



Review

Modulation and Evolution of Animal Development through microRNA Regulation of Gene Expression

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Abstract: microRNAs regulate gene expression by blocking the translation of mRNAs and/or promoting their degradation. They, therefore, play important roles in gene regulatory networks (GRNs) by modulating the expression levels of specific genes and can tune GRN outputs more broadly as part of feedback loops. These roles for microRNAs provide developmental buffering on one hand but can facilitate evolution of development on the other. Here we review how microRNAs can modulate GRNs during animal development as part of feedback loops and through their individual or combinatorial targeting of multiple different genes in the same network. We then explore how changes in the expression of microRNAs and consequently targets can facilitate changes in GRNs that alter development and lead to phenotypic evolution. The reviewed studies exemplify the key roles played by microRNAs in the regulation and evolution of gene expression during developmental processes in animals.

Keywords: microRNA; development; evolution; gene regulation

1. Introduction

microRNAs are short (19–25 nucleotides) non-coding transcripts that reduce the expression levels of protein-coding genes post-transcriptionally. They act by binding to complementary seed sequences in the mRNAs of target genes in a ribonucleoprotein complex to block translation of the target mRNA and/or promote its degradation (for a recent review see [1]). The hardwiring of microRNAs into GRNs can help to directly regulate particular switches and, consequentially, developmental decisions, and/or to provide more global robustness to the outputs of GRNs in the face of environmental or genetic perturbation [1–3].

In animals, microRNAs are thought to target the transcripts of thousands of genes and they have even been predicted to target the majority of mRNAs in humans [1,4–6]. This means that microRNAs are likely involved in the regulation of most developmental processes in animals [1]. The importance of microRNAs is demonstrated by the fact that the removal of most conserved microRNA families in animals like *Drosophila melanogaster* and *Mus musculus* produces strong phenotypes, often affecting a range of traits, although the loss of others, particularly newer or lineage-specific microRNAs has more subtle or no detectable phenotypes [1,7]. It is clear, therefore, that the fine-tuning of gene expression by microRNAs is not only very important for the regulation of specific individual target genes but also the interactions within and outputs of developmental GRNs more generally.

It follows that changes in microRNA expression or function can lead to phenotypic evolution [8,9]. The expression, processing and functionality of microRNAs, and the evolution and roles of microRNA families in metazoans have been comprehensively covered in many excellent reviews (e.g., [1,3,8–12]).

Here instead we focus on how microRNAs can function in feedback loops and act as switches to target key nodes or multiple components in GRNs to help regulate developmental processes. We also review how changes in microRNAs have facilitated phenotypic evolution and provide a perspective on the roles microRNAs may have played in the evolution of development and the diversification of animals.

2. microRNAs in Regulatory Loops

Computational analyses of GRNs have revealed over-represented motifs involving microRNAs [13]. They often act in feed-forward loops (FFL) in which a microRNA and its target gene are regulated by the same transcription factor (TF) [14]. FFLs are categorised into incoherent and coherent FFLs, depending on whether the upstream TF has the same or opposite effects (i.e., activation or repression) on microRNA and target (Figure 1A). This topology determines if a microRNA acts as a buffer for reduction of transcriptional noise [15] or as a so-called ‘expression switch’ (reviewed in [13]). These loops are likely abundant in mammalian GRNs since it has been shown that 44–69% of microRNAs are coordinately regulated with their targets [14].

An incoherent FFL (Figure 1B) has been described during development of *Caenorhabditis elegans*, where expression oscillations of the developmental regulator *lin-14* are dampened by pulsatile transcription of the microRNA *lin-4* [16]. *lin-14* is expressed in a temporally graded fashion. However, Kim and colleagues found that its expression becomes periodic in *lin-4* mutants. The periodicity coincides with the pulses of *lin-4* expression. Consequently, it was proposed that the temporal co-expression of *lin-4* and its target *lin-14* leads to the buffering of the expression output. This generates a temporal *lin-14* expression gradient from pulsatile transcription [16].

A developmental switch that determines left-right asymmetry of the two taste receptor neurons in *C. elegans* is also controlled by microRNAs [17,18]. In this case, a double negative feedback loop (Figure 1C) induces the transition from an equipotent precursor state to the fixed bistable expression of specific markers. The two microRNAs *lsy-6* and *miR-273* repress the expression of each other’s transcriptional activators, *die-1* and *cog-1* [19].

The coupling of expression of a target gene and its microRNA could be problematic under circumstances where down-regulation of the target is not desired. It might then be necessary to enable decoupling of the expression of both in order to allow for the derepression of the target gene. This is easily achieved when microRNA and target gene expression are controlled by distinct enhancers that only lead to co-expression under certain circumstances. However, it is more complicated when an intronic or exonic microRNA targets its own host gene (Figure 1D). Bioinformatic analyses in human, mouse, *Drosophila*, and *C. elegans* revealed that indeed 33–52% of microRNAs are located in introns and 0.6–9% in exons of protein-coding genes [20,21] and that 20% of human intragenic microRNAs are predicted to target their own host gene [20]. The transcription of intronic microRNAs is usually thought to be directly linked to that of their host gene [22–25]. However, 35% of intronic microRNAs have independent regulatory elements [26,27], and their expression can, therefore, differ from that of their host gene. Examples for cases of independent regulation include microRNAs *miR218-1* and *miR218-2* (host genes: *SLIT2* and *SLIT3*) in human and zebrafish [28] and *miR-634* (host gene: *PRKCA*) in human [29]. A well-understood example for a microRNA being co-expressed with its host gene is the *miR-92* family in *Drosophila*, which target their own host gene *jing-interacting gene regulatory 1* (*jigr1*) in order to promote the correct self-renewal of neuroblasts [30]. Expression of *miR-92a* and *miR-92b* is correlated with transcription of *jigr1*. However, alternative mRNA isoforms also allow for expression of *jigr1* alone. Thus, transcription of microRNA and target can be uncoupled when the target gene is expressed as alternative mRNAs which exclude or include the microRNA (Figure 1D). It has indeed been shown that intragenic microRNAs are preferentially located in the 5′ region of their host genes, and that host genes contain more introns than genes without intronic microRNAs [20]. Moreover, the 5′ introns are significantly longer than in a cohort of randomly sampled genes [20]. These findings indicate that alternative transcriptional start sites and regulatory regions could be used in genes with intronic microRNAs to decouple the expression of the microRNA and the host gene.

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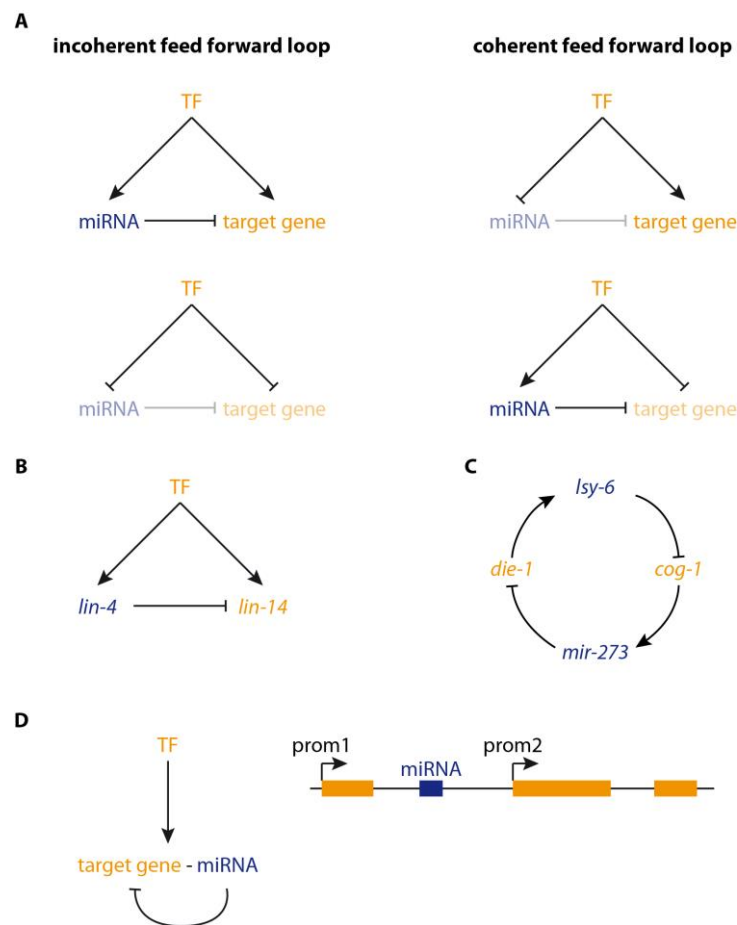


Figure 1. Gene regulatory network (GRN) sub-circuits involving microRNAs. Protein coding genes are coloured orange, microRNAs are blue, with dark and light colouring of the text indicating whether the expression is repressed or not. (A) Feed-forward loops (FFs) are represented within GRNs, expression of microRNAs and its target gene and its target gene regulated by the same upstream (Transcription factor (TF)). In the incoherent FFs, the TF has the same effect on the microRNA and target gene, and in the coherent FFs, the TF has the opposite effect on the microRNA and target gene. (B) In the feed-forward loop, the TF activates both the microRNA and the target gene, and the microRNA inhibits the target gene. (C) In the feed-forward loop, the TF activates both the microRNA and the target gene, and the microRNA inhibits the target gene. (D) In the feed-forward loop, the TF activates both the microRNA and the target gene, and the microRNA inhibits the target gene. The diagram illustrates various GRN sub-circuits involving microRNAs. Panel A shows two feed-forward loops: incoherent (TF activates both miRNA and target gene, miRNA inhibits target gene) and coherent (TF inhibits both miRNA and target gene, miRNA inhibits target gene). Panel B shows a feed-forward loop where TF activates lin-4 and lin-14, and lin-4 inhibits lin-14. Panel C shows a regulatory network with Isy-6, cog-1, mir-273, and die-1. Panel D shows a gene with two promoters, prom1 and prom2, where prom1 transcribes a miRNA and prom2 transcribes a target gene, both regulated by a TF.

3. microRNA Targets in GRNs

There are many examples where individual microRNAs have been experimentally demonstrated to regulate the transcripts of particular individual genes in a given context. For example, *mir-2* regulation of *Kr-h1* during metamorphosis in hemimetabolous insects [32,33], the requirement of

miR-57 repression of *nob-1* for posterior specification in *C. elegans* [34], and *miR-133* regulation of *Gli3* during vertebrate skeletal myogenesis [35].

Some genes—so-called “target hubs”—have been shown to be targeted by several microRNAs [36]. Interestingly, the target hub gene set is enriched for TFs and developmental processes [36]. This suggests that groups of microRNAs target important nodes in GRNs to regulate their outcome. Especially in cases where a gene is expressed in different tissues or under control of a ubiquitous enhancer, targeting by several microRNAs can help to fine-tune this gene’s expression level in different contexts. For example, *p21* encodes a tumour suppressor that is required for cell cycle arrest under different conditions. Expression of *p21* is in vitro down-regulated by 28 different microRNAs [37]. Computational analysis and modelling suggest that indeed, *p21* is repressed by different microRNAs in different contexts to allow progression of the cell cycle [38]. Several of these microRNAs are strongly expressed in different types of cancers [39–44], which might, in turn, modulate *p21* levels and thus lead to cancer progression. The expression level of *p21* in different non-pathological contexts could also be regulated by different microRNAs.

Multiple microRNAs have also been found to act in concert during epithelial to mesenchymal transition (EMT). Cursons and colleagues (2018) demonstrated that microRNAs act in combination with TFs to reinforce transcriptional changes which are required for EMT, and to buffer those changes which are not required [45]. Moreover, the authors showed that multiple microRNAs act in a combinatorial fashion on transcripts. Overexpression of single microRNAs resulted in the non-specific targeting of genes not involved in EMT and, thus, had off-target effects. On the other hand, low-level expression of microRNA combinations was sufficient to induce EMT [45]. These results indicate that synergistically acting microRNAs can reinforce each other and, thus, ensure the required posttranscriptional regulation. Moreover, only a low level of each individual microRNA is necessary which could reduce potential off-target effects of stronger microRNA expression.

As well as multiple microRNAs targeting particular genes in GRNs, individual microRNAs often target multiple genes. Indeed, some microRNAs are predicted to have hundreds of targets, although there are likely to be false positives depending on the stringency of search criteria [1,5,46]. This illustrates the importance of individual microRNAs for certain developmental processes since they can have different targets in various tissues at different stages of development. Moreover, individual microRNAs have been shown to target the transcripts of multiple genes in the same GRN. This may provide robustness to GRNs to ensure precise outputs under different physiological or environmental conditions or in different genetic backgrounds [47–49].

In vertebrates, one of the roles of *miR-9* is to regulate the transition of progenitor cells from non-neurogenic to neurogenic by promoting differentiation and repressing proliferation (reviewed in [50]). It is thought that *miR-9* does this through regulation of multiple target genes in this GRN including the TFs *Hes1*, *FoxG1*, *Gsx2*, *Zic5* and the nuclear receptor *Tlx/Nr2e1*, which promote proliferation [50–56]. Moreover, *miR-9* appears to target genes with different functions in this context including factors that help modulate chromatin modifications like repressor-element-1 silencing transcription factor [50,57]. Such targeting at multiple levels might ensure robustness to the overall process.

In *Drosophila*, *miR-9a* also targets different genes in the same gene regulatory pathway to ensure robust control of cell fate. In this case, *miR-9a* is expressed in non-sensory organ precursor cells and helps to specify the correct number of sensory organ precursor (SOP) cells [47,50,58]. Loss of *mir-9a* results in the production of extra sensory neurons [58]. *miR-9a* promotes non-SOP fate through direct repression of pro-neural genes including *senseless* and *Drosophila LIM-only (dLMO)* [47,58–60]. Interestingly, other members of the *miR-9* family may target other genes during this cell fate decision in *Drosophila* to provide further robustness [50].

Also in *Drosophila*, the *miR-92* family is involved in the regulation of several developmental processes including circadian rhythm, germline specification, neurogenesis, and trichome patterning, and some of its target genes have been identified [7,30,61–63]. In *Drosophila* second legs, *miR-92a*

region of the femur—the so-called ‘naked valley’ [61,64]. It was shown that *miR-92a* targets the mRNA of *shavenoid* (*sha*) to block trichome formation [61,63]. However, over-expression of *sha* does not produce completely normal trichomes and in addition *CG14395*, another likely direct target gene of *miR-92a*, appears to be required [65]. Intriguingly, several other genes involved in trichome formation that are directly activated by the TF Shavenbaby (*Svb*) [66,67] are also predicted to be *miR-92a* targets (Franke, Arif, Kittelmann and McGregor unpublished data) using TargetScan [4] (Figure 2). This suggests that in the GRN for leg trichome patterning, *miR-92a* targets multiple genes with different roles in the production of trichomes to ensure robust repression of these structures, thus playing an antagonistic role to *Svb*. Recently it has also been shown that along with *miR-1ab4* [68] and *miR-1ab8* [69,70], *miR-310C* regulates *Ultrabithorax* (*Ubx*) during haltere development in *Drosophila* [71]. While this indicates that *Ubx* is regulated by multiple microRNAs during the development of this appendage, *miR-310C* has *Ubx*-independent roles in haltere growth and patterning suggesting that it regulates multiple genes of trichomes to ensure robust repression of these structures, thus playing an antagonistic role to *Svb* in the GRN for haltere development.

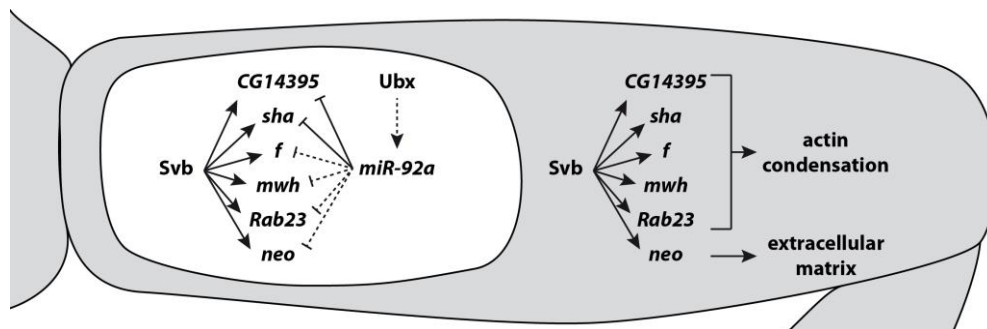


Figure 2. Trichome formation on *Drosophila* legs is repressed by *miR-92a*. The TF Shavenbaby (*Svb*) controls the expression of multiple genes whose products lead to the formation of trichomes (grey) via promotion of actin condensation and changes in the extracellular matrix. Some of these genes (*sha* and *CG14395*) have been shown to be repressed by *miR-92a*, and others are likely targets. The targeting of these genes by *miR-92a* results in a robust suppression of trichome development in the proximal region of the femur where *miR-92a* is expressed (the naked valley, white). *miR-92a* expression is possibly activated by *Ubx*.

4. Evolution of microRNAs and Targets Leading to Phenotypic Change *miR-iab8* [69,70], *miR-310C* regulates *Ultrabithorax* (*Ubx*) during haltere development in *Drosophila* [71]. While this indicates that since the discovery of microRNAs and their role in regulating gene expression, it has been thought that evolutionary changes in microRNA genes have made an important contribution to the diversification of animals [8,9]. These changes may lead to variation in their expression, copy number, arm usage, and seed sequences allowing them to acquire new targets or altering the expression of pre-existing target genes [8]. Indeed, many studies have found an association between the evolution of microRNAs and phenotypic changes among animals including the diversification of cichlids [72] and *Leptodactylus* [73] and even brain function in humans, e.g., [74,75]. However, there are relatively few described cases of phenotypic change in animals where the causal evolutionary changes have been localized to microRNAs.

One exception is again the role of *miR-92a* in trichome patterning in *Drosophila*. The size of the naked valley (see above) varies among different *Drosophila* species and between strains of *D. melanogaster* [61,64]. Genetic mapping combined with analyses of gene expression and function has shown that intra-species variation is caused by changes in the spatial expression of *miR-92a*. Expression of *miR-92a* is proximally restricted and represses *sha* and other trichome genes only in the proximal part of the femur (Figure 2). Further proximal restriction of the expression pattern in some *Drosophila* strains results correspondingly in a smaller naked valley [61]. Although the causative nucleotides have not yet been identified, it is thought that the expression variation has been caused by cis-regulatory changes in *miR-92a* enhancers [69].

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Given that changes in the expression of TFs underlie many examples of phenotypic evolution (reviewed in [76,77]), the question arises why are there are so few known examples to date of changes in microRNA genes causing phenotypic diversification among animals? The hardwiring of microRNAs into GRNs allows them to provide robustness and so it may follow that the GRN is likely to be robust to changes in the expression of a given microRNA. In addition, the effect of a microRNA on the expression of individual target genes is often thought to be relatively subtle [78,79], and so it is unlikely that changes in an individual interaction, for example through the evolution of the location or level of expression of the microRNA, will have a detectable phenotypic effect.

Perhaps then it is only in specific developmental contexts with GRNs of particular topography where a microRNA targets the mRNAs of multiple genes required for a given developmental outcome that changes in the expression of the microRNA could result in phenotypic evolution [65]. As Bartel (2018) has surmised, our understanding of the functions of many microRNAs is usually based on experimental evidence of their effect on one or a few target genes [1]. Therefore, it is probable that, as in the cases of *miR-92a* in trichome development and *miR-9a* in SOP specification, more examples will emerge of microRNAs targeting multiple genes in the same GRNs. Such a better understanding of microRNA targets in GRNs combined with higher resolution genetic mapping of phenotypic changes could reveal many more examples of changes in microRNAs causing developmental and phenotypic evolution.

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