

MicroRNA functions in animal development and human disease

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Summary

Five years into the 'small RNA revolution' it is hard not to share in the excitement about the rapidly unravelling biology of microRNAs. Since the discovery of the first microRNA gene, *lin-4*, in the nematode *Caenorhabditis elegans*, many more of these short regulatory RNA genes have been identified in flowering plants, worms, flies, fish,

frogs and mammals. Currently, about 2% of the known human genes encode microRNAs. MicroRNAs are essential for development and this review will summarise our current knowledge of animal microRNA function. We will also discuss the emerging links of microRNA biology to stem cell research and human disease, in particular cancer.

Introduction

MicroRNAs (miRNAs) are about 22-nucleotide, short, non-coding RNAs that are thought to regulate gene expression through sequence-specific base pairing with target mRNAs. Hundreds of microRNAs have been identified in worms, flies, fish, frogs, mammals and flowering plants using molecular cloning and bioinformatics prediction strategies (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001; Lim et al., 2003a; Llave et al., 2002; Reinhart et al., 2002; Watanabe et al., 2005). MicroRNAs are transcribed as long RNA precursors (pri-miRNAs) that contain a stem-loop structure of about 80 bases. Pri-miRNAs are processed in the nucleus by the RNase III enzyme Drosha and DGCR8/Pasha, which excises the stem-loop to form the pre-miRNA (Denli et al., 2004; Gregory et al., 2004; Han et al., 2004; Landthaler et al., 2004; Lee et al., 2003). Pre-miRNAs are exported from the nucleus by Exportin-5 (Bohnsack et al., 2004; Lund et al., 2003; Yi et al., 2003). In the cytoplasm, another RNase III enzyme, Dicer, cuts the pre-miRNA to generate the mature microRNA as part of a short RNA duplex. The RNA is subsequently unwound by a helicase activity and incorporated into a RNA-induced silencing complex (RISC). For more information on microRNA biogenesis and maturation, please see the accompanying article by Du and Zamore (Du and Zamore, 2005).

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'-untranslated region (3'UTR) of target mRNAs (Bartel, 2004). The underlying mechanism is still poorly understood, but it appears to involve the inhibition of translational initiation (Pillai et al., 2005). MicroRNA targets are largely unknown, but estimates range from one to hundreds of target genes for a given microRNA, based on target predictions using a variety of bioinformatics approaches (Brennecke et al., 2005; John et al., 2004; Kiriakidou et al., 2004; Lewis et al., 2005; Lewis et al., 2003; Rajewsky and Socci, 2004; Stark et al., 2003; Xie et al., 2005). In addition, at least one microRNA, miR-196, can cleave a target mRNA, HOXB8, in the same manner as a short

interfering RNA (siRNA) does during the process of RNA interference (RNAi) (Mansfield et al., 2004; Yekta et al., 2004). This mechanism is the preferred one for plant microRNAs (Meins et al., 2005). MicroRNAs may also play a role in AU-rich element-mediated mRNA degradation (Jing et al., 2005). See Du and Zamore for a further discussion of this (Du and Zamore, 2005). Finally, the involvement of microRNAs in transcriptional gene silencing (TGS), which has been observed in plants, remains an intriguing possibility (Baulcombe, 2004).

This review focuses on the function of animal microRNAs only. For a recent review of our current understanding of the roles of microRNAs in plants, please see (Kidner and Martienssen, 2005), and for recent accounts of the history of this field, please see recent articles by the pioneers of the field themselves (Lee et al., 2004a; Ruvkun et al., 2004). Here, we describe how microRNAs contribute to different aspects of animal development and what we know of their involvement in human disease (see Table 1).

Developmental timing

Interest in the genes controlling developmental timing in *C. elegans* (Ambros and Horvitz, 1984; Chalfie et al., 1981; Horvitz and Sulston, 1980) led to the cloning of the first microRNA, *lin-4* miRNA (Lee et al., 1993), and the identification of the first microRNA target, *lin-14* mRNA (Wightman et al., 1993). The developmental-timing, or heterochronic, pathway regulates stage-specific processes during *C. elegans* larval development. For a recent, detailed review of this pathway, please see Rougvie (Rougvie, 2005). One focus of the study of the heterochronic pathway in *C. elegans* has been the developmental fate of several stem cells in the lateral hypodermis, collectively known as the seam cells. The seam cells undergo a cell division pattern that is synchronised with the four larval molts of the animal (Fig. 1A). Only at the adult stage will the seam cells exit mitosis and terminally differentiate. In *lin-4* mutant animals, the seam cells repeat the cell division pattern that characterises the first larval stage (L1) and fail to differentiate. This mutant phenotype has been interpreted as a heterochronic change with the

Table 1. Function of animal microRNAs in vivo

Process	microRNA	Targets	Function	Evidence	Key citations
<i>C. elegans</i>					
Developmental timing	<i>lin-4</i> microRNA	<i>lin-14</i> , <i>lin-28</i>	Stem cell differentiation	LOF	Lee et al., 1993; Wightman et al., 1993
Developmental timing	<i>let-7</i> microRNA	<i>lin-41</i> , <i>hbl-1</i> , <i>daf-12</i> , <i>pha-4</i>	Stem cell differentiation	LOF	Reinhart et al., 2000; Abrahante et al., 2003; Lin et al., 2003; Slack et al., 2000; Grosshans et al., 2005
Developmental timing	miR-48, miR-84, miR-241	<i>hbl-1</i>	Stem cell differentiation	LOF	Abbott et al., 2005; Lin et al., 2005
Developmental timing	miR-48, miR-84	Unknown	Cessation of molting	LOF	Abbott et al., 2005
Organogenesis	miR-84	<i>let-60</i>	Differentiation/proliferation	GOF	Johnson et al., 2005
Differentiation	<i>lisy-6</i> microRNA	<i>cog-1</i>	Left-right asymmetry	LOF	Johnston and Hobert, 2003
Differentiation	miR-273	<i>die-1</i>	Left-right asymmetry	GOF	Chang et al., 2004
<i>D. melanogaster</i>					
Growth control and programmed cell death	<i>bantam</i> microRNA	<i>hid</i>	Proliferation/programmed cell death	LOF	Brennecke et al., 2003
Programmed cell death	miR-14	Unknown	Programmed cell death	LOF	Xu et al., 2003
Patterning and embryogenesis	miR-2a, -2b, -6, -7	<i>E(spl)/bHLH</i> , <i>bearded</i> families	Notch signalling	GOF	Brennecke et al., 2005; Lai, 2002; Lai et al., 2005; Stark et al., 2003
Embryogenesis and programmed cell death	miR-2, -6, -11, -13, -308	Unknown	Programmed cell death	2-O-Me-RNA	Leaman et al., 2005
<i>D. rerio</i>					
Differentiation and organogenesis	miR-430	Unknown	Neurogenesis	Dicer rescue	Giraldez et al., 2005
<i>M. musculus</i>					
Differentiation and organogenesis	miR-1	Hand2	Angiogenesis	GOF	Zhao et al., 2005
Differentiation and organogenesis	miR-181	Unknown	Hematopoiesis	GOF	Chen et al., 2004
Insulin secretion	miR-375	<i>Myotrophin (Mtpn)</i>	Exocytosis	2-O-Me-RNA	Poy et al., 2004
Human disease	miR-17, -18, -19a, -20, -19b-1, -92-1	Unknown	Tumorigenesis	GOF	He et al., 2005; O'Donnell et al., 2005
<i>H. sapiens</i>					
Human disease	miR-32	PFV-1	Viral defense	LNA	Lecellier et al., 2005

This table includes all microRNAs that have been analyzed in vivo using loss-of-function studies, and microRNAs for which a likely function has been demonstrated by using an indirect approach, e.g. mis-expression experiments. It table does not contain microRNAs for which a target mRNA has been predicted and validated using overexpression experiments, but for which no further functional characterization at the cellular or organismal level has been carried out.

PFV-1, primate foamy virus type 1; E(spl)/bHLH, Enhancer of Split/basic helix loop helix transcription factor cluster; LOF, microRNA has been studied using loss-of-function mutations; GOF, microRNA has been studied using gain-of-function approaches only; 2-O-Me-RNA, microRNA has been studied by depletion using 2' O-Methyl antisense oligoribonucleotides; LNA, microRNA has been studied by depletion using locked oligonucleotides (LNA); Dicer rescue, microRNA has been studied using rescue experiments in a Dicer mutant background.

developmental clock being stuck at the L1 stage, resulting in developmental 'retardation'. Gain-of-function mutations in the *lin-4* miRNA target *lin-14* lead to an identical phenotype, whereas loss-of-function mutations in *lin-14* result in an opposite, 'precocious' phenotype, where the seam cells skip the cell division of the first larval stage. The *lin-4* and *lin-14* gene products therefore act as a developmental switch that controls the L1 to L2 transition (Fig. 1A,B).

Three microRNAs of the *let-7* family, *mir-48*, *mir-84* and *mir-241* act redundantly to control the next developmental transition, from the L2 to the L3 stage (Fig. 1B) (Abbott et al., 2005; Lau et al., 2001; Lin et al., 2005; Reinhart et al., 2000). Loss-of-function mutations in these three microRNAs lead to the repetition of the cell division pattern of the second larval stage, whereas a gain-of-function mutation in *mir-48* results in a precocious phenotype. A likely target of *mir-48*, *mir-84* and *mir-241* during this transition is the *C. elegans* hunchback orthologue *hbl-1*. The microRNA *let-7*, the second microRNA to be identified (Reinhart et al., 2000), controls the transition from the fourth larval stage to the adult stage, and two of its

targets in the heterochronic pathway are the *lin-41* and *hbl-1* genes, both of which are also heterochronic genes (Abrahante et al., 2003; Lin et al., 2003; Slack et al., 2000). More recently, two additional *let-7* target genes, the transcription factor genes *daf-12* and *pha-4*, were identified using a combination of bioinformatics-based prediction and RNAi analyses (Grosshans et al., 2005). *daf-12* is also a regulator of the heterochronic pathway controlling seam cell fate (Antebi et al., 1998). Finally, the two *let-7* family microRNAs *mir-48* and *mir-84* also control cessation of the larval molting cycle at the adult stage, with *mir-48*; *mir-84* double mutant animals undergoing a supernumerary molt at the adult stage (Fig. 1A,B) (Abbott et al., 2005).

It is striking that at least two microRNA families and at least four microRNAs are involved in the control of developmental timing in *C. elegans*. As the *lin-4* and *let-7* microRNA families are conserved, they might play similar roles in other organisms. This notion is supported by the temporal regulation of *let-7* expression in several species (Pasquinelli et al., 2000). However, at least one potential role for *let-7* family

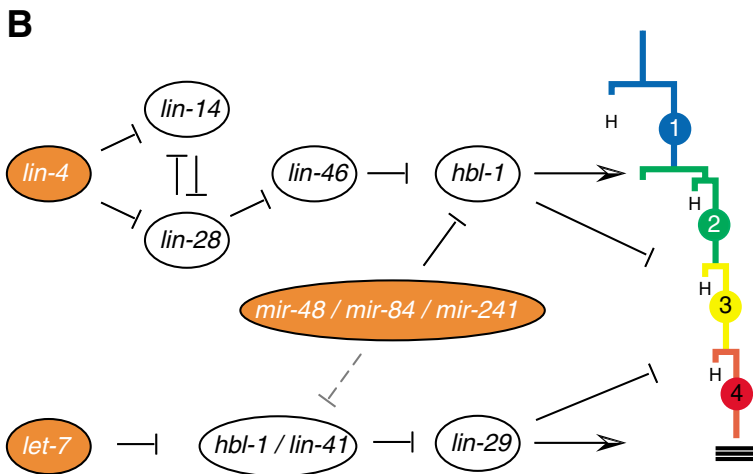
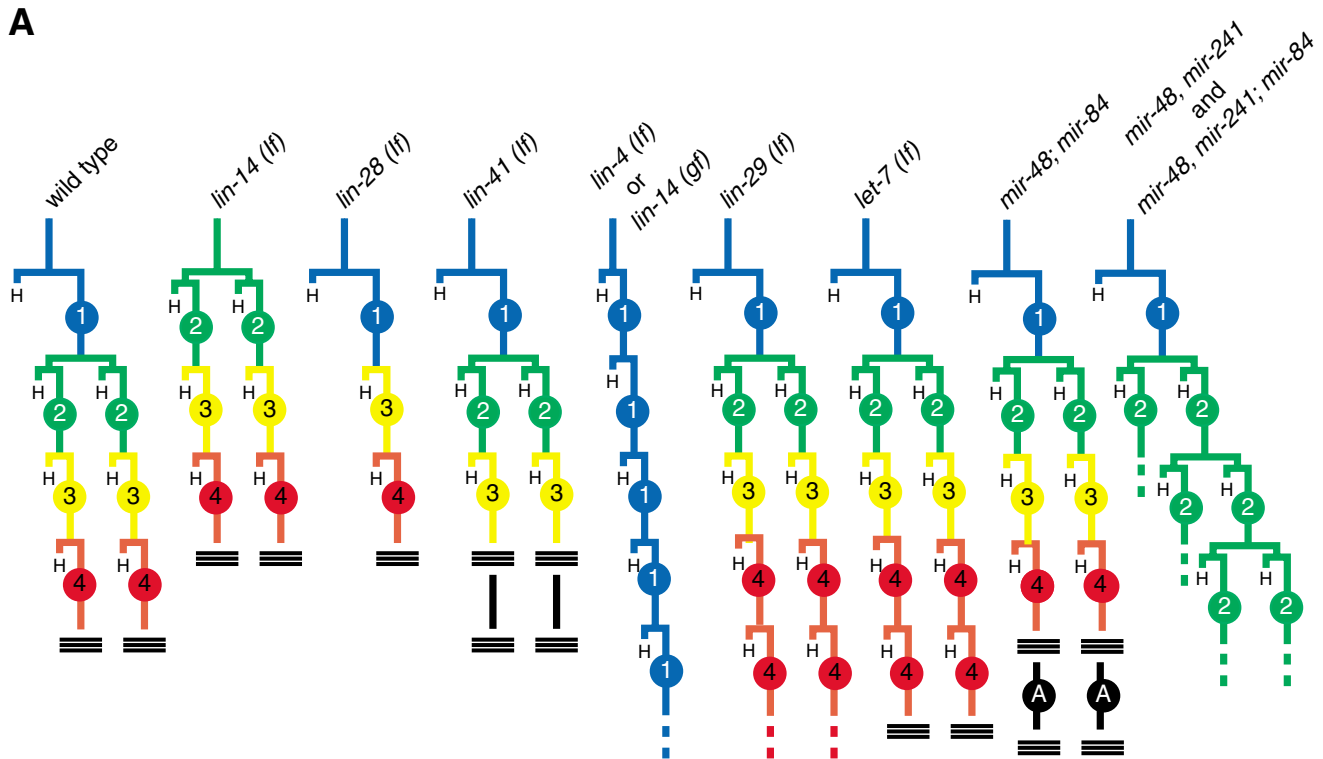


Fig. 1. MicroRNAs control developmental timing. (A) *C. elegans* cell lineage diagram for the seam cells V1-V4 and V6. Developmental time is the vertical axis. 1 to 4, larval stages L1 to L4; A, adult stages. Colours refer to developmental stage. H, cell fused to hypodermis. Triple black line, terminal differentiation signified by lateral alae. Dotted lines, continued cell division according to the same pattern. (B) Simplified genetic pathway of the heterochronic genes in *C. elegans*. Dashed line represents possible late role for *mir-48*, *mir-84* and *mir-241* during development.

microRNAs outside of the heterochronic pathway has been reported, as discussed below (Johnson et al., 2005).

Patterning and embryogenesis

In the absence of loss-of-function mutants for most microRNA genes, organisms with defective microRNA biogenesis are a useful tool for investigating the biological roles of microRNAs, particularly during embryogenesis and early development. This is because such organisms allow one to study the roles of the first set of microRNAs required during development. Dicer knockout mutants have been particularly useful in this regard, although RNAi, and other biological processes as well as microRNA function, might be de-regulated in these mutants. Dicer was first analyzed genetically in *C. elegans*, where it is called DCR-1, and it was found to be essential for germline

development (Knight and Bass, 2001). A similar phenotype was observed for *C. elegans* mutants of the other RNase III enzyme required for microRNA biogenesis, Drosha (DRSH-1) (Denli et al., 2004). As *dcr-1* mutants are sterile, all homozygous animals had to be derived from heterozygous mothers. It is likely that maternal contribution of DCR-1 masks earlier abnormal phenotypes. This is supported by the occurrence of additional abnormal phenotypes when *dcr-1* mRNA is inactivated by RNAi (Grishok et al., 2001). RNAi of *dcr-1* mRNA results in a mixed phenotype that includes embryonic lethality and developmental timing defects that are reminiscent of the *lin-4* and *let-7* mutants (Grishok et al., 2001). These observations suggest that microRNAs have essential roles in *C. elegans* embryogenesis. This hypothesis was further supported by RNAi knockdown of the mRNA

Box 1. miRNA knockdown

To study loss-of-function phenotypes of miRNAs in the absence of knockout strains, miRNA knockdown approaches are being developed. Two strategies are based on artificial oligonucleotides, 2' O-Methyl RNA and locked nucleotide RNA (LNA). The principle for both reagents is the same: an excess of modified RNA complementary to a miRNA is injected or transfected so that it can compete for the miRNA interaction with its target mRNAs. 2' O-Methyl RNA offers an increased in vivo half-life over RNA and was initially used in human cells and in *C. elegans* (Hutvagner et al., 2004; Meister et al., 2004; Poy et al., 2004) for the sequence-specific inhibition of small RNAs for a limited time-span, but it suffers from some pleiotropic effects (Leaman et al., 2005). LNA is a modified ribonucleic acid in which the ribose ring is constrained by a methylene bridge between the 2' O and the 4' C atoms. This modification leads to a higher thermal stability and discriminative power due to a greater difference in the melting temperature of a Watson-Crick base pair versus a mismatch (Petersen and Wengel, 2003).

transcripts for the two *C. elegans* argonaute proteins required for miRNA biogenesis, ALG-1 and ALG-2 (Grishok et al., 2001). RNAi-treated worms showed a mixed phenotype that included embryonic and larval lethality and heterochronic defects.

The fruitfly *Drosophila melanogaster* has two Dicer genes, *Dicer-1* and *Dicer-2*, and genetic analysis suggests that *Dicer-1* is the major Dicer gene required for microRNA biogenesis (Lee et al., 2004b). Although the phenotype of *Dicer-1* mutant *D. melanogaster* has not been fully reported, it appears that *Dicer-1* is required for wild-type development of both somatic tissues and the germline (Hatfield et al., 2005; Lee et al., 2004b) (for details see below).

In the zebrafish *Danio rerio*, a likely null allele of Dicer leads to a developmental arrest at 7 to 10 days post-fertilization (Wienholds et al., 2003). This late terminal phenotype is again likely to be due to maternal provision of Dicer and/or of microRNAs. Indeed, removal of the maternal Dicer contribution through the generation of germline clones leads to a more severe defect (Giraldez et al., 2005). In maternal-zygotic Dicer mutants, axis formation and early differentiation are normal, but many embryos have morphogenesis defects affecting gastrulation, brain formation, somitogenesis and heart development.

In the mouse, *Mus musculus*, *Dicer1* mutants die around 7.5 days of gestation (Bernstein et al., 2003). A maternal contribution of Dicer is likely to have a much smaller effect in *M. musculus* due to the much smaller size of the egg. Homozygous *Dicer1* null mutants from heterozygous mothers die around 7.5 days of gestation (Bernstein et al., 2003). Mutant embryos have defects in axis formation and gastrulation, and are depleted of Oct4-positive stem cells (Bernstein et al., 2003). In all cases where only *Dicer1* mutants have been analysed, one cannot easily distinguish between defects that are due to a loss of microRNA processing and those that are due to a loss of endogenous RNAi or other pathways regulated by Dicer. For example, Dicer appears to have important roles in heterochromatin formation and chromosome segregation in the fission yeast *Schizosaccharomyces pombe*, in the ciliated protozoan

Tetrahymena and in vertebrate cells (Fukagawa et al., 2004; Mochizuki and Gorovsky, 2005; Provost et al., 2002). As *S. pombe* does not encode any known microRNAs, these defects are unlikely to be caused by their loss.

A more direct approach to investigating the role of microRNAs during embryogenesis has been taken in *D. melanogaster*, where 2' O-Methyl antisense oligoribonucleotides were used in microRNA depletion studies (see Box 1) (Leaman et al., 2005).

A single injection of 2' O-Methyl antisense oligoribonucleotides complementary to the 46 microRNAs known to be expressed in the *D. melanogaster* embryo resulted in a total of twenty-five different, abnormal phenotypes. These phenotypes included defects in blastoderm cellularization and patterning, morphogenesis and cell survival. Increased programmed cell death was observed in embryos injected with 2' O-Methyl antisense oligoribonucleotides that targeted the *D. melanogaster* miR-2 family, and miR-6, miR-11 and miR-308.

Clearly, our current understanding of microRNA function during embryogenesis is only rudimentary. However, it is noteworthy that the only evidence for a role of miRNAs in tissue patterning during embryogenesis to date comes from depletion studies of miR-31 in *D. melanogaster* (Leaman et al., 2005). Could pattern formation be largely independent of regulation by microRNAs? With this question in mind, it will be exciting to see functional studies of mammalian miR-196, a microRNA that is located in a HOX cluster and can cleave HOXB8 mRNA (Mansfield et al., 2004; Yekta et al., 2004).

Differentiation and organogenesis

The heterochronic phenotypes of the *lin-4* microRNA and the *let-7* family of microRNAs in *C. elegans* are clear examples of cell differentiation defects. However, an example of a microRNA regulating differentiation that is uncoupled from cell division was first uncovered through the study of left-right asymmetry in *C. elegans* (Johnston and Hobert, 2003). In the worm, two bilateral taste receptor neurons, ASE left (ASEL) and ASE right (ASER), display a left/right asymmetrical expression pattern of *gcy-5*, *gcy-6* and *gcy-7*, three putative chemoreceptor genes (Chang et al., 2003; Hobert et al., 2002) (Fig. 2). In a genetic screen for mutants in which the normally ASEL-specific expression of *gcy-7* is disrupted, the microRNA gene *lisy-6* was isolated (Chang et al., 2003). In *lisy-6* mutants, ASEL neurons do not express *gcy-7*, but instead express the ASER-specific *gcy-5* gene. Genetic interaction and GFP reporter studies showed that *lisy-6* is a negative regulator of the NKX-type homeobox gene *cog-1*, which was identified in the same genetic screen. Interestingly, a second microRNA, miR-273, might act upstream in the same pathway as a regulator of *die-1*, which encodes a C2H2 zinc finger transcription factor (Chang et al., 2004). The transcription factor *die-1* shows ASEL-specific expression and acts upstream of *lisy-6*.

Evidence for a role of microRNAs in organogenesis has come from studies of vulval development in *C. elegans*. The *C. elegans* vulva is a ring-like structure that forms the connection between the hermaphrodite gonadal arms and the exterior, and is essential for egg-laying and sperm entry. It derives from a group of cells in the ventral hypodermis that are induced to undergo a series of cell divisions and differentiation by a signal from the gonadal anchor cell (Sulston and Horvitz, 1977). Vulval induction requires RAS/LET-60 signalling

(Beitel et al., 1990). *let-7* loss-of-function mutants die by bursting at the vulva (Reinhart et al., 2000; Slack et al., 2000), and this bursting is suppressed by RNAi against the *C. elegans* RAS orthologue LET-60 (Johnson et al., 2005). Furthermore, overexpression of the *let-7* family microRNA miR-84 suppresses the *let-60* gain-of-function phenotype. 3'UTR reporter experiments suggest that RAS/LET-60 expression levels are regulated post-transcriptionally and may be directly regulated by the *let-7* family of microRNAs (Johnson et al., 2005).

In *D. melanogaster*, the discovery of an important role for post-transcriptional control of the Notch signalling pathway predates the discovery of the first microRNA in *D. melanogaster* by over a decade. The Notch signalling pathway is an evolutionary conserved signal transduction cascade that is required for patterning and normal development (Lai, 2004). Two clusters of Notch signalling target genes exist in *D. melanogaster*: the Enhancer of split-Complex and the Bearded-Complex, which encode transcription factors of the basic helix-loop-helix repressor and the Bearded families, respectively (Knust et al., 1992; Lai et al., 2000a; Lai et al., 2000b; Wurmbach et al., 1999). Gain-of-function alleles in members of these gene families were found to be due to short deletions in conserved regions of their 3'UTRs (Knust, 1997; Leviten et

al., 1997). These 6- to 7-nucleotide motifs were named the GY-box, the Brd-box and the K-box. Some of these motifs have been shown to post-transcriptionally control the Enhancer of split-Complex and the Bearded-Complex genes (Lai et al., 1998; Lai and Posakony, 1997). More recently, it has been noted that GY-, Brd- and K-box sequences are complimentary to the newly identified *D. melanogaster* microRNAs (Lagos-Quintana et al., 2001; Lai, 2002). Candidate microRNAs regulating these motifs include three microRNA families and the following microRNAs: miR-2, miR-4, miR-5, miR-6, miR-7, miR-11 and miR-79 (Brennecke et al., 2005; Lai, 2002; Lai et al., 2005; Stark et al., 2003). Although no loss-of-function analyses of any of these microRNAs have been carried out, overexpression of some of them causes defects reminiscent of Notch loss-of-function mutants. These include notched wings and wing vein abnormalities, an increased number of micro- and macrochaetes (small and large bristles) in the adult notum, tufted sternopleural bristles and an increase in sensory organ precursor cells in imaginal discs (Lai et al., 2005). Interestingly, the list of microRNAs that might regulate Notch overlaps considerably with the microRNAs that were found to play important roles during embryogenesis in the 2' O-Methyl antisense oligoribonucleotides microRNA depletion studies previously discussed (Leaman et al., 2005).

As outlined above, the analysis of *Dicer* mutant animals suggests that microRNAs have important roles to play during differentiation and organogenesis in *D. rerio*. In particular, neurogenesis, somitogenesis, and ear, eye and heart development were found to be disrupted in maternal-zygotic *Dicer* mutants (Giraldez et al., 2005). It is as yet unclear whether all of these defects are due to the lack of microRNAs or other *Dicer*-dependent processes. No microRNA knockout zebrafish strains are currently available; however, rescue experiments in maternal-zygotic *Dicer* mutant animals suggest an important role for the miR-430 family of microRNAs in neurogenesis (Giraldez et al., 2005). Maternal-zygotic *Dicer* mutant embryos have severe neurulation defects. Formation of the neurocoel and neural tube is impaired in these mutants, as is the formation of the brain ventricles and the midbrain-hindbrain boundary. Surprisingly, the injection of a single microRNA, miR-430, a member of a large family of microRNAs that is related to the miR-302 and miR-17 families (see below), rescued some aspects of this mutant phenotype. Injection of miR-430, but not of an unrelated microRNA, resulted in normal brain ventricles and a rescue of the midbrain-hindbrain boundary. In addition, miR-430 injection led to a partial rescue of gastrulation, retinal development and somatogenesis, but not of heart or ear development. Together, these findings clearly demonstrate that at least a subset of the abnormal phenotypes of maternal-zygotic *Dicer* mutant animals are due to the loss of microRNAs. In the absence of microRNA knockout strains, the maternal-zygotic *Dicer* knockout provides a unique system in which to study the function of other microRNAs in the zebrafish. The recent description of microRNA expression patterns in developing *D. rerio* using in situ hybridization provides a potential resource for candidate microRNA selection (Wienholds et al., 2005).

Similar to *D. rerio*, and with the same caveats, *M. musculus* *Dicer1* mutant animals indicate that microRNAs have wide-ranging roles in differentiation and development during mouse embryogenesis (Bernstein et al., 2003) (see above). In the

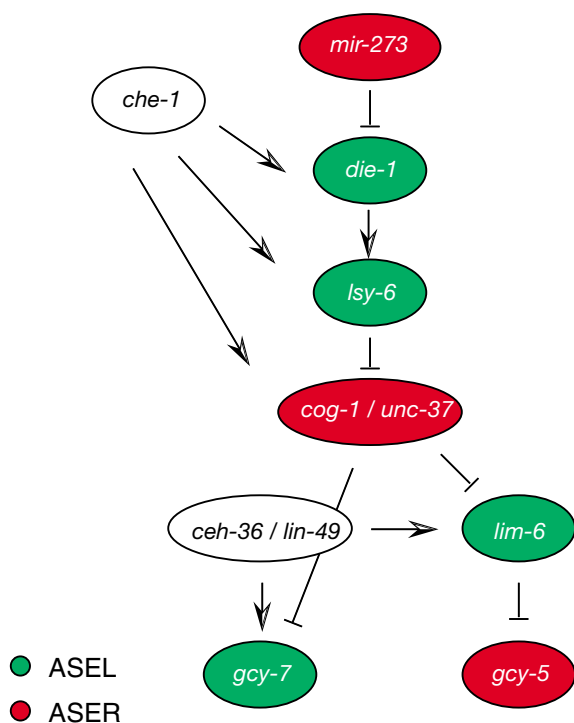


Fig. 2. MicroRNAs control differentiation. Neuronal specification of ASEL and ASER cells by *lsy-6* microRNA and miR-273. *che-1*, *unc-37* and *ceh-36/lin-49* are required for the neuronal determination of the pair of ASE neurons. The ASE cells differ in the asymmetric expression of two candidate chemoreceptor genes, *gcy-7* (in ASEL) and *gcy-5* (in ASER), which also define different chemosensory functions. The microRNA *lsy-6* represses the translation of *cog-1* mRNA, which results in the expression of *lim-6* and *gcy-7* (shown in green). In ASER, miR-273 represses the translation of *die-1* mRNA, which leads to the expression of *cog-1* and *gcy-5* (shown in red). It is still unclear where *che-1* acts in this pathway.

mouse, additional insights can be gained from studying conditional-knockout strains, which allow one to study the requirement for Dicer and microRNAs in different tissues at different developmental time points. In vitro, Dicer mutant embryonic stem (ES) cells, derived from conditional gene targeting, have severe differentiation defects (Kanellopoulou et al., 2005). One study that analyzed the effects of genetically inactivating *Dicer1* early during T-cell development found evidence for the functioning of microRNAs in $\alpha\beta$ cell, but not in CD4/CD8, lineage commitment (Cobb et al., 2005). Another study found that knocking out *Dicer1* during T-cell development blocked peripheral CD8⁺ T-cell development, whereas CD4⁺ T cells, although reduced in numbers, were viable; however, upon stimulation, these CD4⁺ T cells proliferated poorly and underwent increased programmed cell death (Muljo et al., 2005).

The particular caveat with these conditional-knockout studies in mice is that it is often unclear how efficiently Dicer and any existing microRNA pools are depleted upon the somatic deletion of *Dicer1*. Indeed, microRNAs seem to persist for some time (Cobb et al., 2005). One specific microRNA that has been directly implicated in B-cell development is miR-181 (Chen et al., 2004). This microRNA is highly expressed in B-lymphoid cells of mouse bone marrow. When overexpressed in hematopoietic progenitor cells, it leads to an increase in the fraction of B-lineage cells in in vitro differentiation experiments and in vivo in adult mice. Conditionally inactivating *Dicer1* in discrete areas of the limb mesoderm in mice led to severe growth defects in the limbs of mutant embryos, but no defect in basic limb patterning or in tissue-specific differentiation was observed (Harfe et al., 2005). This is a striking finding that is somewhat reminiscent of the *Dicer1* knockout in *D. rerio*. However, it remains unknown whether residual Dicer activity or microRNA pools could have disguised earlier roles of microRNAs in limb development.

MicroRNA expression analysis has led to the discovery of a potential role for the microRNA miR-1 in mammalian heart development. The microRNA miR-1, which is the product of two genes, *mir-1-1* and *mir-1-2*, is highly expressed in mouse heart and muscle (Lagos-Quintana et al., 2001; Lee and Ambros, 2001). An analysis of the upstream-regulatory sequence of these two genes has led to the identification of serum response factor (Srf), myocardin, Mef2 and Myod as transcriptional regulators of miR-1 expression in vitro (Zhao et al., 2005). Of these, Srf was found to be required for miR-1 expression in the developing mouse heart, using a conditional Srf-knockout strain (Zhao et al., 2005). Overexpression of miR-1 under the β -myosin heavy chain promoter resulted in developmental arrest at embryonic day 13.5, after heart failure. Transgenic embryos developed thin ventricle walls and ventricular cardiomyocyte proliferation defects. One candidate target for miR-1 in myocardial development is the transcription factor *Hand2*, which was found to be reduced in transgenic mice overexpressing miR-1 without an apparent change in *Hand2* mRNA levels (Zhao et al., 2005).

Growth control and programmed cell death

The *D. melanogaster bantam* gene was identified in a gain-of-function screen for regulators of cell growth (Hipfner et al., 2002). Overexpression of *bantam* causes the overgrowth of

wing and eye tissue, whereas *bantam* loss-of-function mutant animals are smaller than wild-type animals and have reduced cell numbers. *bantam* was found to interact with the growth regulatory gene *expanded*, but was epistatic to the *CycD/Cdk4* pathway. Subsequent cloning of *bantam* identified it as a microRNA-encoding gene (Brennecke et al., 2003). The *bantam* microRNA regulates tissue growth cell autonomously. Overgrowth phenotypes due to overexpression of *bantam* do not result in increased levels of programmed cell death, and *bantam* overexpression rescued programmed cell death induced by overexpression of the transcription factor E2A element-binding factor (E2F) and its dimerisation partner (DP). *bantam* overexpression also blocked programmed cell death induced by overexpression of the pro-apoptotic gene *hid/Wrinked* (Fig. 3). Furthermore, *Hid* was shown to be the likely direct target of *bantam* mRNA (Brennecke et al., 2003). It is unclear whether the small body size of *bantam* mutant flies is due to the increased activity of *Hid* or the de-regulation of other target genes. A second *D. melanogaster* microRNA, miR-14, was also found to suppress programmed cell death (Xu et al., 2003). Whether this is a direct effect of the de-regulation of pro-apoptotic genes remains to be determined. Increased levels of programmed cell death were also found in depletion experiments using 2' O-Methyl antisense oligoribonucleotides targeting the *D. melanogaster* miR-2 family, miR-6, miR-11 and miR-308 (Leaman et al., 2005) (see previous discussion). Finally, the deletion of *Dicer-1* in *Drosophila* results in a growth defect in germline stem cells (Hatfield et al., 2005) (see below). Together, these observations suggest an important role for microRNAs in growth control during *D. melanogaster* development.

Additional links between microRNAs, growth control and programmed cell death have also come from other species. The phenotype of *mir-48; mir-84; mir-241* mutants in *C. elegans* is one of cellular overgrowth (Abbott et al., 2005). In *D. rerio*, the zygotic removal of Dicer results in a larval growth arrest (Wienholds et al., 2003) (see above). And, finally, in *M. musculus*, removal of Dicer in the limb mesoderm leads to a dramatic programmed cell death in the developing limb (Harfe et al., 2005).

Stem cells and the germline

The first two microRNAs to be identified, the *C. elegans* microRNAs *lin-4* and *let-7*, control cell divisions in the hypodermal blast lineage (Ambros and Horvitz, 1984; Chalfie et al., 1981; Horvitz et al., 1983). In the absence of either gene, this stem cell lineage fails to differentiate and continues its proliferative cycle. More recently, other *let-7* family members have also been shown to be involved in the differentiation of this stem cell lineage (Abbott et al., 2005; Lin et al., 2005). In the mouse, Dicer is required for embryonic stem cell differentiation in vitro (Kanellopoulou et al., 2005) (see above). And in early *Dicer1* mutant mouse embryos, the pool of pluripotent stem cells that is required for the proliferation of cells in the inner cell mass of the blastocyst is diminished, as assayed by in situ hybridization using probes against *Oct4* mRNA (Bernstein et al., 2001). It is unclear whether the pool of *Oct4* mRNA-positive cells fails to expand, differentiates or undergoes programmed cell death. The identities of any microRNAs that may be required for stem cell maintenance in the mouse are currently unknown. However, expression studies

in ES cell lines and in mouse embryoid bodies have identified microRNAs (miR-302 family) that are specifically expressed in ES cells but not in adult mouse tissues (Houbaviy et al., 2003; Suh et al., 2004). These microRNAs may be candidates for stem cell renewal factors.

Suggestions for a role of microRNAs in the germline initially came from the analysis of Dicer loss-of-function mutants in *C. elegans* (Knight and Bass, 2002). However, it remained unclear whether microRNA-processing defects were the underlying cause of the sterility. Subsequently, Droscha was also shown to be required for fertility in *C. elegans* (Denli et al., 2004). No detailed analysis of either phenotype has been reported to date. However, such an analysis has been carried out in *D. melanogaster* *Dicer-1* mutants. *Dicer-1* is one of two Dicer genes in the fly and is the one that is largely required for microRNA biogenesis (Lee et al., 2004b), as previously discussed. It is unclear whether *Dicer-1* is also required for other RNA-dependent processes. *Dicer-1* mutant animals have somatic and germline defects (Lee et al., 2004b). The generation of germline mutant clones has revealed that defects exist in the maintenance of the germline stem cell pool (Hatfield et al., 2005). A *Dicer-1* mutant germline had reduced germline cyst production. Based on cell-cycle marker analysis and epistasis experiments, germline stem cells appeared to be blocked in the G1/S transition phase. No microRNA required for germline stem cell proliferation has yet been identified.

Human disease

Many of the functional roles of microRNAs discussed above hint at the potential involvement of microRNAs in human disease. For example, the *lin-4* and *let-7* mutant phenotypes observed in *C. elegans* can be interpreted as growth defects (Ambros and Horvitz, 1984; Chalfie et al., 1981; Horvitz et al., 1983). The *let-7* family of microRNAs may also be regulators of the proto-oncogene *RAS*. In *D. melanogaster*, the *bantam* microRNA and miR-14 are required for growth control; for example, through the regulation of programmed cell death (Brennecke et al., 2003; Xu et al., 2003). If microRNAs are major regulators of growth and proliferation, is there also evidence for roles of microRNAs in human cancer? Many microRNAs are de-regulated in primary human tumours (Calin et al., 2002; Calin et al., 2004a; He et al., 2005; Lu et al., 2005). Moreover, many human microRNAs are located at genomic regions linked to cancer (Calin et al., 2004b; McManus, 2003). 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 (He et al., 2005). Overexpression of the *mir-17* cluster was found to co-operate with *Myc* to accelerate tumour development in a mouse B-cell lymphoma model. Further evidence for such a link between *Myc* and the *mir-17* cluster has come from microarray expression studies, which showed that *mir-17* cluster gene expression was induced by the overexpression of *Myc* (O'Donnell et al., 2005). Predicted targets for the *mir-17* cluster microRNAs include members of the E2F and retinoblastoma families (Lewis et al., 2003); *mir-17* cluster

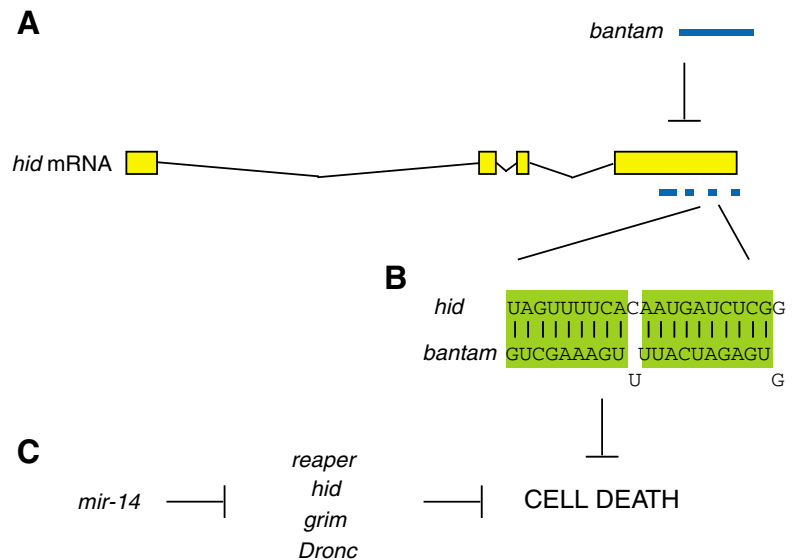


Fig. 3. MicroRNAs control programmed cell death. The *D. melanogaster* microRNA *bantam* and miR-14 inhibit programmed cell death. (A) The mRNA of the pro-apoptotic gene *hid* (yellow) has five predicted target sites for the *bantam* microRNA (blue) in the 3'UTR. (B) The alignment of the *bantam* microRNA with one of these sites is shown in green. (C) miR-14 also inhibits programmed cell death, possibly by regulating several pro-apoptotic genes such as *reaper*, *hid/wrinkled*, *grim* and *Dronc*.

microRNAs have been found to downregulate E2F1 expression (O'Donnell et al., 2005).

Another potential link between microRNAs and human disease comes from the identification of an essential co-factor for the microRNA biogenesis enzyme Droscha. This cofactor is encoded by DGCR8, which maps to chromosomal region 22q11.2, which is commonly deleted in DiGeorge syndrome (Denli et al., 2004; Gregory et al., 2004; Han et al., 2004; Landthaler et al., 2004; Lee et al., 2003; Lindsay, 2001; Shiohama et al., 2003). Haploinsufficiency of this region accounts for over 90% of individuals with DiGeorge syndrome, a disorder that affects 1 in 3,000 live births and results in heterogeneous defects including heart defects, immunodeficiency, schizophrenia and obsessive-compulsive disorder, among others. If indeed haploinsufficiency of DGCR8 contributes to DiGeorge syndrome, reduced levels of specific miRNAs may be to blame.

Potential roles of microRNAs in the development of the immune system have been discussed above; however, microRNAs might also be involved in immune defence. A cellular microRNA, miR-32, can regulate primate foamy virus type 1 (PFV-1) proliferation in cell culture (Lecellier et al., 2005). In addition, large DNA viruses of the herpesvirus family, including EBV (Pfeffer et al., 2005; Pfeffer et al., 2004) and SV40 (Sullivan et al., 2005), encode viral microRNA genes. These viral microRNAs have no apparent homologues in host genomes and their function is currently not understood.

Evolution

The labelling of *C. elegans* *lin-4* and *let-7* as heterochronic genes (Ambros and Horvitz, 1984) was highly provocative, suggesting potential roles for these genes during evolutionary change, but was well received by the evolutionary biology

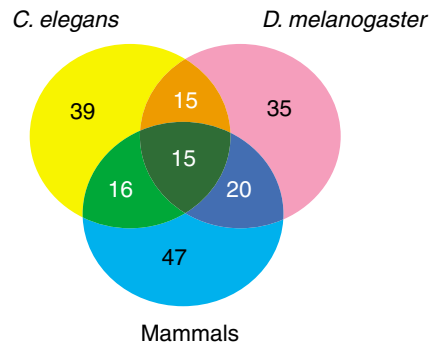


Fig. 4. MicroRNA family conservation. MicroRNAs can be grouped into families according to primary sequence similarity. As a cut-off, we used less than five conserved nucleotides out of the first eight. This Venn diagram shows the conservation of microRNA families between *C. elegans*, *D. melanogaster* and mammals. Primary sequence data was obtained from MiRBase version 7.0 (Griffiths-Jones, 2004).

community (Gould, 2000). With the identification of *lin-4* and *let-7* as microRNAs, their small size added only to the attractiveness of an evolutionary role for these genes. But how quickly do microRNAs themselves evolve? One of the early, exciting findings in the field was the realisation that the *let-7* microRNA is 100% conserved between *C. elegans* and humans (Pasquinelli et al., 2003). Overall, about 40% of the *C. elegans* microRNA families are conserved in humans (Lim et al., 2003b) (Fig. 4). By contrast, many primate-specific microRNAs that have no counterparts in the mouse have been identified (Berezikov et al., 2005), and several microRNA-encoding genes occur in highly repetitive and fast-evolving regions of the genome, such as in LINE-2 transposable elements (Smalheiser and Torvik, 2005). For the *mir-17* cluster and the microRNAs in the HOX gene cluster, an evolutionary analysis has been reported (Tanzer et al., 2005; Tanzer and Stadler, 2004). These initial studies suggest that microRNA families, rather than single microRNAs, are evolutionary conserved. One of the next questions to answer will be how the interactions between microRNAs and their targets evolve.

Conclusions

Research over the past five years has put microRNAs at centre stage. Early cloning experiments (Lagos-Quintana et al., 2001; Lau et al., 2001; Lee and Ambros, 2001) combined with expression studies (e.g. Wienholds et al., 2005), global approaches (such as Dicer knockouts) and selected functional studies have generated a tremendous amount of excitement about microRNAs in many areas of biology (see Table 1). In parallel, many new tools for the study of microRNAs have been developed (see Du and Zamore, 2005). We expect that soon we will appreciate that what we have learned about microRNAs to date is just the tip of the iceberg.

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