BMB
Reports

BMB Rep. 2017; 50(11): 554-559
www.bmbreports.org

Invited Mini Review

Rules for functional microRNA targeting

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MicroRNAs (miRNAs) are ~22nt-long single-stranded RNA molecules that form a RNA-induced silencing complex with Argonaute (AGO) protein to post-transcriptionally downregulate their target messenger RNAs (mRNAs). To understand the regulatory mechanisms of miRNA, discovering the underlying functional rules for how miRNAs recognize and repress their target mRNAs is of utmost importance. To determine functional miRNA targeting rules, previous studies extensively utilized various methods including high-throughput biochemical assays and bioinformatics analyses. However, targeting rules reported in one study often fail to be reproduced in other studies and therefore the general rules for functional miRNA targeting remain elusive. In this review, we evaluate previously-reported miRNA targeting rules and discuss the biological impact of the functional miRNAs on gene-regulatory networks as well as the future direction of miRNA targeting research. [BMB Reports 2017; 50(11): 554-559]

CANONICAL SITE TYPES

It has been widely accepted that more than 60% of the entire human mRNAs are directly regulated by miRNAs (1-4). Accordingly, miRNAs participate in numerous biological processes, and their activity can lead to various human diseases (5-10). Although understanding the complete rules of how miRNAs recognize and regulate their target mRNAs is essential to learn the biological roles of miRNAs, comprehensive rules for functional miRNA targeting are yet to be determined.

miRNAs interact with their target mRNAs through Watson-Crick base pairing (WCP) at their 5′ ends (2, 11-15). Numerous empirical computational analyses have shown that perfect WCPs between the 2-7 nucleotide region at the 5′ end of the miRNA and its complementary target site on the mRNA are crucial for miRNA targeting (11, 13, 16). This 6nt region of miRNA is referred to as "seed", and an additional base pairing at the 8nt position of miRNA or the existence of adenine on the mRNA side corresponding to the 1st nucleotide position of miRNA further improves the miRNA targeting efficacy. Based on these findings, four canonical site types (CSTs) were determined and are indicated as 8mer, 7mer-m8, 7mer-A1, and 6mer, respectively (14, 17).

To measure the impact of CSTs on the whole transcriptome, microarrays were utilized to monitor changes in the transcriptome after ectopic introduction of miRNAs. Accordingly, the widespread impact of the CSTs on the transcriptome was observed as a large number of mRNA targets were directly downregulated (3, 14). Also, whole proteomic analyses and ribosome profiling showed that miRNAs downregulate gene expression mainly through mRNA destabilization rather than translational repression (4, 15, 18). Lewis *et al*. (15) conducted comparative genomic analyses and found that many target sites of the CSTs were conserved across the species (11). Friedman *et al*. (1) used an extended list of vertebrate genomes to show that more than 60% of mammalian genes are conserved targets of miRNAs (1).

In terms of the molecular details of targeting mechanisms, a structural analysis by Schirle *et al*. (19) elucidated the functional mechanism of the CSTs by proposing a mechanistic model for seed pairing. This model includes a pocket to recognize adenine on the mRNA side, which explains why the adenine residue affects the miRNA targeting efficacy (19). The theory of the molecular mechanism for miRNA target recognition was reinforced by a single-molecule study that utilized a fluorescence resonance energy transfer (FRET) assay on human AGO2 (20). The researchers used their results to propose a stepwise model for miRNA target recognition that consists of the initial binding of AGO2 to a target site with WCPs for the miRNA 2-4nt region, which is referred to as the sub-seed recognition motif, and a subsequent step of lateral diffusion for the formation of complete seed pairing.

The broad impact of miRNA targeting and the conservation of miRNA target sites strongly indicate that the CSTs of miRNAs may play biologically important roles. Nonetheless, the response of the transcriptome cannot be fully explained only by the CSTs (12), implying that additional functional site types may exist in addition to the four CSTs.

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https://doi.org/10.5483/BMBRep.2017.50.11.179

Received 6 September 2017

Keywords: Canonical site types, microRNA, miRNA targeting, miRNA, Noncanonical site types

ISSN: 1976-670X (electronic edition)

PREVIOUSLY REPORTED NONCANONICAL SITE TYPES

Accumulated evidence from studies over the past decade have expanded the miRNA targeting rules and have led to the discovery of noncanonical site types (NSTs). Aside from CSTs, two NSTs were identified: centered site and offset 6mer (Table 1) (1, 21). Compared to CSTs, NSTs lack perfect WCPs between the seed site of miRNA and the target site of mRNAs and exhibit weaker but significant effects in downregulating target mRNAs.

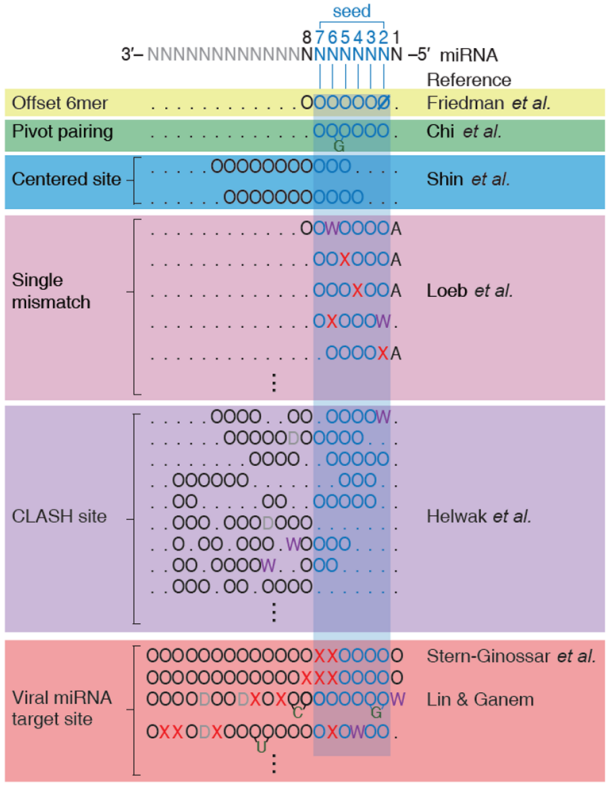
Recently, a powerful biochemistry technique called AGO crosslinking immunoprecipitation and high-throughput sequencing (AGO CLIP-seq) was developed (22, 23). AGO CLIP-seq provides precise information of AGO protein binding regions on mRNAs. Using this technique, two additional NSTs were identified: the pivot pairing and single mismatch site types (STs) (24, 25). Pivot pairing ST contains a guanine bulge on the mRNA target site of the seed pairing region, and the single nucleotide mismatch ST includes a single nucleotide mismatch or a wobble pairing on the seed region. The detection accuracy was improved using photoactivatable-ribonucleoside-enhanced CLIP (PAR-CLIP), which incorporates photoreactive nucleoside analogs to facilitate cross-linking (23, 26-29).

Even though AGO CLIP-seq can effectively identify functional NSTs, the technique suffers from a few limitations. First, since this method detects the association among AGO, miRNAs, and mRNAs, it can only provide indirect evidence for the interactions between individual miRNAs and mRNAs. Second, although binding of AGO to the target mRNA is a necessary condition for downregulation of the target mRNA, it is not sufficient for detectable downregulation (22, 24, 29, 30). For instance, a portion of AGOs have the ability to bind to the ORF as well as 3'UTR (22, 25, 27, 31-33). Those ORF-bound AGOs can be detected by AGO CLIP-seq, but most of the mRNAs with ORF-bound AGOs would not be downregulated since the ORF-bound AGOs are likely to get bumped out by translating ribosomes (14, 34, 35). Therefore, the miRNA targets detected by AGO CLIP-seq tend to include a high rate of false positives.

To overcome the first limitation, an advanced technique, CLASH (CrossLinking, ligAtion, and Sequencing of Hybrids), that aims to identify direct interactions between AGO-bound miRNAs and mRNAs was developed (36, 37). CLASH includes an additional step of ligation for a miRNA and its target mRNAs, and it is thus able to provide a more direct profile of miRNA-mRNA interactions. Additional NSTs were proposed to be functional by analyzing improved binding information, generated by CLASH, among AGO protein, mRNA, and miRNA (Table 1) (37, 38). However, even with CLASH, the second limitation still remains unsolved.

Based on the interactions between viral miRNAs and mRNA targets, several additional NSTs that contain an imperfect seed pairing and additional complementary WCPs were reported (Table 1) (39, 40). These results imply the prevalence and potential contribution of the NSTs in functional miRNA targeting and thus emphasize the need to expand the miRNA targeting rules beyond the previously-accepted CSTs. Nonetheless, whether these proposed NSTs truly serve as general rules for functional miRNA targeting remains uncertain because of the aforementioned limitations.

Table 1. Previously reported noncanonical site types



The residue 'O' indicates a Watson-Crick base pairing between miRNA and mRNA target and 'Ø' represents all the other interactions other than a Watson-Crick base pairing. 'W' and 'X' indicate wobble pairing and mismatch, respectively. For the mRNA region responding to the 1st nucleotide position of miRNA, 'A' is assigned if adenine is in that position. A bulge on mRNA side is depicted as a protrusion of a nucleotide on the mRNA strand, and a bulge on miRNA side is represented as the residue 'D'.

SYSTEMATIC EVALUATION OF PREVIOUSLY REPORTED NSTS

In contrast with the prior results that indicate the widespread importance and functional roles of NSTs, a recent study claimed that almost all previously-identified NSTs are in fact not functional (41). In this study, researchers re-examined the efficacies of previously-reported NSTs by observing transcriptome

changes after knocking out, knocking down, and ectopically expressing miRNAs. After careful and systematic evaluations of the transcriptome data, they concluded that even though NSTs were detected in AGO CLIP-seq studies, most of the NSTs except offset 6mer are non-functional and do not show any detectable downregulation of their target mRNAs (24, 36, 37), suggesting that these NSTs may be conditionally functional for a specific cell type or with specific miRNAs.

Although almost all previously-reported NSTs were found to be non-functional, one critical question still remains unexplored: are there any additional functional NSTs? Compared to the astronomical number of interactions that can possibly occur between miRNAs and mRNAs, previous studies have evaluated only a tiny fraction of possible STs (42). The limited scope of the examination could be the reason for past failures in detecting functional STs, calling for a systematic and exhaustive evaluation of all possible interactions between miRNAs and mRNAs based on direct evidence and the discovery of comprehensive rules for functional miRNA targeting.

COMPREHENSIVE EVALUATION OF FUNCTIONAL SITE TYPES

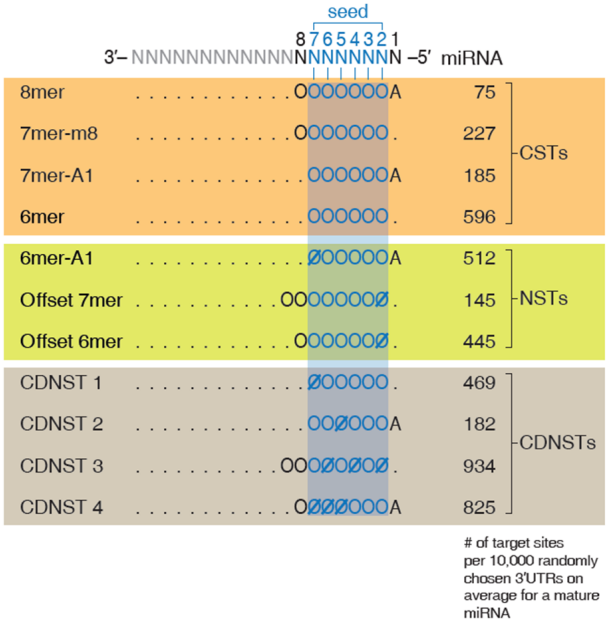
Kim *et al*. (42) systematically determined all possible interactions that can occur between miRNAs and the target mRNAs to expand the number of evaluated STs and utilized large-scale microarray data that measured the transcriptome response when miRNAs are ectopically introduced to evaluate whether these interactions are functional (42). The authors statistically evaluated whether each of the $>$ 2 billion STs is enriched in genes that are highly downregulated when miRNAs are overexpressed. Since the approach the authors adopted was to examine an astronomical number of STs based on direct evidence of actual transcriptome response to miRNA overexpression, their research is free from the limitations of studies based on AGO CLIP-seq.

Through a massive-scale bioinformatics search, the authors discovered three functional NSTs in addition to the CSTs. The newly discovered NSTs consist of previously-identified offset 6mer ST, a novel NST termed as offset 7mer, and another novel NST termed as 6mer-A1 (42). Offset 7mer contains an additional WCP compared to the offset 6mer ST and 6mer-A1 is similar to canonical 7mer-A1 ST with an exception that it is one nucleotide shorter. Kim *et al*. (42) observed that local contexts that are known to affect miRNA targeting, such as local AU content of the surrounding region of the target site, 3′UTR length of the target mRNA, the target site abundance, and the thermodynamic pairing stability between miRNA and mRNA (11, 14, 17, 43-48), also have significant impact on the proficiency of the three newly-discovered NSTs. The authors searched for additional STs whose target sites with good contexts exhibit detectable downregulation and identified four additional functional NSTs. They named these NSTs context-dependent noncanonical site types (CDNSTs). When compared to CSTs, the seven newly-discovered NSTs and CDNSTs elicit weaker-but-still-significant target repression (42). Also, NSTs and CDNSTs have more target sites than CSTs, indicating they may exert considerable influence on the regulation of the transcriptome (Table 2).

The newly-discovered NSTs were thoroughly validated through various experiments and computational analyses. In the luciferase assay, an overall 70% of the target mRNAs of NSTs and CDNSTs exhibited significant repression, which confirms their functionality *in vivo* (42). Also, independent microarray data obtained from various human cell lines was evaluated by monitoring the transcriptome response against the overexpressed miRNA to further demonstrate that these NSTs and CDNSTs are generally functional. In addition, the biological significance of the NSTs and CDNSTs was validated by analyzing the miRNA knock-out and knock-down microarray data, which strongly indicates they effectively downregulate their target mRNAs in an endogenous environment.

The impact of the NSTs and CDNSTs on the transcriptome was assessed by estimating the overall amount of mRNA repression mediated by CSTs, NSTs, and CDNSTs. The analysis showed that even though the individual impact of NSTs and CDNSTs was relatively weak, when added together, the

Table 2. Comprehensive rules for functional microRNA targeting



An expanded view of functional miRNA targeting (modified from Fig. 3B of Kim *et al*.). The normalized numbers of targets for each site type are shown in right side and the representation of interactions follows notations described in Table 1.

overall impact of the NSTs and CDNSTs on the transcriptome was comparable to that of CSTs (42). Moreover, a comparative genomics analysis confirmed that the target sites in 10 out of 11 functional STs are evolutionarily conserved across the vertebrate genome (42). Therefore, novel NSTs and CDNSTs may have physiologically important functions, and the influence of NSTs and CDNSTs should be carefully considered when identifying miRNA targets.

In summary, a massive-scale computational search revealed seven novel functional noncanonical interactions that were validated by multiple lines of strong evidence, suggesting that these NSTs and CDNSTs may serve in important roles in the miRNA-mRNA regulatory network.

DISCUSSION

Since the discovery of miRNA, numerous scientists have attempted to understand miRNA in terms of its biogenesis, functions, and significance. In 2005, Lewis *et al*. discovered CSTs and verified that they are functional *in vivo* (11). This discovery was a scientific breakthrough because CSTs not only exert substantial influence on the whole transcriptome and proteome, but are also evolutionarily conserved, suggesting their biological significance (1, 3, 15). The accumulation of genome-wide data and the development of advanced technologies, such as AGO CLIP-seq and CLASH, have led to a discovery of additional NSTs involved in miRNA targeting (1, 21, 24, 25, 37, 39, 40). Although there are large numbers of previously reported NSTs, these NSTs are not fully accepted as a part of general miRNA targeting rules due to inconsistent results found in various studies (41). Therefore, a recent study made an attempt to systematically and comprehensively evaluate miRNA-target interactions by employing a massive-scale bioinformatics approach (42). In this study, seven potentially functional NSTs and CDNSTs were discovered. Validations via luciferase assays and analyses of independent data suggest that most of these NSTs and CDNSTs may be functional, and the evolutionary conservation and estimated regulatory effect on the transcriptome of NSTs and CDNSTs clearly indicate that expanded miRNA targeting rules could potentially play biologically relevant roles.

A deeper understanding of miRNA targeting rules raises important issues. One major issue is the lack of research on RNA-binding proteins (RBPs) that act as determinants of miRNA targeting and the mechanisms through which these RBPs regulate miRNA targeting proficiencies. Several unique cases were reported in which RBPs influence the proficiency of repression of miRNA target mRNAs (49, 50), but a comprehensive model depicting the interplay between RBPs and miRNA targeting remains to be evaluated. Another issue is the lack of complete understanding of the biological consequences of miRNA targeting on translational regulation. Guo *et al*. (2010) showed that miRNA-mediated gene silencing in a steady state is mainly mediated by mRNA destabilization and that translational repression contributes little to the overall downregulation (18). However, in a transient state, the translational control appears to be a major mechanism of miRNA targeting (51), and even in the steady state, translational control may play more prominent roles for specific miRNAs (52-54). Hence, discovering miRNA targeting determinants associated with translational repression would provide valuable knowledge to understand miRNA targeting mechanisms more completely.

An expanded repertoire of functional miRNA targets implies that miRNA-target mRNA interactions and their regulatory networks are far more intricate than are currently understood. The comprehensive rules of miRNA targeting revealed in recent studies may lead to a deeper understanding of the complex gene-regulatory network controlled by miRNAs, reduction in the off-targeting effects when designing siRNA/shRNA libraries, and an improvement in the accuracy of miRNA target prediction algorithms.

ACKNOWLEDGEMENTS

This work was supported by the Institute for Basic Science (Grant number IBS-R008-D1), and by the National Research Foundation, funded by the Ministry of Science, ICT & Future Planning, Republic of Korea (Grant numbers 2014M3C9A 3063541 and 2012M3A9D1054622).

CONFLICTS OF INTEREST

The authors have no conflicting interests.

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