OPINION

Micromanagers of gene expression: the potentially widespread influence of metazoan microRNAs

David P. Bartel and Chang-Zheng Chen

We propose that the microRNA milieu, unique to each cell type, productively dampens the expression of thousands of mRNAs and provides important context for the evolution of all metazoan mRNA sequences. For genes that should not be expressed in a particular cell type, protein output is lowered to inconsequential levels. For other genes, dosage is adjusted in a manner that allows for customized expression in different cell types while achieving a more uniform level within each cell type. In these ways, the microRNAs add an extensive layer of gene control that integrates with transcriptional and other regulatory processes to expand the complexity of metazoan gene expression.

MicroRNAs (miRNAs) are endogenous ~22-nucleotide RNAs, some of which are known to pair with the mRNAs of protein-coding genes to specify their post-transcriptional repression[1–4]. The first miRNAs to be discovered were the *lin-4* and *let-7* RNAs, which were identified genetically on the basis of their roles in controlling the timing of *Caenorhabditis elegans* larval development[5,6]. In the past year, the list of reported miRNA functions has grown rapidly to include the control of cell proliferation, cell death and fat metabolism in flies[7,8], neuronal patterning in nematodes[9], modulation of haematopoietic lineage differentiation in mammals[10], and the control of leaf and flower development in plants[11–14]. The list of interesting genes that are targeted by miRNAs with unexplored biological consequences has also increased markedly[15–18]. Both the biogenesis and the action of miRNAs rely on components of the RNA-INTERFERENCE (RNAi) machinery, but miRNAs are distinct from the SMALL INTERFERING RNAs (siRNAs) of RNAi in several respects: miRNAs are generally conserved

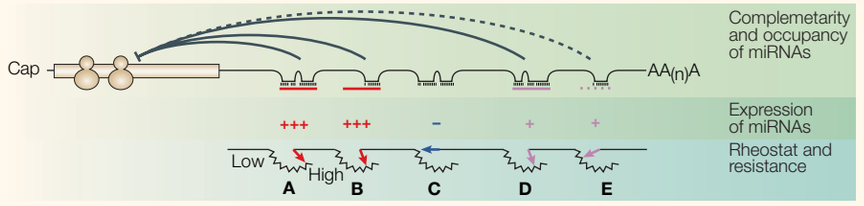

Figure 1 | **The combinatorial rheostat analogy.** Each microRNA (miRNA) complementary site within the mRNA 3′-untranslated region is analogous to a rheostat — that is, an adjustable resistor (bottom of figure; zig-zag arcs indicate a resistor; arrows indicate the adjustment). Rheostats A, B, D and E function together to dampen the productive translation of the mRNA to achieve the optimal protein level for this cell type. Rheostats A and D are adjusted to high resistance by the extensive complementarity to the corresponding miRNAs (top of figure), which ensures that the complementary sites are fully occupied, even in the case of rheostat D, in which the 'purple' miRNA is expressed at only moderate levels in this cell type (middle of figure). Rheostat B is also adjusted to high resistance, in this case by the high level of the 'red' miRNA expression in this cell type, which ensures that the complementary site is almost fully occupied despite its weak complementarity to the 'red' miRNA. Rheostat C is adjusted to no resistance, despite strong complementarity to the 'blue' miRNA, by the absence of the 'blue' miRNA in this cell type. Rheostat E is adjusted to low resistance by the moderate expression of the 'purple' miRNA coupled with weak complementarity to this miRNA, which together lead to intermittent occupancy of the complementary site (indicated as a dashed purple line). Intermittent occupancy, if integrated over time and multiple messages in the cell, results in low but detectable resistance. For this mRNA, the resistance changes in cell types with different effective concentrations of 'red', 'blue' or 'purple' miRNAs. It also changes over the course of evolution as the miRNA complementary sites adapt to increase or decrease their pairing with these miRNAs to fine-tune the level of protein that is produced.

in evolution, they direct the silencing of genes that are unrelated to the loci that encode the miRNAs themselves, and each miRNA comes from a gene that is dedicated to the production of a particular ~22-nucleotide RNA[4].

MiRNAs are among the more abundant gene-regulatory molecules in the animal cell, constituting almost 1% of the predicted genes in humans, worms and flies[19–21]. Many are differentially expressed during the course of development and differentiation[1–3,5,10,20,22–27], leading to the prospect that each cell type might have a unique miRNA profile. To specify repression, metazoan miRNAs seem to require only short stretches of complementarity to an mRNA[16,28,29]. Multiple miRNAs might need to bind to a particular untranslated region to achieve repression, but, in principle, this could be accomplished by the combinatorial action of different miRNA species. Considering the abundance, differential expression and potential promiscuity of metazoan miRNAs (and often borrowing from established concepts of transcriptional regulation), we propose that the miRNA milieu, unique to each cell type, could provide a crucial context for the evolution of all mRNA sequences and could modulate the use of a substantial fraction of the metazoan transcriptome. For genes that should not be expressed in a particular cell type, protein output is lowered to inconsequential levels. For other genes, dosage could be adjusted to in a manner that allows for customized expression in different cell types but a more uniform level within each cell type. We refer to this widespread, often subtle and customized, influence of miRNAs on mRNA expression as the 'micromanager model' for miRNA function.

Micromanaging gene expression

In our model, each miRNA complementary site is a component of the metazoan gene-regulatory circuitry that can be likened to a dimmer switch, or to an adjustable resistor, known as a rheostat, in an electric circuit (FIG. 1). The resistor analogy seems fitting because, for all known cases in which gene expression responds to the presence of a miRNA, gene expression decreases[5–7,11–14,16–18,28,30–32] just as a resistor that is placed into an electric circuit causes the current to decrease. Furthermore, in the instances of metazoan miRNA response in which the protein that is translated from the targeted mRNA has been measured and reported, the protein level decreases but is not extinguished[28,33,34], again analogous to a resistor, which dampens but does not eliminate the flow of current.

In the rheostat analogy, the 'resistance' that is imparted by a miRNA is adjusted in two different ways (FIG. 1). It adjusts as the miRNA-expression profiles change during cellular differentiation and development. Each cell type expresses dozens of different miRNAs, and the identities or expression levels of many of these miRNAs change from cell type to cell type, thereby adjusting the amount of resistance. The resistance also adjusts over evolutionary time. During the course of evolution, each miRNA target site adapts to increase or decrease its pairing to the miRNA, and in this way, fine-tunes the degree of repression in cells that express the corresponding miRNA species. Further heritable variation in gene expression arises with the emergence of new resistors, either in the form of new complementary sites in the messages or even entirely new miRNA genes. The relative simplicity of miRNA genes, together with the diverse sequences of miRNA gene families in both plant and animal lineages[20,35,36], indicates that *de novo* emergence of new miRNA genes with their consequent impact on mRNA regulation might occur relatively frequently in evolution.

In the micromanager model, the miRNA targets fall into three categories, which we call 'switch targets', 'tuning targets' and 'neutral targets' (FIG. 2). Some messages should not be expressed in a particular cell type. These mRNAs, which include the first miRNA targets to be identified — *lin-14*, *lin-28* and *lin-41* in *C. elegans*[5,6,28,30] — take advantage of the miRNAs to dampen protein production to inconsequential levels in specific cell types or developmental stages. These mRNAs are classified as switch targets because the result is equivalent to a discrete off switch. Among the switch targets are mRNAs that linger from a previous environmental or developmental state and perhaps mRNAs that arise from leaky transcription. A second potential class of targets comprises mRNAs for proteins that are optimally expressed at only low levels in particular cell types. Such mRNAs could take advantage of the miRNA milieu, fine-tuning their complementarity to the relevant miRNAs to achieve optimal expression in each cell type. We refer to messages of this type as tuning targets. The third class of targets comprises mRNAs that fortuitously pair with miRNAs, but their consequent downregulation of protein production is tolerated or offset by feedback mechanisms. If there is no selective pressure to maintain or decrease pairing, these 'bystander' mRNAs are neutral targets.

Because all the mRNAs of the cell are exposed to the miRNA milieu, the miRNAs are expected to directly influence the evolution of a subset of both the targets and the non-targets (FIG. 3). Switch and tuning targets are under selective pressure to maintain pairing to the relevant miRNAs, although tuning targets must also avoid too much complementarity to the miRNAs. With regard to non-targets, mRNAs for abundant proteins must avoid fortuitous complementarity to the multitude of miRNAs that are present in the cells in which they are expressed. Such complementarity could dampen their expression and titrate the miRNAs away from their correct targets. These non-targeted mRNAs with sequences

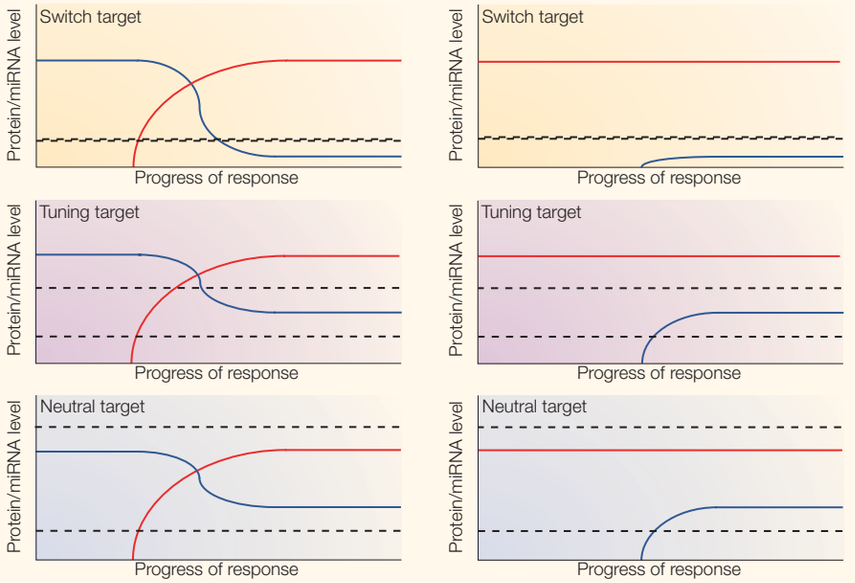

Figure 2 | **Three categories of microRNA targets.** The dashed lines indicate critical thresholds of protein expression; the upper line indicates the level that would be undesirably high in the cells that express the microRNA (miRNA), the lower line indicates the level below which the protein no longer exerts its effect. In the top panel, the two dashed lines overlap because these two thresholds are at the same level. Left panels: with the onset of a new developmental stage or environmental cue, cells induce miRNA expression (red), which in turn dampens protein expression (blue) of targets that were expressed before the developmental/environmental change. Right panels: protein expression of analogous targets can also be dampened by pre-existing miRNAs. Switch targets are repressed to inconsequential levels[6]. Tuning targets are dampened to functional but not undesirably high levels. Neutral targets are repressed but remain within the optimal range. More complex categories that involve different target classifications with respect to particular miRNAs are expected as sequences for mRNAs that are expressed in different cell types adapt to the miRNA milieu of each cell type.

that are under selective pressure to repel miRNAs are classified as anti-targets. In total, many mRNAs are anticipated to be influenced by miRNAs, either in terms of their expression (switch targets, tuning targets and neutral targets) or their evolution (switch targets, tuning targets and anti-targets).

The rheostat is combinatorial because multiple miRNA species can regulate a single mRNA, analogous to rheostats placed in series in a circuit (FIG. 1). Indeed, miRNA-like regulation has been shown to be cooperative, further enhancing the influence of multiple miRNA complementary sites[37]. Although only a small fraction of the miRNA–mRNA regulatory pairs are known in animals, there are already instances — for example, for the *lin-4* and *let-7* targets (*lin-14*, *lin-28*, *lin-41* and *hbl-1* mRNAs) — in which different miRNA species have been proposed to regulate the same targets[6,31,32,38]. These examples, and the analogy to other biological regulatory systems — most notably, transcriptional regulation — have led to the general expectation that as the list of known miRNA–mRNA regulatory interactions becomes more comprehensive, combinatorial control will be common, if not the norm.

Previous models for miRNA-based regulation in both animals and plants imply that as miRNA expression begins, the protein that is encoded by the targeted mRNA diminishes to a negligible amount[6,15]. This discrete off switch describes the relationships between miRNAs and their classical targets well, because in these cases, the protein seems to drop below the threshold of functional significance, even if it does not disappear completely[5,6,28,30]. With the rheostat analogy, the micromanager model extends the previous models to allow for tuning targets, which are proposed to be actively involved in the cell even while their expression is being dampened by miRNAs. A second extension of the previous models is the idea that metazoan miRNAs modulate the expression of a substantial fraction of protein-coding genes. Because the ideas of tuning targets and of many targets are the important differences from previous models, these features are discussed in more detail below. At the same time, we emphasize that our model is not mutually exclusive with models that are based on the discrete switch; instead, it encompasses the switch while accommodating more subtle, but more pervasive, layers of gene regulation.

Why post-transcriptional tuning?

Post-transcriptional gene control seems wasteful at first glance. It would seem more economical to save resources by the efficient use of fewer mRNAs. Nonetheless, biology is replete with evidence that evolution does not necessarily optimize efficiency, and the added opportunity for gene control that is afforded by miRNAs could by itself justify the emergence of the miRNA-based regulatory system. Furthermore, as has been proposed for plant miRNAs[15], the targeted clearing of regulatory mRNAs from daughter-cell lineages — particularly those messages that specify the undifferentiated state — might be important during cellular differentiation (for example, to allow rapid daughter-cell differentiation without having to depend on regulatory genes having constitutively unstable messages). In this situation, greater overall efficiency might be achieved because the mRNAs of genes that are targeted by miRNAs could be more stable in the absence of the miRNA and would not need to be constantly replenished in the cells in which they function.

We suggest that there are important advantages to dampening the use of some mRNAs, apart from the need to turn off expression during differentiation. As is commonly noted, post-transcriptional control could be more responsive than transcriptional control, in terms of both speed and reversibility. Furthermore, some genes, particularly regulatory genes, might have a narrow window of optimal expression — several-fold more or less would have undesired consequences. If, in a particular cell, efficient use of a single mRNA would produce too much protein, then there would be an obvious need to dampen the use of this mRNA in translation. Even if efficient use of a single mRNA produced the optimal level of protein, it would be difficult for the cell to maintain this optimal level because this would require precisely one mRNA to be in that cell at all times. For an unstable protein, expression of zero, two or three copies of its mRNA would lead to substantially different protein levels. Expressing multiple copies of the mRNA while simultaneously dampening their use would provide a means to smooth out the stochastic fluctuations in gene expression to achieve a more constant protein level.

There are constitutive mechanisms for dampening mRNA use, including suboptimal KOZAK SEQUENCES, upstream start codons, mRNA structure and the use of rare codons. The miRNA-based gene-regulatory system provides a more flexible and conditional option that would be particularly useful when mRNA expression must be fine-tuned to different levels in different cell types. The usefulness of

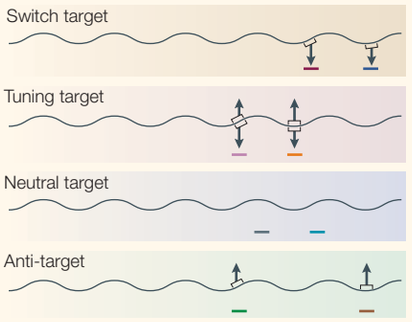

Figure 3 | **The evolutionary pressure that strengthens or weakens mRNA pairing to the microRNAs.** Upward arrows (pointing away from the microRNAs (miRNAs)) represent a selective advantage for mRNA-sequence changes that disrupt fortuitous pairing with the miRNAs. Downward arrows (pointing towards the miRNAs) represent selective pressure for maintaining or improving pairing to the miRNAs. Similar concepts would hold for accessory proteins that might help to mediate miRNA specificity.

miRNA-based regulation would increase with the number of cell types, perhaps providing an explanation for why miRNAs have so far not been identified in single-cell organisms. An alternative would make use of constitutive dampening mechanisms and would achieve differential expression in different cell types exclusively by altering transcriptional output. However, in metazoans with many cell types, such transcriptional fine-tuning might require excessively complex promoters. With the added layer of miRNA regulation, the system becomes more modular, allowing each individual component to be simpler — each promoter can be less complex because transcriptional regulation is distributed among the promoters of both the protein-coding genes and the miRNA genes. In summary, the post-transcriptional dampening of gene expression by miRNAs offers both a mechanism for more uniform gene expression for cells of a particular type and a simple means to customize this expression level for each distinct cell type.

Many regulatory targets

The idea that all mRNAs are evolving in the context of the miRNA milieu predicts that many genes — particularly those with low optimal expression levels — are taking advantage of, or are at least tolerant of, miRNAs dampening their expression. To micromanage so many targets, many miRNA molecules would need to be present in each cell. This is indeed the case: certain *C. elegans* miRNAs, such as *miR-2*, are present at an average of 50,000 molecules per cell, an abundance that is hundreds, if not thousands, of times greater than that of typical mRNAs[20]. In fact, the abundance of *miR-2* and several other miRNAs is at least equivalent to that of U6 snRNA, showing that, overall, the abundance of all the miRNAs in the cell far exceeds that of the SPLICEOSOME. This implies that the miRNAs, together with their associated proteins, comprise one of the more abundant ribonucloeprotein complexes in animal cells. It is therefore plausible that a single miRNA species could modulate the expression of a hundred mRNA targets, and that the miRNAs of an animal could cooperate to dampen the expression of thousands of targets.

What then are these miRNA targets? Before considering the metazoan targets, it is useful to consider the known plant miRNA target relationships, which so far provide scant evidence of either combinatorial rheostats or the micromanagement of many targets. Each plant miRNA apparently regulates a single target or one or two closely related families of targets by pairing to a single site within each mRNA[15,35]. These sites are extensively complementary to the miRNA[15], such that a single site mediates efficient cleavage of the messages[39,40]. The complementary sites are also highly conserved in orthologues of the targets, which indicates that their disruption could lead to a substantial decrease in fitness[15]. Plants do have some tuning targets. The miRNA targeting of *ARGONAUTE1* and *DICER-LIKE1*, two genes that are implicated in plant miRNA function and biogenesis, respectively, indicate that negative-feedback pathways might tune expression of these two genes[15,41]. Such feedback, which itself relies on the function of the repressed gene, could prevent overexpression of the gene, but would have intrinsic difficulty in sustaining repression below the functional threshold. Despite the presence of these and possibly other instances of tuning, the overall picture in plants is currently one in which each miRNA functions non-combinatorially to repress just a handful of related targets. Perhaps fine-tuning gene expression is not as important for plants — compared with animals, they seem to be much more tolerant to chromosome trisomies and other aberrations that would affect gene dosage[42]. Alternatively, micromanagement of many targets might be present in plants but will be revealed only after the application of more sophisticated methods to computationally identify targets in plants.

Regulatory targets of animal miRNAs can be computationally identified but with more effort (and less confidence)[16,17]. These mRNAs are identified on the basis of their unusual propensity to have multiple short conserved segments of complementarity to miRNAs in their 3′-untranslated regions. Using cutoffs that achieve a signal-to-noise ratio of 3.2:1.0, approximately six predicted targets are reported for each mammalian miRNA — a modest number that is well within the range of the previous models[16]. There are, however, reasons to suspect that these predicted targets represent only a small fraction of the total targets. Neutral targets and species-specific targets would be invisible in this and other analyses that depend on evolutionary conservation. More importantly, the computational methods are rudimentary and are expected to miss many of the conserved targets. A set of vertebrate Hox genes is an example of conserved targets that were previously missed because they did not satisfy the particular pairing criteria that were implemented to achieve an acceptable signal-to-noise ratio[18]. Many others have probably been missed because of the preference given to multiple complementary sites to a single miRNA but not to multiple miRNA species.

Although improved computational methods will be required to reveal the full breadth of metazoan miRNA control, the microman-

Glossary

KOZAK SEQUENCE
A consensus sequence element within the 5′-untranslated region of eukaryotes that enhances the recognition of a nearby start codon.

RNA INTERFERENCE
(RNAi). Post-transcriptional gene silencing in animals, triggered by dsRNA that corresponds to the target gene. The dsRNA is processed to small interfering RNAs that serve as guide RNAs for the recognition and cleavage of complementary mRNAs. Analogous silencing processes occur in plants, some fungi and other eukaryotes. The molecular machinery of RNAi is also required for RNA-mediated DNA silencing and for the biogenesis and function of microRNAs.

SMALL INTERFERING RNAS
(siRNAs; also known as short interfering RNAs). Small RNAs, typically 21–23 nucleotides in length, that act as guide RNAs to specify the cleavage of mRNAs during RNA interference (RNAi). Heterochromatic siRNAs are also implicated in the RNAi-related process that silences DNA. siRNAs differ from microRNAs in several respects: they are generally not conserved in evolution, they naturally come from long RNA duplexes that are processed such that many siRNA species come from each duplex and they are typically derived from mRNAs, transposons, viruses or heterochromatic DNA, all of which can be targeted for silencing by the siRNAs.

SPLICEOSOME
The intron-removing apparatus in eukaryotic nuclei.

U6 SNRNA
One of five integral RNA components of spliceosomes, U6 snRNAs are riboprotein complexes that assemble on primary transcripts of eukaryotic mRNAs to catalyse the excision of introns.

ager model is consistent with numerous observations from the initial computational efforts[16,17,43]. The idea that many mRNA targets are subtly regulated by each miRNA helps to explain the observation that the animal miRNAs are much more conserved than is the typical miRNA complementary site: a mutation in the miRNA leading to the sub-optimal dosage of many targets would be far less tolerated than a mutation in a particular complementary site. For most genes that are subject to the rheostat, an mRNA mutation that renders the message unresponsive to the miRNA would be expected to lead to sub-optimal dosage, but would have an effect on fitness that is sufficiently small to allow the emergence of compensatory changes, including increased complementarity to another miRNA. The idea of many regulatory targets for each miRNA is also consistent with the observation that short stretches of complementarity between the miRNA and the mRNA seem to be sufficient for miRNA-specified repression. Such non-stringent pairing requirements would make possible a large and diverse set of regulatory targets, and would facilitate evolutionary acquisition of new or enhanced miRNA control. The idea that each complementary site represents more of a rheostat than a switch explains why miRNAs influence the expression of reporter genes that contain computationally identified complementary sites only modestly (typically between two- and tenfold)[16].

Conclusion

How could this proposed widespread influence on metazoan gene expression have gone unnoticed? A similar question can be asked of the miRNAs themselves, which have only recently been discovered to comprise such an abundant class of gene regulators[1–3]. Many factors seem to have conspired to delay this discovery[44]. Only three miRNAs have been uncovered genetically through hypomorphic or null lesions[5,6,9], which can partly be explained by the greater difficulty in hitting the smaller-sized miRNAs by random mutagenesis, the absence of nonsense mutations as a source of null mutants, the potential preference for investigators to pursue lesions that fall within open reading frames and the potential for related miRNAs to have redundant or overlapping functions (an especially acute concern in *Arabidopsis*, in which nearly all known miRNAs have an identical or close potential paralogue[35]). MiRNA-mediated micromanagement might be even more difficult to expose by forward genetics. Although each miRNA might be participating in fine-tuning the expression of many genes, the loss of a typical miRNA (even one that is highly conserved) would be expected to lead to pleiotrophic but perhaps subtle or impenetrant phenotypic consequences. Now that the abundance of this gene class has been uncovered, understanding the scope of its functions cannot be far off.

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Competing interests statement

The authors declare that they have no competing financial interests.

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FURTHER INFORMATION

David Bartel's laboratory:
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