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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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 specific genes in the two taste receptor neurons. (C) Intragenic microRNAs are usually co-regulated with their host gene, which is often also a target gene. Expression of the microRNA can be avoided if the host gene has different promoters (prom1, prom2) that can be regulated individually and lead to the expression of different host gene isoforms.

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 gene is expressed or not, respectively. (A) Incoherent feed-forward loop involving a microRNA and its target gene. The expression of the microRNA is induced by the same transcription factor (TF) that induces the target gene. (B) Repression of target gene expression by the microRNA. (C) Feed-forward loops (FFLs) are over-represented within GRNs. Expression of target genes is regulated by the same upstream transcription factor (TF). In incoherent FFLs, the TF has the same effect on microRNA and target gene expression. In coherent FFLs, the TF has different effects on microRNA and target gene expression, leading to mutually exclusive expression. Such FFLs have been implicated in expression switches where, e.g., transcriptional repression of a target gene is reinforced by the activation of a microRNA and, thus, results in the transition from a cycling expression pattern to a stable temporal expression gradient. For further examples of FFLs see [31].
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 repressed by miR-92a (Franke, Arif, Kittelmann and McGregor unpublished data) using TargetScan [4] (Figure 2). This suggests that the miR-92a targets multiple genes in the GRN for haltere development.

Ultrabithorax (Ubx) controls the expression of many genes whose products lead to the formation of trichomes (grey via promotion of actin condensation and changes in the extracellular matrix) in those regions of the leg where trichomes are found (Figure 2). Expression of Ubx is also repressed by CG14395, another target of miR-92a, which appears to be required [65]. The targeting of these genes (miR-92a and Ubx) by miR-92a has been shown to be necessary and sufficient to cause the fully-repressed state of the expression of these structures, thus playing an antagonistic role to Svb.

Since the discovery of microRNAs and their role in regulating gene expression, it has been thought that evolutionary changes in microRNA genes may lead to variation in the expression of these genes in animals. This is supported by the observation that the evolutionary diversification of animals is associated with changes in the expression of microRNAs [69]. However, these changes may lead to variation in their expression, copy number, and targets, and thus may lead to the evolution of new phenotypes.

Svb controls the expression of many genes whose products lead to the formation of trichomes in the so-called ‘naked valley’ (white). 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 repressed by miR-92a (Franke, Arif, Kittelmann and McGregor unpublished data) using TargetScan [4] (Figure 2). This suggests that the 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.

Figure 2. Trichome formation in Drosophila legs is repressed by Svb. (a) Svb controls the expression of many genes whose products lead to the formation of trichomes (grey). This includes the promotion of actin condensation and changes in the extracellular matrix in those regions of the leg where trichomes are found (grey via promotion of actin condensation and changes in the extracellular matrix). (b) In the so-called ‘naked valley’ (white), CG14395 is expressed, and other targets of Svb are repressed, thus suggesting a role for Svb in the repression of trichome formation in this region. (c) Expression of Svb is also repressed by CG14395 and another target of miR-92a, which appears to be required [65]. The targeting of these genes (miR-92a and Ubx) by miR-92a has been shown to be necessary and sufficient to cause the fully-repressed state of the expression of these structures, thus playing an antagonistic role to Svb.

4. Evolution of microRNAs and Targets Leading to Phenotypic Change

miR-92a (Figure 2) is one of the most evolutionarily conserved microRNAs, and has been shown to control various aspects of development in animals including the diversification of organs and tissues [69,70]. It has been shown that evolutionary changes in microRNA genes may lead to variation in their expression, copy number, and targets, and thus may lead to the evolution of new phenotypes.

The 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 the same species, due to changes in the expression of miR-92a. This suggests that the causative nucleotides have been conserved and have been recombined with a new part of the genome.
not yet been identified, it is thought that the expression variation has been caused by cis-regulatory changes in miR-92a enhancers [61].

Given that changes in the expression of TFs underlie many examples of phenotypic evolution (reviewed in [76,77]), the question arises why are there 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 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.

Acknowledgments: We thank other members of the McGregor lab and Saad Arif for discussion and comments on the manuscript.

Conflicts of Interest: The authors declare no conflict of interest.

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