Re-thinking miRNA-mRNA interactions: Intertwining issues confound target discovery

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Despite a library full of literature on miRNA biology, core issues relating to miRNA target detection, biological effect, and mode of action remain controversial. This essay proposes that the predominant mechanism of direct miRNA action is translational inhibition, whereas the bulk of miRNA effects are mRNA based. It explores several issues confounding miRNA target detection, and discusses their impact on the dominance of miRNA seed dogma and the exploration of non-canonical binding sites. Finally, it makes comparisons between miRNA target prediction and transcription factor binding prediction, and questions the value of characterizing miRNA binding sites based on which miRNA nucleotides are paired with an mRNA.

Keywords:
biotin pull-down; centered sites; direct interactions; miRNA; mRNA destabilization; non-canonical sites; seed sites; translational inhibition

Introduction

MicroRNAs (miRNAs) are small non-coding RNAs that are best known for their role as guide molecules in the RNA induced silencing complex (RISC). The biogenesis of mature miRNAs (roughly 22 nucleotides long) is diverse: multiple pathways and multiple steps lead to the generation of RNA hairpins (so named because of the resemblance to "hair pins" or "bobby pins;" Fig. 1). These hairpins are further processed into a shortened, single-stranded product, and loaded into an AGO protein, and the miRNA associated AGO – together with various protein binding partners – form the functional RISC. The entire biogenesis pathway is highly regulated, and has recently been reviewed in depth [1].

By regulating the expression of protein-coding genes, miRNAs are involved with almost every biological process in eukaryotes [2], and the dysregulation of miRNA expression has often been associated with human disease [3, 4]. It is not surprising then, that a substantial body of research has been dedicated to understanding how these miRNAs function and what their interactions with various targets achieves biochemically and biologically. Like most disciplines at the center of a research frenzy, there is still substantial debate in the field over almost every aspect of miRNA activity, and even how best to measure this activity.

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Abbreviations:
EBV, epstein-Barr virus; **HITS-CLIP,** high-throughput sequencing of cross-linked immunoprecipitation; **miRNA,** microRNA; **mRNA,** messenger RNA; **PAR-CLIP,** photoactivatable-ribonucleoside-enhanced crosslinking and immunoprecipitation; **RISC RNA,** induced silencing complex; **rRNA,** ribosomal RNA; **UTR,** un-translated region.

The predominant mechanism of direct miRNA action is translational inhibition, whereas the bulk of miRNA effects are mRNA based

Several mechanisms have been reported as to how miRNAs exert their effect on the overall protein production from genes. The first is by interfering directly with protein synthesis, either at the point of initiation [5–7] or during elongation [8, 9]. The second is by mRNA destabilization, where the poly-A tails of mRNA are shortened, leading to a higher turnover of the mRNA product by degradation [10–12]. Finally, extensive complementarity allows AGO proteins with slicer activity (only AGO2, in humans [13]) to specifically cleave mRNA transcripts in a manner analogous to siRNAs [14, 15]. Regardless of which mechanism dominates, the interactions are mediated through base-

Think again

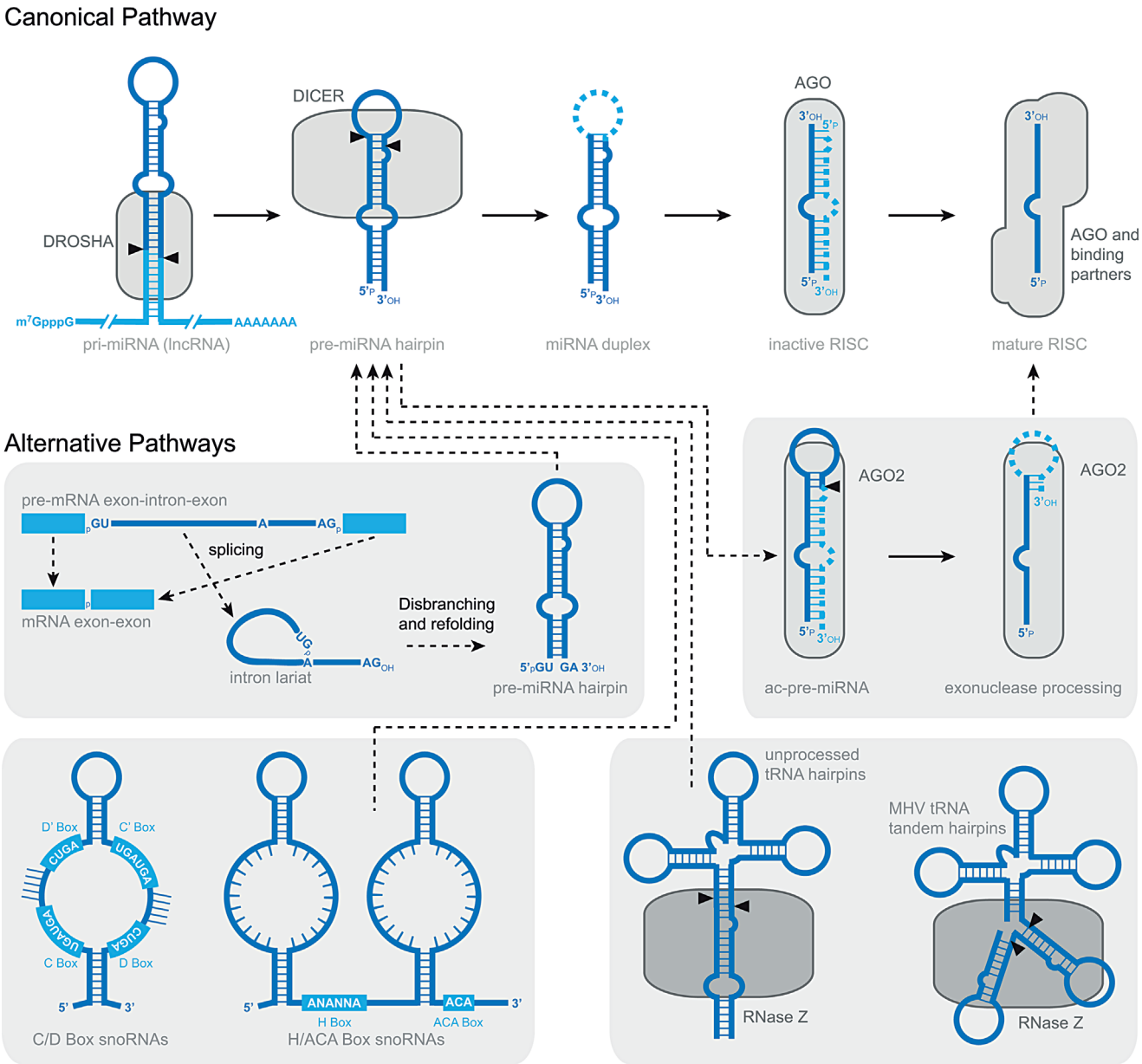

Figure 1. Multiple paths to mature miRNAs. This schematic illustrates the major enzymatic steps required to process primary transcripts into mature miRNAs via the canonical and alternative pathways [108].

pairing of the target, and therefore it is not strictly necessary to determine which mechanism is more prevalent in order to understand how the interaction happens. However as the assumptions around the dominant mechanism affect the selection of assays to validate miRNA-mRNA interactions, and not all assays can detect all kinds of interactions (see "The targets you see depend on the targets you look for"), it is useful to examine the context in which these conclusions have been reached.

When first investigated, it was thought that miRNAs inhibited the translation of proteins without affecting the level of mRNAs [16, 17]. A large number of biochemistry-based investigations concluded that miRNA-associated mRNA destabilization was concurrent with translational inhibition at the initiation stage [10, 18–20], and that this could be explained by sequestration of mRNAs inhibited by miRNAs to the P-bodies, where the silenced mRNA would be degraded [21, 22]. Although this model accounted for all biochemical and cell biology based evidence to date, it didn't explain the observation that miRNAs induced large-scale transcriptional changes, well in excess of what was observed associated with translational inhibition [23, 24]. Genome researchers favored a different model, based on evidence from whole genome comparison of both mRNAs and proteins after either increasing or decreasing the amount of specific miRNAs [25, 26]. These studies demonstrated that while hundreds of targets could be directly repressed without detectable mRNA destabilization [25], proteins that were highly repressed also had a substantial

Box 1

Understanding the limitations of miRNA target detection protocols

For any given experimental design, the miRNA targets detected will be dependent upon the assumptions made and the targets that are measured. In this context, it is useful to examine the limitations and benefits of each methodology (Fig. 2), before reaching conclusions about how miRNAs generally interact with mRNA targets.

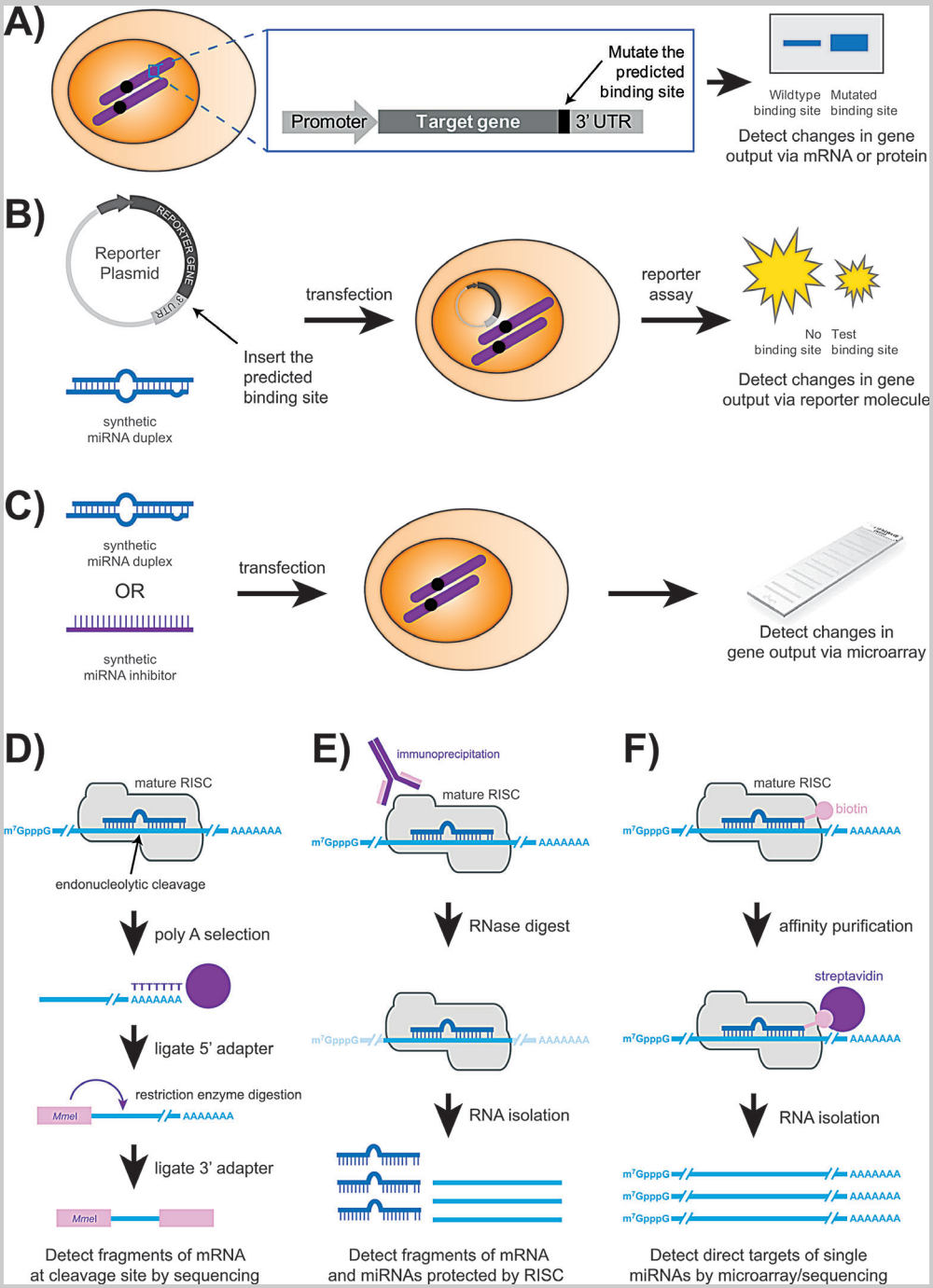

Figure 2. Experimental methods of miRNA target detection. This figure illustrates the six major methods of experimentally determining miRNA binding sites in mRNAs. **A:** genome editing of predicted binding sites. **B:** reporter gene assays. **C:** gene-expression after miRNA modulation. **D:** degradome sequencing. **E:** cross-linked immuno-precipitation. **F:** biotin-linked chromatography [110].

Genome editing of predicted binding sites

Probably the most definitive assay for determining whether a miRNA and a gene interact in a cell type of interest is to edit the genome to destroy the predicted binding site, and compare the protein or mRNA levels before and after editing (Fig. 2A). This method allows you to answer the question of whether an interaction occurs between a miRNA and an mRNA in an endogenous context. It also has the potential to detect both translational inhibition and transcriptional degradation if protein levels are measured. Although such a strategy has been used [91], it is not common, because of the labour intensiveness, expense, and inability to scale to high throughput.

Reporter gene assays

Often considered to be the gold standard of the in vitro assays, cloning either the entire 3'UTR or a fragment surrounding a predicted binding site downstream of a reporter gene (typically luciferase [41, 48], but also fluorescent proteins [92, 93]), and then measuring the output of that reporter in the presence or absence of a miRNA (Fig. 2B) allows one to answer the question: is this miRNA capable of interacting with this sequence? Importantly, this does not reveal whether such an interaction occurs endogenously, and unless care is taken with the miRNA concentration and promoter selection for the reporter, it does not reveal whether it could happen under physiological conditions. Although this approach is still expensive and laborious, it can be scaled to a higher throughput than editing genomes, and has the benefit of being agnostic to the mode of miRNA action, in that both mRNA destabilization and translational inhibition will be detected.

Gene-expression after miRNA modulation

After the hypothesis that miRNAs act to destabilize mRNA was proposed, a flurry of publications used microarray technology (Fig. 2C) to profile the effects of miRNA modulation on the transcriptome [24, 25, 60]. Although this assay cannot distinguish between primary and secondary transcriptional effects, and will mask any interactions mediated by translational inhibition, depending on the specific biological question to be answered this may not be problematic. These assays are relatively inexpensive (for the amount of data that is returned), and allow a global understanding of the biological pathways in which miRNAs are involved. However, if these assays are used to determine specific sequence motifs of interaction, then there is likely to be a substantial amount of noise from secondary effect genes (whose relative changes may be greater than the original targets), hence lowering the sensitivity of the analysis.

Degradome sequencing

Another approach used after the mRNA destabilization hypothesis gained traction was to look at signatures left in the transcriptome after cleavage of mRNA products – so called "degradome sequencing" [94] (Fig. 2D). In mammals, these approaches may be slightly less informative than assays detecting gene expression changes, because only one out of four AGO proteins (AGO2) has the ability to cleave mRNA targets, although all AGOs mediate functional interactions [13, 95]. However, unlike microarrays, this assay will allows the location of sites of cleavage on an individual transcript, and although it will miss translationally inhibited targets as well as those where the mRNA has been destabilized without cleavage, it will provide superior results for examining miRNA binding sites for this specific mode of action.

RNA immuno-purification

One of the most powerful ways to examine miRNA-RISC occupancy on mRNA targets is to perform Cross-linked immuno-precipitation, followed by high-throughput sequencing (HITS-CLIP; Fig. 2E) [96–98]. The procedure is lengthy, but elegant: (i) mRNAs and miRNA-RISC are cross-linked by exposing intact cells to UV light; (ii) exposed RNA regions that are not protected by protein complexes are degraded by introducing RNases; (iii) using an antibody to AGO2 (as the only available pan-AGO antibody has substantial cross-reactivity to another protein radixin [99]), RISC-miRNA-mRNA complexes are isolated and purified; and (iv) the pool of miRNAs and mRNA fragments are sequenced *en masse*. Another protocol, PAR-CLIP [85], is conceptually similar, but differs by feeding photoactivatable nucleosides to the cells prior to reversible cross-linking with UV light.

While sensitive, the protocols have several shortcomings for understanding the nature of direct miRNA-mRNA interactions. Firstly, the results are limited to, and potentially biased by, the restriction of this technique to AGO2. Although AGO2 is the only human argonaute protein to have slicer activity [13], it is not the only argonaute to mediate productive miRNA interactions [95]. The necessary exclusion of other argonautes from this procedure could result in substantial biases regarding the type of interactions that are detected. Secondly, the protocol necessarily disassociates all miRNAs from their mRNA targets, and therefore interactions between them need to be computationally inferred. This obviously limits the interactions detected to those that are assumed to exist, and introduces substantial amounts of noise when examining closely related miRNA species. A variant on the HITS-CLIP method, known as CLASH [40], gets around this problem by ligating the miRNA to the mRNA fragments prior to sequencing, but current protocol only has very low efficiency ($< 2\%$ of reads are miRNA/mRNA pairs) and is likely to be biased towards longer miRNA/mRNA fragments due to steric hindrance of RISC.

Biotin-linked chromatography

More commonly referred to as a biotin pull-down (Fig. 2F), biotin-linked chromatography involves synthesizing a miRNA duplex with the mature strand labelled with a biotin molecule at its 3′ end [100]. By transfecting this duplex into host cells, it will incorporate into RISC and target mRNAs in the usual manner. However, the cells can be lysed, and RISC complexed with the target mRNAs can be captured and purified using streptavidin-coated magnetic beads. Like reporter gene assays and cross-linked immuno-

precipitation, biotin-linked chromatography is agnostic to whether the functional effect happens via mRNA destabilization or translational inhibition. The main advantage of this approach is the ability to precisely identify those miRNA targets associated with a single miRNA species, and it is therefore the most advanced high-throughput technique for indentifying direct interactions between individual miRNAs and mRNAs. First used to identify the mechanism of action of siRNAs [101–105], and later adapted to the task of target identification [100, 106], this protocol is easily the most controversial in the field.

There are three major concerns that are typically (and correctly) voiced about this procedure [107]: (i) that the pull-down of miR-10a by Ørom et al. [106] did not enrich for either transcripts with miR-10a seed sites or transcripts known to be targets of this miRNA; (ii) that the dramatic over-expression of an exogenous miRNA could alter the stoichiometry of detected miRNA-mRNA interactions; and (iii) that the dramatic over-expression of an exogenous miRNA could alter the transcriptional network of the cells, leading to RNA changes that could confound interpretation of these experiments. All three points have been addressed by procedural changes. More modern versions of this protocol [38, 49, 50] using this same miRNA did not observe an enrichment of ribosomal mRNAs, and additionally observed an enrichment of both miR-10a seed sites and previously confirmed targets [38], suggesting that the Ørom protocol had essentially failed. The very low amounts of biotin duplex used in do not appear to substantially alter the transcriptional landscape of the cells, as known phenotypes of proliferative miRNAs are not observed when transfecting at low levels [48, 49], and the transcriptome of cells transfected with two unrelated miRNAs are indistinguishable [38]. Finally, when using high concentrations of transfected biotin duplexes, it is possible that all high-affinity binding sites could be occupied, and the distribution of binding could be shifted to include a higher proportion of low-affinity binding sites. In low concentrations, we would expect the stoichiometry to remain virtually unchanged from the endogenous distribution.

reduction of the corresponding transcript [26], a finding at odds with the biochemical evidence.

Attempting to resolve this controversy, detailed experiments examining the polysome were conducted [27, 28]. These experiments profiled both ribosome number and density (two key parameters of translation) using microarrays [27], and RNA-seq [28], which was a far more sensitive way to search for changes in translation than direct proteomics techniques. Both studies found that translation of hundreds of genes was directly impacted by miRNA activity, but that the effects of translation were relatively modest. The authors concluded that the effects of mRNA changes were more pronounced than the effects on translation, and that the predominant direct effect of miRNAs was to cause mRNA destabilization. A major confounder with these approaches was the reliance upon surveying transcript abundance without accounting for transcript isoforms [29]. Mammalian genes produce as many as a dozen different transcripts per locus [30], and many of these have alternative 5′ and 3′ UTRs, or alternative polyadenylation sites, none of which were captured in the composite measurements made by polysome profiling [27, 28]. When a set of transcripts previously found not to be under translational control was examined in detail, accounting for transcriptional complexity, the substantial effect of translational control became apparent [29].

Thus, as neither the "mRNA destabilization" nor "translation inhibition" camps are able to adequately explain all the data, the subject remains controversial. Both models have merit. For precise and accurate control of protein levels, it makes intuitive sense to directly regulate translation. It also makes sense that changes in mRNA quantity would be a more efficient way of driving larger changes in protein levels with fewer resources. However, the greatest efficiency of all would come from not making mRNA in the first place, rather than by making it and silencing it, or by making it and destroying it. A second major (but not often discussed) confounder with these genome-wide approaches is that they are ultimately unable to differentiate between primary and secondary effects. That is, the measured changes in mRNAs could occur because of either a direct interaction with a miRNA, or as a secondary consequence of a miRNA targeting, for example, a transcription factor. As changing the level of a transcription factor can have dramatic transcriptional changes within 1–2 hours [31–35], the 12 hour assay times reported in the Guo [28] and Hendrickson [27] papers could easily be measuring indirect effects of miRNA action. Both groups attempted to restrict their analyses only direct interactions using the presence of a seed site (see The Dominance of the Seed Site), and while this is the best marker we have for predicting genuine miRNA targets, these predictions are notoriously inaccurate, being wrong more often than they are right (when you include false negatives [36–40] as well as false positives [41–43]).

If we assume that there are both direct and indirect transcriptional consequences of miRNA modulation, then it becomes easy to envisage a model where all genomic and biochemical data is accounted for. The predominant direct effect of a miRNA would be through translational inhibition, whereas the predominant indirect effect would affect protein levels through large-scale transcriptional regulation. If such a model were true, we would expect that miRNAs predominantly target genes encoding transcription factors. Indeed, an early study into predicted miRNA targets seems to support this, finding that nuclear signaling components were significantly more likely to be targets of a miRNA than cell surface or extracellular signaling components [44], whilst other studies find enrichment for transcriptional regulators amongst the targets of miRNAs [38, 45–49] (including the Hendrickson study [27]). This evidence supports the hypothesis that the major direct effect of

miRNA action is to inhibit translation, but the bulk of miRNA effects come from changing the transcriptional landscape.

The targets you see depend on the targets you look for

Understanding the predominant mode of action for miRNAs has substantial implications for the methodology used to determine direct interacting mRNA partners, and subsequently determining their mode of binding. However, having a robust understanding of the mode of miRNA binding is unequivocally required in order to understand the predominant mode of action and identifying miRNA targets. Thus, the relationship between the concepts is often circular, and a cause for confusion – teasing apart the limitations of each technology (Box 1) allows us to evaluate the results that are generated from them. For indirect assays (those that measure the effect of the miRNA, not the interaction itself), methodologies that rely on the detection of a protein make fewer assumptions about the predominant mode of action than those that rely on the detection of mRNAs. If protein levels are measured, then changes by either mRNA destabilization or translational inhibition can be detected; however, if only mRNA levels are measured, translational inhibition will not be detected. A second confounder is trying to distinguish between primary and secondary effects of miRNA action. Experimental designs that look only at correlative changes (such as microarray profiling; Box 1), will have a lower sensitivity when determining specific sequence motifs of interactions due to secondary miRNA effects.

Direct assays are not without their problems either. Methods such as HITS-CLIP (Box 1) are by far and away the most sensitive way to understand where endogenous miRNA binding sites are located on an mRNA transcript. However, the analysis of binding site types relies on computational inference because the miRNA-mRNA relationship is not preserved in the protocol. On the other hand, biotin-linked chromatography (Box 1), while preserving the miRNA-mRNA relationship, necessarily involves the use of an exogenous molecule. This limits the protocol to cells that are easily transfectable, and has the potential to alter both the transcriptional profile of the cells and the distribution of occupied miRNA binding sites if the amount of exogenous miRNA is not very low [50]. Some researchers have also highlighted the potential of direct-assay technologies to form a specific type of false positive, where complexes between RNA and proteins form after cell lysis [51, 52]. In preliminary experiments, human cells were independently transfected with an EBV miRNA not normally expressed in cells, and a labeled AGO protein. Both cell populations were then mixed after lysis, and co-immunoprecipitations identified interactions between the two molecules that could not have happened in the cells [51]. However, the authors chose to use detergent at ten-times less than is required to prevent non-specific association of rRNA [38], so it is unclear whether these interactions were specific or not. Regardless, it is standard scientific rigor to validate scientific claims by multiple methods, and orthogonal approaches should be used to increase confidence in the findings. Deciding upon appropriate techniques will depend very much on the specific biological question that needs to be answered, and what level of precision is required to answer it.

What is a target anyway?

It is essential to realize that both stoichiometry and stochasticity have critical roles to play in the translation of target interactions to biological effect. Although we think about interactions happening at the level of individual miRNAs and mRNAs, spending a great deal of time worrying about the exact nucleotides involved, we typically define miRNA targets based on phenomenological effects from a population of cells. It is certainly possible that miRNAs can interact with a transcript without causing a repressive effect – similar results have been observed with transcription factors due either to limiting concentrations of required cellular co-factors, or to inherent non-productive (or redundant) binding [53–57]. Even if the miRNA binding is productive, full or partial inhibition of individual transcripts does not mean that the overall level of a protein changes substantially. Cells only need to increase the level of mRNA to overcome the effect of miRNA targeting [48, 58]. Finally, repression of an individual protein does not necessarily alter the biological trajectory of a cell or tissue. These considerations mean that the definition of a miRNA target becomes somewhat fuzzy. However, until the technology for analyzing these aspects of miRNA biology becomes more robust, the working definition of miRNA targets (any detectable and stably interacting mRNA) will need to suffice.

Studying individual cells rather than populations may help us tease apart these issues. Although restricted to exogenous interactions, studies at the single cell level revealed a threshold of mRNA expression beyond which miRNA inhibition of protein production became less efficient [58], presumably by the sequestration of all available active and compatible RISC. In a given population of cells, this means that the underlying variation in mRNA expression could lead to substantially different levels of proteins in different cells.

It is unclear how much this stochasticity has impacted the determination of miRNA targets to date. On the one hand, it implies that dramatic over-expression of miRNA could alter the stoichiometric ratio so that all high-affinity binding sites could be occupied. This would mean the distribution of interactions could be shifted to include low-affinity sites that might not otherwise be targeted, generating false positives. On the other hand, reporter assays also typically over-express non-endogenous transcripts driven by strong promoters, which could either negate the effect of miRNA over-expression, or even lead to false negatives if care is not taken with the experimental design. Understanding the variation of both miRNAs and mRNAs at the single cell level can help to better define the parameters of miRNA targeting [59].

The dominance of the seed site is a consequence of prediction

By far and away the most understood interaction between a miRNA and an mRNA revolves around nucleotides

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2–8 in the 5′ end of a miRNA, called the "seed" region.

Even though the first miRNA binding sites reported were not true seed sites [17, 36, 60, 61], seeds were found to have more predictive value for finding miRNA targets than any other interaction type [2, 43, 62], and the 5′ region of the miRNAs were more conserved than any other region. As experimental techniques of the time were both laborious and expensive (see "The targets you see depend on the targets you look for"), quite reasonably, experimental validation has focused around those interactions most likely to yield positive results, leading to a positive reinforcement in the literature dominance of the seed site. However, the presence of a seed site in a mRNA does not guarantee occupancy by a given miRNA even when that miRNA is over-expressed [63], and even the best prediction algorithms have false positive rates of between 20–50% [25, 26, 43, 48]. It's important to note that target prediction programs restrict the output of their programs to increase the accuracy of their predictions, and whilst some restrictions are modeled on biology, others are modeled on abstractions that are unlikely to reflect what actually happens on the inside of a cell. For example, filtering predicted targets based on the folding structure of the mRNA [64–71] is likely to model something that happens in a cell, because RISC will be unable to access binding sites that are hidden away in a 3D structure. In contrast, RISC has no means to assess the evolutionary conservation of a given miRNA binding site in situ. While filters based on comparative genomics approaches [2, 43, 69, 70, 72–75] can tell us a lot about the similarities of miRNA networks within an evolutionary clade, the relevance for rapidly evolving or recently acquired miRNAs is substantially less [76]. Additional problems with the restriction of target output related to the use of shorter RefSeq 3'UTRs when very often UTRs are substantially longer [77], and many functional miRNA binding sites exist outside of the 3'UTR [39, 46, 78–82]. Therefore, whilst seeds in the 3'UTRs of genes make up the vast majority of validated target sites in the literature, this is largely the result of an early entrenched software bias rather than a biological requirement [83].

Experimental protocols reveal alternative modes of interaction

The relatively poor performance of prediction algorithms and the recent availability of high-throughput approaches have driven the experimental exploration of non-seed mediated miRNA – target interactions (Fig. 3). Using "orphan" HITS-CLIP data (RISC binding sites where there was no match to a miRNA seed site), Chi et al. [37] were able to identify an enrichment of sites containing an extra G in the mRNA between positions five and six of the miRNA. These "G-bulge" sites were not unexpected, having previously been shown as possible in a DNA-based experiment [84], but their occupancy of 15% of binding sites was previously unappreciated. Non-seed interactions were also explored using motifs enriched above background in PAR-CLIP data [85], finding relatively infrequent (<7%) instances of seed mismatches and bulges in the seed region, and weak enrichment of four nucleotide matches to positions 13–15 of the miRNA. In both studies, the results were confounded by the necessary inference of miRNA binding sites (see Box 1, "RNA immuno-purification"), an area addressed by a genetically controlled differential HITS-CLIP experiment where miR-155 knock-out cells were compared with wild-type miR-155 cells [86]. This experiment showed greatly increased sensitivity for non-seed sites, revealing that 40% of sites were mediated by non-canonical interactions. The evolutionary conservation of the central region of miRNAs [60] prompted the search for "centered sites," and Shin et al. [87] reported that the rare (<5% prevalence) 11nt sites with perfect Watson-Crick complementarity to mRNAs were functional, but that the more abundant sites containing mismatches or GU wobble were not. In this case, poor selection of the target validation method (microarrays after miRNA modulation) based on the assumption that most targets would have destabilized mRNA masked the functionality of these far more common sites (up to 50% of all miRNA-miRNA interactions). Imperfectly centered sites were eventually discovered to

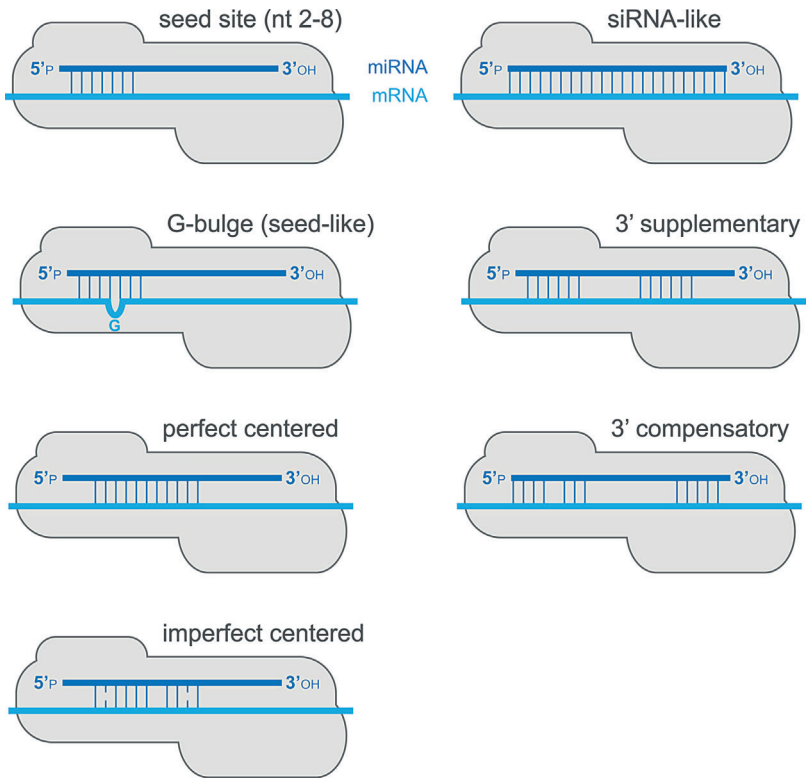

Figure 3. The anatomy of miRNA-mRNA interactions. Although most published interactions involve the miRNA seed, many other modes of miRNA binding have been detected [109].

be functional using more suitable biotin-linked chromatography and reporter assay validation [38].

Together these studies highlight the vast array of non-canonical miRNA-mRNA interactions, and strongly hint that the variability of functional interacting sites is far more extreme than first indicated by comparative genomics studies. It is not unreasonable to consider the possibility that any accessible six nucleotides, whether contiguous in the mRNA or not, is capable of mediating a functional interaction with a miRNA.

There is little value in the classification of binding site combinatorics

Regardless of the progress made into the exploration of non-seed sites, it remains true that the predictive power of the various new categories is likely to be very small [2, 38, 43, 62]. Although some have argued that a lack of predictive power for non-canonical seed sites casts doubt upon the biological validity of such interactions [42], this seems like a bold call considering the strength of the accompanying biological validation, and that our understanding of how RISC occupancy correlates with the strength of an interaction is far from complete [25].

Interesting parallels can be drawn between the prediction of miRNA targets and the prediction of transcription factor binding sites. Both are attempting to discern very short regulatory signals in a large amount of statistical noise, and both are trying to model the truth in a biological system when that truth is not yet understood [88]. It is therefore noteworthy to observe a strong shift away from using computational techniques to predict transcription factor binding sites and guide experimental validation to instead using computational techniques to understand the signals from less biased high-throughput techniques such as ChIP-seq [88–90]. As the tools for dissecting miRNA targets become more sophisticated and more widely accepted, it seems probable that the miRNA field will head in the same direction. When this happens, the value of classifying the different types of miRNA binding sites based on which miRNA nucleotides are involved will be questionable.

Conclusions and outlook

One thing that is becoming clear with the uncovering of these different interaction types is that miRNAs stably interact with hundreds to thousands of mRNAs, and some consideration needs to be given to separating biological noise from functionally-driven signal. Differentiating direct from indirect consequences of miRNA action makes it easier to specifically examine the mechanisms by which miRNAs interact with their mRNA targets, but this requires more scientific rigor if it is to disentangle the functional consequences of those interactions. Issues of miRNA target detection and miRNA mode of action are intertwined with discussion of the major effect and roles of miRNAs in the cell. And, as our understanding of miRNA biology improves, so too will our ability to predict and model target interactions. Although prediction tools have strongly dictated the way we studied miRNAs in the past, their relevancy will change as our repertoire of experimental tools improve. This will in turn shape our understanding of how, where, and why miRNAs function, ideally converging on a model that incorporates all of the data, not just those that are convenient.

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