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MicroRNAs and Developmental Timing

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Abstract

MicroRNAs regulate temporal transitions in gene expression associated with cell fate progression and differentiation throughout animal development. Genetic analysis of developmental timing in the nematode *C. elegans* identified two evolutionarily conserved microRNAs, *lin-4/mir-125* and *let-7*, that regulate cell fate progression and differentiation and in *C. elegans* cell lineages. MicroRNAs perform analogous developmental timing functions in other animals, including mammals. By regulating cell fate choices and transitions between pluripotency and differentiation, microRNAs help to orchestrate developmental events throughout the developing animal, and to play tissue homeostasis roles important for disease, including cancer.

Introduction

The roles for microRNA pathways in developmental timing were revealed by genetic analysis of worm mutants with particular kinds of defective larval cell lineages, in which events that re ordinarily restricted to specific stages of larval development occur at abnormal stages[1]. Cloning of the genes identified by these so-called heterochronic mutants of *C. elegans* led to the identification of the microRNA gene products of *lin-4* [2] and *let-7* [3]. *lin-4* and *let-7* regulate the timing of a wide variety of distinct developmental events in diverse cell lineages by progressively down regulating particular downstream targets (Figure 1), including the transcription factors LIN-14, HBL-1 and the TRIM protein LIN-41 [4]. MicroRNAs act post-transcriptionally on messenger RNA (mRNA) targets to which they base pair and repress production of the target protein. As post-transcriptional regulators with the ability to affect subtle changes in gene activity, or major microRNAs may be particularly suited for the regulation of the timing of events in diverse cell types and hence for coordinate the robust execution of temporal patterns of events throughout a developing organism.

While *lin-4* and *let-7* each exerts its effects on cell fate progression in worm larvae by down regulating a major target (LIN-14 and LIN-41, respectively), a different sort of developmental progression is managed by miR430 in the fish embryo. miR430 expression rises rapidly to very high levels at about 4 hours of embryonic development, and targets hundreds of maternal mRNAs for deadenylation and destruction. Thus, in this case a microRNA triggers a major developmental transition by coordinating the elimination of mRNAs whose function is complete [5]

Interestingly, the involvement of microRNAs in developmental timing is reprised in plants in a fashion quite analogous to *C. elegans* (reviewed in [6]). Heterochronic mutants of corn exhibit global developmental timing defects reminiscent of those in worms [7]([8]. One of

these corn mutants, Corngrass1 was found to result from over expression of the microRNA miR156 [9]. The miR156 microRNA, along with other microRNAs, also controls developmental transitions in Arabidopsis[10] [11]. Plant microRNAs are not related to animal microRNAs, and so these parallel roles for microRNA pathways in plant and animals represent independent evolutionary adaptations of microRNAs to developmental timing roles.

Here I will review recent advances in understanding the microRNA pathways controlling developmental timing in *C. elegans*, and how those studies are illuminating principles of animal microRNA function in general. Emphasis will be placed on relating the functions of worm *lin-4* and *let-7* microRNAs to the functions of their orthologous microRNAs in mammals (*mir-125* and *let-7*, respectively). I will also discuss findings showing that in vertebrates, other microRNAs (unrelated to *lin-4/mir-125* or *let-7*) function analogously to the *C. elegans* heterochronic microRNAs to control the temporal progression of cell fates within cell lineages, and transitions between pluripotency and differentiation.

Complex microRNA pathways control developmental timing in *C. elegans*

One overarching feature of the timing of developmental events in *C. elegans* lineages is the extreme robustness of the normal pattern, which is completely invariant among wild type worm. MicroRNAs play critical roles in posttranscriptional regulation of a set of key transcription factors, LIN-14, HBL-1 and LIN-29 that orchestrate coordinated stage-specific transcription programs throughout the developing larva. The *lin-4*-LIN-14 steps in the cascade occur cell-autonomously [12], so the coordination of events across the animal probably is not the consequence of extracellular traffic of microRNAs, but more likely involves a temporally coordinated activation of the microRNAs and/or communication by conventional hormones at later steps in the pathway [13], [14].

The temporal progression of cell fates in the lateral hypodermal cell lineages of the worm represents a simple model for stem cell lineages in general, which are characterized by regulated self-renewal and proliferative cell division patterns and the regulated production of differentiated cell types (Figure 1). A single proliferative division occurs in the *C. elegans* lateral hypodermal lineages, and is restricted to the L2 stage as a result of the stage-specific down regulation of the transcription factor HBL-1 (Figure 1). HBL-1 is high in the L1 and L2 stage, and then is down regulated in the L3. The down-regulation of HBL-1 is accomplished by semi-redundant activity of members of the *let-7* family microRNAs (*let-7-Fam*), including *mir-48*, *mir-84* and *mir-241* [15]. Single-gene mutations of *let-7-Fam* microRNAs do not result in appreciable perturbation of the timing of lateral hypodermal events, but simultaneous mutation of two or more results repetition of the L2 proliferative division and delay of adult lateral hypodermal fates[15].

The complexity of the gene regulatory pathways in which *let-7-Fam* microRNAs function in *C. elegans* includes a feedback circuit involving *let-7-Fam* miRNAs and the DAF-12 transcription factor [14]. This circuit pathway involves both positive feedback and negative feedback between the microRNAs, whose transcription in regulated by DAF-12, and in turn DAF-12 is regulated by the *let-7-Fam* microRNAs. This circuit functions to integrate environmental signals and developmental timing, and to coordinate developmental quiescence with cell fate specification in the hypodermal lineages (Figure 1).

Another prominent role of *let-7* in *C. elegans* is in terminal differentiation of the lateral hypodermal lineages in conjunction with the final larva-to-adult molt [3]. The terminal differentiation of these cells (termed the “larval-to-adult switch”) is mediated by up regulation of the *let-7* microRNA in the L4 stage, which down regulates LIN-41 and thereby causes the up regulation of the LIN-29 transcription factor (Figure 1). The timing of *let-7* up

regulation is coupled to completion of previous larval development in part by a feed forward circuit wherein *let-7* transcription is repressed by HBL-1 at earlier stages; full *let-7* transcription in the L4 is permitted only after completion of the down regulation of HBL-1 by *let-7* and her sisters during the L3 stage [16].

The larval-to-adult switch involves terminal differentiation of hypodermal cells, which is primarily triggered by *let-7* via LIN-41 and LIN-29, and also the cessation of the cycle of molts (Figure 1). The conserved nuclear hormone receptors NHR-23 and NHR-25 control molting in the worm[17], and the cessation of larval molting results from the direct targeting of NHR-23 and NHR-25 by *let-7-Fam* microRNAs [18].

Integration of temporal information with other developmental signals

The heterochronic pathway microRNAs regulate, via their downstream target genes, a variety of distinct cellular behaviors. For example, *lin-4* acts via its major target, LIN-14, to affect the timing of certain events in development of the worm nervous system -- in particular, in the timing of neural outgrowth in a neuronal type that matures postembryonically [19]. MicroRNAs also help coordinate differentiation and proliferation in other cell lineages, including cell cycle progression and cell fate commitment for vulval precursor cells (VPCs) [20]. Vulval development involves a precisely orchestrated temporal and spatial program of sequential signaling events involving an EGF organizer signal, transduced by the Ras pathway in the so-called 1° VPC, and a LIN-12/Notch lateral signal from the 1° VPC to its 2° VPC neighbors. The timing of Ras-mediated signaling in the 1° VPC is modulated by *mir-84*, a member of the *let-7* family of microRNAs [21]. The Ras-activated fate of this cell includes sending a LIN-12/Notch lateral inhibitory signal to its neighbors, where the *lin-4*-LIN-14 circuit interfaces with the LIN-12/Notch gene expression program to help coordinate steps in cell cycle progression and 2° cell fate commitment [22]. LIN12/Notch activation in the 2° cells engages a feedback loop involving another (non-*let-7*- family) microRNA, *mir-61*. *mir-61* down-regulates Ras signaling in the 2° VPC to help ensure mutual exclusivity of Ras and LIN-12/Notch signaling [23].

Modulation of the activities of temporal microRNAs

The distinctive developmental phenotypes associated with developmental timing microRNA pathways in *C. elegans* offers a powerful system for employing genetic screens to identify cofactors that regulate microRNA biogenesis or activity. RNAi screens for proteins that genetically interact with *let-7-Fam* microRNAs and modify their developmental timing phenotypes identified the conserved TRIM/NHL protein NHL-2, which functions as a positive co-factor for the activity of *let-7-Fam* microRNAs and other microRNAs [24]. The vertebrate and fly orthologs of NHL-2 have similar conserved microRNA-associated functions [25], suggesting that TRIM/NHL proteins could function widely to adjust the activity of *let-7* and other microRNAs in the context of the physiology of the developing animal.

Another interesting cofactor for *let-7* activity, also identified by genetic modifier screens in *C. elegans*, is the ribosomal protein RPS-14[26]. Reduction of RPS-14 by RNAi in the worm results in elevation of *let-7* activity. The RPS-14 protein could be co-immunoprecipitated with the nematode miRISC Argonaute, ALG-1, suggesting a possible direct role for RPS-14 in miRISC activity. It is not know if the microRNA-associated activity of RPS-14 occurs in physical constituent of the ribosome, or in the context of a hypothetical extra-ribosomal function for RPS-14. Consistent with the theme of ribosome-miRISC functional interactions, another ribosome-associated protein, RACK1, has been found to genetically interact with microRNAs in *C. elegans*, and seems to physically associate with miRISC to promote microRNA activity in worms and mammalian cells [27].

RNAi screens for modulators of *lin-4* control of developmental timing in *C. elegans* identified a conserved RNA binding protein gene *rbm-28*, which appears to affect the accumulation of *lin-4* microRNA [28]. RMB-28 is homologous to the human RBM28, a nucleolar protein which has been implicated in diseases associated with defects in spliceosomal and/or ribosome biogenesis [29] [30] [31], suggesting a possible intersection of nucleolar RNP function and the regulation of *lin-4* accumulation.

Conserved Functions of Developmental Timing MicroRNAs

The finding that *let-7* microRNA is conserved in sequence and developmental expression across wide evolutionary distance [32] was a watershed discovery that set in motion searches for other small RNAs like *let-7* and *lin-4* (the only microRNAs known at the time). Rapidly thereafter, scores of microRNAs were identified in animals [33],[34], [35], and then plants [36]. An immediately apparent evolutionarily conserved characteristic of *let-7* microRNA is its temporal up regulation in conjunction with advancing embryonic development and differentiation, and the absence of *let-7* from pluripotent cells [32]. The evolutionary conservation of developmental timing roles for microRNAs, particularly the *let-7* family of microRNAs, has been extensively reviewed [37], [38], [39], [40], [41]. Of particular note is the deep conservation of the direct negative feedback loop between *let-7* and the pluripotency factor LIN-28 (Figure 2 A, Figure 3A). LIN-28 binds to *pre-let-7* and inhibits production of the *let-7* mature microRNA [42], which in turn directly represses LIN-28 production by base-pairing to the *lin-28* mRNA, [43], [41]. Similarly, *let-7* targeting of LIN-41 is conserved between nematodes and mammalian cells (Figure 2A), and the expression pattern of *let-7* and *mir-125/lin-4* microRNAs is inversely correlated with LIN-41 in mouse [44]. In *C. elegans* a *let-7* family microRNA regulates Ras (LET-60) in the context of development of the vulval primordium (Figure 2A), and in mammals *let-7* targets Kras in a range of cell types to inhibit proliferation [21]. These deeply conserved microRNA-target relationships seem to reflect core functions of the microRNA that are intimately engaged in fundamental regulatory circuitry of all animal cells.

A hallmark of the conservation of *let-7* function as a differentiation factor and tumor suppressor is the fact that the target repertoire of *let-7* displays remarkable evolutionary fluidity, while at the same time exhibiting a core set of conserved targets discussed above (LIN-28, Ras, LIN-41). Interestingly, the non- -conserved targets of *let-7* are also set in the theme of temporal control of cell fate (Figure 2A). For example, in Drosophila, one of the temporal transitions regulated by *let-7* is a reorganization of the neuromusculature of the fly during metamorphosis[45],[46]. A key *let-7* target in this event is Abrupt[45], which is not a target of *let-7* in worms or mammals. Similarly, a key target of *let-7* in mammals is the oncogene HMG2A [47], orthologs of which are not targets of *let-7* in worms or flies.

Similarly consistent with a conserved temporal control function, the mammalian *lin-4* homolog miR-125b seems to regulate the proliferation of hematopoietic stem cells and also affects the balance of cell fates during lymphoid development, in part probably by acting as a lineage-specific anti-apoptotic factor [48]. miR-125b also plays analogous roles in the temporal progression of neuronal differentiation in humans by repressing multiple targets [49]. The apparent conserved roles for *let-7* and *miR-125/lin-4* microRNAs as a temporal regulators of cell fate transitions could reflect ancestral roles for these microRNAs.

Noteworthy advances around the subject of microRNAs in neural development include roles for the miR-183 family and for miR-96 in the development of sensorineural fates in the inner ear[50], [51], [52]. Particularly exciting is the finding that mutations in the miR-96 seed sequence are responsible for progressive hearing loss in certain human families[52]. Moreover, mice carrying miR-96 seed mutations exhibit a similar deafness [51], and the

underlying developmental defect in these mice seems to be an arrest in the developmental progression program for inner and outer hair cells, as well as blocks in steps of auditory neural wiring [53]. Thus, mir-96 (which is not related in sequence to lin-4/miR-125 or let-7), controls a program of developmental progression for mammalian inner ear cells in a fashion analogous to the roles of *C. elegans lin-4* and *let-7* microRNAs in promoting developmental progression in worms cell lineages.

Developmental timing and Cancer

Consistent with an analogy between temporal progression of cell fates in *C. elegans* larval development, which is controlled by microRNA pathways, and cancer progression, *lin-4/miR-125* and *let-7* family microRNAs figure prominently in tumorigenesis (reviewed by [41]). Change in the level of miR-125 expression is a common characteristic of leukemia, and experimental support for a direct contribution miR-125 to leukemogenesis comes from mouse experiments. Over expression of miR-125 in transplanted mouse fetal liver results in elevated neutrophils and monocytes, and eventual B-cell acute lymphoblastic leukemia, T-cell acute lymphoblastic leukemia, or myeloproliferative disease[54]. These and other findings implicate miR-125b activity in specifying early stages of hematopoietic cell lineages. Targets for miR-125 in the context of hematopoesis and leukemia have not been identified, although miR-125 is predicted to target pro-apoptotic transcripts [55],[56], and p53 (at least in humans) {Le, 2009 #17150

Transitions between Pluripotency and differentiation

MicroRNAs participate in the regulated transitions of progenitor cells from a multipotent, self-renewal status towards differentiation in numerous cell lineages and tissues of vertebrate embryos. The roles of microRNAs in the development of mammalian skin [57] include the action of mir-203 to promote differentiation by repressing stemness [58].

A possible inverse relationship between microRNA expression and pluripotency of Embryonic Stem (ES) cells emerged from the finding that LIN-28 could act, together with three other proteins, to induce the reprogramming of human somatic cells to pluripotent stem cells {Yu, 2007 #6568}. LIN-28 inhibits expression of microRNAs associated with differentiation, including *let-7* (Figure 2; Figure 3A). MicroRNAs that target LIN-28 (including miR-125/*lin-4* and *let-7*)[59] are expressed during differentiation of cell lineages from ES cells in a fashion inversely correlated with LIN28 expression [60]. Certain microRNAs, such as miR-145 [59], or *let-7* [61] can inhibit reprogramming of somatic cells to induced pluripotent stem (iPS) cells.

However, recent findings provide evidence for a direct role for microRNAs in the pluripotency of Embryonic Stem (ES) cells. First, microRNA-depleted ES cells are incapable of producing differentiated cells, indicating that although they are viable, they do not possess the developmental potential characteristic of normal ES cells [62] [63]. Second, a distinct set of microRNAs are expressed in ES cells [64], and evidence indicates that these ES cell microRNAs (ESmirs, Figure 3) help maintain the pluripotency and self-renewal capacity of ES cells. Third, certain Myc-induced microRNAs can replace Myc in the generation of induced pluripotent cells [65], providing evidence for a potentially direct role for microRNAs in promoting pluripotency. Finally, expression of the miR302/miR367 microRNA locus from a viral vector has been shown to be sufficient to reprogram mouse or human fibroblasts to induced pluripotent stem (iPS) cells [66] (Figure 3B). The fact that reprogramming of somatic cells to induced pluripotency can be triggered by expression of just two microRNAs suggests that these microRNAs exert enormous leverage upon key gene regulatory network hubs that orchestrate bidirectional transitions between pluripotency and differentiation in mammals.

Conclusions

The *C. elegans* model system continues to be a valuable tool for discovering and characterizing microRNA pathway components involved in the organized developmental progression of cell lineages from earlier, more pluripotent stages, towards differentiation. Much work needs to be done, employing model organisms such as *C. elegans*, in conjunction with mouse and human genetics, to understand how microRNAs are temporally regulated in particular cell lineages, and how they engage specific targets in specific cell types in the context of developmental progression. Of particular interest in the near future are the apparently powerful roles of microRNAs in transitions between pluripotency and differentiation that are fundamental to developmental progression, tissue homeostasis, and human disease.

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Highlights

- C. elegans heterochronic gene pathway is a model for temporal control of cell fates.
- MicroRNAs have evolutionarily conserved functions in developmental timing.
- MicroRNAs exert powerful roles in pluripotency and differentiation.

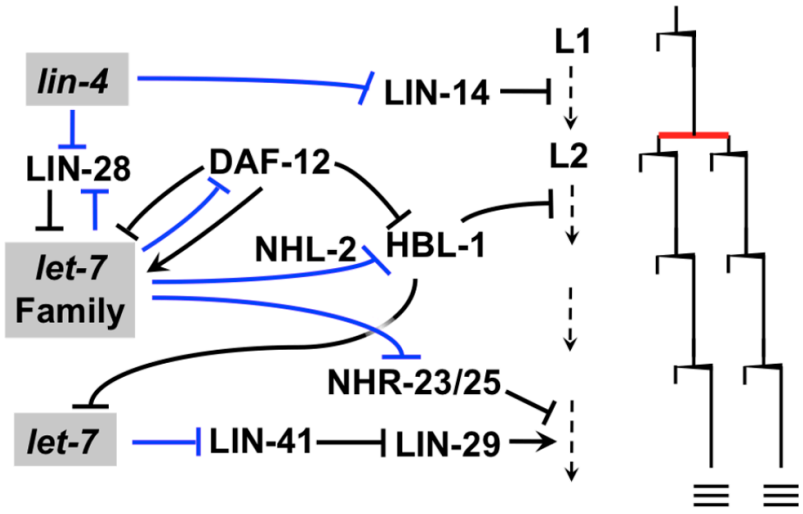

Figure 1. MicroRNAs and developmental timing in *C. elegans*

MicroRNAs (shaded text boxes) of the *lin-4* and *let-7*-Family control the temporal progression of cell fates in the lateral hypodermal "seam" cell lineages of developing *C. elegans* larvae. In each of stages L1-L4, seam cells undergo a single round of stem cell-like self-renewal divisions (wedge-shaped bars), with a single symmetric division (red bar) interposed in the L2 stage. At the L4 molt, seam cells exit the cell cycle and terminally differentiate (triple bars). MicroRNAs post-transcriptionally regulate key target mRNAs by direct interactions (blue lines) with to 3′ UTR sequences. Down regulation of the transcription factor LIN-14 by *lin-4* microRNA is required for progression from the asymmetric L1 division pattern to symmetric division in the L2. Progression from the L2 to the L3 fate is caused by the down regulation of the transcription factor HBL-1 through the redundant activity of microRNAs of the *let-7* family, which includes *let-7*, *mir-48*, *mir-84*, and *mir-241* [15]. *let-7*-Family microRNA activity is modulated positively by the TRIM/NHL protein NHL-2 [24]. The L2 to L3 transition also involves down regulation of the RNA binding protein LIN-28 by *lin-4* microRNA; LIN-28 acts upstream of the *let-7*-Family microRNAs [15]. The nuclear hormone receptor is the hub of a complex set of interactions that integrate microRNA and steroid hormone inputs to coordinate temporal cell fates with a decision to enter an optional diapause after the L2 stage [14]. Progression from a cycling status to terminally-differentiation at the L4 molt is conferred by a dramatic up-regulation of *let-7* in the L4, resulting in down-regulation of the TRIM/NHL protein LIN-41, and consequent up regulation of the transcription factor LIN-29. HBL-1 represses *let-7* transcription, ensuring that the up regulation of *let-7* microRNA occurs only after completion of earlier steps. The cessation of molting after the L4 stage involves in part the down regulation, by *let-7* family microRNAs, of the nuclear hormone receptor molting factors NHR-23 and NHR25 [17].

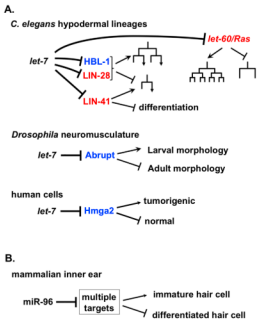

Figure 2. Evolutionary conservation of developmental timing roles for microRNAs

A. In nematodes, insects and mammals, *let-7* family microRNAs control progression from earlier, or more proliferative states, to later, more differentiated states. These conserved activities in developmental progression can involve explicitly conserved targets (red), and non-conserved targets (blue). *C. elegans let-7* family microRNAs act in several cell types to control early-to-late cell fate progression. Examples of targets that are conserved between *C. elegans* and mammals and insects include LIN-28, LET-60/Ras and LIN-41. Nonconserved targets of let-7 can nevertheless mediate roles for let-7 in promoting transitions from more primitive to more differentiated developmental states: examples include in *Drosophila* the down regulation of Abrupt in the control of a reorganization of the neuromusculature at metamorphosis [45],[46], and in humans the down regulation of the oncogene HMGA2 [67].

B. MicroRNAs of families other than *let-7* can also control temporal developmental transitions, such as the case of miR-96, which is required for a program of differentiation in mammalian inner ear hair cells [53]. There could be multiple relevant targets of miR-96 in this context, since many mRNAs are deregulated in mir-96 mutant mice [51].

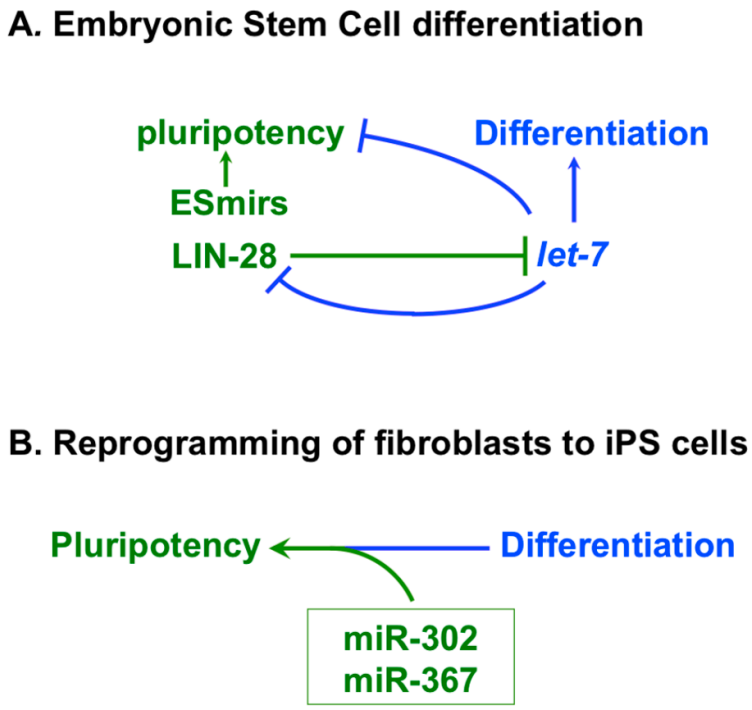

Figure 3. MicroRNAs and transitions between pluripotency and differentiation

A. An evolutionarily conserved reciprocal repression between *let-7* microRNA and LIN-28 results in mutually exclusive expression of LIN-28 between ES cells and differentiated cells, respectively. ES cell microRNAs (ESmirs) promote pluripotency and self-renewal together with other factors, including LIN-28, which acts in part by preventing expression of *let-7*. **B.** MicroRNAs miR-302 and miR-367 are expressed in stem cells of various types, including ES cells. Under certain conditions, experimental expression of miR-302 and miR-367 can be sufficient to reprogram mouse or human fibroblasts to induced pluripotent stem (iPS) cells [66].