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MicroRNAs: a developing story

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Considering the intense genetic efforts applied to understanding development, it is surprising that a relatively large class of regulatory genes has newly surfaced. The first microRNA gene and its developmental role were described more than ten years ago, but only recently have we fully appreciated the broad and abundant presence of such genes. MicroRNAs are ~22 nucleotide RNAs that use antisense complementarity to inhibit expression of specific mRNAs. Recent studies of restricted expression patterns and functional roles have implicated specific microRNAs in complex genetic pathways regulating embryogenesis, hematopoiesis, neuronal differentiation and Hox-mediated development.

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Current Opinion in Genetics & Development 2005, **15**:200–205

This review comes from a themed issue on
Chromosomes and expression mechanisms
Edited by Barbara Meyer and Jonathan Widom

Available online 5th February 2005

0959-437X/\$ – see front matter

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DOI 10.1016/j.gde.2005.01.002

Introduction

The development of a multicellular organism requires specific and coordinated control mechanisms. Genetic screening approaches, particularly in plants, flies and worms, have revealed complex regulatory networks that orchestrate precise cell division and differentiation patterns. An extraordinary outcome of such endeavors was the discovery of a novel gene-regulatory mechanism. A little over ten years ago, the Ambros and Ruvkun [1,2] laboratories found that larval development of the nematode *Caenorhabditis elegans* requires a tiny RNA to inhibit the expression of a protein-coding gene. The *C. elegans lin-4 (lineage)* gene produces a 21 nucleotide RNA that recognizes complementary sites in the 3' untranslated region (3'UTR) of the *lin-14* messenger. In doing so, it downregulates the translation of *lin-14* during the transition from the first to the second larval stage of development. We now recognize hundreds of tiny RNA genes called microRNAs (miRNAs), which populate the genomes of plants and animals [3]. Similar to the founding miRNA *lin-4*, many of these newly identified miRNAs might function in regulating development. Some might

be essential for directing cell fates, whereas others might help fine-tune the complex genetic network that builds a multicellular organism.

In this review, we describe the current understanding of miRNA biogenesis and regulatory mechanisms. We also discuss specific examples of miRNA genes proposed to control developmental fates such as pluripotency of embryonic stem cells, differentiation during hematopoiesis, asymmetric gene expression in neurons, and *Hox* gene-mediated patterning.

Expression and function of microRNAs

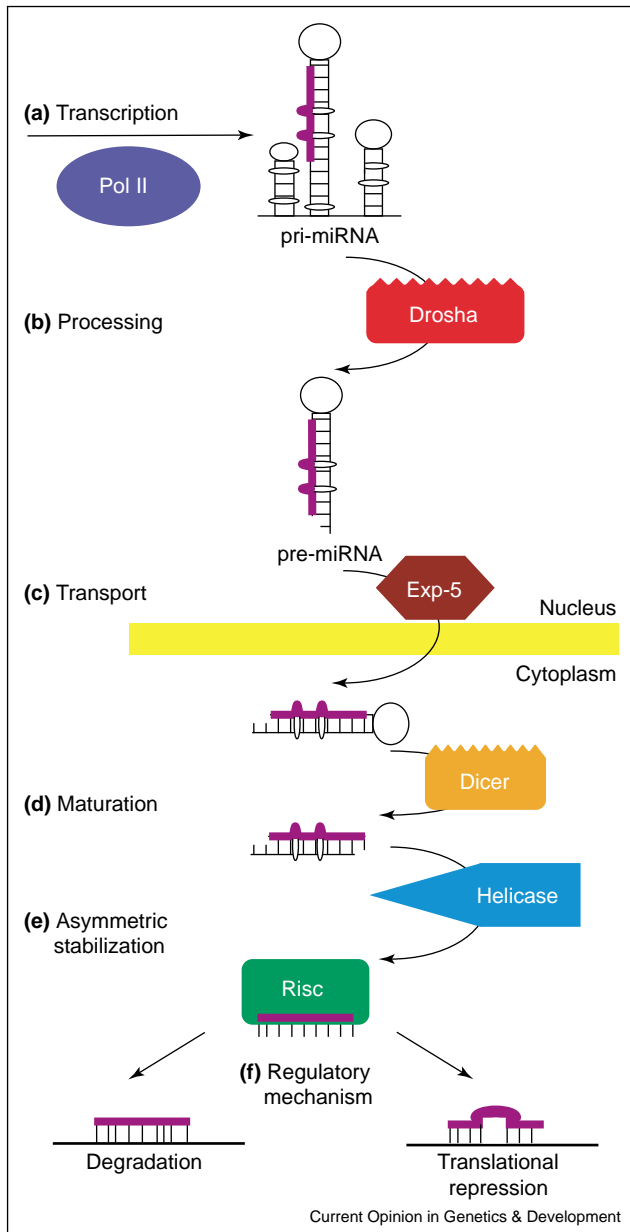
The functional ~22 nucleotide forms of miRNAs are generated by elaborate processing of longer primary transcripts (pri-miRNAs) (Figure 1). The nascent transcripts for several plant and animal miRNAs contain the hallmarks of RNA polymerase II (Pol II) synthesis [4–6] but, as yet, little is known about the factors that regulate precise spatial and temporal expression patterns of specific miRNA genes. The general model depicted in Figure 1 awaits refinement, and broad questions remain: how are pri-mRNA transcripts recognized and cleaved by specific processing factors? Are miRNAs subject to regulated processing and cellular localization? How are the transcription and stabilization of specific miRNAs controlled?

Although remarkably small, miRNAs harbor enough sequence content to be relatively specific. Generally, if a miRNA–target duplex contains imperfect complementarity, protein expression is inhibited without target mRNA destruction. However, if the duplex has nearly perfect base-pairing, then the mRNA target is marked for degradation (Figure 1) [7,8]. The Argonaute proteins present in the RNA-induced silencing complex (RISC) appear to dictate the mode of regulation elicited by the miRNA–target duplex. Recruitment of specific Argonaute proteins can catalyze cleavage of mRNA sequences perfectly base-paired to the miRNA, or inhibit translation of mRNAs that form an imperfect duplex with the miRNA [9–13]. This general paradigm has exceptions already. Near-perfect base-pairing between plant miR172 and its target sequence in *APETALA2 (AP2)* would be expected to confer degradation of the *AP2* mRNA. Instead, *AP2* protein expression is inhibited without a detectable decrease in *AP2* mRNA levels [5,14].

Elucidation of microRNA regulatory pathways

Despite the abundance of miRNA genes in multicellular organisms, relatively few are known to underlie mutant phenotypes. Based on their small product size, miRNA

Figure 1



Potential regulatory points during expression and function of animal miRNAs. Long, primary miRNA transcripts (pri-miRNAs) are recognized by the nuclear RNase Drosha for processing to the hairpin precursor forms (precursor miRNAs), which are then transported from the nucleus by exportin-5 (Exp-5). The RNase Dicer and a putative helicase activity produce the mature single-stranded miRNA, which is then loaded into the RNA-induced silencing complex (RISC), where it can direct regulation of target mRNAs containing antisense sequences [7**]. **(a)** Transcription is believed to be the major regulator of miRNA expression, but the *cis*- and *trans*-acting factors that control miRNA transcription at a particular time or place are yet to be defined. **(b–d)** The findings that plant *DICER* and *ARGONAUTE* genes are themselves regulated by specific miRNAs [45,46] and that miRNA intermediates can accumulate *in vivo* upon depletion of processing factors [4,6,7**,8**] indicate that miRNA maturation could be subject to differential control. **(e)** Generally, one strand of the Dicer product is maintained, but it is unknown how the unselected half is degraded.

genes were originally predicted to be elusive targets of mutagenesis screens. However, it now seems likely that many miRNA primary transcripts will rival mRNAs in length [4–6] and, thus, be just as statistically vulnerable to mutagens. Some miRNAs might control subtle or non-essential regulatory pathways. Within an organism, families of miRNAs that harbor homologous sequences also might share related or overlapping regulatory targets. Thus, a mutant phenotype in one miRNA gene might be diluted by the presence of other highly related miRNAs. For example, humans express identical mature *let-7* (lethal) RNAs from three different genomic loci, and at least nine other highly homologous *let-7* miRNAs [3**]. If multiple members of a miRNA family regulate common targets, then combinations of genetic mutations in the miRNA genes might be required to generate interpretable phenotypes.

In contrast to the known animal miRNAs, plant miRNAs typically match antisense sequences in their targets and, thus, direct mRNA degradation [7**,8**]. This feature greatly facilitates target prediction and validation for plant miRNAs [15,16*]. As such, rapid progress has been made in assigning developmental functions to specific miRNAs in *Arabidopsis*. A thorough discussion of plant miRNA expression and examples of regulatory pathways has been recently published [8**].

Although mutant analyses of specific miRNA genes are emerging slowly, there is ample evidence that members of this new class of RNAs will have crucial roles in regulating development. In both plants and animals, depletion of the ribonuclease (RNase) Dicer or specific Argonaute proteins results in embryonic lethality [17–21]. The essential role of these factors in miRNA biogenesis implies that production of mature miRNAs is vital for embryonic development of all multicellular organisms. Additionally, limited or post-embryonic depletion of miRNA processing activities results in pleiotropic defects, highlighting the broad importance of miRNAs in regulating all developmental stages [17,21–25]. Many of the miRNA targets revealed by computational approaches are known developmental genes, and validation of these predictions might situate specific miRNAs in key regulatory positions (Table 1) [26,27].

MicroRNAs in early development

The lethality associated with defects in miRNA processing is probably due to inadequate expression of embryo-

In addition, the rapid disappearance of some mature miRNAs during development could indicate targeted degradation of miRNAs. **(f)** Typically, miRNAs that form perfect duplexes with their targets direct degradation and those that support partial duplexes inhibit protein expression. The large RNA–protein complexes that mediate miRNA function are potential targets of regulation. In fact, several plant viruses express specific proteins to block host miRNA function [8**].

Table 1

Select miRNAs and proposed regulatory targets and developmental pathways.

Pathway group	miRNA	Organism	Expression	Specific pathway	Gene target ^b
Embryogenesis	miR-290 (in cluster 290–295) [29**]	Mouse	ES cells	Eye development	<i>Six6</i> , <i>Pax6</i> [27]
	miR-290 [29**]	Mouse	ES cells	Tail and neuronal development	<i>Hoxb13</i> [27]
	miR-302 [28**,29**]	Human, mouse	ES cells	Neuronal development	<i>Sox-11</i> [27]
	miR-154 [28**,30**]	Human, mouse (imprinted region)	ES cells, embryo, brain	Muscle development	<i>Gata-6</i> [27]
Hematopoiesis	miR-181 [32**]	Vertebrates	Brain, lung, spleen thymus	Hematopoiesis	<i>LH9</i> [27]
	miR-223 [32**]	Vertebrates	Bone marrow	Hematopoiesis	<i>SNRK</i> [27]
	miR-142 [32**]	Mammals	Bone marrow, spleen, thymus	Hematopoiesis	<i>Gata-3</i> [27]
Neuronal differentiation	<i>lcy-6</i> [33**]	<i>C. elegans</i>	ASEL neuron	Left/right neuronal asymmetry	<i>cog-1</i> (homeobox transcription factor) ^a [33**]
	miR-273 [34**]	<i>C. elegans</i>	ASER neuron	Left and right neuronal asymmetry	<i>die-1</i> (zinc-finger transcription factor) ^a [34**]
Hox-mediated development	miR-10 [43**,44**]	Vertebrates, insects	Embryo posterior	Anterior and posterior patterning	<i>Sox-21</i> [27]
	miR-196a [43**,44**]	Vertebrates	Embryo posterior	Anterior/posterior patterning	<i>Hoxb8^a</i> , <i>Hoxc8^a</i> , <i>Hoxd8^a</i> , <i>Hoxa7^a</i> [43**,44**]

Specific miRNA genes for each of the pathways described in this review are paired with predicted target genes that encode transcription-related factors. Note that only some of the miRNA–target pairings have been experimentally validated and that the remainder are computational predictions, yet to be tested. ^aIndicates experimental validation of target regulation by the miRNA. ^bThe selected target examples are not necessarily the highest-ranking prediction for the indicated miRNA [27].

specific miRNAs, of which there are numerous examples in worms, flies and vertebrates [3**]. Two recent studies [28**,29**] report examples of mammalian miRNAs that are downregulated in differentiating embryonic stem (ES) cells, suggesting that they are involved in the maintenance of a pluripotent state (Figure 2a). The mouse ES cell-specific miR-290–295 genes cluster within 2.2 kb of each other and are homologous to the cluster of human ES cell-specific miR-371–373 genes [28**,29**]. Proximal genomic locations and polycistronic transcription of some ES cell-specific miRNAs might facilitate the coordinated regulation of miRNAs involved in global control of cellular differentiation. Assignment of direct functional roles for the miRNAs expressed in ES cells is complicated by the fact that none of them appear to exhibit exact complementarity to mRNA sequences. Nonetheless, intriguing targets for some of these ES cell-specific miRNAs have been predicted using computational methods (Table 1) [26,27]. Validation of such miRNA target interactions in ES cells could establish miRNAs as key regulators of the stem cell undifferentiated state.

Glossary

Imprinting – this is an epigenetic mechanism whereby specific genetic loci demonstrate parent-specific gene expression.

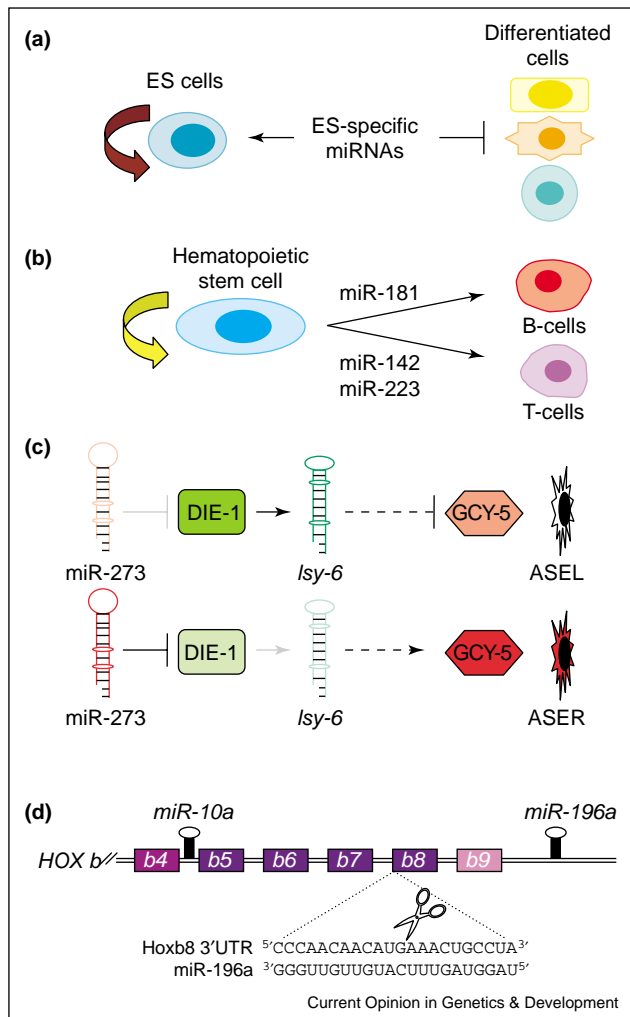
Imprinting control element – these *cis*-acting regulatory sequences typically direct differential methylation of the maternal or paternal chromosomes, resulting in parental origin-specific gene expression.

Interestingly, several of the miRNA genes expressed in mouse ES cells are located within a ~40 kb genomic region on chromosome 12 that is regulated by imprinting (see Glossary) [30**]. Seitz *et al.* [30**,31] demonstrate that several of the miRNAs within this region, which potentially encodes dozens of miRNAs, are only expressed from a maternally inherited chromosome that contains an intact imprinting control element (see Glossary). Deletion of the imprint control element on the maternal chromosome results in embryonic lethality [31]. Although the relevance of regulated miRNA expression at this locus is unclear, a provocative possibility is that they function in establishing or maintaining the epigenetic control of gene expression, which is essential for mammalian development.

Orchestration of hematopoiesis by microRNAs

As first observed for *lin-4* and *let-7*, the founding miRNA genes, specific miRNAs are also involved in post-embryonic developmental decisions. A recent analysis of miRNA profiles in mouse hematopoiesis revealed lineage-specific expression patterns for miR-181, miR-223, and miR-142 (Figure 2b) [32**]. Ectopic expression experiments support roles for miR-181 in directing B-cell lineages, and miR-223 and miR-142 in regulating T-cell fates [32**]. Moreover, transplantation of miR-181-expressing progenitor cells into lethally irradiated mice gave rise to a full complement of immune cells strongly biased toward the B-cell lineages [32**]. Now the

Figure 2



An overview of developmental pathways regulated by miRNAs. (a) Mammalian ES cells contain a distinct repertoire of miRNAs, many of which are downregulated upon cellular differentiation [28^{••}–30^{••}]. (b) Specific miRNAs are expressed during mouse hematopoiesis and help define particular cell lineages [32^{••}]. (c) In *C. elegans* the transcription factor DIE-1 activates expression of *Lsy-6* miRNA, which results in inhibited expression of the gene encoding chemoreceptor GCY-5 in the ASE left (ASEL) neuron [33^{••},34^{••}], as indicated in the abbreviated genetic pathways. By contrast, *miR-273* expression in ASE right (ASER) ultimately results in inhibition of *Lsy-6* and activation of *gcy-5* expression [33^{••},34^{••}]. (d) The vertebrate *HOX b* cluster includes *miR-10a* and *miR-196a* genes [41,42]. Base-pairing between *miR-196a* and a sequence in the 3'UTR of *Hoxb8* mRNA results in cleavage of this target [43^{••},44^{••}].

challenge is to identify the targets of these miRNAs and fit them into the elaborate regulatory networks that control hematopoietic differentiation (Table 1).

MicroRNA control of neuronal diversity

An elegant genetic pathway regulated by miRNAs has been uncovered through studies of nervous system development in *C. elegans* (Figure 2c). Analysis of the genes

responsible for establishing neuronal asymmetry led the Hobert laboratory to a previously unidentified miRNA gene [33^{••}], called *Lsy-6* (*laterally symmetrical*) because of the phenotype associated with mutations in this gene. Further investigation of this same cell-fate pathway uncovered another miRNA, *miR-273*, revealing a cascade of regulation by miRNAs and transcription factors that ultimately dictates cell-specific patterns of chemoreceptor expression [34^{••}]. In addition to these apparently neuronal-specific miRNAs, multitudes of other miRNAs have been identified in neuronal-type cells from a variety of organisms [35–40]. Considering the diverse profile of neuronal miRNAs, it is likely that many of them will also be intimately involved in the complex networks that regulate development and differentiation of animal nervous systems.

MicroRNAs in *Hox* gene developmental pathways

Hox gene regulation is also intimately involved with miRNAs (Figure 2d). Embedded in the mammalian *Hox* clusters are the *miR-10* and *miR-196* families of miRNA genes [41,42]. Furthermore, the expression pattern of *miR-10a* shows striking overlap with that of its flanking gene *Hoxb4*, indicating that these genes might be under common regulatory controls [43^{••}]. Even more remarkable, *miR-196a* exhibits an expression pattern that is inverse to that of *Hoxb8*, which is actually a target for negative regulation by this miRNA [43^{••},44^{••}]. The *Hoxb8* 3'UTR contains a nearly perfect complementary sequence to that of *miR-196a*, enabling the miRNA to direct mRNA cleavage and degradation [43^{••},44^{••}]. Although common in plants, this is the first example of an animal miRNA possessing sufficient base complementarity to target an mRNA for destabilization. Additional *Hox* genes contain 3'UTR sequences that support partial duplexes with *miR-196* and, thus, mediate repression through the translational inhibition pathway (Table 1) [44^{••}]. Taken together, these studies indicate that microRNAs help define the regions where *Hox* genes are expressed, thereby contributing to the precise spatial and temporal patterns by which *Hox* genes regulate developmental processes.

Conclusions

It is not surprising that organismal development involves complex regulatory mechanisms that target every aspect of gene expression. Post-transcriptional control of gene expression by miRNAs offers a new level to consider. The examples cited in this review lay the foundation for matching particular animal miRNAs to specific developmental pathways. However, the possibility that different miRNAs might target common genes and that multiple mRNAs might be recognized by the same miRNA complicates predictions of how isolated miRNA–target pairings might ultimately affect a biological pathway. Methodical testing of miRNA function at the phenotypic

and molecular levels is crucial for establishing bona fide roles for specific RNA genes. The extensive target predictions for particular miRNAs might be filtered by analyzing the level of expression of the miRNA and of both target mRNA and protein in particular cell types or stages of development. Finally, elucidation of how miRNAs themselves are regulated is paramount to understanding their potential for participating in the elaborate gene networks that orchestrate development.

Acknowledgements

We thank Katlin Massirer and Dr Shveta Bagga for critical reading of the manuscript and apologize to colleagues whose work we could not cite because of space limitations. Research support in the authors' laboratory is provided by the National Institutes of Health (GM071654-01) and the Searle Scholar, Peter Gruber and V Foundations.

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