Cell Research (2008) 18:1157-1159.
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www.nature.com/cr

miRNAs: small changes, widespread effects

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Cell Research (2008) **18**:1157-1159. doi: 10.1038/cr.2008.311; published online 1 December 2008

MicroRNAs (miRNAs) are endogenously encoded small RNA molecules that can bind to cognate messenger RNAs, thereby impairing protein expression. miRNAs are transcribed from the genome as long precursor molecules that are processed via multiple steps into their mature form of ~22 nt. Embedded in an RNA Induced Silencing Complex (RISC), miRNAs can bind to target RNAs by base complementarity, after which the target is degraded and/or translation is terminated. miRNA-based regulation has been shown to function in a wide variety of processes, such as development, stress response, and fine-tuning of physiological pathways.

Since their discovery, the challenge has been to identify genuine miRNA targets. There are two major complicating factors in this process. First, in contrast to RNA interference (RNAi) where small interfering RNAs require perfect target-complementarity to function, miRNAs recognize their targets with only partial sequence complementarity, making *in silico* target-prediction very challenging. Although the seed sequence of the miRNA (nt 2-8) requires a high degree of complementarity with the target RNA, overall miRNA::mRNA binding allows for several mismatches. Even more, for the vast majority of miRNAs, a key characteristic of miRNA::target pairing is imperfect matching outside the seed. In flies, it has been shown that the degree of overall complementarity plays an important role in the way the messenger is silenced by the miRNA. If miRNA and target bind perfectly, the target is cleaved and degraded; if not, the target is not cleaved, but translation is inhibited [1]. This mechanism has also been suggested for mammalian systems [2]. Secondly, experimental identification of targets is compromised by difficulties to systematically co-isolate or capture native miRNAs in combination with their endogenous target mRNAs, which may be due to rapid processing of the miRNA::mRNA duplex by the RISC complex. Indirect identification of miRNA targets by first manipulating endogenous miRNA levels (*e.g.* by overexpression or morpholino- or LNA-based knockdown) followed by systematic analysis of the consequences on gene expression levels, *e.g.* by transcriptome microarrays, has been performed successfully in some cases (*e.g.* [2]). However, these studies rely on the assumption that miRNA binding exclusively influences target RNA stability and neglect the potential of miRNAs to regulate protein expression by affecting mRNA translation directly.

While it has been possible to address miRNA regulation at the protein level for individual targets, the existing methods do not allow for large-scale target verification. Two recent papers in the journal *Nature* [2, 4] have addressed this problem and now provide a very interesting insight in miRNA-based regulation at both the mRNA and protein level. Using Stable Isotope Labeling with Amino acids in Cell culture (SILAC), in combination with state-of-the-art mass spectrometry (MS), the authors were able to determine expression levels of thousands of proteins in the presence or absence of a certain miRNA. The outcome of these two studies provides the first high-throughput proteomics evidence of miRNA effects.

Both groups show that upon introduction (or knockdown) of a miRNA, the levels of hundreds of proteins are affected, but that effects are mild, with few proteins decreasing by more than 50%. As might be expected, messengers that encoded the reduced proteins were enriched for seed matches (for the introduced miRNA) in their 3′ UTR. Both groups also determined mRNA levels, enabling them to distinguish between the two proposed modes of miRNA action: mRNA degradation and translational inhibition. Both groups concur that although the studied miRNAs can affect mRNA levels, this alone cannot explain the observed effect on the protein levels, indicating that miRNAs should affect the translational process as well.

Analysis of the data revealed several additional features of miRNA silencing. Seed matches of 8 nt had stronger effects on protein levels than 7 nt (less strong effect) and 6 nt (minor effect) seed matches. This also holds true for the effect at the mRNA level [3]. In addition, more seed-matching sites in a single messenger also results in stronger

silencing, with a higher synergistic effect for seeds spaced <40 nt from each other. Selbach, *et al.* also show that only seed-containing mRNAs with at least one mismatch between nt 9-11 of the miRNA are repressed at the protein level. This would make sense, as such messengers cannot be cleaved by the RISC-complex (like in RNAi), thus favoring translational inhibition over mRNA degradation. Surprisingly, however, protein production from seed-containing mRNAs with perfect base pairing from nt 9-11 are indistinguishable from mRNAs lacking seed matches. Baek, *et al.* do not elaborate on potential mismatches in the nt 9-11 region, but they do appreciate that the messengers with the lowest expression level are relative immune to altered miRNA levels, whereas protein translation of these mRNAs is responsive to miRNA level manipulation. These observations suggest that the primary mode of miRNA action is translational inhibition and that mRNA degradation might be a consequence of translational silencing. This idea is reinforced by the observation by Selbach, *et al.* that shortly after introduction of *mir-1* (8 h) mRNA levels are relatively stable compared to protein levels, whereas after 32 h mRNA levels mimic the effects seen at the protein level. A second interesting observation by Selbach, *et al.* is that *let-7b* targets with strong translational repression (relative to RNA degradation) encode proteins predicted to be synthesized by endoplasmic-reticulum (ER)-associated ribosomes. They speculate that while such messengers can be translationally inhibited, they cannot be detached to be moved to sites for RNA degradation (i.e. P-bodies), in contrast to transcripts associated with free ribosomes. Since the ER is considered to lack proteolytic activity, this also excludes degradation of the nascent polypeptide chain as the mode of miRNA action. Although this model is tempting, they did not extend this analysis to data sets for other miRNA targets. Additional genetic and biochemical analysis is needed to verify this potential mechanism underlying miRNA-based gene silencing.

Although imperfect binding of miRNAs and their target is the most striking feature of miRNA function, additional criteria for miRNA binding have been described that affect silencing, *e.g.* secondary structure of the target, allosteric hindrance by other RNA binding elements, positioning on the 3′ UTR [5-6]. To facilitate miRNA target predictions, a number of algorithms have been created in the past, that take one or several of these features into consideration [7-9]. TargetScan, for example, offers the possibility to include contextual characteristics of potential targets as described in [5]. However, none of the existing algorithms take spatial or temporal expression of miRNA and target into consideration. To investigate the performance of target prediction algorithms, both groups compared their identified protein targets with the results generated by these programs. Not surprisingly, all programs achieve poorly with respect to the absolute number of targets, however TargetScan, Pictar and Diana-MicroT3.0 achieve reasonable results when it comes to discriminating true targets from background: in the best cases, only 40% of the predicted proteins were not identified. However, the number of falsely predicted targets is huge (up to thousands) and the number of missed targets also ranges from hundreds to thousands. Part of this can be explained by the fact that this proteomics approach, like the approach using microarrays, cannot discriminate between direct and indirect effects of miRNAs. However, this does not explain the high number of false positive predictions of the algorithms. This "reverse proteomics" approach made Baek, *et al.* realize that not all mRNAs with seed matches were comparably responsive: they observed that miRNA targets with an A opposite position 1 of the miRNA favors miRNA-mediated protein repression, irrespective of the complementarity to the first nucleotide of the miRNA, a feature implemented in TargetScan. Overall comparison of the algorithms shows that TargetScan is currently the algorithm of choice when it comes to target predictions.

Taken together, the proteomic approaches used by the two groups for the first time allow for systematic miRNA target identification at the protein level. The most striking observation of both studies is that individual miRNAs regulate the levels of hundreds of proteins, although levels do not change dramatically. Most proteins do not change by more than 2 fold, while there is hardly any protein changing by >4 fold upon miRNA interference. This implies that miRNAs fine-tune gene expression, rather than induce dramatic changes. This is in agreement with the fact that hardly any genetic miRNA knockout in *C. elegans* results in an obvious phenotype [10]. One should consider, however, that most targeted messengers are predicted to contain sites for multiple miRNAs. Several different miRNAs could very well contribute independently to the repression of a single target, or even cooperatively, which could result in more dramatic effect. To test this concept, however, complicated combinatorial miRNA knockdowns or overexpression experiments will have to be performed. Given the high number of regulated proteins per miRNA [2, 4], the fact that mRNAs often contain many miRNA target-sites [11] , and the observation that there may exist hundreds or even thousands of different miRNAs [12], the impact of miRNA-based regulation on biological processes is likely to be substantial, while its complexity should not be underestimated.

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