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How microRNAs control cell division, differentiation and death

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After the milestone discovery of the first microRNA in 1993, the past five years have seen a phenomenal surge of interest in these short, regulatory RNAs. Given that 2% of all known human genes encode microRNAs, one main goal is to uncover microRNA function. Although it has been more difficult to assign function to microRNAs in animals than it has been in plants, important roles are emerging: in invertebrates, microRNAs control developmental timing, neuronal differentiation, tissue growth and programmed cell death. Functional studies in zebrafish and mice point toward important roles for microRNAs during morphogenesis and organogenesis. Finally, microRNAs might regulate viral infection and human cancer.

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Current Opinion in Genetics & Development 2005, **15**:563–568

This review comes from a themed issue on Differentiation and gene regulation
Edited by Tony Kouzarides and Andrew J Bannister

Available online 15th August 2005

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DOI 10.1016/jgde.2005.08.005

Introduction

Interest in the genes controlling developmental timing in the roundworm *Caenorhabditis elegans* [1–3] led to the cloning of the first microRNA, *lin-4*, [4] and the identification of the first microRNA target, *lin-14* [5]. The cloning of a second microRNA, *let-7*, which is involved in the same pathway in *C. elegans* [6], and the realization that *let-7* was conserved from worms to mammals [7] led to the identification of many microRNAs in worms, flies, mammals and flowering plants, using cloning and prediction strategies [8–12]. MicroRNAs are short, ~22 nucleotide, non-coding RNAs that are thought to regulate gene expression through sequence-specific base-pairing with their targets. The precise number of microRNA genes in the human genome is still unknown, but current estimates range from 500 to 1000 [13]. This review focuses only on animal microRNAs; the roles of microRNAs in plants have been reviewed recently elsewhere [14,15].

MicroRNA biogenesis and mechanisms of action

MicroRNA genes are found in intergenic regions as well as in introns. When located within introns, they can be on either the coding or the non-coding strand. Co-regulation of microRNAs and their host genes has been observed in some cases [16]. The majority of microRNA genes are probably transcribed by RNA polymerase II. Primary microRNA (pri-miRNA) transcripts can be hundreds of bases to several kilobases in length and might contain a single (monocistronic) or several (polycistronic) miRNAs. Pri-miRNAs have a 5′ 7-methylguanosine cap and a 3′-polyadenosine tail, and they might or might not contain introns that are spliced. For a detailed review of microRNA transcription, see [17].

Pri-miRNAs contain ~80-nucleotide stem–loop structures, named pre-miRNAs, with the mature miRNAs located on at least one of their arms. In the nucleus, pre-miRNAs are processed from pri-miRNAs by the RNase III enzyme Drosha and DGCR8/Pasha [18–22] and are exported to the cytoplasm by a mechanism that involves Exportin-5 [23]. In the cytoplasm, a second RNase III enzyme, Dicer, cuts the pre-miRNA to generate the mature microRNA as part of a short RNA duplex. This RNA is subsequently unwound by a helicase activity and incorporated into an RNA-induced silencing complex (RISC). Detailed reviews of microRNA biogenesis can be found elsewhere [17,24–27].

The mature microRNA is chemically identical to small interfering RNAs (siRNAs; see Glossary) that are generated during RNA interference (RNAi; see Glossary); for example, in experimental conditions and during viral infection (Figure 1). Although most microRNAs in animals are thought to function through the inhibition of effective mRNA-translation of target genes through imperfect base-pairing with the 3′UTR (3′ untranslated region) of target mRNAs [25], the underlying mechanism is poorly understood. In addition to this 'classic' mechanism, at least one microRNA, miR-196, can cleave a target mRNA, HOXB8, in a similar fashion to cleavage by siRNA [28•,29]. MicroRNAs might also play a role in AU-rich element-mediated mRNA degradation [30•]. Finally, the involvement of microRNAs in transcriptional gene-silencing, which has been observed in plants, remains a possibility [14,31].

This review describes the recent advances in understanding microRNA function in animals. As this is very much a work in progress, I also point out new strategies to further our understanding of microRNA biology. All those animal microRNAs for which a function has been demonstrated *in vivo* are listed in Table 1. Table 2 summarizes all

Glossary
Exocytosis: Release of proteins and neurotransmitters, etc, from cells by the fusion of vesicles with the plasma membrane.
RNA interference (RNAi): Sequence-specific mRNA-degradation pathway. Requires small interfering RNAs to target specific mRNAs.
Small interfering RNAs (siRNA): Short, single-stranded RNAs of approximately 22 nucleotides that can cause the degradation of target mRNAs by perfect base-pairing with their target.
Target prediction: Bioinformatic approach to predict microRNA targets on the basis of sequence complementarity of microRNAs and microRNA binding sites in 3′UTRs of target mRNAs.

microRNAs for which a biological role has been proposed and substantiated by indirect means.

Roles of microRNAs in invertebrates

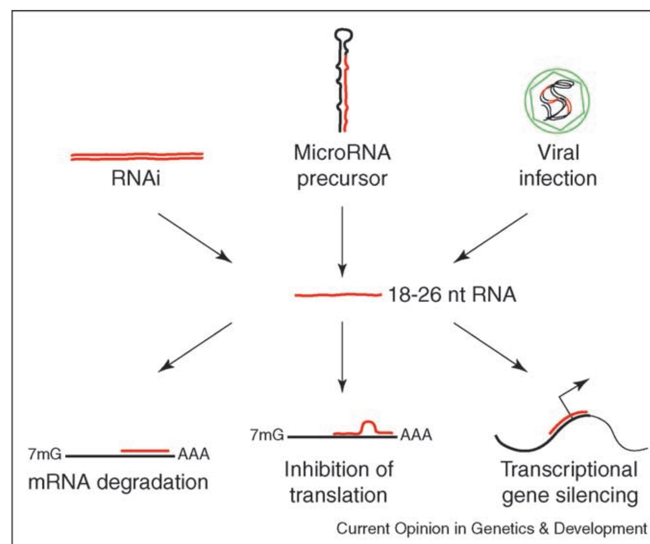
The developmental timing (heterochronic) pathway in *C. elegans* involves at least two microRNAs, *lin-4* and *let-7*. Both *lin-4* and *let-7* are required for endothelial cell fate decisions during larval development [1,2]. When *lin-4* or *let-7* is inactivated, specific endothelial cells undergo additional cell divisions instead of their normal differentiation. The two known target genes of *lin-4*, *lin-14* and *lin-28*, were identified through genetic interaction studies (see Table 1) [4,5,32]. This is also the case for the first two known *let-7* target genes, *lin-41* and *hbl-1* [33–35]. More recently, two additional *let-7* target genes, the transcription factor genes *daf-12* and *pha-4*, were identified with a combination of target prediction (see Glossary) using bioinformatics and RNAi [36••]. A *let-7*-related microRNA, miR-84, was recently identified as a putative regulator of vulval development in *C. elegans* (see Table 2) [37]. miR-84 is proposed to act through *let-60*, a *RAS* homologue [37]. MicroRNAs are also regulators of neuronal differentiation in *C. elegans*. The microRNA *lsy-6* is required for asymmetrical expression of a putative taste-receptor in a pair of sensory neurons, ASEL and ASER [38]. The *lsy-6* microRNA target is the Nkx-type homeobox gene *cog-1*. Both *lsy-6* and *cog-1* were identified in forward genetic screens for loss of asymmetry of ASEL and ASER. Interestingly, a second microRNA, miR-273, might act upstream of *lsy-6* and *cog-0031* as a direct regulator of *die-1*, which also encodes a transcription factor [39].

The first microRNA to be identified in the fruit fly, *Drosophila melanogaster*, is encoded by the *bantam* locus, which had previously been identified in a screen for deregulated tissue growth [40]. The *bantam* microRNA stimulates cell proliferation and reduces programmed cell death. It directly regulates the pro-apoptotic gene *hid/wrinkled*. A second *D. melanogaster* microRNA, miR-14, also suppressed programmed cell death [41]. Its target(s) remain to be determined. Additional roles for microRNAs in *D. melanogaster* can be inferred from conserved regulatory motifs in the 3′UTRs of Notch target genes, termed GY-, Brd- (Bearded) and K-boxes, and from microRNAs with complementarity to these motifs [42]. Candidate microRNAs regulating these motifs include miR-2, miR-4, miR-5, miR-6, miR-7, miR-11 and miR-79 [42–45].

Roles of microRNAs in vertebrates

Little direct evidence for *in vivo* roles for vertebrate microRNAs has been obtained to date. Not a single

Figure 1



Multiple roles for short RNAs. Primary microRNA gene transcripts contain stem–loop structures (pre-miRNAs) that are processed by the RNase III enzyme Dicer to give rise to the mature microRNA. Dicer is also required for the processing of double-stranded viral RNA and for generating siRNAs during RNAi. Mature microRNAs and siRNAs are chemically indistinguishable. Short RNAs are thought to have three modes of action: translational inhibition, mRNA degradation and transcriptional gene-silencing at the level of chromatin. In animals, most microRNAs are thought to inhibit effective target mRNA translation; however, microRNAs might use all three mechanisms to regulate target-gene expression.

Table 1

Animal microRNAs for which a function has been demonstrated *in vivo*.

MicroRNA	Known targets	Organism	Biological process	Cellular process	References
lin-4 microRNA	*lin-14, lin-28*	*C. elegans*	Developmental timing	Differentiation/proliferation	[4,5,32]
let-7 microRNA	*lin-41, hbl-1, daf-12, pha-4*	*C. elegans*	Developmental timing	Differentiation/proliferation	[6,33–35,36••]
lsy-6 microRNA	*cog-1*	*C. elegans*	Left-right asymmetry	Differentiation	[38]
bantam microRNA	*hid*	*D. melanogaster*	Growth control	Proliferation/programmed cell death	[40]
miR-14	Unknown	*D. melanogaster*	Programmed cell death	Programmed cell death	[41]
miR-430[1]	Unknown	*Drosophila rerio*	Neurogenesis	Unknown	[46••]

This table includes only those microRNAs that have been analyzed using loss-of-function studies. Functional roles for a number of other microRNAs have been inferred from overexpression and mis-expression studies; these data are summarized in Table 2. [1]miR-430 function was inferred from rescue experiments in a *Dicer*-mutant background.

microRNA knockout has been reported. However, in zebrafish (*Danio rerio*) experiments in which both maternal and zygotic Dicer product was removed, re-introduction of a single microRNA, miR-430, led to a dramatic improvement of severe brain morphogenesis defects [46••]. This observation suggests an important function for miR-430, or a related microRNA, in brain morphogenesis in the zebrafish.

In the mouse, miR-181 has been implicated in haematopoietic lineage differentiation, and overexpression of miR-181 leads to an increase in B lymphoid cells *in vitro* and *in vivo* [47]. miR-375, which is specifically expressed in mouse pancreatic islet cells, might regulate insulin secretion at the level of exocytosis (see Glossary) [48]. *Myotrophin* was identified as one direct target for miR-375.

Finally, the *miR-17* microRNA cluster and *miR-32* have been linked to human disease and are discussed below [49,50••].

A role for microRNAs in human disease: cancer and viral infection

A potential involvement of microRNAs in human disease might be inferred from the biological roles of microRNAs reported to date. Indeed, the *lin-4* and *let-7* phenotypes observed in *C. elegans* can be interpreted as proliferative defects [1,2], and the roles of *bantam* and *miR-14* in *D. melanogaster* also point to defects in proliferation [40,41]. Therefore, one might speculate that microRNAs might be de-regulated in proliferative diseases, such as cancer. Indeed, a large number of microRNAs appear to be de-regulated in primary human tumours [50••,51,52,53•], and many human microRNAs are located at fragile sites and genomic regions involved in cancer [54,55]. Of particular interest is the *miR-17* microRNA cluster, which is in a region on human chromosome 13 that is frequently amplified in B-cell lymphomas [50••]. Over-expression of the *miR-17* cluster was found to co-operate with *c-Myc* to accelerate tumour development in a mouse B-cell lymphoma model. Although the direct targets of members of the *miR-17* cluster of microRNAs are unknown, their collective role might be to inhibit excessive programmed cell death [50••]. In a separate study, the *miR-17* cluster was found to be up-regulated by over-expression of c-Myc, leading to the de-regulation of E2F1 expression [56•].

Another link between microRNAs and human disease comes from the identification of microRNAs encoded by large DNA viruses of the herpesvirus family, including the Epstein-Barr virus [57,58•]. These viral microRNAs have no apparent homologues in host genomes, and their function is currently not understood. Conversely, a cellular microRNA, miR-32, was shown to play an important

Table 2

Proposed *in vivo* roles for additional microRNAs in animals.

MicroRNA	Targets	Organism	Biological process	Cellular process	References
miR-273	*die-1*	*C. elegans*	Left–right asymmetry	Differentiation	[49]
miR-84	*let-60*	*C. elegans*	Vulval development	Differentiation/proliferation	[37]
miR-2a, -2b, -6, -7	*E(spl)/bHLH*, *Bearded* families[1]	*D. melanogaster*	Notch signalling	Unknown	[42–45]
miR-181	Unknown	*Mus musculus*	Hematopoeisis	Differentiation	[47]
miR-375	*Myotrophin (Mtpn)*	*M. musculus*	Insulin secretion	Exocytosis	[48]
miR-17, -18, -19a, -20, -19b-1, -92-1	Unknown	*M. musculus*	Tumourigenesis	Proliferation	[50••]
miR-32	*PFV-1*	*Homo sapiens*	Viral defense	Viral defense	[49]

This table lists microRNAs for which a probable function has been demonstrated using an indirect approach, for example mis-expression experiments. This table does not contain microRNAs for which a target mRNA has been predicted and validated using over-expression experiments but for which no further functional characterization at the cellular or organismal level has been carried out. Additional validated microRNA–target pairs can be found elsewhere [36••,43–45,70•]. [1]Family of Notch target transcription factors with GY-, Brd- and K-box motifs. Abbreviations: bHLH, basis helix–loop–helix; PFV-1, primate foamy virus type 1.

role in regulating the proliferation of the primate foamy virus type 1 (PFV-1) in cell culture [49].

Approximating microRNA function through the analysis of *Dicer* mutants

The RNase III enzyme Dicer is essential for the processing of microRNAs and for RNAi. Therefore, the analysis of *Dicer*-knockout strains might point toward biological roles of microRNAs, with the caveat that RNAi and possibly other biological processes might be de-regulated. Dicer was first analyzed genetically in *C. elegans* (*dcr-1*), in which it was found to be essential for germline development [59]. It is likely that maternal contribution of *dcr-1* masks earlier phenotypes, and this notion is supported by additional phenotypes that are revealed by the inactivation of *dcr-1* using RNAi [60]. RNAi of *dcr-1* results in embryonic and developmental timing defects that are reminiscent of *lin-4* and *let-7* mutants [60], thereby suggesting additional roles for microRNAs in *C. elegans* development.

In the zebrafish, a knockout of the *Dicer1* gene leads to a developmental arrest 7 to 10 days post-fertilization [61]. An earlier role for Dicer is probably masked by maternal Dicer contribution. Indeed, removal of maternal Dicer by the generation of germ line clones leads to more severe defects: axis formation and early differentiation are normal, but gastrulation, brain formation, somitogenesis and heart development are abnormal [46••].

Dicer-mutant mice have defects in axis formation and gastrulation, are depleted of stem cells and die at around 7.5 days of gestation [62]. A conditional knockout of *Dicer* early during T-cell development suggests a role for microRNAs in αβ-cell lineage [63]. Finally, a knockout of *Dicer* in the chicken–human hybrid DT40 cell line suggests a role for the enzyme in heterochromatin formation [64].

Approximating microRNA function through mRNA target prediction

MicroRNA target prediction offers an attractive route to the discovery of microRNA function. Several algorithms have been applied to *C. elegans*, *D. melanogaster* and mammalian genomes to predict microRNA targets [43,44,65–69]. In a complementary effort, conserved sites in 3′UTRs of human genes have been identified [70•]. For a subset of these predictions, the use of overexpression and green fluorescent protein- or luciferase-reporter assays has demonstrated that the microRNA can regulate the target candidate. It remains an open question as to how many microRNA targets that have been verified in these heterologous systems are important targets *in vivo*.

Approximating microRNA function through expression analysis

Initially, microRNA expression was monitored using northern blotting [4]. Next, cloning of microRNAs from specific tissues was used to approximate expression levels [71]. Over the past two years, microRNA microarray technologies have been developed for the high-throughput profiling of microRNA expression [15,52,53•,72,73]. The most exciting discovery to date is that many microRNAs are tissue-specifically expressed. It is to be expected that microRNA expression profiles will help to guide functional studies, as exemplified by the analysis of the pancreatic islet-specific microRNA miR-340 [48]. Finally, the recent development of microRNA *in situ* technology has the potential to revolutionize microRNA expression analysis [74••].

Conclusions

We are just beginning to unravel the roles of microRNAs in animals, but the examples listed here already demonstrate the great importance of this class of short RNAs. Many of the functions elucidated to date point to important roles for microRNAs in cell fate determination, including the control of cell division, differentiation and death. Few loss-of-function studies have been reported to date, but the tool kit is expanding with devices for global de-regulation of microRNAs (*Dicer* knockouts), target predictions and expression patterns at our disposal. The most exciting time for microRNAs lies ahead.

Acknowledgements

E Miska acknowledges support from a Cancer Research UK Programme Grant (C13474/A4613) and would like to thank Greg Hannon for sharing information before publication.

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