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.

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-
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 allow 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 transected 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.

think again
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 miRNAs. 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 cell 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 miRNA 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 miRNA 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
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, RISChas 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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References

1. Ha M, Kim VN. 2014. Regulation of micro-RNA biogenesis. Nat Rev Mol Cell Biol 15: 509–524.
2. Friedman RC, Farh KK-H, Burge CB, Bartel DP. 2009. Most mammalian miRNAs are conserved targets of microRNAs. Genome Res 19: 92–105.
3. Lujambio A, Lowe SW. 2012. The micro-cosmos of cancer. Nature 482: 347–355.
4. Im H-I, Kenny PJ. 2012. MicroRNAs in neuronal function and dysfunction. Trends Neurosci 35: 325–334.
5. Humphreys DT, Westman BJ, Martin DIK, Preiss T. 2005. MicroRNAs control translation initiation by inhibiting eukaryotic initiation factor 4E/cap and poly(A) tail function. Proc Natl Acad Sci USA 102: 16961–16966.
6. Pillai RS, Bhattacharyya SN, Artus CG, Zoller T, et al. 2005. Inhibition of translational initiation by Let-7 MicroRNA in human cells. Science 307: 1540–1543.
7. Mathonnet G, Fabian MR, Svitkin YV, Parsyan A, et al. 2007. MicroRNA inhibition of translation initiation in vitro by targeting the cap-binding complex eIF4F. Science 317: 1764–1767.
8. Nottrott S, Simard MJ, Richter JD. 2006. Human let-7a microRNA blocks protein production on actively translating polyribosomes. Nat Struct Mol Biol 13: 1106–1114.
9. Maroney PA, Yu Y, Fisher J, Nilsen TW. 2006. Evidence that microRNAs are associated with translating messenger RNAs in human cells. Nat Struct Mol Biol 13: 1102–1107.
10. Eulalio A, Huntzinger E, Nishihara T, Rehwinkel J, et al. 2009. Deadenylation is a widespread effect of miRNA regulation. RNA 15: 21–32.
11. Giraldez AJ, Mishima Y, Rihel J, Grocock RJ, et al. 2008. Zebrafish Mir-430 promotes deadenylation and clearance of maternal miRNAs. Science 312: 75–79.
12. Wu L, Fan J, Belasco JG. 2006. MicroRNAs direct rapid deadenylation of mRNA. Proc Natl Acad Sci USA 103: 1573–1576.
13. Meister G, Landthaler M, Patkaniowska A, Dorsett Y, et al. 2004. Human argonaute2 mediates RNA cleavage targeted by miRNAs and siRNAs, Mol Cell 15: 185–197.
14. Hammond SM, Bernstein E, Beach D, Hannon GJ. 2000. An RNA-directed nuclelease mediates post-transcriptional gene silencing in Drosophila cells. Nature 404: 293–296.
15. Tuschi T, Zamore PD, Lehmann R, Bartel DP, et al. 1999. Targeted mRNA degradation by double-stranded RNA in vitro. Genes Dev 13: 3191–3197.
16. Olsen PH, Ambros V. 1999. The lin-4 regulatory RNA controls developmental timing in Caenorhabditis elegans by blocking LIN-14 protein synthesis after the initiation of translation. Dev Biol 216: 671–680.
17. Wightman B, Ha I, Ruvkun G. 1993. Posttranscriptional regulation of the heterochronic gene lin-14 by lin-4 mediates temporal pattern formation in C. elegans. Cell 75: 855–862.
37. Chi SW, Hannon GJ, Darnell RB. 2012. An alternative mode of microRNA target recognition. Nat Struct Mol Biol 19: 321–327.

38. Martin HO, Charles S, Steptoe AL, Krishna K, et al. 2014. Imperfect centered miRNA binding sites are common and can mediate repression of target miRNAs. Genome Biol 15: R51.

39. Tay Y, Zhang J, Thomson AM, Lim B, et al. 2008. MicroRNAs to Nanog, Oct4 and Sox2 coding regions modulate embryonic stem cell differentiation. Nature 455: 1124–1128.

40. Helwak A, Kudla G, Dudnakova T, Tollervey D. 2013. Mapping the human miRNA interactome by CLASH reveals frequent noncanonical binding. Cell 153: 654–665.

41. Lewis BP, Shih I, Jones-Rhoades MW, Bartel DP, et al. 2003. Prediction of mammalian microRNA targets. Cell 115: 785–798.

42. Lewis BP, Burge CB, Bartel DP. 2005. Conserved seed pairing, often flanked by adenosines, indicates that thousands of human genes are microRNA targets. Cell 120: 15–20.

43. Krek A, Grün D, Poy MN, Wolf R, et al. 2005. Combinatorial microRNA target predictions. Nat Genet 37: 495–500.

44. Cui Q, Yu Z, Pattabiraman DR, Wang E. 2006. Principles of microRNA regulation of a human cellular signaling network. Mol Syst Biol 2: 46.

45. Krishnan K, Steptoe AL, Martin HC, Pattabiraman DR, et al. 2013. MiR-139–5p is a regulator of metastatic pathways in breast cancer. RNA 19: 1767–1780.

46. Krishnan K, Steptoe AL, Martin HC, Wani S, et al. 2013. MicroRNA-182–5p targets a network of genes involved in DNA repair. RNA 19: 230–242.

47. Lai A, Thomas M, Altschuler G, Navarro F. 2011. Capture of microRNA-bound mRNAs identifies the tumor suppressor mir-34a as a regulator of growth factor signaling. Proc Natl Acad Sci U S A 108: 14039–14044.

48. Livak K, Yario T, Steitz J. 2012. Association of Argonaute proteins and microRNAs can occur after cell lysis. RNA 18: 1581–1585.

49. Mili S, Steitz J. 2004. Evidence for reassocation of RNA-binding proteins after cell lysis: Implications for the interpretation of immunoprecipitation analyses. RNA 10: 1692–1694.

50. Liu X, MacArthur S, Bourgon R, Nix D, et al. 2008. Transcription factors bind thousands of active and inactive regions in the Drosophila blastoderm. PLoS Biol 6: e27.

51. Paris M, Kaplan T, Li XY, Villalta JE, et al. 2013. Extensive divergence of transcription factor binding in Drosophila embryos with highly conserved gene expression. PLoS Genet 9: e1003748.

52. Cusanovich D, a Pavlovic, Pritchard JK, Gilad Y. 2014. The functional consequences of variation in transcription factor binding. PLoS Genet 10: e1004226.

53. Fisher WW, Li JJ, Hammonds AS, Brown JB, et al. 2012. DNA regions involved at low occupancy by transcription factors do not drive patterned reporter gene expression in Drosophila. Proc Natl Acad Sci USA 109: 21330–21335.

54. Spivakov M. 2014. Spurious transcription factor binding: non-functional or genetically redundant. BioEssays 36: 798–806.

55. Mukherji S, Ebert MS, Zheng GY, Tsang JS, et al. 2011. MicroRNAs can generate thresholds in target gene expression. Nat Genet 43: 854–859.

56. White AK, Vaninbesghere M, Petrov OI, Hamidi M, et al. 2011. High-throughput microfluidic single-cell RT-qPCR. Proc Natl Acad Sci USA 108: 13999–14004.

57. Grimson A, Farh KK-H, Johnston WK, Garrett-Engele P, et al. 2007. MicroRNA targeting specificity in mammals: determinants beyond seed pairing. Mol Cell 27: 91–100.

58. Bartel DP. 2009. MicroRNAs: target recognition and regulatory functions. Cell 136: 215–223.

59. Stark A, Brennecke J, Bushtali N, Russell P, et al. 2005. Animal MicroRNAs confer robustness to gene expression and have a significant impact on 3’UTR evolution. Cell 123: 1133–1146.

60. Didiano D, Hobert O. 2006. Perfect seed pairing is not a generally reliable predictor for microRNA-target interactions. Nat Struct Mol Biol 13: 849–851.

61. Kertesz M, Iovino N, Unnerstall U, Gaul U, et al. 2007. The role of site accessibility in microRNA target recognition. Nat Genet 39: 1278–1284.

62. Long D, Lee R, Williams P, Chan CY, et al. 2007. Potent effect of target structure on microRNA function. Nat Struct Mol Biol 14: 297–304.

63. Marin RM, Vanick J. 2011. Efficient use of accessibility in microRNA target prediction. Nucleic Acids Res 39: 19–29.

64. Mücke S, Tofer H, Hackemüller J, Bernhart SH, et al. 2006. Thermodynamics of RNA-RNA binding. Bioinformatics 22: 1177–1182.

65. Busch A, Richter AS, Backofen R. 2008. InSilicoRNA: efficient prediction of bacterial sRNA targets incorporating target site accessibility and seed regions. Bioinformatics 24: 2849–2856.

66. Robins H, Press W. 2005. Human microRNAs target a functionally distinct population of genes with AT-rich 3’ UTRs. Proc Natl Acad Sci USA 102: 15557–15562.

67. Hammell M, Long D, Zhang L, Lee A, et al. 2008. MiR-WIP: microRNA target prediction based on microRNA-containing ribonucleo-protein-enriched transcripts. Nat Methods 5: 813–819.

68. Ragan C, Cloonan N, Grimmond SM, Züker M, et al. 2009. Transcriptome-wide prediction of microRNA targets in human and mouse using FASTH. PLoS ONE 4: e5745.

69.icaidatzis D, van Nimwegen E, Haussler J, Zavolan M. 2007. Inference of microRNA targets using evolutionary conservation and pathway analysis. BMC Bioinfo 8: 69.

70. Maragakis M, Alexiou P, Papadopoulos GL, Rezczko M, et al. 2009. Accurate microRNA target prediction correlates with
protein repression levels. BMC Bioinfo 10: 295.
74. Stark A, Brennecke J, Russell RB, Cohen SM. 2003. Identification of Drosophila microRNA targets. PLoS Biol. 1: E60.
75. Murphy E, Vanícek J, Robins H, Shenk T, et al. 2008. Suppression of immediate-early viral gene expression by herpesvirus-coded microRNAs: implications for latency. Proc Natl Acad Sci USA 105: 5453–5458.
76. Marin RM, Vanícek J. 2012. Optimal use of conservation and accessibility filters in microRNA target prediction. PLoS ONE 7: e32208.
77. Kolle G, Ho M, Zhou Q, Chy HS, et al. 2009. Identification of human embryonic stem cell surface markers by combined membrane-polyosome translation state array analysis and immunotranscriptional profiling. Stem Cells 27: 2446–2456.
78. Duursma AM, Kedde M, Schrier M, de Sage C, et al. 2008. MiR-148 targets human DNMT3b protein coding region. RNA 14: 872–877.
79. Forman J, Coller H. 2010. The code within the code: microRNAs target coding regions. Cell Cycle 9: 1533–1541.
80. Forman JJ, Legesse-Miller A, Coller HA. 2008. A search for conserved sequences in coding regions reveals that the let-7 microRNA targets Dicer within its coding sequence. Proc Natl Acad Sci USA 105: 14879–14884.
81. Rigoutsos I. 2009. New tricks for animal microRNAs: targeting of amino acid coding regions at conserved and nonconserved sites. Cancer Res 69: 3245–3248.
82. Lytle JR, Yario TA, Steltz JA. 2007. Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5' UTR as in the 3' UTR. Proc Natl Acad Sci USA 104: 9667–9672.
83. Brodersen P, Voinnet O. 2009. Revisiting the principles of microRNA target recognition and mode of action. Nat Rev Mol Cell Biol 10: 141–148.
84. Wang Y, Juraneck S, Li H, Sheng G, et al. 2008. Structure of an argonaute silencing complex with a seed-containing guide DNA and target RNA duplex. Nature 456: 921–926.
85. Hafner M, Landthaler M, Burger L, Khorshid M, et al. 2010. Transcriptome-wide identification of RNA-binding protein and microRNA target sites by PAR-CLIP. Cell 141: 129–141.
86. Loeb GB, Khan A, a Canner, Hiatt JB, et al. 2012. Transcriptome-wide miR-155 binding map reveals widespread noncanonical microRNA targeting. Mol Cell 48: 760–770.
87. Shin C, Nam J-W, Farh KK-H, Chiang HR, et al. 2010. Expanding the microRNA targeting code: functional sites with centered pairing. Mol Cell 38: 789–802.
88. Tompa M, Li N, Bailey TL, Church GM, et al. 2005. Assessing computational tools for the discovery of transcription factor binding sites. Nat Biotechnol 23: 137–144.
89. Farey TS. 2012. ChIP-seq and beyond: new and improved methodologies to detect and characterize protein-DNA interactions. Nat Rev Genet 13: 840–852.
90. Johnson DS, Mortazavi A, Myers RM, Wold B. 2007. Genome-wide mapping of in vivo protein-DNA interactions. Science 316: 1497–1502.
91. Bassett AR, Azzam G, Wheatley L, Tibbit C, et al. 2014. Understanding functional miRNA-target interactions in vivo by site-specific genome engineering. Nat Commun 5: 1–11.
92. Loya CM, Lu CS, Van Vactor D, Fulga TA. 2009. Transgenic microRNA inhibition with spatiotemporal specificity in intact organisms. Nat Methods 6: 897–903.
93. Baccarini A, Brown B. 2010. Monitoring microRNA activity and validating microRNA targets by reporter-based approaches. In: Monticelli S editor., MicroRNAs and the Immune System SE - 15. Humana Press. pp 215–233.
94. German MA, Luo S, Schroth G, Meyers BC, et al. 2009. Construction of parallel analysis of RNA ends (PARE) libraries for the study of cleaved miRNA targets and the RNA degradome. Nat Proto 4: 356–362.
95. Su H, Trombly M, Chen J, Wang X. 2009. Essential and overlapping functions for mammalian Argonautes in microRNA silencing. Genes Dev 23: 304–317.
96. Licatalosi DD, Mele A, Fak JJ, Ule J, et al. 2008. HITS-CLIP yields genome-wide insights into brain alternative RNA processing. Nature 456: 464–469.
97. Chi SW, Zang JB, Mele A, Darnell RB. 2009. Argonaute HITS-CLIP decodes microRNA-mRNA interaction maps. Nature 460: 479–486.
98. Macias S, Plass M, Stajuda A, Michlewski G, et al. 2012. DGCIR HITS-CLIP reveals novel functions for the Microprocessor. Nat Struct Mol Biol 19: 760–766.
99. Nelson PT, Planell-saguer M, De Lamp-rinaki, Kiriakidou M, et al. 2007. A novel monoclonal antibody against human Argo-naute proteins reveals unexpected charac-teristics of miRNAs in human blood cells. RNA 13: 1787–1792.
100. Orom UA, Lund AH. 2007. Isolation of microRNA targets using biotinylated synthetic microRNAs. Methods 43: 162–165.
101. Martinez J, Patkaniowska A, Urlaub H, Lührmann R, et al. 2002. Single-stranded antisense siRNAs guide target RNA cleavage in RNAi. Cell 110: 563–574.
102. Rand TA, Petersen S, Du F, Wang X. 2005. Argonaute2 cleaves the anti-guide strand of siRNA during RISC activation. Cell 123: 621–629.
103. Chu C, Rana TM. 2006. Translation repression in human cells by microRNA-induced gene silencing requires RCK/p54. PLoS Biol 4: e210.
104. Ameres SL, Martinez J, Schroeder R. 2007. Molecular basis for target RNA recognition and cleavage by human RISC. Cell 130: 101–112.
105. Kedde M, Strasser MJ, Boldajipour B, Oude Vrielink JAF, et al. 2007. RNA-binding protein Dnd1 inhibits microRNA access to target mRNA. Cell 131: 1273–1286.
106. Orom UA, Nielsen FC, Lund AH. 2008. MicroRNA-10a binds the 5'UTR of ribosomal protein mRNAs and enhances their translation. Mol Cell 30: 460–471.
107. Thomas M, Lieberman J, Lal A. 2010. Desperately seeking microRNA targets. Nat Struct Mol Biol 17: 1169–1174.
108. Cloonan N. 2014. Multiple pathways to mature miRNAs. doi: 10.6084/m9.figshare.1222671.
109. Cloonan N. 2014. The anatomy of miRNA- mRNA interactions. doi: 10.6084/m9.figshare.1222718.
110. Cloonan N. 2014 Experimental methods of miRNA target detection. doi: 10.6084/m9.figshare.1222672.