problem[62–65,67].

An underlying and overwhelming limitation of studies assessing ceRNA activity, in particular mathematical modelling studies, is that they are wholly dependent upon the application of miRNA–target prediction algorithms. Limitations and shortfalls of miRNA prediction algorithms are widely appreciated[77,115,135–138]. For example, many algorithms include only the 3′ UTR of coding transcripts for target predictions as is the case for TargetScan[139], Probability of Interaction by Target Accessibility (PITA)[140], miRanda[141] and TargetProfiler[142]. In addition, many algorithms rely on conservation between species[139,143], rarely take expression levels into account[139,140,143], and often only small numbers of transcripts are investigated when looking at ceRNA networks.

Concluding remarks

The exciting prospect of uncovering lncRNA function through the ceRNA mechanism has generated substantial interest. Key to its success is its potential as a global mechanism. However, this is proving to be a double-edged sword: although it can provide a mechanism explaining the function for any RNA, it must also be scrutinized in the context of the entire transcriptome (FIG. 2).

Understanding how RNA–RNA competition takes place in the context of all cellular transcripts has necessitated the application of transcriptome-wide approaches to understand how miRNA sponges function within physiological limits. Furthermore, understanding the stoichiometry of both the miRNA and ceRNA is crucial given that miRNA efficacy is determined by its cellular abundance[22] or, more accurately, its abundance within RISC complexes[122]. The implication of this is that the required number of competing transcripts is much higher for more active miRNAs.

Recent studies address the viability of miRNA-sponge interactions on a transcriptome-wide scale. Optimal ceRNA interactions have been modelled to occur when miRNA and target levels are near equimolar[7,9,16]. Supporting this, it has been shown that lowly expressed miRNAs can be susceptible to ceRNAs[5]. Equally, the need for large changes in target abundances diminishes the ability of individual transcripts to disrupt the activity of highly expressed miRNAs[4,5]. This is particularly relevant when considering non-coding RNAs that typically contribute to only a tiny fraction of the total pool of miRNA targets[5].

Conversely, clarification of models of enhanced ceRNA function may explain how ceRNA activity could be manifested beyond solely as a function of its expression, such as scenarios in which ceRNA concentration is spatially or temporally enriched to meet the high expression requirement for ceRNA activity or if RNA stability is increased. There is still considerable speculation around these models, although progress is being made in describing molecular pathways that may explain how minor transcriptomic changes elicit larger downstream responses through positive feedback loops[57] or pathway divergence though transcription factor regulation[128].

Limitations to experimental manipulations to study ceRNA interactions require more critical evaluation. Many studies have used miRNA overexpression to study ceRNA interactions, using transfected oligonucleotide inhibitors or expression vectors. As these experiments are typically outside of the realms of physiological expression, they overestimate the potential activity of a ceRNA. A clearer physiological picture is observed when overexpression is controlled within physiological ranges[4] or when miRNA suppression experiments support these claims, which has been performed using miRNA inhibitors, but ideally would be done with gene knockout technologies such as CRISPR, thus avoiding potential saturation of RISC complexes.

Considering the evidence collectively, the ceRNA hypothesis shows greatest utility in accounting for pathologies associated with exceptional transcriptome changes, such as the widespread remodelling of 3′ UTRs and polyadenylation dynamics observed in cancer[144], rather than as a general mechanism to predict the function of individual non-coding genes, such as lncRNAs and pseudogenes.

Finally, this Review has focused on the potential for competition between miRNAs and miRNA targets; however, the insights gained from work in this area have far-reaching impact as new examples of RNA competition are being discovered in biological processes as diverse as miRNA suppression from alternative RNA-binding proteins[145] to RNA competition during retrotransposition[146].

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Competing interests statement
The authors declare no competing interests.

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