MicroRNA

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# MicroRNAs Repress Mainly through mRNA Decay

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MicroRNAs (miRNAs) are a class of small noncoding RNAs that regulate gene expression post-transcriptionally. They serve as specificity subunits and help RISC (the RNA-induced silencing complex) to find its targets as a result of their potential to base-pair with complementary messenger RNAs, leading to repression (Figure 1 A). This system is related to—and in fact in part identical with—the process of RNA interference (RNAi).[1,2] miRNA-dependent gene regulation is essential during embryonic development, and even throughout adult life many important processes cannot function properly in the absence of the miRNA system.[3] While the biological importance of miRNAs cannot be questioned, there is quite some confusion about the predominant repressive mechanism of RISC (translational repression versus mRNA degradation) and the in vivo mRNA targets which are recognized by each miRNA.[4] It was already known that many mRNAs are subject to miRNA-dependent control, but which ones are really *direct* targets? Furthermore, while reporter–gene studies were quite successful in identifying target sites one at a time, it was impossible to draw quantitative conclusions on the extent of repression which these sites confer on the endogenous target gene that is located in the genome rather than on a transfected plasmid. Two groups have now started to address this conundrum for at least a few miRNAs by taking an approach that combines large-scale protein quantification and transcriptome analysis.[5,6]

Both teams had identical goals: To quantify the influence of miRNA-mediated regulation on the entire proteome and then compare this directly with the corresponding changes observed on mRNA level. If translational repression prevails, then the protein amounts should change more strongly than the mRNA levels. On the other hand, if mRNA degradation is the more important mechanism the inverse will be true. It is relatively straightforward to measure mRNA levels genome-wide with microarrays, but how can entire proteomes be quantified? The key is SILAC technology (stable isotope labeling with amino acids in cell culture), where the origin of a given protein sample is encoded by an isotope label that can

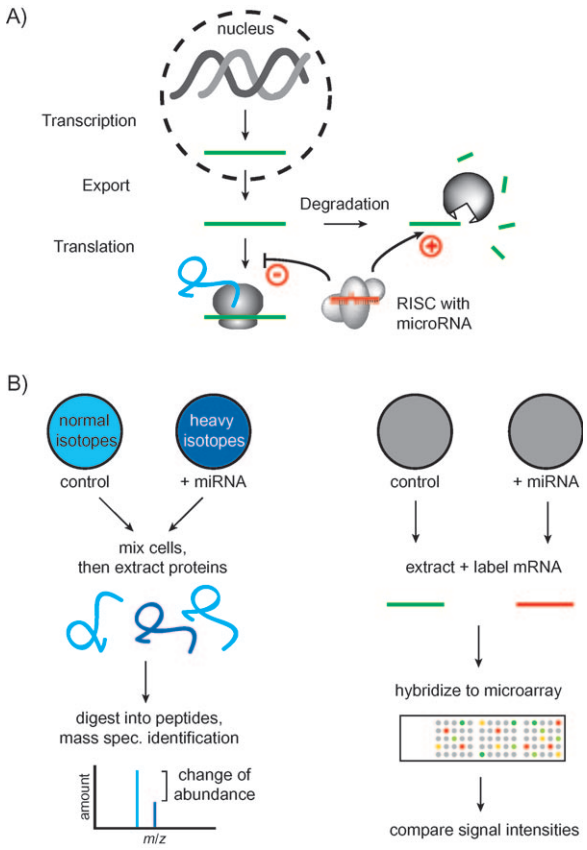


***Figure 1.*** A) Post-transcriptional gene regulation by miRNAs. The RNA-induced silencing complex (RISC) can either repress translation or stimulate the degradation of a targeted mRNA (shown in green). Previously, the exact contribution of each of these activities to overall silencing was unknown. B) Proteome and transcriptome quantification. Left: In the SILAC approach (see text) proteome expression states are encoded with the help of isotopically labeled amino acids in the cell culture dish. The cells are then mixed in a 1:1 ratio and processed together during all subsequent preparation steps, thus eliminating experimental errors. Right: Microarray experiments can reliably quantify changes in the transcriptome between two RNA samples. In the two-color format, RNA is isolated from differentially treated cells and then labeled with different fluorescent dyes (green and red in the picture). After competitive hybridization to microarrays with complementary sequences representing all genes, the signal intensities for each fluorescence channel are a quantitative measure of gene expression. An overlay of the pictures also allows the visual inspection of expression differences according to the composite color.

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be distinguished in modern mass spectrometers (Figure 1B). Experiment and reference are thus pooled very early during sample processing, and the relative changes between the two states become visible after data analysis.[7] A previous study had already exploited this strategy but "only" detected about 500 proteins.[8] The publications by Baek et al. and Selbach et al. now increased this number by a factor of up to 10, depending on the experiment. As if this were not enough, Selbach and colleagues developed a pulse-labeling strategy with a three-state isotope code that allowed them to quantify not only steady-state protein amounts but the actual protein synthesis rates on a proteome-wide scale. This is an advantage especially when one measures the early changes induced by miRNAs since it is not necessary to wait until the new equilibrium between synthesis and decay of a target protein has been reached.

To generate the experimental samples, the two teams employed the same approach and transfected synthetic RNA molecules mimicking miRNA biogenesis intermediates into cultured cells. This significantly increased the active amount of a certain miRNA or even introduced the miRNA into cells that previously did not express it at all. After the cultured cells were given sufficient time to respond to this challenge, the resulting changes in the proteome and transcriptome were analyzed. Even though the actual miRNAs that were overexpressed differed in the two studies, several general conclusions could be drawn immediately. As expected, many proteins changed in response to an increased miRNA dose, but the magnitude of this change was rather small (less than twofold, on average). This is a surprise, given the strong and specific phenotypes of mutant animals with an impaired miRNA biogenesis.[9–16] Furthermore, the changes on the mRNA level were even smaller, which demonstrates that translational repression is indeed occurring in many cases.

A straightforward interpretation of all observed changes is not appropriate because the experiments potentially also recorded secondary effects. These are indirectly caused by the miRNA through the resulting deregulation of a direct miRNA target. For example, if the primary target is a transcription factor, then many observed changes are a result of altered transcriptional output resulting from the hyperrepression of the transcription factor. Thus, they are a bona fide consequence of miRNA transfection but not because the messages are recognized by the miRNA itself. To estimate the contribution of indirect targets to the observed changes in their data sets, both research groups made use of the realization that not all bases of a miRNA contribute equally to the base-pairing interactions with target mRNAs. Throughout the so-called seed sequence, corresponding to positions 2–7 of the miRNA (counting from the 5′-end), perfect complementarity is particularly important, whereas elsewhere mismatches are tolerable.[17–19] Unbiased searches for enriched 6- to 8-mer sequences within the mRNAs coding for proteins that responded to miRNA transfection revealed the correct seed-match for the given miRNA in all cases. This proves that the experiment clearly detects changes of direct miRNA targets. In fact, up to 40%[6] or 60%[5] of all proteins that responded with a decrease of 30% or more to the miRNA transfection contained at least one good match to the miRNA seed sequence within the nontranslated portion at the 3′-end of their mRNA, a known "hot spot" for miRNA target sites. Especially when one considers only mRNAs with a seed-match to the given miRNA, the changes in the transcriptome can account for most of the amplitude of changes at the protein level. This is the first major conclusion and settles a long debate in the miRNA field: Yes, there is translational repression but degradation "takes the cake". There are exceptions to this simple rule, since a few proteins changed without any variation in the abundance of their transcripts. In particular, translational repression seemed stronger for mRNAs translated at ribosomes associated with the endoplasmic reticulum rather than cytosolic ribosomes.

So far, all the results were obtained by artificially increasing the dose of a miRNA within the cell. To see what happens when the level of a particular miRNA is reduced, Selbach et al.[5] introduced an antisense inhibitor of the miRNA *let-7* into cultured cells. In this experimental setup, the abundance of *let-7* target genes will therefore increase. This was indeed true, and summing up all their analyses, Selbach and colleagues state that all effects reported for the *let-7* overexpression experiment also hold true for knockdown of *let-7*. Thus, overexpression experiments with miRNAs can yield physiologically relevant results. Baek and colleagues[6] also wanted to validate their findings in a miRNA loss-of-function setup. Their strategy was to use bone marrow cells derived from wild-type or miR-223 knockout mice and then differentiate these into neutrophils in vitro. In the wild-type case, miR-223 expression is induced strongly during this differentiation. After comparing the proteomes and transcriptomes, the authors also came to the conclusion that the miRNA knockout experiment confirmed the targeting rules derived from overexpression studies.

Since the recognition of an mRNA by a miRNA follows the principles of base-pairing, it should be possible to predict miRNA/target pairs from genomic sequence data. However, because perfect base-pair matches often do not extend beyond the seed sequence, additional criteria such as evolutionary conservation, position within the mRNA, and local sequence context need to be taken into account in order to reduce the false-positive rate in miRNA target prediction algorithms. Can this help us to extrapolate from the now-published experiments to other miRNAs? To answer this question, both teams compared their large sets of experimentally validated targets with the predictions obtained from a variety of programs. This must have been a very rewarding task because they had developed the two top-scoring algorithms (TargetScan and PicTar)! But despite this success, up to two-thirds of the predicted targets turned out to be false positives.

From a practical point of view, the two studies point out several important aspects. First of all, since most of the repression is occurring at the level of the mRNA, an experimental identification of miRNA targets can—at least as a first step—rely on the well-established and sensitive techniques for mRNA quantification. In addition, a miRNA overexpression experiment, which is often easier to achieve than complete inhibition or a genetic knockout, apparently identifies the physiological targets. This should be taken with

  

a note of caution, though, since few direct comparisons exist. Good target prediction programs can certainly help to generate new hypotheses, but experimental validation is indispensable. The most important message from the two papers is, however, that we have to stop thinking about miRNAs primarily as translational repressors. Rather, they are versatile recognition platforms that can attract a variety of activities to their targets—in some cases they might even be able to stimulate translation.[20] With such a complex outcome for the mRNAs upon targeting by a miRNA, the new question is how to distinguish between cause and consequence. Was the translation of all degraded messages initially repressed, or are mRNA decay and translational control operating completely independently?

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