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MINI-REVIEW

MicroRNA rules: Made to be broken

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Abstract **MicroRNAs (miRNAs) are important post-transcriptional regulators of gene expression. For over a decade the deluge of research describing the biogenesis and activity of miRNAs has lead researchers to postulate rules to help make sense of the enormous amount of data produced. These rules are repeated in miRNA research papers and reviews. While these rules have been helpful one must be conscious of their limitations or risk missing future breakthroughs. Here we describe some of the most commonly stated rules, the reasoning behind their formation, their uses, and limitations.**

Keywords microRNA, post-transcriptional, gene regulation, mRNA, 3' UTR, conservation rule, seed pairing rule, biogenesis rule, mechanism of action rule

MicroRNAs (miRNAs) are short, 17–26 nucleotide single strand RNAs that regulate expression at the post-transcriptional level. They are found in almost every eukaryotic organism. It is estimated that > 60% of all mammalian mRNAs are regulated by miRNAs (Friedman et al., 2009), including many mRNAs that control important cellular processes such as proliferation, apoptosis, and differentiation (Bartel, 2009; Carthew and Sontheimer, 2009; Krol et al., 2010). Furthermore, extracellular miRNAs accumulate in biofluids such as blood, urine, and saliva and the level of this accumulation can be altered by disease (Tsui et al., 2002; Mitchell et al., 2008; Weber et al., 2010). Thus, miRNAs are important for understanding gene regulation as well as for therapeutic and diagnostic purposes.

Although the first miRNA was discovered in *Caenorhabditis elegans* by the Ambros and Rufkin laboratories in 1993, miRNAs were considered to be little more than a curiosity until the year 2000, when the *C. elegans'* let-7(a) miRNA was discovered to be nearly 100% sequentially and functionally conserved in a variety of organisms including humans (Lee et al., 1993; Wightman et al., 1993; Pasquinelli et al., 2000, 2003; Reinhart et al., 2000). Since then, a number of revelations on the importance and utility of miRNAs to cellular biology and human disease has caused a research feeding frenzy between many academic and industrial laboratories. As with any young and active field, new data on miRNAs have been quickly synthesized into "rules" describing their form and function. Such rules can be valuable as they clarify the current state of knowledge, facilitate the transfer of information, and help researchers formulate new hypothesis. However, for rapidly evolving fields, it is easy for early observations to be overly interpreted and generalized into hard and fast "rules." This leads to misconceptions that can hinder progress. Having rigorously defined and up-to-date rules is especially important for fields that extensively use bioinformatics, as bioinformatic computer algorithms are basically codified biological rules.

Here we review several commonly repeated "rules" describing miRNA form and function. We note their value, their exceptions, and how they can help or hinder progress (see Table 1). We focus on data derived from studies of *Caenorhabditis elegans*, *Drosophila melanogaster*, zebrafish, and mammalian cell culture.

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The conservation rule

"MiRNAs are highly conserved between species." This

Table 1

Rule	General belief	Exceptions	Consequences	References
The conservation rule	"miRNAs are highly conserved between species."	Numerous examples of lineage and species specific miRNAs.	1) Missed non-conservered, novel miRNAs. 2) Under appreciation of miRNA contributions to species distinctiveness.	Berezikov et al., 2006a; Berezikov et al., 2006b; Brameier, et al., 2010; Niwa & Slack, 2007; Sun et al., 2013; Du et al., 2013; Zhang et al., 2008.
The biogenesis rules	"miRNAs are produced by the sequential cleavage of pri- and pre-miRNAs by Drosha and Dicer."	Dicer independent miRs, Drosha-independent miRs, Mirtrons, snoRNA-derived miRs, tRNA-derived miRs and Simtrons.	1) Missed non-canonical novel miRNAs. 2) Over-interpreted Drosha and Dicer knockout studies.	Miyoshi et al., 2010; Yang & Lai, 2011; Havens et al., 2012.
The seed pairing rule	"miRNAs function requires a near perfect match within the seed region with their target mRNA."	Many examples of miRNA-mRNA binding sites without perfect seed paring.	Missed targets	Bartel, 2009; Lee & Shin, 2012; Shin et al., 2010; Grimson et al., 2007; Elefant et al., 2011.
3' UTR location rule	"miRNA target sites are located in the 3' UTR of mRNAs."	Exonic and 5' UTR miRNA target sites.	Missed targets	Lee and Shin, 2012; Lim et al., 2005; Grimson, 2007; Baek, 2008; Chi et al., 2009; Hafner et al., 2010; Schnall-Levin et al., 2011.
The mechanism of action rule	"Animal miRNAs function by inhibiting mRNA translation and/or by decreasing mRNA stability, depending on the amount of target sequence complementarity."	Most animal miRNA-mRNA interactions inevitably lead to significant mRNA degradation.	Widespread use of Western blot verification of targets as opposed to more reliable RT- PCR	Baek et al., 2008; Selbach, 2008; Hendrickson et al., 2009; Guo et al., 2010.

statement is often repeated as part of the definition of "miRNA" and stems from multiple spectacular examples of miRNAs being nearly 100% conserved from *C. elegans* to humans. For instance, the aforementioned let-7 is not only 100% conserved in sequence across species from *C. elegans* to *D. melanogaster* to human but also is somewhat conserved in function (Roush and Slack, 2008). Let-7 was first discovered in *C. elegans* as a development gene, regulating stem-cell differentiation. Blast searches revealed homologs that contribute to neuromusculature development in *D. melanogaster* and limb development in mice. In humans, hsa-let-7a similarly regulates differentiation by acting as a tumor suppressor in a variety of tissues. Another example is mir-1, which is expressed in the muscle of organisms from *C. elegans* to human and has been found to be involved in muscle development in several organisms (Brennecke et al., 2005; Sokol and Ambros, 2005; Nguyen and Frasch, 2006).

Following the successful discovery of let-7 and mir-1 homologs in many species, dozens more miRNAs were discovered by simple blast searches. Even more miRNAs were subsequently discovered with more sophisticated computer algorithms that used a variety of methods but which usually included a bioinformatics step calling for conservation between species. For instance, several machine learning algorithms found miRNAs by searching genomic sequence for hairpins that exhibited pre-miRNA characteristics. Such algorithms produced millions of hits (Bentwich et al., 2005) which were typically winnowed down to manageable numbers by looking for subsets of hits that are conserved across multiple species (Yousef et al. 2006 and references therein). Xie et al. (2005) developed a particularly clever method for finding novel miRNAs using species conservation. They looked for acutely conserved sequences in the 3′ UTRs of conserved mRNAs. They reasoned that such sequences would represent target sites for conserved miRNAs and then used reverse genetics to find the corresponding novel miRNAs.

Thus, for several years, the majority of miRNAs discovered were found due to their conservation across species. Reviewers who then analyzed miRBase submissions and concluded that most miRNAs are conserved across species were engaging in circular reasoning. More recent methods for finding new miRNAs do not rely as heavily on sequence conservation. Analysis of miRNAs discovered by these unbiased methods makes it increasingly clear that many miRNAs are not well conserved across species. In fact, miRNA evolution is particularly dynamic. For instance, extensive cloning and RAKE analysis discovered over 400 new miRNAs in mice and humans without bias for species conservation (Berezikov et al., 2006). Most of these new

miRNAs are not conserved beyond mammals and ~10% are taxon-specific. Berezikov et al. (2006) used next generation sequencing to identify 447 novel miRNAs in the brains of humans and chimpanzee. Given the > 98% genomic sequence conservation between the two species it is striking that of the 447 new miRNAs, 51 are human specific and 25 are chimpanzee specific.

Several bioinformatics analyses have found many lineage and species-specific miRNAs. Brameier (2010) looked for homologs of 693 human miRNAs from miRBase in chimpanzee, orangutan, and rhesus and found that even in this biased (see above) data set, 35 of these human miRNAs could only be found in 1 of the 3 other primates and 12 were human specific. Other groups have used comparative genomic analysis to follow the evolution of clusters or families of miRNAs finding that even in conserved clusters there is significant expansion/diversification (Zhang et al., 2008; Sun et al., 2013). Interestingly, Niwa and Slack (2007) note that Kingdom-wide surveys of miRNAs show that increases in miRNA number and diversity appear to be associated with major body-plan innovations and phenotypic variation in closely related species.

So not only are there numerous exceptions to the "rule" of miRNA conservation but in fact they are more diverse than protein genes. Propagation of this definition undermines a complete appreciation for the power of this class of regulators to drive evolutionary change and hinders efforts to discover novel miRNAs.

The biogenesis rules

"miRNAs are produced by the sequential cleavage of pri- and pre-miRNAs by Drosha and Dicer." The canonical pathway of miRNA biogenesis states that miRNAs are first transcribed as pri-miRNAs that can be several thousand bases long and often contain multiple miRNAs. A protein complex containing the ribonuclease Drosha along with DGCR8/Pasha recognize hairpins with a characteristic structure cutting the base of the structure to liberate a 60–70 nucleotide pre-miRNA which is exported from the nucleus by Exportin 5. In the cytoplasm, a complex containing the ribonuclease Dicer removes the terminal loop of the pre-miRNA hairpin, leaving a short duplex RNA. One strand of the duplex representing the mature miRNA is selected and loaded into the RISC complex where it carries out its function.

The canonical pathway was rapidly worked out by several laboratories and applied to many of the known miRNAs cataloged in miRBase (Krol and Krzyzosiak, 2004; Carthew and Sontheimer, 2009; Kim et al., 2009). However, in a typical mammalian cell containing dozens of known ribonucleases (D'Alessio and Riordan, 1997) and where > 90% of the genome is transcribed it would be surprising if this was the only way to make and then skin a pre-miRNA. Indeed, two recent reviews describe at least a half dozen alternative pathways (Miyoshi et al., 2010; Yang and Lai, 2011). Pre-miRNA structures can come from many sources including, snoRNAs, pre-tRNAs or tRNA-like sequences, endo-siRNA precursors, or small introns known as mirtrons. These alternative pre-miRNAs can be processed in a variety of ways and by a variety of different enzymes including Dicer (without Drosha), Drosha (without Dicer), splicing factors, and tRNAseZ. Beyond these known alternative pathways there are almost certainly more yet to be discovered. For instance, Havens et al. (2012) have recently shown that a subset of mirtrons, called simtrons, are produced by an unknown splicing-independent pathway that uses Drosha but not Dicer, DGCR8, or Argonaute 2.

The consequences of ignoring these alternative pathways are twofold: 1) it will be more difficult to discover novel miRNAs as bioinformatics programs searching for novel miRNAs will not look for alternative pre-miRNA structures and 2) as Yang and Lai have pointed out, Drosha and Dicer knockout studies will continue to be over interpreted. That is, the persistence of a phenotype in a Drosha knockout mouse does not mean the phenotype is independent of miRNAs.

The seed pairing rule and 3′ UTR location rules

"Animal MiRNAs bind to mRNAs at, conserved, imperfectly complementary sites in the 3′ UTRs, with a perfect match to the miRNA's seed region (5′ nucleotides 2-8) being critical for activity." This statement which contains two rules for miRNA-target mRNA interaction was initially proposed based on the interaction of the first miRNA, lin-4, with several conserved sites within the 3′ untranslated region (UTR) of the lin-14 mRNA (Lee et al., 1993; Wightman et al., 1993). The idea has proven useful as, to some extent, it is followed by many animal miRNAs, it is supported by biochemical and structural data, and it has led to correct predictions both specific and broad (reviewed by Lee and Shin in 2012). For instance, the seed region is indeed more conserved than the rest of the miRNA among related miRNAs (Lim et al., 2003) and retrieves the largest number of conserved target sites above background when used in target prediction programs (Lewis et al., 2003). Also, several genome-wide studies demonstrate that changes in miRNA levels do lead to changes in the expression of genes that contain corresponding seed matches (Krützfeldt et al., 2005; Baek et al., 2008; Selbach et al., 2008). Interestingly, and again consistent with the primacy of the seed sequence for determination of targets, crystal structures of Ago proteins from several species bound to nucleic acids reveal that the seed region is more accessible to interaction with the target RNA than the rest of the miRNA (Wang et al., 2008; Elkayam et al., 2012; Schirle and MacRae, 2012).

A number of miRNA target prediction programs have been written that are based, to various degrees, on the seed rule and

3′ UTR location (Bartel, 2009). Such programs have proven helpful in finding some targets however, in most cases; it is necessary to supplement the prediction programs with both wet laboratory data and common sense. For instance, a common research arc is 1) a miRNA is linked to biological process, 2) a list of hundreds of prospective target genes are generated by target prediction programs based on the existence of conserved seed matches in the 3′ UTR, 3) from this list, researchers pick prospective target genes linked to the biological process under study, 4) miRNA regulation of these genes is confirmed through overexpression of the miRNA followed by monitoring either the proposed target's expression directly or the activity of a 3′ UTR linked luciferase construct.

Although the protocol stated above has proven useful, an unbiased assessment of 5 of these seed-rule-reliant miRNA target prediction programs reveals high false positive and false negative rates (precision of ~ 50% and sensitivity of 6%–12%) (Alexiou et al., 2009). Furthermore, there are now numerous exceptions to both the seed pairing rule and the 3′ UTR location rule. Bartel and others (Grimson et al., 2007; Bartel, 2009; Shin et al., 2010; Elefant et al., 2011) classify miRNA binding sites into multiple groups including: 1) canonical sites representing both the seed rule and the 3′ UTR location rule; 2) 3′-supplementary seed sites containing matching seeds plus significant matching at the 3′ end of the miRNA; 3) 3′-compensatory sites with mismatches in the seed that are compensated by binding at the 3′ end; 4) centered sites with little matching at either end of the miRNA but having 11-12 contiguous Watson-Crick pairs in the center; 5) cleavage sites, like plant miRNA-mRNA and siRNA-mRNA interactions with nearly perfect complementarity throughout the site; 6) bulged sites with a 1 nt unpaired nucleotide at position 5-6 of the mRNA, with the unpaired nucleotide being able to pivot and pair at the miRNA position 6. Finally, as Lee and Shin review (2012), there are an increasing number of experimentally identified miRNA-target interactions that do not fall into any of these categories.

There is also increasing variability being discovered in the location of the miRNA binding sites. Initially, 3′ UTR binding sites were the focus of discovery efforts for historical reasons and because inter-species conservation studies are easier to perform in the more variable 3′ UTR. Also, studies with artificial miRNA binding sites show 3′ UTR positioned sites are more efficient, most likely due to interference in miRNA action by translational machinery (Grimson et al., 2007; Gu et al., 2009). Like the seed pairing rule, the 3′ UTR location rule has proven useful, as many sites have been found in this region. Also, predictions based on this rule, such as the prediction that the relative length of a transcript's 3′ UTR inversely correlates with the relative level of its expression, have been proven correct (Sandberg et al., 2008; Mayr and Bartel, 2009). However, genome-wide differential miRNA expression studies show a significant number of miRNA binding sites are located in ORFs in addition to 3′ UTRs (Lim et al., 2003; Grimson et al., 2007; Baek et al., 2008). Schnall-Levin et al. (2011) have shown that several miRNAs can efficiently target mRNAs via exonic target sites if those sites are repetitive. Also, CLIP (cross-linking immunoprecipitation) studies that map Ago binding sites suggest that nearly half of miRNA target sites are outside of the 3′-UTR, although it is difficult to know how many of these binding sites are active (Chi et al., 2009; Hafner et al., 2010).

Given all of the diversity in binding sites and location how much value is there to classifying them? Inclusion of additional non-canonical binding site groups and exonic locations in target prediction programs will certainly increase already high false positive rates (Elefant et al., 2011). Thus, more characterization of binding sites may be needed before inclusion into target prediction algorithms. One way to reduce false positives generated from including a broader definition of binding sites and locations in target prediction programs could be to include annotated genome wide expression and/or Ago binding site data.

The mechanism of action rule

"Animal MiRNAs function by inhibiting mRNA translation and/or by decreasing mRNA stability, depending on the amount of target sequence complementarity."

Again early studies on lin-4/lin-14 set a precedent that was quickly applied to other animal miRNAs. In this case, it was shown that the lin-4 miRNA represses the translation of the lin-14 mRNA without significantly affecting its stability (Olsen and Ambros, 1999). However, numerous studies quickly showed that many mRNAs targeted by miRNAs were indeed destabilized in a process including deadenylation (Bagga et al., 2005; Rehwinkel et al., 2005; Behm-Ansmant et al., 2006; Giraldez et al., 2006). The observations that plant miRNAs, designed siRNAs, and the occasional animal cleavage site miRNA (see above) typically have a much higher degree of complementarity with their targets and also promote the destruction of the mRNA (Zamore et al., 200; Elbashir et al., 2001; Llave et al., 2002; Yekta et al., 2004), led to the widely quoted rule that a mRNA can be either degraded or translationally blocked depending on the extent of complementarity with the miRNA. That is, if an endogenous animal miRNA shows complete or nearly complete complementarity to its target then it will degrade that target in an Ago-dependent manner. If it shows the more typical partial complementarity then it will repress expression either by translational inhibition or degradation including deadenylation or some combination of the two. Furthermore, the relative contribution of both processes will vary widely from message to message (Behm-Ansmant et al., 2006).

A practical consequence of this rule is that when trying to determine if a particular gene is regulated by a particular miRNA, with depletion or overexpression studies, it is best to assay protein levels as RT-PCR may miss a purely

translational effect. However, studies by Bartel and others suggest that most, if not all, animal miRNAs-mRNA interactions affect translation to varying degrees but inevitably lead to significant mRNA degradation (Baek et al., 2008; Selbach et al., 2008; Hendrickson et al., 2009; Guo et al., 2010). Interestingly, kinetic studies of some miRNAs in zebrafish and Drosophila S2 cells show that a translation blockade occurs first and thus may trigger mRNA degradation (Bazzini et al., 2012; Djuranovic et al., 2012). It remains to be seen if this is true for mammalian systems. Also, more studies are needed to ascertain if the extent or timing of mRNA degradation varies depending on the number and/or type of miRNA binding sites. Nevertheless, researchers can take heart that the more laborious, more expensive, and less reliable protein level assays can be replaced with RT-PCR for validating a miRNA's effect on a putative target's steady-state expression.

Less common but more dramatic exceptions to this rule have also been described. For instance several cases of miRNA stimulation of expression have been reported. Vasudevan et al. (2007, 2008) found that multiple miRNAs that inhibit expression in proliferating mammalian cells have the opposite effect during cell cycle arrest, by activating translation. Binding of the miRNA with the mRNA is required for both inhibition and stimulation in this system. The exact mechanism for this and other examples of miRNA stimulation of translation is unknown but involves cellular localization, RNA sequence context, and the recruitment of associated protein complexes (Lee and Vasudevan, 2013). Another example is human mir-122's stimulation of Hepatitis C viral (HCV) replication. Apparently, viral replication is so dependent on mir-122 that mir-122 expression levels explain HCV viral tropism (Jopling et al., 2005). In this case, the miRNA is thought to function at the level of RNA stability as it binds to and stabilizes the 5′ end of the viral transcript (Machlin et al., 2011).

Summary

In the few short years since miRNAs became widely studied much has been learned about their biogenesis, function, and mechanism of action. However, given the growing number of exceptions to the above described rules, researchers must take care when using them to interpret wet laboratory or *in silico* data. For miRNA novel discovery, one must factor in non-canonical miRNA biogenesis pathways and perhaps deemphasize species conservation. Likewise a miRNA's function is more than the sum of its target binding sites. Clearly, the context and locations of the binding sites, the presence of other protein and miRNA regulators, and the state of the cell all contribute to a given miRNAs effect on a given set of mRNAs.

Going forward, the rules of miRNA character and behavior will continue to be refined, strengthened, or cast aside. As more miRNAs are discovered their inclusion in future Kingdom-wide surveys will elucidate the evolutionary consequences of miRNA diversification. Likewise, biochemical studies will further define the miRNA biogenesis and target-miRNA interaction rules. Codification of such rules will not only simplify the description of miRNA characteristics and make it easier to interpret experimental results but could also lead to bioinformatics protocols that will facilitate the discovery of novel miRNAs, novel targets, and their functions.

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Compliance with ethics guidelines

Tom Volpe declares no conflict of interest. P. Shannon Pendergrast works for Ymir Genomics LLC, a company performing non-coding RNA discovery. This manuscript is a review article and does not involve a research protocol requiring approval by an IRB or ethics committee.

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