

# The evolving role of microRNAs in animal gene expression

Katlin B. Massirer and Amy E. Pasquinelli\*

## Summary

MicroRNAs (miRNAs) constitute an abundant family of 22-nucleotide RNAs that base-pair to target mRNAs and typically inhibit their expression. To assess the global impact of animal miRNAs on gene regulation, the expression of predicted targets and their cognate miRNAs was extensively analyzed in mammals and *Drosophila*.<sup>(1,2)</sup> In general, targets are co-expressed at relatively low or undetectable levels in the same tissues as the miRNAs predicted to regulate them. Additionally, genes that are highly co-expressed with miRNAs usually lack target sites. The authors conclude that many animal genes are under evolutionary pressure to maintain or avoid complementary sites to miRNAs.<sup>(1,2)</sup> Thus, the miRNA pathway broadly contributes to the complex gene regulatory networks that shape animal tissue development and identity. *BioEssays* 28:449–452, 2006.

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## miRNAs regulate target genes that contain complementary sequences

The turn of the millennium ushered in a new and surprisingly abundant class of RNA regulatory genes known as microRNAs (miRNAs). The first miRNA gene, *lin-4*, was discovered as an essential regulator of development in the nematode *Caenorhabditis elegans*.<sup>(3)</sup> The function of the 22-nucleotide RNA product expressed from this gene might have been inexplicable if genetic studies had not already suggested that *lin-4* was responsible for negatively regulating the protein-coding gene *lin-14* via its 3' untranslated region (3' UTR).<sup>(4)</sup> Simple visual comparison of the *lin-4* RNA sequence with elements in the 3' UTR of the *lin-14* messenger RNA (mRNA) revealed a direct mechanism for regulation: the *lin-4* RNA could base-pair to multiple sites in the *lin-14* 3' UTR, which would then inhibit expression of LIN-14 protein.<sup>(3,5)</sup> This first

example of miRNA function is the basis for predicting how miRNAs recognize and regulate specific target genes.

Now that we know of hundreds of distinct miRNA genes, there is the enormous task of matching them to direct targets and understanding their biological roles. Curiously, plant miRNAs often share near perfect complementarity with their targets, greatly facilitating the task of matching them to partners of regulation. In contrast, perfect base-pairing between the known animal miRNAs and target genes is extremely rare. Instead, it appears that, in many cases, strong base-pairing between the 5' half of the miRNA and its target may be sufficient to mediate regulation in animals.<sup>(6–8)</sup> Specifically, nucleotides 2 to 7 at the 5' end of the miRNA have been christened the “seed” and this relatively small amount of sequence information can impart specificity and functionality to a miRNA–target interaction.<sup>(9)</sup> Armed with the observation that most miRNAs exhibit higher conservation at their 5' ends and a handful of experiments supporting the functional importance of the 5' end of the miRNA, many groups devised computational approaches to predict direct targets of specific miRNAs, generally by searching for seed complementarity in mRNA 3' UTRs.<sup>(10)</sup> The requirement for cross species conservation of predicted target sites narrows the results, but still leaves hundreds of potential targets for most miRNAs, which is not surprising given the short length of the seed. This parameter may also exclude genuine target sites that are functional but not shared in orthologues across species. The abundance of miRNAs and predicted targets suggests that this regulatory system has a significant influence on gene expression and evolution. This idea is persuasively bolstered by recent papers that combine extensive miRNA and mRNA expression data with target prediction methods.<sup>(1,2)</sup> The authors conclude that miRNAs have had a widespread influence on spatial and temporal gene expression patterns in mammals and insects.<sup>(1,2)</sup>

## miRNAs downregulate the expression of target mRNAs

Animal miRNAs function to inhibit protein expression of their direct targets, but it has been unclear if this downregulation coincides with a substantial reduction in target mRNA levels. The first indication that human miRNAs might broadly regulate the levels of their target mRNAs came from microarray experiments comparing changes in gene expression patterns

Department of Biology, University of California, San Diego, La Jolla, California

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\*Correspondence to: Amy E. Pasquinelli, Department of Biology, University of California, San Diego, La Jolla, CA 92093-0349.

E-mail: apasquin@ucsd.edu

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in HeLa cells after transfection of specific miRNAs.<sup>(11)</sup> Introduction of either mir-1 or mir-124 miRNAs resulted in downregulation by at least 1.5-fold of dozens of genes. Remarkably, the majority of repressed genes contained sequences complementary to the seed of the specific transfected miRNA and, consequently, were predicted to be direct targets of regulation. Another common feature of the downregulated genes is that they tended to share tissue-specific expression patterns distinct from that of the miRNA. For example, mir-1 is preferentially expressed in heart and skeletal muscle and introduction of this miRNA into HeLa cells resulted in downregulation of 96 genes that are generally detected at lower levels in heart and skeletal muscle compared to other tissues. Complementarity to the mir-1 seed was found in 88% of the 3'UTRs of downregulated genes. A similar pattern of downregulated transcripts containing mir-124 sites was observed when this brain-specific miRNA was transfected into HeLa cells. The authors concluded that some miRNAs may contribute broadly to tissue identity by negatively regulating the steady-state levels of many target mRNA transcripts.<sup>(11)</sup>

The apparent ability of a single miRNA to sway the gene expression pattern of HeLa cells towards that of a specific tissue prompted the question of whether miRNAs exert this power in normal tissue development and specification. Farh, Grimson and colleagues tackled this problem by using the mouse expression atlas to compare the expression patterns for predicted targets of six different tissue-specific miRNAs.<sup>(1)</sup> They found that mRNAs containing target sites for a given miRNA were generally expressed at low levels in the tissues enriched for the miRNA. Mirroring the effect of mir-1 in HeLa cells,<sup>(11)</sup> endogenous predicted targets of mir-1 tended to be downregulated specifically in skeletal muscle.<sup>(1)</sup> Most of the mir-1 targets were detected in muscle tissue but they were expressed at lower levels than in other tissues, implying that the presence of a miRNA is related to the relative expression levels of its targets.

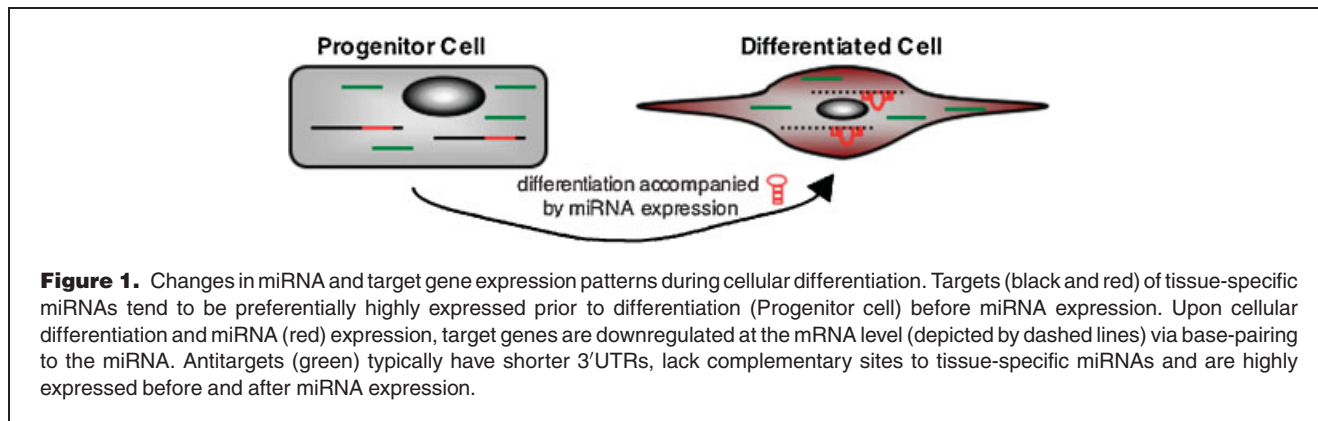
But is the inverse correlation between miRNA and target mRNA levels in a given tissue caused by expression

of the miRNA (Fig. 1)? Evidence in favor of this idea emerged from mRNA expression analyses of differentiating myoblasts. In general, predicted targets of mir-1 were highly expressed in myoblast cells but upon differentiation into myotubes, which coincides with induction of mir-1, the levels of these same targets plummeted.<sup>(1)</sup> Comparable changes in miRNA and target expression levels were observed during mouse embryogenesis, strengthening the authors' conclusion that miRNAs downregulate the levels of target transcripts to help define tissue-specific gene expression patterns.<sup>(1)</sup>

The trend of miRNAs being enriched in tissues where their targets are diminished extends to *Drosophila*. Stark, Brennecke and colleagues compared their sets of predicted miRNA targets to mRNA in situ hybridization data for *Drosophila* gene expression patterns during embryogenesis.<sup>(2)</sup> At the resolution of in situ experiments, it appeared that miRNAs and their targets have mutually exclusive expression patterns in *Drosophila*, leading the authors to conclude that miRNAs contribute to the accuracy of tissue-specific gene expression patterns.<sup>(2)</sup>

**Antitargets avoid miRNA complementary sites**

If target genes conserve sequences that support regulation by specific miRNAs, then do genes that are co-expressed with tissue-specific miRNAs lack such sites? The Bartel group addressed this question by searching for specific miRNA target sites in transcripts that were enriched in the same mouse tissue as the miRNA.<sup>(1)</sup> Genes found to be highly co-expressed with a miRNA typically lacked target sites for that miRNA. Their results support the concept of antitargets - genes that are depleted of sites for a specific miRNA. *Drosophila* genes also appear to be constrained to avoid specific miRNA target sites in genes co-expressed with the miRNA.<sup>(2)</sup> Broadly expressed genes, such as ribosomal associated genes, seem to avoid miRNA targeting by exclusion of target sites and maintenance of relatively short 3'UTRs.<sup>(1,2)</sup> Together these studies indicate that avoidance might be as important as conservation of



**Figure 1.** Changes in miRNA and target gene expression patterns during cellular differentiation. Targets (black and red) of tissue-specific miRNAs tend to be preferentially highly expressed prior to differentiation (Progenitor cell) before miRNA expression. Upon cellular differentiation and miRNA (red) expression, target genes are downregulated at the mRNA level (depicted by dashed lines) via base-pairing to the miRNA. Antitargets (green) typically have shorter 3'UTRs, lack complementary sites to tissue-specific miRNAs and are highly expressed before and after miRNA expression.

miRNA target sites in the evolution of tissue-specific gene expression patterns.

### **miRNAs exert evolutionary constraints on gene expression patterns**

Typically, thousands of mammalian genes contain a sequence complementary to the 7-nucleotide seed of a given miRNA, but only one tenth of these target sites are conserved across species.<sup>(12,13)</sup> To determine if non-conserved sites are functional, Farh, Grimson and colleagues generated reporter genes fused to the 3'UTRs of genes containing conserved or non-conserved target sites.<sup>(1)</sup> Transfection of the reporter genes along with their cognate miRNAs into HeLa cells resulted in comparable levels of repression mediated by conserved or non-conserved sites. However, targets in these two categories are distinguished by their general tissue-specific expression patterns. Genes with non-conserved target sites tended to be expressed in tissues lacking the miRNA that would recognize that site. The authors propose that emergence of new miRNA target sites will only be conserved if tissue-specific regulation via that site offers selective advantages.<sup>(1)</sup> Likewise, there is evolutionary pressure to avoid complementarity to specific miRNAs in genes highly co-expressed with that miRNA.<sup>(1,2)</sup>

### **Regulation by miRNAs is widespread and diverse**

The ability of miRNAs that partially base-pair with their targets to cause downregulation at the mRNA level was not widely recognized until this past year. In fact, the degree of complementarity of a miRNA with its target appeared distinguishing at the mechanistic as well as the organismal levels. In many cases, plant miRNAs can pair perfectly with their targets to direct cleavage and degradation of the mRNA.<sup>(14)</sup> However, the first plant miRNA identified by genetic methods guides cleavage of its targets through recognition of sites that include four or five mismatches,<sup>(15)</sup> and similar examples of non-stringent base-pairing for plant miRNA function were reported last year.<sup>(16)</sup> Specific examples of animal miRNAs regulating the level of mRNAs containing partially complementary target sites also recently emerged.<sup>(17,18)</sup> Thus, an outcome of regulation by some miRNAs appears to be degradation of the target mRNA.

Large-scale microarray analyses that compare changes in gene expression in response to the presence of specific miRNAs show that many targets are subject to downregulation at the mRNA level.<sup>(1,2,11,16,19)</sup> Although mRNA degradation may be a secondary consequence of translational repression,<sup>(20)</sup> it provides a robust signature of miRNA regulation for numerous targets. In many cases, target mRNA levels diminish but do not disappear.<sup>(1,11)</sup> Moderate downregulation at the mRNA level, possibly in combination with translational

repression, may be sufficient to maintain tissue or developmental gene expression patterns. Thus, the evolution of perfect target complementarity to direct immediate cleavage may be limited to select genes.

Moreover, a delay or inability to degrade select miRNA targets may be advantageous to sequester repressed transcripts for the possibility of re-activation. This flexibility in regulatory mechanisms could contribute to the rapid changes in gene expression demanded in neuronal function, for example. This idea is supported by the recent report that a brain-specific rat miRNA inhibits a kinase gene important for dendritic spine development.<sup>(21)</sup> Extracellular signaling releases the miRNA target from translational repression in the dendritic spines.<sup>(21)</sup> Curiously, the Bartel group observed complex expression patterns for the conserved targets of two different brain-specific miRNAs.<sup>(1)</sup> Unlike the patterns for other tissue-specific miRNAs, targets of the brain miRNAs did not conform to the expressed but downregulated trend. The authors suggest that heterogeneity of cell types in brain could contribute to this discrepancy between the expression pattern in brain compared to that of other tissues.<sup>(1)</sup> It is also tempting to speculate that brain miRNAs might avoid degradation of certain target mRNAs to prepare those transcripts for rapid translation in response to external cues.

### **Conclusion and outlook**

From the extensive comparison of temporal and spatial expression patterns of miRNAs and mRNAs in mammals and *Drosophila* emerges the conclusion that gene expression and evolution are heavily shaped by miRNAs.<sup>(1,2)</sup> There appears to be selection for conserved target sites in mRNAs downregulated by tissue-specific miRNAs and against such sites in genes highly co-expressed with miRNAs. Now that the general trend for the effect of miRNAs on target expression is established, the exceptions should be carefully considered. Are predicted targets that maintain relatively high expression levels in the same tissue as the miRNA not genuine targets? Or are they subject to repression exclusively at the translational level or through a feedback loop that obscures changes in mRNA levels? The general mechanism for miRNA-mediated repression remains poorly understood, but a careful examination of specific miRNAs and their targets in their natural tissue contexts may shed more light on the molecular functions of these tiny regulatory RNAs. Considering the potential impact of single miRNAs on hundreds of target genes, an outstanding challenge is to determine how temporal and tissue-specific expression patterns for miRNAs are achieved. The profound influence of miRNAs on evolution and gene expression is unquestionable, and researchers now have the daunting task of incorporating these RNAs into specific gene regulatory networks.

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