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Title: Rules for functional microRNA targeting

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ABSTRACT

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 how miRNAs recognize and repress their target mRNAs is of utmost importance. To determine the functional miRNA targeting rules, previous studies extensively utilized various methods including high-throughput biochemical assays and bioinformatics analyses. However, in many cases targeting rules reported in one study fail to be reproduced in other studies, leaving the general rules for functional miRNA targeting elusive. In this review, we evaluate previously reported rules of miRNA targeting and discuss the biological impact of the functional miRNAs on gene-regulatory networks and the future direction of miRNA targeting research.

CANONICAL SITE TYPES

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

miRNAs interact with their target mRNAs through Watson-Crick base pairing (WCP) at their 5' ends (2, 11-15). Numerous empirical computational analyses showed the perfect WCPs between 2-7 nucleotide region at the 5' end of the miRNA and its complementary target site on the mRNA are crucial in miRNA targeting (11, 13, 16). This 6nt region of miRNA is called "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 enhances the miRNA targeting efficacy. Based on these findings, four canonical site types (CSTs) were determined and termed 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 the transcriptome changes after the 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). Based on comparative genomic analyses, Lewis *et al*. (2005) found that many target sites of the CSTs were conserved across the species (11). With an extended list of vertebrate genomes, Friedman *et*

al. (2009) showed 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.* (2014) elucidated the functional mechanism of the CSTs by proposing the mechanistic model for seed pairing. This model includes a pocket for recognition of adenine on mRNA side, which explains why the adenine residue affects the miRNA targeting efficacy (19). The theory on the molecular mechanism of miRNA target recognition was reinforced by a single-molecule study that utilized a fluorescence resonance energy transfer (FRET) assay on human AGO2 (20). From these results, the researchers proposed a stepwise model for miRNA target recognition that consists of the initial binding of AGO2 to a target site with WCPs for miRNA 2-4nt region, which is called 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 there may exist additional functional site types in addition to the four CSTs.

PREVIOUSLY REPORTED NONCANONICAL SITE TYPES

Cumulated evidence from various studies over the past decade expanded the miRNA targeting rules and led to the discovery of noncanonical site types (NSTs). Aside from CSTs, two NSTs, called centered site and offset 6mer, were identified (Table 1) (1, 21). When 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 the single mismatch site types (STs) (24, 25). Pivot pairing ST contains a guanine bulge on 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 by 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 it is a method that detects the association among AGO, miRNAs, and mRNAs, it can only provide indirect evidence for interactions between individual miRNAs and mRNAs. Second, although binding of AGO to 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 has 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), which 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 thus is 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 to be unsolved.

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

SYSTEMATIC EVALUATION OF PREVIOUSLY REPORTED NSTS

In contrast to the previous results which propose widespread importance and functional roles of NSTs, a recent study claimed that almost all of the previously identified NSTs are in fact not functional (41). In this study, the 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 at specific cell type or with specific miRNAs.

Although almost all of the previously reported NSTs were found to 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 examination could be the reason for the past failure 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 EVALUAION OF FUNCTIONAL SITE TYPES

In order to expand the number of evaluated STs, Kim *et al.* (2016) systematically determined all of the possible interactions that can occur between miRNAs and the target mRNAs and evaluated whether these interactions are functional by utilizing large-scale microarray data, that measured the transcriptome response when miRNAs are ectopically introduced (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 astronomical number of STs based on direct evidence of actual transcriptome response to miRNA overexpression, their research is free from the limitations of the studies based on AGO CLIP-seq.

Through the massive-scale bioinformatics search, the authors discovered three functional NSTs in addition to the CSTs. The newly discovered NSTs consists 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*. (2016) 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 impacts on the proficiency of the three newly discovered NSTs. Searching for additional STs whose target sites with good contexts exhibit detectable downregulation, the authors identified four additional functional NSTs, naming these NSTs context-dependent noncanonical site types (CDNSTs). Compared to the 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 by various experiments and computational analyses. In luciferase assay, overall 70% of target mRNAs of NSTs and CDNSTs exhibited significant repression, which confirms their functionality *in vivo* (42). Also, evaluation of independent microarray data obtained from various human cell lines by monitoring the transcriptome response against the overexpressed miRNA further demonstrated that these NSTs and CDNSTs are generally functional. Additionally, the biological significance of NSTs and CDNSTs were validated by analyzing miRNA knock-out and knock-down microarray data, which strongly indicates they effectively downregulate their target mRNAs in the 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, and NSTs, and CDNSTs. The analysis showed that even though the individual impacts of NSTs and CDNSTs are relatively weak, when added together, the overall impact of NSTs and CDNSTs on the transcriptome is comparable to that of CSTs (42). Moreover, 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 influences of NSTs and CDNSTs should be carefully considered when finding miRNA targets. In summary, a massive-scale computational search revealed seven novel functional noncanonical interactions which are validated by multiple lines of strong evidence, suggesting that these NSTs and CDNSTs may serve as important roles in the miRNA-mRNA regulatory network.

DISCUSSION

Since the discovery of miRNA, numerous scientists 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 the CSTs not only exert substantial influence on the whole transcriptome and proteome, but also are evolutionarily conserved, suggesting their biological significance (1, 3, 15). Accumulation of genome-wide data and development of advanced technologies such as AGO CLIP-seq and CLASH led to the discovery of additional NSTs that are 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 the inconsistent results found in various studies (41). Therefore, a recent study attempted to systematically and comprehensively evaluate miRNA-target interactions employing a massive-scale bioinformatics approach (42). In this study, seven potentially functional NSTs and CDNSTs were discovered. Validations through luciferase assays and analyses of independent data suggest that most of these NSTs and CDNSTs may be functional. 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.

The deeper understanding of miRNA targeting rules raises important issues that need to be solved. One major issue is the lack of research on RNA-binding proteins (RBPs) that act as determinants of miRNA targeting and by which mechanisms these RBPs regulate miRNA targeting proficiencies. Several unique cases have been reported where RBPs influence the proficiency of repression of miRNA target mRNAs (49, 50), but the comprehensive model that depicts the interplay between RBPs and miRNA targeting still remains to be unevaluated.

Another issue is the lack of complete understanding of the biological consequences of miRNA targeting on the translational regulation. Guo *et al*. (2010) showed that miRNA-mediated gene silencing in steady state is mainly mediated by mRNA destabilization and translational repression contributes little to the overall downregulation (18). However, in a transient state, the translational control appears to be the major mechanism of miRNA targeting (51) and, even in the steady state, the translational control may play more prominent roles for specific miRNAs (52-54). Hence, discovering the miRNA targeting determinants that are associated with translational repression would provide valuable knowledge that would help understand miRNA targeting mechanisms more completely.

An expanded repertoire of functional miRNA targets implies that the 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 deeper understanding of the complex gene-regulatory network controlled by miRNAs, the reduction of off-targeting effects when designing siRNA/shRNA libraries, and the improvement on the accuracy of miRNA target prediction algorithms.

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CONFLICTS OF INTEREST

The authors have no conflicting financial interests.

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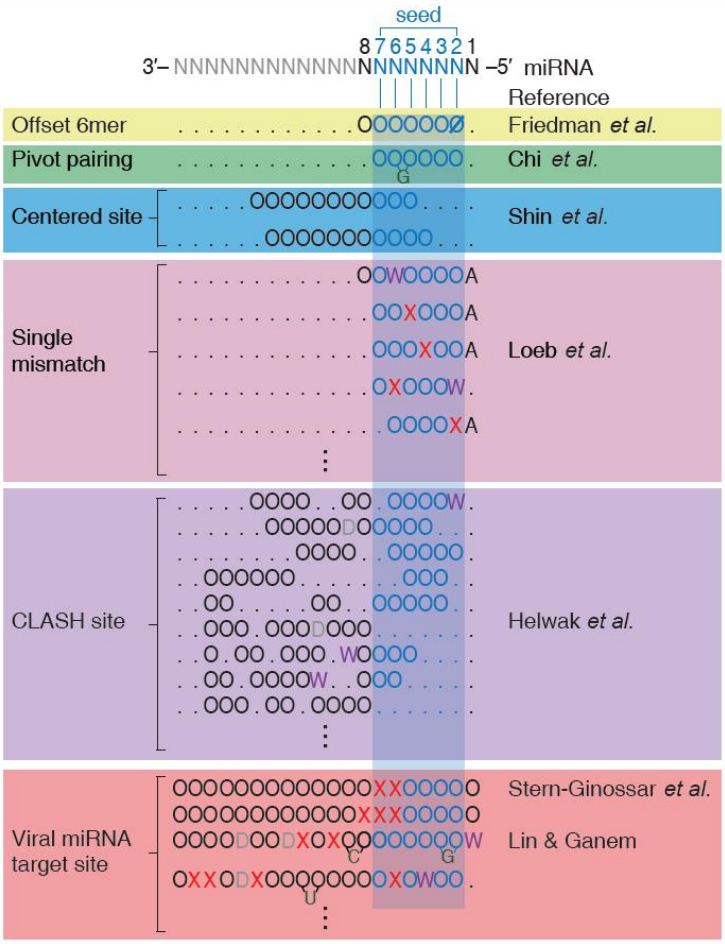
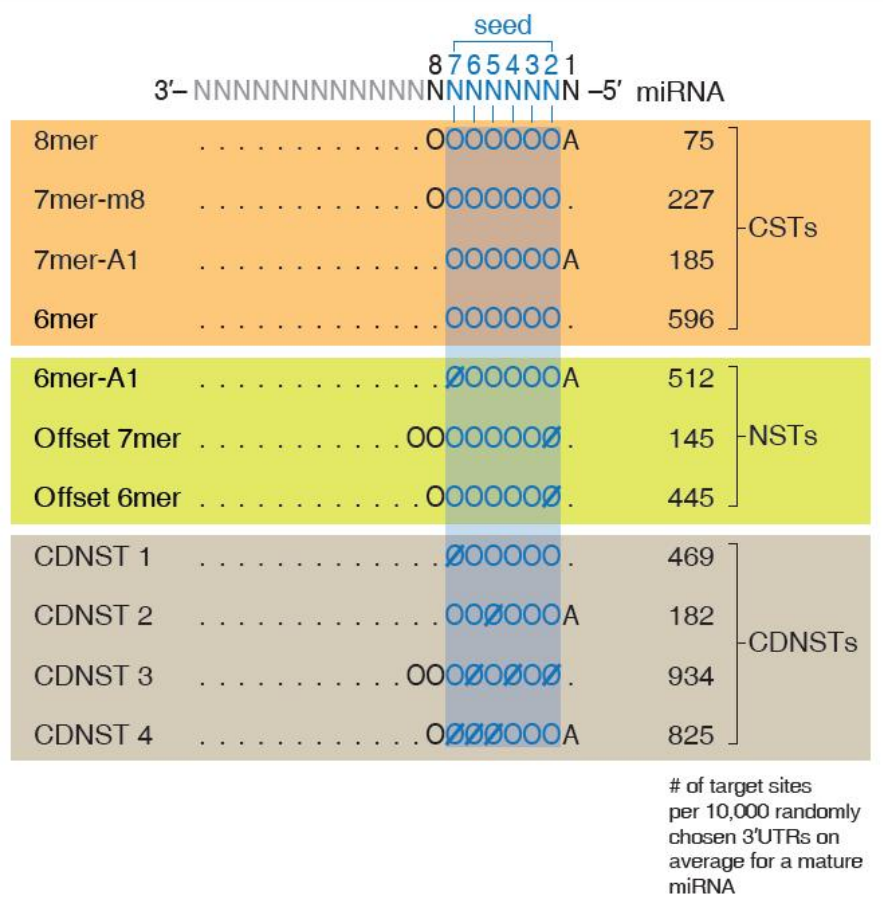
TABLES

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’.

Table 2. Comprehensive rules for functional microRNA targeting.



An expanded view of functional miRNA targeting (modified from Figure 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.